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THE MOLECULAR DIFFERENTIATION OF RENIBACTERIUM SALMONINARUM ISOLATES

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THE MOLECULAR DIFFERENTIATION OF *RENIBACTERIUM SALMONINARUM* ISOLATES

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THE MOLECULAR DIFFERENTIATION OF *RENIBACTERIUM SALMONINARUM* ISOLATES

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ABSTRACT

Studies were undertaken to examine the extent of molecular variation among isolates of the fish pathogen *Renibacterium salmoninarum*. As many isolates as possible were gathered from diagnostic laboratories within the UK, and checked for viability and contamination. The isolates were derived mainly from infected rainbow trout and Atlantic salmon that were farmed in England, Scotland and Wales, subject to the requirements of statutory legislation. Incomplete histories were available for the sources of the isolates, which spanned a time scale of 36 years, from 1962-1998. Molecular variation between the isolates was examined using two strategies. Firstly, defined regions of the genome were examined for polymorphisms. PCR analysis of previously characterised genes, *msa*, *hly*, and *rsh*, revealed no length polymorphisms among 43 UK isolates of *R. salmoninarum*. The 23S and 5S rRNA genes were sequenced and sequence analysis of the 16S-23S (ITS1) and 23S-5S (ITS2) rRNA regions was performed. Sequence analysis confirmed the correct taxonomic placement of *R. salmoninarum* within the Micrococcus/Arthrobacter subdivision of the Actinomycetes. Some isolates possessed small sequence variations within ITS1 that can provide an indication of their geographical origins. Sequence variation also exists in the ITS2 region but was found only within a single isolate from an outlying region of Canada. Ribotyping was found to be of limited use for discriminating among isolates of *R. salmoninarum* probably because *R. salmoninarum* possesses only two copies of the rRNA operon with no length polymorphisms in the ITS regions. Additionally, the discovery and analysis of a genetic locus containing a 51bp exact tandem repeat unit (designated ETR-A), revealed that some isolates of *R. salmoninarum* could be distinguished by the possession of one rather than two copies of this repeat unit. Finally, PCR amplification of length polymorphisms in the tRNA gene spacer regions (tDNA-PCR) was developed using consensus tRNA gene primers to enable tRNA genes and spacer regions to be cloned and sequenced. Subsequently, specific tRNA gene primers were designed and enabled the discrimination of 43 UK isolates into 15 groupings. tDNA-PCR proved to be one of the most powerful typing methods applied to this organism. Secondly, typing methods that analysed the whole genome for the presence of molecular variation were employed. A putative insertion sequence IS*994*, was used as a probe in a RFLP-based study to discriminate between 52 isolates of *R. salmoninarum* from different countries. This method showed great potential for distinguishing between isolates and separated the 52 isolates that were examined into 12 groupings. Randomly amplified polymorphic DNA (RAPD) was also used to examine 28 isolates of UK origin. This method was found to be highly discriminatory, with 28 isolates generating 12 different banding patterns, which appeared to reflect geographical source. Pulsed field gel electrophoresis was also used to investigate the genomic diversity of isolates. Due to technical difficulties in obtaining pure, undamaged DNA the preparation of suitable macro restriction profiles was never achieved. However, preliminary findings suggested that the *R. salmoninarum* chromosome was linear and approximately 4.5-6Mb in size. Finally, repetitive element PCR was evaluated using 3 different approaches (ERIC, REP and BOXA-2) but proved to have a limited capacity for defining molecular variation between different isolates. Ultimately, RAPD, tDNA-PCR and IS*994* RFLP profiling proved to be most useful for the molecular differentiation of *R. salmoninarum*. A comparison of the groupings that resulted from each of these methods revealed substantial areas of agreement. The use of a multifactorial approach not only resulted in a greater discrimination of isolates but also provided increased confidence in the outcome. It is recommended that for typing purposes such an approach should be adopted. Epizootiological interpretations of groupings were hampered by the general lack of background information attached to each isolate. However, the application of multiple typing methods reveal that, despite the highly conserved nature of this bacterium, UK isolates of *R. salmoninarum* possess genetic diversity, with geographically related isolates often being grouped together. Overall there was little evidence to suggest the regular introduction of genetically distinct *R. salmoninarum* into the UK and the results suggest that some isolates may be relatively localised despite the international trade in fish stocks.

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PUBLICATIONS

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Grayson, T. H., Alexander, S. M., Cooper, L. F., and Gilpin, M. L. 2000. *Renibacterium salmoninarum* isolates from different sources possess two highly conserved copies of the rRNA operon. Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology 78[1], 51-61.

Grayson, T. H., Atienzar, F. A., Alexander, S. M., Cooper, L. F., and Gilpin, M. L. 2000. Molecular diversity of *Renibacterium salmoninarum* isolates determined by randomly amplified polymorphic DNA analysis. Applied and Environmental Microbiology 66[1], 435-438.

Rhodes, L. D., Grayson, T. H., Alexander, S. M., and Strom, M. S. 2000. Description and characterization of IS*994*, a putative IS*3* family insertion sequence from the salmon pathogen, *Renibacterium salmoninarum*. Gene 244[1-2], 97-107.

CONFERENCE POSTERS

Alexander, S.M., Grayson, T. H., Cooper, L. F., Atienzar, F. A., Knowles, M. R., and Gilpin, M. L. 1999. Molecular differentiation of *Renibacterium salmoninarum* isolates from worldwide locations. Proceedings of the Society for General Microbiology 143rd Ordinary Meeting, Edinburgh (April 1999).

Rhodes, L. D., Grayson, T. H., Alexander, S. M., and Strom, M. S. 2000. Description and characterization of IS*994*, a putative IS*3* family insertion sequence from the salmon pathogen, *Renibacterium salmoninarum*. Western Fish Disease Conference, Seattle, USA (June 2000).

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CONFERENCES ATTENDED

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Signed:.....

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ABBREVIATIONS

bp	base pair
BKD	bacterial kidney disease
BOX-A2	*Streptococcus pneumoniae* repetitive DNA sequence
BSA	bovine serum albumin
CHEF	contour-clamped homogenous field
CSS	cell suspension solution
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
ddH_20	distilled and deionised water
Da	dalton
Dig-11-dUTP	digoxigenin deoxyuracil triphosphate
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
DIG	digoxigenin
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERIC	enterobacterial repetitive intergenic consensus
ETR	exact tandem repeat
FAT	fluorescent antibody test
FIGE	field inversion gel electrophoresis
g	gram
kg	kilogram
mg	milligram
µg	microgram
h	hour
hly	*R. salmoninarum* haemolysin gene
IFAT	indirect fluorescent antibody test
IPTG	isopropyl-β,D-thiogalactopyranoside
ITS	intergenic spacer region
ITS1	16S-23S rRNA intergenic spacer region
ITS2	23S-5S rRNA intergenic spacer region
IS	insertion sequence
kb	kilobase
kDa	kilodalton
L	litre
LBA	Luria-Bertani agar
LBB	Luria-Bertani broth
M-PCR	multiplex PCR
Mb	megabase
MF-FAT	membrane-filtration fluorescent antibody test
min	minute
msa	*R. salmoninarum* major soluble antigen gene
nt	nucleotide

NBT	nitroblue tetrazolium
PAGE	polyacryamide gel electrophoresis
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PMSF	phenylmethylsulfonylfluoride
RAPD	randomly amplified polymorphic DNA
REP	repetitive extragenic palindromic
RFLP	restriction fragment length polymorphisms
RNA	ribonucleic acid
rRNA	ribosomal RNA
rsh	*R. salmoninarum* hemolysin gene
RT	room temperature
RT-PCR	reverse transcription PCR
s	seconds
SKDM	selective kidney disease medium
SSC	salt sodium citrate
tRNA	transfer RNA
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
TSA	tryptone soya agar
TSB	tryptone soya broth
U	unit
UV	ultra violet
V	volts
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
X-phosphate	5-bromo-4-chloro-3-indolylphosphate

Chapter One

INTRODUCTION

Renibacterium salmoninarum is a slow growing gram-positive bacterium representing a genospecies that is phylogenetically placed within the Micrococcus/Arthrobacter subdivision of the actinomycetes (Sanders *et al.*, 1980; Goodfellow *et al.*, 1985; Stackebrandt *et al.*, 1988; Gutenberger *et al.*, 1991). It is an obligate pathogen and the causative agent of bacterial kidney disease (BKD), a chronic systemic infection of salmonid fish. BKD is geographically widespread and is responsible for significant losses in farmed and wild salmonids, globally (Evelyn, 1993). Control of BKD has proved to be difficult because the pathogen survives intracellularly and can be transmitted vertically inside the ova as well as horizontally between cohabiting fish (Mitchum & Sherman, 1981; Bullock *et al.*, 1978). Currently there is no effective vaccine or chemotherapy and sub-clinical infections make control by eradication less effective (Evenden *et al.*, 1993). The detection of BKD and investigations of the pathogenicity of *R. salmoninarum* have also been hindered because the organism is difficult to culture on laboratory media, requiring periods of 6 weeks or more for growth (Wolf & Dunbar, 1959; Goodfellow *et al.*, 1985).

Until recently very little was known about the epidemiology of BKD due to a lack of practical and simple typing methods. Traditional typing methods, such as biochemical profiles, serological typing and multilocus enzyme electrophoresis, have failed to provide a reliable means for discriminating between isolates of the pathogen due to a remarkable degree of biochemical and serological uniformity (Starliper, 1996; Bruno & Munro, 1986; Bullock *et al.*, 1974; Getchell *et al.*, 1985; Wiens & Kaattari, 1989; Daly & Stevenson, 1990). A number of molecular tools that enable the analysis of genetic diversity amongst

isolates of *R. salmoninarum* have recently been developed. Grayson *et al.* (1999) developed two molecular methods, randomly amplified polymorphic DNA (RAPD) and 16S-23S rRNA intergenic spacer region (ITS) sequencing, that were useful for the differentiation of *R. salmoninarum* isolates. RAPD was found to be a reliable and reproducible means for differentiating between isolates of *R. salmoninarum*, while intergenic spacer regions were of limited use but provided some indication of the geographical origin of isolates.

Commonly, when different typing systems are applied to a set of bacterial isolates, each method generates different groupings. This is to be expected because different typing systems analyse different loci or different traits. However, dependence on only one or a few properties for typing purposes may lead to serious errors in the relationships that are obtained (Maslow & Mulligan, 1996). With respect to *R. salmoninarum* and BKD, this means that despite the development of two reproducible and reliable typing methods further characterisation of specific markers of variation will facilitate a better understanding of the genetic relatedness between isolates that have been sourced from the same farm, river or lake.

Bacteriological typing is based on the principle that epidemiologically related isolates are derived from the clonal expansion of a single precursor or parent isolate within the same species (Olive & Bean, 1999). Therefore, the outbreak strain or isolate shares characteristics with its precursor that may differ from those of epidemiologically unrelated isolates. Ultimately typing systems aim to identify characteristics that allow discrimination below the species level (LiPuma, 1998). As a result, typing methods are most commonly used in cases of contact tracing to confirm or disprove hypotheses regarding the possible routes of transmission of bacterial infections (Cobben *et al.*, 1996; Das *et al.*, 2002; Ling *et*

al., 2002; Nishikawa *et al.*, 2001; Wei & Chiou, 2002). Typing techniques have also been proven to be useful in the management and diagnosis of persistent bacterial infections where they can distinguish cases of relapse (due to the original isolate) from cases of reinfection (infection with a new isolate) (Kato *et al.*, 1996; Wain *et al.*, 1999). Some of the more common typing methods currently used include pulsed field gel electrophoresis (PFGE) (Tenover *et al.*, 1995), rRNA analysis (Stull *et al.*, 1988; Gurtler & Stanisich, 1996), randomly amplified polymorphic DNA (Power, 1996; Welsh & McClelland, 1990), multi-locus sequence typing (MLST) (Spratt, 1999), insertion sequence analysis by restriction fragment length polymorphisms (Stanley *et al.*, 1996), amplified fragment length polymorphisms (AFLP) (Janssen *et al.*, 1996), and PCR analysis of repetitive elements on the bacterial genome (rep-PCR) (Versalovic *et al.*, 1993; Koeuth *et al.*, 1995).

1.1 Aims

The aim of this research was to develop and apply new and existing molecular typing techniques for the differentiation of *R. salmoninarum* isolates. The availability of such techniques will assist future studies in the epizootiology of BKD.

1.2 Objectives

1) To evaluate the following typing techniques for their ability to discriminate between *R. salmoninarum* isolates:

i) Specific gene polymorphisms.

ii) Randomly amplified polymorphic DNA (RAPD).

iii) Sequence analyses of rRNA intergenic spacer regions.

iv) Examination of the whole rRNA operon of *R. salmoninarum*.

viii) tRNA intergenic spacer regions (tDNA-PCR).

vi) Insertion sequence analysis.

vii) Pulsed field gel electrophoresis (PFGE).

viii) The use of enterobacterial repetitive intergenic consensus (ERIC)-PCR, repetitive extragenic palindromic (REP)-PCR and BOXA2-PCR.

2) To compare the molecular typing methods that were investigated for their ability to differentiate *R. salmoninarum* isolates and to consider their future use either alone or in combination.

Chapter Two

LITERATURE REVIEW

2.1 The organism

Renibacterium salmoninarum is a small (0.5 X 0.8μm), gram-positive, aerobic, non-motile, fastidious, rod-shaped bacterium that often appears as a diplococcus. It is an obligate pathogen, which is capable of intracellular survival and causes bacterial kidney disease (BKD) in salmonid fish (Evelyn, 1993). The organism was first isolated on Dorset's medium from the kidneys of infected fish by Ordal & Earp (1956), who classified it as *Corynebacterium* on the basis of morphology. Sanders & Fryer (1980) recognised the unique biochemical properties of this bacterium including the guanine plus cytosine ratio of its DNA and the cell wall composition, and proposed the name *Renibacterium salmoninarum.*

Numerous studies of the biochemistry, morphology, physiology (Sanders & Fryer, 1980; Goodfellow *et al.*, 1985; Kusser & Fiedler, 1983; Fiedler & Draxl, 1986), 16S rRNA (Stackebrandt *et al.*, 1988; Gutenberger *et al.*, 1991; Banner *et al.*, 1991; Koch *et al.*, 1994), lipid and menaquinone composition (Embley *et al.*, 1983), growth characteristics and pathogenic status of the bacterium have supported the integrity of the genus *Renibacterium* and its correct placement within the *Micrococcus-Arthrobacter* subdivision of the actinomycetes (Evenden *et al.*, 1993). Interestingly, most members of the *Micrococcus-Arthrobacter* group tend to be commensals and are widely distributed in the environment, although there are reports that *Micrococcus luteus* can cause disease in rainbow trout fry (Austin & Stobie, 1992).

2.2 Virulence factors and pathogenicity

The most studied product and potential virulence factor of *R. salmoninarum* is a 57kDa soluble protein that is mainly localised on the bacterial cell surface. This protein, which has been termed antigen F, major soluble antigen (*msa*) and p57, has been shown to agglutinate mammalian erythrocytes and leukocytes, and salmonid sperm (Daly & Stevenson, 1989; Wiens & Kaattari, 1991). It has been speculated that p57 may have a role in attachment to host cells and perhaps also in cell entry (Fevolden *et al.*, 1993).

A study carried out on an attenuated strain of *R. salmoninarum*, MT239, suggested that the reduced virulence of this isolate may have been attributed to the absence of p57 (Bruno, 1988). Grayson *et al.* (1995c) showed that both attenuated and virulent isolates of *R. salmoninarum*, including MT239, produce p57 as a cell-associated molecule and also in the extracellular products. A more recent study found that while the amount of cell-associated p57 was reduced in MT239, the level of p57 in the culture supernatant was nearly equivalent to that of a virulent isolate. In the same study it was determined that *R. salmoninarum* possesses two copies of *msa*, the gene encoding p57, which are identical in sequence between isolates (O'Farrell & Strom, 1999). This was confirmed in a study by Senson & Stevenson (1999), which concluded that the virulence of *R. salmoninarum* depends not only on the production of p57, but also on its direct association with the bacterial cell surface.

A number of other components have also been identified that may have an as yet undefined role in pathogenicity. Rockey *et al.* (1991b) identified a serine protease that is secreted by *R. salmoninarum* although no clear role for the enzyme could be determined. *In vitro* and *in vivo* studies of extra-cellular products by Bandin *et al.* (1991) found a paucity of extra cellular proteins in a variety of different isolates of *R. salmoninarum* and concluded that

most were poor producers of proteolytic enzymes. However, Evenden *et al.* (1990) cloned and expressed a gene that encoded a haemolytic activity (*rsh)* and Grayson *et al.* (1995b), cloned and sequenced a gene, *hly*, encoding another haemolysin from this pathogen, which showed strong similarity to secreted bacterial zinc-metalloproteases (Evenden, 1993). It was demonstrated that iron modulates the expression of *hly* (Grayson *et al.*, 1995b).

Immunoelectron microscopic studies of *R. salmoninarum* have demonstrated that this organism is capsulated although it has not been demonstrated whether this is related to virulence (Dubreuil *et al.*, 1990). Other investigations have highlighted the role of iron reductase as an important component of the iron acquisition mechanism of *R. salmoninarum*, which clearly has role in the pathogenicity of this organism (Grayson *et al.*, 1995a). More recent work by Maulen *et al.* (1996) involved the isolation of a recombinant clone, containing an *R. salmoninarum* DNA fragment that apparently conferred an invasive phenotype on *Escherichia coli.* Further analysis of this fragment revealed that the gene encoded a product with substantial sequence identity to bacterial glucose kinase (Concha & Leon, 2000).

2.3 Culture and growth

R. salmoninarum is a slowly growing fastidious organism requiring rich laboratory media containing cysteine and serum for growth (Ordal & Earp, 1956). Growth is optimal between 15°C and 18°C, very slow at 5°C and 22°C and is absent at 30°C. *R. salmoninarum* was for many years routinely cultivated on Mueller-Hinton medium supplemented with 0.1% L-cysteine hydrochloride, although growth is slow often requiring several weeks of incubation (Wolf & Dunbar, 1959; Goodfellow *et al.*, 1985). In 1977, an improved kidney disease medium (KDM2) which incorporated serum was developed and techniques for improving consistent results and for accelerating growth when using this

medium have been identified (Evelyn, 1977; Evelyn *et al.*, 1989; Evelyn *et al.*, 1990). As yet there is no defined medium for culturing *R. salmoninarum* although a semi-defined medium containing tryptone (Embley *et al.*, 1982) and a 'selective kidney disease medium' (SKDM) have been developed (Austin *et al.*, 1983). There have been conflicting reports in the literature as to which growth medium is the most effective. Gudmundsdottir *et al.* (1991) regarded SKDM as best for initial isolation from clinical samples although Olsen *et al.* (1992) preferred KDM2 for this purpose.

Starliper *et al.* (1998) performed an investigation into 14 different broth media, 13 of which were serum free and found that no medium performed noticeably better than any other and that they all performed well. A further development in the culturing of *R. salmoninarum* has involved the employment of a fish cell line. *R. salmoninarum* was found to maintain virulence when cultured within this cell line rather than on agar plates (McIntosh *et al.*, 1997). Ultimately the slow growth and fastidious requirements of this pathogen mean that the process of isolation and culture are time consuming and expensive and cultures are prone to contamination. Consequently, the diagnosis of BKD and investigations into the pathogenicity of *R. salmoninarum* has been hampered by these difficulties.

2.4 Bacterial kidney disease (BKD)

BKD is geographically widespread and is responsible for significant losses in farmed and feral salmonids. The first reported case of BKD was in Atlantic salmon (*Salmo salar*) in Scotland (Mackie *et al.*, 1933). In the USA, the disease was first reported by Belding & Merrill (1935) who described gross external and internal pathology in brook trout (*Salvelinus fontinalis*). Because of the abscesses presented during BKD it has sometimes

been referred to as white boil disease. Other names include Dee disease, corynebacterial kidney disease, and salmonid kidney disease (Fryer & Sanders, 1981).

2.5. Pathology

2.5.1 External signs

BKD is a systemic disease that progresses slowly and can spread from foci of infection to affect most of the vital organs. External signs of BKD are variable and often not evident until the disease is well established. In some cases, fish that are chronically infected with BKD can be asymptomatic. However, in most cases one or more of the following symptoms are usual: pale gills (anaemia), superficial skin blisters, exopthalmos, shallow ulcers, haemorrhagic areas, deep abscesses, swollen abdomen and, in more extreme cases, cavitations in the muscle (Fryer & Lannan, 1993; Fryer & Sanders, 1981; Evenden *et al.*, 1993).

2.5.2 Internal signs

Internal damage caused by BKD is often extensive. Early signs of the disease are small granulomatous lesions on the kidneys, and often also on the liver and spleen. Focal granulomas also occur in the muscle lining in the peritoneum, heart, swim bladder, pancreas and eyes. Commonly, in advanced cases of BKD the lesions increase in number, size and eventually progress to form large shallow ulcers. The kidney is generally the organ that is most severely affected with swelling, whitening and necrosis often occurring. This is thought to be a result of the accumulation of leucocytes, bacteria, and host cell debris (Evenden *et al.*, 1993; Fryer & Lannan, 1993; Starliper *et al.*, 1997).

BKD diseased fish have also been reported to have a false white membrane over liver, spleen, and gonads. This layer consists of fibrin, collagen, leucocytes and *R. salmoninarum* cells (Smith, 1964; Snieszko & Griffin, 1955; Bell, 1961). The formation of this membrane

has been shown to be linked to an increase in water temperate and it has been suggested that it may be an attempt by the host to encapsulate the pathogen (Smith, 1964; Bruno, 1986). In advanced cases, when much of the kidney tissue is destroyed, haematopoietic and excretory functions are both affected. Death is ultimately attributed to the obliteration of normal kidney and liver tissue, which leads to organ failure and, possibly, heart dysfunction (Bruno, 1986).

At the cellular level, the tissue destruction brought about by this disease is thought to be caused by the release of hydrolytic and catabolic enzymes from lysed phagocytic cells infected with *R. salmoninarum*. However, the hosts defence mechanisms including cell-mediated immunity (CMI) and reactive oxygen species generated by phagocytes are also thought to aggravate the damage (Bandin *et al.*, 1993; Bruno, 1986; Fryer & Sanders, 1981).

2.6 Detection and diagnostic methods

2.6.1 Identification of clinical symptoms and direct observations

Because no effective treatment exists for BKD, rapid and sensitive techniques for the detection of *R. salmoninarum* in asymptomatic fish are essential for successfully controlling the spread of the disease. The presumptive identification of BKD has relied on clinical symptoms and the presence of gram-positive diplobacilli in infected tissue (Earp *et al.*, 1953). However, gram stain is not sensitive, requiring 10^7-10^9 cells per gram of tissue for a positive identification (Bullock *et al.*, 1980; Sakai *et al.*, 1987). The presence of melanin granules in kidney tissues also obscures the presence of the pathogen and reduces the sensitivity of this technique (Bruno & Munro, 1982).

2.6.2 Culture

The definitive diagnosis of BKD depends on the culture of *R. salmoninarum* from infected tissues and confirmation by the application of an additional technique. Suspected cultures can be confirmed with a PCR-based technique or comparison with the characteristic profile generated using an API test (Sakai *et al.*, 1993). However, with initial isolations of *R. salmoninarum* taking 6-8 weeks and sometimes up to 20 weeks, it is a very time consuming, expensive and labour intensive way of screening large numbers of asymptomatic fish (Benediktsdottir *et al.*, 1991). In addition to the possibility of false culture negatives, these difficulties have greatly hindered the effectiveness of control programs.

2.6.3 Serological detection

A number of immunodiagnostic procedures have been developed for the diagnosis of BKD including immunodiffusion (Chen *et al.*, 1974; Kimura *et al.*, 1978), direct fluorescent antibody tests (DFAT) (Bullock *et al.*, 1980), indirect fluorescent antibody test (IFAT) (Bullock & Stuckey, 1975), enzyme linked immunosorbent assay (ELISA) (Pascho & Mulcahy, 1987), filtration FAT (Elliott & Barila, 1987), and a monoclonal antibody based ELISA (Dixon, 1987; Rockey *et al.*, 1991a). However, some of these techniques are reported to be prone to false positives (Bullock *et al.*, 1980; Evelyn *et al.*, 1984; Austin *et al.*, 1985; Yoshimizu *et al.*, 1987). Enzyme immunoassays employing monoclonal antibodies have been shown to be the most specific, sensitive and reproducible of the serological techniques (Meyers *et al.*, 1993; Pascho *et al.*, 1987). These assays can detect very low levels of *R. salmoninarum* antigen in subclinical infections and can be used to establish the distribution and abundance of *R. salmoninarum* in fish populations.

2.6.4 Molecular detection

2.6.4.1 PCR

A number of PCR-based assays have been developed to assist in the detection of *R salmoninarum* e.g. using a randomly cloned 149bp fragment (pMAM29) as the basis for a PCR assay from infected kidney tissues (Etchegaray *et al.*, 1991; Leon *et al.*, 1994b; Butendieck *et al.*, 1995). This PCR was very sensitive, allowing the detection of 22 cells of *R. salmoninarum,* however the probe was found to cross-react with *Corynebacterium striatum.* Several PCR-based methods that target the p57 gene, *msa,* have been developed and their sensitivity assessed in rainbow trout lymphocytes (McIntosh *et al.*, 1996), salmonid eggs (Brown *et al.*, 1994), and ovarian samples (Pascho *et al.*, 1998). Further developments using *msa* for the detection of *R. salmoninarum* have involved a nested PCR for use with infected kidney samples (Chase & Pascho, 1998) and nested reverse transcription PCR assays (Cook & Lynch, 1999). A nested reverse transcription PCR assay of the 16S ribosomal rRNA operon was tested and could detect as few as 1-10 cells in ovarian fluids but the technique took 1-2 days and was found to be unreliable with kidney tissues possibly due to the inhibition of *Taq* polymerase (Magnusson *et al.*, 1994). Rhodes *et al.* (1998), investigated this PCR further and found that the sensitivity could be increased 10-fold by probing Southern blot of PCR products with a specific DNA probe.

Until recently, serological rather than molecular detection methods have been applied when investigating BKD outbreaks. PCR is more sensitive and specific than serological tests, but both methods have a role to play in the diagnosis of BKD (Brown *et al.*, 1995; Pascho *et al.*, 1998). Both molecular and serological methods of detection are potentially prone to the problems of false positives and negatives because both techniques have the potential to detect dead, live, dormant, damaged, and inhibited *R. salmoninarum* cells or cells of an unrelated but cross-reacting organism. Consequently, it has been recommended

that positive results should always be interpreted with caution and wherever possible the isolation of the organism in pure culture should be used for confirmation. Similarly, negative outcomes should always be questioned, particularly when salmonid farms or fish stocks have a history of BKD or a low level of unexplained mortalities.

2.6.4.2 Oligonuclotide probes

Some *R. salmoninarum* detection systems have involved the development of specific molecular probes. Glover and Harris (1998) developed a *R. salmoninarum* probe that hybridised to a portion of the 16S rRNA gene, although the specificity of this probe was questioned as it was found to cross-hybridise with *Arthrobacter globiformis.* Another probe was developed to a different region of the *R. salmoninarum* 16S rRNA sequence, however this probe was found to be more specific and did not cross react with the other 22 bacterial species against which it was tested (Mattsson *et al.*, 1993). Other probes have also been developed including a biotinylated probe (Hariharan *et al.*, 1995), and a digoxygenin-labelled specific probe from an unknown region of the *R. salmoninarum* genome (Leon *et al.*, 1994a).

2.7 Epizootiology

2.7.1 Host species

Natural occurrences of BKD are restricted to members of the Salmonidae, namely, salmon (*Salmo*), trout (*Oncorhynchyus)*, grayling (*Thymallus)* and char (*Salvelinus*) (Evenden, 1993; Fryer & Sanders, 1981). There is also evidence that resistance to BKD within a species has a genetic basis (Withler & Evelyn, 1990), although some studies have found otherwise (Beacham & Evelyn, 1992; Fevolden *et al.*, 1993).

Although it is unlikely that non salmonid fishes serve as a natural reservoir for *R. salmoninarum*, BKD infections have been established in a number of species under laboratory conditions including Pacific herring (*Clupea harengus pallasi*), sablefish (*Anoplopoma fimbria*), shiner perch (*Cymatogaster aggregata*), common shinner (*Notropis cornutus*) and flathead minnow (*Pimephales promelas*) (Bell *et al.*, 1988; Bell *et al.*, 1990; Bell & Traxler, 1986);(Evelyn [unpublished data]: cited in (Evelyn, 1993). However, experiments to infect lamprey (*Lampetra tridentata*) and the common carp (*Cyprinus carpio*) have failed (Bell & Traxler, 1986; Sakai *et al.*, 1989) but serological tests have been able to detect *R. salmoninarum* antigens in greenling (*Hexagrammos otakii*), Japanese sculpin (*Cottus japonicus*) and scallops (*Patinopecten yessoensis*) that were taken from the vicinity of netpens containing BKD infected coho salmon (Sakai & Kobayashi, 1992). *R. salmoninarum* has also been detected by visual inspection and FAT in the soft tissue of mussels that had been artificially challenged with the pathogen (Starliper & Morrison, 2000).

2.7.2 Transmission

BKD can be transmitted directly by feeding from the infected tissues of diseased fish (Wood & Wallis, 1955), horizontally between cohabiting fish (Mitchum & Sherman, 1981; Bell *et al.*, 1984; Balfry *et al.*, 1996) and vertically from mother to progeny via the infected ova (Bullock *et al.*, 1978; Evelyn *et al.*, 1984; Evelyn *et al.*, 1986b).

An early extensive investigation into the distribution of *R. salmoninarum* in water, sediment and fish samples from freshwater farms in England and Wales was carried out by Austin & Rayment (1985). Water and sediment samples taken from all of the 56 sites examined were negative for the presence of *R. salmoninarum*, strongly suggesting that *R. salmoninarum* is an obligate pathogen and not a member of the normal microbial flora.

Although there was no evidence that BKD was widespread in England and Wales, two rainbow trout (*Salmo gairdneri*) farms were found to contain culture positive fish. Surprisingly, in fish sampled from one farm *R. salmoninarum* was recovered from the kidneys of 39 asymptomatic animals, which were found to be negative by IFAT. The same study also reported that *R. salmoninarum* excreted in the faeces of infected fish could survive for up to 21 days although the bacterium could only exist unprotected in freshwater for up to 4 days (Austin & Rayment, 1985). This work reinforces the belief that *R. salmoninarum* is unlikely to exist as a free-living organism in the aquatic environment and that the only known reservoir of infection is salmonid fish.

2.7.3 Predisposing factors

It has been suggested that *R. salmoninarum* is endemic in salmonid fish populations and only presents a problem when infected fish are stressed, thereby tipping the balance in favour of the pathogen and the development of clinical disease (Jonsdottir *et al.*, 1998). Although BKD is thought to occur mainly in freshwater salmonids, infected fish are more likely to die of BKD during migration or after entry into salt water (Pascho *et al.*, 1993). The physiological stress of transition from freshwater to saltwater is thought to accelerate BKD related mortality (Sanders *et al.*, 1992; Elliott *et al.*, 1995; Mesa *et al.* 1999). In addition, the progression of BKD can be affected by temperature, salinity, food availability and water quality (Pirhonen *et al.*, 2000). Outbreaks of BKD during the spring and summer months was first related to increases in water temperature by Belding & Merrill (1935), and this phenomenon has been echoed in several other studies. However, a more recent study that examined the relationship between stress and BKD suggested that physical stress, as distinct from physiological stress, does not increase BKD related mortality or even exacerbate the disease. Mesa *et al.* (2000) subjected *R. salmoninarum* infected and non-infected fish to a series of multiple stressors (handling, hypoxia and mild agitation) to

establish if physical stress increases infection and ultimately leads to increased mortality. The results of this study indicated that the stressors that were applied did not lead to higher infection levels or increased mortality, although it was observed that fish with a moderate to high level of *R. salmoninarum* infection were unable to elicit significant hyperglycaemia in response to a stress (Mesa *et al.*, 2000).

2.7.4 Geographical distribution

BKD is geographically widespread and is thought to occur wherever there are susceptible populations of salmonids present. The known range of BKD in farmed fish includes Canada, Chile, Denmark, England, France, Germany, Iceland, Italy, Japan, Scotland, Spain, Turkey, United States, Norway and Yugoslavia. In addition, BKD has been reported to occur in wild fish populations from USA, Iceland, Canada, Europe and Eastern Asia (Elliott *et al.*, 1997; Jonsdottir *et al.*, 1998; Kent *et al.*, 1998; Sakai *et al.*, 1992). BKD has not been reported in Australasia, and Russia (Evelyn, 1988).

2.7.5 Interaction between feral and farmed salmonids in the transmission of BKD

Although outbreaks of BKD are generally only reported in farmed salmonids, BKD has been detected sporadically in populations of wild salmonids. In Wyoming USA, the BKD status of feral populations of brook trout, brown trout and rainbow trout along a river system was examined. The original source of infection in the river system was thought to be infected hatchery trout, which were stocked into the stream from a station on the river. It was found that BKD was responsible for all the dead fish collected from three stations and, in live fish sampled by FAT, the incidences of BKD ranged from 83% at the upstream station to only 3% at the downstream station (Mitchum *et al.*, 1979). In the same river system, Mitchum & Sherman (1981) reported that newly stocked hatchery salmonids were infected with *R. salmoninarum* that had been transmitted from the infected wild salmonids

which inhabited the river system. However, this route of infection was speculated rather than scientifically established.

R. salmoninarum antigens have been detected by IFAT in 5% of migrating feral chum salmon (*Oncorhynchus keta*) taken from Japanese waters. The feral fish had migrated into a bay where cultured coho salmon that were known to be infected with *R. salmoninarum* were present (Sakai *et al.*, 1992). It has been suggested that BKD was first introduced into Japan via infected eggs and reports of BKD in Japan have been associated with salmonid hatcheries (Kimura & Awakura, 1977). Unfortunately, in the former study no isolates of *R. salmoninarum* were cultured and due to the lack of simple typing systems no further investigations into the sources of the outbreak were made.

In a recent Icelandic study, in which 22 populations of wild Arctic char and nine populations of wild brown trout were screened by ELISA, it was determined that none of the populations were free of *R. salmoninarum* antigens and the prevalence of infection was found to be 46% for Arctic char and 35% for brown trout (Jonsdottir *et al.*, 1998). None of the wild fish that were examined in the study showed signs of BKD, and it was suggested that *R. salmoninarum* has been endemic for a long time within Icelandic fish populations and exists as a normal or low density resident in the fish. Because the patterns of infection were found to differ between the two fish species, even between coexisting populations from the same lake, it was proposed that the dynamics of infection and the internal proliferation of *R. salmoninarum* were quite different between the char and the trout (Jonsdottir *et al.*, 1998). It is possible that this could be explained by differences in host innate immunity and resistance to the presence of *R. salmoninarum*. Alternatively, differences in the virulence or pathogenicity of isolates of *R. salmoninarum* may have evolved as a consequence of adaptation to a different host fish. Many of the

epizootiological questions that have been posed as a consequence of these studies could have been resolved by an appropriate typing system.

2.7.6 Treatment and control

2.7.6.1 Chemotherapy

Control of BKD has proved to be very difficult, and it appears that the intracellular nature of the pathogen and intra ovum transmission, place it beyond the reach of most antimicrobials (Evelyn *et al.*, 1986a). Erythromycin is currently the most popular antibiotic used to treat BKD, either by immersion, injection or via the oral route. The majority of published reports indicate that applications of erythromycin are effective in significantly reducing mortality in BDK outbreaks but mortality often occurs after the treatment is terminated (Moffitt & Kiryu, 1999; Peters & Moffitt, 1996; Elliott *et al.*, 1995; Austin, 1985; Groman & Klontz, 1983). Despite the effectiveness of erythromycin in reducing mortalities it does not eliminate the pathogen from the host (Fryer & Sanders, 1981). Disturbingly, erythromycin resistance can be induced in *R. salmoninarum* under laboratory conditions, and raises the possibility that inappropriate use of antibiotics in the field could have undesirable consequences (Bell *et al.*, 1988).

2.7.6.2 Vaccination

Despite substantial efforts to produce a BKD vaccine, immunization of fish has not been widely accepted as an effective method for controlling this disease. A variety of preparations including heat killed suspensions, cell wall fractions and a range of *R. salmoninarum* antigens and products have been delivered by feeding, injecting intraperitoneally, spraying and immersion (Griffiths *et al.*, 1998; Kaattari & Piganelli, 1997). Although many of the vaccines have been shown to produce an immune response in fish, long term protective ability has been lacking (Fryer & Lannan, 1993; Evelyn, 1988;

Evenden *et al.*, 1993). Recently, a vaccine has been licensed for use in Canada that is based on the killed whole cells of an unidentified *Arthrobacter* sp. The vaccine reduces mortalities in laboratory challenges by up to 60% and is also effective under field conditions. The protective component has been identified as the cell wall polysaccharide, which shares antigenic identity with the cell wall polysaccharide of *R. salmoninarum* (Griffiths *et al.*, 1998).

2.7.6.3 Diet and fish management

Diet has been shown to influence the outcome of BKD by Paterson *et al.* (1985) who found that diets containing high levels of iodine and fluorine appeared to reduce the prevalence of clinical BKD compared to control fish. There are also reports that diets containing vitamin E and selenium can have similar benefits and that survival of BKD-infected fish is inversely proportional to levels of vitamin C in diets with low levels of zinc and manganese (Thorarinsson *et al.*, 1994; Bell *et al.*, 1984). There is also evidence that the pathogen is sensitive to chlorine which can be used to disinfect hatcheries (Pascho *et al.*, 1995).

The improved management of fish stocks by maintaining high water quality, avoiding sudden fluctuations in water temperature and reducing unnecessary or poor handling, stocking densities and movements of fish, often leads to a reduction in disease outbreaks and transmission (Banks, 1994; Mazur *et al.*, 1993; Mesa *et al.*, 2000; Mesa *et al.*, 1998).

2.7.6.4 Control of BKD within the UK

Due to the significant mortality in both wild and farmed salmonid stocks caused by BKD, its prevalence in both the UK and the European union (EU) is tightly monitored. BKD is one of the eight notifiable fish diseases within the UK and is a "list III" fish disease, with

"list I and II" diseases being restricted to very severe fish diseases that have never been found with the UK. Legislation governing the control of fish farms and fish diseases are subject to three legal acts. Primarily "The Diseases of Fish Act, 1937" (amended by the Disease of Fish Act 1983), and two additional bills from the European Union, "Diseases of fish (control) regulations, 1994 (SI 1994; No. 1441)" and "Fish health regulation, 1997 (SI 1997; No. 1881)". Ultimately these acts make it a legal requirement to notify the presence or suspicion of BKD to the Department of Environment, Food and Rural Affairs (DEFRA), with failure to do so resulting in possible criminal prosecution. Additionally, this legislation ensures that DEFRA inspectorates have the power to remove random samples of fish, gametes and foodstuffs from commercial fish farms for the examination of notifiable infectious agents. Upon suspicion of a BKD outbreak the inspectorate has the power to serve a temporary notice enforcing quarantine on the farm in question by the delivery of site and livestock movement restrictions which in the case of a positive identification will become a permanent notice which will only be lifted when livestock is destroyed and a certification of disinfection is provided (Department for Food Environment and Rural Affairs, 2002).

The current legislation exists to reduce the outbreaks of notifiable fish diseases within the UK and other EU member states by ensuring that imports of live fish from the salmon family are prohibited, and that eggs or gametes are licensed and certified. This legislation is primarily policed by the Fish Health Inspectorate (FHI), who administer and enforce national legislation and implement EU fish health directives. It is a requirement that commercial fish farmers register with the FHI and keep detailed records regarding fish movements including fish incomings, outgoings and mortalities records. So that in the event of a BKD outbreak (or the outbreak of any other notifiable disease) routes of infection can be traced and the further spread of the disease prevented (Department for

Food Enviroment and Rural Affairs, 2001). Clearly, the successful development and application of reliable typing systems could assist this process.

2.8 Molecular epidemiological typing

2.8.1 How typing systems can assist in understanding BKD

Despite the importance of BKD, the lack of practical and simple typing systems for *R. salmoninarum* have, until recently, constrained useful epizootiological studies (Bruno & Munro, 1986; Starliper, 1996). Recently, several molecular typing techniques for *R. salmoninarum* have been under development and there are now some promising methods including randomly amplified polymorphic DNA (RAPD) and 16S-23S rRNA intergenic spacer sequence analysis (Grayson *et al.*, 1999; Grayson *et al.*, 2000b).

The development of a number of different typing systems that can reliably differentiate between different isolates of *R. salmoninarum* could greatly aid studies of the epizootiology of BKD and allow key questions about the disease to be answered. Primarily, it would be possible to delineate patterns of transmission of one of more epidemic clones of *R. salmoninarum* enabling routes of infection (such as infected fish stocks or eggs from farms) to be conclusively established. BKD transmission between farmed and feral salmonid stocks could be investigated and measures aimed at reducing the spread of disease between the farming industry and pristine river systems could be introduced. Other issues that could be addressed include i) determining whether certain isolates of *R. salmoninarum* are more prevalent in disease situations, ii) whether particular isolates or clones are associated with a specific fish host, iii) whether fish hosts carry more than one isolate of *R. salmoninarum*, iv) how rapidly *R. salmoninarum* can spread and to determine the major vehicles and mechanisms of dispersal. Arguably of major importance is gaining a greater understanding of whether and how rapidly *R. salmoninarum* can evolve

and adapt to a changing environment, such as may result from antibiotic and chemical treatments and also altered farming and farming-related practices. In addition to gathering epizootiological information, typing techniques have the potential to provide phylogenetic and taxonomic information, clarifying genetic relationships, and leading to a better understanding of the population genetic structure of *R. salmoninarum* and, indeed, the epizootiology of BKD. Ultimately this information could be used to develop and implement effective strategies for the diagnosis, treatment, control and, possibly, the eradication of the disease.

2.8.3 Successful application of typing systems

A large number of studies have detailed the successful application of typing systems for establishing epidemiological routes and determining the transmission of bacterial infections. Typing methods are most commonly applied to answer specific questions, often being used to confirm or disprove a hypothesis. For example an investigation carried out by Cobben *et al.* (1996) examined 21 cases of pulmonary infection caused by *Pseudomonas aeruginosa* in patients with obstructive airways. Several possible sources of *P. aeruginosa* were established but the relationship between the bacterial isolates sourced from the patients and from contaminated nebulizers was confirmed by the application of several typing methods including serotyping, phage typing and arbitrary-primed PCR (AP-PCR). In this situation the application of typing methods resulted in the successful identification of the source of the infection as well as aiding control in the form of sterilisation of the contaminate nebulizers, which in turn led to the termination of the outbreak. Typing has also proved to be very important in tracing routes of infection in food poisoning cases. For example, in Singapore the application of pulsed field gel electrophoresis (PFGE), serotyping and phage typing were used to prove a link between *Salmonella enterica* that had infected 33 individuals and the consumption of imported

dried anchovy (Ling *et al.*, 2002). Typing methods have been successfully applied and epidemiological links confirmed in a wide range of other studies including tracing a case of *Staphylococcus aureus* food poisoning in 10 school children to the hand lesion of a specific food handler (Wei & Chiou, 2002). An increase in the number of infections due to a multiple antibiotic resistant strain of *Acinetobacter* was detected in a Birmingham hospital. This outbreak was solved by employing PFGE to analyse numerous isolates of the bacterium that were sourced from the hospital environment. The PFGE profiles revealed that dry fabrics, such as the curtains within the ward, were an important reservoir for the dissemination of this organism and rigorous cleaning was employed to eradicate this source of infection (Das *et al.*, 2002). However, in some epidemiological investigations typing methods have failed to identify the source of an outbreak strain. For example, an investigation carried out by Nishikawa *et al.* (2001), which used phage typing and PFGE analysis to examine isolates of enterohemorrhagic *Escherichia coli* O157 isolates from an apparently sporadic infections within Osaka city in Japan. This typing study revealed that 57% of isolates were of the same clone and were isolated from numerous patients over a three-week period. However, an epidemiological link to a possible common source was not established (Nishikawa *et al.*, 2001).

In addition to establishing routes of infection and contact tracing, bacteriological typing systems have also proved to be important in the management and diagnosis of a disease within a single patient. Typing systems are commonly used in patients with persistent infectious diseases to establish whether the infection is the result of relapse (due to the original isolate) or reinfection (infection with a new isolate). Examples include the utilisation of both immunoblotting and PFGE to examine several *Clostridium difficile* isolates sourced from ten-year-old patient on several occasions after four episodes of colitis. The application of these typing systems revealed that the second colitis episode was

a result of relapse from the first infection and that the third and fourth events were the result of reinfection by other isolates (Kato *et al.*, 1996). In a much large study, isolates of *Salmonella enterica* serovar Typhi, which had been sourced from 322 Vietnamese patients with typhoid fever were examined by PFGE and phage typing. This study found that the majority of the 5% of patients that suffered from subsequent bouts of typhoid fever was the result of relapse caused by the same isolate. However, in a minority of cases individuals were re-infected with a presumably newly acquired isolate (Wain *et al.*, 1999).

Other successful applications of typing methods have established whether bacterial infections are the result of either single or multiple isolates of the same species. A typing study carried out Slutsky *et al.* (1994), for example, employed PFGE to examine *Mycobacterium avium* isolates from AIDS patients and revealed that 14-20% of patients were infected by two distinctly different strains, concurrently (Slutsky *et al.*, 1994). However, a more recent study carried out by Picardeau *et al.* (1997) that employed both serotyping, IS*1245* RFLP, and in some cases PFGE to examine 196 isolates of *Mycobacterium avium* sourced from 93 French AIDS patients revealed that 95.2% of infections were monoclonal.

2.8.4 Principles and properties of a molecular typing system

The separation and accurate recognition of subtypes within a bacterial species and the use of subtype identification methods to determine sources of infection, rates of spread of infection and mechanisms of transmission is referred to as typing. Typing is based on the premise that an outbreak of an infectious disease is the result of exposure to a pathogen that is derived from a single cell or clone, the progeny of which will be genetically identical or closely related to the source organisms. Typing assumes that at the species level sufficient genetic diversity exists so that organisms isolated from different sources

and/or at different times and locations can be classified as a either a different isolate or strain (Olive & Bean, 1999). Where, the term 'isolate' is defined as a pure bacterial culture, presumed to be derived from a single cell for which no other information is necessarily available other than its genus and species (Tenover *et al.*, 1995). The term 'strain' is used to describe a well characterised isolate or group of isolates that can be distinguished from other isolates of the same genus or the same species by phenotypic and/or genotypic characteristics (Tenover *et al.*, 1995).

The typing of microorganisms can be achieved using a variety of different techniques, which must meet certain criteria. The techniques should be standardised and reproducible so that they can be carried out in any laboratory and give informative and comparable data, that is applicable to all isolates. The ideal typing system should be sufficiently discriminatory to distinguish a reasonable number of types amongst which isolates can be roughly equally distributed. A typing system should be able to differentiate unrelated strains such as those which are geographically distinct from the source organisms but at the same time demonstrate the relationships that exist between organisms isolated from individuals infected through the same source (Olive & Bean, 1999). Although typing systems should be discriminatory they should also be reproducible and stable, at least over the time frame of the investigation. The same result must be produced when a strain or isolate is repeatedly examined and the methods should be easy to execute and relatively inexpensive in order to allow for this possibility (Towner & Cockayne, 1994).

Of equal importance to the technique that is used is the process of interpreting the data that is obtained. The development of highly discriminatory typing methods, such as PFGE where the resolution is so high that the majority of the isolates of bacterial species can be shown to differ (Pestel-Caron *et al.*, 1999; Vadivelu *et al.*, 1997), illustrates the need for

the careful interpretation of such data. Questions such as "how different is different?" must be addressed and guidelines need to be established (Tenover *et al.*, 1995). In contrast, some epizootiological investigations have been complicated by the lack of clonality in some microbial populations because a high degree of genetic material is transferred horizontally (Yakubu *et al.*, 1999).

2.9 Traditional typing methods

2.9.1 Biochemical profiling of *R. salmoninarum*

R. salmoninarum isolated from widely separated sources has been shown to possess a remarkable degree of biochemical uniformity. A comprehensive taxonomic study of 44 isolates of *R. salmoninarum* and 12 other representatives of other actinomycete groups was undertaken in 1985 by Goodfellow *et al.* (1985). One hundred and four characters were assessed based on the results of an array of biochemical, morphological and growth tests. The 44 isolates of *R. salmoninarum* were found to be homogeneous, and the study justified the taxonomic placement of the organism. Remarkable similarities were also found in the biochemical properties of 26 recent isolates of *R. salmoninarum* from the UK and North America in a study carried out by Bruno & Munro (1986). A wide biochemical profile was established, including haemolytic and DNase activities, and total uniformity in all of the properties was recorded (Bruno & Munro, 1986). Isolate uniformity was also confirmed when the biochemical and immunochemical properties of the cell surface of *R. salmoninarum* was investigated by Fiedler & Draxl (1986). In a taxonomic study performed by Austin & Rodgers (1980) in which 25 gram-positive bacterial isolates sourced from the infected kidneys of salmonid fish from Europe and North America were subjected to morphological, biochemical and serological analysis, isolate diversity was apparent. One group of 6 strains was related to *Corynebacterium pyogenes*, a second group of 12 strains represented a new taxon, and 7 strains did not fall into either group. However,

the true identities of these isolates were never confirmed. Bruno (1988) studied the hydrophobicity of the cell surfaces of eight *R. salmoninarum* isolates using a salt aggregation method. A positive correlation between the virulence and the cell surface hydrophobicity of isolates was proposed suggesting that diversity does exist within the *R. salmoninarum* species.

Starliper *et al.* (1997) examined the virulence of 23 isolates of *R. salmoninarum* in 8 different salmonid hosts species and while host species varied in their susceptibility to BKD there was no evidence for isolate-related virulence differences. In contrast, a challenge study carried out by Dale *et al.* (1997) did demonstrate significant differences between isolates and the authors concluded that differences in virulence may reflect changed selective pressure on *R. salmoninarum* when introduced from feral stocks into the environment of fish farms. Thus, while there appears to be some evidence for the existence of pathogenic diversity among isolates of *R. salmoninarum* there is no information about whether this is reflected in the genetics of the bacterium. Consequently, biochemical profiling of *R. salmoninarum* appears to be of limited value in understanding the nature of any genetic variation which may exist and the absence of a minimal or defined medium makes further investigations of this type particularly difficult.

2.9.2 Serological profiling

Serological studies of *R. salmoninarum* have encompassed whole-cell serology (Bullock *et al.*, 1974), counter immunoelectrophoresis (Getchell *et al.*, 1985) and monoclonal and polyclonal antibody analysis of surface proteins (Wiens & Kaattari, 1989; Daly & Stevenson, 1990). These studies showed that isolates of *R.salmoninarum* from different sources are serologically identical.

2.10 Molecular typing techniques

2.10.1 Multilocus enzyme electrophoresis (MLEE)

One of the first investigations into the molecular diversity of *R. salmoninarum* used multilocus enzyme electrophoresis (MLEE) of whole cell lysates from 40 North American isolates (Starliper, 1996). MLEE is a method that examines the mobility of a selection of metabolic enzymes; variations in mobility are attributed to isozymes or allozymes due to differences in amino acid substitutions in the polypeptide sequences. MLEE is a technique that has been very useful for studying bacterial population genetics and has been used to show that some bacterial populations are predominantly clonal (e.g. *Salmonella*) whereas others (e.g. *Neisseria gonorrhoeae*) are panmictic (recombination between isolates is common). MLEE is more commonly used to examine long-term epizootiology on a global scale. In these applications the advantage of MLEE rests with its lack of discriminatory ability so that it does not detect minor differences between related strains (micro-evolution), which is required when examining local outbreaks of infection (Yakubu *et al.*, 1999). Interestingly, in the very comprehensive assessment by Starliper (1996) where 26 loci from 40 isolates were scored the two most diverse groups were found to come from isolates which had been sourced from different host species, coho and chinook salmon. Starliper (1996) concluded that there was a relatively low genetic diversity within *R. salmoninarum.*

2.10.2 Randomly amplified polymorphic DNA analysis (RAPD)

Typing methods that examine variation throughout the whole genome are often more discriminatory than techniques that examine variation at a specific locus, particularly in studies of highly conserved species. Randomly amplified polymorphic DNA (RAPD) is one such method that is a PCR-based technique employing a single, short oligonucleotide primer of arbitrary sequence that binds to any region in the genome bearing the

complementary sequence (Welsh & McClelland, 1990; Williams *et al.*, 1990). PCR amplifies complementary genomic DNA sequences and the products are separated and visualised by standard electrophoresis. RAPD has been applied to discriminate between isolates from a wide range of bacterial species including *Helicobacter pylori, Lactobacillus helveticus* and *Salmonella enterica* (Akopyanz *et al.*, 1992; Drake *et al.*, 1996; Hilton & Penn, 1998).

RAPD fingerprinting is an attractive typing technique in clinical microbiology and the method is applicable to any organism containing DNA. It is more rapid and less technically demanding that most other molecular typing methods and no prior DNA sequence information is required. RAPD also requires only a small amount of template DNA, (typically < 20 ng) which is a much smaller quantity than RFLP based typing techniques (which typically require 0.5μg - 1μg). This makes it ideal for the analysis of an organism like *R. salmoninarum* where sequencing data is limited and, because of the difficulty in culturing the organism, only small quantities of DNA are often available.

There have been two short reports of the application of RAPD to *R. salmoninarum.* Griffiths *et al.* (1998) used arbitrary primed or AP-PCR, a technique that is similar to RAPD, to distinguish between *R. salmoninarum* which had been isolated from vaccinated fish. The technique was applied to identify the presence of the vaccine strain and the challenge strain that were used in the experiment (Griffiths *et al.*, 1998). Berg *et al.* (1994) referred to the application of RAPD to studies of *R. salmoninarum* in a methods paper. They reported that testing 14 independent *R. salmoninarum* isolates with 18 RAPD primers only one isolate generated a different RAPD profile.

Two more comprehensive studies have been carried out examining the role of RAPD for discriminating between *R. salmoninarum* isolates. Grayson *et al.* (1999) used two different RAPD protocols and eight primers to differentiate between 19 isolates of *R. salmoninarum*. The methods proved to be highly reproducible and it was found that none of the isolates produced identical RAPD patterns with all eight primers. The study placed the isolates into three arbitrary groups; one consisted of Canadian, English, Scottish and two American isolates; a second group contained Icelandic isolates and a third group contained the remaining American isolates. A further RAPD study by Grayson *et al.* (2000b) examined an additional 41 isolates of *R. salmoninarum* and grouped 60 isolates into 21 banding patterns. However, 28 isolates from unrelated sources were indistinguishable. It was concluded that the clusters were not representative of biological source, although isolates sourced from areas of the world regarded as isolated from intensive mainstream salmonid culture showed genetic divergence and formed their own distinctive groups.

2.10.3 Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis uses restriction enzymes to digest genomic DNA and the resulting fragments are separated according to size by agarose gel electrophoresis. Because of the complexity of the resulting pattern the DNA is transferred to a membrane and probed by DNA hybridisation using a labelled probe, a process called Southern blotting. The probe hybridises with bands that possess the complementary DNA sequence so that only particular bands are visualised, simplifying the RFLP profile. Consequently, variations that are observed between the RFLPs of the DNA from different isolates using this technique reflect differences in the positions of restriction enzyme recognition sites. Often rRNA gene probes (section 2.10.3.1) or insertion sequences (2.10.3.2) have been used for this analysis. However, probes for other areas of the genome have been successfully developed including the development of a randomly cloned probe for the subtyping of *Legionella*

pneumophila and a probe for exotoxin A for the subtyping of *Pseudomonas aeruginosa* (Tran *et al.*, 1991; Loutit & Tompkins, 1991). In some studies multiple probes have been used (Grundmann *et al.*, 1995). It should be noted that whilst RFLP has been very successfully applied to the typing of bacterial species and strains, Southern blotting is relatively time consuming and expensive.

2.10.3.1 Ribotyping

Ribotyping is a form of RFLP analysis using a DNA probe based on the rRNA operon, usually part of the 16S or 23S rRNA genes. The use of rRNA probes for isolate discrimination was first reported by Grimont & Grimont (1986), and the appeal of this method is due to i) all known bacterial species possess at least one rRNA operon so all are typeable, ii) the highly conserved nature of this operon means that commercially available universal rRNA probes developed from *Escherichia coli* are available, iii) a large number of microbial species possess multiple rRNA operons which increases the chances that variation in this region between isolates will be observed, and iv) the hybridisation patterns that are generated are reproducible, stable and easy to interpret. There are some disadvantages of the technique including i) some microbial species particularly obligate pathogens, have only one or two copies of the rRNA operons thereby decreasing the chance that variation between isolates will be observed, and ii) only variation within or around the rRNA operon on the bacterial genome will be observed rather than variation throughout the whole genome (Grimont & Grimont, 1986). Despite the disadvantages, ribotyping is a widely applied technique that has been successfully applied to typing studies of a wide range of bacterial species including *P. aeruginosa*, *Yersinia enterocolitica*, and *Burkholderia pseudomallei* (Ferrus *et al.*, 1998; Lobato *et al.*, 1998; Trakulsomboon *et al.*, 1997; Stull *et al.*, 1988).

2.10.3.2 Insertion sequence analysis

Insertion sequence analysis is another RFLP-based technique that has been used widely and successfully for subtyping bacterial species. Insertion sequences are defined as "small phenotypically cryptic segments of DNA with a simple genetic organisation and capable of inserting at multiple sites in a target molecule" (Mahillon & Chandler, 1998). Insertion sequences have been shown to form an integral part of the chromosome of many bacterial species and are responsible for significant changes in the bacterial host's genome. Insertion sequences replicate and transpose independently of chromosome replication, as such insertion patterns are subject to continuous variation during the life span of a bacterial strain. Following integration of IS elements at defined places in the bacterial genome elements can be used as markers in RFLP analysis for subtyping bacterial species and epizootiological surveillance. Insertion sequences probes have been use to type a number of important bacterial species including IS*6110* in *M. tuberculosis*, IS*901* in *M. avium* complex, IS*200* in *Salmonella* and IS*1167* in *Streptococcus pneumoniae* (Stanley & Saunders, 1996; Luna *et al.*, 2000).

The first and only known IS in the *R. salmoninarum* genome was only very recently identified when Rhodes *et al.* (2000) presented details of IS*994*, a member of the group IS*51* and of the IS*3* family of insertion sequences. It was found that IS*994* shared substantial sequence homology with the two open reading frames in IS*6110*, an insertion sequence of *M. tuberculosis*. It was conservatively estimated that this insertion sequence had integrated with the *R. salmoninarum* genome at least 17 times. Initially, a small RFLP study was undertaken using 8 isolates of *R. salmoninarum* and several restriction enzymes but the hybridisation patterns exhibited little variation between isolates (Rhodes & Strom, 1998). However, during this study only a few isolates were screened, and the true potential

of this insertion sequence as an epizootiological marker for *R. salmoninarum* has yet to be fully explored.

2.10.4 rDNA intergenic spacer (ITS) sequence analysis

The genes composing a typical bacterial ribosomal rRNA operon are usually arranged in a characteristic formation, 16S, 23S and 5S. These genes are separated by intergenic spacer regions (termed the 16S-23S rRNA ITS and the 23S-5S rRNA ITS region) which are known to be more variable in both size and nucleotide sequence than the flanking structural genes. Sequences that have been determined for the highly conserved structural genes, particularly the 16S rRNA can be used for phylogenetic and taxonomic purposes. Sequences that have been obtained for the ITS regions have been shown to be variable and are often used to distinguish between closely related bacterial species and, sometimes, between strains and isolates (Gurtler & Stanisich, 1996; Jensen *et al.*, 1993). The potential of the ITS regions to act as tools for studying molecular systematics and also for subtyping bacterial species is substantial, but the presence of multiple non-identical rRNA operons poses problems. Although most organisms have multiple copies of rRNA genes and sequence uniformity is usual, there are notable exceptions among prokaryotes where at least three exceptions have been reported so far, the archaeon *Haloarcula marismortui* (Dennis *et al.*, 1998) and the actinomycetes *Thermobispora bispora* and *Thermomonospora chromogena* (Wang *et al.*, 1997; Yap *et al.*, 1999). Nevertheless, the extent of these differences remains small, from 4-5% variation between operons.

Heterogeneity in the nucleotide sequence of the 16S-23S rRNA ITS region (ITS1) has formed the basis for the development of identification and typing systems in a wide range of bacteria. In addition to the ITS1 region, sequence variation in the 23S-5S rRNA intergenic spacer regions (ITS2) has been examined in only a few studies. This spacer is

smaller in length than ITS1, and consequently less variation is observed between bacterial isolates. Despite this, in a few cases ITS2 has been used to identify subtypes of a bacterial species and there is evidence that the region may prove useful for designing species-specific probes and establishing the relationships between closely related species (Yoon *et al.*, 1997).

Examination of the 16S-23S rRNA ITS region of *R. salmoninarum* has been carried out by Grayson *et al.* (1999, 2000b). A PCR based analysis of ITS1 in 74 isolates of *R. salmoninarum* that were sourced from mainland United States, Alaska, Canada, Sweden, England, Scotland, Norway and a range of fish hosts found evidence of only a single ITS1 region, which was of a uniform size in all of the isolates. Sequence analysis of the PCR products from 17 isolates was performed revealing the presence of four sequence variants (SV) which differed by a maximum of three nucleotide substitutions. Ten of the isolates that were examined had the same nucleotide sequence, designated sequevar 1 (SV1). Four isolates, three sourced from Iceland and one from Japan, were found to possess SV2 and a single isolate from the Northwest Territories of Canada possessed SV3. Interestingly, three other isolates, two from Scotland and one from Norway, possessed a fourth sequence variation termed SV4. Although 16S-23S rRNA spacer sequence analysis of *R. salmoninarum* is of limited use for typing purposes the analysis of this region can offer clues to the geographic origins of some isolates.

2.10.5 tDNA-PCR analysis

Examining length polymorphisms in the spacer regions that separate tRNA genes is a PCR-based strategy that has been used to explore the degree of relatedness between different taxa. The arrangement of tRNA gene clusters in multiple tandem-repeat units on the bacterial genome (Green & Vold, 1993; Vold, 1985) allows the amplification of

intergenic length polymorphisms (tDNA-ILPs) by PCR employing consensus primers that are annealed at low stringency. Welsh and McClelland developed four 'universal' tRNA gene primers from multiple alignments of tRNA genes from a wide variety of sources. The primers were designed to face outwards from the end of the tRNA genes in order to amplify a tDNA-ILP fingerprint that is determined by the arrangement of tRNA genes on the bacterial genome. Often the order and arrangement of tRNA genes is highly conserved on the genome and consequently the patterns that are generated using consensus primers are often characteristic of a particular species and can be identical or nearly identical within every species (Welsh & McClelland, 1991; Welsh & McClelland, 1992). tDNA-PCR fingerprinting has been shown to reliably differentiate between bacterial species including *Acinetobacter, Entercococcus spp., Listeria spp.*, and *Staphylococcus* sp. (Ehrenstein *et al.*, 1996; Baele *et al.*, 2000; Vaneechoutte *et al.*, 1998; Maes *et al.*, 1997). Furthermore, specific tRNA gene primers can be developed from the DNA sequences of the PCR products that are amplified by consensus primers and this approach was used to distinguish between streptococci on the basis of tRNA gene spacer length polymorphisms (Honeycutt *et al.*, 1995).

However, whilst the arrangement of tRNA genes on bacterial genomes is highly conserved the non-coding spacer regions between the genes can vary in length and nucleotide sequence. Interestingly, tRNA genes and the flanking DNA in a wide range of bacteria have been reported to be prone to disruption and insertion by a wide range of genetic elements including insertion sequences, tandem repeats, pathogenicity islands and phage and plasmid DNA (Campbell, 1992; Cole *et al.*, 1998; Dundon *et al.*, 1999; Folkesson *et al.*, 1999; Kunze *et al.*, 1991; Moss *et al.*, 1999). Consequently, tDNA-PCR has been used to discriminate between sub-species and, in some cases, isolates. Consensus tRNA primers have been used to generate divisions below species level in studies of a variety of bacteria

including *Bacteroides ovatus*, *Bacillus licheniformis*, *Bilophila wadsworthia* and *Pseudomonas solanacearum*. In each of these studies, tDNA-PCR products were visualised on standard agarose gels and provided a means of separating isolates into two or more sub-groups (Daffonchio *et al.*, 1998; Claros *et al.*, 1997; Seal *et al.*, 1992).

2.10.6 Pulsed field gel electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE) is an extremely sensitive and sophisticated method that has been widely employed for the purpose of subtyping bacterial pathogens. The method uses intact genomic DNA, which is digested with infrequently cutting restriction enzymes and separated in agarose using specialist equipment that allows the separation of large DNA fragments between 50kb and 10Mb in size. In practice, this involves the use of high-purity chemical reagents and is achieved by embedding whole bacteria in agarose plugs, lysing them *in situ*, removing the proteins and digesting the intact genomic DNA (Smith & Cantor, 1987; Stern *et al.*, 1984; Schwartz & Cantor, 1984). Differences may be observed between the length of the restriction fragments in different isolates and these arise as a result of differences in the restriction sites that may be caused by point mutations (resulting in a creation or removal of a restriction site), and insertions or deletions of DNA which can alter either the sizes or the numbers of restricted fragments (Tenover *et al.*, 1995).

PFGE is widely regarded as one of the most powerful methods for typing bacteria that are currently available. It is a widely used technique that has been successfully applied to solve some of the most difficult typing questions. Epizootiological investigations into *Staphylococcus aureus* isolates using PFGE generally involves digestion of the DNA with the restriction enzyme *Sma*I. Although the technique has greatly aided epizootiological knowledge, there have been problems with standardisation and reproducibility (Goering &

Duensing, 1990; van Belkum *et al.*, 1998c). In a comparative study of typing techniques, 30 isolates of *Clostridium difficile* were separated into 26 types by PFGE (Chachaty *et al.*, 1994). PFGE also plays an important role in the subtyping of many other bacterial species including *Salmonella typhimurium*, *Vibrio cholerae*, *Listeria monocytogenes* and members of the *Brucella* and Enterococcal genera (Tsen *et al.*, 2000; Arakawa *et al.*, 2000). The widespread use of PFGE has been hampered by the length of time that is required to complete the analysis and the cost of equipment, labour and reagents. Although the procedure is complex, the data that can be obtained is highly reproducible and easy to interpret. Unlike PCR-based methods, such as RAPD, where banding patterns can be ambiguous and band intensities confusing, PFGE analysis can be readily interpreted by computerised gel scanning and analysis software (Olive & Bean, 1999). Indeed, reference databases are now available over the internet for a variety of important bacterial pathogens enabling research groups around the world to share data http://www.cdc.gov/ncidod/dbmd/pulsenet/pulsenet.htm (Food and Drug Administration, 1997).

In addition to providing an extremely valuable tool for subtyping bacteria PFGE has proved to be a great aid in the field of genome research. Due to the capacity of this technique to separate large DNA fragments PFGE has been applied to the identification of multiple chromosomes, linear chromosomes and, indeed, large plasmids in many bacteria. PFGE has been used to determine the genome size of a wide range of bacteria including *Mycoplasma genitalium,* which possesses the smallest known genome size, 0.6Mb, and *Myxococcus xanthus* which has the largest known genome at 9.5Mb (Chen *et al.*, 1991; Colman *et al.*, 1990). In addition, PFGE has been successfully applied in studies designed to unravel the organisation of bacterial genomes in order to create comprehensive genomic maps (Fonstein & Haselkorn, 1995). Consequently, physical genome maps now exist for a

variety of bacterial species including *Streptococcus pneumoniae*, *Bacillus thuringiensis* and *Neisseria gonorrhoeae* (Gasc *et al.*, 1991; Carlson & Kolsto, 1993; Dempsey *et al.*, 1991).

When using a technique as sensitive as PFGE the interpretation of the restriction fragment patterns must take into account the random nature of the genetic changes that can occur, albeit infrequently, such as point mutations, insertions, mutations and deletions, because these could generate slightly different fingerprints between two isolates of the same strain (Maule, 1998). In order to overcome problems of establishing degrees of 'relatedness', Tenover *et al.* (1995) proposed a system for standardising PFGE patterns. They proposed that isolates which generated identical patterns were to be considered the same strain whereas isolates that differed by a single genetic event, representing a difference of one to three restriction fragment bands, should be considered as closely related. When isolates differ by four to six bands, and consequently have two independent genetic changes, they are considered to share a possible relationship, and isolates differing by more than six restriction fragment bands are considered to be unrelated. These criteria have been adopted as a general guide for estimating the relationship between isolates in a number of PFGE studies but problems are sometimes encountered e.g. in a PFGE study of *Enterococcus faecalis* using a *Sma*I digest seven different restriction fragments were generated in isolates that were closely related due to the insertion of a transposon (Thal *et al.*, 1997).

2.10.7 PCR analysis of repetitive prokaryotic regions

Repetitive element sequence-based PCR (rep-PCR) refers to a number of subtyping methods that use oligonucleotide primers specific to short repetitive sequence elements, which are dispersed throughout the bacterial genome. Some forms of rep-PCR include

repetitive extragenic palindromic (REP-PCR), enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), and BOX-PCR.

2.10.7.1 Repetitive extragenic palindromic (REP)-PCR

Repetitive extragenic palindromic (REP) sequences are repetitive elements that appear to be distributed throughout prokaryotic genomes. The REP sequence was the first repetitive sequence in the prokarotyic genome to be characterised and was originally identified from sequence information from the chromosomes of *Escherichia coli* and *Salmonella typhimurium.* The REP sequence consists of a conserved 33bp inverted repeat and it is thought that approximately 500 copies are present in the genome of *Escherichia coli* and *Salmonella typhimurium* (Stern *et al.*, 1984). The precise role of the REP sequence in the prokaryotic genome is currently unknown although the sequences have been shown to stabilise upstream mRNA and influence gene expression (Newbury & Higgins, 1987; Newbury *et al.*, 1987), affect transitional coupling between genes (Stern *et al.*, 1988), terminate transcription (Gilson *et al.*, 1986) and serve as a region that facilitates recombination (Shyamala *et al.*, 1990).

Versalovic *et al.* (1991) designed consensus opposing PCR primers complementary to the REP sequence in order to amplify sequences between adjacent repetitive extragenic elements (Versalovic *et al.*, 1993). This method highlights differences in band sizes between different bacterial isolates, which represent length polymorphisms between repetitive elements of the genomes. This technique, termed REP-PCR, has been use in subtyping to generate strain-specific DNA fingerprints that can easily be visualised by agarose gel electrophoresis. REP-PCR is very simple to perform and has been used successfully to subtype both gram-negative and gram-positive bacterial species including

Xanthomonas fragariae and *Actinobacillus seminis* (Pooler *et al.*, 1996; Appuhamy *et al.*, 1998),

2.10.7.2 Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR)

ERIC sequences are a novel family of repetitive elements that were first identified in the genomes of *E. coli* and *Salmonella typhimurium*. The sequence is 126bp long and has been shown to be highly conserved containing an inverted repeat sequence which is thought to generate a stem and loop structure when transcribed into RNA. ERICs are restricted to transcribed regions of the genome, either in the intergenic regions of polycistronic operons or in the untranslated regions that lie upstream or downstream of open reading frames (de Bruijn, 1992). Although numerous functions have been proposed for ERIC sequence no single function has yet emerged which can satisfactorily and fully explain the distribution and high degree of sequence conservation of the ERIC element (Hulton *et al.*, 1991).

Hulton *et al.* (1991) estimated that there are at least 30 copies of this element on the *E. coli* genome and approximately 150 copies on the genome of *S. typhimurium*. Versalovic *et al.* (1991), designed consensus opposing PCR primers complementary to the ERIC sequence in order to amplify the intervening sequences, and although ERIC sequences are highly conserved, their chromsomal locations are known to vary between different bacterial species, strains and even isolates making ERIC-PCR ideal for molecular subtyping. Even though ERIC sequences were initially discovered and characterised in gram-negative bacteria (de Bruijn, 1992) they have been used to subtype several methicillin-resistant *S. aureus* strains (van Belkum *et al.*, 1998c). Since then ERIC-PCR has been applied to studies of many bacterial species including *Haemophilus influenzae* (Gomez-De-Leon *et al.*, 2000), *Mycobacterium tuberculosis* (Sechi *et al.*, 1998) and *Vibrio cholerae* (Gomez-De-Leon *et al.*, 2000; Rivera *et al.*, 1995).

2.10.7.3 BOX-PCR

The BOX element was first identified in *Streptococcus pneumoniae* and is an interspersed repetitive DNA sequence that is found throughout the bacterial genome. BOX elements have been shown to consist of different combinations of three subunits termed boxA, boxB, and boxC. Sequence analysis revealed that boxA was highly conserved in the bacterial kingdom, whilst boxB and boxC were highly variable and found only within *S. pneumoniae.* Consequently, primers that can be used in subtyping many bacteria have been designed to the boxA sequences and PCR reactions usually employ a single box primer to amplify these regions (Koeuth *et al.*, 1995).

BOX-PCR has been used to differentiate between human and non-human isolates of *E. coli* (Dombek *et al.*, 2000), and closely related *Streptomyces* strains which were indistinguishable by other classification methods (Appuhamy *et al.*, 1998; Sadowsky *et al.*, 1996). The method has been used as a polyphasic tool in the identification of different taxa of the genus *Bifidobacterium* (Gomez Zavaglia *et al.*, 2000) and as a highly discriminatory typing method in *Streptococcus pneumoniae* (Hermans *et al.*, 1995).

2.10.8 Variable number tandem repeat

Variable number tandem repeats are nucleotide sequences that occur in specific regions of a genome where there are variations in the number of repeat units (VNTR loci) (van Belkum, 1999). The number of repeat units that occur at a locus can vary from strain to strain due to slipped strand mispairing, where sequence identity between neighbouring pairs of repeat units results in errors in based pairing lead to the introduction or deletion of a whole repeat region. VNTR analyses use PCR primers complementary to stable flanking

sequences and polymorphisms in the numbers of repeat units are determined by agarose gel electrophoresis (van Belkum, 1999; van Belkum *et al.*, 1998b).

VNTR's are very important for examining relatedness within *M. tuberculosis* isolates and are particularly useful for typing isolates which have a low number of IS*6110* insertions and can not be typed by IS analysis (Frothingham & Meeker-O'Connell, 1998; Barlow *et al.*, 2001). Recently, a tetranucleotide repeat sequence was identified in genome of *Yersinia pestis*, the causative agent of the plague, which occurs at nine alleles on the genome and has great diversity within the 35 strains that were examined. It is hoped that this VNTR marker will provide a high-resolution tool for epizootiological analysis (Adair *et al.*, 2000). Anther important pathogen where VNTR loci have proved to be a useful epizootiological tool is *Bacillus anthracis.* VNTR loci are also important in eukaryotic typing scenarios, most famously they are used to determine human relatedness in cases of paternity testing and for forensics evidence (van Belkum, 1999).

Unlike ERIC-PCR, BOX-PCR and REP-PCR, VNTR sequences are sequence specific for each bacterial species and may occur at only a single locus. Whilst VNTRs have proven to be very valuable in many applications the technique has yet to be applied to many bacterial species due to the paucity of available sequences information. However, with the recent dramatic expansion in the numbers of partial and complete microbial sequences that are available on public databases it is likely that VNTR will be of increasing importance in bacterial typing.

Chapter Three

GENERAL MATERIALS AND METHODS

3.1 Chemicals and reagents

Unless otherwise stated all general laboratory chemicals were purchased from Sigma, Oxoid, Roche, BDH, Gibco BRL or ICN and, if possible, were of molecular biology grade. PCR-related reagents were obtained from Roche or MBI Fermentas. Recipes for all of the buffers that were used and the suppliers' details are in outlined in Appendix 1. Details of specific reagents can be found in the Materials and Methods section of individual chapters.

3.2 Bacteriology

3.2.1 Isolates of *Renibacterium salmoninarum*

The designation codes of the *R. salmoninarum* isolates used in this study and their countries of origin and sources of isolation are listed in Table 3.1. Unless otherwise stated the isolates were held as freeze-dried stocks in the University of Plymouth culture collection, Department of Biological Sciences. In-depth analysis was performed on 43 of the UK isolates for which further details, if known, are outlined in Table 3.2 and included one or more of the following techniques: randomly amplified polymorphic DNA (RAPD), tRNA intergenic length polymorphism analysis (tRNA-ILP), insertion sequence IS*994* restriction fragment length polymorphism (RFLP), pulsed field gel electrophoresis (PFGE), ribotyping, 16S-23S and 23S-5S rRNA intergenic spacer (ITS) sequence analyses and repetitive element sequence-based PCR (REP-PCR).

3.2.2 Culture of *R. salmoninarum*

All *R. salmoninarum* isolates were recovered from freeze-dried stocks and were cultured for 6-8 weeks at 15°C in 20ml volumes of selective kidney disease medium (SKDM) broth

with shaking (70 rpm). SKDM media was prepared as stated in Appendix I.4. Numerous *R. salmoninarum* cultures were received by workers at the University of Plymouth prior to and during the course of this project. Purity was confirmed by the visual inspection of cultures spread onto SKDM agar following long growth periods (over six weeks) and gram staining.

3.2.3 Additional bacterial species

Bacterial strains other than *R. salmoninarum* that were used in this study are listed in Table 3.3.

3.2.4 Culture of additional bacterial strains

The media on which each of the additional bacterial strains were grown is stated in Table 3.3. and the formulation is detailed in Appendix I.1. Cultures that were grown on tryptone soya agar or tryptone soya broth (TSA or TSB) were incubated at 25°C for 3 days. *Escherichia coli* was grown on Luria-Bertani (LB) medium overnight at 37°C.

3.3 Genomic DNA extraction

Genomic DNA was isolated from *R. salmoninarum* cultures using a 'Puregene D-6000 DNA isolation kit for gram-positive bacteria' according to the instructions of the manufacturer (Gentra Systems Inc., 'DNA Isolation Kit Instruction Manual', pages 57-58). The contents of all solutions that were used in this procedure can be found in Appendix I.1. *R. salmoninarum* broth culture was suspended fully and transferred to a 50ml centrifugation tube on ice. The culture was centrifuged (1000 x g, 10min, 4°C) and the cell pellet was resuspended in 6ml of cell suspension solution with gentle pipetting. In the case of gram-positive bacteria, such as *R. salmoninarum,* 30µl of lytic enzyme solution was added to the cell suspension and the tube was inverted 25 times and placed at 37°C for 30min to digest cell walls. The suspension was then centrifuged (2000 x g, 10min, 4°C),

the supernatant was discarded and 6ml of cell lysis solution was added and the mixture was pipetted up and down to facilitate cell lysis. A 30µl volume of RNase A solution was added, the tube was inverted 25 times and placed at 37°C for 1 hour. Following this, the sample was cooled to room temperature, 2ml of protein precipitation solution was added and the tube was vortexed vigorously at high speed for 20 seconds to mix the protein precipitation solution uniformly with the cell lysate. The sample was centrifuged (2000 x g, 10min, 4°C), the supernatant containing the DNA was then transferred to a fresh 1.5ml microfuge, the volume was measured and the DNA was precipitated by adding an equal volume of isopropanol. The sample was mixed by 50 inversions of the tube, centrifuged (2000 x g, 3min, 4°C), the supernatant was discarded and the DNA pellet was washed with 70% ethanol, dried at 37°C, resuspended in 50µl-100µl of TE buffer and stored at 4°C.

The above procedure was also used to extract genomic DNA from all of the additional bacterial strains that were used in this study but with the following modifications. For species other than *R. salmoninarum* the DNA was extracted using 5ml broth cultures and only half of the stated reagent volumes were used. For gram-negative bacteria, the lytic enzyme stage was omitted and, instead, bacterial cells were placed at 80°C for 5min.

3.4 DNA quantification

The quality and quantity of genomic DNA was established by agarose gel electrophoresis (section 3.6). Images of each gel were captured with a Gel Documentation system (Uvi-Tech, UK), and the DNA concentration was determined for each isolate by comparison with a range of the following known amounts of λ DNA standards: 0.500µg, 0.250µg, 0.125µg and 0.063µg and using 'U.V. Photo' software (UVi-tech).

Table 3.1: Description of the *R. salmoninarum* isolates that were used in this study.

Isolate	Geographic origin	Biological source[A]	Isolate	Geographic origin	Biological source[A]
980036-150	Wales	Rainbow trout(f)	MT1880	Scotland	Atlantic salmon (f)
980036-87	Wales	Rainbow trout (f)	MT2118	Scotland	Atlantic salmon (f)
980297#97	England	Rainbow trout (f)	MT2119	Scotland	Atlantic salmon (f)
980109F3	England	Rainbow trout (f)	DR143[SP1]	Alberta, Canada	Brook trout (f)
980109F47	England	Rainbow trout (f)	DR384	British Columbia, Canada	Coho salmon (f)
980109F60	England	Rainbow trout (f)	3784	British Columbia, Canada	Sockeye salmon (f)
980109F82	England	Rainbow trout (f)	980002	British Columbia, Canada	Chinook salmon (f)
980109F85	England	Rainbow trout (f)	960023	British Columbia, Canada	Coho salmon (f)
980109F95	England	Rainbow trout (f)	960046	British Columbia, Canada	Coho salmon (f)
970083-88	Southern England	Rainbow trout (f)	GC96-1	British Columbia, Canada	Sockeye salmon (w)
970083-102	Southern England	Rainbow trout (f)	DR-128	British Columbia, Canada	Rainbow trout (w)
980106 #1.1.5[SP1]	Southern England	Rainbow trout (f)	RS -TSA	Nova Scotia, Canada	Atlantic salmon (f)
970419-1.2.3[SP1]	Southern England	Atlantic salmon (w)	AcF6-1[SP3]	North West Territory's, Canada	Arctic char (w)
970153-19[SP1]	Southern England	Grayling (w)	F-120-87 (P-2)	Iceland	Atlantic salmon (f)
A6	Southern England	Rainbow trout (f)	F-130-87 (P-4)	Iceland	Rainbow trout (f)
A80	Southern England	Rainbow trout (f)	F-138-87 (0-78)	Iceland	Atlantic salmon (f)
W2	Northern England	Rainbow trout (f)	F-260-87 (P-16)	Iceland	Atlantic salmon (f)
W6[SP1]	Northern England	Rainbow trout (f)	F-273-87 (P-19)	Iceland	Atlantic salmon (f)
WMV1	Southern England	Rainbow trout (f)	F-283-87 (P-10)[SP2]	Iceland	Atlantic salmon (f)
MT409	Scotland	Unknown	F-358-87 (P-13)[SP2]	Iceland	Atlantic salmon (w)
MT417[SP1]	Scotland	Atlantic salmon (f)	S-182-90 (P-7)[SP2]	Iceland	Atlantic salmon (f)
MT239	Scotland	Atlantic salmon (f)	Siletz	Oregon, USA	Coho salmon (f)
MT426	Scotland	Unknown	Marion Forks[SP1]	Oregon, USA	Chinook salmon(f)
NCIMB1111	Scotland	Atlantic salmon(w)	Little Goose	Washington, USA	Chinook salmon(f)
NCIMB1112	Scotland	Atlantic salmon(w)	CCM6205	Washington, USA	Coho salmon(f)
NCIMB1113	Scotland	Atlantic salmon(w)	84-019-OC	Washington, USA	Chinook salmon(w)
NCIMB1114[SP1]	Scotland	Atlantic salmon(w)	SS-ChS-94-1	Oregon, USA	Chinook salmon
NCIMB1115	Scotland	Atlantic salmon(w)	Cow ChS94 P22	Washington, USA	Chinook salmon(f)
NCIMB1116[SP1]	Scotland	Atlantic salmon(w)	Idaho 91-126	Idaha, USA	Sockeye salmon(f)
2119#5	England	Rainbow trout (f)	RFL-643.94 #1	Washington, USA	Sockeye salmon(f)
3258#15	England	Rainbow trout (f)	CCM6206	Oregon, USA	Chinook salmon(f)
3506W134	England	Rainbow trout (f)	Round Butte	Oregon, USA	Chinook salmon(f)
3506W132	England	Rainbow trout (f)	NCIMB2196	Wyoming., USA	Brook trout(f)
MT420	Scotland	Atlantic salmon(f)	ATCC33209[SP1]	Oregon, USA	Chinook salmon(f)

			(NCIMB2235)		
MT452	Scotland	Rainbow trout(f)	D-6	Oregon, USA	Coho salmon
MT1363	Scotland	Rainbow trout(f)	Cole River	Oregon, USA	Unknown
MT410	Scotland	Unknown	Looking Glass	Oregon, USA	Unknown
FT-10	Scotland	Atlantic salmon	BY1996[SV1]	Alaska, USA	Chinook salmon(f)
BA99	Scotland	Atlantic salmon	RS9[SV1]	Sweden	Rainbow trout
MT419	Scotland	Atlantic salmon(f)	RS19	Sweden	Atlantic salmon
MT425	Scotland	Rainbow tout(f)	RS61	Sweden	Arctic char
MT444	Scotland	Atlantic salmon(f)	RS116	Sweden	Grayling
MT839	Scotland	Atlantic salmon(f)	RS122	Sweden	Rainbow trout(f)
MT1261	Scotland	Atlantic salmon(f)	RS125	Sweden	Rainbow trout
MT1351	Scotland	Atlantic salmon(f)	RS126	Sweden	Rainbow trout
MT1469	Scotland	Rainbow trout(f)	3015-86[SV1]	Norway	Atlantic salmon
MT1470	Scotland	Rainbow trout(f)	4451-86[SV1]	Norway	Atlantic salmon
MT1511	Scotland	Rainbow trout(f)	Iwate[SV2]	Japan	Coho salmon
MT1770	Scotland	Atlantic salmon(f)			

[A] Isolates were obtained from wild fish (w) or farm (captive reared) fish (f) sources. The complete histories of some isolates are unknown.

[SV1] Isolates for which the 16S-23S rRNA intergenic spacer region (ITS1) was previously sequenced and are designated sequevar 1 isolates (Grayson *et al.*, 1999).

[SV2] ITS1 sequevar 2 isolates (Grayson *et al.*, 1999).

[SV3] ITS1 sequevar 3 isolates (Grayson *et al.*, 1999).

[SV4] ITS1 sequevar 4 isolates(Grayson *et al.*, 2000b).

Table 3.2: *R. salmoninarum* isolates of UK origin which were subject to additional typing analysis.

Number	Isolate	Geographic Origin	Biological source	Details of isolation
1	970083-88	Southern England	Rainbow trout(f)	Farm A, tank A (1997)
2	970083-102	Southern England	Rainbow trout(f)	Farm A, tank A (1997)
3	980106 #1.1.5^{SVI}	Southern England	Rainbow trout(f)	Farm B raceway (1998)
4	980036-150	Wales	Rainbow trout(f)	Farm C, pond (1998)
5	980036-87	Wales	Rainbow trout(f)	Farm C, raceway (1998)
6	970419-1.2.3	Southern England	Atlantic salmon(w)	Unknown (1997)
7	970153-19 ^{SVI}	Southern England	Grayling(w)	Unknown (1997)
8	A6	Southern England	Rainbow trout(f)	Farm D (1998)
9	A80	Southern England	Rainbow trout(f)	Farm D (1998)
10	980297#97	England	Rainbow trout(f)	Hatchery E, raceway (1998)
11	980109F95	England	Rainbow trout(f)	Farm A, tank B (1998)
12	980109F85	England	Rainbow trout(f)	Farm A, tank B (1998)
13	980109F82	England	Rainbow trout(f)	Farm A, tank C (1998)
14	980109F60	England	Rainbow trout(f)	Farm A, tank D (1998)
15	980109F47	England	Rainbow trout(f)	Farm A, tank E (1998)
16	980109F3	England	Rainbow trout(f)	Farm A, raceway (1998)
17	NCIMB1111	Scotland	Atlantic salmon(w)	River Dee (1962)
18	NCIMB1112	Scotland	Atlantic salmon(w)	River Dee (1962)
19	NCIMB1113	Scotland	Atlantic salmon(w)	River Dee (1962)
20	NCIMB1114^{SV4}	Scotland	Atlantic salmon(w)	River Dee (1962)
21	NCIMB1115	Scotland	Atlantic salmon(w)	River Dee (1962)
22	NCIMB1116^{SV4}	Scotland	Atlantic salmon(w)	River Dee (1962)
23	3258#15	England	Rainbow trout (f)	Farm F (2000)
24	3506W134	England	Rainbow trout (f)	Farm G (2000)
25	3506W132	England	Rainbow trout (f)	Farm G (2000)
26	2119#5	England	Rainbow trout (f)	Farm H (2000)
27	MT239	Scotland, Strathclyde	Atlantic salmon (f)	Farmed, (20/2/86)
28	MT419	Scotland	Atlantic salmon(f)	Farmed, freshwater (3/1/88)
29	MT425	Scotland	Rainbow trout (f)	Unknown Farm (7/3/88)

30	MT426	Scotland	Rainbow trout (f)	Unknown Farm (7/3/88)
31	MT444	Scotland, Western Isles	Atlantic salmon (f)	Farmed, Seawater (20/5/88)
32	MT452	Scotland, Dumfries	Rainbow trout (f)	Farmed, Freshwater (30/5/88)
33	MT839	Scotland, Rosshire	Atlantic salmon (f)	Farmed, freshwater (31/1/90)
34	MT861	Scotland	Atlantic salmon (f)	Farmed, Freshwater (25/4/90)
35	MT1261	Scotland, Sutherland	Atlantic salmon (f)	Farm, freshwater (8/5/92)
36	MT1351	Scotland, Ullapool	Atlantic salmon (f)	Farmed, Seawater (10/6/93)
37	MT1469	Scotland, Perthshire	Rainbow trout (f)	Farmed, Freshwater (31/5/94)
38	MT1470	Scotland, Perthshire	Rainbow Trout (f)	Farmed, Freshwater (26/5/94)
39	MT1511	Scotland, Strathclyde	Rainbow Trout (f)	Farmed, freshwater (13/5/94)
40	MT1770	Scotland, Highland	Atlantic salmon (f)	Farmed, Seawater (3/4/96)
41	MT1880	Scotland, Strathclyde	Atlantic salmon (f)	Farmed, Seawater (2/11/96)
42	MT2118	Scotland, Strathclyde	Atlantic salmon (f)	Farmed, Seawater (3/7/98)
43	MT2119	Scotland, Strathclyde	Rainbow trout (f)	Farmed, Freshwater (24/7/98)

[A] Isolates were obtained from wild fish (w) or farm (captive reared) fish (f) sources. The complete histories of some isolates are unknown.

[SV1] Isolates for which the 16S-23S rRNA intergenic spacer region (ITS1) was previously sequenced and are designated sequevar 1 isolates (Grayson *et al.*, 1999).

[SV4] The 16S-23S rRNA ITS (ITS1) region has previously been determined for these isolates (sequevar 4) (Grayson *et al.*, 2000b).

Table 3.3: Description of additional bacterial strains that were used in this study.

Strain	Code	Growth Media[a]
Aeromonas hydrophila[1]	NCIMB 1134	TSB
Aeromonas salmonicida[1]	CM30	TSB
Yersinia ruckeri[1]	13/86	TSB
Pseudomonas fluorescens[1]	NCIMB 1953	TSB
Pseudomonas anguilliseptica[1]	NCIMB 1949	TSB
Rhodococcus equi[1]	NCTC 1621	TSB
Brevibacterium linens[1]	NCIMB 8546	TSB
Micrococcus luteus[1]	NCTC 7563	TSB
Streptomyces griseus[1]	NCIMB 8136	TSB
Nocardia cellulans[1]	NCIMB 8868	TSB
Arthrobacter Sp.[2]	ZAT 012	TSB
Arthrobacter globiformis[1]	NCIMB 10267	TSB
Arthrobacter polychromogenes[1]	NCIMB 8907	TSB
Arthrobacter Sp.[3]	S23H2	TSB
Arthrobacter Sp.[2]	ZAT 277	TSB
Escherichia coli[1]	DH5α	LB

[a] Organisms were grown on either tryptone soya broth/agar (TSB/TSA) or Luria-Bertani broth (Appendix I.3).

[1] These organisms are part of the University of Plymouth culture collection and were recovered from liquid nitrogen.

2 These strains were kindly provided by from the Professor David Balkwill from the Nuclear Research program, Tallahassee on TSA agar slopes. The organisms were subcultured on TSA and placed at -80°C in a glycerol stock.

[3] This strain was kindly provided by Professor James T. Staley from the University of Washington on TSA agar slops. The organism was then subcultured on TSA and placed at -80°C in a glycerol stock.

3.5 Polymerase chain reaction (PCR)

PCR reactions were performed with a DNA thermal cycler (TC1, Perkin-Elmer; MWG Primus 96 Plus, MWG Biotech; or Cyclogene Thermal Cycler, Techne). Each reaction mixture contained 1U of *Taq* polymerase (Roche), 1 x reaction buffer (Appendix I.1) containing 1.5mM $MgCl_2$ (Roche), 24pmol of each primer, each deoxynucleoside triphosphate at a concentration of 0.2mM, 10ng of bacterial DNA and the reaction volume was made up to 50µl using PCR grade water. Negative controls were set up in exactly the same way except that the addition of DNA template was replaced with an equivalent volume of PCR grade water.

Reactions that were placed into the TC1 thermal cycler were overlaid with mineral oil, due to the absence of a heated lid. Unless otherwise stated, reaction conditions were as follows: samples were incubated at 96°C for 2min, and then subjected to 35 cycles consisting of 96°C for 30s, 65°C for 30s, and 72°C for 90s, in the final cycle the extension time was 5min.

3.5.1 PCR primer design

PCR primers were designed with the aid of DNA-sis (Hitachi Software) and Amplify software downloaded from the internet at www.wisc.edu/genetics/catg/amplify/index.html (Engels, 1993). Primers consisting of 24 bases were selected on the basis of a 50% G+C ratio, the presence of 2-3 guanine or cytosine bases at the 3' end of the primer, a lack of secondary structure and primer dimer formation and their proximity in relation to the desired target sequence.

3.5.2 Purification of PCR products

DNA was purified using the 'Prep-A-Gene DNA purification kit' (BioRad) according to the manufacturers' instructions. Details of the individual solutions included in this kit can

be found in Appendix I.6. The aqueous phase of PCR reactions, usually between 20-50µl in volume, were transferred to a clean 1.5ml microfuge tube. To facilitate the maximum recovery of DNA, 10µl of Prep-A-Gene Matrix was added to all PCR reaction products. Prior to this step, a volume of DNA purification buffer was mixed with the PCR reaction products that was equal to three times the combined volumes of the sample plus matrix. The Prep-A-Gene matrix was then added and the mixture was vortexed and incubated for 10min at room temperature (RT). The Prep-A-Gene matrix containing the DNA was centrifuged (13,000-16,000 x g, 30s, RT), the supernatant was discarded and the pelleted matrix was rinsed by resuspending it gently in 250µl of DNA binding buffer. The sample was centrifuged (13,0000-16,000 x g, 30s, RT) and the matrix was washed with 250µl of wash buffer, twice. Following the second wash all traces of liquid were removed from the sample tube and the bound DNA was eluted at 42°C for 5min following resuspension of the Prep-A-Gene matrix in 10-40µl of elution buffer. The sample was centrifuged (13000-16000 x g, 30s, RT) and the supernatant containing the purified PCR products was transferred to a clean tube.

3.5.3 DNA sequencing and sequence analysis

Unless otherwise stated both strands of all PCR products were sequenced directly using a cycle sequencing method by MWG-Biotech Ltd. PCR products that had been cloned into a plasmid vector (section 6.2.2) were also sequenced by MWG-Biotech Ltd., using a primer extension method. Pairwise alignments of the complementary nucleotide sequences were performed directly over the internet using the 'Blast 2 sequences' program at www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html (Tatusova & Madden, 1999). Any ambiguities in the sequences were resolved by visual inspection of the primary sequence data supplied by MWG Biotech and when necessary the DNA templates were re-sequenced.

Comparison of sequences obtained from different isolates was achieved using the multiple alignment program provided by Baylor College of Medicine http://dot.imgen.bcm.tmc.edu.9331/multi-align/multi-align.html. Sequences were also compared with those from other organisms on the GenBank database using the gapped BLAST program at www.ncbi.nlm.gov.html (Altschul *et al.,* 1997).

3.6 Agarose gel electrophoresis

The details of all solutions referred to in this section can be found in Appendix I.2. Unless otherwise stated, agarose gel electrophoresis was performed using 1% agarose gels made with 1 x TBE buffer. Two sizes of agarose gel were used, 60ml or 250ml volume approximately 80 x 100mm or 200 x 200mm slabs. Agarose gels of 250ml were electrophoresed at 90V for 6h, while 60ml gels were run for 90min at 120V. Prior to electrophoresis, an appropriate volume of gel loading solution was added to all samples. The sample volumes and concentrations varied according to each analysis and are stated in the Material and Methods section of each chapter. The sizes of the DNA bands that were resolved on agarose gels were estimated by comparison with either 100bp or 1kb standards (0.3μg) and these are detailed in Appendix I.4. Following electrophoresis, the DNA bands were stained by immersing the gel for 20min in a solution containing 1μg of ethidium bromide per ml of TE buffer. The stained bands were visualised with UV light using a transiluminator (Chromatono-UVe cabinet, Module: CC-60 UVP inc) and gels were recorded as digital images using a gel documentation system (Uvi-Tech).

3.7 Restriction enzyme digests

Restriction enzyme digestion of genomic and plasmid DNA was performed using enzymes purchased from either Roche or New England Biolabs. Usually, between 0.3μg-1.5μg DNA was incubated with 10-20 units of enzyme, using the buffer supplied by the

manufacturer. Unless otherwise stated, digests were left overnight at 37°C to ensure complete digestion.

3.8 Restriction fragment length polymorphism: Southern blotting

The details of all solutions referred to in this section can be found in Appendix I.4. Genomic DNA digests were separated according to size by agarose gel electrophoresis and stained and examined as described in section 3.6. After electrophoretic separation, DNA fragments were transferred to positively charged nylon membranes (Roche) according to the method of Southern (1975). Agarose gels were placed in 300ml of depurination solution for 10min, then washed with ddH_20 and placed in 300ml of denaturing solution for 1h with gentle constant shaking. Finally, the gel was immersed in 300ml of neutralisation solution and left for 1h with constant shaking. Capillary transfer was carried out using a Southern blotting apparatus (Scot-Lab). The wick was hydrated with 150ml of 95% ethanol, washed several times with 'Analar' water and finally saturated with transfer buffer (10 X SSC): 50ml was applied directly to the wick and 500ml was placed in the buffer reservoir in the base. The wick was covered in 3MM Whatmann Filter paper, upon which the agarose gel was placed. A positively charged nylon membrane which had been cut to the same dimensions as the gel was hydrated in distilled water and then soaked in 10 x SSC for 10min to equilibrate. Exposed areas of the wick were covered with cling-film to prevent "short circuiting" and the treated nylon membrane, a sheet of filter paper and paper towels were placed on top of the agarose gel, in that order. Transfer was allowed to proceeded overnight and total transfer was confirmed by examining the gel under UV light.

Following DNA transfer, nylon membranes were washed in sterile water for 5min. DNA was fixed to the membranes following exposure to short wave UV-light for 3min thereby cross-linking the DNA and the nylon (Chromatono-UVe cabinet, Module: CC-60 UVP inc). The following steps were performed in a hybridisation oven (Techne, UK).

Membranes were pre-hybridised at 68°C in a 32ml volume of prehybridsation solution for 1h. DNA probes were prepared as described in sections 3.8.2 and 3.8.3. Prior to use, the probe was denatured at 95°C for 10min then immediately placed on ice. Following pre-hybridisation, the solution was removed and replaced with hybridisation solution consisting of 2µl of denatured DIG-labelled probe diluted in 20ml of pre-warmed pre-hybridisation solution [final probe concentration was approximately 10ng/ml]. Hybridisation was allowed to proceed at 68°C, overnight. Following hybridisation, the membranes were washed twice for 5min with 50ml of 2 x SSC at room temperature followed by two washes with 0.1 X SSC for 15min at 68°C.

3.8.1 Immunological detection of DNA-DNA hybrids

Details of all buffers referred to in this section can be found in Appendix I.4. Immunological detection of DNA hybrids was performed according to the protocol provided in the Roche instruction manual 'DNA Labelling and Detection [Non radioactive]'. Membranes prepared as described (section 3.8) were submerged for 1min in 200ml of buffer 1 with constant gentle agitation, and then placed in 200ml buffer 2 for 30min. The membranes were rinsed with buffer 1 then placed in a solution containing anti-digoxygenin antibody conjugated to alkaline phosphatase diluted 1:10,000 in buffer 1, for 2h at room temperature. Any unbound antibody was subsequently removed by washing the membranes twice for 15min in buffer 1. The membranes were equilibrated for 2min in 100ml buffer 3 and then immersed in 70ml of developing solution. The development reaction was allowed to proceed in the dark until a colour precipitate formed. The reaction was terminated by washing the membrane several times with TE buffer. The developed membranes were photographed by the staff at the University of Plymouth Repro Graphics Unit. Photographs were subsequently scanned to capture digital images of the blots using a Scanjet 3400C (Hewlett Packard).

3.8.2 Digoxigenin (DIG) labelling of DNA probes using PCR

Incorporation of DIG-dUTP into DNA probes using PCR was performed as described in section 3.5 but with the following modifications. Deoxynucleoside triphosphate concentrations were as follows: 2mM dATP, 2mM dGTP, 2mM dCTP, 1.3mM dTTP, and 0.7mM alkali-labile DIG-11-dUTP. In addition, 10ng of purified PCR amplicon (section 3.5.2) was used as template to ensure that only the desired DNA sequence was labelled. The identity of the PCR amplicon was confirmed by DNA sequencing (section 3.5.3). The PCR reaction conditions were otherwise the same as outlined in section 3.5 except that 40 amplification cycles were used. Following amplification, the probe was purified as described in section 3.5.2 and stored at –20°C until required.

3.8.3 Digoxigenin (DIG) labelling of DNA probes using the random priming method

Randomly primed labelling of DNA probes was performed according to the protocol provided in the Roche instruction manual 'DNA Labelling and Detection [Non radioactive']. Details of all reagents referred to in this section can be found in Appendix I.5. The reaction was performed in a 0.5ml microfuge tube containing the following reagents: between 0.4-1μg of template DNA, 2μl of hexanucleotide mixture (stock concentration 1.56mg/ml), 2μl dNTP labelling mixture, the mixture was made up to 19μl with the addition of 'Analar' water and then 1μl of Klenow enzyme (2 U/μl), was added. The tube was vortexed, centrifuged briefly (13000-16000 x g, 5s, RT) and incubated at 37°C for at least 1h. Following incubation 2μl of 0.2M EDTA solution was added to stop the reaction.

Chapter Four

IDENTIFICATION AND SPECIFIC GENE POLYMORPHISM ANALYSIS OF *RENIBACTERIUM SALMONINARUM* USING THE POLYMERASE CHAIN REACTION (PCR)

4.1 Introduction

There are numerous PCR-based techniques that have been developed to assist in the detection and identification of bacterial pathogens isolated in pure culture or directly from clinical samples (White *et al.*, 1992; Toze, 1999; Tang *et al.*, 1997; Rodriguez, 1997; Harris & Griffiths, 1992). One of these techniques is multiplex PCR (M-PCR), in which multiple primer pairs that are designed to amplify specific sequences within the bacterial genome are included in the same reaction. M-PCR's have been developed for various purposes, including (i) the detection of multiple pathogens from a single clinical sample, (ii) to establish different biotypes within a given bacterial species e.g. toxinotyping of *Clostridium perfringens*, and (iii) the co-amplification of two or more specific genes from a single bacterial species to unequivocally confirm its presence (Mahony *et al.*, 1995; Yoo *et al.*, 1997; Kariyama *et al.*, 2000). Because multiple amplification is performed in a single reaction, M-PCR may offer savings in time and money, and consequently the development and application of M-PCR is widely reported.

In order to confirm the origin of genomic DNA that had been extracted from *R. salmoninarum* and to examine possible polymorphisms at specific regions of the genome, Grayson *et al.* (1999) designed 6 sets of PCR primers specific to 3 *R. salmoninarum* genes, namely *msa* (Chien *et al.*, 1992), *rsh* (Evenden *et al.*, 1990;

Evenden, 1993), and *hly* (Grayson *et al.*, 1995b). The primers were found to be highly specific for the detection of this pathogen by PCR and were used in the study to confirm the origin of the DNA that was extracted from 74 different isolates of *R. salmoninarum*. For each primer set, all of the isolates that were examined produced a single unambiguous amplicon of the expected size demonstrating that these regions were highly conserved and had limited potential for isolate discrimination. This chapter describes the development of M-PCR for the identification of *R. salmoninarum* based on the PCR primers previously developed by (Grayson *et al.*, 1999).

4.2 Materials and Methods

4.2.1 Genomic DNA extraction

Genomic DNA was extracted from each of the isolates as stated in section 3.2 and was resuspended in 50µl of TE buffer. For DNA quantification, 1µl of each extract was analysed using 0.8% agarose gels as outlined in sections 3.3 and 3.6.

4.2.2 PCR amplification of specific *R. salmoninarum* genes in separate reactions

The reaction conditions for PCR amplification of *R. salmoninarum* genes *msa*, *hly*, and *rsh* in separate reactions are described in section 3.5. However, 25µl rather than 50µl reaction volumes were used and consequently half the stated volume of each reagent was added. Details of the primers that were used are provided in Table 3.2. PCR products were visualised by electrophoresis of a 12.5µl sample volume, in 1.2% agarose gels as described in section 3.6.

4.2.3 Development of M-PCR

A method for M-PCR was developed using different combinations of the 6 primer sets described in Table 3.2. The PCR reactions were performed in a 50µl volume as described in section 3.5, each primer was used at 24pmol concentration. The M-PCR products were visualised by electrophoresis of 5µl volumes of the reactions in a 0.8% agarose gel as described in section 3.6.

Table 4.2: Primers that were used to amplify specific sequences from the *R. salmoninarum* genes *msa*, *hly*, and *rsh* (Grayson *et al.*, 1999).

Primer	Product size (bp)	Designation	Sequence (5'-3')
msa	487	Rs57+127	TCGCAAGGTGAAGGGAATTCTTCC
		Rs57-611	GGTTTGTCTCCAAAGGAGACTTGC
	297	Rs57+155	CAACAGTACAAGGCTTCAGCAGTG
		Rs57-449	CCGAAACCTACGTTTAGAGTCGTC
hly	542	RsMP+338	ATCGGCTCAGACTAGCGCCATAAT
		RsMP-877	GCTTCAAGATCGATGACCTTCGAG
	336	RsMP+487	TTACTGAGGTCCTTGATGGTCAGG
		RsMP-820	CGATCGGTGCGGTCATTCAAGATA
rsh	572	RsH+231	TCCGGTCATCATGCTTTCTTCGCT
		RsH-800	ATTGCCACCAAGCTGAAGTACCTG
	374	RsH+401	TGCCCAATCTGAAGACAGCGACTA
		RsH-772	GGTCGATAATGCTCGTCATGCCTA

4.3 Results

4.3.1 Genomic DNA extraction

Genomic DNA was extracted from 43 isolates of *R. salmoninarum* and 15 additional bacterial species. The quantity and quality of DNA was assessed by running 1µl of the extract on a 0.8% agarose gel and comparing the intensities of the bands with known standards. Two examples of these gels are provided in Figure 4.1, and, the remaining gel photographs are displayed in Appendix III. The concentration of DNA extracted from *R. salmoninarum* isolates was estimated to be as follows: MT444, 0.10µg/µl; MT452, 0.65µg/µl; MT839, 0.04µg; MT861, 0.04µg, MT1351, 0.01µg/µl; MT1469, 0.05µg/µl (Fig: 4.1A). For species other than *R. salmoninarum* the concentration of DNA was estimated to be as follows: *Yersinia ruckeri*, 0.05µg/µl; *Pseudomonas fluorescens*, 0.03µg/µl; *Arthrobacter* S23H2, 0.02µg/µl; *Aeromonas hydrophila*, 0.06µg/µl; *Rhodococcus equi*, 0.01µg/µl; *Brevibacterium linens*, 0.02µg/µl; *Pseudomonas anguilliseptica*, 0.03µg/µl (Fig: 4.1B).

There was no evidence for the presence of extrachromosomal DNA in any isolate of *R. salmoninarum*. However simple DNA extraction did provide evidence of plasmids in *A. hydrophila* where 2 bands of extra chromosomal DNA were observed.

4.3.2 PCR amplification of *R. salmoninarum* genes in separate reactions

In addition to purity checks (by visual inspection and regular gram staining) the origin of the genomic DNA extracted from cultures of *R. salmoninarum*, was confirmed by 6 different pairs of primers that were used in separate reactions to amplify specific sequences that are known to exist in the genome of the pathogen (Grayson *et al.*, 1999; Woese, 1987).

Using template DNA from each of the isolates, each primer set produced an amplicon of the expected size (Table 3.2). The results for some isolates are shown in Figure 4.2, whilst the remaining gel images are shown in Appendix III. In reactions containing DNA template where no amplification product was visible, the PCR was repeated and a band of the expected size was amplified.

4.3.3 M-PCR amplification of *R. salmoninarum* genes in a single reaction

In order to develop M-PCR for use in the detection of *R. salmoninarum,* different combinations of the PCR primers designed by Grayson *et al.* (1999) were used together in a single tube (M-PCR) to determine whether amplicons from each of the 3 well characterised genes could be observed. A small study was undertaken, where template DNA from 2 *R. salmoninarum* isolates, MT444 and MT426, was screened using 4 different combinations of the 6 primer sets. Primer combinations RS57+127 & RS57-611, RSMP+338 & RSMP–877 and RSH+231 & RSH–800 failed to produce the expected 3 bands, amplifying 2 bands of approximately 487bp and 542bp. A 572bp band was not visible. Similarly, the primer combinations RS57+155 & RS57–449, RSMP+487 & RSMP–820, and RSH+401 &RSH–772 amplified 2 bands of 336bp and 297bp. But not the expected 374bp band. Primer combination RS57+127 & RS57-611, RSMP+338 & RSMP–877 and RSH+401 & RSH–772 also amplified 2 bands, of 534bp and 374bp but the expected band of 487bp was not present. However, when primers RS57+155 & RS57–449, RSMP+487 & RSMP–820, and RSH+231 & RSH–800 were used in combination 3 clear bands of the expected sizes were produced, although the bands were not as bright when compared with those that were obtained using the same primers in separate PCR reactions (Fig: 4.3 and 4.4). Confirmation of the identities of the M-PCR products would require nucleotide sequencing. Further optimisation of the M-PCR may improve the level of amplification of each of the products and hence the sensitivity of the method.

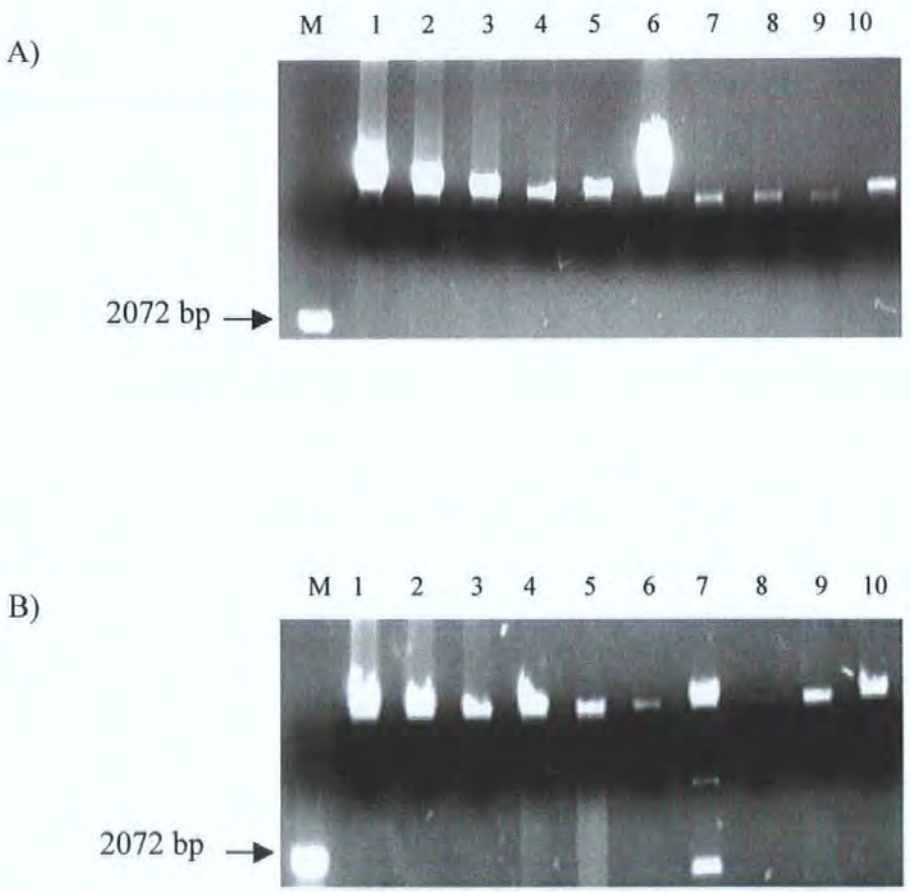


Figure 4.1: Quantification of DNA extracted from *R. salmoninarum* and other bacterial cultures. Panel A: λ DNA standard concentrations and DNA extracted from several *R. salmoninarum* cultures. Lanes 1-10 correspond to the following 1; 0.5μg of λ standard DNA, 2; 0.25μg of λ DNA, 3; 0.125μg of λ DNA, 4; 0.06μg of λ DNA, 5; 1μl of DNA from *R. salmoninarum* isolate MT444, 6; 1μl of DNA from *R. salmoninarum* isolate MT452, 7; 1μl of DNA from *R. salmoninarum* isolate MT839, 8; 1μl of DNA from *R. salmoninarum* isolate MT861, 9; 1μl of DNA from *R. salmoninarum* isolate MT1351, 10; 1μl of DNA from *R. salmoninarum* isolate MT1469. Lane M: 100bp ladders (0.3μg). Panel B: λ DNA standard concentrations and DNA extracted from several different bacterial cultures. Lanes 1-10 correspond to the following 1; 0.125μg of λ DNA, 2; 0.06μg of λ DNA, 3; 0.03μg of λ DNA, 4; 1μl of *Yersinia ruckeri* DNA, 5; 1μl of *Pseudomonas fluorescens* DNA, 6; 1μl of *Arthrobacter sp.* S23H2 DNA, 7; 1μl of *Aeromonas hydrophila* DNA, 8; 1μl of *Rhodococcus equi* DNA, 9; 1μl of *Brevibacterium linens* DNA, 10; 1μl of *Pseudomonas anguilliseptica* DNA. Lane M: 100bp ladders (0.3μg), only a single marker (arrowed) is visible.

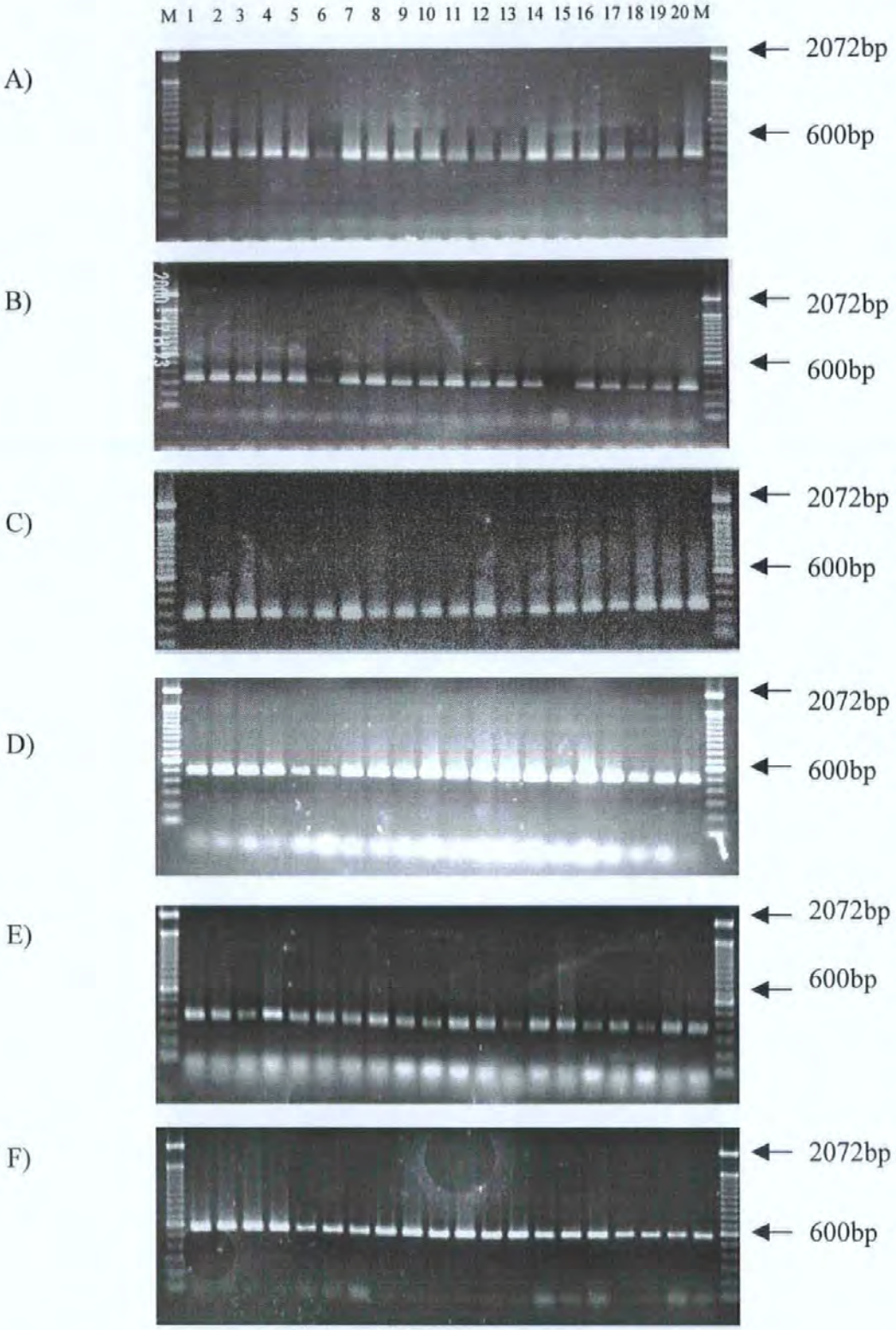


Figure 4.2: PCR amplification of *R. salmoninarum* DNA using primers RSMP+338 & RSMP-877 (panel A), RSMP+487 & RSMP-800 (panel B), RS57+155 & RS57-449 (panel C), RS57+127 & RS57-611(panel D), RSH+401 & RSH-774 (panel E), RsH+231 & RsH-800 (panel F). Lanes 1-20 correspond to the following isolates: 970083-88, 970083-102, 980036-150, 980036-87, 970419-1.2.3, 970153-19, A6, NCIMB1111, NCIMB1112, NCIMB1113, NCIMB1114, NCIMB1115, NCIMB1116, MT239, MT419, MT425, MT426, MT444, MT452, MT839. Lane M: 100 bp ladders (0.3µg). The sizes of selected markers are indicated by arrows on the right.

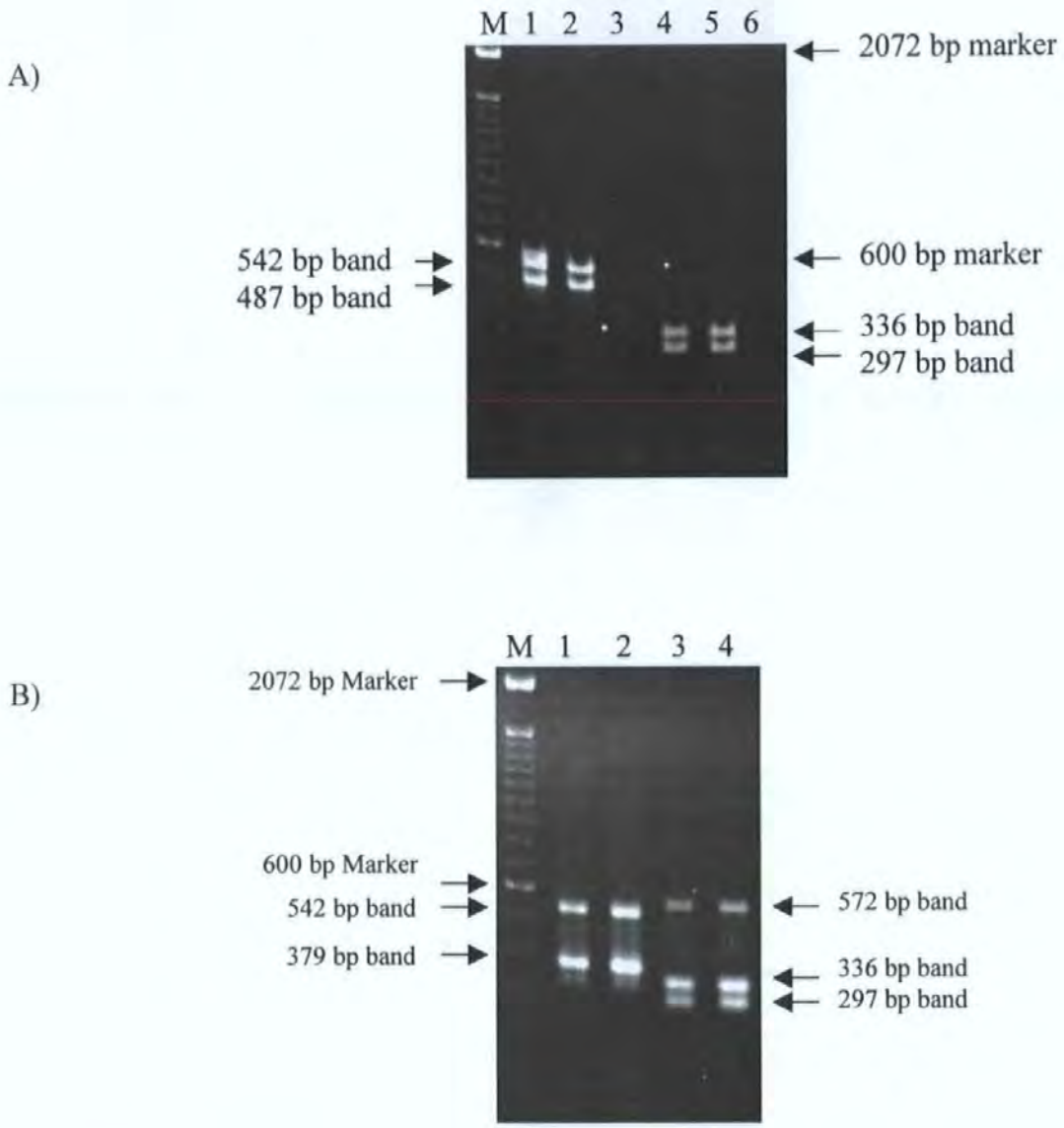


Figure 4.3: The results for M-PCR using genomic DNA extracted from *R. salmoninarum*. isolates MT426 (panel A: lanes 1 and 4, panel B: lanes 1 and 3) and MT444 (panel A: lanes 2 and 4, panel B: lanes 2 and 4). Panel A) Lanes 1 and 2: results using primers RS57+127 & RS57–611, RSMP+338 & RSMP–877, RSH+231 & RSH–800. Panel A) Lanes 4 and 5: results using primers: RS57+155 & RS57–449, RSMP+487 & RSMP–820, RSH+401 & RSH–772. Panel A) Lanes 3 and 6: negative controls. Panel B) Lanes 1 and 2: contained the following primers: RS57+127 & RS57–611, RSMP+338 & RSMP–877, RSH+401 & RSH–772. Panel B) Lanes 3 and 4: results using primers: RS57+155 & RS57–449, RSMP+487 & RSMP–820, RSH+231 & RSH–800. Amplified bands and markers are labelled by size. Lane M: 100bp ladders.

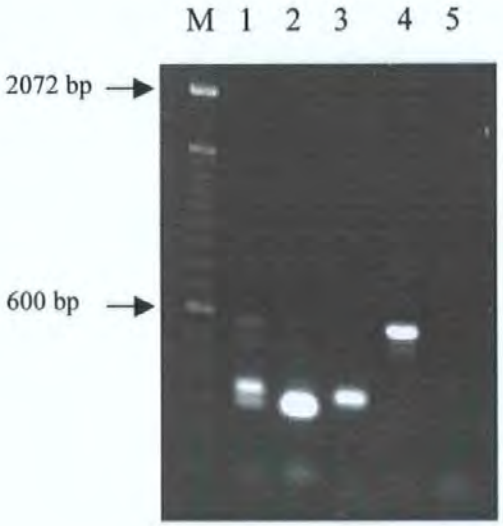


Figure 4.4 M-PCR analysis of genomic DNA extracted from *R. salmoninarum* isolate MT426. Lane 1; M-PCR reaction products obtained using primers RS57+155 and RS57–449, RSMP+487 and RSMP–820, RSH+231 and RSH–800 (5µl of amplification product). Lane 2; PCR reaction products obtained using primers RS57+155 and RS57–449 (5µl of amplification product). Lane 3; PCR reaction products obtained using primers RSMP+487 and RSMP–820 (5µl of amplification product). Lane 4; PCR reaction products obtained using primers RSH+231 and RSH–800 (5µl of amplification product). Lane 5; Negative control containing primers RS57+155 and RS57–449, RSMP+487 and RSMP–820, RSH+231 and RSH–800. Lane M: 100 bp ladders, the size of selected markers are indicated by arrows on the left.

4.4 Discussion

4.4.1 PCR with primer pairs in separate reactions

When primers that are complementary to sequences contained within the genes *msa*, *hly*, and *rsh* were used in separate PCR reactions a single band of the expected size was amplified (Fig 4.2). No obvious variation was observed in the lengths of the amplicons that were obtained using genomic DNA templates from a wide range of isolates. This supports previous studies carried out by Grayson *et al.* (1999) who found no evidence for length polymorphisms in the same regions of the genomes of 74 isolates of *R. salmoninarum* from worldwide locations.

Various studies have reported the presence of polymorphisms in either housekeeping or virulence genes in a range of bacteria, enabling the relationships between species, strains and even isolates to be examined. For example, simple PCR analysis of the gene encoding toxin A (*tcd*A) in *Clostridium difficile* highlights length polymorphisms that enable some strains to be distinguished from others (Rupnik *et al.*, 1998). Similarly, a PCR to amplify capsule genes can be used to discriminate between isolates of *Haemophilus influenzae* (Falla *et al.*, 1994). Other PCR-based techniques have been used to define mutations in the *gyr*A gene of *Salmonella enterica* by PCR-hybridisation, to examine a variable tandem repeat region in the *rpo*T gene in *Mycobacterium leprae*, and to examine single base changes in several genes by multilocus sequence typing (MLST) of a number of important pathogenic bacteria, such as *Campylobacter jejuni* and *Neisseria meningitidis* (Walker *et al.*, 2001; Matsuoka *et al.*, 2000; Dingle *et al.*, 2001; Maiden *et al.*, 1998).

Despite the lack of obvious length polymorphisms in the regions of genes *msa*, *hly*, and *rsh* that were examined here, it is possible that variations in the nucleotide sequence may be present. An investigation of this possibility would require DNA sequence analysis of the

genes from a wide range of isolates. Whilst this is possible, though time-consuming and expensive, it may prove to be of limited value in a highly conserved bacterium such as *R. salmoninarum*.

PCR-RFLP is another technique for examining variation at a specific locus (Gaydos *et al.*, 1992; Navarrow *et al.*, 1992). The method relies on the PCR amplification of a specific product that subsequently is digested with 1 or more restriction enzymes. Different restriction patterns for different isolates are visualised by agarose gel electrophoresis. PCR-RFLP has been successfully applied to studies of a range of bacteria including analysis of the *hrp* gene in *Ralstonia solanacearum, rpo*B gene in *Bartonella* sp., and the P1 cytoadhesin gene in *Mycoplasma pneumoniae* (Poussier *et al.*, 2000; Renesto *et al.*, 2001; Bereswill *et al.*, 1999). Additionally, a PCR-RFLP detection system has been used on clinical samples to investigate the *spo*T gene of the unculturable plant pathogen *Canadidatus* Phlomobacter *fragariae* (Foissac *et al.*, 2000).

One disadvantage of PCR-RFLP is the lack of discriminatory power as only the changes arising at specific restriction sites are detected and these are often located in genes that are highly conserved (Poussier *et al.*, 2000). Despite this, PCR-RFLP could be useful in investigating diseases, such as BKD, where the causative agent may never be isolated, provided that a specific gene was investigated, the technique was carefully standardised and corroborative nucleotide sequence data was obtained where possible. It could be possible not only to detect *R. salmoninarum* but also to gain information about epizootiology without even isolating the pathogen. The *rsh*, *msa* and *hly* amplicons have multiple restriction sites and it would be very interesting if future work were to investigate whether these sites differ between a diversity of isolates. The successful development of a PCR-RFLP method could greatly aid epizootiological studies of BKD.

4.4.2 Multiplex PCR

M-PCR, using primers RS57+155 & RS57–449, RSMP+487 & RSMP–820, and RSH+231 & RSH–800, in a single PCR reaction produced 3 bands that correspond with the sizes of amplicons that are obtained when the matching primers are used in separate reactions. Before routinely applying M-PCR to clinical samples it is necessary to confirm the identity of the amplicons by nucleotide sequencing. It is interesting that other combinations of the primer sets only produced 2 bands, despite the care taken to check the possibility of undesirable primer interactions, and the reasons for this outcome remain obscure (Engels, 1993).

A variety of PCR-based methods have been developed for the detection of *R. salmoninarum*, all of which have been shown to be totally specific and to amplify only a single unambiguous band from the tissues for BKD infected fish (Brown *et al.*, 1994; Butendieck *et al.*, 1995; Chase & Pascho, 1998; McIntosh *et al.*, 1996; Miriam *et al.*, 1997; Magnusson *et al.*, 1994; Brown *et al.*, 1995; Leon *et al.*, 1994a; Leon *et al.*, 1994b). However, M-PCR has the potential to provide a confirmatory test for *R. salmoninarum* particularly in cases where these are concerns about the specificity of diagnosis (Austin *et al.*, 1985; Brown *et al*, 1995; Etchegaray *et al.*, 1991). Further optimisation of M-PCR using clinical samples and nucleotide sequencing to confirm the identities of the amplicons may lead to the development of a useful diagnostic tool.

Chapter Five

RANDOMLY AMPLIFIED POLYMORPHIC DNA ANALYSIS OF *RENIBACTERIUM SALMONINARUM*

5.1 Introduction

Randomly amplified polymorphic DNA (RAPD) is a PCR-based technique that is used for genomic analysis. The method employs a single, short oligonucleotide primer that binds to any region in the genome bearing the complementary sequence (Welsh & McClelland, 1990; Williams *et al.*, 1990). PCR amplifies complementary genomic DNA sequences and the products can be separated and visualised by standard electrophoresis. RAPD analysis has been used for differentiating between strains of a wide range of bacterial species including *Burkholderia pseudomallei, Borrelia burgdorferi, Vibrio vulnificus* and *Flavobacterium psychrophilum* (Norton *et al.*, 1998; Wang *et al.*, 1998; Arias *et al.*, 1998; Chakroun *et al.*, 1997). The procedure is unique in that no prior sequence information is needed and it relies upon small quantities of genomic DNA, which makes it ideally suited to the study of slowly growing and fastidious organisms like *R. salmoninarum* where only limited sequence information is available.

Grayson *et al.* (1999) used RAPD to reproducibly differentiate between *R. salmoninarum* isolates. A total of 60 isolates, sourced from world-wide locations were examined using 2 different methods and 8 individual primers (Grayson *et al.*, 1999; Grayson *et al.*, 2000b). The isolates were grouped into 21 banding patterns, which did not reflect either biological source or geographical location. This chapter describes how this method was applied to 28 isolates that were obtained from a variety of sources within the United Kingdom.

5.2 Materials and Methods

5.2.1 RAPD Analysis

RAPD analysis was performed using 28 isolates according to the 2 methods developed by Grayson *et al.* (1999). The first method employed a Ready-To-Go RAPD Analysis Beads kit (Amersham Pharmacia Biotech) containing 6 distinct random 10-mer primers, including primer P1 (GGTGCGGGAA), primer P2 (GTTTCGCTCC), primer P3 (GTAGACCCGT), primer P4 (AAGAGCCCGT), primer P5 (AACGCGCAAC), and primer P6 (CCCGTCAGCA) and was used according to the manufacturer's instructions. Ready-To-Go RAPD analysis beads contain a pre-mixed, pre-dispensed reaction bead which, when resuspended in a 25µl volume, contained 1U AmpliTaq DNA polymerase and Stoffel fragment, 0.4mM each dNTP, 2.5 µg BSA, and buffer [3mM $MgCl_2$, 30mM KCl and 10mM Tris, (pH8.3)]. To each reaction bead, 23µl of PCR grade water (Sigma-Aldrich), 25pmol of primer (1µl) and 10ng of template DNA (1µl) was added. The reactions conditions were as follows: 1 cycle consisting of 95°C for 5 min and then 45 cycles consisting of 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min.

A second method originally described by Atienzar *et al.* (1998), was also performed. Two primers were selected from the 10 primers in a kit obtained from Operon Technologies Inc.: primer OPA9 (GGGTAACGCC) and primer OPB1 (GTTTCGCTCC). Each 25µl reaction mixture contained 10mM Tris-HCl (pH 8.3), 50mM KCl, 5.11mM $MgCl_2$, 0.1% Triton X-100, 0.1% gelatin, each deoxynucleoside triphosphate at a concentration of 0.33mM, 2µM primer, 2.5µg of BSA, 2.8U of *Taq* DNA polymerase (Immunogen International), and 25ng of template DNA. The reaction conditions were as follows: 1 cycle consisting of 95°C for 5min, 39 cycles consisting of 95°C for 1min, 50°C for 1min, and 74°C for 1min, and 1 cycle consisting of 95°C for 1min, 50°C for 1min, and 74°C for

10min. All RAPD products were analysed by electrophoresis of 12.5µl of each reaction mixture in 1.2% agarose gels as described in section 3.6.

5.2.2 RAPD dendrogram analysis

A dendrogram showing the relationship between *R. salmoninarum* isolates was generated with the aid of "Tools for population genetic analysis" software package (Miller, 1997). Fifty-eight bands, many of which were uniformly present in the majority of isolates, were recorded in a similarity matrix according to their presence or absence. Faint bands were excluded from the analysis. A band was scored as absent only if no visible band was present within a 2% size range. The patterns generated with each of the primers were combined for each isolate, and distances were calculated by the unweighted pair group method by arithmetic averaging (UPGMA) algorithm using Nei's original distance (Nei, 1972; Nei, 1978; Sneath & Sokal, 1973).

5.3 Results

5.3.1 RAPD analysis

Twenty-eight isolates of *R. salmoninarum* from the UK were examined by RAPD analysis using 8 primers and 2 different protocols (Fig: 5.1 – 5.8). A dendrogram was constructed to determine the relationship between these isolates using 58 major bands combined from the data gathered from all of the primers that were utilised. Forty of the 58 bands that were scored were present (invariant) in all 28 isolates examined. It is clear from examining the dendrogram that this technique generated 5 main clusters of *R. salmoninarum* isolates with 7 other isolates possessing distinct and unique profiles (Fig: 5.9). Some of the isolates are grouped according to the place of isolation. Six of the isolates (980109F60, 980109F3,

980109F82, 980109F85, 980109F95, 980109F47) were recovered from diseased fish from farm A in England. These isolates formed 2 distinct clusters, 1 and 2, that distinguished them from the 21 remaining isolates due to the presence of unique bands that were generated with primer OPA9 (Fig: 5.2, band 10) and primer P3 (Fig: 5.5, band 6). Interestingly, these clusters also included another English isolate (980297#97) sourced during the same year, not from farm A but from Hatchery E.

RAPD analysis revealed that all of these 7 isolates produced identical RAPD profiles using primers P1, P2, P4, P5, and OPA9. However, the RAPD patterns that were obtained using primers P3, P6 and OPB1 did reveal differences between some of the isolates. Using primer P3, isolates 980109F3, 980109F60, 980109F82 and 980109F85 possessed stronger bands of 600bp and 750bp compared with isolates 980297#97, 980109F47 and 980109F95 (Fig: 5.5). Primer P6 generated a stronger band of 1.25kb in isolate 980109F3 which was weak or absent in the other isolates (Fig: 5.8). These differences were not registered in the band scoring matrix that was used to generate the UPGMA dendrogram because only amplicons which are uniformly present in the majority of isolates are scored and band intensity is not taken into account. However, these isolates were placed into 2 clusters using primer OPB1 where a 1.15 kb band (Fig: 5.1, band 5) that is strongly present in isolates 980297#97, 980109F3 and 980109F60, 980109F82 is absent from isolates 980109F47, 980109F85 and 980109F95. Four other English isolates that were placed in clusters 3 and 4 were identical apart from the presence of a 750bp amplicon (Fig: 5.5 band 6) in isolates 3506W134 and 2219#5, which is absent from isolates 3258#15 and 3506W132. Cluster 4 also contained some isolates of Scottish origin.

The majority of Scottish isolates were placed in clusters 4 and 5. Those isolates placed in cluster 4 differed from those in cluster 5 by the presence of a single amplicon (Fig: 5.7,

band 4). Isolates MT239, MT861, MT1261, MT1351, MT1469, MT1470, and MT1770 all generated unique profiles based upon different combinations of the presence or absence of the bands scored and were placed into unique clusters 6, 7, 8, 9, 10, 11 and 12, respectively. Interestingly, Scottish isolates MT1351 and MT861 both generated similar but not quite identical profiles (Fig: 5.6 and 5.7), which were uniquely distinctive from the remaining 26 isolates.

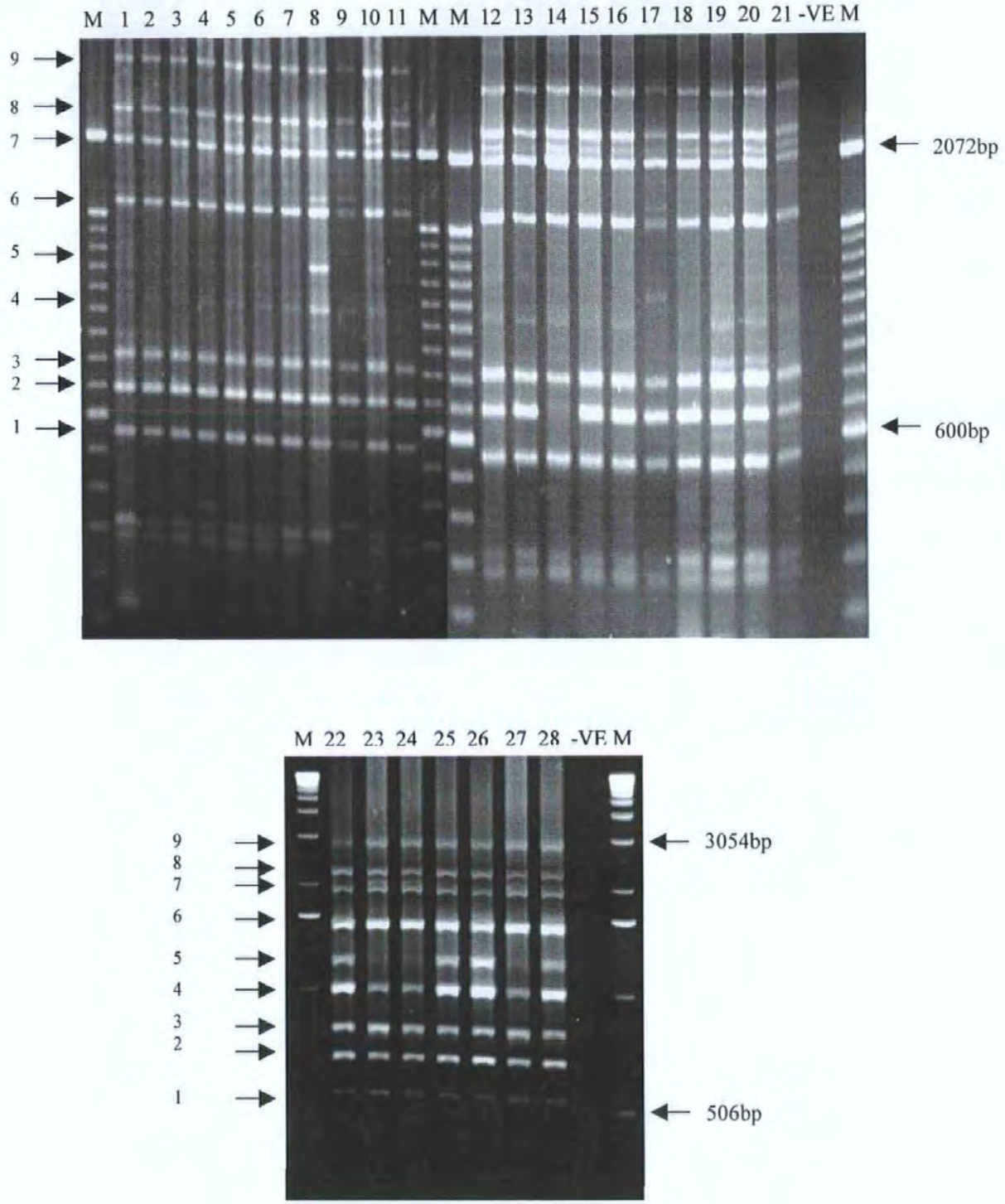


Figure 5.1: RAPD analysis of 28 isolates of *R. salmoninarum* using primer OPB1. Lanes 1-28 correspond to the following isolates MT239, MT419, MT425, MT426, MT444, MT452, MT839, MT861, MT1261, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132, 2119#5, 980297#97, F95, F85, F82, F60, F47, and F3 respectively. Lanes labelled –VE represent negative controls. Arrows on the left indicate the bands that were scored for dendrogram construction. Lane M: 100bp or 1 kb ladders, with the sizes of selected markers indicated by arrows on the right.

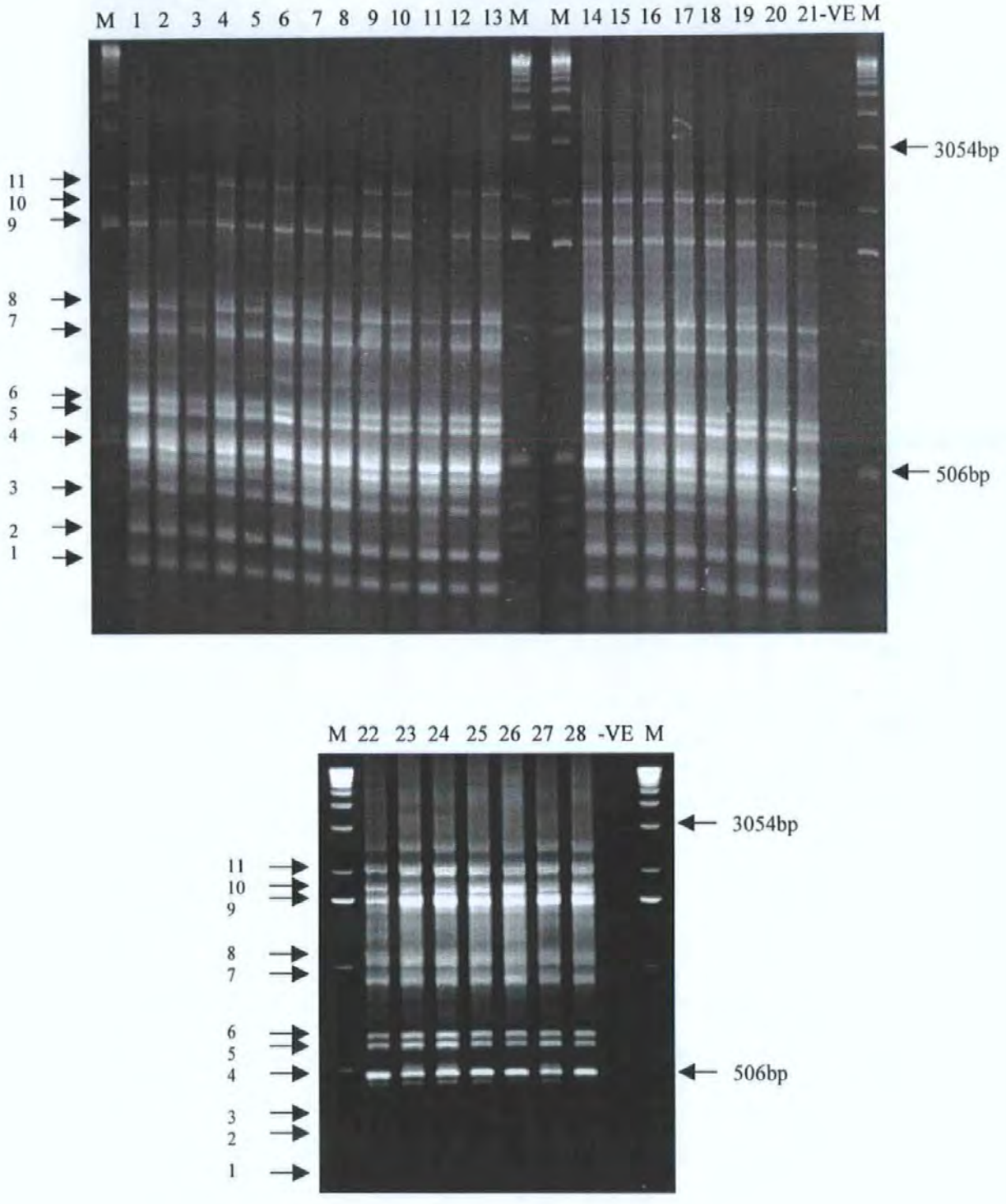


Figure 5.2: RAPD analysis of 28 isolates of *R. salmoninarum* using primer OPA9. Lanes 1-28 correspond to the following isolates MT239, MT419, MT425, MT426, MT444, MT452, MT839, MT861, MT1261, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132, 2119#5, 980297#97, F95, F85, F82, F60, F47, and F3, respectively. Lanes labelled –VE represent negative controls. Arrows on the left indicate the bands that were scored for dendrogram construction. Lane M: 1 kb ladders. The sizes of the selected markers are indicated by arrows on the right.

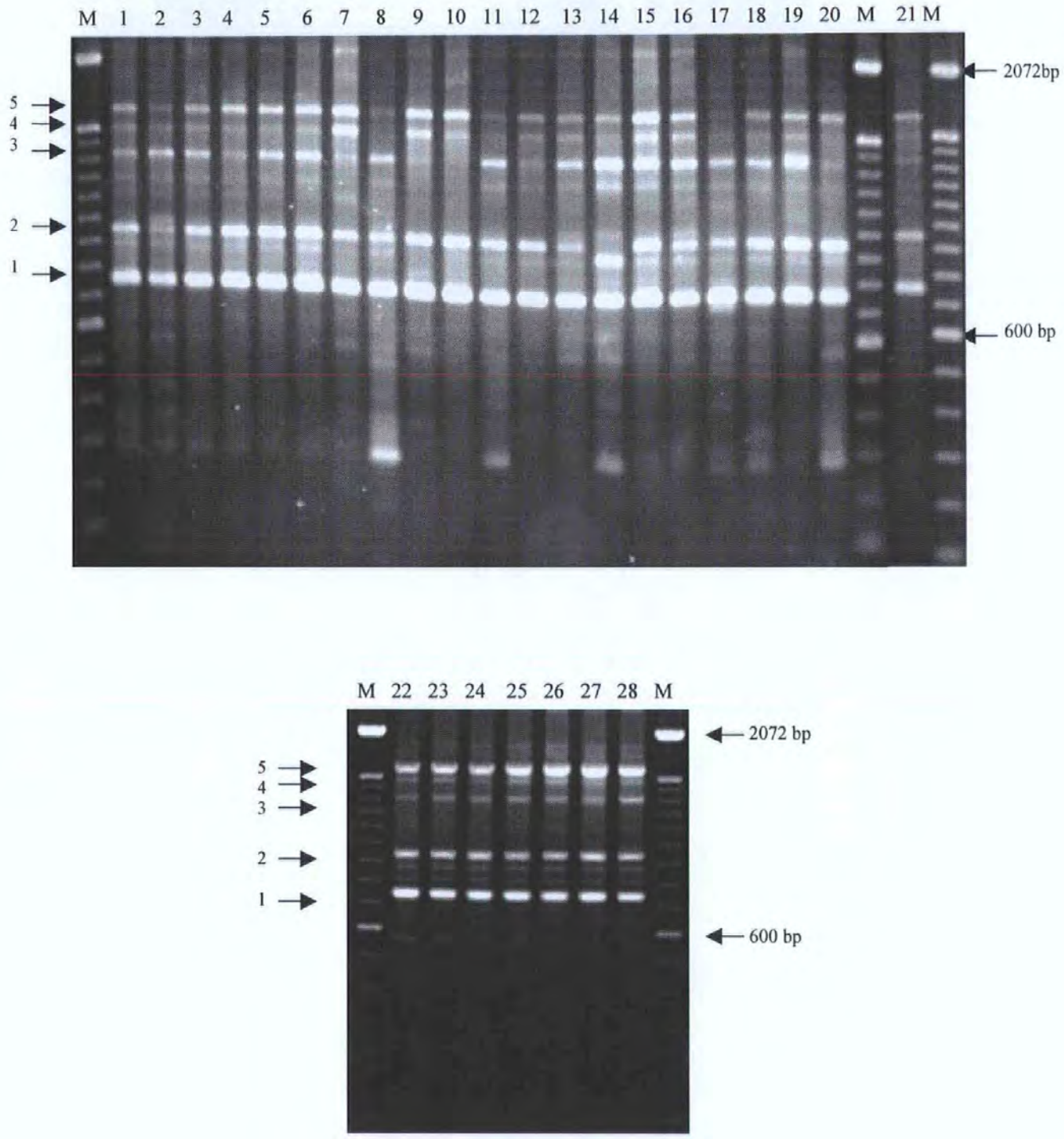


Figure 5.3: RAPD analysis of 28 isolates of *R. salmoninarum* using primer P1. Lanes 1-28 correspond to the following isolates MT239, MT419, MT425, MT426, MT444, MT452, MT839, MT861, MT1261, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132, 2119#5, 980297#97, F95, F85, F82, F60, F47, and F3, respectively. Arrows on the left indicate bands that were scored for dendrogram construction. Lane M: 100bpbp ladders (0.3µg), the sizes of selected markers are indicated by arrows on the right.

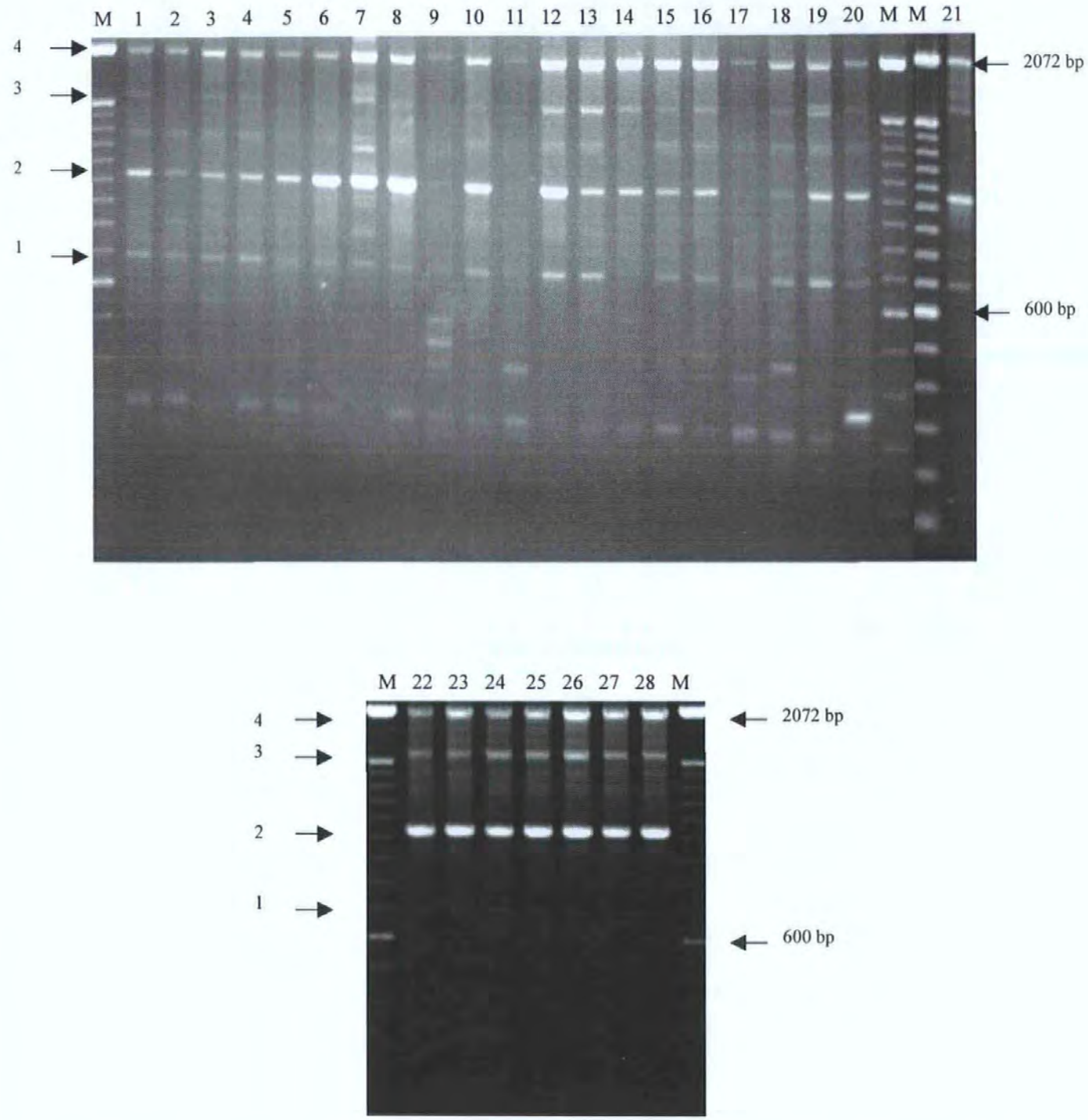


Figure 5.4: RAPD analysis of 28 isolates of *R. salmoninarum* using primer P2. Lanes 1-28 correspond to the following isolates MT239, MT419, MT425, MT426, MT444, MT452, MT839, MT861, MT1261, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132, 2119#5, 980297#97, F95, F85, F82, F60, F47, and F3, respectively. Arrows on the left indicate the bands that were scored for dendrogram construction. Lane M: 100bp ladders, the sizes of selected markers are indicated by arrows on the right.

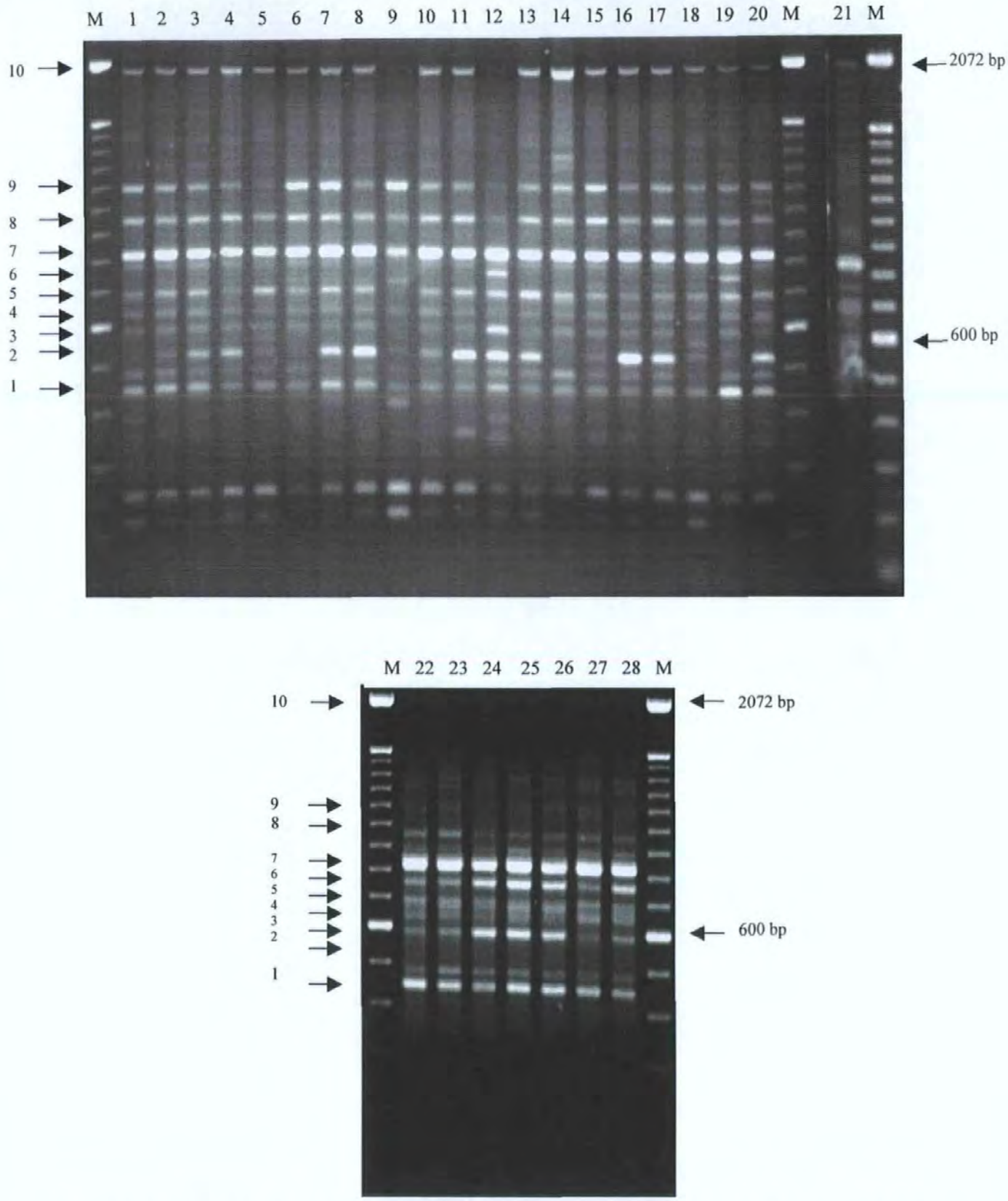


Figure 5.5: RAPD analysis of 28 isolates of *R. salmoninarum* using primer P3. Lanes 1-28 correspond to the following isolates MT239, MT419, MT425, MT426, MT444, MT452, MT839, MT861, MT1261, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132, 2119#5, 980297#97, F95, F85, F82, F60, F47, and F3, respectively. Arrows on the left indicate the bands that were scored for dendrogram construction. Lane M: 100bp ladders, the sizes of selected markers are indicated by arrows on the right.

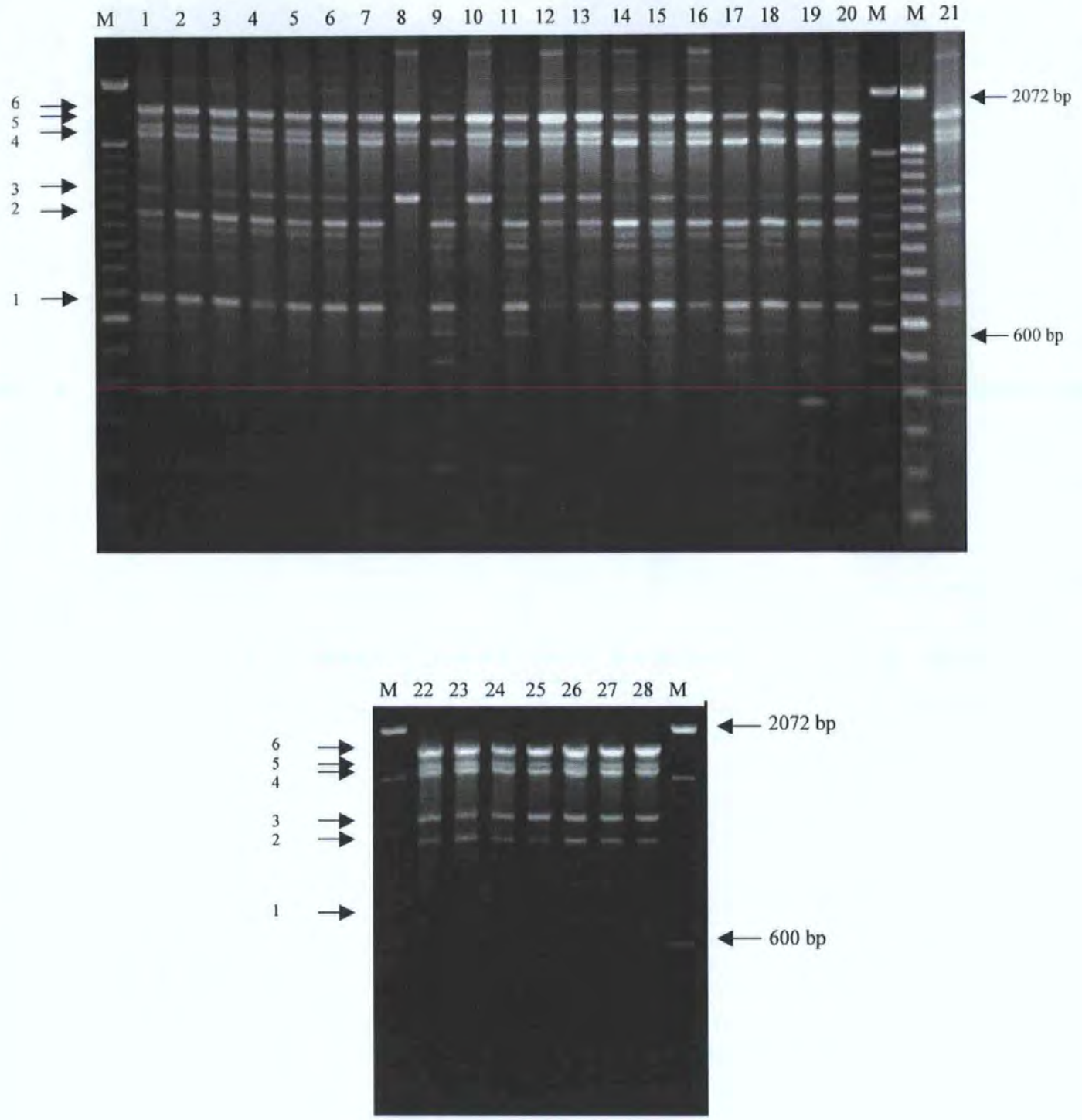


Figure 5.6: RAPD analysis of 28 isolates of *R. salmoninarum* using primer P4. Lanes 1-28 correspond to the following isolates MT239, MT419, MT425, MT426, MT444, MT452, MT839, MT861, MT1261, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132, 2119#5, 980297#97, F95, F85, F82, F60, F47, and F3, respectively. Arrows on the left indicate the bands that were scored for dendrogram construction. Lane M: 100bp ladders (0.3μg), the sizes of selected markers are indicated by arrows on the right.

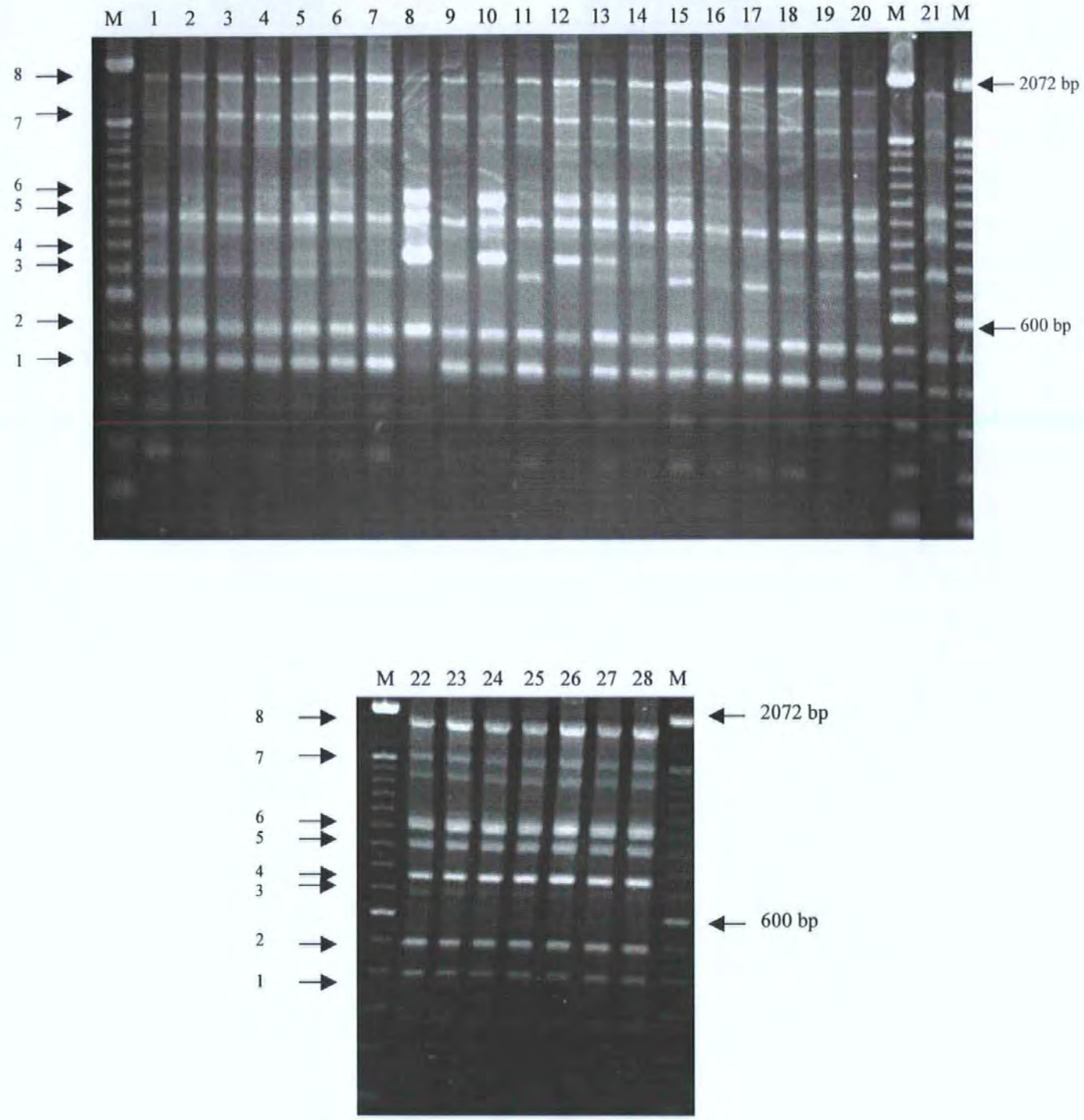


Figure 5.7: RAPD analysis of 28 isolates of *R. salmoninarum* using primer P5. Lanes 1-28 correspond to the following isolates MT239, MT419, MT425, MT426, MT444, MT452, MT839, MT861, MT1261, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132, 2119#5, 980297#97, F95, F85, F82, F60, F47, and F3, respectively. Arrows on the left indicate the bands that were scored for dendrogram construction. Lane M: 100bp ladders (0.3μg), the sizes of selected markers are indicated by arrows on the right.

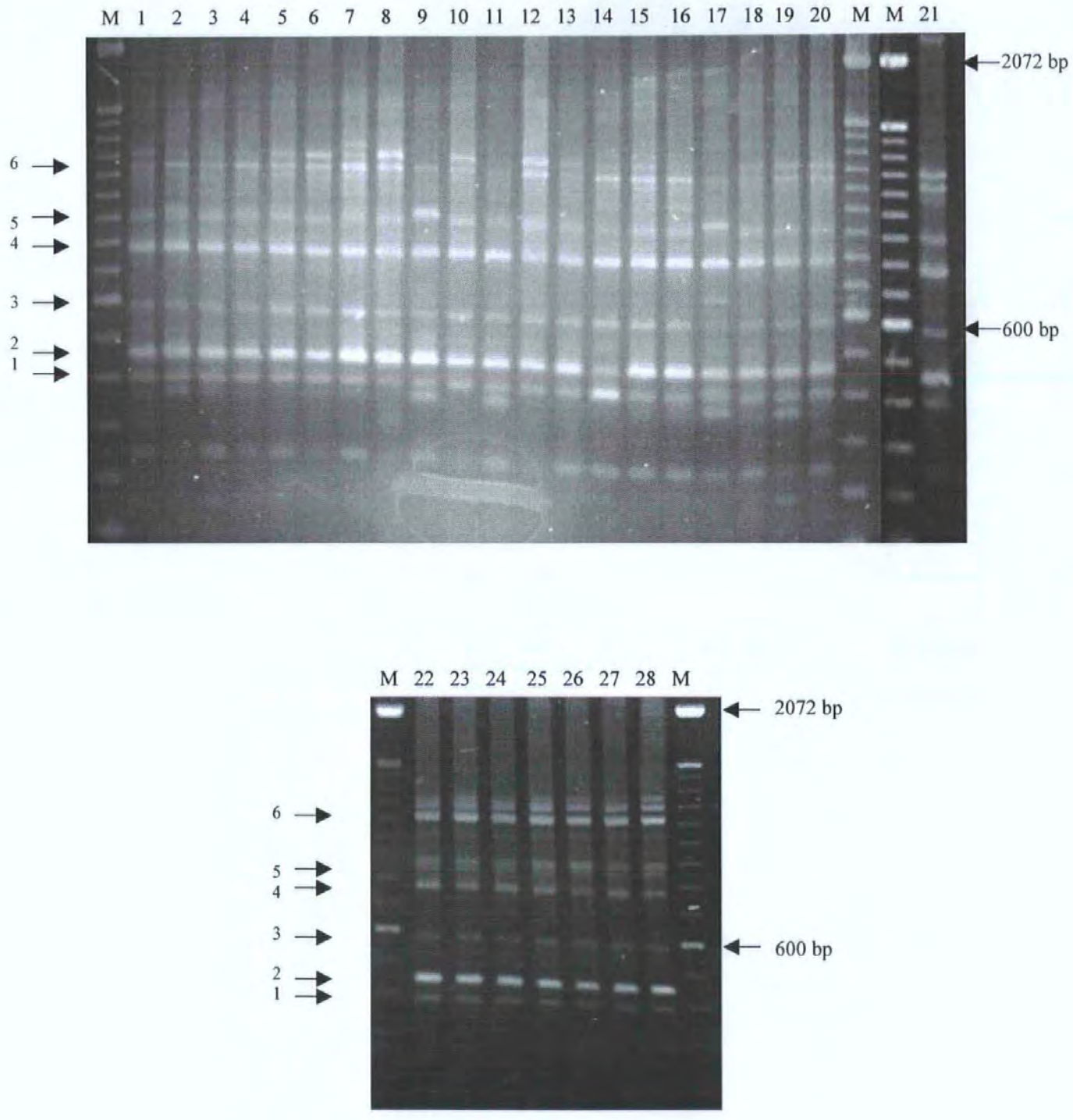


Figure 5.8: RAPD analysis of 28 isolates of *R. salmoninarum* using primer P6. Lanes 1-28 correspond to the following isolates MT239, MT419, MT425, MT426, MT444, MT452, MT839, MT861, MT1261, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132, 2119#5, 980297#97, F95, F85, F82, F60, F47, and F3 respectively. Arrows on the left indicate the bands that were scored for dendrogram construction. Lane M: 100bp ladders (0.3μg), the sizes of selected markers are indicated by arrows on the right.

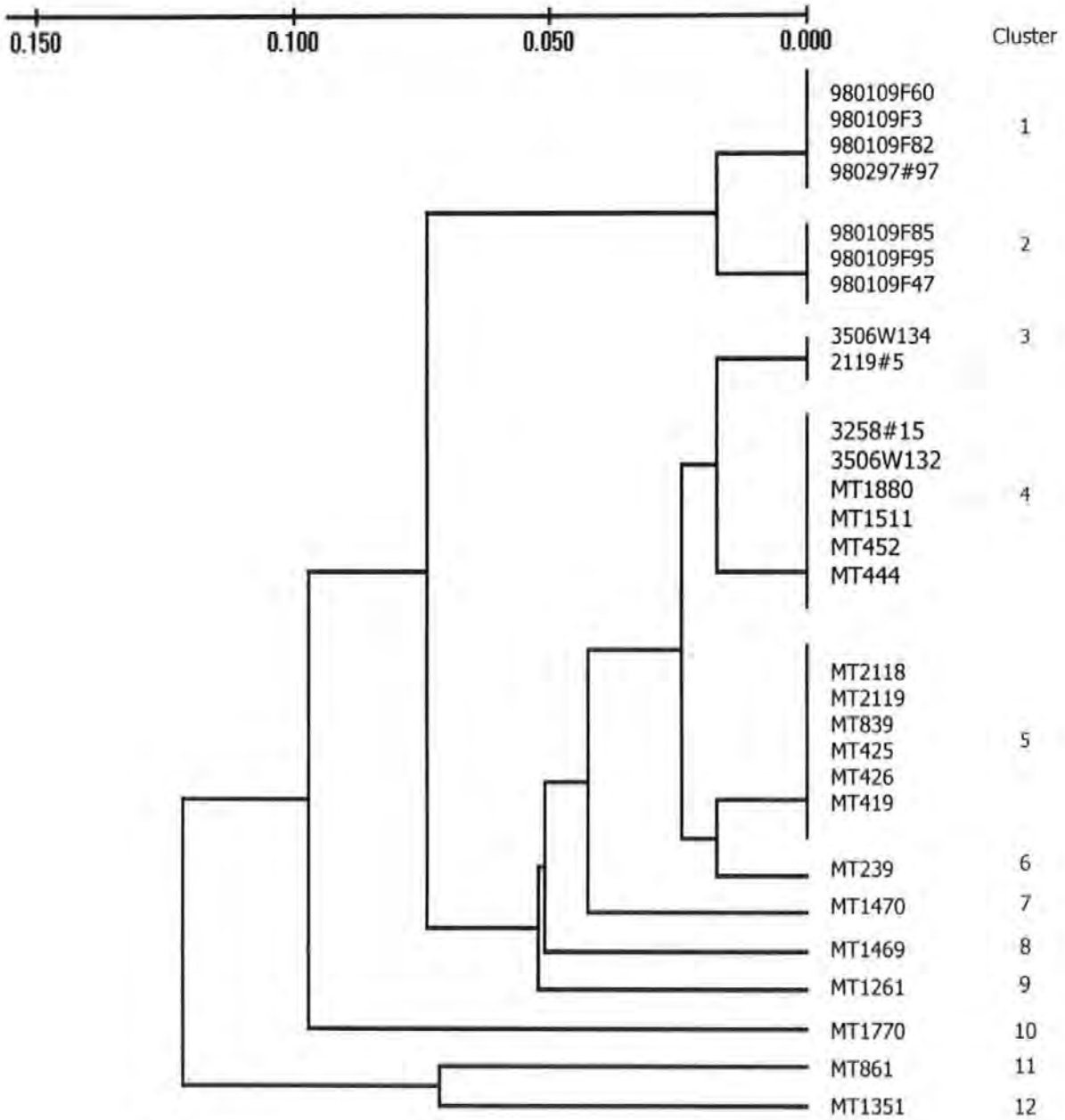


Figure 5.9: A UPGMA generated dendrogram from RAPD analysis of 28 isolates of *R. salmoninarum* from UK locations. The scale bar denotes a relative difference between the isolates where 0.01 is equivalent to 1% in branch lengths.

5.4 Discussion

In this study, 28 isolates of *R. salmoninarum* were examined by RAPD, and were grouped into 12 clusters, some of which reflected a common origin (Fig: 5.9). Seven of the 28 isolates, which were placed into clusters 1 and 2, were all isolated from infected fish in the same year, 6 of which were from the same farm (Farm A) and 1 isolate was from an infected hatchery (Hatchery E). Interestingly, although Hatchery E did not supply Farm A with eggs or hatchlings it was known to supply another farm (Farm B) that is owned by the same company. It is unknown if any transfer or interaction of livestock occurred between the 2 farms. The geographical position of farms and hatcheries from which isolates were sourced was not revealed, which makes predictions of possible routes of infection difficult. Four other *R. salmoninarum* isolates sourced in 2000 from 3 English farms were placed in clusters 3 and 4, with cluster 4 also containing some Scottish isolates. Furthermore, 2 isolates (3506W134 and 3506W132) from the same fish farm were found to be different suggesting that this farm could have more than one route of infection or may have been supplied with livestock from an establishment infected with multiple *R. salmoninarum* isolates. It is interesting that these 2 English isolates were also grouped with 4 Scottish isolates from an earlier outbreak of BKD. It is possible that these isolates could be the same or a similar isolate derived from the same clone. Alternatively, they could be unrelated and this typing method has failed to discriminate between them. However, because only incomplete information is available regarding the histories of the isolates any epizootiological relationships remain purely speculative.

Comparing the RAPD data presented here with the more extensive studies that have been carried out, some important differences should be noted. Grayson *et al.* (1999, 2000b) carried out an extensive study on 60 isolates of *R. salmoninarum* isolates from worldwide locations. While the same methods for scoring bands were employed a total of 86 bands

were scored in comparison to the 58 that were scored in this studies. This discrepancy in band scoring may have occurred because this study concentrated on a very limited number of *R. salmoninarum* isolates, which were of UK origin. Grayson *et al.* (1999, 2000b) examined a greater number of isolates from more diverse locations and consequently this diversity may help explain the greater numbers of bands that were scored. Further evidence in favour of this suggestion is provided by Grayson *et al.* (2000b) who found that only 11 of the total of 86 bands that were scored were present in all of the isolates. In contrast, in this study it was found that 40 out of a possible 56 bands were present in all isolates. Additionally, of the 60 isolates that were examined by Grayson *et al.* (1999), 22 were of UK origin and the bulk of these isolates were grouped into 4 distinct clusters while some of the remainder were grouped with American isolates or formed unique profiles. Clearly, the results of this study are in good agreement with these previous works (Grayson *et al.*, 2000b).

Examining the RAPD profiles reveals that subtle differences do exist between some isolates that have been placed into the same cluster in the dendrogram. To quantitatively and objectively analyse RAPD profiles, data distance matrices are often employed. Unfortunately, due to the binary nature of distant matrices and in order to remove subjectivity, bands are scored as either present or absent and the intensity of different bands is not taken into account. In addition, fainter bands are often excluded and bands within a 2% size range are scored as the same size. For example, examining Fig: 5.8 (band 5) it can be seen that the amplicon generated by isolates MT1261 and MT2119 was greater in size but within the 2% size range and so was scored as present in both isolates. Such interpretation of RAPD profiles is necessary in order to avoid the possibility of subjective bias but sometimes subtle but important differences between isolates can be lost as a consequence. Some methods for scoring RAPD profiles are available that take account of

band intensity by employing complex computer technology. However, the intensity of bands on ethidium bromide stained agarose gels is subject to many variables, consistency and reproducibility may be compromised and difficult to maintain (Johansson *et al.*, 1995).

RAPD has been the subject of criticism with reports that the method of DNA extraction, brand of *Taq* polymerase, colony age, type of thermal cycler and primer, magnesium and DNA template concentrations can affect the fidelity of the assay (Aded *et al.*, 1995; Meunier & Grimont, 1993; Bassam *et al.*, 1992; Ellsworth *et al.*, 1993). For example, an RAPD based study carried out on a series of *M. tuberculosis* isolates showed that the patterns obtained varied greatly in response to minimal changes in the amplification; and the reproducibility of the experiment was found to be low (Linton *et al.*, 1994). However, many of these criticisms apply to any PCR-based technique and some variables, such as the ages of cultures and template concentrations and purity apply to every diagnostic method. To combat poor reproducibility a number of suggestions were put forward by Welsh & McClelland (1990) including the use of 2 different DNA concentrations and analysis of DNA from different batches of culture extracted at different times. These were both performed by Grayson *et al.*, (1999), who found that RAPD analysis was a highly reproducible and discriminatory method for the typing of *R. salmoninarum* isolates. This is reflected in the finding that 3 *R. salmoninarum* isolates (MT239, MT426 and MT452) that were included in this study, as well as previous work by Grayson *et al.*, (2000b) were found to be different and were placed into different clusters, in both of the studies.

In RAPD analysis primers bind at random to unknown areas on the bacterial genome generating a series of amplicons, which are then separated according to size. However, some studies examining different bacterial strains have shown that bands of the same size, (inferring similarity between them) can differ in nucleotide sequence because they have

been derived from different regions of the genome (Oakey *et al.*, 1998). This co-migration of RAPD amplicons has been a further cause for criticism of the technique. If RAPD is being assigned as a basic tool to differentiate between dissimilar bacterial strains or isolates, rather than to confirm relationships, this is not a cause for concern as the basic principle can be applied that if bacterial isolates generate different profiles they are definitely different while those isolates that generate identical fingerprints are not necessarily the same. However, in more complex strain and isolate differentiation studies such as this one, where RAPD fingerprints are used to generate a dendrogram inferring degrees of phylogenetic similarity between isolates, this is a bigger problem. Consequently, the results of RAPD analysis require careful interpretation.

A further aspect of RAPD analysis that has been a cause of criticism but is also one of the methods biggest appeals is that differences between bacterial fingerprints are of unknown origin. It has been suggested that, ideally, a typing system should identify functional and significant differences between isolates that reflect real and relevant population diversity. The reasoning behind such an ideal is that a functional difference between isolates would be more likely to be stable during the time frame of the investigation (Burr *et al.*, 1998). Despite the criticisms of RAPD as a typing method the development and application of this technique to analyse important bacterial strains is continually being reported in peer-reviewed journals (Nigatu *et al.*, 2001; Ramasoota *et al.*, 2001; Hendson *et al.*, 2001). The attractiveness of the method is due to the high degree of discrimination, ability to examine anonymous genomes, cost effectiveness and ease of execution.

Ultimately this chapter has described the application of a previously developed technique to analyse 28 isolates of *R. salmoninarum* from UK sources. Data from the RAPD fingerprints were used to generate a dendrogram and the isolates were placed into 12

clusters, which in some cases reflected a common geographical origin. Additionally, this chapter supports previous studies (Grayson *et al.*, 1999; Grayson *et al.*, 2000b) that have demonstrated that RAPD, despite its limitations is a highly reproducible, quick and simple technique which has been proven to be a useful additional technique for the discrimination of *R. salmoninarum* isolates.

Chapter Six

CHARACTERISING THE rRNA OPERON OF *RENIBACTERIUM SALMONINARUM:* SEQUENCING, SEQUENCE ANALYSIS, AND RIBOTYPING

6.1 Introduction

Bacterial ribosomal RNA genes (rRNA) are present in all known bacteria and are commonly arranged in a defined order (16S, 23S and 5S). They are highly conserved, making them ideal for the classification, identification and typing of isolates (Woese, 1987; Gutenberger *et al.*, 1991; Ludwig & Schleifer, 2001). The 16S rRNA gene (Olsen *et al.*, 1991) and the 5S rRNA gene (Dams *et al.*, 1987; Park *et al.*, 1991; Syzmanski *et al.*, 1998), have been almost invariably used to establish phylogenetic relationships between a wide variety of organisms. The intergenic spacer (ITS) regions of the rRNA operon, particularly the 16S-23S rRNA ITS region (ITS1), show the most variation, in sequence and length (Dennis *et al.*, 1998; Gurtler & Stanisich, 1996; Leblond-Bourget *et al.*, 1996; Wang *et al.*, 1997) and this variation can be used for distinguishing between closely related bacterial species and, sometimes, between strains (Barry *et al.*, 1991; Jensen *et al.*, 1993; Gurtler & Stanisich, 1996; Leblond-Bourget *et al.*, 1996). Most organisms have multiple copies of rRNA genes and sequence uniformity is usual, although among procaryotes there are at least three exceptions which have been reported so far, the archaeon *Haloarcula marismortui* (Dennis *et al.*, 1998) and the actinomycetes *Thermobispora bispora* (Wang *et al.*, 1997) and *Thermomonospora chromogena* (Yap *et al.*, 1999).

In addition to the 16S-23S rRNA ITS region, the rRNA operon also possesses another spacer which separates the 23S and 5S genes. There have been few published works examining variation in bacterial 23S-5S rRNA ITS (ITS2) although there is some evidence that ITS2 may prove useful for designing species-specific probes and establishing the relationships between closely related species (Nour, 1998; Ji *et al.*, 1994).

Currently, incomplete 16S rRNA gene sequences are known for 2 isolates of *R. salmoninarum* (Gutenberger *et al.*, 1991; Magnusson *et al.*, 1994) and a 112 bp common insertion fragment of the 23S rRNA gene has been sequenced (Roller *et al.*, 1992). Based on the known region of the 16S rRNA it has been established that *R. salmoninarum* is phylogenetically intermixed with species of the genus Arthrobacter and certain Micrococcus species (*Micrococcus luteus*, and *Micrococcus lylae*) (Stackebrandt *et al.*, 1995). In addition, the 16S-23S rRNA ITS (ITS1) has been determined for 19 *R. salmoninarum* isolates from a wide variety of locations. Four sequence variants (sequevars) of the ITS1 region have been identified with the majority of isolates (11) possessing an identical sequence, which was designated sequevar 1, SV1 (ITS1-1). For the remaining 7 isolates, 3 sequevars were identified. Three isolates from Iceland and 1 from Japan were shown to possess an alternative sequevar, SV2 (ITS1-2), which differed from SV1 by 3 base substitutions. A sole unique isolate sourced from the North West Territories of Canada possessed an alternative sequevar, which differed from SV2 by 2 base substitutions and was designated SV3 (ITS1-3). The fourth sequevar which differed from SV1 by a single nucleotide substitution was possessed by 2 Scottish and 1 Norwegian isolate and was designated SV4 (ITS1-4)(Grayson *et al.*, 1999; Grayson *et al.*, 2000b).

This chapter describes i) the sequencing of the previously unknown regions of the *R. salmoninarum* rRNA operon, the 23S rRNA gene, 23S-5S rRNA ITS region and the 5S rRNA gene, ii) an investigation of sequence variation in the 16S-23S rRNA ITS region of *R. salmoninarum* isolates using a method developed by Grayson *et al.* (1999), iii) an investigation of sequence variation in the 23S-5S rRNA ITS region of *R. salmoninarum* isolates, iv) an investigation of the usefulness of ribotyping as a tool for differentiating between isolates of *R. salmoninarum* and, finally, v) an investigation into the use of the 23S-5S rRNA ITS region as a *R. salmoninarum* specific probe.

6.2 Materials and Methods

6.2.1 Sequencing of the rRNA genes of *R. salmoninarum* ATCC33209

Sequencing of the 23S rRNA gene, and 5S rRNA gene of *R. salmoninarum* ATCC33209 was achieved by PCR amplification (section 3.5), using nine sets of PCR primers (Table 6.1). Primers were designed as outlined in section 3.5.1 in order to amplify a series of overlapping fragments that would cover the entire length of the 23S rRNA gene, 5S rRNA gene, and 23S-5S rRNA ITS region. The primers were designed from highly conserved regions that were identified following the alignments of rRNA sequences from bacteria that are known to be closely related to *R. salmoninarum*. The sequences (and their GenBank accession numbers) included those for *Micrococcus luteus* (X06484), *Rhodococcus erythropolis* (AF001265), *Rhodococcus facians* (Y11196), *Streptomyces griseus* (X55435; X61478), *Streptomyces rimosus* (X62884) and *Frankia* sp. (M88466).

The primers were used in the following combinations to generate the amplicons displayed in Figure 6.1: R23SF1+205 & R23SR2-537 (~330bp product; A1), R23SF2+549 &

R23SR3-1334 (~780bp product: A2), R23SF3+1351 & R23SR4-2149 (~800bp product: A3), R23SF4+2105 & R23SR5-2925 (~820bp product: A4), R23SF5+2930 & R23SR7-3624 (~700bp product: A5). In order to resolve areas of sequence ambiguity, primers R23SF1+205 & R23SR3-1334 (~1129bp product; A6) were used and additional primers F3A+1113 & R3A-1451 (~338; A7), were designed. This strategy ensured that almost the entire DNA sequence spanning the 23S rRNA gene, the 5S rRNA gene and the ITS2, was derived from both strands of 2 different templates.

6.2.1.1 Nucleotide sequencing and sequence analysis

Nucleotide sequencing and sequence analysis of PCR products was performed as described in section 3.5.3. *R. salmoninarum* sequences were compared with those from other organisms on the GenBank database using the gapped BLAST program (Altschul *et al.*, 1997).

6.2.1.2 Dendrogram Construction

A dendrogram examining the phylogenetic relationships among members of the actinomycetes and related organisms based on 5S rRNA sequences was generated using a bootstrapped clustered algorithm. Sequence data was obtained from the GenBank database the following organisms (GenBank accession number is depicted in brackets): *Renibacterium salmoninarum* (AF143477), *Arthrobacter polychromogenes* (X08001), *Arthrobacter globiformis* (M16173), *Micrococcus luteus* (K02682), *Cellulomonas biazotea* (M16172), *Rhodococcus fascians* (X15126), *Dactylosporangium auranticum* (X55267), *Nocardia asteroides* (X55261), *Flavobacterium breve* (M33888), *Streptomyces rimosus* (X62884), *Streptomyces griseus* (V01347), *Streptomyces ambifaciens* (M27245), *Streptomyces lividans* (X58874), *Frankia sp.* (M55343), *Mycobacterium tuberculosis*

(Z73902), *Mycobacterium leprae* (X56657), *Bacillus subtilis* (D11460), *Escherichia coli* (D12500).

Initially, a multiple alignment of 5S rRNA data from the above organisms was generated using an online 'ClustalW' computer program available at the European Bioinformatics Institute at http://www.ebi.ac.uk. The multiple alignment data was then placed into the online 'Tree-Top phylogenetic tree prediction program', available at http://www.genebee.msu.su/services/phtree-reduced.html, to generate a bootstrapped clustered algorithm and a dendrogram showing phylogenetic relationships.

6.2.2 16S-23S rRNA intergenic spacer (ITS1) analysis

The analysis of ITS1 was performed in accordance with the method of Grayson *et al.* (1999). Primer sets RS+1002 (5'-CCGTCCAAGTCACGAAAGTTGGTA-3') & ML-1329 (5'-ATCGCAGATTCCCACGTCCTTCTT-3') and RS+1002 & ML-1469 (5'-GTGGGTACTGAGATGTTTCAGTTC-3'), which span ITS1 and have previously been shown to amplify in each case single unambiguous products of 751bp and 895bp, respectively, from *R. salmoninarum* DNA, were used in separate PCR reactions (section 3.5). Products were analysed on a 1.2% agarose gel (section 3.6).

Sequence analysis was performed (section 3.5.3) on the 751bp ITS1 amplicon from the following 28 isolates: 970083-88, 970083-102, 980036-150, 980036-87, 970419-1.2.3, A6, A80, 980297#97, 980109F95, 980109F85, 980109F82, 980109F60, 980109F47, 980109F3, NCIMB1111, NCIMB1112, NCIMB1113, NCIMB1115, 3506W132, 2119#5, MT419, MT839, MT861, MT1261, MT1351, MT1469, MT1470 and MT1770 (Table 3.2).

6.2.3 23S-5S rRNA intergenic spacer (ITS2) sequence analysis

Primers R5+118 (5'-CTGACCGGTACTAATAGGCCAACA-3') and R5-433 (5'-GTCTTAGCTTCCGGGTTCGAGATG-3') were designed (as stated in section 3.5.1) to the 3' end of the 23S rRNA gene and the 5' end of the 5S rRNA gene, respectively, thereby flanking the ITS2 region of the rRNA operon of *R. salmoninarum.* PCR amplification (section 3.5) was performed on 84 isolates of *R. salmoninarum* (Fig: 6.5 and Appendix III). The reaction products were analysed on, 1.2% agarose gels (section 3.6).

The nucleotide sequences of the ITS2 amplicons were determined (section 3.5.3), for the following 33 isolates: 980036-150, 970083-88, 970083-102, 980106#1.1.5, 970153-19, 980036-87, 970419-1.2.3, A6, A8, NCIMB1111, NCIMB1112, NCIMB1113, NCIMB1114, NCIMB1115, NCIMB1116, 980297#97, 980109F95, 980109F85, 980109F82, 980109F60, 980109F47, 980109F3, S-182-90(P-7), Marion Forks, ATCC33209, Iwate, AcF6-1, MT861, MT1470, MT1770, MT1880, 3506W132 and 2119#5 (Table 3.1 & 3.2).

6.2.4 Ribotyping

Genomic DNA extracted from *R. salmoninarum* (section 3.3) was digested in separate reactions with *BamHI, XhoI, StuI, SpeI* and *AvaI* (section 3.7). The fragments of digested DNA were separated by agarose gel electrophoresis (section 3.6), transferred to nylon membranes (section 3.8) and probed by DNA hybridisation, using 2 different digoxygenin rRNA probes (Fig: 6.1), which were prepared as described in section 3.8.2. Firstly, a DNA probe spanning 1.2kb of the 5' end of the *R. salmoninarum* 23S rRNA gene was prepared

using primers RsF1+184 (5'-GAACGTGGGGAACTGAAACATCTC-3') and R3-1358 (5'-CTTTCGCTACTCATGCCTGCATTC-3'). Secondly, a DNA probe spanning 724bp of the *R. salmoninarum* 16S rRNA gene was prepared using primers R16+774 (5'-CAGGATTAGATACCCTGGTAGTCC-3') and R16-1491 (5'-TACGACTTAGTCCCAATCGCCAGT-3').

Table 6.1: Primer combinations that were used to amplify the 23S and 5s rRNA genes and 23S-5S rRNA ITS region of *R. salmoninarum.* Product name corresponds to the amplicons in Fig: 6.1. Note that amplicon A6 was generated from using primer combinations R23SF1+205 and R23SR3-1334.

Primer	Sequence 5'→ 3'	Product
R23SF1+205	GAACGTGGGGAACTGAAACATCTC	A1
R23SR2-537	CTTTCGCTACTCATGCCTGCATTC	
R23SF2+549	TGACCGATAGCGGATCAGTACCGT	A2
R23SR3-1334	CTTTCGCTACTCATGCCTGCATTC	
R23SF3+1351	ACGAGTGAGAATGCAGGCATGAGT	A3
R23SR4-2149	AAGTCGTTACTCCATTCGTGCAGG	
R23SF4+2105	GGCGGTGGTAACTATAACCATCCT	A4
R23SR5-2925	CCGCTTAGATGCTTTCAGAGGTTA	
R23SF5+2930	TAGCTACGTTCGGGATGGATAACC	A5
R23SR7-3624	ATTGGCGACTTTCGTAGATTCGCC	
F3A+1113	TGTGCTAAGTGGGAAAGGATGTGG	A7
R3A-1451	TTAGCTTGACCCTGGAACCCTTGA	
R23SF4+2105	GGCGGTGGTAACTATAACCATCCT	A8
R23SR6-3344	AATGTGTCACGGCGGCGACCTACT	
R5+118	CTGACCGGTACTAATAGGCCAACA	ITS2
R5-433	GTCTTAGCTTCCGGGTTCGAGATG	

6.2.5 Development of a 23S-5S rRNA ITS *R. salmoninarum* specific probe

6.2.5.1 Bacterial isolates

Fifteen bacterial species were used in this study (Table 6.2). All organisms were recovered from storage and grown on TSA at 25°C for 3 days. Genomic DNA was extracted from broth cultures of each of the bacteria (section 3.2) and 100ng of this DNA was used to test the specificity of the DNA probe. Ten-fold dilutions of genomic DNA (100ng-1pg) that had been extracted from 4 isolates of *R. salmoninarum* 970083-102 (SV1), MT1770 (SV2), ACF6-1 (SV3) and NCIMB1114 (SV4) isolates (section 3.2) were prepared in TE buffer in order to examining the sensitivity of the ITS2 probe.

6.2.5.2 Dot blot

DNA was transferred onto positively charged nylon membrane by using a Minifold I 96-well dot-blot system (Schleicher & Schuell). DNA was cross-linked to the membrane using UV light (section 3.8).

6.2.5.3 Probe development

A digoxygenin-labelled probe was generated in a PCR DIG-labelling reaction using primers RSPROBE+12 (5'-GATAAACCCACCCTTCAAAAGGGG-3') and RSPROBE-217 (5'-TTTTTTACCGGTCCGCACCCTGTT-3') that were designed to amplify a 210bp product, spanning almost the entire 23S-5S rRNA ITS spacer region. Genomic DNA from isolate NCIMB 1111 was used as a template for this reaction and the resulting product was sequenced to confirm its identity (Appendix II.2). The blot was developed using the anti-digoxygenin alkaline phosphatase conjugate and colour development kit (Roche) as described in section 3.8.1.

Table 6.2: Bacterial strains that were used in this study. All organisms were grown on TSA at 25°C for 3 d.

Strain	Code	Location in Dot Blotter
Aeromonas hydrophila	NCIMB 1134	E4 100ng
Aeromonas salmonicida	CM30	E2 100ng
Yersinia ruckeri	13/86	E1 100ng
Pseudomonas fluorescens	NCIMB 1953	E3 100ng
Pseudomonas anguilliseptica	NCIMB 1949	E5 100ng
Rhodococcus equi	NCTC 1621	F3 100ng
Brevibacterium linens	NCIMB 8546	F4 100ng
Micrococcus luteus	NCTC 7563	F1 100ng
Streptomyces griseus	NCIMB 8136	F2 100ng
Nocardia cellulans	NCIMB 8868	F5 100ng
Arthrobacter sp.	ZAT 012	G4 100ng
Arthrobacter globiformis	NCIMB 10267	G2 100ng
Arthrobacter polychromogenes	NCIMB 8907	G1 100ng
Arthrobacter sp.	S23H2	G5 100ng
Arthrobacter sp.[2]	ZAT 277	G3 100ng

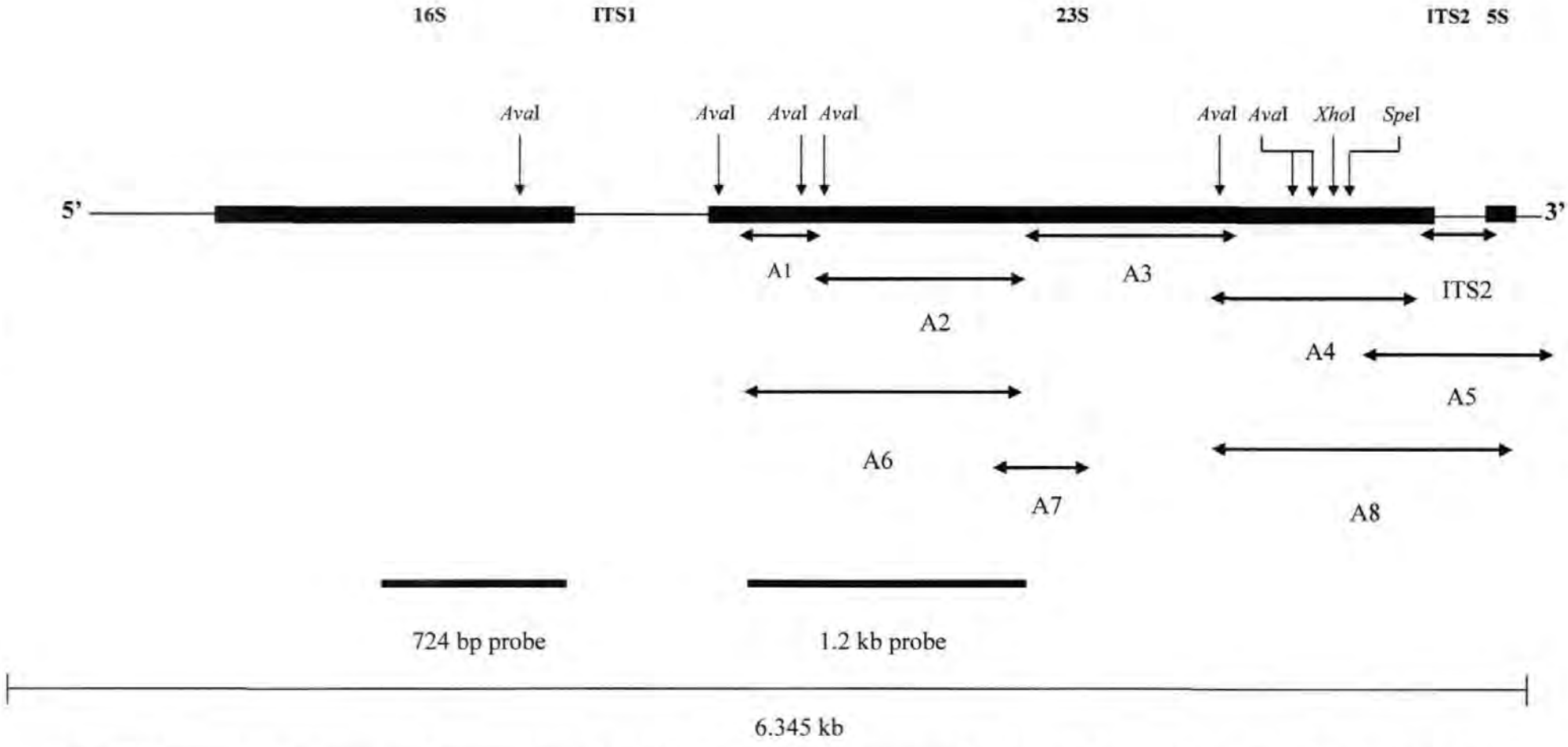


Figure 6.1 Strategy for sequencing and restriction map of the rRNA operon of *R. salmoninarum* ATCC33209. A sequence was obtained from the 5' end of the 23S rRNA gene to the 3' tail of the 5S rRNA gene from both strands of PCR amplicons. The positions of rDNA probes (with sizes) that were used for hybridisation studies are indicated as bold lines.

6.3 Results

6.3.1.1 Nucleotide sequencing and sequence analysis of the rRNA

The complete 23S rRNA, 5S rRNA and 23S-5S rRNA spacer region was sequenced for *R. salmoninarum* ATCC33209, the type strain, by directly sequencing an overlapping series of PCR-amplified products. The assembled sequence can be found in Appendix III. The presence of DNA encoding rRNA genes was confirmed by comparison of the *R. salmoninarum* sequences with nucleotide sequences from the GenBank database. The 5' and 3' ends of the *R. salmoninarum* rRNA genes were assigned on the basis of alignments with the rRNA genes from the most similar organisms. The analysis confirmed that the rRNA genes of *R. salmoninarum* are arranged in the order 16S-ITS1-23S-ITS2-5S (GenBank accession nos. AF143477, AF180950).

The total length of the 23S rRNA gene was determined to be 3135 nucleotides (G+C content 54.1 mol%), separated by a 219bp intergenic spacer (G+C content 48.9 mol%) from the 5S rRNA gene of 120 nucleotides (G+C content 65.0 mol%). An exact match with the nucleotide sequence of a common insertion fragment of 112bp which was previously identified within the 23S rRNA genes of many gram-positive bacteria with a high G+C content, including *R. salmoninarum* (Roller *et al.*, 1992), was located from nucleotides 1537 to 1648 inclusive. No tRNA genes or pseudogenes could be identified either within the ITS2 or within the 176 nucleotide sequence following the 3' end of the 5S rRNA gene. An inverted repeat (5'-aataaaggtcccacaacgacgtgggacctttatt-3') was located 9 bp downstream from the 3' end of the 5S rRNA gene. This region, which could form a hairpin structure consisting of a perfectly matched stem of 14bp and a loop of 6bp, was followed by a U-rich region, which is typical of a transcriptional termination sequence.

6.3.1.2 Phylogenetic analysis of the 23S and 5s rRNA gene sequences

Figure 6.2, provides a graphical picture of the 5S rRNA gene data presented in Table 5.3, showing that *R. salmoninarum* is grouped within the Arthrobacter/Micrococcus sub group of the Actinomycetes. This was also confirmed by the results of pairwise alignments performed using 23S rRNA genes, where the *R. salmoninarum* sequence was found to be most similar (86.7%) to the *M. luteus* sequence. Unfortunately this analysis could not be performed on any of the Arthrobacter species because no 23S rRNA gene sequence data was available.

The 5S rRNA gene data also shows that the remaining high G + C group of the actinomycetes were placed into 2 clusters, one containing members of the genera Flavobacter, Cellulomonas, Rhodococcus, Dactylosporangium, Actinokinetospora and Nocardia and the other containing members of the genera Streptomyces and Frankia. Furthermore *Mycobacterium tuberculosis* and *Mycobacterium leprae* with 89.0% identity were grouped in a distinct cluster. It is of interest that when the 23S rRNA genes were compared, these 2 species were found to share 94.4% identity.

6.3.1.3 Nucleotide sequencing and sequence analysis of the 16S-23S rRNA ITS region (ITS1)

The ITS1 region was amplified by PCR from the genomic DNA of 43 isolates of *R. salmoninarum* using 2 primer sets (Fig: 6.3 and Appendix III). For each of the isolates, the 2 primer sets generated only a single amplicon of the expected size. Sequence analysis was performed on 28 of the isolates examined and all isolates were found to have a ITS1 region of 534bp. Twenty-seven of the isolates examined had the same nucleotide sequence that has previously been designated as sequevar SV1 (Grayson *et al.*, 1999). However, 1

isolate, MT1770, which had been sourced from Atlantic salmon farmed in the Scottish highlands, possessed a sequence which differed from SV1 by 3 base substitutions and has previously been designated as sequevar SV2 (Fig: 6.4).

6.3.1.4 Nucleotide sequencing and sequence analysis of the 23S-5s rRNA ITS region (ITS2)

ITS2 was amplified in a PCR reaction from the genomic DNA of 81 isolates of *R. salmoninarum* using the *R. salmoninarum* sequence specific primers R5+118 and R5-433 which bind close to the 3' end of the 23S rRNA gene and the 5' end of the 5S rRNA gene, respectively. For every isolate only a single band of the same apparent size, 318bp, was detected in 1.2% agarose gels (Fig: 6.5 and Appendix III).

The ITS2 sequences of 33 isolates of *R. salmoninarum* were determined by directly sequencing both strands of PCR amplified products and were found to be the same length, 219bp. Furthermore, 32 isolates possessed an identical nucleotide sequence, which was designated sequevar 2-1 (SV21; GenBank accession nos. AF143477-AF143485). These 32 isolates were sourced from England, Scotland, Wales, Iceland, Japan, and the United States, and from various host species, including rainbow trout, Atlantic salmon, chinook salmon, and grayling. A single isolate, AcF6-1 sourced from Arctic char from the Northwest Territories of Canada, was found to differ at 2 positions and the ITS2 sequence of this isolate was designated SV22 (GenBank accession no. AF143486) (Fig: 6.6)

Interestingly, following an exhaustive search of the GenBank database, the ITS2 sequence was found to be unique, and is highly conserved among *R. salmoninarum* isolates.

Therefore it was decided to investigate the potential of this sequence to act as a specific oligonucleotide probe for *R. salmoninarum*.

6.3.4 Ribotyping and determination of the number of copies of the rRNA gene

With the inclusion of the full sequence of the 16S rRNA gene the size of the *R. salmoninarum* rRNA operon is 6345bp (Gutenberger *et al.*, 1991; Grayson *et al.*, 2000a). The entire operon does not contain either *Stu*I or *Bam*HI sites so in order to determined the number of copies of the rRNA operon blots of either *Stu*I or *Bam*HI digested genomic DNA from isolates of *R. salmoninarum*, were probed using a 1.2kb 23S rRNA probe. Digestion of *R. salmoninarum* genomic DNA with *Stu*I revealed that there was a single band present in all of the isolates which was estimated to be approximately 25kb in size (Fig: 6.8). Southern blots of *Bam*HI digests revealed that 2 bands were present in each of the 10 isolates that were examined (Fig: 6.7, panel A). The largest of the *Bam*HI bands was estimated to be 19.7kb in all of the isolates and the estimated size of the smaller band was 8.3kb in 8 isolates (970083-88, 980036-150, 980036-87, AcF6-1, DR143, F-273-87 (P-19), RS61, BY1996) 7.2kb in the type strain ATCC33209, and 7.0kb in isolate NCIMB1114.

In order to determine whether digests using enzymes that cut within the rRNA operon would show different restriction patterns, Southern blots of *Xho*I and *Spe*I digested DNA were probed with a 1.2kb 23S rRNA gene probe. Two positive bands were obtained with *Xho*I in all isolates, which varied within the range 10.9-11.1kb and 6.8-7.0kb (Fig: 6.7, panel B). Similarly, 2 bands were obtained with *Spe*I and the sizes of these were estimated to be 9.2kb and 6.6kb in all isolates (Fig: 6.7 panel C). The results show that the *R. salmoninarum* genome contains 2 copies of the *rrn* operon. In order to confirm this a 16S rDNA probe was hybridised with genomic DNA from 40 isolates (obtained from a variety

of countries in Europe and North America) that had been digested with *Ava*I. *Ava*I cleaves the *R. salmoninarum* operon at 7 points, 1 of these lies 118bp within the 3' end of the region to which the 16S rDNA probe hybridises (Fig: 6.9). In every case 3 identical bands were obtained for each isolate. The bands represent 2 fragments of 6.5kb and 3.5kb corresponding to the 2 operons and the third fragment of 775bp includes the 3' end of the 16S rRNA gene and the complete ITS1, which must be of an identical size within each operon (Fig: 6.1). This proves conclusively that *R. salmoninarum* possesses 2 copies of the rRNA operon and confirms previous work which showed that *R. salmoninarum* possesses 1 size of ITS1, 534bp (Grayson *et al.*, 1999).

6.3.5 Development and application of a 23S-5S rRNA ITS (ITS2) oligonucleotide probe

The *R. salmoninarum* ITS2 region shares no substantial sequence similarity with any sequence on the GenBank database so it was decided to investigate its potential use as a *R. salmoninarum* specific probe. Six dilutions of *R. salmoninarum* DNA from 4 different isolates were examined to determine probe sensitivity and the DNA from 15 distinct bacterial species were employed to determine probe specificity.

6.3.5.1 Specificity of a 23S-5S rRNA ITS (ITS2) oligonucleotide probe

Several bacterial species that were known to be phylogenetically related to *R. salmoninarum* as well as a number of other fish pathogens were chosen to determine probe specificity. The results of the dot blot assay demonstrated that under the conditions employed here the ITS2 probe hybridises with DNA from the 4 isolates of *R. salmoninarum* that were examined and not the DNA of 15 closely related bacterial species.

6.3.5.2 Sensitivity of a 23S-5S rRNA ITS oligonucleotide probe

To determine whether a ITS2 rDNA probe might be a useful tool for further studies of *R. salmoninarum* and BKD, the detection limits of the probe were investigated by using serial dilutions of *R. salmoninarum* DNA. A weak but positive signal was obtained from each of 4 isolates using 100ng of genomic DNA (Fig: 6.10). It appears that the ITS2 rDNA probe gave a stronger signal with the DNA of NCIMB1114 than with the DNA of the remaining 3 isolates. Because the ITS2 regions of each of the isolates has been sequenced and is identical except for 2 single base substitutions in AcF6 it may be that this occurred as the result of an error in preparing the 10 fold serial dilutions when the experiment was being performed. Nevertheless, the probe does bind faintly to 100ng of DNA from the remaining 3 isolates of *R. salmoninarum*. Unfortunately, the lack of signal intensity means that it is difficult to assess whether the sensitivity of the probe may be influenced as a consequence of ITS2 sequence variation.

Table 6.3: Percentage identity calculated from pairwise alignments between 5S and 23S rRNA genes of *R. salmoninarum* and other taxa: 5S rRNA gene (lower left) and 23S rRNA gene (upper right).

Species	1	2	3	4	5	6	7	8	9	10	11
1. *Renibacterium salmoninarum*	-	*	86.7	*	*	*	80.8	83.1	83.7	83.4	80.2
2. *Nocardia asteroids*	87.6	-	*	*	*	*	*	*	*	*	*
3. *Micrococcus luteus*	88.3	82.6	-	*	*	*	81.0	82.1	83.1	82.3	81.4
4. *Flavobacterium breve*	86.8	88.5	84.3	-	*	*	*	*	*	*	*
5. *Brevibacterium helvolum*	91.7	89.3	90.0	88.4	-	*	*	*	*	*	*
6. *Arthrobacter globiformis*	91.8	89.3	90.2	89.3	96.7	-	*	*	*	*	*
7. *Mycobacterium tuberculosis*	79.2	81.8	78.5	88.4	80.3	79.8	-	80.5	81.0	85.1	94.8
8. *Streptomyces griseus*	80.0	81.8	82.5	83.5	82.5	82.8	75.8	-	86.8	83.5	80.2
9. *Frankia sp.*	78.3	80.2	79.8	79.3	81.7	81.1	73.0	86.7	-	84.9	81.0
10. *Rhodococcus fascians*	88.4	92.6	84.3	91.0	87.6	88.6	84.3	86.7	81.8	-	84.7
11. *Mycobacterium leprae*	76.0	77.9	75.2	82.6	77.7	77.9	89.0	78.9	74.0	80.3	-

* = Sequences unavailable on the GenBank database.

The accession numbers of the sequences of the 5S rRNA and 23S rRNA genes used in this analysis are *Renibacterium salmoninarum* (5S & 23S: AF143477), *Nocardia asteroides* (5S: X55261), *Micrococcus luteus* (5S: K02682; 23S: X06484), *Flavobacterium breve* (5S: M33888), *Brevibacterium helvolum* (5S: X08003), *Arthrobacter globiformis* (5S: M16173), *Mycobacterium tuberculosis* (5S & 23S: Z73902), *Streptomyces griseus* (5S: V01347; 23S: M76388), *Frankia sp.* (5S: M55343; 23S: M88466), *Rhodococcus fascians* (5S: X15126; 23S: Y11196) and *Mycobacterium leprae* (5S & 23S: X56657).

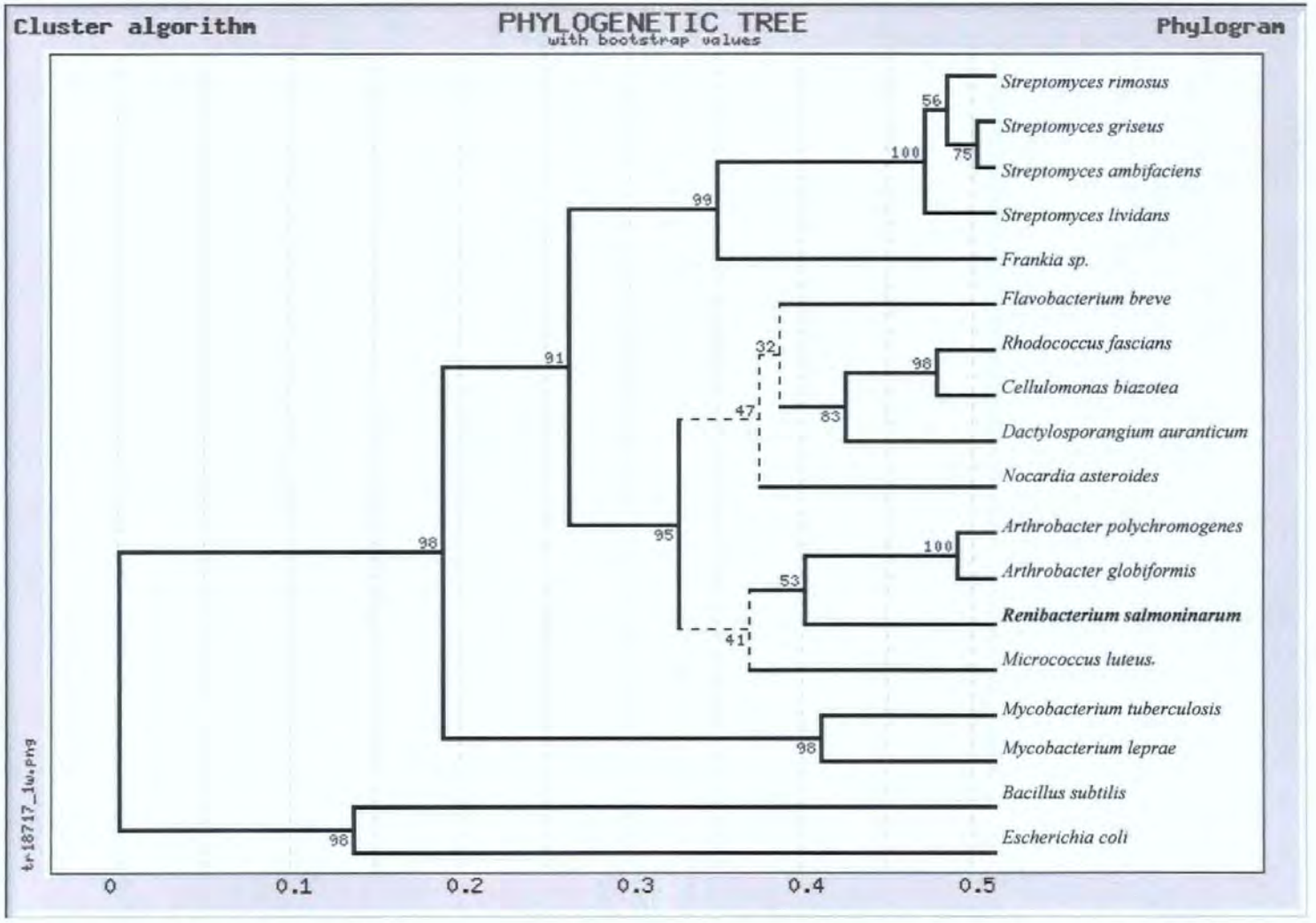


Figure 6.2: Phylogenetic relationships among representatives of the actinomycetes and selected taxa based on 5S rRNA gene sequence data. The phylogram was generated using a bootstrapped cluster algorithm to calculate phylogenetic distances.

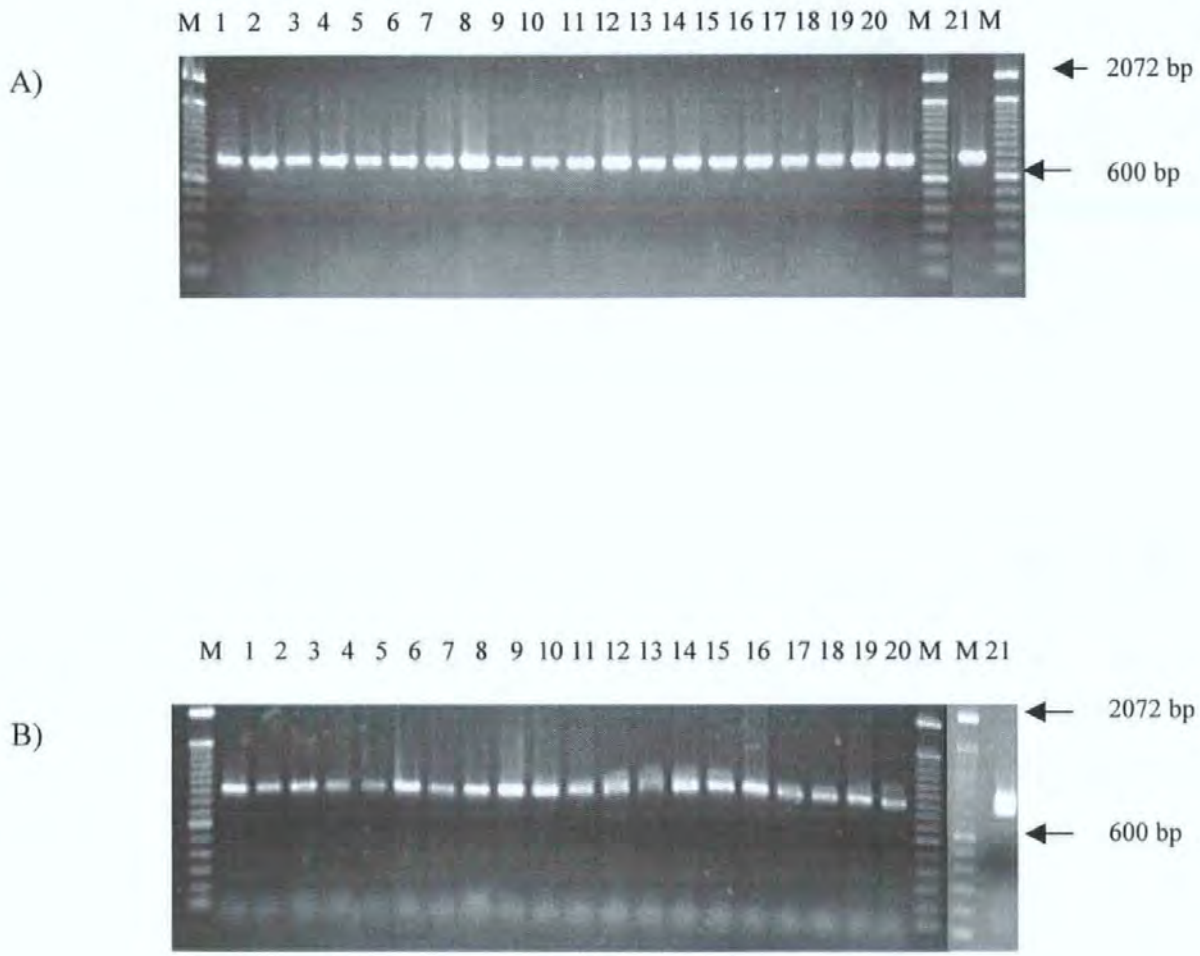


Figure 6.3: PCR amplification of the 16S-23S rRNA ITS region of *R. salmoninarum* using primers RS+1002 & ML-1339 (panel A), RS+1002 & ML-1469 (panel B). Lanes 1-21 correspond to the following isolates: MT239, MT419, MT425, MT426, MT444, MT452, MT839, MT861, MT1261, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132 and 2119#5 respectively Lane M: 100 bp ladders (0.3μg), the sizes of selected markers are indicated by arrows on the right.

```
SV1    AAGGAGCACCAAACACTTGATCAGTTTCCGCATGGTTTCTGGTGTGAGTGTGAGGAGTTAAATCCCATTGCGAGAACATTCGTTTCTCGGTGGGTGCTCAAGGGTGGAATATCAACAAAT   120
SV2    ------------------------------------------------------------G-------------------------------------------------------------

SV1    TTATGCTGAAGTGCTGGTTTTCTGGTGCGGAGGTGTTGTCGCTGGTTCTGTTCTCGGATTGTTGGTTGAGTACGCTGTTTCCTTCGGGGAGTGGTTGGAAAGATTCGATGGTTTGGGAGG   240
SV2    ------------------------------------------------------------------------------------------------------------------------

SV1    TTCTGGTGGGTACAAAACACACTGTTGGGTCCTGAGATAACAGGCGCTCTGCTGATTCTGGTACTGACTTTTGGTTGGTGTTGGAGGTCGGTGGGGTGTGTTTGTTGTTTCTGGTTTCCC   360
SV2    ----------------------------------------------A-------------------------------T-------------------------------------

SV1    TTCTTACTAATCAATCCTTTTCCGTTTGTTCGGTGGGTTGTGTGTGGGAGTGGGTGTTGTTGTTTGAGAACTGTATAGTGGACGCGAGCATCTTAAGAACAAAGCAATTTGTTTAAGAGC   480
SV2    ------------------------------------------------------------------------------------------------------------------------

SV1    AATTTTTTCATCACATCTTTTGGTGTGGTGAGAGTTTTAGCTCTGGTTTTTGTG            534
SV2    --------------------------------------------------
```

Figure 6.4: Nucleotide sequences of two sequevars, SV1 and SV2, of the 16S-23S rRNA ITS of *R. salmoninarum*. The isolates with each sequevar are stated in section 6.3.2.

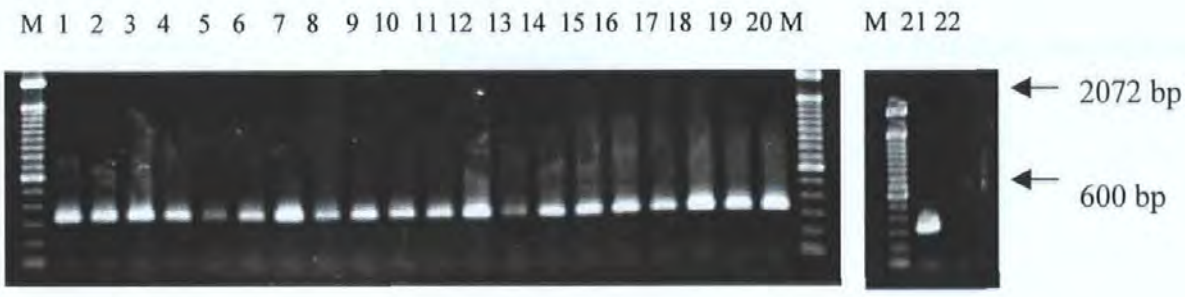


Figure 6.5: PCR amplification of the 23s-5S rRNA (ITS2) region of *R. salmoninarum* using primers R5+118 & R5-433. Lanes 1-22 correspond to the following isolates: MT239, MT419, MT425, MT426, MT444, MT452, MT839, MT861, MT1261, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132, 2119#5 and negative control respectively. Lane M: 100 bp ladders (0.3μg) the sizes of selected markers are indicated by arrows on the right.

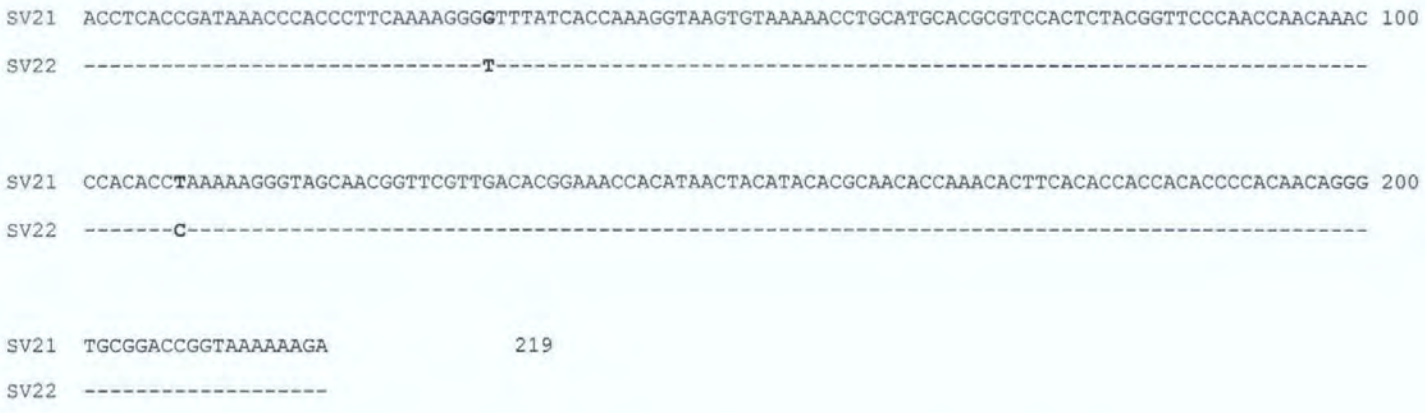


Figure 6.6: Nucleotide sequences of two sequevars, SV21 and SV22, of the 23S-5S rRNA ITS of *R. salmoninarum*. The isolates with each sequevar are stated in section 6.3.3.

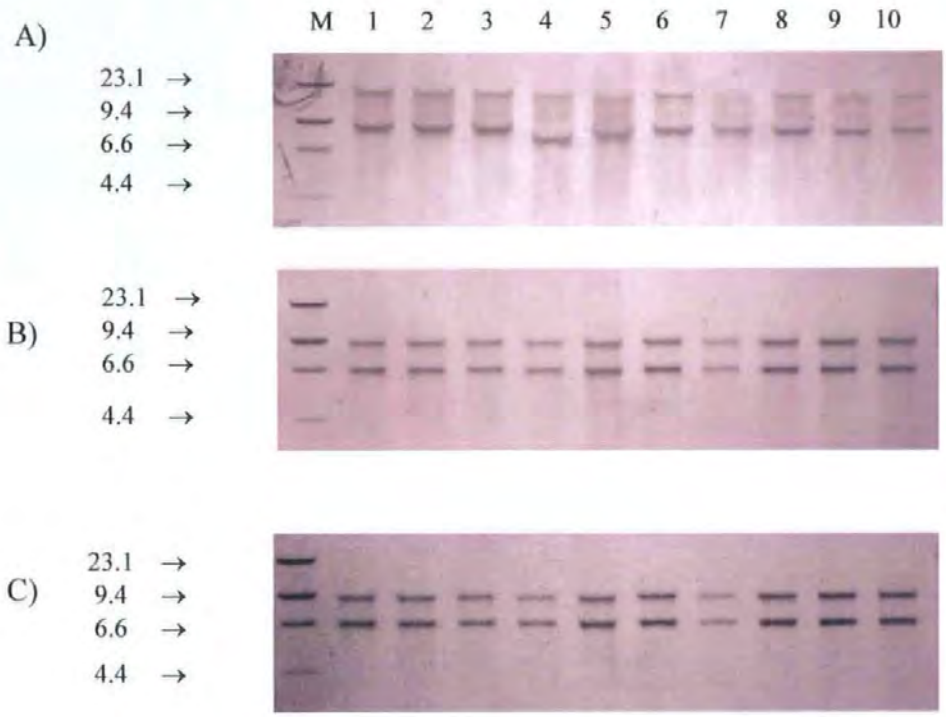


Figure 6.7: Ribotyping of genomic DNA from 10 isolates of *R. salmoninarum*. A 1.2kb 23S rDNA probe was used to hybridises to genomic DNA digested with A) *Bam*HI, B) *Xho*I, C) *Spe*I. Lanes: to 10, refer to isolates 1) 970083-88, 2) 980036-150, 3) 980036-87, 4) NCIMB1114, 5) ATCC33209, 6) AcF6-1, 7) DR143, 8) F-273-87 (P-19), 9) RS61, 10) BY1996, respectively. Lane M, DNA size markers (Roche). The molecular sizes (in kilobases) are indicated on the left.

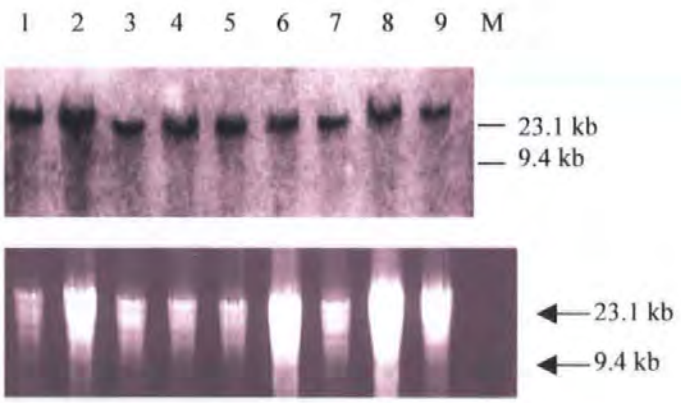


Figure 6.8: Ribotyping of genomic DNA from 9 isolates of *R. salmoninarum*. Panel A; A 1.2kb 23S rDNA probe was used to hybridise to genomic DNA digested with *Stu*I. Lanes 1 to 9, isolates 1) 980106#1.1.5, 2) 970419-1.2.3, 3) NCIMB1114, 4) ATCC33209, 5) AcF6-1, 6) DR143, 7) P-7, 8) RS9, 9) BY1996 respectively. The position of molecular weight markers (Lane M) are indicated on the right. The position of markers on the southern blot has been estimated due to the failure of markers to satisfactory develop. Panel B; Original agarose gel from which blot was generated. Electrophoresis conditions were as follows; 0.7% agarose, 90V, 60 mins.

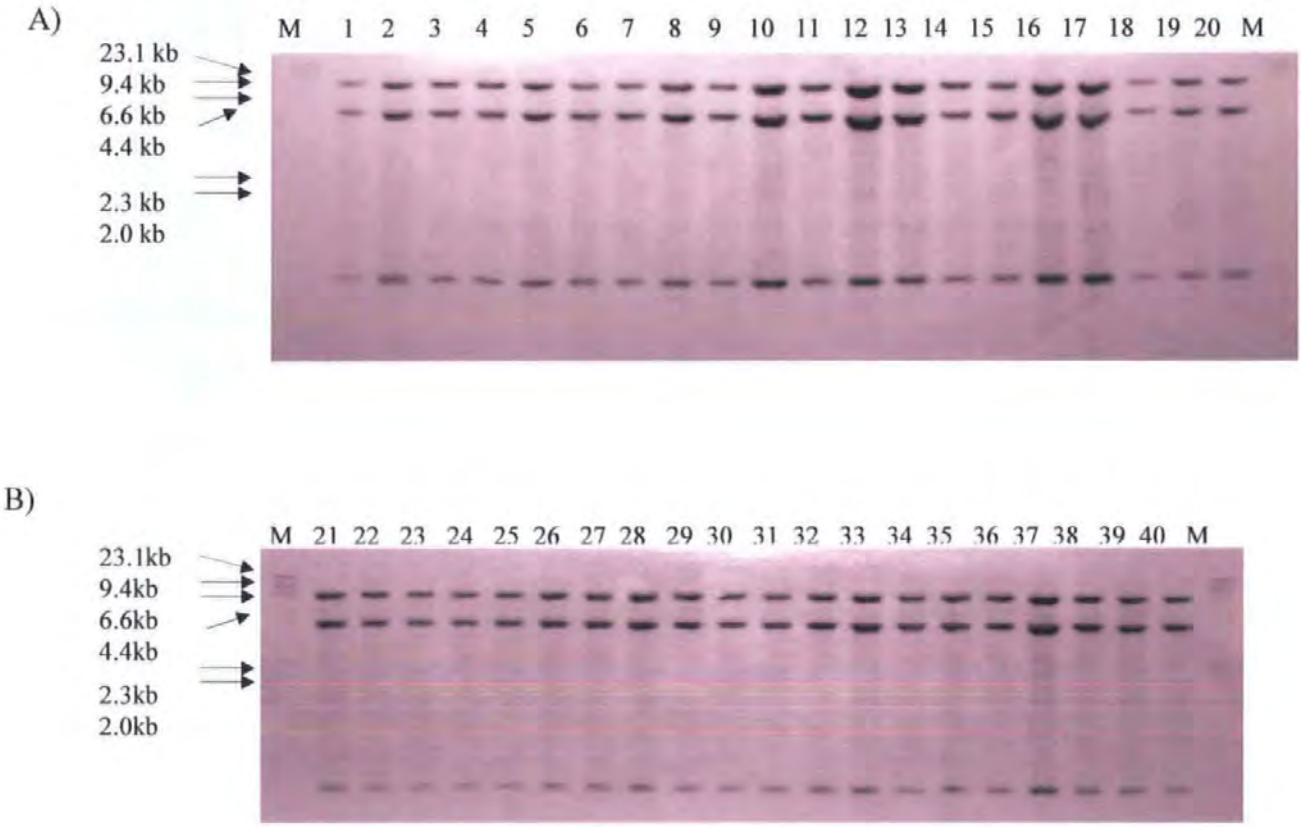


Figure 6.9: Southern blot hybridisation of *Ava*I- digested genomic DNA from 40 isolates of *R. salmoninarum*. A 724bp 16S rDNA probe was used to hybrised to detect DNA hybrids. Molecular sizes are indicated on the right. Lanes: 1, BY1996; 2, Rs 125; 3, Rs 122; 4, Rs 116; 5, Rs 61; 6, Rs 19; 7, S-182-90; 8, F-283-87; 9, F-273-87; 10, F-120-87; 11, 960046; 12, 960023; 13, 980002; 14, DR143; 15, AcF6-1; 16, Round Butte; 17, CCM6206; 18, RFL-643.94#1; 19, Idaho 91-126; 20, Cow ChS 94; 21, SS-ChS-94-1; 22, CCM6205; 23, Siletz; 24, NCIMB1116; 25, NCIMB1115; 26, NCIMB1114; 27, NCIMB1113; 28, NCIMB1112; 29, NCIMB1111; 30, F3; 31, F47; 32, F60; 33, F82; 34, F85; 35, F95; 36, 980297#97; 37, A-80; 38, A-6; 39, 970083-102; 40, 970083-88, Lane M: DNA size markers (Roche).

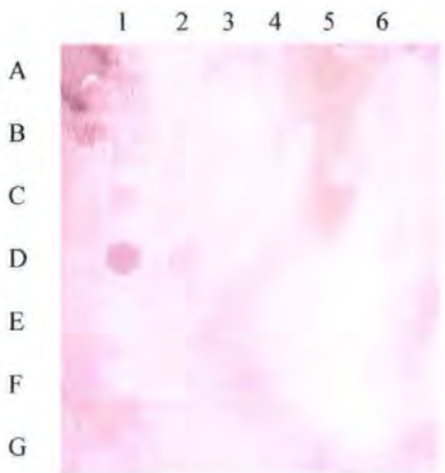


Figure 6.10: Dot blot hybridisation with a 23S-5S ITS probe. Rows A, B, C, and D contain DNA from *R.. salmoninarum* isolates 970083-102 (SV1), MT1770 (SV2), ACF6-1 (SV3) and NCIMB1114 (SV4), respectively. Lanes 1-6 contain DNA from the above isolates at decreasing concentrations: 100ng, 10ng, 1ng, 100pg, 10pg and 1pg. Rows E, F and G contain 100ng of the reference bacteria, their identities and locations are stated in Table: 6.2.

6.4 Discussion

6.4.1 Sequencing of the 23S and 5S rRNA genes and sequence analysis

The complete 23S rRNA, 5S rRNA and 23S-5S rRNA spacer region was determined for *R. salmoninarum* (Yoshimizu *et al.*, 1987). Two copies of the rRNA operon were identified within the genome, and sequencing evidence suggests that both copies are identical or nearly identical in nucleotide sequence. In most prokarytic organisms that possess multiple copies of rRNA genes sequence uniformity is usual and although it should be noted that there are exceptions including *Haloarcula marismortui*, *Thermobispora bispora*, *Thermomonospora chromogena*, *Campylobacter hyointestinalis* and *Escherichia coli* (Dennis *et al.*, 1998; Wang *et al.*, 1997; Yap *et al.*, 1999; Harrington & On, 1999; Martinez-Murcia *et al.*, 1999), the sequence variation between different copies of rRNA operons is almost entirely restricted to the ITS regions. Because the rRNA ITS regions of *R. salmoninarum* are so highly conserved, possessing from 0.19%-0.56% variation in ITS1 and 0.46% variation in the ITS2 in isolates from around the world, it is most likely that the 2 copies are identical or nearly identical. However absolute proof would require both operons to be cloned and individually sequenced.

Pairwise alignment of the *R. salmoninarum* rRNA gene sequences with the nucleotide sequences of 16S, 23S and 5S rRNA genes from other bacteria that are available on the GenBank database confirmed the close relationship with the high G+C group of actinobacteria, particularly *Arthrobacter* species and *M. luteus* (Table 6.2). The close genetic relationship between *R. salmoninarum* and *Arthrobacter* species is reflected in other important shared features, notably obligate aerobic metabolism and the possession of cell walls containing A3α peptidoglycan, lysine as the diamino acid, but lacking mycolic acids (Kusser & Fiedler, 1983; Goodfellow *et al.*, 1985).

Comparison of 5S rRNA gene sequences placed *R. salmoninarum* within the Arthrobacter-Micrococcus sub-group of the actinomycetes (Fig: 6.2). Although many studies using 5S rRNA gene sequence data for phylogenetic analysis have been reported, there have been criticisms raised as to the value of the results (Dams *et al.*, 1987; Park *et al.*, 1991; Syzmanski *et al.*, 1998). The 5s rRNA gene is small, 120 bp, and so non-random changes can magnify and distort phylogenetic distance predictions (Woese, 1987; Hori & Osawa, 1979). Although 16S rRNA gene sequences are available for many other members of the Actinomycetes, including *R. salmoninarum*, (Gutenberger *et al.*, 1991; Stackebrandt *et al.*, 1988; Stackebrandt & Schumann, 2000; Grayson *et al.*, 2000a), the use of 5S rRNA sequence data can help to confirm previous findings.

6.4.2 Sequence variation within the 16S-23S rRNA (ITS1) intergenic spacer

The sequences of the 16S-23S rRNA ITS region were determined for 28 isolates of *R. salmoninarum*. Twenty-seven of these isolates were found to possess sequevar SV1 (ITS1) supporting the findings of previous work that showed all *R. salmoninarum* isolates from England and Wales and the majority of Scottish isolates possess this sequevar (Grayson *et al.*, 1999; Grayson *et al.*, 2000b). In these previous studies it was proposed that isolates possessing sequevars other than SV1 were often sourced from regions of the world that are isolated from intensive salmonid aquaculture.

Previous work has shown that isolates from Iceland, Norway, the North West Territories of Canada, Scotland and Japan possess ITS1 sequences other than SV1. Interestingly, in previous studies the only UK isolates that were found to possess variation in the ITS1

region were the Scottish isolates NCIMB1114 and NCIMB11116. These isolates date back to 1962, before the substantial development associated with Scottish salmonid culture. However, more recently sourced Scottish isolates are SV1. SV1 may be a marker for a more successfully pathogenic or virulent strain of *R. salmoninarum* that has consequently been widely disseminated. Alternatively, the predominance of SV1 may simply reflect infrequent mutational events occurring in the genome of *R. salmoninarum* isolates, which because of their geographical isolation have not subsequently become widely distributed.

Interestingly, during this study 1 isolate, MT1770, which was sourced from a fish farm in the highlands of Scotland in 1996, was shown to be SV2. The only other isolates that are known to display this sequevar were isolated from infected fish in Japan and Iceland. Because of the lack of information, it is unknown if this isolate or indeed the farm from which it was isolated has any links with either Iceland or Japan or whether the isolate was transferred without human intervention, possibly via an infected migrating wild fish.

6.4.4 Sequence variation within the 23S-5S rRNA intergenic spacer (ITS2)

Only limited sequence variation was found to be present in the ITS2 region of *R. salmoninarum*. Of the 33 isolates that were sequenced, only a single isolate from the Northwest Territories of Canada (SV3) showed the presence of 2 single nucleotide substitutions in ITS2. This isolate had previously be shown to have a unique ITS1 sequence (Grayson *et al.*, 1999). Clearly, sequence analysis of the ITS1 region rather than ITS2 is more useful for isolate discrimination and this is probably a reflection of the smaller size of ITS2, 219 bp, compared with that of ITS1, 534 bp.

6.4.5 Ribotyping and determination of the number of rRNA genes

An investigation of the potential of ribotyping to discriminate between *R. salmoninarum* isolates showed that 2 copies of the operon exist within the genome of the pathogen. The rRNA operons were designated *rrnA* and *rrnB* on the basis of the descending sizes of the *Bam*HI bands. Whether both copies of the operon are complete and expressed awaits further study. Many other slowly growing pathogens have only 1 or 2 copies of the rRNA operon (Amikam *et al.,* 1984; Bercovier *et al.,* 1986; Frothingham & Wilson, 1993; Sela *et al.,* 1989; vanderGiessen *et al.,* 1994) and this is in keeping with the nature of *R. salmoninarum* which has a generation time of about 24 hours and is an obligate pathogen of salmonid fish (Fryer & Sanders, 1981).

6.4.6 Development of the 23S-5S rRNA ITS region as an *R. salmoninarum* specific probe

The 23S-5S rRNA ITS sequence was investigated to determine its potential as an *R. salmoninarum* specific probe. Under the conditions employed in this study the probe, although highly specific, was found to only faintly detect a minimum of 100ng of *R. salmoninarum* DNA. This level of sensitivity is insufficient for an environmental or clinical study into the distribution of *R. salmoninarum* and requires further research to elucidate how the sensitivity may be improved. The reasons for the lack of sensitivity using this probe is currently unknown. However, it is possible that the primers used for the probe amplification, RSPROBE+12 and RSPROBE-217 could be useful in a PCR-based detection system for *R. salmoninarum.*

Ultimately this chapter reported that *R. salmoninarum* possesses 2 copies of the rRNA operon, which are most likely identical or nearly identical and are highly conserved

amongst a wide variety of isolates. Whilst the analysis of the rRNA operon and intergenic spacer regions provided some indication of geographical origin there is only a limited capacity for the routine discrimination between clinical isolates of *R. salmoninarum*. The highly conserved and unambiguous nature of the sequences obtained for the *R. salmoninarum* rRNA operon and the evidence provided by DNA hybridisation using 23S and 16S rRNA probes reveals that the 2 copies of the rRNA operon are probably identical or nearly identical amongst isolates from a variety of sources. Consequently ribotyping is probably of limited use for the discrimination of *R. salmoninarum* isolates mainly because ITS1 and ITS2 of *R. salmoninarum* show limited variation in DNA sequence and no variation in length between isolates from diverse sources. This is consistent with studies, which have shown a remarkable uniformity in biochemical, serological and genetic characteristics of *R. salmoninarum* isolates (Bruno & Munro, 1986; Goodfellow *et al.*, 1985; Starliper, 1996). As randomly amplified polymorphic DNA studies have shown that molecular variation does exist between some isolates of *R. salmoninarum* (Grayson *et al.*, 1999; Grayson *et al.*, 2000b) this variation must arise largely outside the rRNA operon.

Chapter Seven

ANALYSIS OF THE tDNA INTERGENIC SPACER REGIONS OF *RENIBACTERIUM SALMONINARUM*

7.1 Introduction

tDNA-PCR is a technique that has been used to examine length polymorphisms in the spacer regions between transfer RNA (tRNA) genes and to explore the degree of relatedness between a range of bacteria. On the bacterial genome, tRNA genes are arranged in clusters which in turn are arranged within multiple tandem repeating units (Green & Vold, 1993; Vold, 1985). This makes them ideal for the PCR amplification of intergenic length polymorphisms by employing consensus primers that are annealed at low stringency. Welsh and McClelland (1991) developed 'universal' tRNA gene primers designed to face outwards from the end of the tRNA genes and these have been shown to amplify a tDNA-PCR fingerprint that is determined by the arrangement of tRNA genes on the bacterial genome. The order and arrangement of tRNA genes is highly conserved and the fingerprints generated by the consensus primers are often characteristic of a particular species. tDNA-PCR fingerprinting has been found to reliably differentiate between species of *Acinetobacter* and *Staphylococcus* sp. (Ehrenstein *et al.*, 1996; Maes *et al.*, 1997). Furthermore, specific tRNA gene primers can be developed from the DNA sequences of PCR products generated using consensus primers and this approach was used to distinguish between streptococci on the basis of tRNA gene spacer length polymorphisms (McClelland *et al.*, 1992).

Whilst the arrangement of tRNA genes on bacterial genomes is highly conserved the spacer regions between the genes can vary in length and nucleotide sequence and tDNA-

PCR can be used to discriminate between sub-species and, in some cases, isolates. Consensus tRNA primers have been used to generate divisions below species level in several studies including *Bacteroides ovatus*, *Bacillus licheniformis* and *Pseudomonas solanacearum*. In each of these studies, tDNA-PCR products were visualised on standard agarose gels and provided a means of separating isolates into 2 or more sub-groups (Daffonchio *et al.*, 1998; Seal *et al.*, 1992; Claros *et al.*, 1997).

The flanking DNA between tRNA genes and indeed the tRNA genes themselves have been reported to be prone to disruption by mobile genetic elements (Section 2.10.5). An area of the genome that is prone to such a high degree of genetic change may have the potential to differentiate between isolates in a highly conserved organism such as *R. salmoninarum*. This chapter describes how specific tRNA gene and intergenic spacer PCR primers were developed for *R. salmoninarum*, and how these primers were applied to discriminate between isolates from UK locations

7.2 Materials and Methods

7.2.1 Amplification of tRNA genes and intergenic spacer regions using consensus primers

PCR reactions were performed on DNA extracted from 59 isolates of *R. salmoninarum* from worldwide locations using 3 consensus tRNA gene primers T5A (5'-AGTCCGGTGCTCTAACCAACTGAG-3'), T5B (5'-AATGCTCTACCAACTGAACT-3'), and T3B (5'-AGGTCGCGGGTTCGAATCC-3'). Genomic DNA from these isolates was kindly provided by Dr. T. H. Grayson. PCR were performed as stated in Chapter 2 except that the primers were annealed at 50°C and 40 amplification cycles were employed (Welsh & McClelland, 1991; Welsh & McClelland, 1994).

7.2.2 Cloning of tRNA genes and intergenic spacers from *R. salmoninarum*

A variety of products that had been amplified in tDNA-PCR reactions using consensus tRNA gene primers T5B & T5A (Fig: 7.1) and T3B & T5A (Fig: 7.2) were purified and cloned into pUC18. The products were chosen from 5 isolates of *R. salmoninarum* namely NCIMB2235, Marion Forks, 970153-19, 980106#1.1.5, and NCIMB1114, that reflected diverse origins.

7.2.2.1 Cloning of PCR products into pUC18

7.2.2.2 Blunting and kinasing reaction

PCR products were individually purified using the Prep-A-Gene kit (BioRad) (Section 3.5.2). A volume of 5-10µl (~0.5µg) DNA was added to 1µl Klenow fragment of DNA polymerase, 1µl of polynucleotide kinase, 2µl of 10 X blunting/kinasing buffer, and sterile dH_2O to make up a final volume of 20µl. The mixture was then incubated at 37°C, 30min. Following blunting and phosphorylating the fragments were purified with the Prep-A-Gene kit, BioRad (section 3.5.2).

7.2.2.3 Ligation of PCR fragments in to pUC18

The "Ready to Go" Sureclone kit (Pharmacia) was then employed to clone the blunted-kinased PCR products into pre-cut pUC18. In a 1.5ml microfuge tube, dephosphorylated and pre-digested pUC18 (1µl = 50ng) was mixed with 5-8µl of the treated PCR product, 10µl of 2 X ligation buffer, 1µl of 0.1M DTT solution, 1µl of T4 DNA ligase and sterile dH_2O to a final volume of 20µl. The mixture was then incubated at 16°C, overnight.

7.2.2.4 Transformation of *Escherichia coli*

7.2.2.5 Transformation of *E. coli* K12 with recombinant plasmid DNA

Recombinant plasmids were transformed in *E. coli* DH5α using calcium chloride treatment to induce competency. *E. coli* DH5α was cultured overnight at 37°C in 10ml LB broth

(Appendix I). A 1.5ml aliquot of this culture was added to a microfuge tube and centrifuged (13,000 x g, 30s, RT). The supernatant was removed and the cells were resuspended in 200µl of pre-chilled 75mM $CaCl_2$, and incubated on ice for 30min. Following incubation the cells were pelleted (13,000 x g, 30s, RT) and then gently resuspended in 100µl of fresh ice-cold $CaCl_2$ (75mM). Approximately 60ng of plasmid DNA in a 20µl volume was added to the cells, mixed gently and the tube was left on ice for 10min. The cells were placed at room temperature for 5mins, ice for 20mins, and then heat shocked in a 42°C water bath for 2min. Pre-warmed (37°C) Luria-Bertani (LB) broth (0.9ml) was added and the transformation mixture was incubated at 37°C for 60min. Following the incubation 50µl, 100µl and 200µl of transformation mix was plated out using the spread plate technique onto LB agar containing 50µg/ml ampicillin and IPTG and X-Gal overlays.

7.2.2.6 Blue/white screening for recombinant clones

Transformants were spread onto LB agar containing ampicillin at a concentration of 100µg/µl and 4ml of a LB agar overlay containing 50µl of X-Gal (24mg/ml) and 50µl of IPTG (24mg/ml) solutions. The plates were incubated at 37°C for 24h, then examined for the presence of blue *(lac+,* non recombinant), and white *(lac-,* recombinant) colonies.

7.2.2.7 Selection and identification of recombinant clones

Following the transformation of *E. coli* DH5α with ligated DNA the recombinants were selected by blue/white screening. Recombinant plasmid DNA was isolated from *E. coli* using the Miniprep plasmid DNA extraction kit (BioRad). Following isolation recombinant plasmids were digested with *EcoRI* and *PstI* (section 3.7) and analysed by agarose gel electrophoresis (section 3.6).

7.2.3 DNA sequencing and sequence analysis of cloned products

Cloned DNA inserts were sequenced by MWG-Biotech Ltd., using a primer extension method. The full nucleotide sequences from all of the cloned amplicons can be found in Appendix II. DNA sequences were compared with sequences from other organisms that were present on the GenBank database (Altschul *et al.,* 1997). tRNA genes were identified using the tRNA-scan program available at http://www.genetics.wustl.edu/eddy/tRNAscan-SE/ (Fichant & Burks, 1991; Lowe & Eddy, 1997).

7.2.4 Amplification of tDNA gene spacer regions using specific primers

Sixteen PCR primers (Table 7.1) were designed on the basis of the nucleotide sequences of amplicons derived from PCR reactions using consensus tRNA primers (Appendix II). The primers were initially tested singly and in all possible pair wise combinations in PCR reactions for their ability to amplify reproducible amplicons which aided the subtyping of *R. salmoninarum*. The initial PCR testing utilised as template the genomic DNA that had been extracted from 7 *R. salmoninarum* isolates (980297#97, 980109F95, 980109F85, 980109F82, 980109F60, 980109F3, 980109F47). Single primers were used at a concentration of 24pmols, while pairwise combinations used 24pmol of each forward and reverse primer. In all reactions using primer T25-128, a PAGE purified primer was employed (Sigma-Genosys). All primers were annealed at 50°C and 40 PCR cycles were used. The isolates of *R. salmoninarum* that were used for tDNA-PCR analysis are listed in Table 3.2 and represent isolates of *R. salmoninarum* from UK locations.

7.2.5 Development of VNTR primers and amplification

An exact tandem repeat locus, ETR-A, was identified from the sequence analysis of tDNA-PCR products that had been amplified using consensus primers. A multiple alignment was

performed using cropped sequences obtained from isolates 980106#1.1.5, and NCIMB1114 (section 7.3). ETR-A and PCR primers 17D+94 and 17D-334 were developed to amplify this region. All PCR products were visualised on 0.8% agarose gels (Fig: 7.4). The ETR-A amplification product was sequenced from isolates 980297#97 and 980109F82 to confirm identity.

7.2.6 Dendrogram analysis

A dendrogram showing the relationship between *R. salmoninarum* isolates using tDNA-PCR and ETR-A analysis was generated with the aid of "Tools for population genetic analysis" software package (Miller, 1997). Six primer sets (ETR-A17D+94 & ETR-A17D-334, T17C+80 & T25E-128, T3C+42 & T25E-128, A35K+754 & T25D-120, A35K+754 & T17C-135, and T25E-128 alone) were selected for the analysis of *R. salmoninarum*. Nineteen bands, many of which were uniformly present in the majority of isolates, were recorded in a similarity matrix according to their presence or absence. Faint bands were excluded from the analysis. A band was scored as absent only if no visible band was present within a 2% size range. The patterns generated with the 6 primer sets were combined for each isolate, and distances were calculated by the unweighted pair group method by arithmetic averaging (UPGMA) algorithm using Nei's original distance (Nei, 1972; Nei, 1978; Sneath & Sokal, 1973).

Table 7.1. PCR primers specific to *R. salmoninarum* DNA sequences that were used to amplify tDNA intergenic spacer regions. Letters in brackets depict the tRNA anticodon.

Designation	Primer Sequence (5'-3')	Source
A35K+754	TTGGTAAGAACGAGGTCACCGGAT	tRNA gene Thr (GGU)
A35K-760	TTACCAAGAACGCGCTCTACCACT	tRNA gene Thr (GGU)
T17C+80	GACAGGATTCGAACCTGTGACCAT	tRNA gene Arg (UCU)
T17C-135	TCAGTTGGATAGAGCATCCGCCTT	tRNA gene Arg (UCU)
T25D+115	CCTTAGTTCGTAGCCAAGTGCTCT	tRNA gene Arg (UCG)
T25D-120	TGGATAGAGCACTTGGCTACGAAC	tRNA gene Arg (UCG)
T35E+128	ACCAACTGCAGCTACAGGGCCTTGC	tRNA gene Asp (GUC)
T35E-94	GTTCAAGTCCCGTCAGGGTCGCTA	tRNA gene Asp (GUC)
A7A+46	GCGTGAAAGATCTTAACCGGTGAG	tRNA ITS region
A7A-309	ATGATCGACGTCAGCTCCATCAAG	tRNA ITS region
A25A+4	CCGGTGCTCTAACCAACTGAGCTA	tRNA ITS region
A25A-247	GGTTGTTGCGTTTAGCTCAAGACG	tRNA ITS region
T3C+42	GGCTCGTGTCAAGACGGTTTTTGA	tRNA ITS region
T3C-123	TGCTCTAACCAACTGAGCTACACC	tRNA ITS region
T25E+19	AACTGAGCTAAGCGCCCTTGAGAA	tRNA ITS region
T25E-128	CGCCATTTTCTAGATCCCTTGTGC	tRNA ITS region
ETR-A17D+95	TCGCGAATAGCTTGGCCATTTTGC	Variable number tandem repeat
ETR-A17D-344	CGTAGCACCGAAGTCAGATAAGAG	Variable number tandem repeat

7.3 Results

7.3.1 PCR amplification using consensus tRNA gene primers

A series of PCR reactions were carried out using 3 consensus tRNA gene primers. These primers were designed to amplify the intergenic spacer region from the 5' and 3' ends of tRNA genes (Welsh & McClelland, 1991; Welsh & McClelland, 1992). PCR amplification of DNA templates from 59 isolates of *R. salmoninarum* resulted in fingerprints that were identical in appearance for many of the isolates regardless of their origin. Using consensus primer set T3B-T5A all of the isolates examined showed identical tDNA-PCR fingerprints consisting of 2 main bands of 105bp and 230bp (Fig: 7.1).

However, using primer combinations T5A and T5B some distinct differences were observed in some of the more diverse isolates (Fig: 7.2). Although these primers produced 2 major bands of approximately 1.1kb and 2.4kb in most of the samples, isolates 970153-19, and Marion Forks showed only a single band of 490bp and 300bp, respectively. Careful examination of the 2.4kb fragment in isolates NCIMB1114, NCIMB116, AcF6-1, F-120-87, F-138-87, S-182-90, F-130-87, F273-87, F283-87, and F283-87 revealed that this fragment was smaller than a similarly placed band that is present in other isolates (Fig: 7.2: band X).

7.3.2 Sequencing *R. salmoninarum* tRNA genes and intergenic spacers

A variety of products that had been amplified in PCR reactions using consensus tRNA gene primers were purified and cloned into pUC18. The products were chosen from 5 isolates of *R. salmoninarum* NCIMB2235, Marion Forks, 970153-19, 980106#1.1.5, and NCIMB1114, and represented some of the variations that were found in the tDNA fingerprints. The full nucleotide sequences for the all of the tDNA-PCR products can be found in appendix II. Sequencing of the clones revealed that many of the products spanned

part of $tRNA^{Ile}$ and all of $tRNA^{Asp}$, anticodon GUC. In a single clone, the $tRNA^{Thr}$ gene preceded a partial $tRNA^{Met}$ gene sequence. Two distinct and complete $tRNA^{Arg}$ genes, anticodons UCU and UCG, were identified and these were flanked by partial $tRNA^{Ala}$ and $tRNA^{Val}$ sequences, respectively (Fig: 7.3). Other partial tRNA gene sequences were found in the primer sites but could not be fully identified.

7.3.3 Specific primers

PCR primers that were specific to *R. salmoninarum* tRNA genes and tRNA ITS regions (Table 7.1) were designed according to the sequence information that was derived from the results of the consensus tDNA-PCR (Appendix II). The primers were complementary to tRNA gene sequences that encode $tRNA^{Thr}$, $tRNA^{Asp}$, and two forms of $tRNA^{Arg}$, and to tRNA intergenic spacer sequences. The primers were tested singly and in paired combinations in a series of PCR reactions in order to determine their potential to discriminate between UK isolates of *R. salmoninarum*. The results of this initial screening showed that most of the isolates possessed identical banding patterns (Appendix II and Fig: 7.4-7.6). Following initial screening 5 primer sets were selected for further analysis. PCR reactions using primers T17C+80 and T25E-128 amplified a single band of 780bp that was present in each isolate (Fig: 7.4). Similarly, primers A35K+754 and T25D-120, which spanned the intergenic region between the $tRNA^{Thr}$ and $tRNA^{Arg}$ (UCG) genes, amplified 2 bands of 650bp and 130bp that were present in all of the 43 isolates examined (Fig: 7.5). In contrast, some primers did reveal differences. Primers A35K+754 and T17C-135 amplify the spacer region between the $tRNA^{Thr}$ and $tRNA^{Arg}$ (UCU) genes. In these reactions all isolates showed 2 bands of 110bp and 680bp but isolate 970153-19 also possessed an additional band of 1.2kb (Fig: 7.6). Furthermore, the profiles produced using primers T3C+42 and T25E-128 showed 2 major bands of 200bp and 870bp in all of the isolates while an additional 300bp fragment was found to be present in 970419-1.2.3,

970153-19, A6, 980297#97, 980109F3, 980109F47, 980109F60, 980109F82 and 980109F95 (Fig: 7.4).

Using primer T25E-128 alone produced a banding pattern of up to 9 amplicons of the following sizes: 320bp, 480bp, 550bp, 660bp, 690bp, 790bp, 850bp, 1250bp and 1600bp. Of these bands a maximum of 6 were only ever present in any individual isolate. The T25E-128 primer was found to have great potential in differentiating between isolates of *R. salmoninarum*. One of the major profiles characterised by 3 of bands 690bp, 1250bp and 1600bp, and was generated by 12 of the 43 isolates that were investigated. Of the 12 isolates, 7 were from England, 2 were from Wales and 3 were from Scotland. Another major pattern was displayed by 15 Scottish isolates and contained 6 bands of 320bp, 480bp, 640bp, 690bp, 1250bp, and 1600bp. Another profile generated by 3 isolates (A6, 980109F47, and 970419-1.2.3) included the possession of 4 bands (320bp, 690bp, 1250bp and 1600bp). Five amplicons (320bp, 480bp, 690bp, 1250bp and 1600bp) were present in the tDNA-PCR fingerprint generated by isolates 980109F82, 980109F3 and 980297#97. Consequently, each of these isolates were grouped together in the dendrogram. The remaining 11 isolates produced unique profiles using the T25E-128 primer that contained different combinations of the above bands (Fig: 7.5).

Interestingly, primer T25E-128 was designed on the basis of the tDNA-PCR sequence that was obtained from the Marion Forks isolate which has been shown to be absolutely unique by RAPD despite belonging to the common SV1 sequevar of rRNA ITS1 (Grayson *et al.*, 2000b). This primer was derived from a sequence of a 220bp PCR product using consensus primers T3B and T5A (Fig: 7.1), which was found to be unique when compared with the other sequences obtained from other products, suggesting that this amplicon contains multiple bands. Interestingly, this sequence lacks homology with any other sequences on

the GenBank database. Closer inspection of the 220bp band that is present in Marion Forks suggests that this band may be smaller than the band that is similarly sized in other isolates (Fig: 7.1).

7.3.4 Identification of the ETR-A locus

Close inspection of the 2.4kb fragment produced from isolates NCIMB1114, NCIMB1116, AcF6-1, F-120-87, F-138-87, S-182-90, F-130-87, F273-87, F283-87, and F283-87 reveals that this fragment appears to be smaller than in neighbouring isolates (Fig: 7.2: band X). Sequence analysis of this fragment revealed a 51bp tandem repeat region. Only 1 copy of this tandem repeat was found in isolate NCIMB1114, while 2 copies of this repeat were identified in the sequences determined from isolates 980106#1.1.5 and ATCC33209^{T}. PCR primers ETR17D+94 and ETR17D-334 flanking this region were designed in order to amplify the locus designated ETR-A, Exact Tandem Repeat-A locus (Fig: 7.7). This allowed a simple visual inspection of the locus, with the amplification of a 301bp product from the genome of isolates possessing 2 copies of the tandem repeat and a 250bp product from isolates possessing 1 copy of the repeat unit. This PCR was then performed on an additional 28 isolates which had not been analysed by consensus tDNA-PCR (Fig: 7.8). It is clear that isolate MT1770 possess only 1 copy of the 51bp tandem repeat while all of the other isolates that were analysed possessed 2 copies. PCR products were sequenced from isolates 980297#97 and 980109F82 to confirm the identity of the region that was amplified.

7.3.5 Dendrogram analysis

Forty-three isolates of *R. salmoninarum* from the UK were examined by specific tDNA-PCR and ETR-A analysis using 6 primer sets (Figs: 7.4-7.8). A dendrogram was constructed to determine the relationship between these isolates, using 20 major bands,

combined from all of the primers that were utilised (Fig: 7.9). Ten of the bands that were scored were present (invariant) in all of the 43 isolates examined. Only clear strong bands were scored for the construction of the dendrogram, fainter bands were excluded. Four main clusters of *R. salmoninarum* isolates were observed with 11 other isolates possessing distinct and unique profiles (Fig: 7.9). Cluster 1 contained isolates from England, Wales and Scotland. Fifteen Scottish isolates were also placed into a separate cluster (Cluster 10) based on the unique profile that they generated using the primer T25E-128. Cluster 8 contained 3 isolates from England, A6, 970419-1.2.3 and 980109F47. Isolate 980109F60, that was the sole member of a distinct group, differed from these isolates by the absence of only 1 band (T25E-128: 320bp band). Cluster 12 was another group containing multiple isolates, 980109F82, 980109F3 and 980297#97, all from England.

The following isolates all generated unique profiles and were placed into separate groups: NCIMB1113, 980109F95, 980109F85, 3258#15, MT1770, MT1470, 970153-19, 980109F60, NCIMB1114 and NCIMB1116. Interestingly, although isolates NCIMB1114 and NCIMB1116 could be discriminated between they were grouped within the same sub-cluster (Fig: 7.9).

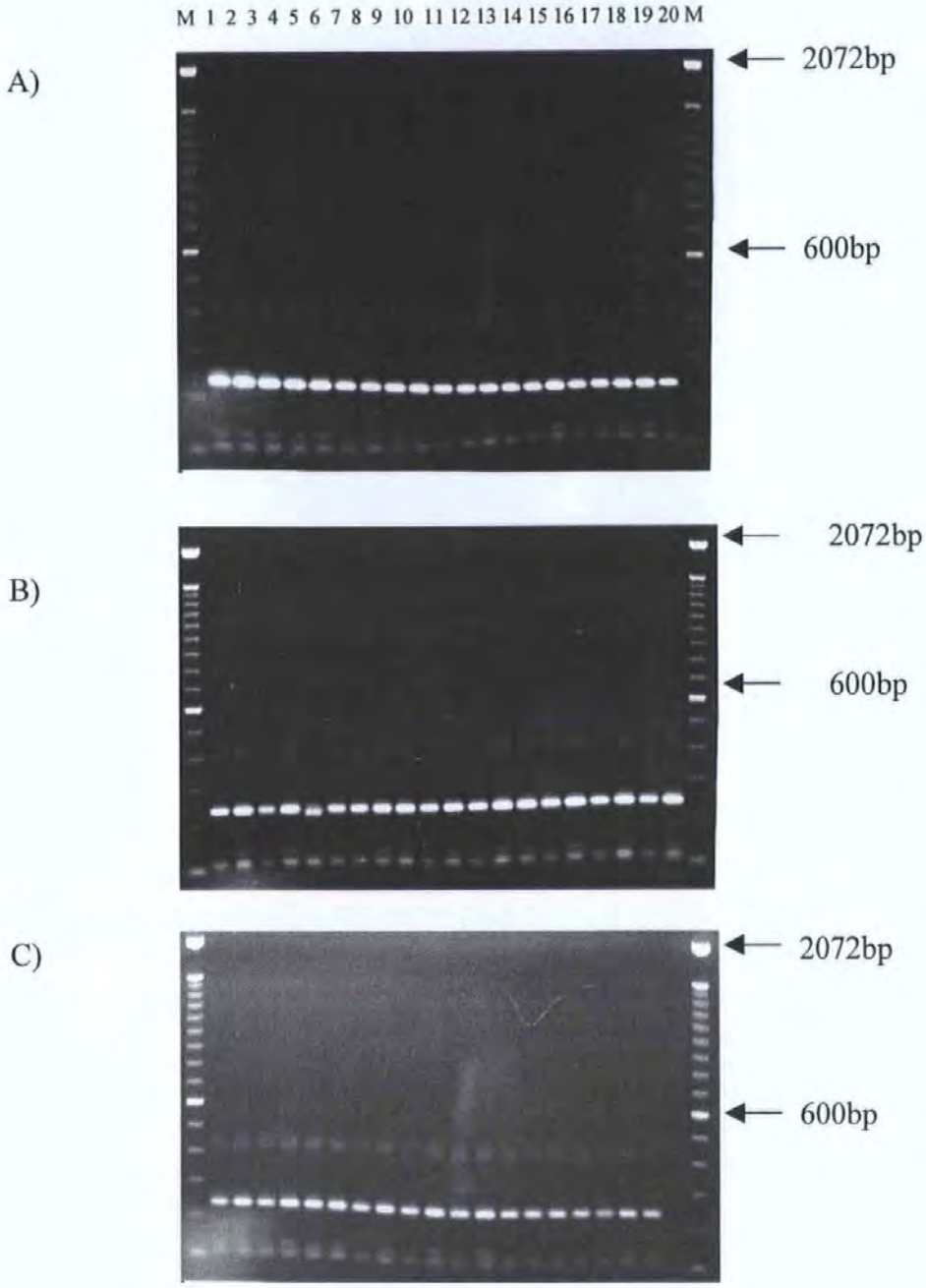


Figure 7.1: tDNA-PCR fingerprints of 59 isolates of *R. salmoninarum* from worldwide locations using primers T3B and T5A. Panel (A) lanes 1-20 correspond to isolates; 970083-88, 970083-102, 980106 #1.1.5, 980036-150, 980036-87, 970419-1.2.3, 970153-19, A6, A80, MT409, MT417, MT239, MT426, NCIMB1111, NCIMB1112, NCIMB1113, NCIMB1114, NCIMB1115, NCIMB1116 and MT420. Panel (B) Lanes 1-20 correspond to isolates MT452, MT1363, MT410, Siletz, Marion Forks, Little Goose, CCM6205, 84-019-OC, SS-ChS-94-1, Cow ChS94 P22, Idaho 91-126, RFL-643.94 #1, CCM6206, Round Butte, NCIMB2235, BY1996, AcF6-1, DR143, DR384, 960023. Panel (C) lanes 1-20 correspond to isolates 960046, F-120-87(P-2), F-130-87(P-4), F-138-87(0-78), F-273-87(P-19), F-283-87(P-10), F-358-87(P-13), S-182-90(P-7), Rs 9, Rs 19, Rs 61, Rs 116, Rs 122, Rs 125, Rs 126, 3015-86, 4451-86, RS-TSA, FT-10 and negative control. Arrows on the right indicate the sizes of selected molecular size markers.

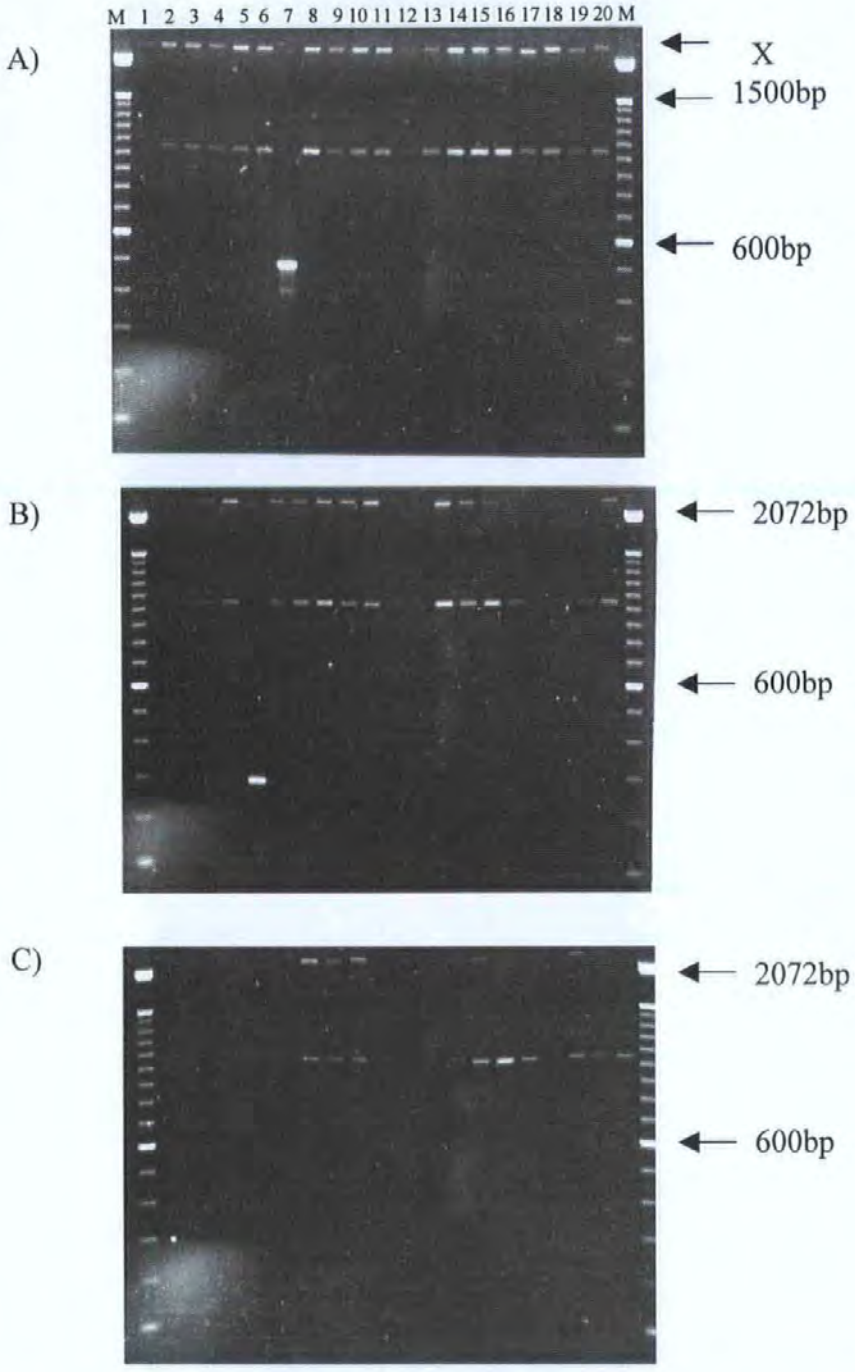


Figure 7.2: tDNA-PCR fingerprints of 60 isolates of *R. salmoninarum* from worldwide locations using primers T5B and T5A. Panel (A) lanes 1-20 correspond to isolates; 970083-88, 970083-102, 980106 #1.1.5, 980036-150, 980036-87, 970419-1.2.3, 970153-19, A6, A80, MT409, MT417, MT239, MT426, NCIMB1111, NCIMB1112, CIMB1113, NCIMB1114, NCIMB1115, NCIMB1116 and MT420. Panel (B) Lanes 1-20 correspond to isolates MT452, MT1363, MT410, Siletz, Marion Forks, Little Goose, CCM6205, 84-019-OC, SS-ChS-94-1, Cow ChS94 P22, Idaho 91-126, RFL-643.94 #1, CCM6206, Round Butte, NCIMB2235, BY1996, AcF6-1, DR143, DR384, 960023. Panel (C) lanes 1-20 correspond to isolates 960046, F-120-87(P-2), F-130-87(P-4), F-138-87(0-78), F-273-87(P-19), F-283-87(P-10), F-358-87(P-13), S-182-90(P-7), Rs 9, Rs 19, Rs 61, Rs 116, Rs 122, Rs 125, Rs 126, 3015-86, 4451-86, RS-TSA, FT-10 and BY1996. Fragment X depicts the 2.4kb fragment that differs in size between isolates by 51bp. Selected molecular size markers are indicated by arrows on the right.

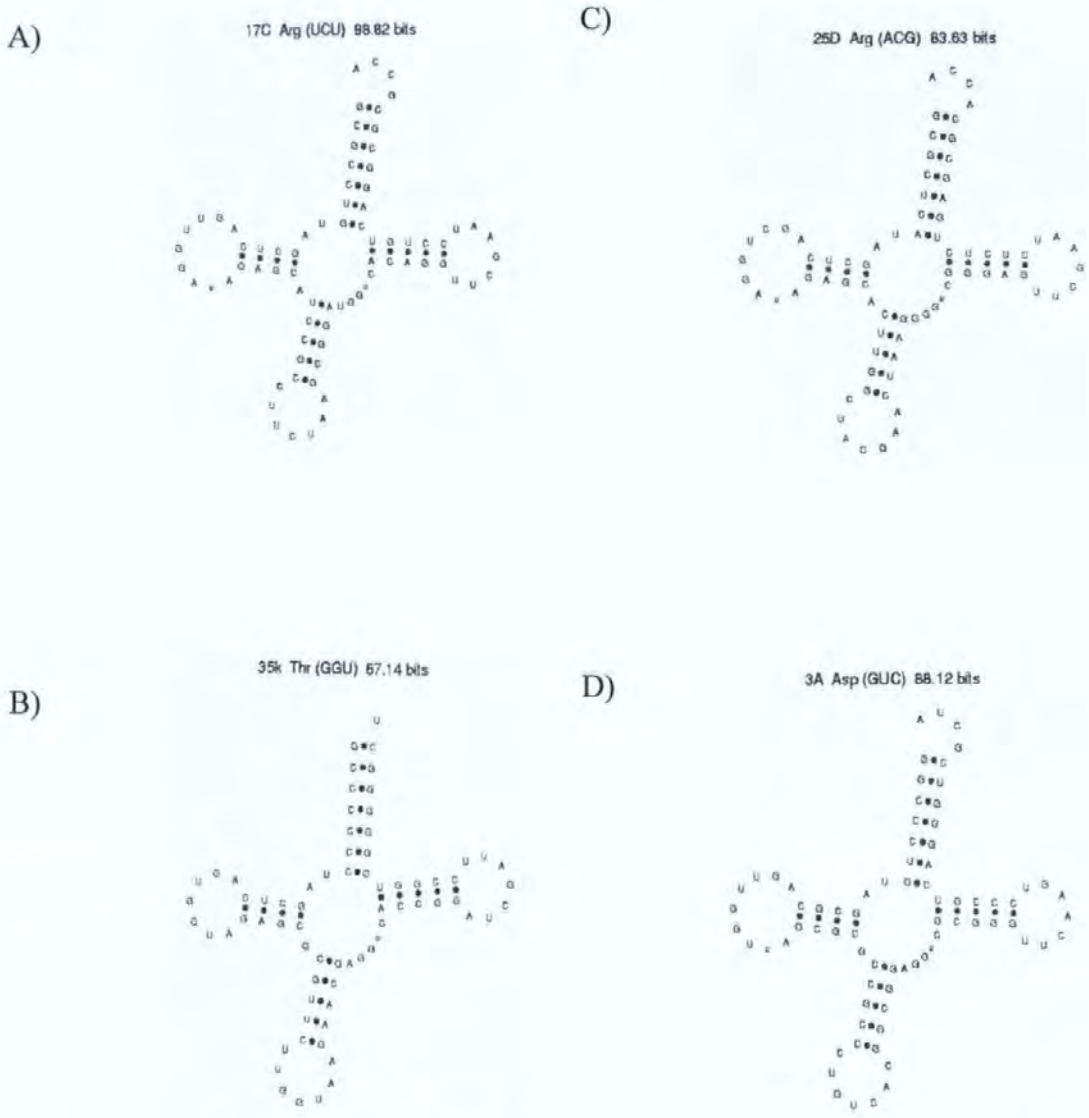


Figure 7.3: tRNA gene models. Produced from the sequences of PCR products amplified using consensus tRNA gene primers. Panel A depicts a $tRNA^{Arg}$ (anti-codon UGU) molecule, which was sequenced from a 350bp PCR product generated using primers T3B and T5A (isolate NCIMB1114). Panel B depicts a $tRNA^{Arg}$ (anti-codon ACG) molecule, which was sequenced from a 250bp PCR product generated using primers T3B and T5A (isoalte Marion forks). Panel C depicts a $tRNA^{Thr}$ molecule (anticodon GGU), which was sequenced from a 950bp PCR product generated using primers T5A and T5B (isolate ATCC2235). Panel D depicts a $tRNA^{Asp}$ molecule (anticodon GGU), which was sequenced from a 350bp product generated using primers T3B and T5A (isolates Marion forks, 970153-19, ATCC2235 and 980106 #1.1.5).

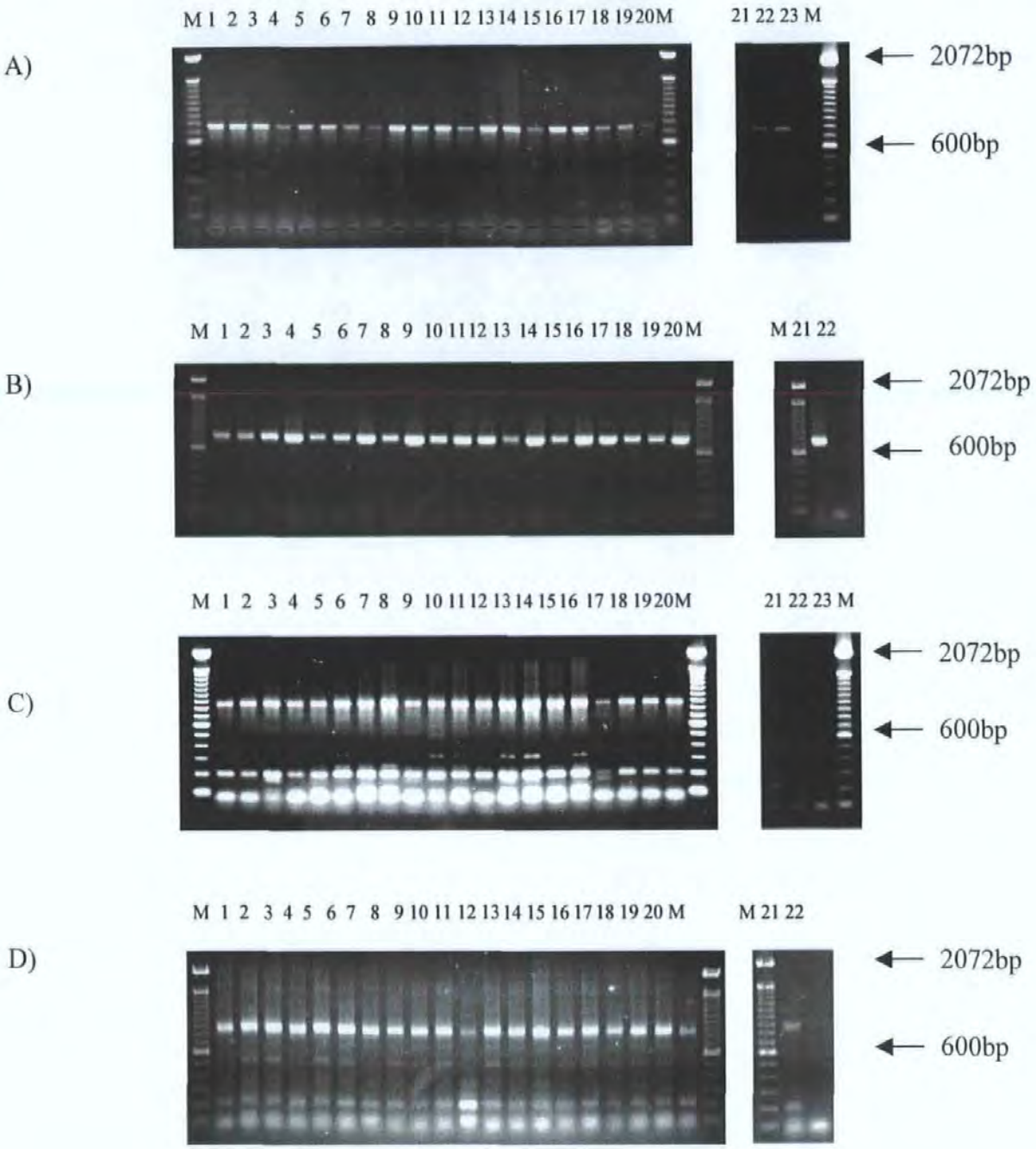


Figure 7.4. tDNA-PCR fingerprints of isolates of *R. salmoninarum* obtained using specific tRNA gene primers. Using primers T17C+80 and T25E-128 (Panel A and B) and T3C+42 and T25E-128 (Panels C and D). Lanes 1-23 in panels A and C correspond to the following isolates:1; 970083-88, 2; 970083-102, 3; 98106#1.1.5, 4; 980036-150, 5; 980036-87, 5; 970419-1.2.3., 7; 970153-19, 8; A6, 9; A8, 10; 980297#97, 11; F95 12; F85, 13; F82, 14; F60, 15; F47, 16; F3, 17; NCIMB 1111, 18; NCIMB 1112, 19; NCIMB1113, 20; NCIMB 1114, 21; NCIMB 1115, 22; NCIMB 1116, 23; negative control. Lanes 1-22 in panels B and D correspond to the following isolates: 1; MT239, 2; MT419, 3; MT425, 4; MT426, 5; MT444, 6; MT452, 7; MT839, 8; MT861, 9; MT1261, 10; MT1351, 11; MT1469, 12; MT1470, 13; MT1511, 14; MT1770, 15; MT1880, 16; MT2118,17; MT2119, 18; 3258#15, 19; 3506W134, 20; 3506W132, 21; 2119#5, 22; negative control. Selected molecular size markers are indicated by arrows on the right.

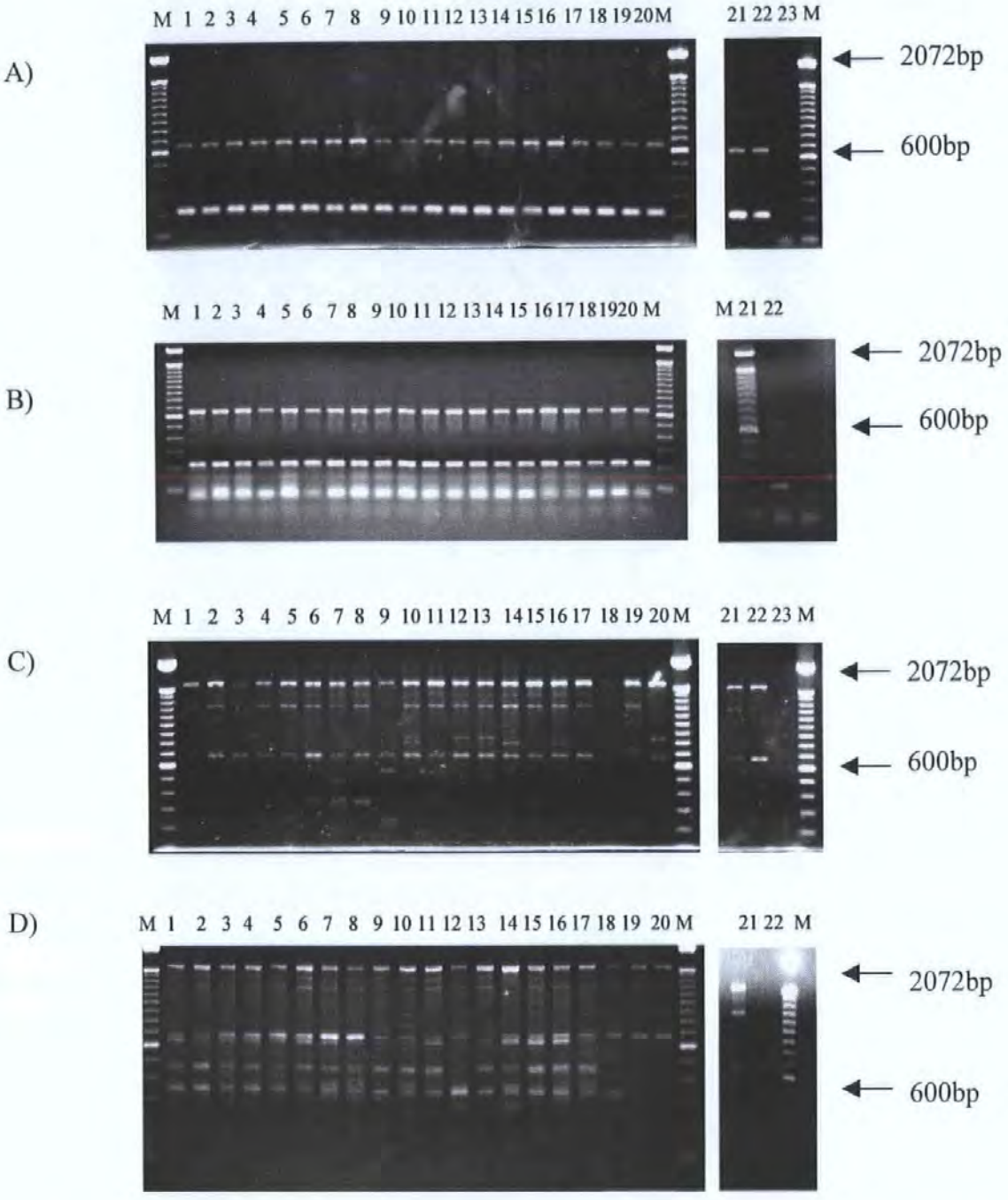


Figure 7.5: tDNA-PCR fingerprints of isolates of *R. salmoninarum* generated using specific tRNA gene primers. Using primers A35K+754 and T25D-120 (Panel A and B) and T25E-128 (Panels C and D). Lanes 1-23 in panels A and C correspond to the following isolates:1; 970083-88, 2; 970083-102, 3; 98106#1.1.5, 4; 980036-150, 5; 980036-87, 5; 970419-1.2.3., 7; 970153-19, 8; A6, 9; A8, 10; 980297#97, 11; F95 12; F85, 13; F82, 14; F60, 15; F47, 16; F3, 17; NCIMB 1111, 18; NCIMB 1112, 19; NCIMB1113, 20; NCIMB 1114, 21; NCIMB 1115, 22; NCIMB 1116, 23; negative control. Lanes 1-22 in panels B and D correspond to the following isolates: 1; MT239, 2; MT419, 3; MT425, 4; MT426, 5; MT444, 6; MT452, 7; MT839, 8; MT861, 9; MT1261, 10; MT1351, 11; MT1469, 12; MT1470, 13; MT1511, 14; MT1770, 15; MT1880, 16; MT2118, 17; MT2119, 18; 3258#15, 19; 3506W134, 20; 3506W132, 21; 2119#5, 22; negative control. Lane M; 100bp DNA ladder [Gibco BRL]. Selected molecular size markers are indicated by arrows on the right.

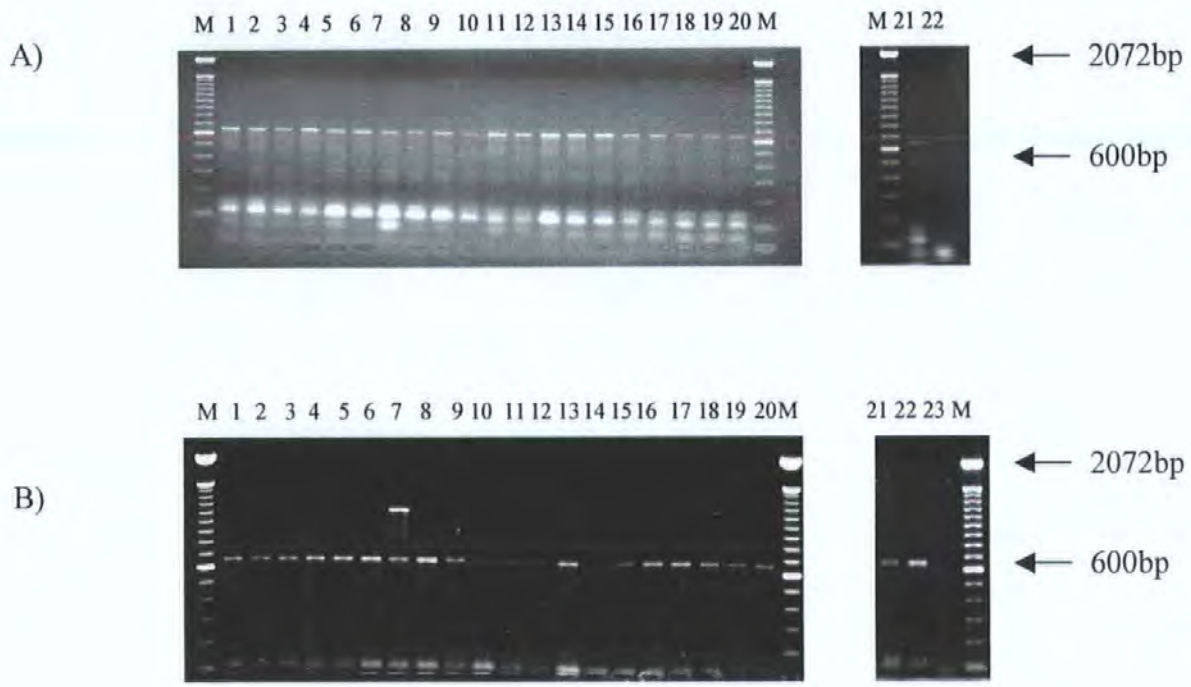


Figure 7.6: tDNA-PCR fingerprints of isolates of *R. salmoninarum* obtained using specific tRNA gene primers. Using primers A35K+754 and T17C-135. Lanes 1-22 in panel A correspond to the following isolates: 1; MT239, 2; MT419, 3; MT425, 4; MT426, 5; MT444, 6; MT452, 7; MT839, 8; MT861, 9; MT1261, 10; MT1351, 11; MT1469, 12; MT1470, 13; MT1511, 14; MT1770, 15; MT1880, 16; MT2118, 17; MT2119, 18; 3258#15, 19; 3506W134, 20; 3506W132, 21; 2119#5, 22; negative control. Lanes 1-23 in panel B correspond to the following isolates:1; 970083-88, 2; 970083-102, 3; 98106#1.1.5, 4; 980036-150, 5; 980036-87, 5; 970419-1.2.3., 7; 970153-19, 8; A6, 9; A8, 10; 980297#97, 11; F95 12; F85, 13; F82, 14; F60, 15; F47, 16; F3, 17; NCIMB 1111, 18; NCIMB 1112, 19; NCIMB1113, 20; NCIMB 1114, 21; NCIMB 1115, 22; NCIMB 1116, 23; negative control. Lane M; 100bp DNA ladder [Gibco BRL]. Selected molecular size markers are indicated by arrows on the right.

Figure 7.7: Nucleotide sequence of the ETR-A locus contains either 1 (ETR-A1) or 2 (ETR-A2) copies of a 51bp tandem repeat. The priming sites of 17D+94 and 17D-334 are denoted in bold.

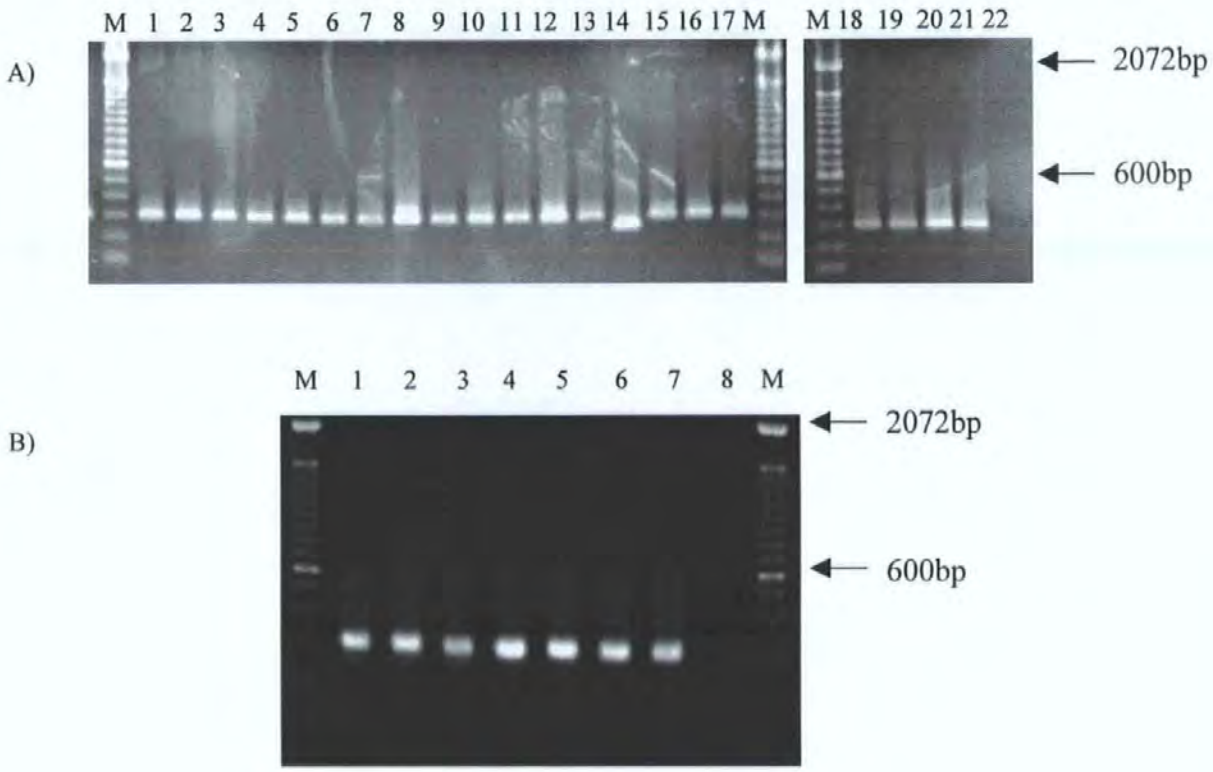


Figure 7.8: PCR amplification of the ETR-A locus (ETR-A17D+94 and ETR-A17D-334) from isolates of *R. salmoninarum* of UK origin. Panel A, Lanes 1-21 correspond to the following isolates, MT239, MT419, MT425, MT426, MT444, MT452, MT839, MT861, MT1261, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132, 2119#5. Lane 22, negative control. Panel B Lanes 1-7 correspond to the following isolates: 980297#97, 980109F95, 980109F85, 980109F82, 980109F60, 980109F47 and 980109F3. Lane 8 negative control. Lane M: 100bp ladders (0.3µg). Selected molecular size markers are indicated by arrows on the right.

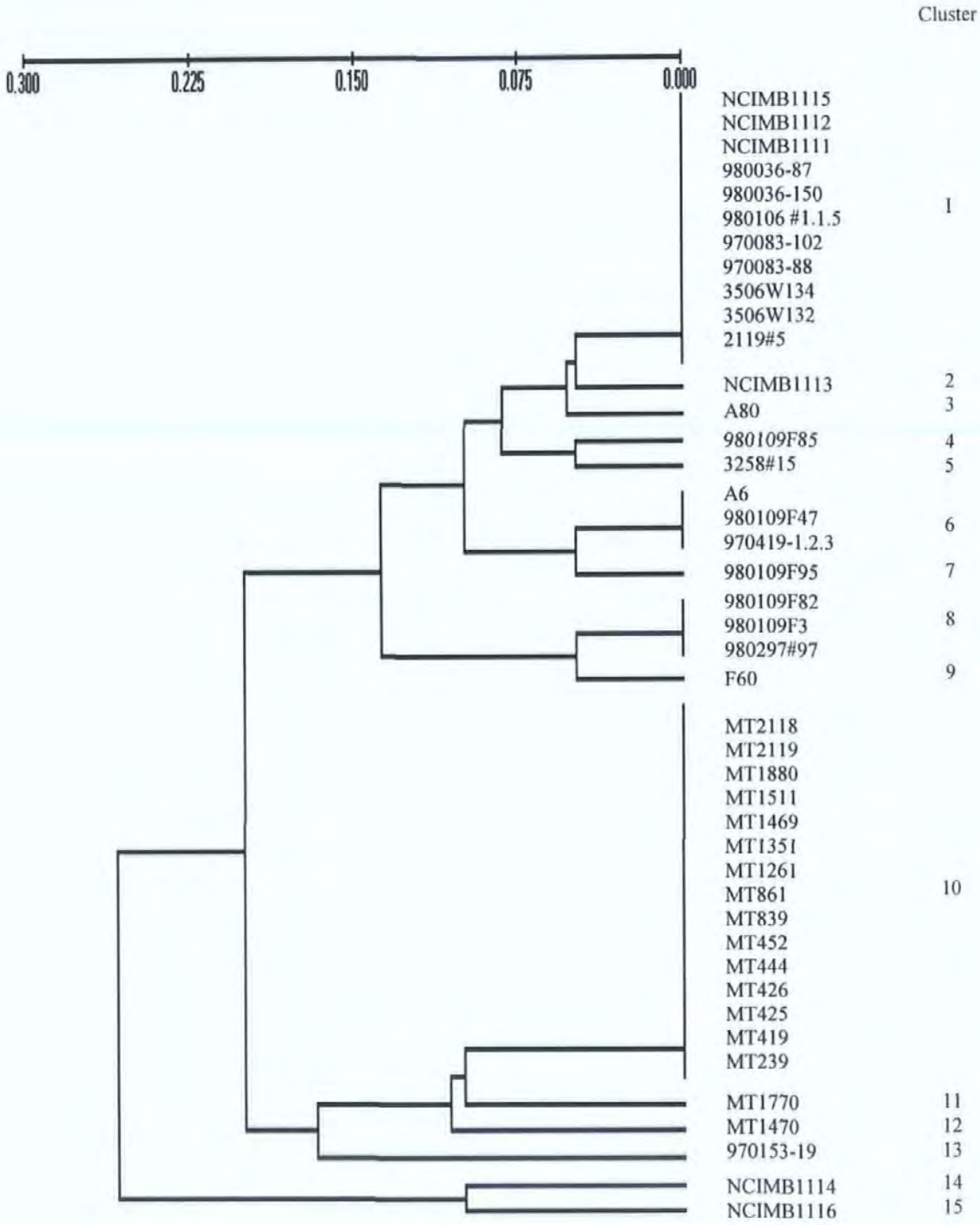


Figure 7.9: A UPGMA generated dendrogram from tDNA-PCR analysis of 43 isolates of *R. salmoninarum* from UK locations. The scale bar denotes a relative difference between the isolates where 0.01 is equivalent to 1% in branch lengths.

7.4 Discussion

This study examined the contribution of tRNA intergenic spacer regions to the genetic variation that is present in the genome of 43 isolates of *R. salmoninarum.* The method employed a PCR-based approach that was simple and reliable, enabling divisions to be made between isolates of *R. salmoninarum* that had been found to be identical using other methods.

7.4.1 Consensus tRNA gene primers

Consensus tRNA gene primers were used to obtain fingerprints from 59 isolates of *R. salmoninarum.* Welsh and McClelland (1991; 1992) speculated that because of the highly conserved nature of tRNA genes and their order on the cistron the products generated using consensus primers are often characteristic of a species or even a genus and this has been apparent in a number of other studies. In this study, 56 isolates of *R. salmoninarum* produced identical profiles using consensus primers. However, isolates 970153-19, Marion Forks, and AcF6-1 produced different fingerprints when using consensus primers. This is in good agreement with previous work by Grayson *et al.* (1999), who demonstrated that all 3 of these isolates produced unique RAPD profiles. Grayson *et al.* (1999), also demonstrated that isolate AcF6-1 has a unique 16S-23S rRNA ITS sequence.

It is clear from examining the profiles generated, that both sets of consensus primers amplified more than 1 major PCR product for each isolate. This probably occurred because the products are derived from a multiple of tRNA genes from the same or different tRNA gene cluster (Honeycutt *et al.,* 1995). Sequence analysis of the cloned PCR products confirmed this. It was also discovered from sequencing that some of the products were not amplified from tRNA genes but were in fact fragments produced by the arbitrary

amplification of unrelated loci. This occurred in a small number of cases only, and is unsurprising as the primers used were designed to consensus sequences.

7.4.2 Specific tDNA-PCR and dendrogram analysis

Primers complementary to specific tRNA genes were designed in order to discriminate between and assist in determining the epizootiological relationships amongst a selection of isolates of *R. salmoninarum* from the UK. Sixteen primers were designed and tested in single and pairwise combinations initially using 7 isolates of *R. salmoninarum.* The results of this initial screening showed that most of the isolates possessed identical banding patterns (Appendix III). Following the initial screening, a further forty-three isolates were examined using a subset of 5 selected primers. The PCR reaction using primers A35K+754 and T17C-135 that amplifies the spacer region between the $tRNA^{thr}$ and $tRNA^{arg}$ genes produced 2 bands of 110bp and 630bp in 42 of the isolates examined. It is possible to generate 2 or more products from primers that correspond to specific tRNA genes because these genes are present in multiple copies within clusters, which are in turn arranged within multiple tandem repeat units. Amplicons can be generated from both within and between these multiple repeats (Green & Vold, 1993; Honeycutt *et al.*, 1995; Seal *et al.*, 1992). Isolate 970153-19 also possessed an additional band, suggesting there is some fundamental difference either in the sequence or the order of the genes encoding tRNA or its associated intergenic spacer regions. Due to their nature, tRNA genes are often targets for the insertion of mobile genetic elements. It is possible that such a process may be responsible for this feature of the 970153-19 genome and has lead to the amplification of this additional band. Nucleotide sequencing and restriction fragment mapping would resolve this issue.

Primer set A35K+754 and T25D-120 generated 2 bands of 220bp and 650bp. These primers were designed to the tRNA genes threonine (anticodon GGU) and arginine (anticodon UCG), respectively, and the size of the amplicons probably reflects the existence of at least 2 different tRNA gene clusters containing these genes. However, this primer set failed to discriminate between the 43 isolates of *R. salmoninarum* that were examined. Similarly, primers T17C+80 and T25E-128 also failed to differentiate between any of the isolates examined.

In contrast, some of the primer sets were found to be more useful in discriminating between isolates of *R. salmoninarum*. The tDNA-PCR profiles that were produced using primers T3C+42 and T25E-128, showed bands of 200bp and 870bp. Nine isolates of UK origin also generated an additional 300bp band. The presence of this band enabled the isolates to be divided into 2 clear groups. However, primer T25E-128 used alone proved to be most useful at discriminating between *R. salmoninarum* isolates and generated 10 different amplification patterns between the 43 isolates. Further visual differences could be observed in addition to the 10 groupings e.g. isolate 980109F95 had a faint 350bp, band that was omitted in the interests of reproducibility when scoring the data for dendrogram construction (Fig: 7.5: Panel C).

A dendrogram was constructed from the data that was obtained from tDNA-PCR and ETR-A analysis. A total of 20 bands were scored of which 10 were invariant and the 43 UK isolates were distributed into 15 clusters, with 29 isolates placed into either 1 of 2 clusters (clusters 1 or 10). Ten of the isolates that were examined generated unique profiles. Although tDNA-PCR is a sufficiently discriminatory typing method that is able to distinguish a reasonable number of groupings, the isolates are not equally distributed across the groups with 67% of isolates being placed into 2 out of a possible fifteen clusters

highlighting that the majority of UK isolates of *R. salmoninarum* are highly conserved. This result also suggests that the introduction of *R. salmoninarum* from external sources has occurred infrequently.

This study has highlighted that tRNA genes and their respective spacer regions represent variable regions on the *R. salmoninarum* genome. tRNA genes have consistently been reported to be sites for the integration of mobile genetic elements (Section 2.10.5). Due to their highly conserved and repeated nature tRNA genes are prone to the insertion of mobile DNA and indeed may play a key role in the evolution of microbial pathogens. It is possible that the variation within tRNA genes and their spacer regions, observed in this study could be a reflection of this process.

During this study it has been shown that tDNA-PCR is highly discriminatory. Of particular interest was the finding that some of the English isolates that had been cultured from fish held on the same farm and sampled at the same time (980109F95, 980109F85, 980109F82, 980109F60, 980109F47 and 980109F3) possessed different tDNA-PCR fingerprints, a difference that has been further confirmed by both RAPD and IS*994* RFLP profiling (Chapter 5 and Chapter 8, respectively). It can be speculated that the groups may have emerged on the farm over the course of time from an initial isolate, in which case the selective pressure of antibiotic and chemical treatments may have played some role in genetic modification. Alternatively, there may have been successive introductions from external sources which are related to differences in the hatchery supply of eggs, the cohorts of fish held on the farm, and the identity of the broodstock. Interestingly, 980106#1.1.5, 980036-87 and 980036-150 were also isolated from fish held on two farms, one in Wales and one in England, with a common hatchery source. It is unfortunate that the full details

of the exact origins of all of the fish stocks from which *R. salmoninarum* was isolated are unavailable.

In some instances however, tDNA-PCR failed to discriminate between a range of isolates. Figure 7.9 shows that 11 isolates from six apparently unrelated sources could not be differentiated and were grouped together into cluster 1. The reason for this grouping remains unclear although the possibility that they may share a common ancestor cannot be excluded. Similarly tDNA-PCR did not discriminate between the more recent Scottish isolates, 15 Scottish isolates were grouped together in cluster 10. This suggests that the recent nature of BKD in Scotland, as in England, does not lend itself to either regular input from external sources or the rapid evolution of new clones. The arrangement of a majority of isolates into 1 or 2 groups probably reflects the highly conserved nature of the bacterium, which has been noted by others (Grayson *et al.*, 1999; Grayson *et al.*, 2000b; Starlipper, 1996) and also the possibility that in some environments the genome of *R. salmoninarum* does not rapidly diverge and may represent the result of the dissemination of a very few clones.

The tDNA-PCR analysis did indicate unique profiles for the Scottish isolates MT1770 and MT1470 and this is in agreement with the results of the RAPD analysis. Interestingly, isolate MT1770 possesses an uncommon sequevar SV2 of the 16S-23S rRNA ITS region and was grouped with a selection of Icelandic rather than Scottish isolates when IS*994* profiling was applied. MT1770 was originally isolated from a seawater farmed Atlantic salmon originating from the highlands of Scotland. No further details are available to shed light on the possible route of transmission of this isolate but it is unique amongst Scottish isolates in possessing this sequevar. A unique tDNA-PCR profile was also generated by isolate MT1470 which was derived from a freshwater fish farm in Perthshire, Scotland.

Interestingly, tDNA-PCR analysis show that this isolate bears no resemblance to isolate MT1469, which was also isolated from a freshwater fish farm in Perthshire only 5 days later. Enquiries with the relevant authorities at Fisheries Research Service, Scotland failed to reveal whether both of these isolates were from the same or different fish farms.

It should be remembered that the specific tRNA primers were initially screened against 7 English isolates with the intention of selecting discriminatory primer sets. This may help explain why the primers that were finally selected for screening a larger isolate collection proved to be more useful for discriminating between English rather than Scottish isolates. It is possible that some of the 16 primers that were initially developed and found to, produce identical profiles when tested against the 7 English isolates could prove to be useful in discriminating between this group of Scottish isolates.

Interestingly when comparing the tDNA-PCR isolate groupings with the groupings generated by some of the other methods used in this thesis they appear to be in relatively good agreement. Some UK isolates that were grouped together by either IS*994* RFLP or RAPD were found to be different using tDNA-PCR fingerprinting and *vice versa*. For example, it was possible to differentiate between isolates NCIMB1114 and NCIMB1116 by using tDNA-PCR (Fig: 7.9) and IS*994* profiling (Chapter 8, Fig: 8.6), even though the isolates were shown to be identical using RAPD (Grayson *et al.*, 2000b). On the other hand, isolates 970419-1.2.3 and A6 were found to be different in the RAPD-based study by Grayson *et al.* (2000b), but were identical using tDNA-PCR. Unfortunately isolate 970419-1.2.3 was not examined by IS994 profiling. These 3 methods for discriminating between isolates of *R. salmoninarum* measure molecular variation in different ways. RAPD and IS*994* analyses measure strain variation throughout the genome while tDNA-

PCR is restricted to variation within the tDNA intergenic spacer regions, and so this outcome is to be expected.

When comparing tDNA-PCR, RAPD profiling, and RFLP IS*994* analysis with regards to *R. salmoninarum* it can be deduced that the techniques are of similar discriminatory power. Although the techniques highlighted differences between some isolates that were found to be identical using an alternative method the results using the 3 methods have been shown to be complementary rather than contradictory. It is recommended that tDNA-PCR, RAPD-profiling and RFLP IS*994* analyses should be performed in order to more fully understand relationships that may exist between isolates of *R. salmoninarum*. Each technique complements the information that can be gained from the other methods and therefore allows more confidence to be attached to the outcome.

7.4.4 Properties of tDNA-PCR as a typing system

This study revealed that tDNA-PCR possesses high discriminatory ability and was easy and cheap to perform. Although these are 3 very important features of a typing system there are others to be considered. One criticism that has been made of many typing systems and can also be applied to this method is that the bacterial fingerprints that are generated do not reflect biological function or any significant difference between isolates that reflects real and relevant population diversity. This method examines the spacer regions between tRNA genes and as these are non-coding it is possible that their stability could be questioned (Burr *et al.*, 1998). The stability of a typing method is the ability to express constant markers over time and generations and the method should be stable for at least the time frame of the investigation. Struelens (1998) stated that when developing a typing system, stability is commonly overlooked and that the accuracy of the epidemiological interpretations generated from the application of the majority of molecular typing systems

developed for bacterial pathogens still requires additional research into the evolution rate of polymorphic loci in bacteria. Clearly the stability of tDNA-PCR has not been examined and if this method is to be routinely applied then this should be explored. The examination of a specific gene locus often provides a more stable region within which to examine polymorphisms. However, in the case of *R. salmoninarum* an examination of specific genes revealed no variation and it is for this reason that unknown (RAPD) or more variable regions of the genome (IS*994* insertion sequence or tDNA ITS regions) have been explored (Chapter 3).

In addition to stability and ease of interpretation, reproducibility is a very important factor of a typing system that should also be considered. Many factors have been shown to affect PCR amplification such as: the method of DNA extraction, brand of *Taq* polymerase, colony age, type of thermo-cycler and primer, magnesium and DNA template concentrations can affect the outcome of a PCR. Using the conditions stated in section 7.2.4 the tDNA-PCR profiles were found to be reliable and reproducible. However if any of the amplification conditions or the reagent concentrations were to be altered the affect on reproducibly should be considered.

Other concerns that could be expressed about the integrity of tDNA-PCR (and indeed has been proved in the case of the consensus primers), is that isolate profiles can be interpreted incorrectly as amplicons derived from different regions of the genome in different isolates could co-migrate. In this study the co-migration of amplicons could present a problem of interpretation as tDNA-PCR has been used not just as a basic tool to differentiate between dissimilar bacterial strains or isolates but also to generate a dendrogram inferring degrees of phylogenetic similarity between isolates. The possibility of band co-migration should always be considered when interpreting tDNA-PCR results and it should remembered that

differences that are observed between isolates reflect real differences, however, those isolates that have generated identical profiles may not necessary be identical.

7.4.5 Role of ETR-A locus in discriminating between *R. salmoninarum* isolates

The ETR-A locus was identified when the use of consensus tRNA primers revealed that there was a size difference in an amplicon of about 2.4kb in length that was amplified from different isolates (Fig: 7.2, Band X). Sequencing of a selection of these PCR products revealed a sequence of 51bp (an exact tandem repeat unit), which was located within a open reading frame. The region was designated the ETR-A locus. The majority of isolates seem to contain 2 copies of this 51bp repeat. However some isolates seem to possess only a single copy. PCR primers spanning this ETR-A locus (ETR17D+95 and ETR17D-3344) were developed and used to analyse those isolates that had not been tested using consensus tRNA primers in order to determine the number of TR regions. The majority of isolates that were examined possessed 2 copies of the 51bp repeat, however notable exceptions included NCIMB1114, NCIMB116 and MT1770. These 3 isolates have been separated from all other UK isolates because none of them possesses the SV1 sequevar of the 16S-23S rRNA ITS spacer region. Isolates NCIMB1114, NCIMB1116, and MT1770 possessed sequevars SV4, SV4, and SV2, respectively. Work by Grayson *et al.* (2000b), who studied a larger number of isolates in this area revealed that a single copy of the tandem repeat locus ETR-A was a marker for isolates possessing the SV1 sequevar of the 16S-23S rRNA ITS. In laboratories where resources are limited the simple visual sizing of a PCR product is much easier and quicker than DNA sequencing.

This chapter explored the potential of tDNA-PCR fingerprinting to discriminate between clinical isolates of *R. salmoninarum*. The technique was found to be very useful, differentiating 43 UK isolates into 15 clusters. Nevertheless, as for other typing methods

the majority of isolates were placed in to 1 of 2 main groups. The groupings that were created were found to be in good agreement with previous divisions made using RAPD profiles and RFLP IS*994* analysis (Chapters 5 and 8). When used alone or in combination with another molecular typing method tDNA-fingerprinting appears to be a useful tool for epizootiological studies of BKD outbreaks in populations of both wild and farmed fish.

Chapter Eight

IS*994* RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF *RENIBACTERIUM SALMONINARUM*

8.1 Introduction

Mobile genetic elements including transposons, plasmids, pathogenicity islands and insertion sequences (IS) can cause substantial changes in bacterial genomes. Bacterial IS are the simplest of the mobile DNA elements and are capable of transposition into diverse sites within the host genome causing changes, such as insertional mutagenesis, alteration of transcriptional activity, and genomic rearrangements. The ability of IS to replicate and transpose independently of chromosome replication means that the insertion patterns are subject to continuous variation during the life-span of a bacterial strain. The restriction fragment length polymorphic (RFLP) analysis of insertion sites has revealed much about the epidemiology and evolutionary genetics of bacterial pathogens and consequently these elements have been used for typing studies in a number of bacterial species (Stanley & Saunders, 1996). For example, IS*6110* has been used to differentiate *Mycobacterium tuberculosis* isolates and IS*1002*, IS*1001* and IS*481* have been applied for the same purpose in *Bordetella* sp. (VanderZee *et al.*, 1997).

Rhodes *et al.* (2000) reported the first and to date the only IS to have been identified in *R. salmoninarum.* The element was termed IS*994* and it is a member of the IS*3* family, group IS*51*. By conservative estimate there are at least 17 chromosomal inserts of IS*994* in the *R. salmoninarum* genome, which strongly suggests that it would be useful as a marker

for isolate differentiation. This chapter investigates how IS*994* was used as a probe in a RFLP-based study to examine the relationships between *R. salmoninarum* isolates from both UK and worldwide locations.

8.2 Materials and Methods

8.2.1 Production of probes

8.2.1.1 Randomly primed labelling of an IS994 oligonucleotide probe

A pZeor-2.1 (Invitrogen) plasmid containing an *Xba*I fragment of *R. salmoninarum* (ATCC type 33209) chromosomal DNA was kindly provided by Dr. L Rhodes (Northwest Fisheries Science Centre, Resource Enhancement and Utilisation Technologies Division, 2735 Montlake Boulevard East, Seattle, Washington 98112, USA). A 1.2kb fragment, which encoded all but 186bp at the 3' end of the IS*994* element, was excised from the plasmid with restriction enzymes *Xba*I and *Eco*RI (section 3.7). The digested products were separated by agarose gel electrophoresis (section 3.6) and the desired fragment was excised from the gel and purified using the Prep-A-Gene Kit (Section 3.5.2).

The purified fragment was labelled with digoxigenin using the randomly primed labelling method (section 3.8.3). The labelled fragment was purified before use with a Prep-a-Gene kit (section 3.5.2).

8.2.1.2 PCR labelling of an IS*994* oligonucleotide probe

An oligonucleotide probe for the detection of IS*994* was prepared and labelled with digoxygenin by PCR using primers IS3+146 (5'-GCGATGCAAAAAGTTGCCCAGCTT-3') and IS3-1241 (5'-GTGGTTTTCTAGCTCTACTGGTGG-3'), which were designed to amplify an

1124bp region of the IS3 IS*994* sequence. The 50µl PCR reaction mixture was prepared as described in section 3.8.2 except that the reaction contained 1% DMSO and 15ng genomic DNA from isolate NCIMB1111. The nucleotide sequence of the probe can be found in Appendix II.

8.2.2 Restriction fragment length polymorphism (RFLP) analysis

Genomic DNA (0.5µg -1µg) from a variety of isolates of *R. salmoninarum* from worldwide locations were digested separately with restriction enzymes *Eco*RV, *Xba*I and *Ava*I according to the methods outlined in section 3.7. The digested DNA was separated by agarose gel electrophoresis (section 3.6), transferred to nylon membranes and probed using a digoxigenin labelled IS*994* oligonucleotide probe (section 3.8).

8.2.3 Dendrogram analysis

A dendrogram showing the relationships between 52 isolates of *R. salmoninarum* was generated with the aid of "Tools for population genetic analysis" software package (Miller, 1997). A total of 39 bands, which were uniformly present in the majority of isolates, were recorded in a similarity matrix as present or absent. Only bands generated from the *Ava*I and *Xba*I digests were scored in this way as these enzymes generated clear and distinct profiles for all of the isolates that were scored. A band was scored as absent only if no visible band was present within a 2% size range. Only bands under 9kb were scored, as separation of bands above this size was unclear. The patterns that were generated with each restriction enzyme were combined for each isolate, and distances were calculated by the unweighted pair group method by arithmetic averaging (UPGMA) algorithm using Nei's original distance (Nei, 1972; Nei, 1978; Sneath & Sokal, 1973)

8.3 Results

8.3.1 RFLP analysis using IS*994*

Fifty-two isolates of *R. salmoninarum* were examined using IS*994* RFLP analysis. Three restriction endonucleases were employed for this analysis: *Eco*RV, which does not cut within the element; *Ava*I, which cuts at nucleotide 735, near the middle of the element and the probe; and *Xba*I, which cuts at nucleotide 1159, near the 3' end of the element, and at the end of the probe.

8.3.1.1 RFLP patterns using *Xba*I digests of genomic DNA

Analysis of the *Xba*I digests yielded the greatest number of variations in RFLP patterns between the isolates. Nine distinct patterns were observed among bands of <9kb (Fig: 8.1 & 8.2). Pattern A (displayed by the majority of isolates) was found among all 5 isolates from Sweden, all 4 isolates from Iceland, 8 of isolates from the USA, ten Scottish isolates and 4 English isolates (Fig: 8.1 & 8.2). Of the remaining 10 English isolates, 6 had an additional 1.2kb band (Fig: 8.1; pattern B; lanes 9 - 15); 4 English isolates lacked a 1.3kb band (Fig: 8.1; pattern C; lanes 31 - 20). Among the 5 isolates from Canada, 3 isolates displayed pattern A, 1 isolate (DR143) displayed pattern C (lane 34), and 1 isolate (AcF6-1) lacked a 1.5kb band (pattern D; lane 35).

The greatest variation in the *Xba*I analysis was found among the 15 isolates from Scotland where 5 different patterns were observed. Eight of the Scottish isolates displayed pattern A (Fig: 8.2; MT444, MT452, MT839, MT1470, MT1511, MT1880, MT2118, MT1770), 1 isolate (NCIMB1111) exhibited pattern B (Fig: 8.1; lane 9), and 1 isolate (NCIMB1112) displayed pattern C (Fig: 8.1; lane 8). The remaining 5 isolates exhibited patterns found only in the Scottish isolates of which 2 isolates (NCIMB1115 and NCIMB1113) lacked both the 1.3kb and 1.5kb bands (pattern E; lanes 15 and 17), 1 isolate (NCIMB1114)

possessed an additional ~0.5kb band (pattern F; lane 16), and 1 isolate (NCIMB1116) possessed both an additional ~0.5kb band and appeared to lack a 1.9kb band (pattern G; lane 24, band). A unique profile (pattern I; lane 1, Fig: 8.2) was also displayed by isolate MT2119. Pattern I was predominantly the same as pattern A except that an additional unique band of 3.2kb was present.

The single US isolate that did not display pattern A exhibited a unique pattern (pattern H; lane 20). Pattern H possessed many bands (greater than 24), with only a few bands appearing to correspond in size with bands observed in the other 8 patterns. Furthermore, no high molecular weight (greater than 20kb) bands were observed in pattern H.

8.3.1.2 RFLP patterns using *Ava*I digests of genomic DNA

The greatest number of bands appeared with the *Ava*I digestion (≥ 25 bands) (Fig: 8.3 & 8.4), but variation was observed only in a single ~4kb band, out of the total of 19 bands that were scored for the construction of the dendrogram. This band was present in the Swedish and the majority of English isolates, absent in the Icelandic isolates, and variable among the Scottish, Canadian, and American isolates.

8.3.1.3 RFLP patterns using *Eco*RV digests of genomic DNA

The *Eco*RV digestion (Fig: 8.5) yielded the fewest number of bands (~13). Only 2 variations were observed with this digestion: A prominent 1.8kb band was missing from Canadian isolate AcF6-1 (lane 36), and a 1.1kb band was missing from American isolate Round Butte (lane 35). However, due to the poor quality of the blots the precise pattern could not be determined for every isolate and so the data was not included in the generation of the dendrogram.

8.3.2 Dendrogram analysis

A total of 39 bands were combined from the *XbaI* and *Ava*I IS*994* RFLPs scored as present or absent for 52 isolates of *R. salmoninarum* and the resulting matrix was used to produce a dendrogram (Fig: 8.6). Out of the total of 39 bands 26 were invariant i.e. present in all of the isolates that were examined. The dendrogram analysis grouped the *R. salmoninarum* isolates into twelve clusters that were not all reflective of geographical origin. The relative difference between most paired groups was less than 0.1, illustrating the high degree of genetic conservation within this species. Only 1 isolate, which generated a completely unique *Xba*I pattern, was sufficiently different to exceed this value.

Almost half of the isolates, 25 out of 52, were grouped into a single cluster, cluster 1. This cluster comprised of isolates from a wide range of geographic origins including England, Scotland, Sweden, Canada and USA and a wide range of fish hosts including grayling, rainbow trout, Atlantic salmon, Arctic char, chinook salmon, sockeye salmon and coho salmon. All of these isolates displayed *Xba*I pattern A and possessed all of the 18 *Ava*I bands scored, including the variable 4.4kb band. Cluster 2 contained only a single isolate MT2119, which possessed the unique *Xba*I profile I (differing from pattern A by only a single band) and also the variable 4.4kb *Ava*I band.

Interestingly, cluster 3 consisted of 6 isolates from Iceland and Scotland although a solitary English isolate was also present. Cluster 3 also contained isolates that displayed *Xba*I pattern A but lacked the variable 4.4kb *Ava*I band. Cluster 4 contained 1 isolate from England and 1 from USA each of which displayed *Xba*I pattern C and lacked the 4.4kb *Ava*I band.

Cluster 5 contained 4 isolates of which 3 had been sourced from 3 different English fish farms and 1 from a wild fish in Scotland. These isolates were grouped together because they possessed *Xba*I pattern C and also the variable 4.4kb *Ava*I band. Cluster 6 contained the 2 Scottish isolates that displayed *Xba*I pattern E. These isolates possessed all of the *Ava*I bands, including the variable 4.4kb band.

Clusters 7 and 8 contained 4 and 3 *R. salmoninarum* isolates, respectively. Cluster 7 contained 3 English isolates, all sourced from the same farm, and 1 Scottish isolate. Cluster 8 also contained 3 English isolates, all of which had been sourced from the same farm as the English isolates in cluster 7. Interestingly, all of the isolates in clusters 7 and 8 possessed *Xba*I pattern B but the isolates in cluster 7 lacked the variable 4.4kb *Ava*I band while those in cluster 8 possessed this band. Clusters 9 and 10 each contained a single isolate, NCIMB1116 and NCIMB1114, respectively. Both isolates lacked the variable 4.4kb *Ava*I band but were placed into separate clusters due to their unique *Xba*I profiles, G and F.

Cluster 11 contained only 1 isolate, AcF6-1, which displayed a unique *Xba*I pattern D and lacked the variable 4.4kb *Ava*I band. Cluster 12 also contained only a single isolate, Cow Chs 94. This cluster was noticeably different to the other 9 clusters due to the unique *Xba*I pattern H that distinguished Cow Chs 94 from all of the other *R. salmoninarum* isolates (Fig: 8.1, Lane 40). Interestingly, this isolate also lacked the variable 4.4kb *Ava*I fragment.

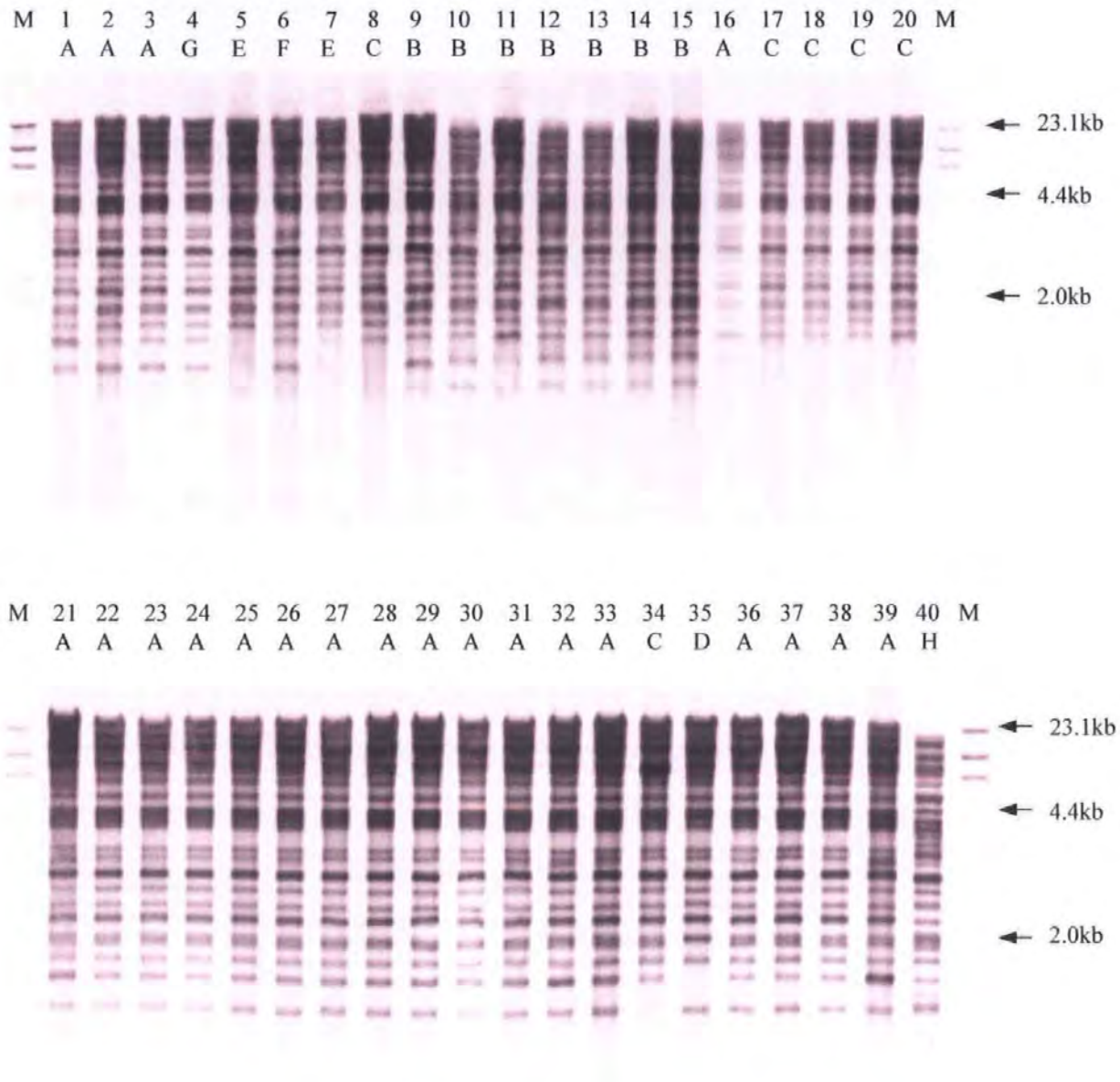


Figure 8.1: RFLP analysis of *Xba*I-digested genomic DNA from forty isolates of *R. salmoninarum*. Genomic DNA (0.5 µg) was digested with *Xba*I, resolved on an 1.2% agarose gel, transferred to nylon membrane, and hybridised with digoxygenin-labelled, randomly primed probe generated from a 1.2kb fragment encompassing 1159 nt of IS*994*. Blots were washed at high stringency, and the hybridised probe was visualised by phosphatase colour development. Lanes 1-40 correspond to the following isolates: SS-ChS-94-1, CCM6205, Siletz, NCIMB1116, NCIMB1115, NCIMB1114, NCIMB1113, NCIMB1112, NCIMB1111, F3, F47, F60, F82, F85, F95, 980297#97, A-80, A-6, 970083-102, 970083-88, BY1996; Rs 125; Rs 122, Rs 116, Rs 61, Rs 19, S-182-90, F-283-87, F-273-87, F-120-87, 960046, 960023, 980002, DR143, AcF6-1, Round Butte, CCM6206, RFL-643.94#1, Idaho 91-126, and Cow ChS 94 respectively. Lane M contains six molecular size markers: 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0kb. Arrows on the right indicate the sizes of selected markers (kb).

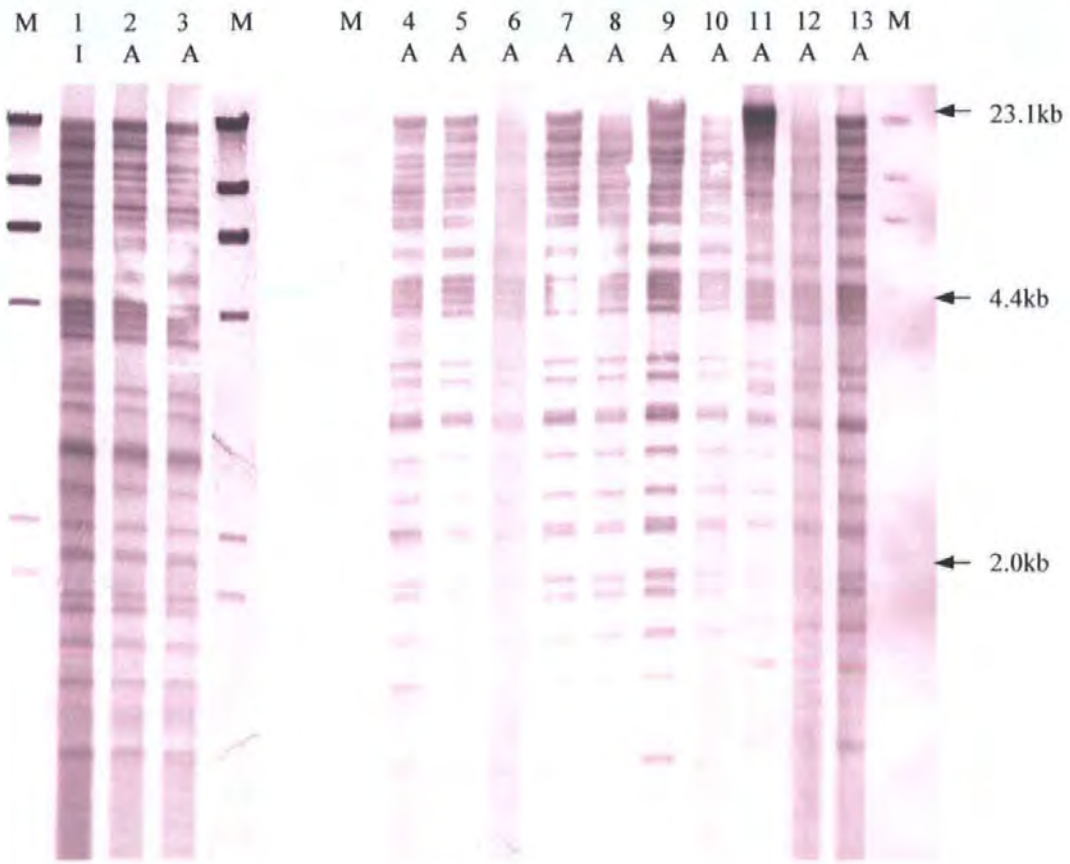


Figure 8.2: RFLP analysis of *Xba*I-digested genomic DNA from thirteen isolates of *R. salmoninarum*. Genomic DNA (0.5 µg) was digested with *Xba*I, resolved on an 1.2% agarose gel, transferred to nylon membrane, and hybridised with a digoxygenin-labelled probe generated by the PCR amplification of a 1.1kb sequence of IS*994*. Blots were washed at high stringency, and the hybridised probe was visualised by phosphatase colour development. Lanes 1-13 correspond to the following isolates: MT2119, MT2118, MT1880, MT1770, MT1511, MT1470, MT1469, MT839, MT452, MT444, 3506W134, 970153-19 and 980036-150, respectively. Lane M contains six molecular size weights 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0kb. Arrows on the right indicate the sizes of selected markers (kb).

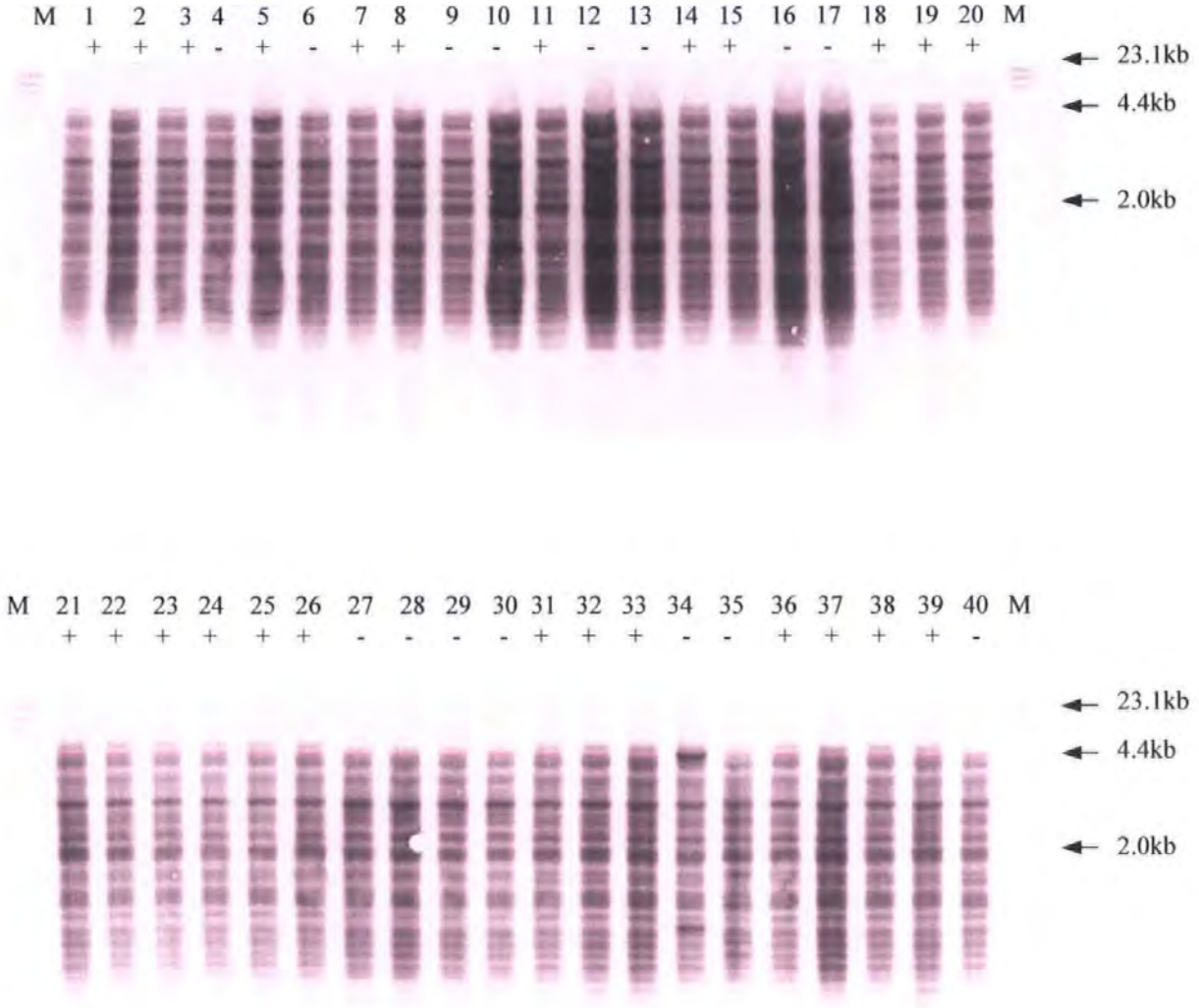


Figure 8.3: RFLP analysis of *Ava* I-digested genomic DNA from forty isolates of *R. salmoninarum*. Genomic DNA (0.5 µg) was digested with *Xba*I, resolved on a 1.2% agarose gel, transferred to nylon membrane, and hybridised with a digoxygenin-labelled, randomly primed probe generated from a 1.2kb fragment of IS*994*. Blots were washed at high stringency, and the hybridised probe was visualised by phosphatase colour development. Lanes 1-40 represent the following isolates: SS-ChS-94-1, CCM6205, Siletz, NCIMB1116, NCIMB1115, NCIMB1114, NCIMB1113, NCIMB1112, NCIMB1111, F3, F47, F60, F82, F85, F95, 980297#97, A-80, A-6, 970083-102, 970083-88, BY1996, Rs 125, Rs 122, Rs 116, Rs 61, Rs 19, S-182-90, F-283-87, F-273-87, F-120-87, 960046, 960023, 980002, DR143, AcF6-1, Round Butte, CCM6206, RFL-643.94#1, Idaho 91-126, and Cow ChS 94, respectively. Lane M contains six molecular size weights 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0kb. Arrows on the right indicate the sizes of selected markers (kb).

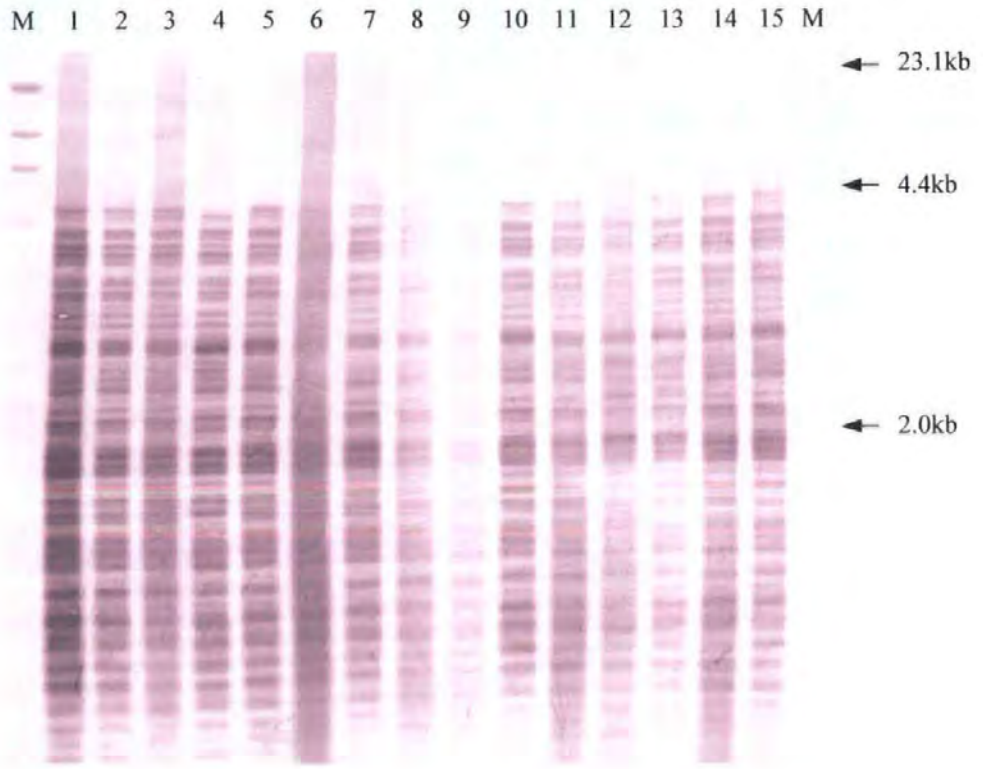


Figure 8.4: RFLP analysis of *AvaI* digested genomic DNA from 15 isolates of *R. salmoninarum*. Genomic DNA (0.5 μg) was digested with *Ava*I, resolved on a 1% agarose gel, transferred to nylon membrane, and hybridised with a digoxygenin-labelled, PCR-generated 1.1kb IS*994* probe. Blots were washed at high stringency, and the hybridised probe was visualised by phosphatase colour development. Lanes 1-15 correspond to the following isolates MT2119, MT2118, MT1880, MT1770, MT1511, MT1470, MT1469, MT839, MT451, MT444, MT239, 3506W134, 3258#15, 970153-19 and 980036-150, respectively. Lane M contains six molecular size markers 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0kb. Arrows on the right indicate the sizes of selected markers (kb).

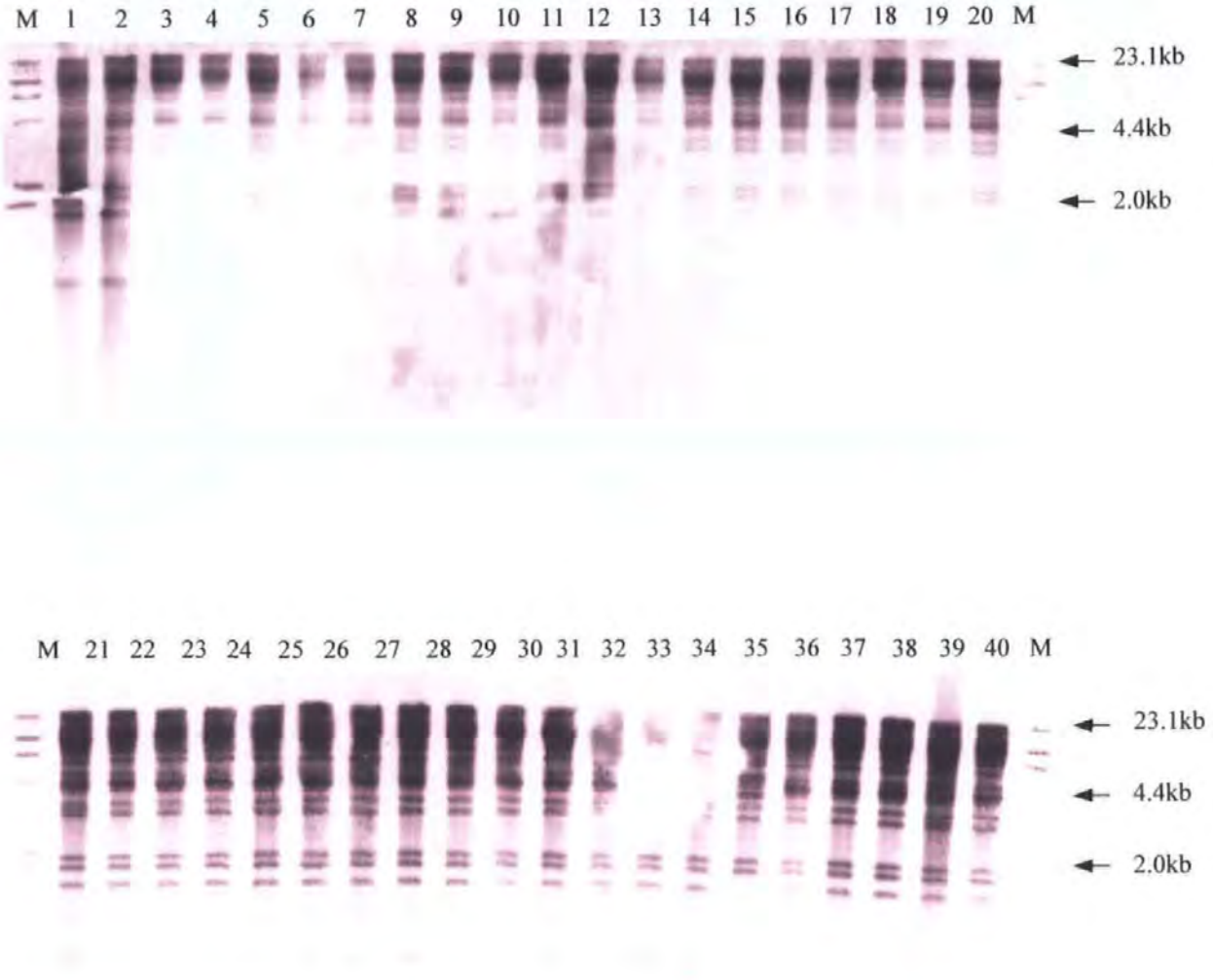


Figure 8.5: RFLP analysis of *Eco*RV digested genomic DNA from 40 isolates of *R. salmoninarum*. Genomic DNA (0.5 µg) was digested with *Eco*RV, resolved on a 0.7% agarose gel, transferred to nylon membrane, and hybridised with a digoxygenin-labelled, randomly primed probe generated from a 1.2kb fragment of *IS994*. Blots were washed at high stringency, and the hybridised probe was visualized by phosphatase colour development. Lanes 1-40 represent the following isolates: SS-ChS-94-1, CCM6205, Siletz, NCIMB1116, NCIMB1115, NCIMB1114, NCIMB1113, NCIMB1112, NCIMB1111, F3, F47, F60, F82, F85, F95, 980297#97, A-80, A-6, 970083-102, 970083-88, BY1996, Rs 125, Rs 122, Rs 116, Rs 61, Rs 19, S-182-90, F-283-87, F-273-87, F-120-87, 960046, 960023, 980002, DR143, AcF6-1, Round Butte, CCM6206, RFL-643.94#1, Idaho 91-126, and Cow ChS 94 respectively. Lane M contains six molecular size markers 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0kb. Arrows on the right indicate the sizes of selected markers (kb).

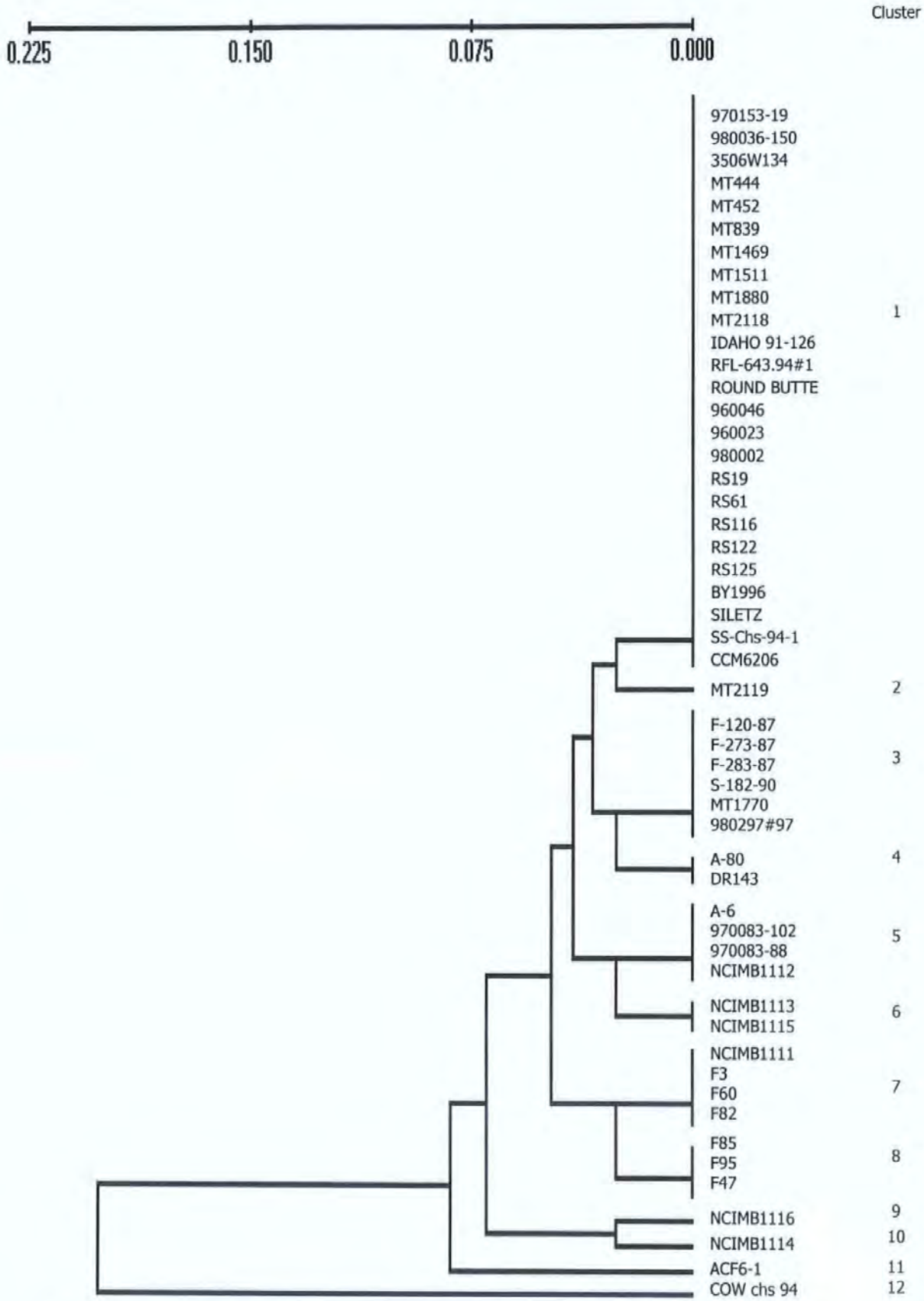


Figure 8.6: A UPGMA generated dendrogram from IS*994* RFLP analysis of *R. salmoninarum* isolates from worldwide locations. The scale bar denotes a relative difference between the isolates where 0.01 is equivalent to 1% in branch lengths.

8.4 Discussion

In this study IS*994*, a putative insertion sequence identified in the genome of *R. salmoninarum* by Rhodes *et al.* (2000), was examined by RFLP analysis in order to determine its potential use as a means for the differentiation of *R. salmoninarum* isolates. IS*994* probes were developed by both PCR and randomly primed labelling for RFLP analysis of the DNA of 52 isolates of *R. salmoninarum* from diverse sources, which had been digested with *Eco*RV, *Xba*I and *Ava*I. The RFLP patterns that were produced were surprisingly homogeneous but there were sufficient differences in the patterns to allow the differentiation of the isolates into 12 distinct clusters.

IS*994* RFLP analysis appeared to be most useful for distinguishing among isolates from Scotland. Fifteen Scottish isolates were placed into 8 clusters, 3 of which were unique to single isolates (NCIMB1114, NCIMB1116 and MT2119). Additionally, another Scottish isolate MT239, which was also included in the work of Rhodes *et al.* (2000) was found to display the pattern appropriate for cluster 1 making it identical in profile to many of the other Scottish isolates that were examined in this study.

It is interesting to compare the groupings of isolates that are proposed here using IS*994* RFLP analysis with those postulated on the basis of 16S-23S rRNA ITS sequence analysis and RAPD analysis by other workers (Grayson *et al.*, 2000b; Grayson *et al.*, 1999). For example, Cow ChS94, which was found to possess a completely unique pattern by *Xba*I IS*994* RFLP analysis, was not distinguished from most of the other isolates from the USA by RAPD analysis (Grayson *et al.*, 2000b). Additionally, of the other 7 USA isolates examined in this study, all were placed in cluster 1, as no differences were readily apparent in their IS*994* RFLP profiles. However, in the RAPD analysis carried out by Grayson *et al.* (2000b) the Alaskan isolate BY1996 generated unique RAPD profile that was distinct from

most other isolates. Furthermore, 2 English isolates, 970153-19 and 980036-150 that have previously been shown to produce unique RAPD profiles were also placed into cluster 1 (Grayson *et al.*, 2000b). Some of the clusters that were established by IS*994* RFLP analysis seem to improve upon the resolution of RAPD analysis. For example, in the study carried out by Grayson *et al.* (2000b) isolates DR143 and A80 were grouped with a number of other *R. salmoninarum* isolates many of which were included in this study (Grayson *et al.*, 2000b). However, when IS*994* profiling was applied these 2 isolates were placed into separate clusters.

IS*994* RFLP analysis placed the Icelandic isolates F-120-87, F-283-87, S-182-90 and F-273-87 together, confirming the broad grouping that was obtained from these isolates by RAPD analysis. However, RAPD analysis did provide a more sensitive means for differentiating between the Icelandic isolates, which placed F-273-87 into a separate subgrouping. Sequence analysis of the 16S-23S rRNA ITS region and PCR amplification of the ETR-A locus in these isolates also grouped the Icelandic isolates (Grayson *et al.*, 2000b; Grayson *et al.*, 1999). Interestingly, IS*994* analysis placed 2 other isolates, MT1770 (Scotland) and 980297#97, with the Icelandic group. Other techniques that have been carried out elsewhere in this thesis such as ETR-A (Chapter 6) and 16S-23S rRNA ITS sequence analyses (Chapter 5), have also shown that the Scottish isolate MT1770 is closely related to the Icelandic isolates. However, none of the studies apart from IS*994* analysis carried out so far have been able to link the English isolate 980297#97 with the Icelandic isolates and the reasons for the association with this group remain obscure.

Although IS*994* RFLP analysis represents an additional means for defining molecular variation among isolates of *R. salmoninarum* it is a relatively expensive and time consuming technique that requires large amounts of genomic DNA. However, IS*994*

analysis could be developed as a PCR assay by designing primers that face outward from the 5' and 3' ends of the sequence and produce a series of bands that reflect the distance between adjacent IS elements provided they are closely located on the bacterial genome. Variation in band size would occur if the IS elements have inserted at different points in the genome of different *R. salmoninarum* isolates. A PCR-based method would be quicker and easier to apply but would require careful development and optimisation. The PCR-IS technique has been successfully applied to a study of genetic variation in *Xanthomonas oryzae* pv. *oryzae*, (Gupta *et al.*, 2001).

It has been suggested that IS have such a profound impact on the genomic arrangement of bacteria that they are responsible for the differences observed between isolate profiles when techniques such as RAPD and PFGE are applied (Gurtler & Mayall, 2001). The results of this study show that IS*994* insertions make a substantial contribution to a molecular variation in *R. salmoninarum*. However, it does not provide a complete explanation and it is likely that there are other as yet unknown sources of variation, including possibly other insertion sequences, present on the *R. salmoninarum* genome that are responsible for the molecular diversity that this and previous studies have identified.

This chapter has presented data obtained from using the insertion sequence IS*994* as a probe to examine restriction fragment length polymorphisms in *R. salmoninarum* from a wide range of geographical locations. IS*994* RFLP profiling was found to have great potential for discriminating between isolates which a number of other techniques had shown to be identical. Consequently, the routine application of this technique to new isolates of *R. salmoninarum* is recommended.

Chapter Nine

PULSED FIELD GEL ELECTROPHORESIS ANALYSIS OF *RENIBACTERIUM SALMONINARUM*

9.1 Introduction

Pulsed field gel electrophoresis (PFGE) is a sophisticated method for the subtyping of bacterial species. Preparations of intact genomic DNA are digested using infrequently cutting restriction enzymes and the resulting large DNA fragments are separated in agarose gels, using highly specialised equipment to switch the direction of the current and permit the separation of DNA fragments sized between 50kb and 10Mb. In practice, this is achieved by embedding the whole organism in question in agarose plugs, lysing the cell walls *in situ*, removing the interfering constituent proteins, and finally, restriction enzyme digestion of the intact genomic DNA (Smith & Cantor, 1987; Schwartz & Cantor, 1984). Visualisation of the DNA banding patterns following electrophoresis and comparison with other strains or isolates of the same species may reveal differences in the number and sizes of DNA fragments that have arisen as a consequence of variation in the positions of restriction sites (Tenover *et al.*, 1995). PFGE has been widely used to subtype a variety of bacterial species, including *Salmonella typhimurium* and *Vibrio cholerae* (Tsen *et al.*, 2000; Arakawa *et al.*, 2000).

Macro-restriction analysis using PFGE has greatly advanced the field of bacterial genomics (Cole & Girons, 1994). Because this technique can separate very large fragments of DNA it has enabled the identification of multiple chromosomes, linear chromosomes and indeed the complex unravelling of the organisation of many bacterial genomes in order to produce

detailed physical maps (Kolsto, 1997; Fonstein & Haselkorn, 1995; Krawiec & Riley, 1990).

At present there has been no published work on the application of PFGE to studies of *R. salmoninarum* and there is no data available on the genomic organisation of this pathogen. This chapter describes the application of PFGE analysis to isolates of *R. salmoninarum* from a variety of sources within the UK with the aim of determining information about the *R. salmoninarum* genome and the potential of PFGE as a method for subtyping isolates.

9.2 Materials and methods

9.2.1 *R. salmoninarum* plug preparation

R. Salmoninarum cells were harvested from either SKDM plates or 10ml SKDM broths. For storage purposes, whole cells were placed into liquid nitrogen in the presence of 20% glycerol and transferred to -80°C. Wherever possible all reagents that were used in this chapter were of PFGE quality. Recipes for all of the buffers that were used in this section are in outlined in Appendix 1.7. *R. salmoninarum* cells were embedded into agarose plugs using three different methods described as follows.

9.2.1.1 Buffer washed cells

The first method that was used to embed *R. salmoninarum* cells into agarose plugs was based on a protocol provided in the "CHEF-DR II Pulsed Field Electrophoresis Systems Instruction Manual" and a protocol by Kaufmann and Pitt (1994);(BioRad, 1993). The cell density of *R. salmoninarum* broths was established using a bacterial cell counter. A volume corresponding to 1 x 10^8 bacterial cells was centrifuged (13,000 x g, 3min, RT) in a 1.5ml

microfuge tube. The supernatant was discarded and the cells were washed twice in 1ml of cell suspension solution (CSS), re-suspended in 500µl of CSS and equilibrated to 50°C. To the cell suspension, a 500µl volume of a molten solution of 2% (w/v) 'Pulsed field electrophoresis sample preparation agarose' prepared in SE buffer at 50°C, was added and mixed gently.

9.2.1.2 Phenol washed cells

The second method for embedding *R. salmoninarum* cells in agarose was based on the method described by Beyazova *et al.* (1995). *R. salmoninarum* cells were washed twice with 0.5M EDTA [pH 8.0] then mixed with 0.5ml tris-saturated phenol [pH 8.0] and incubated at 56°C, for 15min. The phenol mixture was extracted with chloroform-isoamyl alcohol (24:1) three times by adding 500µl of chloroform-IAA and centrifuging at 13000 x g for 5min. The cells were resuspended in 1% (w/v) SDS in HE buffer at 56°C and incubated for 15min. Following this the cells were pelleted (13,000 x g, 3min, RT) and resuspended in 500µl of fresh HE buffer and this washing process was repeated five times. After the final resuspension, the cell density was adjusted to OD_{600} = 1.6 and the solution was then mixed with an equal volume of a molten solution of 2% (w/v) 'Pulsed field electrophoresis sample preparation agarose' (Sigma), which had been prepared in SE buffer and preheated to 50°C. In the first preparation using the phenol method, cells were adjusted to OD_{600} = 1.8. However due to lane overloading, it was decided to reduce this to OD_{600} = 1.6.

9.2.1.3 Formalin washed cells

The third method of embedding that was used has been described by Gibson *et al.* (1994). The cell density of *R. salmoninarum* broths was established using a bacterial cell counter.

A volume corresponding to 1 x 10^8 cells was centrifuged (13,000 x g, 3min, RT), the supernatant discarded and the cell pellet washed twice in 1ml of CSS. After the final wash, the cells were thoroughly re-suspended in 900µl cell suspension solution, to which 100µl of formaldehyde solution (37-40 % w/v) was added and the mixture was incubated for 1h at RT. The cells were washed five times in CSS and following the final wash were resuspended in 500µl of CSS and equilibrated to 50°C. To the cell suspension, a 500µl volume of a molten solution of 2% (w/v) 'Pulsed field electrophoresis sample preparation agarose' (prepared in SE buffer at 50°C) was added and mixed gently.

9.2.2 Lysis and de-proteination of plugs

Following the cell preparation procedures described above, cells were transferred to disposable plug moulds (BioRad) and placed at 4°C for 15mins. Once the agarose had set, the plugs were removed from the moulds and ten plugs were transferred to a 50ml sterile disposable polypropylene tube (Falcon) containing 20ml of lysis buffer (Appendix 1.7), achromopeptidase (30u/ml) and lysozyme (1mg/ml). The plugs were incubated overnight at 37°C with shaking (100 rpm). Following this, the lysis buffer was discarded and the plugs were first washed briefly with 20ml wash buffer and then incubated in 20ml of proteolytic solution containing proteinase K (500µg/ml) for 24h at 50°C with shaking (100 rpm). Following incubation, as much proteolytic solution, as possible was removed and the plugs were washed for 20min in 50ml of wash buffer. This washing step was repeated 5 times. If undigested DNA plugs were required, then no further procedures were performed and the plugs were stored at 4°C.

9.2.3 Restriction enzyme digestions of embedded agarose plugs

Following the above procedure, plugs were treated with a 5mM Pefabloc solution (a PMSF substitute) for 2h at 37°C to inactivate any residual proteinase activity. Plugs were then washed twice with TE buffer and stored in TE buffer at 4°C until required for restriction enzyme digestions. Prior to restriction digestions, the plugs were cut in half and placed into 1.5ml microfuge tubes containing 100µl of the appropriate restriction enzyme reaction buffer (1 x concentration). The tube was placed at 4°C for 30min and then 100µl of reaction buffer containing the restriction enzyme (20U) was added to the reaction tube. The contents were gently mixed and the reaction was incubated overnight at 37°C, unless otherwise stated.

9.2.4 Running conditions and agarose gels

All gels were run at 14°C using a CHEF DR-II (BioRad). The running conditions, percentage of agarose used, and the nature of the running buffer varied according to the expected sizes of the restricted DNA fragments. The details are recorded in the legends for each figure. Following electrophoresis, all PFGE gels were stained for 30min in 300ml of ethidium bromide solution (1µg/ml).

9.2.5 Control organisms

Throughout this experiment two control organisms were used, *Streptomyces griseus* (NCIMB8136) and *Bacillus cereus* (NCIMB11925). PFGE analysis has been successfully applied to studies of both of these organisms and it is known that *B. cereus* possesses a circular genome which was estimated by PFGE to be approximately 5.7Mb, while *S. griseus* possesses a linear genome which has been estimated to be approximately 7.8Mb by PFGE analysis (Kolsto, 1990; Lezhava, *et al.*, 1995). In this experiment, the *B. cereus*

plugs were prepared using the buffer washed cell method (section 9.2.1.1) and *S. griseus* plugs were prepared using the phenol washed cell method (section 9.2.1.2) in accordance with published protocols for these organisms (Kolsto, 1990; Beyazova *et al.*, 1995).

9.3 Results

9.3.1 *R. salmoninarum* plug preparation

Three methods were used to prepare *R. salmoninarum* DNA plugs. The first method used buffer washed cells that were embedded in agarose plugs and resulted in partial to total degradation of the genomic DNA from *R. salmoninarum*. This is illustrated in Figure 9.1 (Gel A, Band X), where a low molecular weight DNA smear can be seen and no intact chromosomal DNA is apparent. One possible cause of this smearing may be the presence of endogenous nucleases from *R. salmoninarum* or from contaminated enzymes used in the preparation process. A similar problem is observed in the preparation of plugs from some strains of *Camplyobacter jejuni*. Some *C. jejuni* strains, like *R. salmoninarum*, produce DNase and it is this enzyme that is thought to responsible for the DNA degradation encountered using standard plug preparation methods (Gibson *et al.*, 1994). In the latter study a wash with formalin was found to reduce the activity of this enzyme. Similarly, Beyazova *et al.* (1995) encountered problems of DNA degradation when preparing actinomycete plugs for PFGE. In this case, cells were pre-treated with phenol solutions prior to embedding in an attempt to eliminate DNA nuclease-mediated degradation. Therefore, it was decided to employ both these methods to try and produce *R. salmoninarum* DNA plugs of a suitable quality for PFGE analysis.

Figure 9.1, Gel B (fragment Y) shows that both the phenol and the formalin method of cell preparation represented an improvement on the buffer washed cells method and enabled DNA of a large size to be examined. However, it should be noted that even when using the

phenol or formalin techniques to prepare DNA plugs for PFGE there was always a substantial quantity of lower molecular weight DNA. It is possible that a large amount, if not all, of this represented degraded chromosomal DNA because when undigested plugs were run on gels using shorter pulse times this DNA was visible as a smear rather than a clear band that would be indicative of extra-chromosomal DNA. However, it is also possible that some of this lower molecular weight smear could contain plasmid DNA that was not fully resolved. In a separate study conducted by Dr. M.L. Gilipin no evidence for the presence of plasmids was found in 96 isolates of *R. salmoninarum* (personal communication). Additionally, there has been no evidence in the literature to show that *R. salmoninarum* possesses extra-chromosomal DNA.

Initially, cell preparations using the phenol method were adjusted to OD_{600} = 1.8 in accordance with the method of Beyazova *et al.* (1995) instead of performing a cell count prior to the procedure. Following the initial run using these plugs it was decided to reduce the OD_{600} to 1.6 as the quantity of DNA was clearly excessive, making it very difficult to estimate the sizes of the DNA fragments (Fig: 9.1, Gel B, Lane 5). It has been documented that erroneous size values can be obtained if the DNA concentration is too high during sample preparation because excess DNA strongly retards band migration (Romling *et al.*, 1996).

In addition to the change in optical density that was required when using *R. salmoninarum* cells it was found that the plugs had a much shorter shelf life than was reported by Beyazova *et al.* (1995) using Acinomycetes from the genera *Streptomyces, Kutzneria, Dactylosporangium, Microtetraspora* and *Actinoplanes*. Beyazova *et al.* (1995) found that plugs that had been prepared using the phenol wash method had a shelf life of at least 18

months. Generally, the plugs prepared in this work using either the formalin or the phenol wash methods only had a shelf life of several weeks before the DNA showed signs of severe degradation.

9.3.2 The *R. salmoninarum* chromosome: size and topology

Figures 9.1 and 9.2 show the presence of a large DNA fragment of 4.5-6Mb (labelled Y) in agarose gels that had been electrophoresed under conditions designed to separate very large DNA fragments. It has previously been documented that only linear DNA up to approximately 10Mb in size or supercoiled circular DNA up to approximately 100-150kb in size will migrate into a PFGE gel. Circular DNA of a greater size than this is retained within the agarose plugs, so, for example, a circular DNA molecule of genomic proportions would not migrate into a PFGE gel. Additionally, although small circular DNA (<100kb) does migrate in PFGE gels it does so in a very different manner to that of linear DNA. It is extremely unlikely that a circular molecule of this size range would migrate along side size markers of megabase proportions (Birren & Lai, 1993; Romling *et al.*, 1996; Pisabarro *et al.*, 1998). Therefore, the results indicated that the 4.5-6Mb fragment Y, represents the *R. salmoninarum* chromosome and, as this molecular has migrated into the PFGE gel, that this chromosome is linear.

In order to test the possibility that fragment Y was the linear chromosome of *R. salmoninarum* a further experiment was undertaken. A procedure used in several studies to determine the linearity of *Streptomyces* and *Saccharopolyspora* chromosomes is to prepare duplicate DNA plugs either in the presence or in the absence of proteinase K. This experiment was based on the principle that a linear chromosome often assumes a circular conformation in the cell due to the presence of a terminal protein that binds both ends of the molecular together. Treatment of the chromosome with proteases will degrade these

proteins and allow the linearised chromosome to migrate into the gel. In the absence of proteases, a linear chromosome will have both ends bound together and, consequently, will remain in the plug (Lin *et al.*, 1993; Lezhava *et al.*, 1995; Reeves *et al.*, 1998). Figure 9.3 clearly shows that the *R. salmoninarum* chromosome did not migrate out of agarose plugs prepared without proteinase K, while migration occurred when plugs were prepared with proteinase K. This provides further evidence that the chromosome of *R. salmoninarum* is linear. Unfortunately the quality of Figure 9.3 is compromised because the migration of the DNA has been hindered by an air bubble in the agarose, which was not captured by the photograph but was visualised by the author.

In addition to the *R. salmoninarum* plugs it can be seen from Figure 9.1 that the DNA from the control organisms *S. griseus* and *B. cereus* behaved as expected. The DNA from *B. cereus* did not migrate from the wells into the gel (Fig: 9.1, Gel B, lane 2). The DNA from *S. griseus,* however, did enter the gel and can be seen in Figure 9.1 just above the *R. salmoninarum* band and the largest *S. pombe* marker (Fig: 9.1, Gel B, Lane 1).

9.3.3 Restriction enzyme digests of genomic DNA

Throughout the six month duration of this PFGE study on *R. salmoninarum* many restriction enzymes were employed with the intention of generating a suitable number of fragments for analysis, with mixed results. Because the *R. salmoninarum* genome has a mol% G+C of 55.5%, restriction enzymes with a recognition sequence of 6-8bp and comprising mainly A and T nucleotides were selected to digest the DNA (*Dra*I, *Ssp*I, *Swa*I, and *Sfi*I) (Banner *et al.*, 1991). In addition to above the restriction enzymes *Not*I was also employed, this enzyme has an 8bp recognition sequence that this entirely comprised of G and C nucleotides. Despite the use of a range of restriction enzymes, none were found to be

adequate. *Dra*I, for example, appeared to generate a partial digest of *R. salmoninarum* and despite several attempts no distinct bands were ever visualised (Fig: 9.4).

It can be seen from Figure 9.5 that when the restriction enzyme *Not*I was employed clear bands were generated. However, it is also apparent from the undigested plugs that were run in parallel that a large quantity of the *R. salmoninarum* DNA was degraded. It is therefore likely that the banding is actually due to digestion of the degraded DNA rather than a true restriction profile of the intact *R. salmoninarum* genome. This would explain why so many bands are present and why no DNA fragments greater than 100kb are observed.

The restriction enzymes *Ssp*I and *Swa*I, were unsuitable as they appeared to digest the *R. salmoninarum* genome many times and only a low molecular weight smear is visible in Figure 9.6. This is perhaps not surprising as *Ssp*I (recognition sequence AATATT) digestion of *S. griseus* DNA resulted in more than 30 bands. The *S. griseus* genome has mol% G+C of 72% (Lezhava *et al.*, 1995). Although the use of different running conditions could potentially have partially resolved this smear it seemed unlikely to provide total separation. Ideally, macrorestriction profiles using PFGE should have fewer than 30 bands as complete separation of all of these fragments can be problematic making the interpretation of the patterns more difficult (Birren & Lai, 1993).

To investigate the reasons for the degradation of *R. salmoninarum* DNA, plugs were prepared from bacterial cells that had been cultured in broth and on agar. It has been observed when growing *R. salmoninarum* within the laboratory that this organism often grows quicker on a solid agar medium rather than in broth culture. This course was chosen in the hope that cultures that had grown on agar would have grown more quickly and,

consequently, would contain less degraded DNA than cells that had been grown in broth. It can be seen from Figure 9.6 that there appears to be less degradation of the DNA of *R. salmoninarum* cells cultured on agar compared with cells cultured in broth. Nevertheless, this did not result in clearer restriction patterns for any of the restriction enzymes. And although less DNA degradation is apparent when using cells cultured on agar there is still a substantial amount present.

In contrast to the results that were obtained for *R. salmoninarum,* Figures 9.4 & 9.6 show that the DNA of the control organism *S. griseus* cut to completion and produced a clear banding pattern. *Dra*I digested the *S. griseus* genome and three bands of approximately 2200kb, 800kb and 400kb were visible. It is highly unlikely that these are single bands as the genome of *S. griseus* has been sized at 7.9Mb and so it is probable that these bands consist of doublets or triplets. The successful digestion of the *S. griseus* DNA reinforces the view that it is the quality of the *R. salmoninarum* DNA rather than the reagents that has resulted in the smearing and obscuring of restriction patterns.

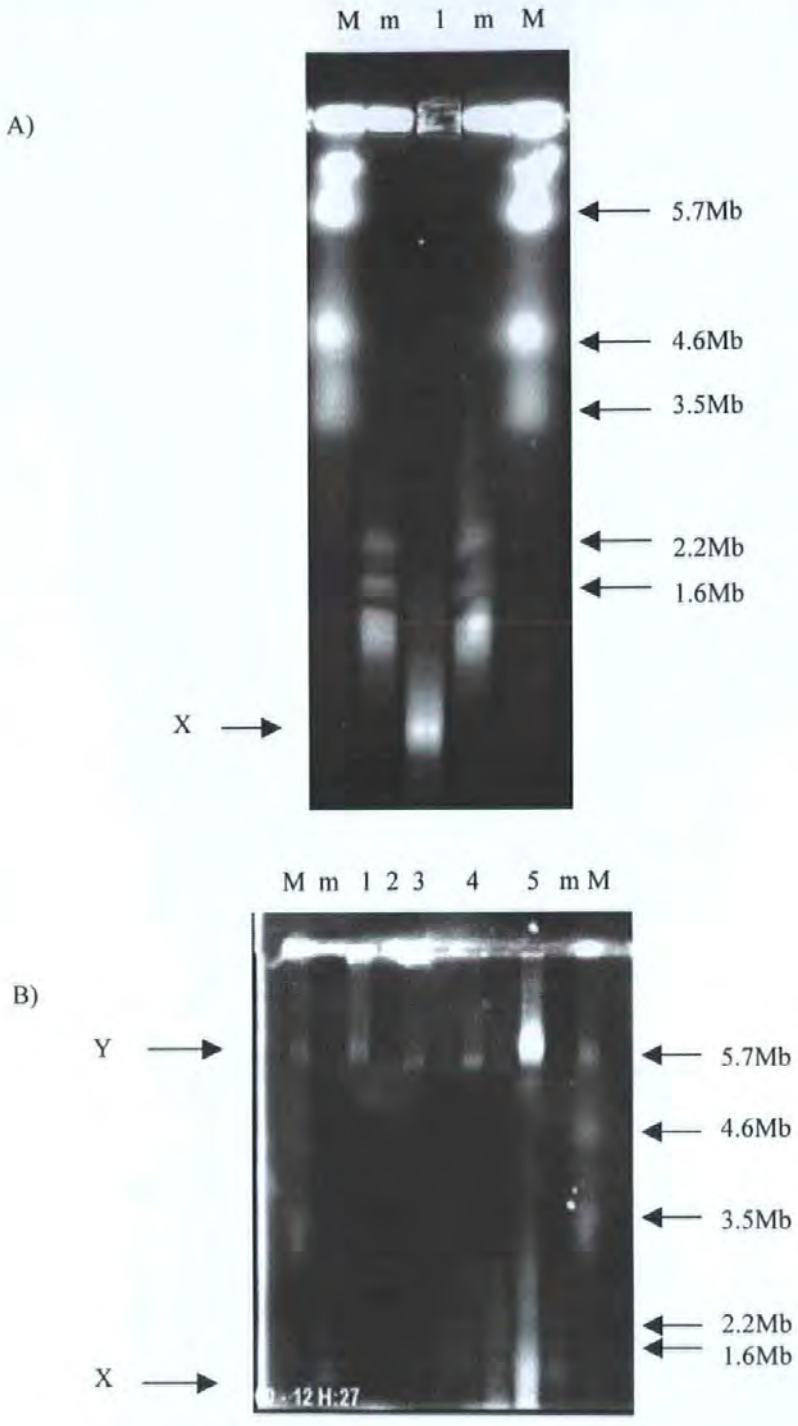


Figure 9.1: PFGE analysis of undigested *R. salmoninarum* DNA prepared using different methods. Total DNAs were subjected to extremely long pulse times to attempt to elucidate the chromosomal topology. Gel A: Lane 1 corresponds to DNA from *R. salmoninarum* using buffer washed cells (isolate MT2119). Gel A was run under the following conditions: 30min pulse, at 65 volts (1.9v/cm), for 80 h (Lezhava, 1995). Gel B lanes1-4 refers to the DNA preparations of the following organisms respectively 1) *Streptomyces griseus* 2) *Bacillus cereus* 3) *R. salmoninarum* formalin treated cells, 4*)* *R. salmoninarum* formalin treated cells, 5) *R. salmoninarum* phenol treated cells. Gel B was run initially under the following conditions: 30min pulse, at 65 volts (1.9v/cm), for 80 h, after which the gel was then run for a further time under the following conditions: 60min pulse, 45 V (1.3 v/cm) for 50 hours (Lezhava, 1995). Lane-m: *Saccharomyces cerevisiae* markers. Lane M *Saccharomyces pombe* markers. Gel B was run under the following conditions 30min pulse, Both gels were made up using 0.7% agarose in 1 x TAE. Arrows on the right indicate the positions and sizes of molecular markers.

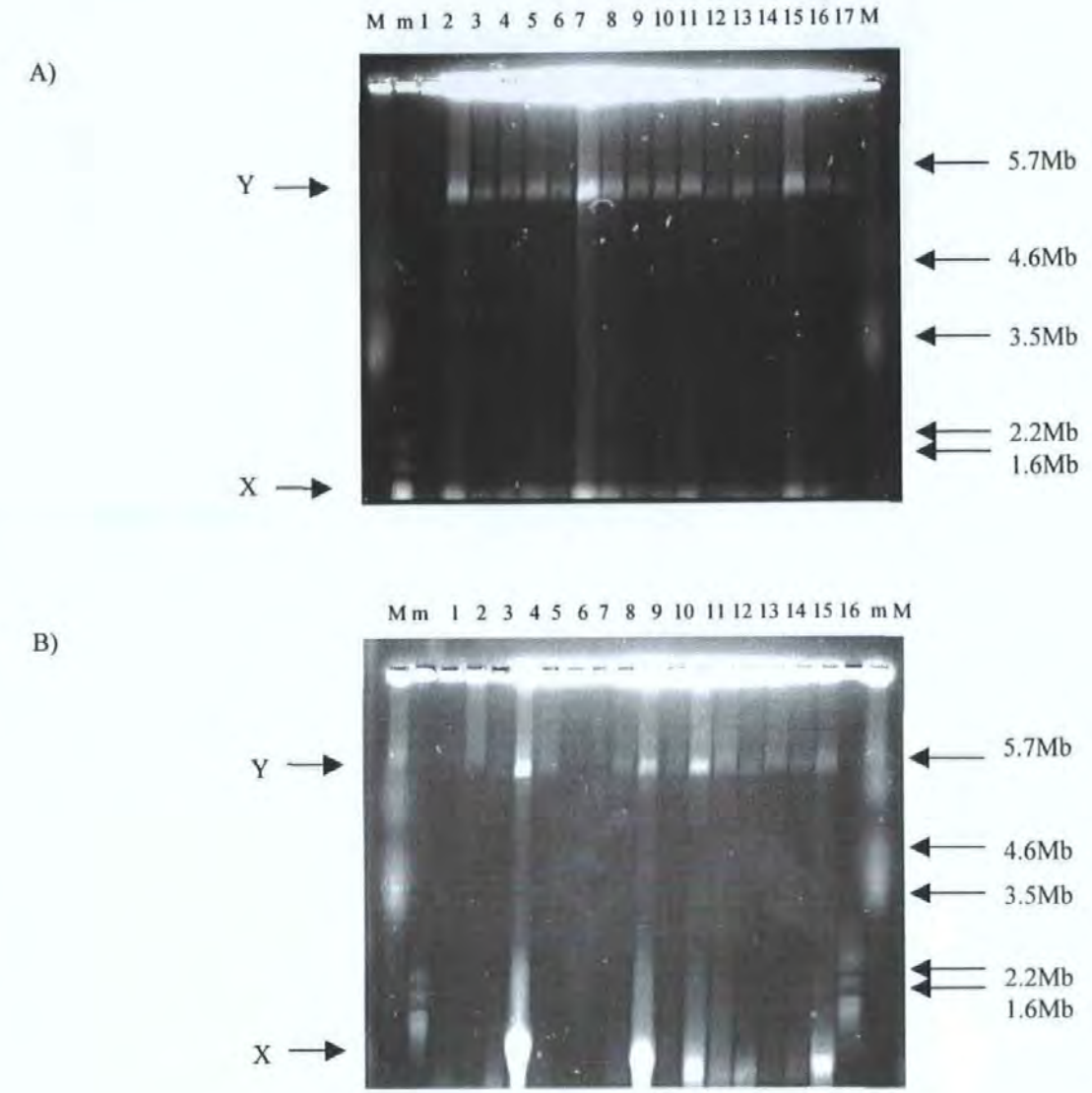


Figure 9.2: PFGE analysis of undigested *R. salmoninarum* DNA prepared using different methods. Total DNAs were prepared using the formalin method and were subjected to extremely long pulse times to attempt to elucidate the chromosomal topology. Gel A lanes 1-16 correspond to DNA preparations from the following *R. salmoninarum* isolates or control organisms: 1; *Bacillus cereus,* 2; MT419, 3; MT426, 4; MT444, 5; MT452, 6; MT839, 7; MT861, 8; MT1261, 9; MT1351, 10; MT1469, 11; MT1470, 12; MT1511, 13; MT1770, 14; MT1880, 15; MT2118, 16; MT2119. Gel B lanes 1-16 refers to the DNA preparations from the following *R. salmoninarum* isolates or control organisms respectively 1: *Bacillus cereus* plug, 2; RS 970083-88, 3; RS A80, 4; RS 970083-102, 5; RS 980036-150, 6; RS 980036-87, 7; RS 970419-1.2.3, 8; 970153-19, 9; RS A6, 10; RS NCIMB 1111, 11; RS NCIMB 1112, 12; RS NCIMB 1113, 13; RS NCIMB1114, 14; RS NCIMB1115, 15; RS NCIMB 1116, 16; RS MT239. Both gels were run initially under the following conditions: 30min pulse, at 65 volts (1.9v/cm), for 80 h, after which the gel was then run for a further time under the following conditions: 60min pulse, 45 V (1.3 v/cm) for 50 hours (Lezhava *et al.*, 1995). Lane m: *Saccharomyces cerevisiae* markers. Lane M *Schizosaccharomyces pombe* markers. Both gels were made up using 0.7% agarose in 1 x TAE. Arrows on the right indicate the sizes and positions of molecular markers.

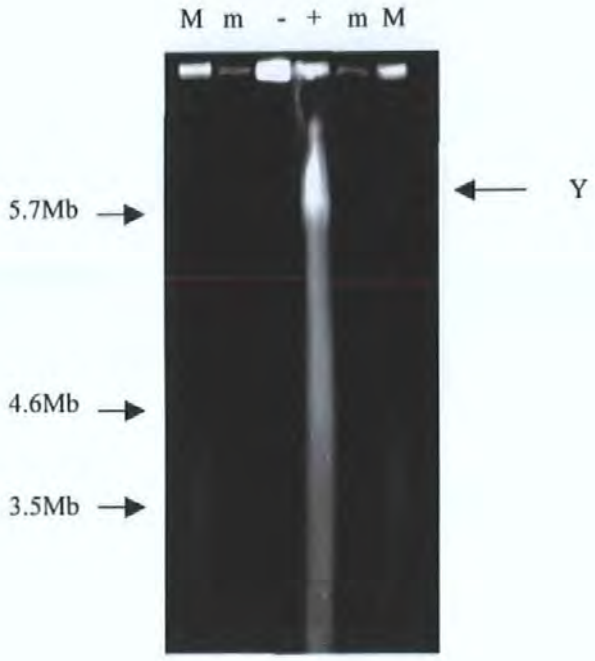


Figure 9.3: PFGE analysis of the undigested chromosome of *R. salmoninarum* isolate MT2119 prepared with (+) and without (-) proteinase K treatment. DNA was subject to extremely long pulse times and the gel was run initially under the following conditions: 30min pulse, at 65 volts (1.9v/cm), for 80 h, after which the gel was then run for a further time under the following conditions: 60min pulse, 45 V (1.3 v/cm) for 50 hours. DNA was run in a 0.7% agarose gel using 1 x TAE as the running buffer. Lane m: *Saccharomyces cerevisiae* markers. Lane M *Schizosaccharomyces pombe* markers. Arrows on the left indicate the sizes and positions of molecular markers.

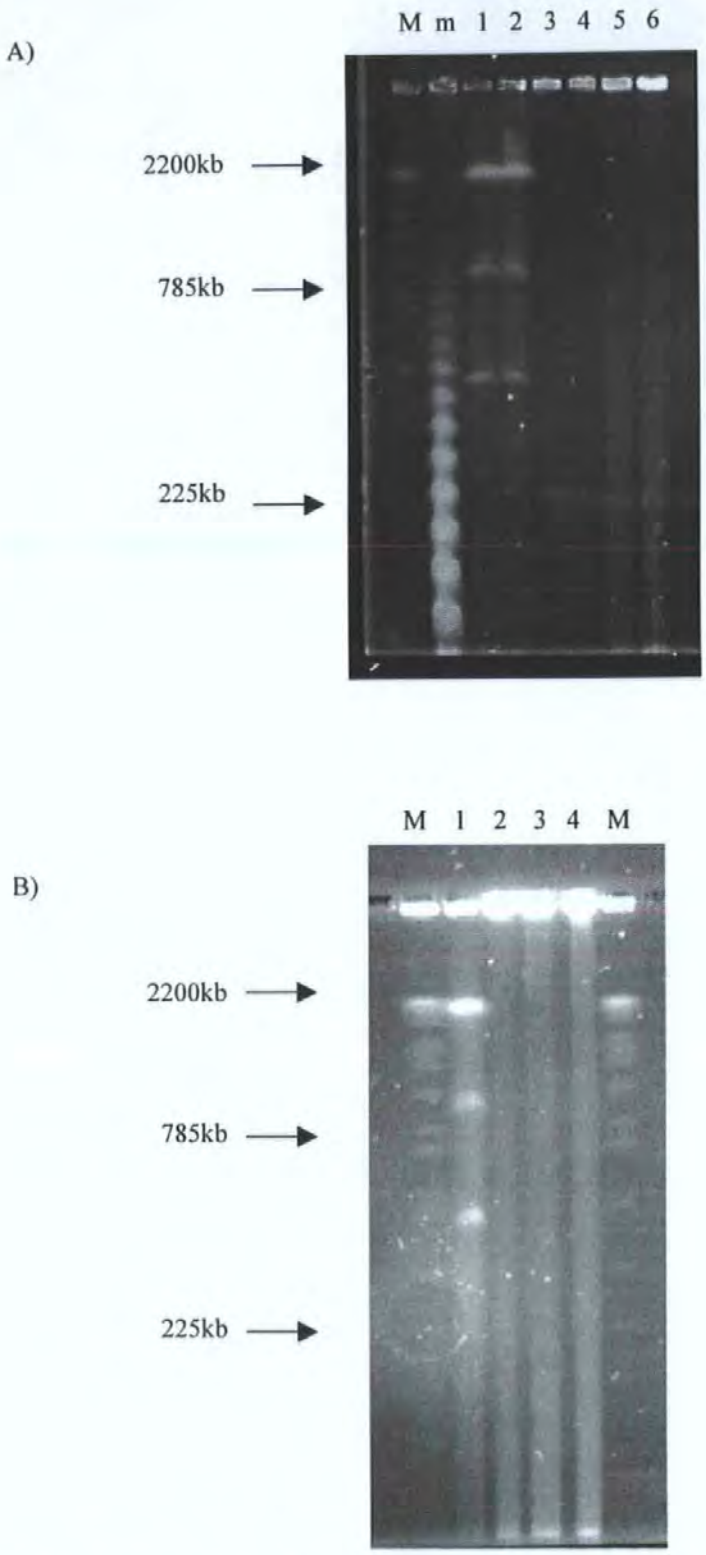


Figure 9.4: PFGE analysis of restriction enzyme digests of the *R. salmoninarum* chromosome. Gel A; lanes 1-2: *Dra*I digests of *S. griseus*, Lanes 3-4: *Dra*I digests of *B. cereus*, Lane 5: *Dra*I digest of RS MT2119 prepared using the formalin method, Lane 6: *Dra*I digests of 970083-88 prepared using the formalin method. Gel B; lane 1: corresponds to *Dra*I digests of *S. griseus*, lane 2: *Dra*I digests of 970083-102 (prepared using the phenol method), lane 3: *Dra*I digest of A6 (prepared using the phenol method), lane 4: *Dra*I digests of NCIMB1112 (prepared using the phenol method). The 1.2% agarose gel was run for 22 hrs under the following conditions, pulse times from 5s to 60s with linear ramping, at 210v (6 v/cm^2) using 0.5 % TBE as a running buffer. Lane M: *S. cerevisiae* markers. Arrows on the left indicate the sizes and positions of molecular markers.

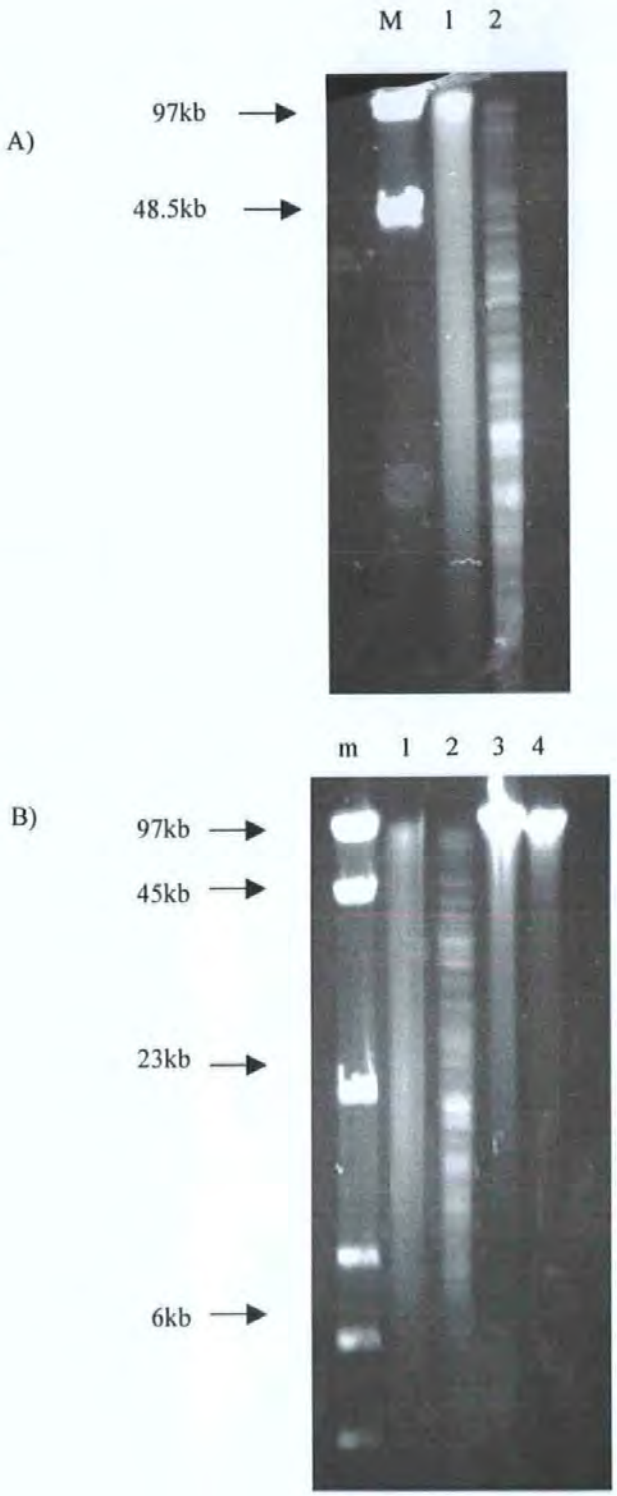


Figure 9.5: PFGE analysis of restriction enzyme digests of the *R. salmoninarum* chromosome. Gel A: Lane 1: uncut genomic DNA from MT2119, lane 2: genomic DNA from MT2119 (prepared using the phenol method) digested with *Not*I. The 1.2% agarose gel was run for 28 hrs under the following conditions, pulse times from 0.6 s to 3 s with linear ramping, at 150v (4.4 v/cm^2) using 0.5 % TBE as running buffer. Gel B; lane 1; MT2119 *Swa*I digest (plug prepared using the phenol method), lane 2; MT2119 *Not*I digest (plug prepared using the phenol method), lane 3; MT2119 uncut genomic DNA (prepared using the phenol method), lane 4; 970083-88 (plug prepared using the phenol method). The 1.2% agarose Gel was run for 22 hrs under the following conditions, pulse times from 1.2 s to 1.6 s with linear ramping, at 210v (6 v/cm^2) using 0.5 % TBE as a running buffer. Lane M: Lambda Ladder. Lane m: PFGE/FIGE markers. Arrows on the left indicate the sizes and positions of molecular markers.

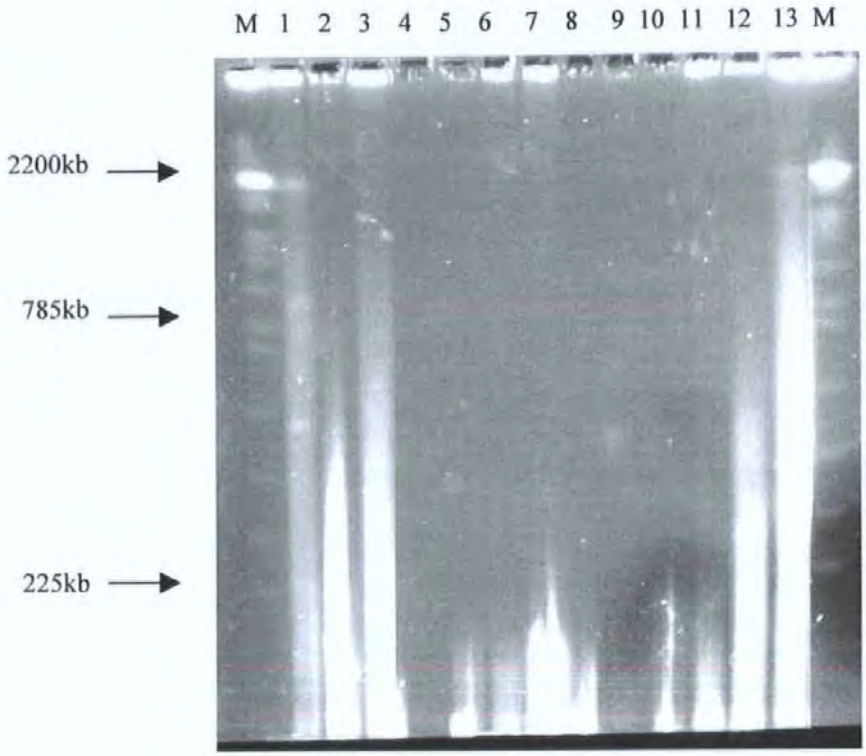


Figure 9.6: PFGE analysis of restriction enzyme digests of *R. salmoninarum*. Plugs were prepared from *R. salmoninarum* 980036-150 cells cultured in broth and on agar using the phenol method. Lane 1; *S. griseus* plug digested with *Dra*I, lane 2; *Dra*I digest from agar cultured cells, lane 3; *Dra*I digest from broth cultured cells, lane 4; *Sfi*I digested *S. griseus* DNA, 5; *Sfi*I digest from agar cultured cells, lane 6; *Sfi*I digest from broth cultured cells, lane 7; *Sfi*I digest from broth cultured cells (at double the normal concentration), lane 8; *Ssp*I digest from agar cultured cells, lane 9; *Ssp*I digest from broth cultured cells, lane 10; *Swa*I digest from agar cultured cells, lane 11; *Swa*I digest from broth cultured cells, lane 12; Undigested plug of agar cultured cells, lane 13; Undigested plug from cultured cells. The 1.2% agarose Gel was run for 22 hrs under the following conditions, pulse times from 5 s to 60 s with linear ramping, at 210v (6 v/cm^2) using 0.5 % TBE as a running buffer. Lane M: *S. cerevisiae* markers. Arrows on the left indicate the sizes and positions of molecular markers.

9.4 Discussion

9.4.1. *R. salmoninarum* DNA plugs

Three different methods for the preparation of DNA agarose plugs were explored to attempt to produce embedded and intact chromosomal DNA of *R. salmoninarum,* that would be suitable for digestion with restriction enzymes. Unfortunately, this was never satisfactorily achieved. The first procedure employed a tris buffer to wash the cells prior to embedding in agarose. Other studies have shown this method to be adequate for the preparation of DNA plugs from a range of other bacteria, including *Escherichia coli*, *Wolbachia* spp. and *Azospirillum* spp. (Schad, 1995; Sun *et al.,* 2001; Martin-Didonet *et al.,* 2000). But when this method was applied to *R. salmoninarum* the complete degradation of DNA was observed (Fig: 9.1). It is likely that residual nuclease activity was responsible for this outcome as *R. salmoninarum* has previously been reported to be DNase positive, and so two alternative methods were explored (Bruno & Munro, 1986). Gibson *et al.* (1994) reported that formalin treatment inactivated the residual nucleases that are produced by some *Campylobacter* stains (Gibson *et al.,* 1994). Similarly, Beyazova *et al.* (1995) used a phenol treatment followed by a chloroform wash to removed nucleases from various actinomycetes prior to PFGE analysis.

The use of either phenol or formalin for preparing *R. salmoninarum* DNA plugs was promising and resulted in the migration of what appeared to be intact chromosomal DNA into the PFGE gel. Nevertheless, a substantial quantity of apparently degraded DNA was present at the bottom of each gel (Fig: 9.1: panel B). It also appeared that the DNA continued to degrade with time, even though the plugs were stored at 4°C. This is consistent with an enzymatic process and it is therefore probable that the treatment of the cells with phenol or formalin failed to inactive all of the nucleases. It is also possible that some of

DNA degradation may have resulted from exposure to the harsh chemical environment. Phenol is known to be capable of inducing cell lysis and this could have resulted in shearing of the DNA during subsequent manipulations thereby producing the lower molecular weight smear (Ruiz-Ordaz *et al.*, 1998).

Another possible explanation for the degradation of the DNA may be that some of the cells that were used to prepare the plugs were not intact. *R. salmoninarum* is a very slowly growing organism in the laboratory with a generation time of from 24-48 h (Benediktsdottir *et al.*, 1991). It is therefore possible that at least some of the cells from the harvested culture were dead or had lysed and that this may have contributed to the degradation. In order to explore this possibility, *R. salmoninarum* cells were cultured in broth and on agar and agarose plugs were prepared from each of these sources. *R. salmoninarum* has been observed to grow more quickly on agar plates than in broth and so it was hoped that less cell degradation would be present. The results indicated that the DNA prepared from cells cultured on agar did appear to be less degraded than the DNA prepared from cells cultured in broth (Fig: 9.6: lanes 12 and 13). Clearly, there is room for further investigations into the best method for preparing *R. salmoninarum* cells for PFGE. In particular, the choice of faster growing isolates of the organism and a means of completely inactivating the endogenous nucleases would prove useful.

9.4.2 The *R. salmoninarum* chromosome

Preliminary findings of PFGE analysis of the whole *R. salmoninarum* chromosome indicated that it was large, approximately between 4.5-6Mb, and of a linear nature. This conclusion was reached because i) circular chromosomes, because of their size, can not migrate out of agarose plugs, and ii) unless the plug is adequately deproteinised the chromosome will not migrate out of the well. Interestingly, there appeared to be no difference in the sizes of the chromosomes of several isolates of *R. salmoninarum* that were

examined. Generally, although genomic size does not differ drastically between different isolates of the same species there are exceptions e.g. the genome size of different mutants of *Streptomyces ambofaciens* can vary between 6.5-8Mb due to deletions of up to 2Mb (Leblond *et al.*, 1991) although whether these mutants should be considered to be the same species was not discussed.

It should be emphasised that this is only a preliminary estimate of the *R. salmoninarum* chromosome for two reasons. First and foremost, whole chromosomal or megabased sized DNA can not be accurately sized by PGFE. This is because it migrates so slowly in the gel that there is only a very small region that allows separation in which migration is related to size. This can be clearly seen from examining Fig: 9.1, Gel B where there is only a very small distance separating the linear genome of *Streptomyces griseus* at 7.9Mb and the *Schizosaccharomyces pombe* molecular weight marker at 5.7Mb. In addition, there are a lack of DNA size markers of megabase proportions, and so it is difficult to accurately size molecules within this very small region of separation (Birren & Lai, 1993). Furthermore, the separation and migration of DNA fragments of this size is at the upper-limit of the capabilities of the equipment that was used in this study (BioRad, 1993). Ideally, estimating the size of a bacterial genome should be based upon the sum of accurately sized digested DNA fragments. Smaller fragments (200kb-1Mb) can be more accurately sized as a greater variety of size standards are available. To ensure maximum precision it is also recommended that the sizes of DNA fragments obtained from at least two different enzymes should be used for the calculation and gels should be run under several different ramped switch times to obtain maximum resolution. Secondly, although PFGE is a popular method of chromosomal sizing, even if all the above criteria are met it can at best only be relied upon to give an estimates of the true size of a genome (Romling *et al.*, 1996). The

only way to determine the precise size of the *R. salmoninarum* chromosome is by complete genomic sequencing.

It is surprising that the size of the *R. salmoninarum* genome was estimated to be 4.5-6Mb. *R. salmoninarum* is an obligate pathogen, unlike the majority of other actinomycetes that are commensals, widely distributed in the environment and possess large genomes. It is generally believed that because of increased parasitism of host-cell metabolism many obligate intracellular pathogens do not require much of the genetic material that is needed by their free living relatives and ancestors and consequently possess smaller genomes (Weisburg, 1989). This holds true in the genus *Ehrlichia* which is composed of obligate intracellular gram-negative bacteria, whose genomes range from 0.8-1.5Mb (Rydkina *et al.*, 1999). Indeed, at 0.58Mb, one of the smallest self-replicating genomes known, is possessed by intracellular pathogen *Mycoplasma genitalium* (Birren *et al.*, 1993; Fraser *et al.*, 1995). However, there are notable exceptions, the intracellular pathogen *M. tuberculosis* that has a genome size of 4.4Mb. A large proportion of this genome is devoted to the production of enzymes involved in lipogenesis and lipolysis (Cole & Girons, 1998).

The results of this study also indicated that the *R. salmoninarum* genome is of a linear nature. Circular bacterial chromosomes, do not migrate into pulsed field gels and with the advent of PFGE many simple experiments have used the migration or lack of migration of a chromosome as an indication of its topology (Schwartz & Cantor, 1984). The results of these and other investigations challenged the traditional belief that bacteria possess only one circular chromosome. Many bacteria have been shown to contain linear genomes (Hinnebusch & Tilly, 1993). At least 10 species of the genus Streptomyces have been reported to possess linear genomes including *S. lividans*, *S. coelicolor*, *S. ambofaciens* and *S. griseus* (Lin *et al.*, 1993; Leblond-Bourget *et al.*, 1996; Lezhava *et al.*, 1995). Linear

genomes have also been reported in other bacteria including the spirochaete *Borrelia burgdorferi*, *Agrobacterium tumefaciens* and *Rhodococcus fascians* (Baril *et al.*, 1989; Ferdows *et al.*, 1992; Allardet-Servent *et al.*, 1993; Crespi *et al.*, 1992) although the latter has been disputed (Pisabarro *et al.*, 1998b).

9.4.3 Restriction enzyme digests

Unfortunately, none of the restriction enzymes that were utilised were able to generate a clear macro-restriction profile of suitably sized fragments from *R. salmoninarum* DNA. The reasons for this are unclear. It is very unlikely that all the enzymes that were used were unsuitable and it is more likely that the *R. salmoninarum* DNA plugs were of poor quality. It seems unlikely that residues of phenol, chloroform or formalin were the cause of this as the DNA contained within the control *S. griseus* plugs did cut to completion and produced very clear banding. It was hoped that a restriction enzyme would be identified that would cut the *R. salmoninarum* genome infrequently thereby generating fragments of between 100-400kb. It should be noted that the macro-restriction profiles of *R. salmoninarum* were only attempted after the intact chromosomal gels were performed and examined. By that stage even more degradation of the DNA would have occurred making the task more difficult. Further PFGE analysis of the *R. salmoninarum* genome should involve the use of fresh plugs, and removing degraded DNA from plugs by electrophoresis prior to restriction digestion may also improve the outcome (McClelland *et al.*, 1994).

This project was undertaken with the high expectation of producing a physical map of the *R. salmoninarum* chromosome and possibly developing another functional typing system for this organism. Because of the difficulties that were experienced in the preparation of DNA of a suitable quality this was never achieved. Further development is required in order to resolve these problems so that the arrangement of the *R. salmoninarum* chromosome and the analysis of isolate diversity can be explored using PFGE.

Chapter Ten

DNA FINGERPRINTING OF *RENIBACTERIUM SALMONINARUM* USING ENTEROBACTERIAL REPETITIVE INTERGENIC CONSENSUS (ERIC)-PCR, REPETITIVE EXTRAGENIC PALINDROMIC (REP)-PCR AND BOX-PCR

10.1 Introduction

ERIC, REP, and BOX fingerprinting are PCR-based typing methods that have been shown to discriminate between strains, pathovars and isolates of some bacterial species (Versalovic *et al.*, 1991; Versalovic *et al.*, 1993; Koeuth *et al.*, 1995). Enterobacterial repetitive intergenic consensus (ERIC) regions are repetitive elements of 126bp that are found in transcribed regions of the chromosome, either in the intergenic regions of polycistronic operons or in the untranslated regions upstream or downstream of open reading frames. ERIC sequences have been identified in the genomes of a number of gram-negative isolates and although ERIC sequences are highly conserved at the nucleotide sequence level, their chromosomal locations differ between species or strains (de Bruijn, 1992; Hulton *et al.*, 1991). Consequently, these elements have been successfully used for molecular typing purposes (Versalovic *et al.*, 1991). ERIC-PCR has been used to examine relationships between bacterial isolates of both gram-positive and gram-negative bacteria, for example *Vibrio cholerae*; *Haemophilus influenzae* and *Listeria monocytogenes* (Rivera *et al.*, 1995; Gomez-De-Leon *et al.*, 2000; van Belkum *et al.*, 1998b; Jersek *et al.*, 1999).

Repetitive extragenic palindromic (REP) sequences are repetitive elements that appear to be distributed throughout prokaryotic genomes (Higgins *et al.*, 1982; Higgins *et al.*, 1988; Stern *et al.*, 1984; Gilson *et al.*, 1984; Gilson *et al.*,1987). The REP sequence consists of a conserved 33bp inverted repeat region and approximately 500 copies of this REP sequence have been found on the chromosomes of bacteria such as *Escherichia coli* and *Salmonella typhimurium* (Stern *et al.*, 1984). Versalovic (1991) designed consensus PCR primers to the REP regions and these have been used to differentiate between closely related strains of bacteria such as *Streptomyces* sp. and *Actinobacillus seminis* (Sadowsky *et al.*, 1996; Appuhamy *et al.*, 1998).

BOX elements, first identified in *Streptococcus pneumoniae* are also repetitive DNA sequences that are interspersed throughout the bacterial genome. There are 3 different sequences termed boxA, boxB, and boxC. Primers have been designed to BOX sequences and PCR reactions usually employ a single box primer to amplify these regions (Koeuth *et al.*, 1995). These primer have been used to highlight differences between strains of several species of bacteria including *Enterococcus faecalis* and *Bifidobacterium sp.* (Malathum *et al.*, 1998; Gomez Zavaglia *et al.*, 2000). This chapter describes how ERIC, REP and BOXA2 primers were applied to analyse 29 isolates of *R. salmoninarum* from locations within the UK.

10.2 Materials and Methods

10.2.1 ERIC, REP and BOX PCR analysis

PCR amplifications were performed as stated in section 3.5 with the following modifications: reactions were performed in 35µl volumes and contained 100ng of genomic DNA, 50pmol of each primer, and 1% DMSO. Reactions were overlaid with 1 drop of

sterile mineral oil. The REP and ERIC PCRs contained both a forward and a reverse primer while the BOXA2 PCR contained only a single primer (Table 10.1).

Each of the PCR methods were initially carried out using 10ng of template DNA. However, these reactions failed to produce a PCR product and it was necessary to increase the amount of template to 100ng per reaction in order to produce visible amplicons.

The cycling conditions for the ERIC-PCR were as follows: an initial denaturation step at 94°C for 2 minutes followed by 35 cycles of 94°C for 45s, 52°C for 1min, and 70°C for 10min and a final extension at 70°C for 20min. Cycling conditions for the BOXA2 and REP-PCR's were identical except that the annealing step was performed at 40°C. All products were analysed by electrophoresis of 12.5µl of each reaction mixture in 1.2% agarose gels as described in section 3.6.

10.2.2 Dendrogram analysis

A dendrogram showing the relationship between *R. salmoninarum* isolates was generated with the aid of "Tools for population genetic analysis" software package (Miller, 1997). Thirty-three bands, many of which were uniformly present in the majority of isolates, were recorded in a similarity matrix according to their presence or absence. Faint bands were excluded from the analysis. A band was scored as absent only if no visible band was present within a 2% size range. The patterns generated with the ERIC, REP and BOX primers were combined for each isolate, and distances were calculated by the unweighted pair group method by arithmetic averaging (UPGMA) algorithm using Nei's original distance (Nei, 1972; Nei, 1978; Sneath & Sokal, 1973).

Table 10.1: Oligonucleotide primers used for PCR-fingerprinting

Primer	Sequence (5' to 3')	Reference
BOXA2	ACGTGGTTTGAAGAGAGATTTTCG	(Koeuth *et al.*, 1995)
REP1R-Dt*	IIINCGNCGNCATCNGGC	(Versalovic *et al.*, 1991)
REP2-Dt	NCGNCTTATCNGGCCTAC	(Versalovic *et al.*, 1993)
ERIC1R	ATGTAAGCTCCTGGGGATTCAC	(Versalovic *et al.*, 1991)
ERIC2	AAGTAAGTGACTGGGGTGAGCG	(Versalovic *et al.*, 1991)

*I = inosine

10.3. Results

10.3.1 ERIC-PCR

ERIC-PCR analysis of the genomic DNA from 29 isolates of *R. salmoninarum* generated multiple amplicons, which ranged in size from 200bp to 3040bp. A total of 9 bands were scored in order to produce the dendrogram, 7 of which were present in all of the isolates (Fig: 10.1 & 10.2). Only 2 bands were found to be variable in the 29 isolates that were examined. A band of approximately 1500bp was found to be absent from isolate 970153-19 and was present in the remaining 28 isolates. Additionally, a 1640bp band was found to be absent from isolates 3506W132, 2119#5, MT1470 and NCIMB1115. A band of approximately 2036bp also appeared to be variable between isolates but because it could not be reliably determined whether this band was truly absent or just faintly present in some isolates it was excluded from the analysis.

10.3.2 REP-PCR

Complex fingerprints were also generated for all of the 29 isolates of *R. salmoninarum* that were examined using REP-PCR (Fig: 10.3 and 10.4). A total of fourteen bands, which ranged in size from 220bp to 3500bp were strongly and reliably amplified in the majority of isolates resulting in intricate profiles. In general, the band patterns of isolates from different sources were very similar, supporting the suggestion that isolates of *R. salmoninarum* are very highly conserved. However, while the fingerprint patterns for *R. salmoninarum* isolates obtained from the same farm were often very similar, they were not always identical. A 3010bp band was found to be absent from isolates 2119#5, 3506W132 and MT1470 but present in the remaining 26 isolates examined.

10.3.3 BOXA2-PCR

A complex pattern comprising of multiple bands was also generated using the BOXA2 primer (Fig: 10.5 & 10.6). The size range of amplicons was between 230bp and 2000bp, a smaller size range than was evident in the ERIC and REP PCR. A total of 10 amplicons were scored for the dendrogram construction and all 10 bands were amplified in all of the 29 isolates of *R. salmoninarum* that were examined. A 350bp band was found to be strongly present in some isolates and only faintly present or possibly absent in a number of other isolates but was considered to be ambiguous and was rejected from the analysis. Additionally, a band of approximately 580bp was clearly present in a number of isolates including MT444, MT452, NT1351, MT1469, MT1770, MT1880, MT2118, MT2119, 970083-102, NCIMB1112 and NCIMB1113. However, the presence or absence of this band could not be reliably determined in the remaining 10 isolates and so this band was also rejected. Ultimately, BOXA2 produced subtle differences in band intensities between *R. salmoninarum* isolates that were not recorded in the dendrogram.

10.3.4 Dendrogram construction

A total of 33 bands combined from ERIC-, REP- and BOXA2- PCR profiles from 29 isolates of *R. salmoninarum* were scored to generate a distance dendrogram. Thirty of the 33 bands were found to be invariant. Combining the profiles for the 3 primer methods resulted in 4 distinct clusters, with 24 isolates placed into a single cluster (Fig: 10.7). Clusters 2 and 3 each contained a single isolate, NCIMB1115 and 970153-19 respectively, while 3 isolates were placed into cluster 4, 3506W132, 2119#5 and MT1470.

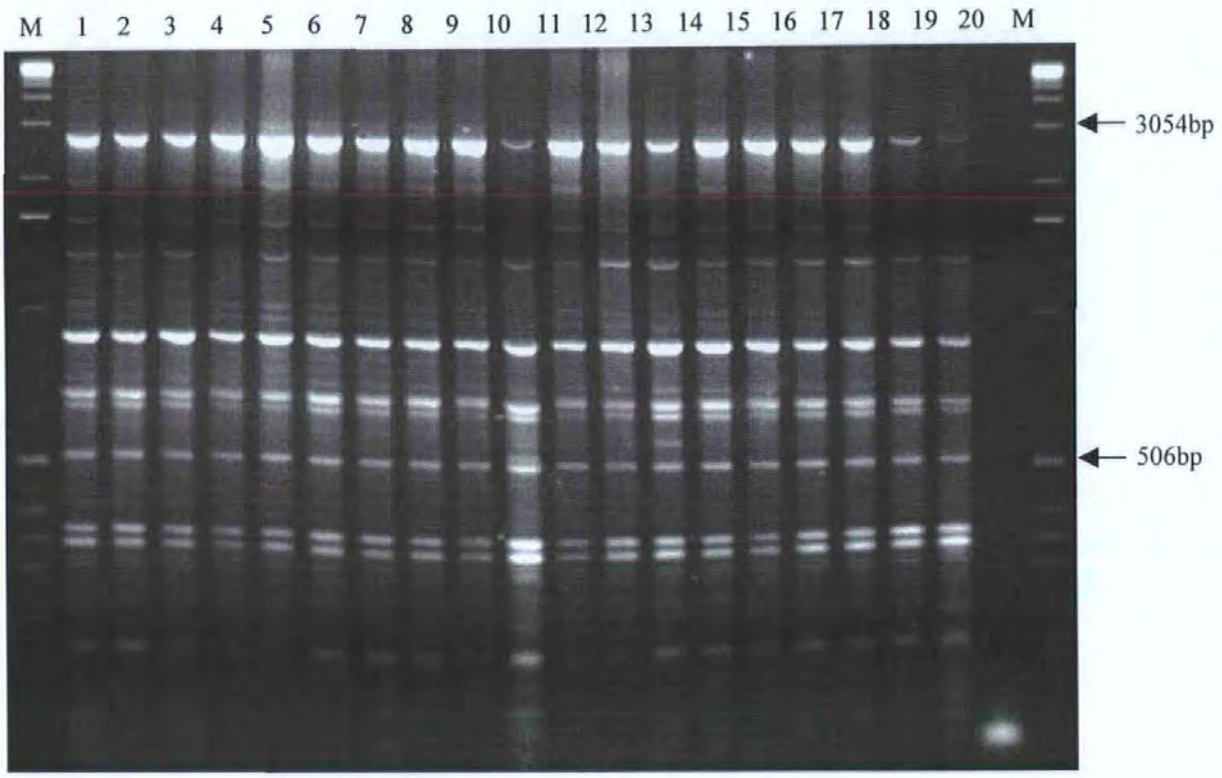


Figure 10.1: ERIC-PCR fingerprints of 19 isolates of *R. salmoninarum*. Lanes 1-19 correspond to the following isolates: MT239, MT419, MT426, MT444, MT452, MT839, MT861, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132 and 2119#5. Lane 20: Negative control. Lane M: 1kb Ladders (Gibco BRL: 0.3µg), the sizes of selected markers are indicated by arrows on the right.

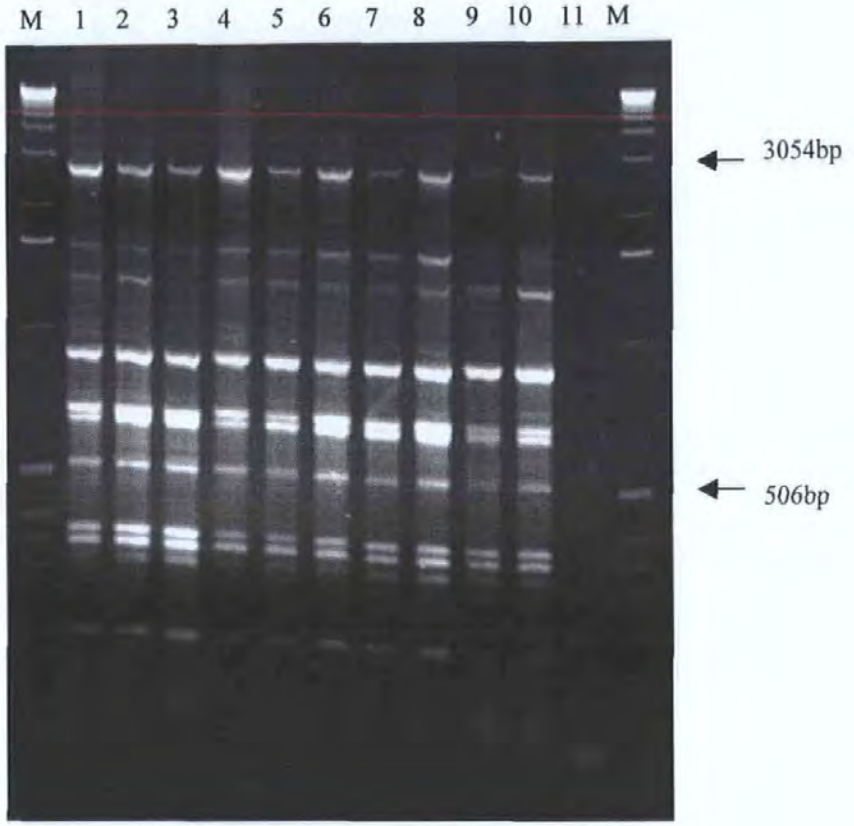


Figure 10.2: ERIC-PCR fingerprints of 10 isolates of *R. salmoninarum*. Lanes 1-10 corresponds to the following isolates: 970083-102, 980036-87, 970153-19, A6, NCIMB1111, NCIMB1112, NCIMB1113, NCIMB1114, NCIMB1115 and NCIMB1116. Lane 11: Negative control. Lanes M: 1Kb Ladders (Gibco BRL: 0.3μg) , the sizes of selected markers are indicated by arrows on the right.

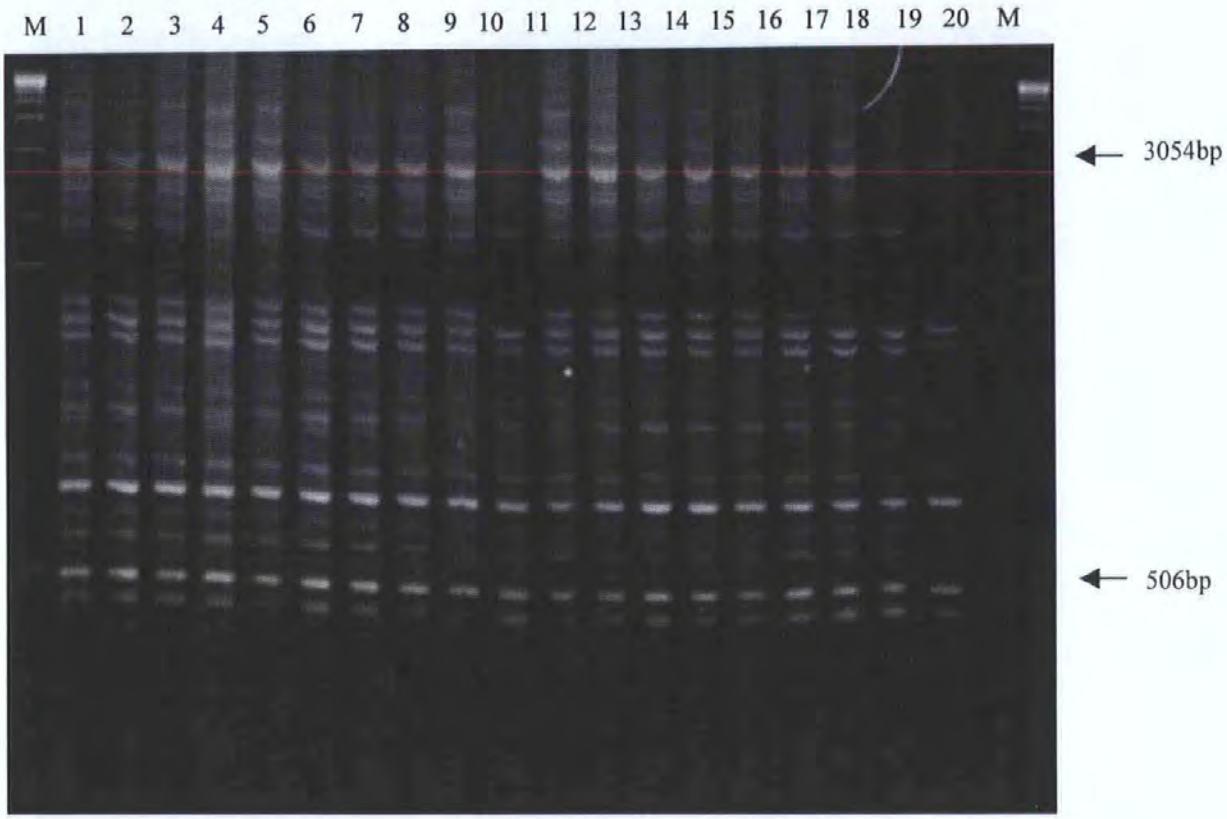


Figure 10.3: REP-PCR fingerprints of 19 isolates of *R. salmoninarum*. Lanes 1-19 correspond to the following isolates: MT239, MT419, MT426, MT444, MT452, MT839, MT861, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132 and 2119#5. Lane 20: Negative control. Lane M: 1Kb Ladders (Gibco BRL: 0.3μg), the sizes of selected markers are indicated by arrows on the right.

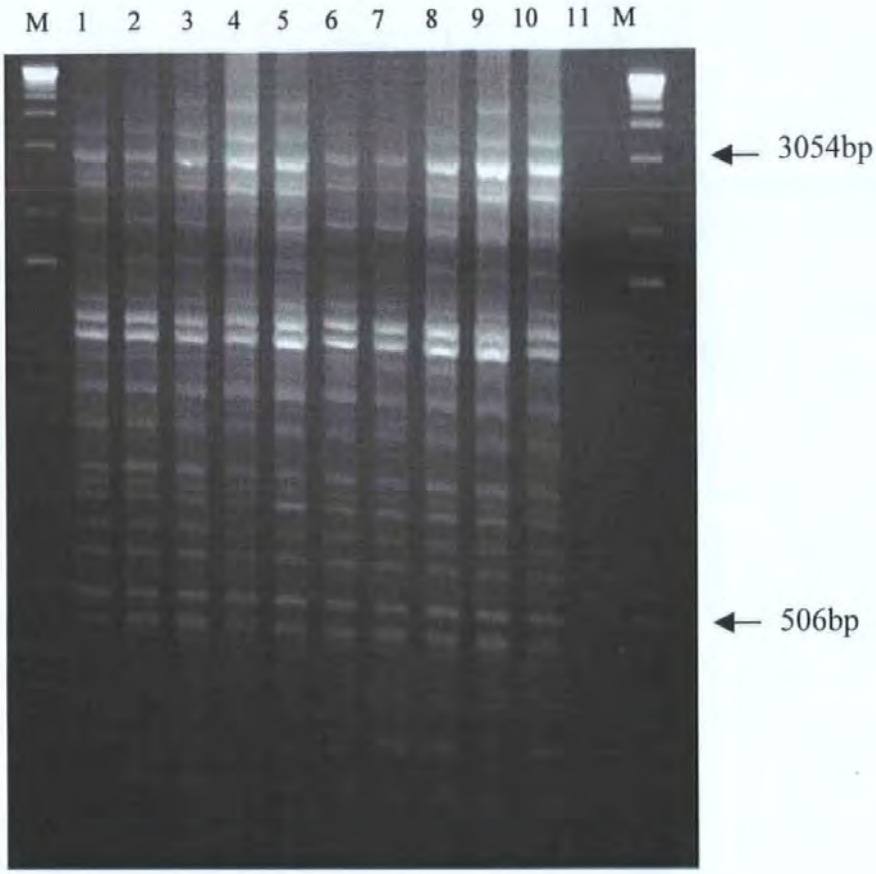


Figure 10.4: REP-PCR fingerprints of 10 isolates of *R. salmoninarum*. Lanes 1-10 correspond to the following isolates: 970083-102, 980036-87, 970153-19, A6, NCIMB1111, NCIMB1112, NCIMB1113, NCIMB1114, NCIMB1115 and NCIMB1116. Lane 11: Negative control. Lane M: 1Kb Ladders (Gibco BRL: 0.3μg), the sizes of selected markers are indicated by arrows on the right.

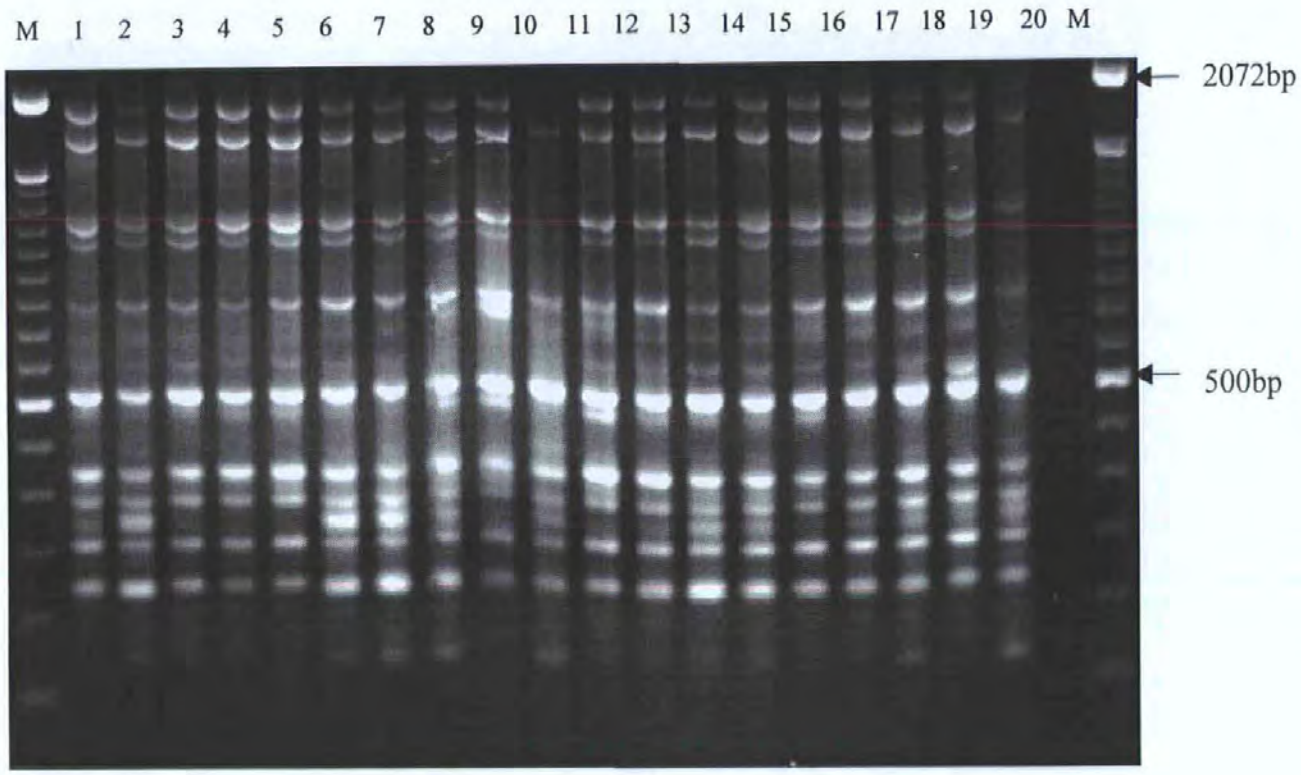


Figure 10.5: BOXA2-PCR fingerprints of 19 isolates of *R. salmoninarum*. Lanes 1-19 correspond to the following isolates: MT239, MT419, MT426, MT444, MT452, MT839, MT861, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132 and 2119#5. Lane 20: Negative control. Lane 1 and 19: 100bp Ladders (Gibco BRL: 0.3μg), the sizes of selected markers are indicated by arrows on the right.

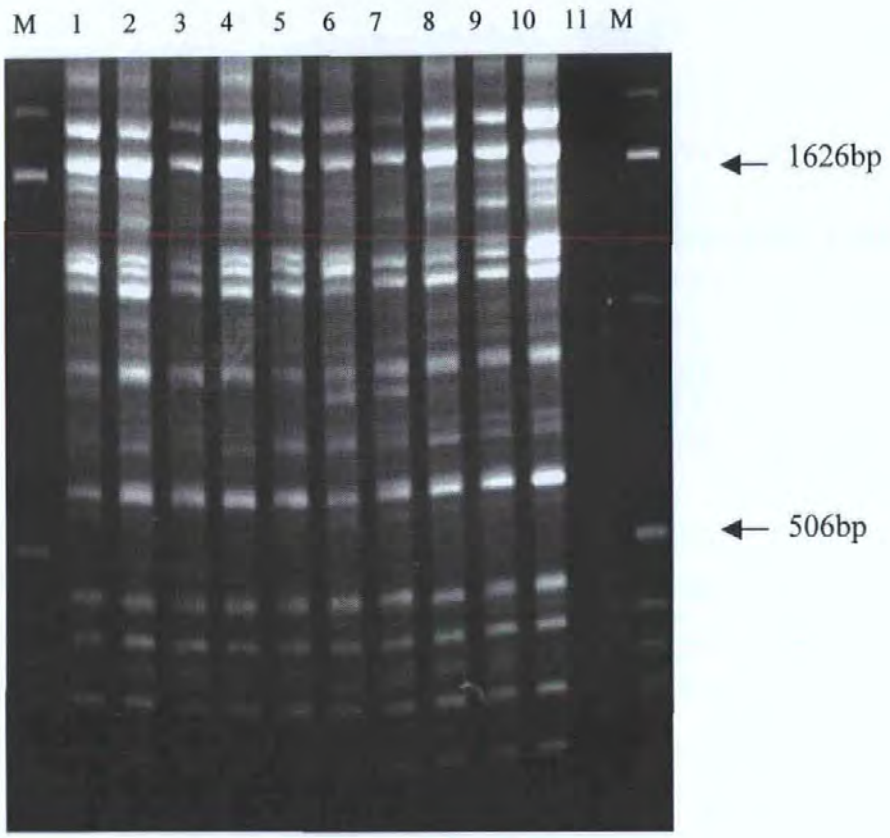


Figure 10.6: BOX-A2 PCR fingerprints of 10 isolates of *R. salmoninarum*. Lanes 1-10 correspond to the following isolates: 970083-102, 980036-87, 970153-19, A6, NCIMB1111, NCIMB1112, NCIMB1113, NCIMB1114, NCIMB1115 and NCIMB1116. Lane 11: Negative control. Lane 1 and 12: 1Kb Ladders (Gibco BRL: 0.3μg), the sizes of selected markers are indicated by arrows on the right.

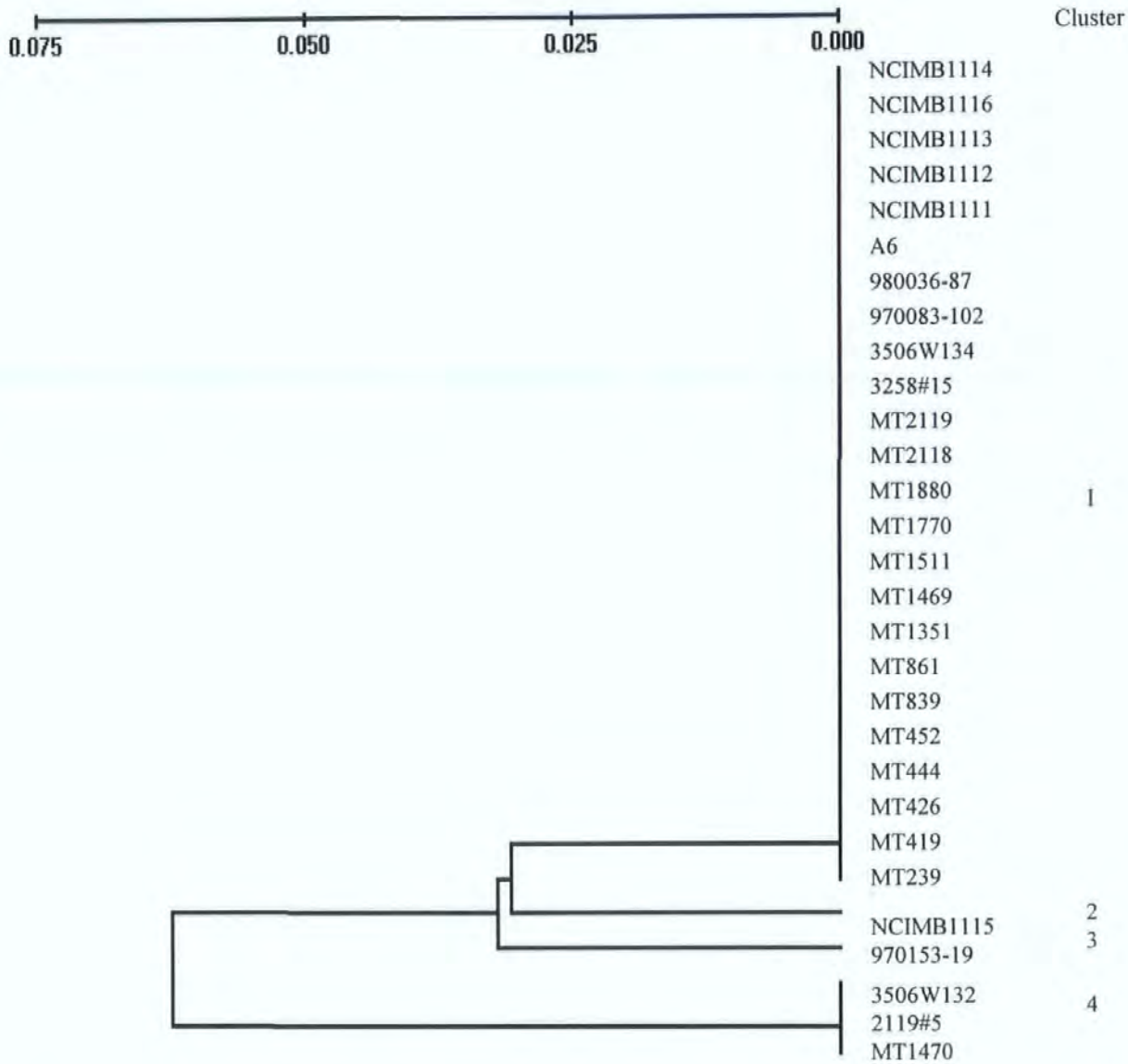


Figure 10.7: A UPGMA generated dendrogram from ERIC, REP and BOX PCR analysis of 29 *R. salmoninarum* isolates from UK locations. The scale bar denotes a relative difference between the isolates where 0.01 is equivalent to 1% in branch lengths.

10.4 Discussion

REP, ERIC and BOXA2 PCRs were applied to 29 isolates of *R. salmoninarum* from UK locations. The 3 techniques produced fingerprints that were very similar for all the 29 isolates examined; 30 of the 33 bands that were used in the analysis were invariant. Nevertheless, some differences were observed between the profiles and on this basis the isolates were grouped into 4 clusters. The majority of isolates, 24, were placed into cluster 1. These were sourced from both wild and farmed fish, from a variety of host species and from a variety of locations within the UK. There was no obvious epizootiological relationship between them (Table 3.2). It has been suggested that ideally a typing system should be able to distribute isolates more or less equally between a reasonable number of categories depending on the size and nature of the samples (Towner & Cockayne, 1994). Clearly, with 24 isolates placed into a single cluster ERIC, REP and BOX fingerprinting does not meet these ideals. In addition, ERIC, REP and BOX fingerprinting required a larger amount of genomic DNA (Timmins *et al.*, 1998) than other PCR-based methods, which is less than ideal when studying a slow growing fastidious organism such as *R. salmoninarum* where often only very small quantities of DNA are available.

Clusters 2 and 3 each contained a single *R. salmoninarum* isolate, NCIMB1115 and 970153-19, respectively. Isolate 970153-19 was initially isolated from the infected kidney of a wild grayling and has also been shown to be unique by RAPD analysis and in other typing techniques presented in this thesis (Grayson *et al.*, 1999;Grayson *et al.*, 2000b). However, isolate NCIMB1115, which was isolated in 1962 from wild Atlantic salmon in Scotland, has previously been grouped with at least 1 other Scottish isolate by RAPD

analysis, IS994 analysis, and tDNA-PCR (Grayson *et al.*, 2000b);(Fig: 8.6, Fig: 7.9). So in this case ERIC fingerprinting may have improved upon the resolution of previous studies.

The 3 isolates that were placed into cluster 4 originated from 2 English fish farms and a farm in Scotland. It is unclear why these isolates have been grouped together as they share no obvious epizootiological relationship. Nevertheless, REP- and ERIC-PCR analysis showed that all 3 lacked the same amplicons that were present in the other isolates. The absence of the amplicons may be due to an unforeseen variation in the concentration of the template DNA which can affect the outcome of these techniques but this seems unlikely because of the care taken to ensure accuracy and also because the intensities of the amplicons that were commonly shared with the remaining isolates were of equal, if not identical intensity. Initially, the reactions were performed using 10ng of template DNA and no products were generated. Because only limited quantities of DNA were available for each *R. salmoninarum* isolate, template concentrations of more than 100ng were not investigated. Therefore, the relationship between template DNA concentration and fidelity is unknown and should be addressed before the assays could be employed routinely.

Although REP and ERIC fingerprinting were able to differentiate between some *R. salmoninarum* isolates, at present, they are unlikely candidates for routine use. The overall similarity in the fingerprints that the 3 assays produced between isolates of *R. salmoninarum* without any obvious epizootiological connection further underlines the high degree of genetic conservation of this bacterium. ERIC primers have been used in other studies to establish clonal relationships between different strains of *M. tuberculosis*, a bacterium that is so highly conserved that it has been suggested that it is a relatively

recently evolved species. Nevertheless, divisions were generated with ERIC primers even within clusters of strains showing identical IS*6110* fingerprints (Sechi *et al.*, 1998).

It has been suggested that PCR protocols that are designed to amplify short interspersed repetitive elements, particularly ERIC, generate profiles by the same mechanisms as RAPD analysis. This is because there is no direct evidence for the existence of ERIC elements in gram-positive bacteria. Furthermore ERIC, REP and BOX primers are annealed at low stringency and can bind to sequences that are not fully complementary but can still initiate polymerisation e.g. ERIC-PCR primers can generate fingerprints from eukaryotic DNA templates (Hulton *et al.*, 1991; Versalovic *et al.*, 1991; Gillings & Holley, 1997). Consequently, the same careful approach to methodology and data interpretation should be employed with ERIC, REP and BOXA2 PCR as with RAPD. Primarily, it should be remembered that although different profiles clearly mean that isolates are different, those isolates that generate identical profiles are not necessarily the same. In addition to this the dendrogram (Fig: 10.6) that was designed on the basis of the ERIC, REP and BOX fingerprints should be interpreted as only a guide to inferring phylogenetic distances between isolates rather than an absolute measure.

This chapter presented the results of ERIC, REP and BOX PCR analysis of the diversity among 29 isolates of *R. salmoninarum* from the UK. Although the ERIC and REP primers were found to have a greater potential for isolate discrimination than the BOX primers, in comparison to other typing methods they have a limited capacity for routinely discriminating between clinical isolates of *R. salmoninarum.*

Chapter Eleven

A COMPARISON AND OVERVIEW OF THE MOLECULAR TYPING METHODS USED IN THIS INVESTIGATION OF *RENIBACTERIUM SALMONINARUM* ISOLATES

11.1 Introduction

In this investigation, several methods that have been commonly used for typing bacterial strains were applied to analyse the fish pathogen *R. salmoninarum* in an attempt to facilitate studies on the epizootiology of BKD. Although etiological conclusions were hampered because in-depth information was in many instances not available for the isolates that were investigated, nevertheless several techniques yielded valuable data about this bacterium. Some of these methods involved the application of PCR-based techniques (RAPD, ETR-A analysis and tDNA-PCR), whilst others, although useful, employed more complex and labour intensive techniques, such as PFGE and RFLP (IS*994* profiling). This chapter aims to review, compare and contrast the properties and results of each to the typing systems that were used in the analysis of *R. salmoninarum*, with particular emphasis on RAPD, IS*994* profiling, and tDNA-PCR, which were found to be the most discriminating of the methods that were investigated.

11.2 Experimental chapters

11.2.1 Specific gene polymorphisms

To confirm the origin of the extracted genomic DNA and to examine 3 specific regions of the genome for the presence of length polymorphisms, genes *msa*, *hly* and *rsh* were examined by PCR in a variety of *R. salmoninarum* isolates from UK locations. No obvious

variation was observed in the lengths of the amplicons that were obtained using genomic DNA template from UK isolates, reinforcing the idea that this organism is highly conserved. This supports previous studies carried out by Grayson *et al.* (1999) who found no evidence for length polymorphisms in the same regions of the genomes of 74 isolates of *R. salmoninarum* from worldwide locations. Despite the lack of obvious length polymorphisms in these regions of the genes it is possible that variations may exist at the nucleotide sequence level and it would be interesting to explore this further. Although PCR examination of the *msa*, *hly* and *rsh* genes for length polymorphism clearly has no potential for the discrimination of *R. salmoninarum* isolates, multiplex PCR of these genes may have a potential role in the positive identification of this pathogen.

11.2.2 Randomly amplified polymorphic DNA (RAPD)

Chapter 5 examined the potential of RAPD to discriminate between 28 isolates of *R. salmoninarum* from the UK. Of the 58 amplified bands that were examined, 40 bands were found to be present in all of the isolates and the presence or absence of the remaining bands was used as the basis for allocating the isolates into 12 clusters. The dendrogram that was constructed from the RAPD profiles revealed the presence of 5 main clusters of *R. salmoninarum* isolates with 7 other isolates possessing distinct and unique profiles (Fig 5.9). Some of these groupings were in good agreement with the clusters that were generated using other techniques. One of the main clusters included 4 English isolates, namely 980109F60, 980109F3, 980109F82 and 980297#97. Interestingly, 3 of these isolates were grouped together using tDNA-PCR with the fourth (980297F60) placed into a closely related sub-cluster. Three of these 4 isolates were also grouped in a distinct cluster when IS*994* profiling was applied, although in this case the fourth isolate (980297#97) was placed into what appeared to be a totally unrelated group. Therefore, of these 4 isolates grouped together using RAPD only 2 isolates 980109F3 and 980109F82 generated

identical profiles by each of these methods suggesting that these 2 isolates are either the same or a very closely related clone.

RAPD profiling also placed 3 other English isolates, 980109F85, 980109F95 and 980109F47, within a single cluster. Interestingly, these isolates were also grouped together using IS*994* profiling, but were differentiated by tDNA-PCR which placed 980109F95 in a different, but closely related, sub-cluster to isolate 980109F47, and isolate 980109F85 was placed into an entirely separate cluster. Additionally, a third cluster from the RAPD analysis containing 2 very recent English isolates, 3506W134 and 2119#5,were grouped together by tDNA-PCR along with other English, Welsh and Scottish isolates, the majority of which were not included in the RAPD study. However, tDNA-PCR analysis did place isolate 3506W132 (isolated from the same fish farm as 3506W134) with these 2 isolates. RAPD analysis provided a means of differentiating between these very similar isolates. Unfortunately, IS*994* profiling was performed on only 1 of these isolates, 3506W134, which was placed into the major cluster containing 24 other isolates from the UK.

RAPD was the only typing method that was capable of discriminating between some of the more recent Scottish isolates (isolates containing the prefix MT), the majority of which were placed into 1 of 2 groups. Cluster 4 contained 2 English isolates (3506W132, 3258#15) and 4 Scottish isolates (MT444, MT452, MT1511 and MT1880). However, tDNA-PCR profiling placed these 2 English isolates in another cluster and IS*994* analysis placed the only one of these English isolates (3506W134) that was included in the study, with these Scottish isolates (MT444, MT452, MT1511 and MT1880) albeit within a major cluster containing 25 apparently unrelated isolates.

A group of 7 Scottish isolates generated a unique RAPD profile. One such representative isolate is MT1770. This is in good agreement with other methods as this isolate is SV2, possesses a unique tDNA-PCR profile and was grouped with other SV2 isolates (which were not included in the other studies) when IS*994* profiling was applied. Additionally, and perhaps due to the resolving power of RAPD, isolates MT861 and MT1351 were found to be very different from the rest of the Scottish isolates and consequently were placed into a separate sub cluster. Neither of these 2 isolates was distinguished by tDNA-PCR or IS*994* profiling although IS*994* profiling was not performed on isolate MT1351. Four other Scottish isolates also possessed unique RAPD profiles (MT1470, MT1469, MT239, and MT1261) but only one was found to possess a unique profile by tDNA-PCR analysis (MT1470). Unfortunately, only one of these isolates, MT1469, was examined by IS*994* profiling and was grouped with the majority of other Scottish isolates.

In both this and other studies, RAPD has proved an important tool for the molecular differentiation of *R. salmoninarum* isolates. It would be a rewarding conclusion to the RAPD study to collaborate with Grayson *et al*, so that the data generated from the 28 UK isolates examined in this study could be merged with the data that was collected from the study that examined 60 isolates of world-wide origin (Grayson *et al.*, 1999; Grayson *et al.*, 2000b). This would result in the construction of a large dendrogram containing many isolates, enabling the RAPD generated relationships between isolates to be more full explored.

11.2.3 rRNA analysis

In this and related studies the rRNA operon of *R. salmoninarum* has been explored for its potential to discriminate between isolates. In a previous study, Grayson *et al.* (1999) sequenced the 16S-23S rRNA ITS region (ITS1) from 19 isolates of *R. salmoninarum* from

diverse locations and found 4 sequence variants (sequevars). It was demonstrated that the majority of isolates sourced from regions of the world that are associated with intensive salmonid culture possessed sequevar 1 (SV1). This was further reinforced by the findings in this study where sequence analysis of the 16S-23S rRNA ITS region from 28 UK isolates of *R. salmoninarum* revealed that 27 of the isolates that were examined possessed SV1. Only a single isolate (MT1770) deviated from this sequevar and possessed SV2 that had previously been reported for all Icelandic isolates as well as a lone Japanese isolate (Grayson *et al.*, 1999). The association between the Scottish isolate MT1770 and the Icelandic isolates was further confirmed by IS*994* profiling which grouped these isolates together. Additionally, this isolate was also shown to possess unique tDNA-PCR and RAPD profiles. However, in these 2 studies the association of MT1770 with the Icelandic isolates could not be confirmed, as Icelandic isolates were not included within this RAPD and tDNA-PCR study.

Interestingly, there was also a direct correlation between the possession of SV1 and the presence of two copies of the 51 bp repeat unit at the ETR-A locus. This was confirmed in an investigation by Grayson *et al.* (2000b) who suggested that ETR-A could be used as a specific marker for rapidly distinguishing SV1 isolates. Although sequence analysis of the ITS1 region from *R. salmoninarum* isolates does not possess a high degree of discriminatory power, it is recommended that this technique be applied to recent clinical isolates, particularly if the isolates under examination possess only one copy of the ETR-A locus. In these cases, sequence analysis of the ITS1 region can provide a powerful means for defining the unique nature of an isolate as well as providing some clues as to the possible geographic origin.

The rRNA operon of *R. salmoninarum* was further examined by analysing the sequence of the 23S-5S rRNA ITS (ITS2) region from 33 diverse isolates. Perhaps unsurprisingly this region was found to be identical in 32 of the isolates examined, of which the majority were sourced from England, Scotland or Wales. However, a few isolates from Iceland, Japan, and the United States were also included in the study. A single isolate (AcF6-1), which was sourced from Arctic char in the Northwest Territories of Canada, was found to differ at two nucleotide positions and the ITS2 sequence of this isolate was designated sequevar 2-2, SV22. This was in relatively good agreement with previously published results as this isolate has not only been shown to demonstrate a unique RAPD profile but also a unique ITS1 sequence, SV3 (Grayson *et al.*, 1999; Grayson *et al.*, 2000b). In this study it was demonstrated that this isolate also generated a unique IS*994* profile (Fig 8.6). Routine sequencing of PCR products could prove to be an expensive procedure and the ITS2 region apparently provides no additional information than is provided by the ITS1 region. For this reason it is not recommended for the routine typing of clinical isolates of *R. salmoninarum*. However, there are a number of rapid, high throughput typing methods that have recently been developed for detecting single nucleotide polymorphisms.

Investigations of the *R. salmoninarum* rRNA operon as a tool for isolate differentiation using ribotyping showed that the *R. salmoninarum* genome contained two rRNA genes and although some isolates showed the presence of different sized fragments using *Bam*H1 there was little other evidence to suggest that the technique would prove to be useful for typing purposes. Evidence from DNA sequences that were obtained from the intergenic spacer regions of the rRNA genes from many isolates showed that these regions, the most variable regions of the rRNA operon, are highly conserved. Although *Bam*H1 ribotyping did distinguish 2 isolates, NCIMB2235 and NCIMB1114, from the others that were examined (Chapter 6; Fig 6.7), the time consuming and expensive nature of ribotyping

combined with the conserved nature of the *R. salmoninarum* rRNA operon suggests that it is not an appropriate means for the routine typing of *R. salmoninarum* isolates.

11.2.4 tDNA-PCR analysis

The application of tDNA-PCR proved to be a very effective means of discriminating between the 43 isolates that were analysed. The 15 groups that were created using this method appeared to bear substantial similarity with the divisions that were made using other typing methods, sometimes improving upon the resolution. There were 2 major clusters of UK isolates, one that contained 11 isolates of Welsh, Scottish and English origins, and a second containing fifteen isolates solely of Scottish origin (Fig 7.9).

As noted above, tDNA-PCR placed isolates 980109F82, 980109F3 and 980297#97 into a single cluster, and isolate 980109F60 into a closely related sub-cluster. This grouping was generally reflected by both RAPD and IS*994* profiling (although this method placed isolate 980297#97 in a unrelated group). The tDNA-PCR analysis also generated unique profiles for the following English isolates A80, 980109F85, 3258#15, 980109F95, 980109F60, and 970153-19. A further sub-cluster contained isolates A6, 980109F47 and 970419-1.2.3.

When comparing these groupings with those that were constructed using other typing techniques some interesting patterns emerge. For example, isolate 970153-19, which was shown to demonstrate a unique tDNA-PCR also demonstrated a unique profile when RAPD analysis was applied. Isolate A80, which again demonstrated a unique tDNA-PCR fingerprint was grouped with many other isolates by RAPD analysis (including some of the Scottish NCIMB isolates). However, IS*994* profiling placed A80 into a separate cluster containing one other Canadian isolate which was not included in the tDNA-PCR study. The reasons why isolates A6, 970419-1.2.3 and 980109F47 were grouped together by

tDNA-PCR is unclear as none of the other techniques that were applied produced this result. Isolates A6 and 970419-1.2.3 generated different profiles during RAPD analysis and IS*994* analysis was not performed on isolate 970419-1.2.3 so the groupings generated using this method remain unknown (Grayson *et al.*, 2000b). Isolates 980109F85, 3258#15, 980109F95, and 980109F60, which have been examined by both RAPD and IS*994* analyses did not generate a unique profile using either technique. Interestingly, although both RAPD and IS*994* profiling have previously placed the 3 English isolates that were sourced from the same fish farm, 980109F85, 980109F95 and 980109F47, together in a single cluster, tDNA-PCR analysis enabled them to be differentiated. This is not entirely unexpected as the specific primers that were selected for tDNA-PCR were initially screened against these isolates for precisely this purpose.

Furthermore, tDNA-PCR analysis did not group any of the NCIMB isolates that were isolated from Scottish salmon in 1962, into the same clusters as the more recently sourced Scottish isolates (prefix MT). This was the general pattern that was also unveiled when IS*994* profiling was applied, where the more recently sourced Scottish isolates were placed in the main cluster of isolates but NCIMB isolates were placed into separate sub-clusters, often grouped along with other English isolates. Unfortunately, although RAPD profiling has been performed on all of these isolates, the NCIMB isolates were examined by Grayson *et al.* (1999; 2000b), while the recent Scottish isolates were examined in this study and so the relationships between these two sets of isolates are not readily comparable.

The tDNA-PCR analysis did not differentiate between most of the more recently sourced Scottish isolates with sixteen placed within the same cluster. A similar result was observed using IS*994* profiling, supporting the notion that genetic change does not occur rapidly

among *R. salmoninarum* populations from Scotland. Although not all of the Scottish isolates were examined using this method, of the 8 that were included, 7 were grouped together. Using IS*994* profiling these 7 isolates were also grouped with numerous other isolates of English, Welsh, Canadian, American and Swedish origin. Two of the English isolates (980036-150, 3506W134) with which the Scottish isolates were grouped by IS*994* profiling were included in the tDNA-PCR study but were placed into a different cluster (Cluster 1). However, IS*994* profiling did produce a unique result for isolate MT2119, which generated a profile similar to many other Scottish isolates during tDNA-PCR analysis, thereby improving upon the resolution that could be achieve by tDNA-PCR fingerprinting alone. In contrast to both IS*994* profiling and tDNA-PCR fingerprinting, RAPD proved to be a much more useful tool at discriminating between these Scottish isolates dividing them into a total of 9 groups of which the majority of isolates were placed into two of the groups (Chapter 5; Fig 5.9). Examination by tDNA-PCR also generated unique profiles for isolates MT1770 and MT1470. As stated above, both of these isolates generated unique profiles using RAPD analysis. Isolate MT1770, has also been shown to be different from the other UK isolates by a number of different techniques.

Clearly, tDNA-PCR fingerprinting provided another method for discriminating between clinical isolates of *R. salmoninarum.* In some cases, tDNA-PCR could differentiate between isolates which were otherwise identical in their rRNA ITS sequences, ETR-A locus and IS*994* patterns, but in other cases this technique could not discriminate between a group of isolates which had been shown to differ in one or more of these respects. The analysis of *R. salmoninarum* using tDNA-PCR fingerprinting appears to provide results that generally agree with the results of RAPD analysis. When used in combination with other molecular typing methods, tDNA-PCR could be a useful tool for epizootiological studies of BKD outbreaks in populations of both wild and farmed fish.

11.2.5 ETR-A analysis

An exact tandem repeat locus, ETR-A, was identified following the sequencing of amplicons derived from tDNA-PCR using consensus primers. ETR-A is a region of variation and, therefore, has the potential to discriminate between isolates of *R. salmoninarum* that differ in the number of copies of the 51 bp repeat that is present at this locus. Specific PCR primers complementary to sequences flanking ETR-A amplify a product that differs in size depending on the number of copies of the repeat that is present. A 250bp amplicon represents a single copy of the repeat and a 301 bp amplicon represents two copies. An investigation of ETR-A in this study and elsewhere (Grayson *et al.*, 2000b) has shown that of the 43 isolates of UK origin that were examined, only 3 were found to possess a single copy of this repeat. Grayson *et al.* (2000b) showed that a single copy of the repeat was present in Scottish isolates NCIMB1114 and NCIMB1116 and, in this study, this was also shown to be the case for isolate MT1770. Grayson *et al.* (2000b) concluded that possession of two copies of the 51 bp repeat unit at ETR-A was a marker for the possession of the SV1 sequevar of the 16S-23S rRNA ITS region. The conclusion was based upon the finding that all of the isolates that were shown to possess a single copy of the repeat unit at ETR-A were also shown to possess one of the less common sequevars, SV2, SV3 or SV4. These findings are supported by the results of this study where isolate MT1770 which, was shown to possess only a single copy 51 bp repeat at ETR-A, is SV2, whereas all of the other 42 isolates are SV1 and possess two copies of the 51 bp repeat at ETR-A. PCR amplification of the ETR-A locus can give a rapid indication of the likely nature of the 16S-23S rRNA ITS region and help define the presence of a clear and specific genetic difference within an otherwise uniform population.

11.2.6 Insertion sequence IS*994* analysis

IS*994* profiling differentiated 52 isolates from a number of different countries into 12 clusters, which generally fitted with the groupings that resulted from the use of other methods of analysis. Interestingly, cluster 1 contained 25 isolates from a variety of countries including England (3 isolates), Scotland (7 isolates), America (7 isolates), Sweden (5 isolates) and Canada (3 isolates). Two of the three English isolates (970153-19 and 980036-150) have previously been shown to produce unique RAPD profiles in a study by Grayson *et al.* (2000b) that also included many of the remaining cluster 1 isolates. Similarly, the Scottish isolate MT1469 was also placed into this cluster although it had been demonstrated to be unique using RAPD profiling (Fig 5.9) but was not unique by tDNA-PCR analysis (Fig 7.9). Of the remaining isolates placed into cluster 1, 5 of the American and all of the Swedish and Canadian isolates were grouped together using RAPD analysis. In contrast, although RAPD analysis did reveal the Alaskan isolate BY1996 to be unique, this was not supported by IS*994* analysis. Furthermore, all of the isolates in group 1 have been shown in either this study or the studies carried out by Grayson *et al.* (1999, 2000b) to possess two copies of the ETR-A locus and when 16S-23S rRNA ITS sequencing has been performed it has been shown that these isolates possess sequevar SV1.

Although many of the results that were obtained from IS*994* analysis were identical to the results from other methods, there were exceptions. Isolate 970153-19, for example, was placed into cluster 1 using IS*994*, a method which has been shown to posses good discriminatory power. This isolate has been shown to generate a unique profile in this study using rep-PCR and tDNA-PCR using both specific and consensus primers. Grayson *et al.* (2000) also showed that isolate 970153-19 possessed a unique RAPD profile. Furthermore, IS*994* profiling placed 7 Scottish isolates into cluster 1 along with 2 English

(970153-19, 3506W134) and 1 Welsh isolate (980036-150). However, tDNA-PCR analysis showed that the Scottish isolates were distinct from these.

Many of the other results of IS*994* analysis improve upon the discriminatory resolution of RAPD analysis. For example, IS*994* profiling placed DR143 (Canada) and A80 (England) together in a distinct cluster, whereas RAPD analysis placed both of these isolates within a single cluster containing a large number of other *R salmoninarum* isolates, many of which were included here (Grayson *et al.*, 2000b). Five of the 52 isolates that were examined by IS*994* analysis were unique (Cow ChS94-P22, ACF6-1, MT2119, NCIMB1114, and NCIMB1116). Paradoxically, Cow ChS94-P22 was not distinguished from most of the other isolates from the USA by RAPD analysis (Grayson *et al.*, 2000b), but tDNA-PCR using a set of consensus primers, like IS*994* analysis, did reveal this isolate to be different from the majority of other isolates that were examined (Alexander *et al.*, 2001). Similarly, MT2119 (Scotland) was distinguished from the other Scottish isolates by IS*994* profiling although tDNA-PCR and RAPD analyses did not generate a unique profile for this isolate. Using either IS*994* profiling or tDNA-PCR it was possible to differentiate between the Scottish isolates NCIMB1114 and NCIMB1116 although these isolates were shown to be identical by RAPD analysis.

As previously mentioned, 2 clusters of UK isolates, one containing isolates NCIMB1111, 980109F3, 980109F60, and 980109F82, and the other containing isolates 980109F95, 980109F95, and 980109F47, proved to be in very good agreement with the results that were obtained by RAPD analysis. Furthermore, with respect to the first cluster, the results were in good agreement with tDNA-PCR. However, the placement of isolate NCIMB1111 with these English isolates was surprising as no other technique used in this study has suggested this association.

IS*994* RFLP analysis placed the Icelandic isolates F-120-87, F-283-87, S-182-90 and F-273-87 together in a cluster that also contained the Scottish isolate MT1770 and the English isolate 980297#97. The grouping of MT1770 with these Icelandic isolates was supported by ETR-A analysis and 16S-23S rRNA ITS sequence analysis (Chapters 5 & 6; Grayson *et al.*, 1999; 2000b). However, the reasons for the association of 980297#97 with the other isolates are unclear and this relationship has not been proposed as a result of any of the other methods of analysis. Interestingly, RAPD analysis, unlike IS*994* profiling, did sub-divide some of the Icelandic isolates, thus F-273-87 was placed into a separate sub group (Grayson *et al.*, 1999; Grayson *et al.*, 2000b).

Using insertion sequence IS*994* as a probe to examine restriction fragment length polymorphisms in *R. salmoninarum* is very useful for typing purposes. IS*994* RFLP profiling provided information that, in many cases, improved upon the results that were obtained by other methods. Because of the additional costs of Southern blotting, in particular the quantities of DNA that are required, the technique will probably be most useful as a means of confirming the results of tests that can be applied directly to tissues sampled from infected fish.

11.2.7 rep-PCR

REP, ERIC and BOX PCR analysis of *R. salmoninarum* placed 29 isolates into 4 clusters with 24 isolates present in the same cluster. Many of these isolates have been demonstrated to be different using RAPD, tDNA-PCR, IS*994* profiling and 16S-23S rRNA ITS sequencing.

Although REP ERIC and BOX fingerprinting were able to differentiate between some *R. salmoninarum* isolates, such as NCIMB1115 and 970153-19, they are unlikely candidates for routine use. This is because of the overall similarity in the fingerprints that the three assays produced between isolates of *R. salmoninarum* without any obvious epizootiological connection. In comparison to other typing methods they have a limited capacity for routinely discriminating between clinical isolates of *R. salmoninarum*.

11.3 Discussion

In the preceding chapters the relationships between different isolates of *R. salmoninarum* were examined using a variety of methods, some of which required substantial development prior to application. The methods were specific gene PCR, RAPD analysis, ribotyping, sequence analysis of 16S-23S and 23S-5S rRNA ITS regions, tDNA-PCR, ETR-A analysis, IS*994* profiling, ERIC-PCR, REP-PCR and BOXA2 PCR. Three techniques, RAPD analysis, tDNA-PCR and IS*994* profiling, were found to be most useful for discriminating between isolates. Each of these 3 techniques highlighted differences between isolates that were found to be identical using the alternative methods. Generally similar genotypic groups were generated by the techniques and the methods were considered to be complementary rather than contradictory.

While 16S-23S rRNA ITS sequencing and ETR-A analysis were in absolute agreement, only a few genotypes could be distinguished in comparison with techniques such as RAPD analysis, tDNA-PCR and IS*994* profiling. This is probably because both tDNA-PCR and RAPD employ a number of different PCR primers and therefore examine a larger and, particularly in the case of RAPD, a more randomly distributed part of the genome. Similarly, IS*994* profiling examines restriction enzyme site polymorphisms around IS*994* loci. A conservative estimate revealed at least seventeen of these sites on the

R. salmoninarum genome, consequently this technique also has the capacity to examine a much larger region for variation than either the 16S-23S rRNA ITS region or the ETR-A locus (Rhodes *et al.*, 2000). Despite the lack of discriminatory power in 16S-23S ITS rRNA sequencing and ETR-A analysis these methods do have an important use. Firstly, because it is possible to design PCR primers that can be shown to be wholly specific to these regions of the *R. salmoninarum* genome, both of these methods can potentially be directly applied to DNA extracted from the tissues of infected fish. This represents a considerable saving in time that is not possible with other methods of analysis that rely on the extraction of DNA from pure cultures of the pathogen. It may take from 6-20 weeks from the time of collecting samples from diseased fish before single colonies of *R. salmoninarum* are available from selective media for testing using either RAPD or tDNA-PCR (Gudmundsdottir *et al.*, 1991). In the case of tests requiring larger quantities of DNA, such as IS*994* profiling, there would be an additional period of 3-6 weeks for the growth of pure cultures of sufficient volume. Such lengthy periods of time place considerable constraints on the implementation of appropriate action during disease outbreaks. Although the results that can be obtained from ETR-A analysis and ITS1 sequencing provide limited information, nevertheless their application could prove useful. In particular in areas such as Scotland, Iceland, Norway and Canada, where variation in ITS1 sequevars exist in populations of fish infected with *R. salmoninarum* and changes in one area could conceivably arise and indicate the possible importation of a new genotype. Where possible it is recommended that both of these techniques be carried out and in laboratories where resources are limited ETR-A analysis could be performed on all isolates and those displaying only a single copy of the tandem repeat could be further investigated using 16S-23S rRNA ITS sequence analysis.

Unfortunately, direct comparisons between the typing techniques that were applied in this investigation was complicated because not all of the isolates were examined by each of the methods. Initially, it was intended that, for consistency, all of the subtyping methods would be applied to a core number of *R. salmoninarum* isolates. This would have enabled the construction of a dendrogram that included all isolates based on all of the typing methods and a comparison of isolate groupings that were generated by each individual method. It was hoped that such an analysis would provide a more accurate representation of the genetic diversity contained within *R. salmoninarum*. Unfortunately, because of the difficulties associated with culturing *R. salmoninarum* it was not possible to have fresh DNA from each isolate available continuously throughout the duration of this study. Future work on the molecular genetics of *R. salmoninarum* will benefit from the application of the typing systems developed here, including the further development of PFGE, and also the identification of more specific markers of variation that can be applied directly to clinical samples. However, to give meaning to epizootiological studies of *R. salmoninarum* the fully recorded histories of the hosts, broodstock and offspring, associated fish stocks, stock movements and farms are required.

Chapter Twelve

GENERAL DISCUSSION

Accurate and reliable methods for the differentiation of *R. salmoninarum* are essential to epizootiological studies of BKD. Traditional methods, such as biochemical or nutritional tests and serotyping, are unable to distinguish between isolates due to the highly conserved nature of this pathogen. This study aimed to apply existing molecular typing methods and also to develop new typing methods to examine *R. salmoninarum* isolates from the UK. It was envisaged that this study would (i) enable routes of infection to be revealed particularly with respect to the interactions between wild and farmed fish, (ii) determine whether specific isolates are linked to particular host fish species, (iii) investigate the possibility that some host fish could be infected with more than one *R. salmoninarum* isolate, and (iv) establish the fluidity of *R. salmoninarum* isolates.

Various molecular-typing techniques were used to analyse many isolates of *R. salmoninarum* throughout the course of this study with varying degrees of success. Specific gene PCR, ribotyping, 23S-5S rRNA ITS sequencing and rep-PCR were found to be of limited use for discriminating among isolates of *R. salmoninarum* although some of the techniques could play an important role in identification. Other techniques, such as 16S-23S ITS rRNA sequencing and ETR-A analysis, although not highly discriminatory were found to be in excellent agreement with one another and to provide a useful means for identifying specific and well defined markers of genetic variation. Ultimately three methods, RAPD analysis, tDNA-PCR and IS*994* profiling, were found to be very useful for defining the molecular variation of *R. salmoninarum*. Some of the groupings that resulted from these typing methods contained isolates from geographically distant sources

and some groupings contained only *R. salmoninarum* isolates sourced from the same fish farm. For example, four English isolates, 980109F3, 980109F60, 980109F82 and 980297#97, of which three were sourced from the same fish farm and one from a supplying hatchery, were always grouped closely together regardless of the typing method. This suggests that they may have been derived from the same clone. Similar associations were also apparent within some groups of English and Scottish isolates. These isolates were distinctively different from isolates sourced from other parts of the UK, suggesting that some *R. salmoninarum* isolates are both geographically and genetically isolated. In order to examine this in greater depth it would be interesting to conduct a study containing a larger number of *R. salmoninarum* isolates from these specific areas and to compare them with isolates taken from areas within the UK with greater genetic diversity. This would be difficult to achieve because prior to this study all available sources of *R. salmoninarum* within the UK (and many outside the UK) were contacted in order to obtain as many isolates as possible.

This study found no obvious association between isolate grouping and host fish species. It is likely that *R. salmoninarum* isolates do not adapt to specific salmonid host fish species. However, it should also be remembered that the vast majority of isolates that were examined in this and other studies (Grayson *et al.*, 1999; 2000b) had been isolated from either rainbow trout or Atlantic salmon. If the relationship between fish host and isolate was to be fully explored more isolates sourced from a wider range of salmonid hosts should be examined. Interestingly, the only isolate studied that had been cultured from a feral grayling, 970153-19, was shown to be genetically distinct. Unfortunately, 970153-19 was the only isolate of this nature that was examined within this study as grayling are not normally associated with intensive salmonid aquaculture within the UK and so isolates such as 970153-19 are not readily available. Although this wild grayling was not

apparently associated with intensive salmonid aquaculture, which may explain why it was host to a more genetically distinct isolate of *R. salmoninarum*, the possibility that it was exposed to the pathogen as a result of fish farming practices on the same river system cannot be excluded. Hence the question as to how and why the differences in the genome of 970153-19 arose remains unanswered. Furthermore, isolates such as ACF6-1 and COW ChS 94 P22, which generated completely unique IS*994* profiles were sourced from an artic char and a chinook salmon, respectively. However, it should be noted that in this work other isolates that were sourced from both of these fish hosts were also included but were not related, again without any clear evidence for an "isolate-fish host" association. The availability of several typing systems will facilitate further investigations of this nature.

The combined results of both this and the study by Grayson *et al.* (1999, 2000b) reveal that the majority of *R. salmoninarum* isolates examined possess both the SV1 sequevar of the ITS1 region in conjunction with two copies of the 51 bp repeat unit at ETR-A. It is unknown whether the possession of these attributes is an indication of some adaptive or pathogenic advantage that has arisen in response to fish farming, for example in response to antibiotic treatments or commercial fish diets, or whether this sequevar was widely disseminated with some of the original stocks of rainbow trout that were distributed to many parts of the world from a few sources in the 19th and 20th centuries. Alternatively, the predominance of isolates possessing SV1 and two copies of the repeat at ETR-A may simply reflect the infrequent occurrence of mutational events in isolates which, because of their geographical isolation, have not become widely distributed. From a phylogenetic and evolutionary perspective it may be that sequevars other than SV1 may have originated clonally from a single ancestral *R. salmoninarum* isolate that lacked two copies of the ETR-A locus and that mutations within the 16S-23S rRNA ITS region gave rise to the different sequevars SV2, SV3, and SV4 that were identified by Grayson *et al.* (1999;

2000b). An alternative, and seemingly less likely, hypothesis is that in certain environments there is a selection pressure on the *R. salmoninarum* genome and a single copy of the ETR-A tandem repeat region and a variation in the ITS1 region have arisen together from different ancestral clones on more than one occasion. Whatever the reasons it should be remembered that because of the nature of this study, the isolates of *R. salmoninarum* were obtained from statutory bodies whose main focus is the management of fish and fisheries. Hence the isolates were not derived from a programme designed to gather samples from a wide range of diverse species and environments, including both wildfish and farmed fish. This situation has undoubtedly led to some bias in the results, particularly as most of the isolates were obtained from veterinary laboratories and were sourced from farmed rainbow trout and Atlantic salmon. In the case of the studies of Grayson *et al.* (1999; 2000b) many of the isolates from the USA and Canada were from either farmed or captive bred Pacific salmon. Those isolates from wild fish were nearly always obtained from stocks in close proximity to aquaculture operations.

It is interesting from an epizootiological perspective that the Scottish isolate MT1770 was linked, using 16S-23S ITS rRNA sequences, IS*994* profiling and ETR-A analysis, with Icelandic isolates. It is possible that the ancestral clone of MT1770, the only SV2 sequevar to have been identified in *R. salmoninarum* from Scottish sources spanning 1962-1998, originated in Iceland, as all of the Icelandic isolates that have been examined to date possess this sequevar and were isolated up to 10 years earlier than MT1770. If this were the case, possible routes of infection of the fish stocks held on a Perthshire farm with an infrequently encountered *R. salmoninarum* clone that may have originated from Iceland are unknown. It may be that this fish farm acquired the isolate as a consequence of purchasing contaminated fish stocks, or alternatively, from exposure to infected migratory feral fish that had contact with this fish farm. Grayson *et al.* (1999) reported the only other known

source of an SV2 isolate from *R. salmoninarum* isolated from an infected coho salmon in Japan.

Previous studies of the detection, epizootiology and control of *R. salmoninarum* have been constrained by the difficulties associated with culturing the organism. In this study, the interpretation and comparison of the results that were obtained using each of the typing methods was made more complicated because not all of the isolates were examined by every method. This occurred because *R. salmoninarum* grows slowly and cultures are sometimes inviable imposing substantial time constraints on the work. Often no other source of a culture was available. Culture of viable organisms is both a definitive test for the presence of this organism and an essential stage if the isolates are to be fully sub-typed and it is likely that future epizootiological studies will also suffer from this problem. This problem will be overcome with the development of more effective culture media and typing methods that can be applied directly to clinical samples.

Currently, the epizootiology of BKD has yet to be fully determined. The epizootiology of this disease, its disease status, the interaction between wild and farmed fish and the relative distribution of *R. salmoninarum* have been questioned. In Iceland it has been suggested that *R. salmoninarum* is a common endemic resident within some feral salmonid populations and host fish are commonly exposed to and are colonised by low levels of this pathogen. In such populations, sub-clinical infections and asymptomatic fish predominate, with clinical BKD commonly being found during the migration season when fish endure periods of stress (Jonsdottir *et al.*, 1998). In contrast, other salmonid populations have been reported to be relatively free of *R. salmoninarum* with the occurrence of small epidemics associated with the importation of infected fish stocks. In many other countries, including the UK, the precise dynamics of this disease and its status as either endemic or epidemic in

both farmed and feral salmonid populations remains unclear. It is envisaged that further studies into the epizootiology of this disease, fish host immunity and isolate detection and diversity will reveal the true extent of the distribution of *R. salmoninarum* and more about the nature of the BKD within the UK. Such information would be used to determine the true extent of *R. salmoninarum* isolate distribution, the fluidity of the isolates that are known to exist and to show whether there is a continued necessity for BKD to be a notifiable disease.

Overall the results of each of the typing methods were in good agreement with one another, indicating that *R. salmoninarum* could be clonal. Currently, *R. salmoninarum* is considered to be an obligate intracellular pathogen that is not known to exist as a free-living organism. It is likely that in this situation there are limited opportunities for genetic exchange to occur between either *R. salmoninarum* isolates or other bacterial species. To date there has been no published information on either the occurrence or the mechanisms that could facilitate conjugation or transformation. Indeed, there is no evidence for the existence of a bacteriophage that is able to infect *R. salmoninarum*, making transduction unlikely. However, this may be a reflection of the limited amount of information that is available for this organism. Much of the genomic polymorphism that has been observed in this study may result from mobile genetic elements and random mutation. It would, however, be very interesting to investigate the clonality of this organism further. Methods, such as multi-locus sequence typing (MLST), that examine the nucleotide sequence of common housekeeping genes could possibly reveal more about the clonality of this organism. MLST has identified mosaic genes (a gene where the nucleotide sequence is recognisable as a recombination of two parental sequences) in several bacterial species revealing that horizontal genetic exchange has played a role in their evolution (Maiden, 1993). It would be interesting to apply a method such as MLST to *R. salmoninarum* not only to determine

its potential as a tool for the discrimination of isolates but also to possibly reveal additional information about the evolution of this pathogen.

Finally, this study involved the development and application of several typing systems, which proved to be reproducible and reliable, and in good agreement with each other. It is hoped that they will be used in future studies to reveal more about the population genetics of *R. salmoninarum* and BKD epizootiology. Many of the typing methods used in this study were both developed and applied in this laboratory. It would be useful to perform a more in-depth analysis with the collaboration of other laboratories. Possibly a double blind study which examines the discriminatory power, reproducibility and epizootiological groupings generated by all of the typing methods. Such studies have been successfully performed for many of the typing methods applied to other pathogenic bacteria and often reveal the true robustness of each of the typing methods, as well as reinforcing or questioning isolate groupings generated in other studies (van Belkum, 1998c).

In addition, it may prove useful to determine the stability of the typing methods that were applied in this study of *R. salmoninarum*. It has previously been suggested that only methods such as MLEE (multilocus enzyme electrophoresis) and MLST (multilocus sequence typing) are suitable for long-term and global epizootiological analysis because these methods index variations that accumulate very slowly and are commonly selectively neutral. Whether this method would be successful remains unclear as MLEE, the less discriminatory alternative to MLST, did not differentiate between American isolates of *R. salmoninarum* (Starliper, 1996). Similarly, there are also reports that some genetically uniform bacterial species, such as *Mycobacterium tuberculosis*, do not contain sufficient diversity at the nucleotide level for MLST to be used and *R. salmoninarum* may well fall into this category (Sreevatsan *et al.*, 1997). It has also been suggested that for many

bacterial pathogens PCR-based methods and methods that rely on differences in the position of restriction sites essentially rely on more highly variable regions of the genome which are more suited to examining micro-variation and, consequently, their stability is more suitable for the examination of local or short-term epizootiology (Spratt, 1999; Yakubu *et al.*, 1999). These statements however were made with reference to more genetically diverse and less clonally constrained bacterial species such as *Neisseria meningitidis* and *Streptococcus pneumoniae*. Due to the conserved nature of *R. salmoninarum* it is unclear whether some of the typing methods that were explored in this study may be suitable for long-term typing of *R. salmoninarum*. Several of the typing studies performed in this investigation included *R. salmoninarum* isolates sourced as far back as 1962 some of which often displayed fingerprints also generated by more recently sourced isolates inferring that the many of the techniques are reasonably stable. This was also indicated in the RAPD study carried out by Grayson *et al.* (2000b), where 28 out of 60 isolates, many of which were sourced from different world-wide locations over a broad time scale, possessed identical RAPD fingerprints inferring that *R. salmoninarum* maintains a stable genetic framework over time. Nevertheless, it would be very interesting to determine whether the techniques are more suitable for either long-term global or short-term local epizootiological studies.

12.2 Conclusion

This study explored the potential of newly developed and existing typing methods to discriminate between different isolates of *R. salmoninarum* with the aim of expanding upon the epizootiological knowledge of BKD. Multilocus typing techniques, including RAPD analysis, IS*994* profiling and tDNA-PCR proved to be very discriminatory and were found to be in good agreement with one another. Similarly single locus typing techniques, including 16S-23S rRNA ITS sequencing and ETR-A analysis, although less

discriminatory were also in good agreement with one another and proved to be important for the typing of *R. salmoninarum*. It is hoped that these methods will be useful tools for further epizootiological studies of BKD outbreaks in populations of both wild and farmed fish.

Some of the other techniques that were applied in this study, including REP-PCR, ERIC-PCR, BOX-PCR, specific gene PCR, 23S-5S ITS rRNA sequencing and ribotyping, proved to be inadequate for isolate discrimination although some of these methods could play an important role in identification. PFGE was also explored, and although the full potential of PFGE was not established due to technical difficulties, the results suggest that the *R. salmoninarum* genome is larger than expected, lying somewhere between 4.5-6 Mb. Due to the great discriminatory power of PFGE, it is recommend that this technique be investigated further for the purposes of characterising the genome of *R. salmoninarum*.

Unfortunately, any epizootiological conclusions that could have been drawn from this study were hampered by the lack of information that was available for each isolate. Due to the sensitive nature of BKD outbreaks the exact locations of affected fish farms, the sources and suppliers of fish stocks, the recorded histories of BKD outbreaks and fish movements were not available. Hence, this project was initially undertaken to study the epizootiology of BKD, but of necessity became a study investigating the population genetics of *R. salmoninarum*. Despite this the application of multiple typing methods established that isolates of *R. salmoninarum*, even from the UK, appear to be genetically diverse. Geographically related isolates of *R. salmoninarum* were often grouped together using the typing methods suggesting that some isolates may be relatively localised despite the international trade in fish stocks.

APPENDIX I

REAGENTS, SOLUTIONS, MEDIA AND SUPPLIERS

I.1 Genomic DNA extraction

Genomic DNA was extracted using the Puregene Kit manufactured by Gentra Systems, 13355 10th Avenue North, Suite 120, Minneapolis, MN55441, USA. Details of the contents of each solution are as follows. The concentration of each components is unknown.

Cell Suspension Solution
Tris [hydroxymethyl] aminomethane
EDTA
Sorbitol

Cell Lysis Solution
Tris [hydroxymethyl] aminomethane
EDTA
SDS

Protein Precipitation Solution
Ammonium acetate

DNA Hydration Solution
Tris
EDTA

Lytic enzyme solution and RNase A stock solution were stored at 4°C. All other Puregene reagents were stored at room temperature (21°C).

I.2 General molecular biology solutions and buffers

TBE buffer

TBE was made up at 5 X strength according to the following recipe and was then diluted to 1 X:

Tris-base	54g
Boric acid	27.5g
EDTA 0.5M [pH 8.0]	20ml

TE buffer

TE buffer was prepared either by diluting X 100 TE supplied by Sigma in sterile 'Analar' water or made up as a 1 x solution according to the following recipe:

Tris-HCL	10mM
EDTA	1mM

pH 8

Autoclave

Gel loading solution

Gel loading solution (X6) was purchased from Sigma:

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)
Sucrose	40% (w/v)

Roche *Taq* polymerase 10 x reaction buffer

Tris-HCl	100mM
$MgCl_2$	15mM
KCl	500mM

pH 8.3

MBI Fermentas *Taq* polymerase 10 x reaction buffer

Tris-HCl	100mM
KCl	500mM
Nonidet P40	0.8%

I.3 Bacteriological media

Selective Kidney Disease Medium (SKDM)
Per litre of ddH_20:

Tryptone T,	10g
Yeast extract,	0.5g
L-cysteine	1g
pH - 6.8	(Adjusted with the addition of NaOH)

Media was sterilised by autoclaving at 121°C, 15psi for 15 minutes.

Once cooled the following supplements were added:

Spent broth culture,	50ml
Newborn calf serum,	50ml
Oxolinic acid (Stock)	1ml (final concentration 2.5µg/ml)
Polymyxin B sulphate (Stock)	1ml (final concentration 2.5µg/ml)

(Evelyn *et al.*, 1990)
(Austin *et al.*, 1983)

SKDM agar was made by the addition of 1.5% (w/v) agar

Preparation of spent broth culture
Spent broth culture was prepared by centrifugation of a stationary *R. salmoninarum* broth culture. The supernatant was decanted and autoclaved. After cooling to 15°C the spent broth was ready to dispense.

Preparation of antibiotic stocks
Oxolinic Acid
25mg oxolinic acid was dissolved in 10ml of 0.1M NaOH
The resulting mixture at a concentration of 2.5mg/ml, was filter sterilised, aliquoted and stored at -20°C until required.

Polymyxin B sulphate
25mg polymyxin B sulphate was dissolved in 10ml of 0.1M NaOH
The resulting mixture was filter sterilised, aliquoted and stored at -20°C until required

Ampicillin
0.5g of amplicillin was dissolved in 10ml of ddH_20.
The resulting solution (50mg/ml) was filter sterilised and stored at 4°C until required.

Tryptone soya broth (TSB)
TSB powder was purchased as pre-made media from Lab M. 30g of media was dissolved in 1L of ddH_20, the pH adjusted to 7.5 and then autoclaved. The formula of the resulting media is as follows:

Tryptone (casein digest USP)	17g
Soya peptone	3g
Sodium chloride	5g
Dibasic potassium phosphate	2.5g
Dextrose	2.5g

Tryptone soy agar (TSA)
TSA powered was purchased as pre-made media from LabM. 37g of media was dissolved in 1L of ddH_20, the pH adjusted to 7.5 and then autoclaved. TSA contained the same components as state in the TSB media although TSA contained 1.5% (w/v) agar.

Luria-Bertani medium (LB)
Per litre of ddH_20:

Tryptone	10g
Yeast extract	5g
Sodium chloride	10g

pH - 7.5
Sterilised by autoclaving

LB agar was prepared as stated above but with the addition of 1.5% w/v agar

Luria- Bertani medium with ampicillin
A litre of LB agar (made as stated above) was melted and placed in a water bath at 45°C. 1ml of ampicillin stock was added to the agar, mixed thoroughly and poured into Petri dishes.

ITPG and X-Gal overlays
Overlays were added to LB agar plates, prepared as above, by dispensing 50µl of IPTG and 50µl of X-Gal stock solution into 5ml of ampicillin LB agar in a bijou bottle. The mixture was pipetted up and down twice to ensure adequate mixing and then poured over a preformed LB agar plate.

IPTG stock
Prepared at a concentration 25µM in ddH_20
96mg of IPTG was dissolved in 4ml of ddH_20. The resulting solution was filter sterilised and stored at -20°C until required.

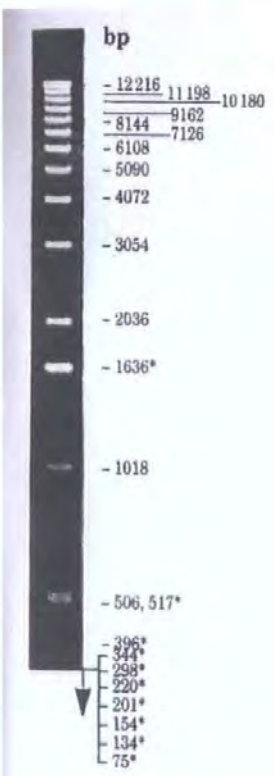
X-Gal stock
Prepared at a concentration of 20µM in DMF
100mg was dissolved in 5ml of DMF and the solution was stored at -20°C until required.

I.4 Details of molecular weight markers

100 bp Ladders 100 – 2072bp (Gibco BRL)

The 100bp ladders range from 100-1500bp in 100bp increments, plus a 2072bp fragment. The 600bp band is 2-3 times brighter than the others.

1kb Ladder 75 - 122216bp (Gibco BRL)

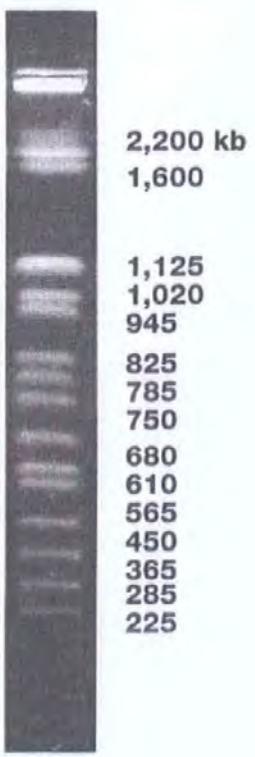


The 1kb ladders range from 75bp to 122216bp in various accruements.

Lambda Ladder 48.5 – 873kb (BioRad)

Lambda ladders range from 48.5kb – 873kb in 48.5kb increments.

***Saccharomyces cerevisiae* Ladders 0.2 – 2.2 MB (BioRad)**



CHEF DNA size standards generated from 15 *S. cerevisiae* chromosomes which range in size from 0.2-2.2Mb.

***Schizosaccharomyces pombe* Ladders 3.5 – 5.7MB (BioRad)**

CHEF DNA size standards generated from 3 *S. pombe* chromosomes which range in size from 3.5-5.7Mb.

I.5 Description of RFLP solutions and buffers

Depurination solution
Per litre of ddH_20:
HCl 0.2M

Denaturing solution
Per litre of ddH_20:
NaCl 0.5M
Tris-HCl 1.5M
pH 8

Neutralisation solution
Per litre of ddH_20:
NaOH 1.5M
NaCl 0.5M

20 X SSC
Per litre of ddH_20:
Sodium chloride 175.32g
Sodium citrate 88.23g
pH 7

The above solution was diluted to 10 x, 5 x, 2 x, or 0.1 x strength as required, with sterile 'Analar' water.

Prehybrisation solution
Made up in 5 x SSC:

Herring sperm DNA (blocking reagent)	1% (w/v)
N-lauroylsarcosine	0.1% (w/v)
SDS	0.02% (w/v)

Immunological detection and development solutions

Buffer 1
Per litre of ddH_20:
Tris-HCl 100 mM
NaCl 150mM
pH 7.5

Buffer 2
Made up in Buffer 1:
Herring sperm DNA (blocking reagent) 0.5% (w/V)

Buffer 3
Tris-HCl 100mM
NaCl 100mM
$MgCl_2$ 50mM
pH 9.5

Development solution

Made up in 70ml Buffer 3:

NBT	33mg
X-phosphate	17mg

Buffer 4

Tris-HCl	10mM
EDTA	1mM
pH 8	

Probe buffers

Hexanucleotide mixture (x 10)

Random hexanucleotides	62.5 A_{260} units/ml (1.56mg/ml)
Tris-HCl	500mM
$MgCl_2$	100mM
Dithioerythritol (DTE)	1mM
BSA	2mg/ml
pH 7.2	

dNTP labelling mixture (x 10)

dATP	1mM
dCTP	1mM
dGTP	1mM
dTTP	0.65mM
Alkali-labile DIG-dUTP	0.35mM
pH 6.5	

Klenow enzyme

2 Units/µl of DNA polymerase I (Klenow enzyme, large fragment, labelling grade from *E.coli*).

I.6 Prep-A-Gene DNA purification kit solutions

DNA was purified using the Prep-A-Gene purification kit supplied by BioRad. Details of the content of each solution are as follows:

Prep-A-Gene DNA purification kit binding buffer

Sodium perchlorate	6M
Tris [pH 8]	50mM
EDTA [pH 8]	10mM

Prep-A-Gene binding matrix

Prep-A-Gene binding matrix is made up of a silica based DNA purification matrix suspended in deionised water.

Prep-A-Gene wash buffer

NaCl	400mM
Tris [pH 7.5]	20mM
EDTA [pH 7.5]	2mM
50% EtOH (v/v)	

I.7 Pulsed field gel electrophoresis buffers

Cell suspension buffer

Tris	10mM [pH 7.2]
NaCl	20mM
EDTA	50mM

Wash buffer

Tris	20mM
EDTA	50mM
pH 8	

Lytic buffer

Tris-HCl	10mM
NaCl	34mM
EDTA	100mM
Brij 58	0.5%
SDS	0.2%
Lauroyl sarcosine	0.5%
pH 8	

Autoclave, cool and add achromopeptidase and lysozyme to a final concentration of 30 u/ml and 1 mg/ml, respectively.

Proteolysis buffer

Tris	10mM
EDTA	1mM
SDS	1%
pH 7.4	

HE buffer

HEPES	10mM
EDTA:	1mM
pH 8.0	

SE buffer

NaCl	75mM
EDTA	25mM
pH 7.5	

Achromopeptidase stock

Achromopeptidase stock solution (3000u/ml) was prepared in sterile distilled water and stored as 200µl aliquots at -20°C.

Proteinase K

Proteinase K stock solution (25 mg/ml) was prepared in sterile distilled water and stored as 400µl aliquots stocks at -20°C.

Running buffers

Tris acetate EDTA

50 x stock:	
Tris base	242g
Glacial acetic acid	57.1 ml
0.5M EDTA	100ml
pH 8	

This solution was diluted immediately to a 1 x working concentration.

Tris-borate EDTA (TBE)

100 x stock:	
Tris base	108g
Boric acid	55g
0.5M EDTA	40ml
pH 8	

This solution was diluted immediately to a 0.5 x working concentration.

I.8 Suppliers details

BioRad Labs Ltd
BioRad House
Maylands Ave
Hemel Hempstead
Hertfordshire
HP2 7TD

Gentra Systems
13355 10th Avenue North
Suite 120
Minneapolis
MN55441
USA
www.gentra.com

ICN Pharmaceuticals Ltd
ICN Elmwood
Chineham
Business park
Basingstoke
Hants

LabM
Topley House
Wash Lane
Bury
England
BL9 6AV

New England Biolabs (UK) Ltd.
73 Knowl Piece
Wilbury Way
Hitchin
Hertfordshire
SG4 OTY
England
www.neb.com

MBI Fermentas / Helena BioSciences
Colima Avenue
Sunderland Enterprise Park
Sunderland
Tyne & Wear
SR5 3XB
England
www.helena-biosciences.com

MWG Biotech
Mill Court
Featherstone
Wolverson Mill South
Milton Keynes
MK12 5RD

Oxoid Ltd
UK Head Office
Wade Rd
Basingstoke
Hampshire
RG24 8PW
UK

Roche Molecular Biochemicals
Bell Lane
Lewes
East Sussex
BN7 1LG
www.biochem.roche.com

Sigma-Aldrich
Fancy Road
Poole
Dorset
UK
England
BH17 7NH
www.sigma-aldrich.com

Thistle Scientific Ltd (formerly ScotLab Ltd)
DFDS House
Goldie Road
Uddingston
Glasgow
G71 6NZ
www.thistlescientific.co.uk

Appendix III

Sequencing Data

II.1 Sequencing data relevant to Chapter 6

Figure II.1: Partial nucleotide sequence of the rRNA operon of *R. salmoninarum* ATCC33209^T. Sequence includes 23S rRNA gene, 23S-5S rRNA ITS (ITS2) region, 5S RNA gene and 176bp sequence of 3' region. GenBank accession numbers: AF143777, AF180950).

>*R. salmoninarum* 23S rRNA gene 3135bp
GTTCAAGTTTTTAAGGGCACACGGTGAATGCCTTGGCATTAGGAGCCGAAGAAGGACGTAGGAATCTGCG
ATAAGCCTCGGGGAGTTGATAACCGAACACTGATCCGAGGATGTCCGAATGGGGAAACCCCGCTGCCTGT
TATGGGTAGTGACCCACATCTGAACACATAGGGTGTGTGGAGGGAACGTGGGGAACTGAAACATCTCAGT
ACCCACAGGAAGAGAAAACAATAGTGATTCCGTAAGTAGTGGCGAGCGAACGCGGAACAGGCTAAACCGT
TCATGTGTGATACCCGGTAGGGGTTGCATGGTCGGGGTTGTGGGACGTACTGTTCCAGTTCTACCGGACT
GGTGAGGTGAGAGTGTGAACATAGGTGAACGGTCTTGAAAGGCCGGCCAGAGAGGGTGTAAGCCCCGTAA
CTGAAATGTTTACACCGCCTGGGACGTATCCCAAGTAGCACGGGGCCCGAGAAATCCCGTGCGAATCTGT
CAGGACCACCTGATAAGCCTAAATACTCCCTAATGACCGATAGCGGACAAGTACCGTGAGGGAAAGGTGA
AAAGTACCCCGGGAGGGGAGTGAAATAGTACCTGAAACCGTGTGCTTACAATCCGTCGGAGCAGTCATGT
GATTTATCACGAGTAACTGTGACGGCGTGCCTTTTGAAGAATGAGCCTGCGAGTTAGTGCTCAGTGGCGA
GGTTAACCCGTGAGGGGCAGCCGTAGCGAAAGCGAGTCTGAATAGGGCGAATGAGTCGCTGGGTCTAGAC
CCGAAGCGGAGTGATCTACCCATGGCCAGGTTGAAGCGACGGTAAGACGTCGTGGAGGACCGAACCCACT
TCAGTTGAAAATGGAGGGGATGAGCTGTGGGTAGGGGTGAAAGGCCAATCAAACTCCGTGATAGCTGGTT
CTCCCCGAAATGCATTTAGGTGCAGCGTTGCGTGTTTCTTACTGGAGGTAGAGCTACTGGATGGCCGATG
GGCCCTACAAGGTTACTGACGTCAGCCAAACTCCGAATGCCGGTAAGTGAGAGCGCAGCAGTGAGACTGT
GGGGGATAAGCTTCATAGTCGAGAGGGAAACAGCCCAGACCACCAACTAAGGTCCCTAAGCGTGTGCTAA
GTGGGAAAGGATGTGGGATTGCTTAGACAACCAGGAGGTTGGCTTAGAAGCAGCCACCCTTGAAAGAGTG
CGTAATAGCTCACTGGTCAAGTGATTCCGCGCCGACAATGTAGCGGGGCTCAAGTACACCGCCGAAGTTG
TGGATTTCATATTATTGGTAAGCCTTCGTGGTTCAGCCGTATGGAGTGGTAGGGGAGCGTCGTGTGGGCA
GTGAAGTCGCGGTGTAAACCAGCGGTGGAGCCTACACGAGTGAGAATGCAGGCATGAGTAGCGAAAGACG
GGTGAGAAACCCGTCCGCCGAATGATCAAGGGTTCCAGGGTCAAGCTAATCTGCCCTGGGTAAGTCGGGA
CCTAAGGCGAGGCCGACAGGCGTAGTCGATGGACAACGGGTTGATATTCCCGTACCGGCGAAGAACCGCC
CATACTGAGCAGGTGATACTAACCGCCAGAAGCATGATCGATCACCCTTGTGGTGTGAGGTTTTTTGTGG
ATCGCGGGACCTTATCCTGGGAGGTAAGCGTATTAACAGGTGTGACGCAGGAAGGTAGCTGAGCCGGGCG
ATGGTTGTCCCGGTCTAAGCAGGTAGGCCGTTCCCTAGGCAAATCCGGGGAACATTAAGGCTGAGACGTG
ATGGGACCCCCTTACGGGGGAATTCAGTGATCCTATGCTGCCAAGAAAAGCATCGACGCGAGGTTCCAGC
CGCCCGTACCCCAAACCGACACAGGTGATCAGGTAGAGAATACTAAGGCGATCGAGAGAATTATGGTTAA
GGAACTCGGCAAAATGCCCCCGTAACTTCGGGAGAAGGGGGGCCCCGACCGTGACACACACTTGCTGTGT
AGAGCGGGCAAGGGCCGCAGAGACCAGGGGGAAGCGACTGTTTACTAAAAACACAGGTCCGTGCGAAGTC
GCAAGACGATGTATACGGACTGACTCCTGCCCGGTGCTGGAAGGTTAAGAGGACCGGTTAGCTCTTCGGA
GCGAAGCTGAGAATTTAAGCCCCAGTAAACGGCGGTGGTAACTATAACCATCCTAAGGTAGCGAAATTCC
TTGTCGGGTAAGTTCCGACCTGCACGAATGGAGTAACGACTTCCCCGCTGTCTCAACCATAAACTCGGCG
AAATTGCACTACGAGTAAAGATGCTCGTTACGCGCAGCAGGACGGAAAGACCCCGAGACCTTTACTATAG
TTTGGTATTGGTGTTCTAAGTGGTTTGTGTAGGATAGGTGGGAGACTTTGAAGCCCGGACGCCAGTTCGG
GTGGAGTCATCGTTGAAATACCACTCTGGCCACTTGGGACTCCTAACTTCGGCCCGTAATCCGGGTCAGG
GACAGTGCCTGATGGGTAGTTTAACTGGGGCGGTTGCCTCCTAAAGAGTAACGGAGGCGCCCAAAGGTTC
CCTCAGCCTGGTTGGCAATCAGGTGTCGAGTGTAAGTGCACAAGGGAGCTTGACTGTGAGAGCGACAGCT
CGAGCAGGGACGAAAGTCGGGACTAGTGATCCGGCGGTACATTGTGGAATGGCCGTCGCTCAACGGATAA
AAGGTACCTCGGGGATAACAGGCTGATCTTGCCCAAGAGTCCATATCGACGGCATGGTTTGGCACCTCGA
TGTCGGCTCGTCGCATCCTGGGGCTGGAGTAGGTCCCAAGGGTTGGGCTGTTCGCCCATTAAAGCGGTAC
GCGAGCTGGGTTTAGAACGTCGTGAGACAGTTCGGTCCCTATCCGCTGCGCGCGCAGGAAATTTGAGAAG
AGCTGTCCTTAGTACGAGAGGACCGGGACGGACGAACCTCTGGTGTGTCAGTTGTACTGCCAAGTGCATC
GCTGATTAGCTACGTTCGGAAGGGATAACCGCTGAAAGCATCTAAGCGGGAAGCCCACTTCAAGATGAGA
TTTCCATACACTTTATGTGTGAGAGGCCCCCAGCCAGACCACTGGGTTGATAGGCCGGACGTGGAAGCAA
GGACTAACGACTTGTGAAGCTGACCGGTACTAATAGGCCAACAACTTACAACCAC

>*R. salmoninarum* 23S-5S rRNA intergenic spacer 219bp
ACCTCACCGATAAACCCACCCTTCAAAAGGGGTTTATCACCAAAGGTAAGTGTAAAAACCTGCATGCACG
CGTCCACTCTACGGTTCCCAACCAACAAACCCACACCTAAAAAGGGTAGCAACGGTTCGTTGACACGGAA
ACCACATAACTACATACACGCAACACCAAACACTTCACACCACCACACCCCACAACAGGGTGCGGACCGG
TAAAAAAGA

>*R. salmoninarum* 5S rRNA gene 120bp
GTTACGGCGGCCATAGCGTGGGGGAAACGCCCGGACCCATCTCGAACCCGGAAGCTAAGACCCACAGCGC
CCATGGTACTGCACTCGCGAGGGTGTGGGAGAGTAGGACACCGCCGGACA

>*R. salmoninarum* 5S rRNA 3' region 176bp
ACCAGTAAGAATAAAGGTCCCACAACGACGTGGGACCTTTATTAATTTAACAAGCTTTTTGTGACTTCAG
TGACCAACCCAACAAAGCATGAGGCTGATCTTATGGAAAGACTTTTCTCACACACAACCGAGAACCGGGG
CAGTTCGCTTTCCTCGCTGTAGTGCCGATTCTGTTT

Figure II.2: Nucleotide sequence of the 23S-5S rRNA ITS DNA probe amplified from the genomic DNA of *R. salmoninarum*NCIMB1114. Sequence in bold depicts primer sites.

>*R. salmoninarum* 23S-5S rRNA ITS DNA probe
GATAAACCCACCCTTCAAAAGGGGTTTATCACCAAAGGTAAGTGTAAAAACCTGCATGCACGCGTCCACT
CTACGGTTCCCAACCAACAAACCCACACCTAAAAAGGGTAGCAACGGTTCGTTGACACGGAAACCACATA
ACTACATACACGCAACACCAAACACTTCACACCACCACACCCCAC**AACAGGGTGCGGACCGGTAAAAAA**

II.2 Sequencing data relevant to Chapter 7

Figure II.3: Nucleotide sequence of 2.3kp PCR product generated from *R. salmoninarum* isolate ATCC33209^{TS} using primers T5A-T5B (Fig: 7.2).

AGTCCGGTGCTCTAACCAACTGAGACACCACAACACGCTCGGTGCCGTTGATAACAAAGGTGCCTTTTTC
GGTCATCAGCGGGAAATCGCCCATGAACACGGTTTGCTGCTTGATTTCACCAGTGTTGTTGTTCATGAAC
TCGGCTTTGACATACAAAGGAGCCGAGAACGTAGCGTCCCGGTCCTTGCATTCAGCCATCGTGTACTTCG
GATCAGCGAACTCCGGCTCCGAGAAGCTCAAGGACATAGTGCCCTGGAAATCCTCGATCGGGGAGATCTC
TTCGAAGATGTCCGACAGACCGGAGGTAGTAGCTACGCTCTCGTCTCCGGCGTCGACGGCCTTCTGCATC
CGGTCCTGCCAGCGCCCGTTACCGACGAGCCAATCAAAGCTCTCGGTCTGAAGGGCAAGCAGATTCGGAA
CATCAAGCGGTTCGTGAATCTTTGCGAATGAGATACGGCGGGTTGCACCATCGGTGCTTTCGACGTTGTT
AGCGGTTTCGTTATTAGAGGCGCTTGAGGCGACCAAGAGGGATCCTTCCACAGACCTTCAGGCTTTGCTG
CGTCTCTCTGCGGCCGGTAAACCGGCGCTACACCACTTGCACATGCCGTCAAGGAGACAACATGGCGAGC
CCACCGCTATATGAAGGCTGAGGGTAAACAGGGAAGACGCGAATATTCAGAATAGCTGATGCATCCAAAT
ATGTCCAGCCCACCAGAATACGCCCCGAAAACCTGGAGCACAAGAGTATCGAAGCAGCGAGTTACCTGCC
TATTGGCTGACAGTTCACTCCCCCGGTAAAGGCTGCACCACTTTTCGGGCTTATCAGCGCCCTTTAAGCC
CGAAAAGTGGTGCAGCGTTTGAAGATGGTTGAGCTTAGAATCCCGTTTGGGCTCTTACCACAGCGGCTTC
ATCGCGCTGGCCACTGTTTTCGAGCAGCTCGGCAAACTCCATACTGACGCCGCTGTGTTCCGGGGTACGG
TGTCCAGCCGTTCAAGGCAAGCCCTAAAAGCGGTGAATGCTTCGTCCGCTCTGGCTACCTGTGACAAGGC
TCGCGCCGCACCAAGCTCGGCCATTGCTGCGGCCATGTGGTCGCCCGTTTCCGCGAACTCATCCGCGGCT
GAAAGCAGCTCGGCAACGCTCTCATCAATACGATCCAACGCCAGTAACCCGCGCCCTTTTGAATCACGAA
TATTAGCGGCTTGCCACAGTGCACCATGTTCGACGGCGAGCGCACGGGCCTCGTCGAAATCGGCCAGTCC
AGTGCCGTCCCCCGGTTCGCAACGGGCCTGTCCTTGCAGTTCAAGACTCTGCACAATCAGTTGCGGGAAA
CCAGAAGCACGCGCCGCCTCAACCGCGATCTCGGCGACTTGAACCGCGCCGGGGCCAGAGGCTCTCAAGA
GTAATGAGACATCATCGATTGCCGCCGCCGCGATAACTTCGGGCTTATCGGTTCCTGTGGCCAGTTCGAT
TGCCTCGGTCCAAGCCTGGTGTGCAACTCCCAAGCTTTCAGCGGCTTTGGCCGCTCGGCCAAACCACAAA
AGCGTTTCAGCGATTGAGGCTGGCTCAACCTCGGCCGCTTCTTCTTGCTGCCGAACGGCGTCGAAATTCT
CCATCGCCAGGCCTGCTTCACCGGACCAGAACTGGTACTGACCCAATTTGAAGCGCAAGCCGGCTAAGTG
CAACGGTTCCAGCCCGGCCATTTCACCGTGCCGGATTGTTTGCTGCGCGCGGATGGCCGCCTGCTCATCT
TGGCCCGCGTCGCTATAGAGCGCTGCAGCAAGTCCCAATGAATCAATGAGTTGTTTCCGGGCTCCGGCGG
CAAGGCAGAGGTCCGCACGGCGGTCTGCGAGCGCTGCCCCCTCGAGGAATTCGCCTTGCCGACCATGCAA
TTGAGCACGCGCTCGTAGCACCGAAGTCAGATAAGAGGCGGGCACCGTGTCGACGGCTAGTGTTGCGTCG
AGCAATCGGCTAGCTTCTGGGATGAGCGAGTCATCTCCGGCCACGGCGAAGAGGCTGCCGAAGAGCCCAC
CAATGACAGCGGCCTGTTCTTTGTCCACTAAGTCCGGCGCTGCGGCGACGGCAATCCGGGCCGTGGCTAC
GGCGTCGTCTAAGTCCGGCGCTGCGGCGACGGCAATCCGGGCCGTGGCTACGGCGTCGTCAATTCGCCCG
GCGCCCCACTGCAAAATGGCCAAGCTATTCGCGATTGAAACTATTGCCTCAGTACTTTCGTTTTGCTGGG
CAGAGCTGAGTTCTTCCCGCAGCAGTTCTTCATGCTCAGTTGGTTAGAGCACCGGACT

Figure II.4: Nucleotide sequence of 2.25kp PCR amplicon from the genomic DNA of *R. salmoninarum* NCIMB114 using primers T5A-T5B (Fig: 7.2).

AGTCCGGTGCTCTAACCAACTGAGACACCACAACACGCTCGGTGCCGTTGATAACAAAGGTGCCTTTTTC
GGTCATCAGCGGGAAATCGCCCATGAACACGGTTTGCTGCTTGATTTCACCAGTGTTGTTGTTCATGAAC
TCGGCTTTGACATACAAAGGAGCCGAGAACGTAGCGTCCCGGTCCTTGCATTCAGCCATCGTGTACTTCG
GATCAGCGAACTCCGGCTCCGAGAAGCTCAAGGACATAGTGCCCTGGAAATCCTCGATCGGGGAGATCTC
TTCGAAGATGTCCGACAGACCGGAGGTAGTAGCTACGCTCTCGTCTCCGGCGTCGACGGCCTTCTGCATC
CGGTCCTGCCAGCGCCCGTTACCGACGAGCCAATCAAAGCTCTCGGTCTGAAGGGCAAGCAGATTCGGAA
CATCAAGCGGTTCGTGAATCTTTGCGAATGAGATACGGCGGGTTGCACCATCGGTGCTTTCGACGTTGTT
AGCGGTTTCGTTATTAGAGGCGCTTGAGGCGACCAAGAGGGATCCTTCCACAGACCTTCAGGCTTTGCTG
CGTCTCTCTGCGGCCGGTAAACCGGCGCTACACCACTTGCACATGCCGTCAAGGAGACAACATGGCGAGC
CCACCGCTATATGAAGGCTGAGGGTAAACAGGGAAGACGCGAATATTCAGAATAGCTGATGCATCCAAAT
ATGTCCAGCCCACCAGAATACGCCCCGAAAACCTGGAGCACAAGAGTATCGAAGCAGCGAGTTACCTGCC
TATTGGCTGACAGTTCACTCCCCCGGTAAAGGCTGCACCACTTTTCGGGCTTATCAGCGCCCTTTAAGCC
CGAAAAGTGGTGCAGCGTTTGAAGATGGTTGAGCTTAGAATCCCGTTTGGGCTCTTACCACAGCGGCTTC
ATCGCGCTGGCCACTGTTTTCGAGCAGCTCGGCAAACTCCATACTGACGCCGCTGTGTTCCGGGGTACGG
TGTCCAGCCGTTCAAGGCAAGCCCTAAAAGCGGTGAATGCTTCGTCCGCTCTGGCTACCTGTGACAAGGC
TCGCGCCGCACCAAGCTCGGCCATTGCTGCGGCCATGTGGTCGCCCGTTTCCGCGAACTCATCCGCGGCT
GAAAGCAGCTCGGCAACGCTCTCATCAATACGATCCAACGCCAGTAACCCGCGCCCTTTTGAATCACGAA
TATTAGCGGCTTGCCACAGTGCACCATGTTCGACGGCGAGCGCACGGGCCTCGTCGAAATCGGCCAGTCC
AGTGCCGTCCCCGGCTTCGCAACGGGCCTGTCCTTGCAGTTCAAGACTCTGCACAATCAGTTGCGGGAAA
CCAGAAGCACGCGCCGCCTCAACCGCGATCTCGGCGACTTGAACCGCGCCGGGGCCAGAGGCTCTCAAGA
GTAATGAGACATCATCGATTGCCGCCGCCGCGATAACTTCGGGCTTATCGGTTCCTGTGGCCAGTTCGAT
TGCCTCGGTCCAAGCCTGGTGTGCAACTCCCAAGCTTTCAGCGGCTTTGGCCGCTCGGCCAAACCACAAA
AGCGTTTCAGCGATTGAGGCTGGCTCAACCTCGGCCGCTTCTTCTTGCTGCCGAACGGCGTCGAAATTCT
CCATCGCCAGGCCTGCTTCACCGGACCAGAACTGGTACTGACCCAATTTGAAGCGCAAGCCGGCTAAGTG
CAACGGTTCCAGCCCGGCCATTTCACCGTGCCGGATTGTTTGCTGCGCGCGGATGGCCGCCTGCTCATCT
TGGCCCGCGTCGCTATAGAGCGCTGCAGCAAGCCCCAATGAATCAATGAGTTGTTTCCGGGCTCCGGCGG
CAAGGCAGAGGTCCGCACGGCGGTCTGCGAGCGCTGCCCCCTCGAGGAATTCGCCTTGCCGACCATGCAA
TTGAGCACGCGCTCGTAGCACCGAAGTCAGATAAGAGGCGGGCACCGTGTCGACGGCTAGTGTTGCGTCG
AGCAATCGGCTAGCTTCTGGGATGAGCGAGTCATCTCCGGCCACGGCGAAGAGGCTGCCGAAGAGCCCAC
CAATGACAGCGGCCTGTTCTTTGTCCACTAAGTCCGGCGCTGCGGCGACGGCAATCCGGGCCGTGGCTAC
GGCGTCGTCAATTCGCCCGGCGCCCCACTGCAAAATGGCCAAGCTATTCGCGATTGAAACTATTGCCTCA
GTACTTTCGTTTTGCTGGGCAGAGCTGAGTTCTTCCCGCAGCAGTTCTTCATGCTCAGTTGGTTAGAGCA
CCGGACT

Figure II.5: Nucleotide sequence of a 1.3kp PCR amplicon from the genomic DNA of *R. salmoninarum* NCIMB114 using primers T5A-T5B (Fig: 7.2).

AGTCCGGTGCTCTAACCAACTGAGGTGTCCGAGATTGATTCGATCGTGCCGGACTGCACATCCAAAGTCA
CAGTTTTTCCATCACGGTCAACAACAGACTCGCCATGCAAGCATCTTTTCAGCGCCACGATGCCCCGCGT
GTTTATTGGCATCCGAGCTGCCAAACTGGCTACTCGACGACGACGGTGAGCTTCCGGATGATGCATCTGC
AGCATAAACCGCGCCTACTCCAACGCTGAGTACTATCCCGGCGGTCGCAGTCGCGATCACGGTATTGCGG
ACCCACCGGCCTCCGGGACGCTTCTTCAGGGCATGAGGATCGTTGTTAATTTCATTGTTCACGGTTGCTC
CTATAAATAGTTCAAGAGCTTCCATCGTGACCTGGCAGCTTGTGATCAACCCCCGATTTGGCCAAGCCCT
GGCTTTGTCATTGCCAAGATCTCTGTGACCACTTCGCAAGTTTTCACAGCCGATACCCTGCTGACGGCGT
TCGTTAGAAAGGAGCTCGGACGGCCTGAACCAGTTTAGGTAAATCAATTTGTTGCGAGGTGTCTAGTTCA
AGAACCGGGGAAAGTTCAATCGGAATGGCGCCTTCGGCAAGCCTGCGCCAATGTGCCATAGTGAATTGAT
CTCCGCTATGCACCGGATGCCGGGCTCTGCTAGCAGCTCGCGCCAGGGCAACGTCGACGTCAACACGACA
ATAGATCTCAACGACCACTTCTAGATTTGCCCGCTGCAAACCTGCGATAGCAAACTCACGGTCTCGTTCA
CGGTGCCAATTCGATTCAATGATTGACGCAGGTACCGCGGTGGCGGCAGTCCAAAGTGCCTCGATTGCCA
GCATTCCCAATGCTTTCGCTGGAATGCCATCCGGACCAAAATCAAAGAACGTCTCTTTGAAATCGTCTTG
ACTGAACAATGGTAAGCCAAGCATCGGCGCCAAGTCTCGAGCTAGAGATGTTTTCCCTGCGCCGCTGATC
CCGTTGATCAACACCACTCGACCCTGCGGCCTACTCAGTTGGTTAGAGCACCGGACT

Figure II.6: Nucleotide sequence of 400bp PCR amplicon from the genomic DNA of *R. salmoninarum* Marion Forks isolate using primers T5A-T5B (Fig: 7.2).

AGTCCGGTGCTCTAACCAACTGAGCTATACACCTGAAGTAAAAGCATAATATTCACTTTTAAAAAGTATG
ACAAGCGCTATTTCTTGTTTTTTAAAGCAGATGCACAAGTTTTAAGCATTCCTTTAAATTGTTGAATATT
TTTTGGATTCAACGTATTGGAATAACGCATTTGTTGATAATAATCAGCTGCCTGATGAAATAAAACTTGC
TGTTCAGGCTTAACGTCTTGAGCTAAACGCAACAACCATTGATTTACCGTTTCGGCATCTTGTTTACGCT
GCTCAGTTGGTTAGAGCACCGGACT

Figure II.7: Nucleotide sequence of a 550bp PCR amplicon from the genomic DNA of *R. salmoninarum* 970153-19 using primers T5A-T5B (Fig: 7.2).

AATGCTCTACCAACTGAACTACAGCCACCATTGCCGCGCGAAGCGGCGTGAAAGATCTTAACCGGTGAGG
GCGCCTTAGGCCGAATCGGTATCCGGAGCGTTATCCAGGTTCGCCACGATCGCGACAAGGTCACTGGTAG
AGGGCCCGGGGTCGGGAACGAACGCCGCGCGCCGGTAATAGCGCAGCTCGCGGATCGACTCCTTGATGTC
GGCGAGGGCCCGGTGGGCCAATCCCTTGTCGGGCTGGCCGAAGTAGATCCGCGGGTACCAGCGGCGGCAG
AGCTCCTTGATGGAGCTGACGTCGATCATCCGGTAATGCAGGAAGTCATCGAGCAGCGGCATATCCCGTG
CGATGAATCCGCGGTCGGTGGCAATGGAGTTCCCGGCCAGGGGAGCCACCTTGGCGGCGGGCACGTGCTG
GCGGATGTAGTCGAGCACCAGCGCCTCGGCCTCTTCCAGGGTGACCGTCGACTTACGGACCTCAGTTGGT
TAGAGCACCGGACT

Figure II.8: Nucleotide sequence of a 2.3kp PCR amplicon from the genomic DNA of *R. salmoninarum* 980106#1.1.5 using primers T5A-T5B (Fig: 7.2).

AGTCCGGTGCTCTAACCAACTGAGACACCACAACACGCTCGGTGCCGTTGATAACAAAGGTGCCTTTTTC
GGTCATCAGCGGGAAATCGCCCATGAACACGGTTTGCTGCTTGATTTCACCAGTGTTGTTGTTCATGAAC
TCGGCTTTGACATACAAAGGAGCCGAGAACGTAGCGTCCCGGTCCTTGCATTCAGCCATCGTGTACTTCG
GATCAGCGAACTCCGGCTCCGAGAAGCTCAAGGACATAGTGCCCTGGAAATCCTCGATCGGGGAGATCTC
TTCGAAGATGTCCGACAGACCGGAGGTAGTAGCTACGCTCTCGTCTCCGGCGTCGACGGCCTTCTGCATC
CGGTCCTGCCAGCGCCCGTTACCGACGAGCCAATCAAAGCTCTCGGTCTGAAGGGCAAGCAGATTCGGAA
CATCAAGCGGTTCGTGAATCTTTGCGAATGAGATACGGCGGGTTGCACCATCGGTGCTTTCGACGTTGTT
AGCGGTTTCGTTATTAGAGGCGCTTGAGGCGACCAAGAGGGATCCTTCCACAGACCTTCAGGCTTTGCTG
CGTCTCTCTGCGGCCGGTAAACCGGCGCTACACCACTTGCACATGCCGTCAAGGAGACAACATGGCGAGC
CCACCGCTATATGAAGGCTGAGGGTAAACAGGGAAGACGCGAATATTCAGAATAGCTGATGCATCCAAAT
ATGTCCAGCCCACCAGAATACGCCCCGAAAACCTGGAGCACAAGAGTATCGAAGCAGCGAGTTACCTGCC
TATTGGCTGACAGTTCACTCCCCCGGTAAAGGCTGCACCACTTTTCGGGCTTATCAGCGCCCTTTAAGCC
CGAAAAGTGGTGCAGCGTTTGAAGATGGTTGAGCTTAGAATCCCGTTTGGGCTCTTACCACAGCGGCTTC
ATCGCGCTGGCCACTGTTTTCGAGCAGCTCGGCAAACTCCATACTGACGCCGCTGTGTTCCGGGGTACGG
TGTCCAGCCGTTCAAGGCAAGCCCTAAAAGCGGTGAATGCTTCGTCCGCTCTGGCTACCTGTGACAAGGC
TCGCGCCGCACCAAGCTCGGCCATTGCTGCGGCCATGTGGTCGCCCGTTTCCGCGAACTCATCCGCGGCT
GAAAGCAGCTCGGCAACGCTCTCATCAATACGATCCAACGCCAGTAACCCGCGCCCTTTTGAATCACGAA
TATTAGCGGCTTGCCACAGTGCACCATGTTCGACGGCGAGCGCACGGGCCTCGTCGAAATCGGCCAGTCC
AGTGCCGTCCCCCGGTTCGCAACGGGCCTGTCCTTGCAGTTCAAGACTCTGCACAATCAGTTGCGGGAAA
CCAGAAGCACGCGCCGCCTCAACCGCGATCTCGGCGACTTGAACCGCGCCGGGGCCAGAGGCTCTCAAGA
GTAATGAGACATCATCGATTGCCGCCGCCGCGATAACTTCGGGCTTATCGGTTCCTGTGGCCAGTTCGAT
TGCCTCGGTCCAAGCCTGGTGTGCAACTCCCAAGCTTTCAGCGGCTTTGGCCGCTCGGCCAAACCACAAA
AGCGTTTCAGCGATTGAGGCTGGCTCAACCTCGGCCGCTTCTTCTTGCTGCCGAACGGCGTCGAAATTCT
CCATCGCCAGGCCTGCTTCACCGGACCAGAACTGGTACTGACCCAATTTGAAGCGCAAGCCGGCTAAGTG
CAACGGTTCCAGCCCGGCCATTTCACCGTGCCGGATTGTTTGCTGCGCGCGGATGGCCGCCTGCTCATCT
TGGCCCGCGTCGCTATAGAGCGCTGCAGCAAGTCCCAATGAATCAATGAGTTGTTTCCGGGCTCCGGCGG
CAAGGCAGAGGTCCGCACGGCGGTCTGCGAGCGCTGCCCCCTCGAGGAATTCGCCTTGCCGACCATGCAA
TTGAGCACGCGCTCGTAGCACCGAAGTCAGATAAGAGGCGGGCACCGTGTCGACGGCTAGTGTTGCGTCG
AGCAATCGGCTAGCTTCTGGGATGAGCGAGTCATCTCCGGCCACGGCGAAGAGGCTGCCGAAGAGCCCAC
CAATGACAGCGGCCTGTTCTTTGTCCACTAAGTCCGGCGCTGCGGCGACGGCAATCCGGGCCGTGGCTAC
GGCGTCGTCTAAGTCCGGCGCTGCGGCGACGGCAATCCGGGCCGTGGCTACGGCGTCGTCAATTCGCCCG
GCGCCCCACTGCAAAATGGCCAAGCTATTCGCGATTGAAACTATTGCCTCAGTACTTTCGTTTTGCTGGG
CAGAGCTGAGTTCTTCCCGCAGCAGTTCTTCATGCTCAGTTGGTTAGAGCACCGGACT

Figure II.9: Nucleotide sequence of a 2.4kp PCR amplicon from the genomic DNA of *R. salmoninarum* 980106 #1.1.5 using primers T5A-T5B (Fig: 6.2).

AGTCCGGTGCTCTAACCAACTGAGACACCACAACACGCTCGGTGCCGTTGATAACAAAGGTGCCTTTTTC
GGTCATCAGCGGGAAATCGCCCATGAACACGGTTTGCTGCTTGATTTCACCAGTGTTGTTGTTCATGAAC
TCGGCTTTGACATACAAAGGAGCCGAGAACGTAGCGTCCCGGTCCTTGCATTCAGCCATCGTGTACTTCG
GATCAGCGAACTCCGGCTCCGAGAAGCTCAAGGACATAGTGCCCTGGAAATCCTCGATCGGGGAGATCTC
TTCGAAGATGTCCGACAGACCGGAGGTAGTAGCTACGCTCTCGTCTCCGGCGTCGACGGCCTTCTGCATC
CGGTCCTGCCAGCGCCCGTTACCGACGAGCCAATCAAAGCTCTCGGTCTGAAGGGCAAGCAGATTCGGAA
CATCAAGCGGTTCGTGAATCTTTGCGAATGAGATACGGCGGGTTGCACCATCGGTGCTTTCGACGTTGTT
AGCGGTTTCGTTATTAGAGGCGCTTGAGGCGACCAAGAGGGATCCTTCCACAGACCTTCAGGCTTTGCTG
CGTCTCTCTGCGGCCGGTAAACCGGCGCTACACCACTTGCACATGCCGTCAAGGAGACAACATGGCGAGC
CCACCGCTATATGAAGGCTGAGGGTAAACAGGGAAGACGCGAATATTCAGAATAGCTGATGCATCCAAAT
ATGTCCAGCCCACCAGAATACGCCCCGAAAACCTGGAGCACAAGAGTATCGAAGCAGCGAGTTACCTGCC
TATTGGCTGACAGTTCACTCCCCCGGTAAAGGCTGCACCACTTTTCGGGCTTATCAGCGCCCTTTAAGCC
CGAAAAGTGGTGCAGCGTTTGAAGATGGTTGAGCTTAGAATCCCGTTTGGGCTCTTACCACAGCGGCTTC
ATCGCGCTGGCCACTGTTTTCGAGCAGCTCGGCAAACTCCATACTGACGCCGCTGTGTTCCGGGGTACGG
TGTCCAGCCGTTCAAGGCAAGCCCTAAAAGCGGTGAATGCTTCGTCCGCTCTGGCTACCTGTGACAAGGC
TCGCGCCGCACCAAGCTCGGCCATTGCTGCGGCCATGTGGTCGCCCGTTTCCGCGAACTCATCCGCGGCT
GAAAGCAGCTCGGCAACGCTCTCATCAATACGATCCAACGCCAGTAACCCGCGCCCTTTTGAATCACGAA
TATTAGCGGCTTGCCACAGTGCACCATGTTCGACGGCGAGCGCACGGGCCTCGTCGAAATCGGCCAGTCC
AGTGCCGTCCCCCGGTTCGCAACGGGCCTGTCCTTGCAGTTCAAGACTCTGCACAATCAGTTGCGGGAAA
CCAGAAGCACGCGCCGCCTCAACCGCGATCTCGGCGACTTGAACCGCGCCGGGGCCAGAGGCTCTCAAGA
GTAATGAGACATCATCGATTGCCGCCGCCGCGATAACTTCGGGCTTATCGGTTCCTGTGGCCAGTTCGAT
TGCCTCGGTCCAAGCCTGGTGTGCAACTCCCAAGCTTTCAGCGGCTTTGGCCGCTCGGCCAAACCACAAA
AGCGTTTCAGCGATTGAGGCTGGCTCAACCTCGGCCGCTTCTTCTTGCTGCCGAACGGCGTCGAAATTCT
CCATCGCCAGGCCTGCTTCACCGGACCAGAACTGGTACTGACCCAATTTGAAGCGCAAGCCGGCTAAGTG
CAACGGTTCCAGCCCGGCCATTTCACCGTGCCGGATTGTTTGCTGCGCGCGGATGGCCGCCTGCTCATCT
TGGCCCGCGTCGCTATAGAGCGCTGCAGCAAGTCCCAATGAATCAATGAGTTGTTTCCGGGCTCCGGCGG
CAAGGCAGAGGTCCGCACGGCGGTCTGCGAGCGCTGCCCCCTCGAGGAATTCGCCTTGCCGACCATGCAA
TTGAGCACGCGCTCGTAGCACCGAAGTCAGATAAGAGGCGGGCACCGTGTCGACGGCTAGTGTTGCGTCG
AGCAATCGGCTAGCTTCTGGGATGAGCGAGTCATCTCCGGCCACGGCGAAGAGGCTGCCGAAGAGCCCAC
CAATGACAGCGGCCTGTTCTTTGTCCACTAAGTCCGGCGCTGCGGCGACGGCAATCCGGGCCGTGGCTAC
GGCGTCGTCTAAGTCCGGCGCTGCGGCGACGGCAATCCGGGCCGTGGCTACGGCGTCGTCAATTCGCCCG
GCGCCCCACTGCAAAATGGCCAAGCTATTCGCGATTGAAACTATTGCCTCAGTACTTTCGTTTTGCTGGG
CAGAGCTGAGTTCTTCCCGCAGCAGTTCTTCATGCTCAGTTGGTTAGAGCACCGGACT

Figure II.10: Nucleotide sequence of a 950bp PCR amplicon from the genomic DNA of *R. salmoninarum* ATCC33209^{TS} using primers T5A-T5B (Fig: 6.2). Complete sequence for tRNA^{thr} is depicted red, partial sequence for tRNA^{asp} is depicted in blue.

TGTTCCCGGTGAAAATCCATTTGTATGACGAAATCTCTTGGAACTCGCAATTTTGGCCCATAGCATCGGG
TTGTCATGCGGACTGTATGACGTAACAAGTTCGACGACGACGAAGCTTTAGTTCGTCGAGCTCAGACGGT
TGGAAAGTATTTCTTATGGGACTTTTCGATGACATCAAGAAGAATGTCGAAGATGTAGCCAAGAATGTGG
GCGATGCAGTGGGGGACGTGTTCAACCCGAACCACAACCAGAACCAGTCCGAGCAGGCTCCGGCCGAGCA
GGAATCCCCGGCAGCAGCCAACGACGACGTCGCTGCTGAGCCGGTTGCTGTAGACGCTCCGGTTGAGGTC
GCACCCGTGGCTGAAGAAGTTGCACCGGTTGAAGCCTCTCCGGTCACTGAAGAGGCTCCTGTGGCCCGTC
AGGTGACTGTTGCTGAAGGAGATAGCCTTTCCGCTATCGCTGAAGCTAACGGCGTGGACCTGCAGGCTCT
GATCAGCACCAACGGCATCGAGAACCCGGACCTGATTTTCCCGGGCCAGGTTTTGACGCTTCCGTAGGTT
TTTCGCGCAAATAGCAACGCAGGATTCCTCGCCAAAAGGCCCGTTCAAGGGGCTAGTAGATGGCGTGGGG
ATCCTGCGTTGCTGCTTGTGGAATCGCTTTTCTGGAAGTAGCATTTGGCCGATTTCCCCCAGGCGAAATC
CACTGGTAAAGTATTCATCTGTT**GCCCCCCTAGCTCAGTGGTAGAGCGCGTTCTTGGTAAGAACGAGGTC**
ACCGGATCGATTCCGGTGGGGGGCTCGCAATGGGTAGGCTCGTGTCAAGACGGTTTTTGACCGGCGAAGA
CACGGGCCAATCCCATTTATGGCGG**TGTAGCTCAGTTGGTTAGAGCACCGGACT**

Figure II.11: Nucleotide sequence of a 1.1kp PCR amplicon from the genomic DNA of *R. salmoninarum* ATCC33209^{TS} using primers T5A-T5B (Fig: 7.2).

AGTCCGGTGCTCTAACCAACTGAGGTGTCCGAGATTGATTCGATCGTGCCGGACTGCACATCCAAAGTCA
CAGTTTTTCCATCACGGTCAACAACAGACTCGCCATGCAAGCATCTTTTCAGCGCCACGATGCCCCGCGT
GTTTATTGGCATCCGAGCTGCCAAACTGGCTACTCGACGACGACGGTGAGCTTCCGGATGATGCATCTGC
AGCATAAACCGCGCCTACTCCAACGCTGAGTACTATCCCGGCGGTCGCAGTCGCGATCACGGTATTGCGG
ACCCACCGGCCTCCGGGACGCTTCTTCAGGGCATGAGGATCGTTGTTAATTTCATTGTTCACGGTTGCTC
CTATAAATAGTTCAAGAGCTTCCATCGTGACCTGGCAGCTTGTGATCAACCCCCGATTTGGCCAAGCCCT
GGCTTTGTCATTGCCAAGATCTCTGTGACCACTTCGCAAGTTTTCACAGCCGATACCCTGCTGACGGCGT
TCGTTAGAAAAGAGCTCGGACGGCCTGAACCAGTTTAGGTAAATCAATTTGTTGCGAGGTGTCTAGTTCA
AGAACCGGGGAAAGTTCAATCGGAATGGCGCCTTCGGCAAGCCTGCGCCAATGTGCCATAGTGAATTGAT
CTCCGCTATGCACCGGATGCCGGGCTCTGCTAGCAGCTCGCGCCAGGGCAACGTCGACGTCAACACGACA
ATAGATCTCAACGACCACTTCTAGATTTGCCCGCTGCAAACCTGCGATAGCAAACTCACGGTCTCGTTCA
CGGTGCCAATTCGATTCAATGATTGACGCAGGTACCGCGGTGGCGGCAGTCCAAAGTGCCTCGATTGCCA
GCATTCCCAATGCTTTCGCTGGAATGCCATCCGGACCAAAATCAAAGAACGTCTCTTTGAAATCGTCTTG
ACTGAACAATGGTAAGCCAAGCATCGGCGCCAAGTCTCGAGCTAGAGATGTTTTCCCTGCGCCGCTGATC
CCGTTGATCAACACCACTCGACCCTGCGGCCTACTCAGTTGGTTAGAGCACCGGACT

Figure II.14: Nucleotide sequence of a 100bp PCR amplicon from the genomic DNA of *R. salmoninarum* NCIMB1114 using primers T3B and T5A (Fig: 7.1). The sequence depicted in blue represents a partial $tRNA^{asp}$ gene. Sequence depicted in red represents a partial $tRNA^{ile}$ gene. Note: This fragment is a partial fragment of the PCR products displayed in Figs: II.16 – II.19.

AGTCCGGTGCTCTAACCAACTGAGCTACAGAGCCTTGGTGAACTTGCCACCGGATTCCCCGAAAGGAACC
TAGCGACCCTGACGGGATTCGAACCCGCGACCTGG

Figure II.15: Nucleotide sequence of a 100bp PCR amplicon from the genomic DNA of *R. salmoninarum* 980106 #1.1.5 using primers T3B and T5A (Fig: 7.1). Sequence depicted in red represents a partial $tRNA^{ile}$ gene. Note: This fragment is a partial fragment of the PCR products displayed in Figs: II.16 – II.19.

AGTCCGGTGCTCTAACCAACTGAGCTACAGAGCCTTGGTGAACTTGCCACCGGATTCCCCGAAAGGAACC
T

Figure II.16: Nucleotide sequence of a 100bp PCR amplicon from the genomic DNA of *R. salmoninarum* 970153-19 using primers T3B and T5A (Fig: 7.1). Sequence depicted in red represents a partial $tRNA^{ile}$ gene. Note: This fragment is a partial fragment of the PCR products displayed in Figs: II.16 – II.19.

AGTCCGGTGCTCTAACCAACTGAGCTACAGAGCCTTGGTGAACTTGCCACCGGATTCCCCGAAAGG

Figure II.17: Nucleotide sequence of a 350bp PCR amplicon from the genomic DNA of *R. salmoninarum* Marion Forks using primers T3B and T5A (Fig: 7.1). Sequence depicted in red represents a complete $tRNA^{asp}$ gene. Sequence depicted in blue represents partial $tRNA^{Ile}$ gene.

AGTCCGGTGCTCTAACCAACTGAGCTACAGAGCCTTGGTGAACTTGCCACCGGATTCCCCGAAAGGAACC
TAGCGACCCTGACGGGACTTGAACCCGCGACCTCCGCCGTGACAGGGCGGCGCGCTAACCAACTGCGCTA
CAGGGCCTTGCTTTTTACAAGGCTTCCAGCTTACCAGCTCTCACCGGATACTTTTGCCGTGCGTACCCCC
AACGGGATTCGAACCCGCGACCT

Figure II.18: Nucleotide sequence of a 350bp PCR amplicon from the genomic DNA of *R. salmoninarum* 970153-19 using primers T3B and T5A (Fig: 7.1). Sequence depicted in red represents a complete $tRNA^{asp}$ gene. Sequence depicted in blue represents partial $tRNA^{Ile}$ gene.

AGTCCGGTGCTCTAACCAACTGAGCTACAGAGCCTTGGTGAACTTGCCACCGGATTCCCCGAAAGGAACC
TAGCGACCCTGACGGGACTTGAACCCGCGACCTCCGCCGTGACAGGGCGGCGCGCTAACCAACTGCGCTA
CAGGGCCTTGCTTTTTACAAGGCTTCCAGCTTACCAGCTCTCACCGGATGCTTTTGCCGTGCGTACCCCC
AACGGGATTCGAACCCGCGACCT

Figure II.19: Nucleotide sequence of a 350bp PCR amplicon from the genomic DNA of *R. salmoninarum* ATCC33209^{TS} using primers T3B and T5A (Fig: 7.1). Sequence depicted in red represents a complete tRNA^{asp} gene. Sequence depicted in blue represents partial tRNA^{Ile} gene.

AGTCCGGTGCTCTAACCAACTGAGCTACAGAGCCTTGGTGAACTTGCCACCGGATTCCCCGAAAGGAACC
TAGCGACCCTGACGGGACTTGAACCCGCGACCTCCGCCGTGACAGGGCGGCGCGCTAACCAACTGCGCTA
CAGGGCCTTGCTTTTTACAAGGCTTCCAGCTTACCAGCTCTCACCGGATGCTTTTGCCGTGCGTACCCCC
AACGGGATTCGAACCCGCGACCT

Figure II.20: Nucleotide sequence of a 350bp PCR amplicon from the genomic DNA of *R. salmoninarum* 980106 #1.1.5 using primers T3B and T5A (Fig: 6.1). Sequence depicted in red represents a complete tRNA^{asp} gene. Sequence depicted in blue represents partial tRNA^{Ile} gene.

AGTCCGGTGCTCTAACCAACTGAGCTACAGAGCCTTGGTGAACTTGCCACCGGATTCCCCGAAAGGAACC
TAGCGACCCTGACGGGACTTGAACCCGCGACCTCCGCCGTGACAGGGCGGCGCGCTAACCAACTGCGCTA
CAGGGCCTTGCTTTTTACAAGGCTTCCAGCTTACCAGCTCTCACCGGATGCTTTTGCCGTGCGTACCCCC
AACGGGATTCGAACCCGCGACCT

Figure II.21: Nucleotide sequence of a 250bp PCR amplicon from the genomic DNA of *R. salmoninarum* Marion Forks using primers T3B and T5A (Fig: 7.1). Sequence depicted in red represents a complete tRNA^{arg} gene. Sequence depicted in blue represents partial tRNA^{val} gene.

AGTCCGGTGCTCTAACCAACTGAGCTATGAGCGCATTACTTGTGTGCCAATTCAGGCGATACGTTACATA
CAGTTTACTTATTGGTGCGCTCAGAGAGATTCGAACTCCCGACCCCTTAGTTCGTAGCCAAGTGCTCTAT
CCAGCTGAGCTATGAGCGCGACATAAGTAAAGTTGGTTAGAGCACCGGACT

Figure II.22: Nucleotide sequence of a 220bp PCR amplicon from the genomic DNA of *R. salmoninarum* Marion Forks using primers T3B and T5A (Fig: 7.1). Sequence depicted in red represents a partial complete tRNA^{arg} gene. Sequence depicted in blue represents partial tRNA^{asn} gene.

AGTCCGGTGCTCTAACCAACTGAGCTAAGCGCCCTTGAGAAATAAAAATAGCATCGCTATTTTTATGATG
CAAGAAAGAAATCTTGCTTGATTCCATTTACTAGCACAAGGGATCTAGAAAATGGCGCAGCGGACGGGAT
TCGAACCCG

Figure II.23: Nucleotide sequence of a 350bp PCR amplicon from the genomic DNA of *R. salmoninarum* NCIMB1114 using primers T3B and T5A (Fig: 7.1). Sequence depicted in red represents a complete tRNA^{arg} gene. Sequence depicted in blue represents partial tRNA^{ala} gene.

AGTCCGGTGCTCTAACCAACTGAGCTACATCCACCATAACGTGTCGGTTCTTAATACCAAGCTACCTAAT
GGCGCGCCTGACAGGATTCGAACCTGTGACCATCCGCTTAGAAGGCGGATGCTCTATCCAACTGAGCTAC
AGGCGCATGACCAATAATACGAGGTATTATGGATAACCTTGCCTTGAAGAATTGGTCGGAGCAGTAGGAT
TCGAACCCGCGACCT

Figure II.24: Nucleotide sequence of a 350bp PCR amplicon from the genomic DNA of *R. salmoninarum* ATCC33209^{TS} using Primers T3B and T5A (Fig: 7.1). The sequence below is thought to be a PCR artefact as it contains three primer sequences fused together.

AGGCTCTGTAGCTCAGTTGGTTAGAGCACCGGACTCAAGGCTCTGTAGCTCAGTTGGTTAGAGCACCGGA
CTCAGTTGGTTAGAGCACCGGACT

Figure: II.25 Nucleotide sequence of a 120bp PCR amplicon from the genomic DNA of *R. salmoninarum* 980106 #1.1.5 using primers T3B and T5A (Fig: 7.1). The sequence depicted in red represents a partial tRNA^{ile} gene.

AGGTCGCGGGTTCGAATCCGGTGGGGGGCTCGCAATGGGTAGGCTCGTGTCAAGACGGTTTTTGACCGGC
GAAGACACGGGCCAATCCCATTTATGGCGGTGTAGCTCAGTTGGTTAGAGCACCGGACT

II.3 Sequencing data relevant to Chapter 8

Figure II.26: Nucleotide sequence obtained from the PCR amplicon from the genomic DNA of *R.salmoninarum* NCIMB1111. The identity of the product was confirmed following an alignment with known IS*994* sequences from the GenBank database. Primer sites are not depicted.

CGGTGCGTAAATGGGTCCGGCAAGCCGAGATCGATGTTGGTACTAGAACTGGAACAACGAGCACGGAATC
GGCCGAGCTGAAACGGTTACGGCGTGAGAACGCTGAGCTGAAACGGGCGAACGCGATCCTTCGGAGTGCT
TCAGCTTTTTTCGCGGTCGAACTCGACCGCCACAACACTGATCGTGAAATACATCAAGGACCATGCCGGT
CACCGCGAGAATAATGGATTGCGGTGGGGTGTCGAGTCGATCTGCCAGGTGCTTACTGGGACGGGGTGAA
GACCACCCCGTCCACGTACTACGAATGGGTGGATAAAACACGATCTCACCGAGAACAACGTGATGAGGTG
CTCAAGCCCGTGATCCAGAAGGTGTATGCCGCTAATTACGGGGTTTACGGCACCAGGAAAGTCTGGTTGG
CGATGAACCGTGAAGGTGTGCCGGTGGCCAGGTGCACGGTAGAACGGCTCATGGGGTTACTTGGCATACA
GGGTGCGGTCCGTGGCAAGGTCAAACGCACCACGATCAAAGACTCGAAGGCGGCCCGAGCGAAGGACTTG
GTCCGCCGTGATTTCACACCAACGGCACCGGATCGGCTATGGGTAGATGATTTCACCTATGTTTCGACCT
GGTCCGGGTGGGTCTATGTTGCCTTCGTGATCGATGCTTACTCTCGGAGGATCCTGGGCTGGTCAGCGAG
TGCTTCTATGAACACCGTGCTAGTGCTCAACGCAGTTAATCAGGCAATCTGGAGTCGTGAACGGGCCGGG
GCTGAGATTTCCGGGGTGATTCATCATCACGATGCCGGGGCTCAATACGCCTCCTTGGCCTTCACCGAAC
GCCTGGCCCAGGCCGGTATCCGCCCCTCGATCGGTTCTGTGGGTGATAGTTACGACAACGCCTTGGCGGA
AACCATCAACGGGCTTTATAAGACCGAGCTGATCAAACCCGGCAATCCCTGGCGGACTCTAGAAGAAGTC
GAAATCGGCACCGCTGAATGGGCCGATTGGTACAACCACCGAAGCTCTACCAGTACTGCT

Appendix III

Additional Gel Images

III.1 Quantification of genomic DNA extracted from *R. salmoninarum*

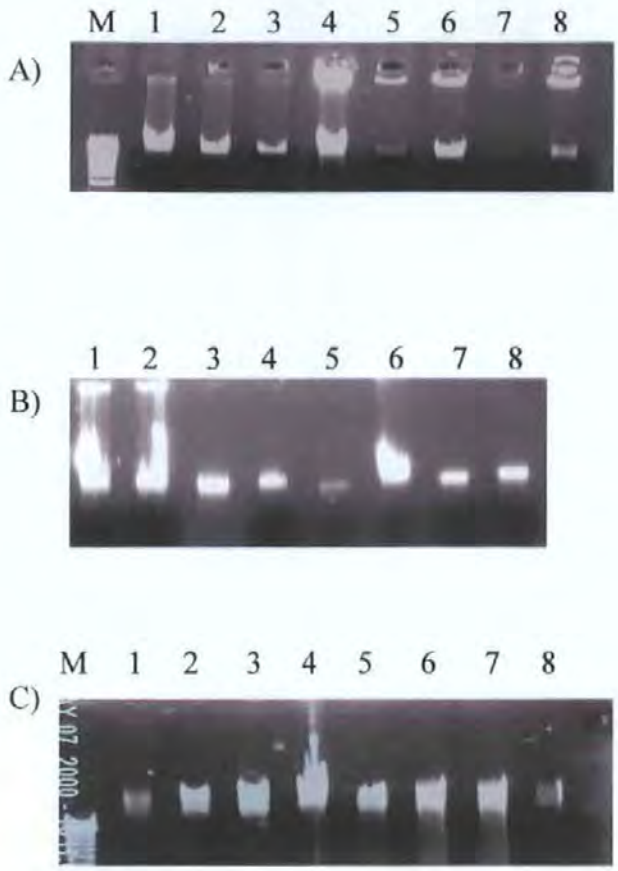


Figure III.1: Quantification of DNA extracted from *R. salmoninarum* cultures. Panel A: lanes 1-8 correspond to the following, 1; 0.250μg of λ standard DNA, 2; 0.125μg of λ DNA standard, 3; 0.063μg of λ standard DNA, 4; DNA from isolate 9700083-102, 5; DNA from isolate 980036-150, 6; DNA from isolate A6, 7; DNA from isolate 970419-1.2.3, 8; DNA from isolate NCIMB1111. Lane M: 1 kb ladders (0.3μg). Panel B lanes 1-8 correspond to the following, 1; 0.500μg of λ standard DNA, 2; 0.250μg of λ standard DNA, 3; 0.125μg of λ standard DNA, 4; DNA from isolate 970083-88, 5; DNA from isolate A80, 6: DNA from isolate 970153-19, 7; DNA from isolate 980036-87, 8 DNA from isolate NCIMB1112. Panel C lanes 1-8 correspond to the following 1; 0.063μg of λ standard DNA, 2; 0.125μg of λ DNA, 3; 0.250μg of λ standard DNA, 4; 0.500μg of λ standard DNA, 5; DNA from isolate 3258#15, 6; DNA from isolate 3506W134, 7; DNA from isolate 3506W132, 8; DNA from isolate 2119#5. Lane M: 1kb ladders (0.3μg).

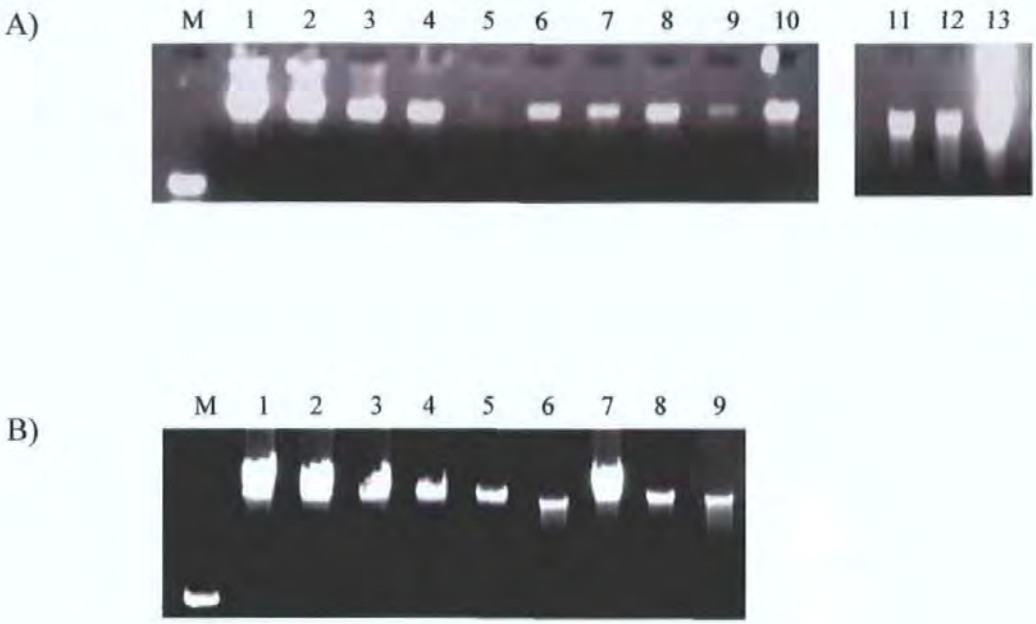


Figure III.2: Quantification of DNA extracted from *R. salmoninarum* cultures. Panel A: Lanes 1-13 correspond to the following 1; 0.500μg of λ standard DNA, 2; 0.250μg of λ DNA, 3; 0.125μg of λ DNA, 4; DNA from isolate MT1770, 5; DNA from isolate MT1261, 6; DNA from isolate MT1880, 7; DNA from isolate MT239, 8; DNA from isolate MT419, 9; DNA from isolate MT425, 10; DNA from isolate MT426, 11; DNA from isolate MT2118, 12; DNA from isolate MT2119 13; DNA from isolate MT1511 Lane M: 100bp ladders (0.3μg). Panel B lanes 1-9 correspond to the following 1; 0.500μg of λ standard DNA, 2; 0.250μg of λ DNA, 3; 0.125μg of λ DNA, 4; 0.063μg of λ standard DNA, 5; DNA from isolate MT1470, 6; DNA from isolate NCIMB 1113, 7; DNA from isolate NCIMB1114, 8; DNA from isolate NCIM1115, 9; DNA from isolate NCIMB1116.

Table III.1: Genomic DNA concentrations determined from agarose gels (Figs. III.1 & III.2 & 4.1). Note: Isolates that were use in this study but are not featured in this table were kindly provided by Dr. Grayson as purified DNA at a known concentration.

Isolate	DNA conc. (ng/µl)	Isolate	DNA (ng/µl)
MT444	100	NCIMB1112	75
MT452	650	MT1770	115
MT839	40	MT1261	10
MT861	40	MT1800	95
MT1351	10	MT239	105
MT1469	50	MT425	20
9700083-102	243	MT426	130
980036-150	15	MT419	120
A6	135	MT2118	235
970419-1.2.3	None detected	MT2119	254
NCIMB1111	50	MT1511	720
970083-88	90	3458#15	100
A80	30	3506W134	260
9700153-19	265	3506W132	260
MT1470	50	NCIMB1114	310
NCIMB1113	40	NCIMB1115	45
980036-87	75	2119#5	60
NCIMB1116	40		

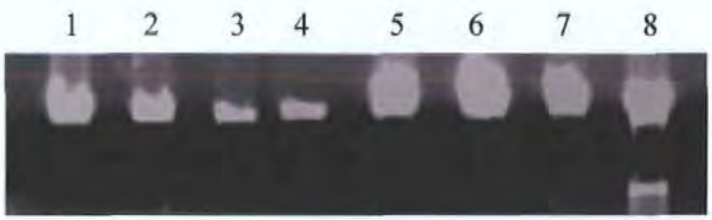


Figure III.3: Quantification of DNA extracted from additional bacterial cultures. Panel A: Lanes 1-8 correspond to the following 1; 0.125μg of λ standard DNA, 2; 0.063μg of λ standard DNA, 3; 0.032μg of λ standard DNA, 4; 1μl of DNA from *Nocardia cellulans* (44 ng/μl), 5; 1μl of DNA from Arthrobacter ZAT012 (320 ng/μl), 6; 1μl of DNA from *Arthrobacter globiformis* (360ng/μl), 7; 1μl of DNA from *Arthrobacter polychromogenes* (210ng/μl) 8; 1μl of DNA from *Aeromonas salmonicida* (180ng/μl).

III.2 Additional gel images relevant to Chapter 4

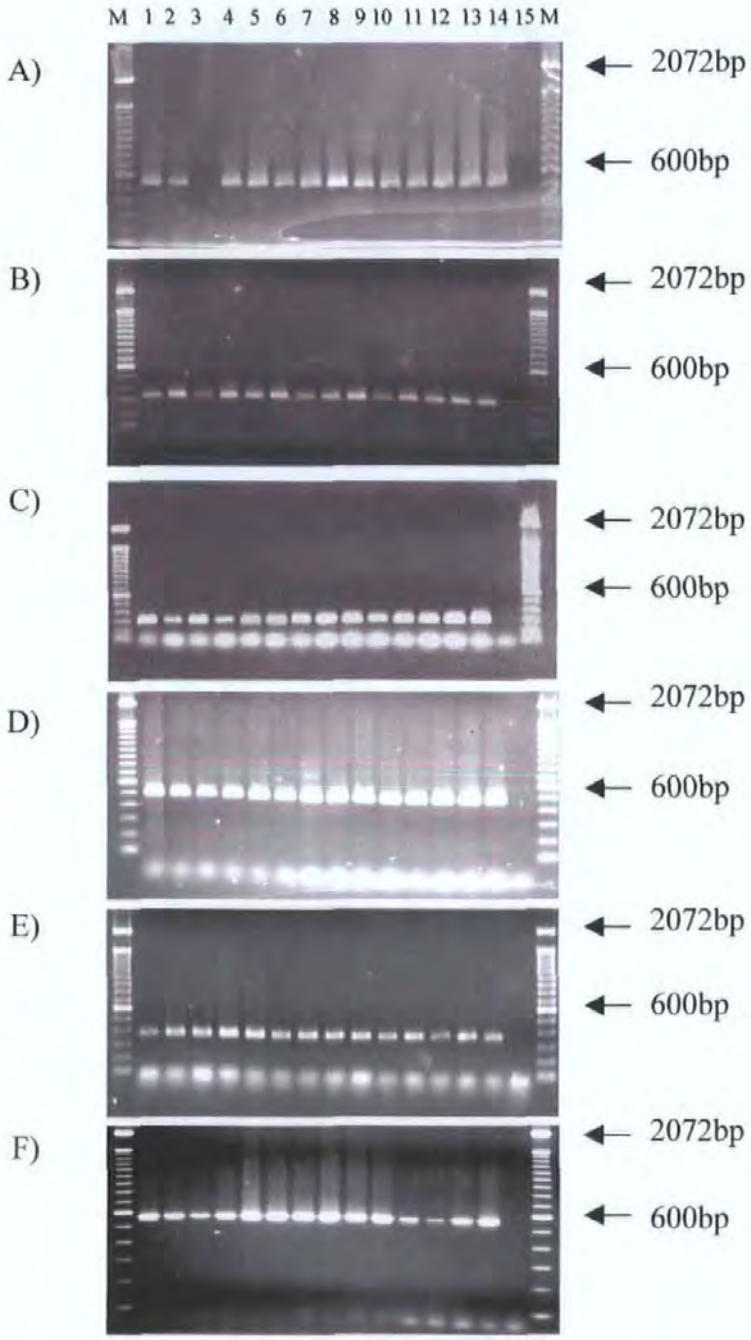


Figure III.4: PCR amplification of *R. salmoninarum* DNA. PCR reactions using specific primers RSMP+338 & RSMP-877 (panel A), RSMP+487 & RSMP-800 (panel B), RS57+155 & RS57-449 (panel C), RS57+127 & RS57-611(panel D), RSH+401 & RSH-774 (panel E) and RsH+231 & RsH-800 (panel F). Lanes 1-15 correspond to the following isolates: MT861, MT1261, MT1351, MT1469, MT1470, MT1511, MT1770, MT1880, MT2118, MT2119, 3258#15, 3506W134, 3506W132, 2119#5 and negative control, respectively. Lane M: 100 bp Ladders (0.3µg), the size of selected markers are indicated by arrows on the right.

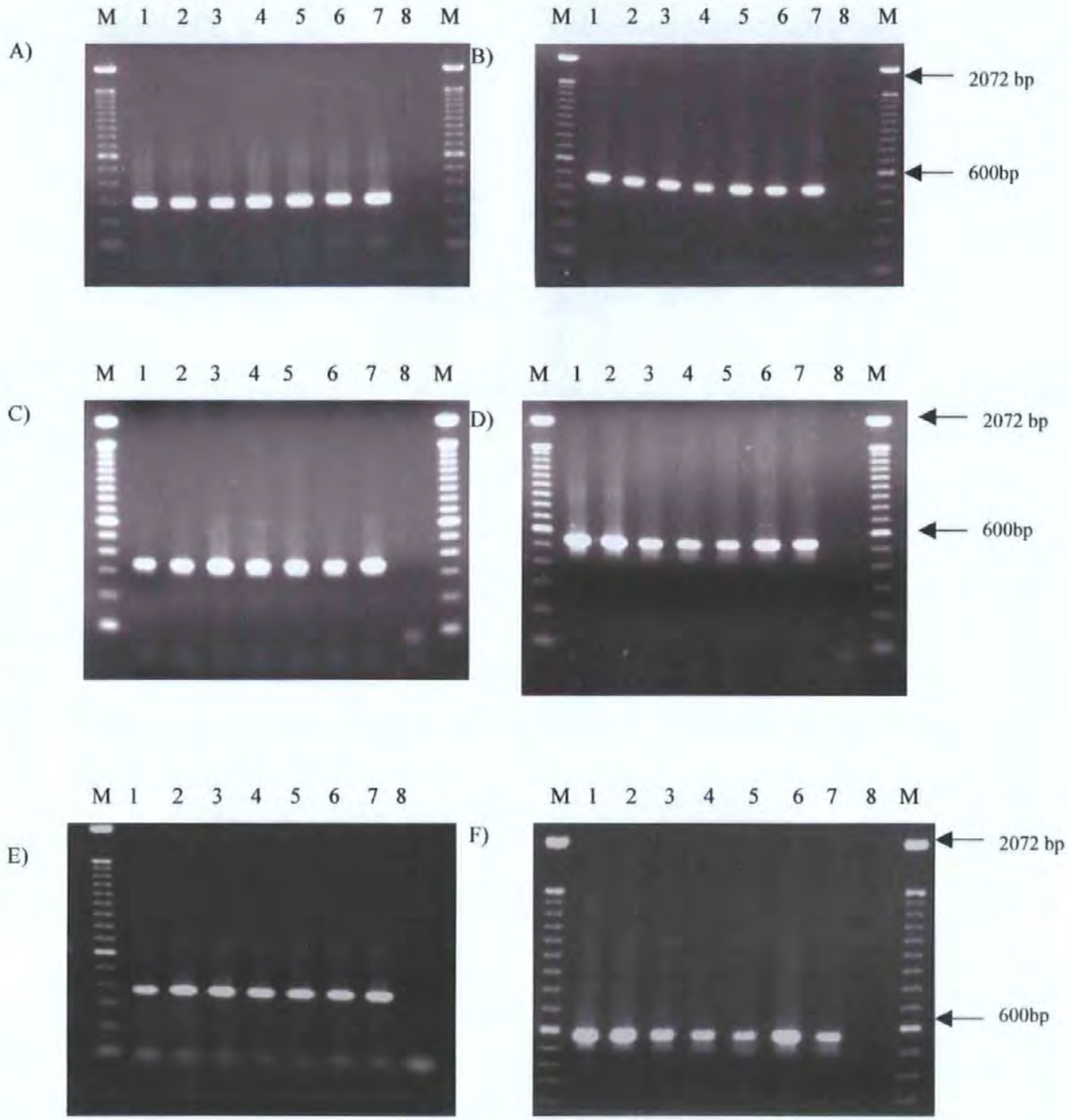


Figure III.5: PCR amplification of *R. salmoninarum* DNA. PCR reactions using primers RS57+155 & RS57–449 (panel A), RS57+127 & RS57-611 (panel B), RSMP+487 & RSMP-820 (panel C), RSMP+338 & RSMP-877 (panel D), RSH+401 & RSH-772 (panel E), RSH+231 & RSH-800 (panel F). Lanes 1-8 corresponds to isolates 980297#97, 980109F95, 980109F85, 980109F82, 980109F82, 980109F60, 980109F47, 980109F3 and negative control, respectively. Lane M: 100 bp Ladders (0.3µg), the size of selected markers are indicated by arrows on the right.

III.3 Additional gel images relevant to chapter 6

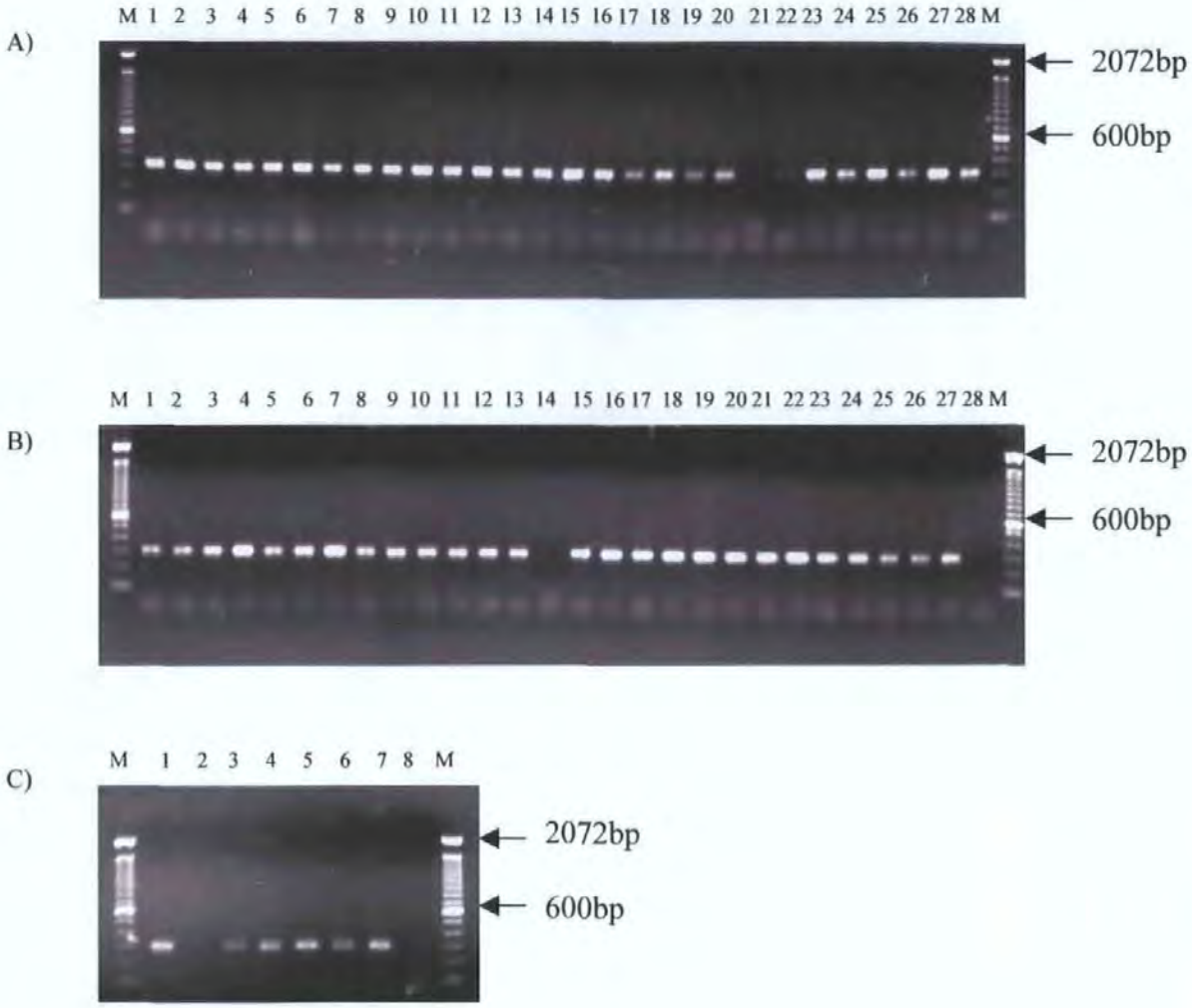


Figure III.6: PCR amplification of the 23S-5S rRNA ITS (ITS2) region of *R. salmoninarum* using primers R5+118 & R5-433. Panel A lanes 1-28 correspond to the following isolates; 970083-88, 970083-102, 980106 #1.1.5, 980036-150, 980036-87, 970419-1.2.3, 970153-19, A6, A80, MT409, MT417, MT239, MT426, NCIMB1111, NCIMB1112, NCIMB1113, NCIMB1114, NCIMB1115, NCIMB1116, MT420, MT452, MT1363, MT410, Siletz, Marion Forks, Little Goose, CCM6205 and 84-019-OC. Panel B lanes 1-28 correspond to the following isolates; SS-ChS-94-1, Cow ChS94 P22, Idaho 91-126, RFL-643.94 #1, CCM6206, Round Butte, NCIMB2235, AcF6-1, DR143, DR384, 960023, 960046, F-120-87(P-2), F-130-87(P-4), F-138-87(0-78), F-260-87 (P-16), F-273-87(P-19), F-283-87(P-10), F-358-87(P-13), S-182-90(P-7), Rs 9, Rs 19, Rs 61, Rs 116, Rs 122, Rs 125, Rs 126, and 3015-86. Panel C lanes 1-8 correspond to the following isolates: 4451-86, RS-TSA, FT-10, BY1996, Iwate, K-28, K-70 and negative control. Lane M: 100 bp Ladders (0.3µg), the size of selected markers are indicated by arrows on the right.

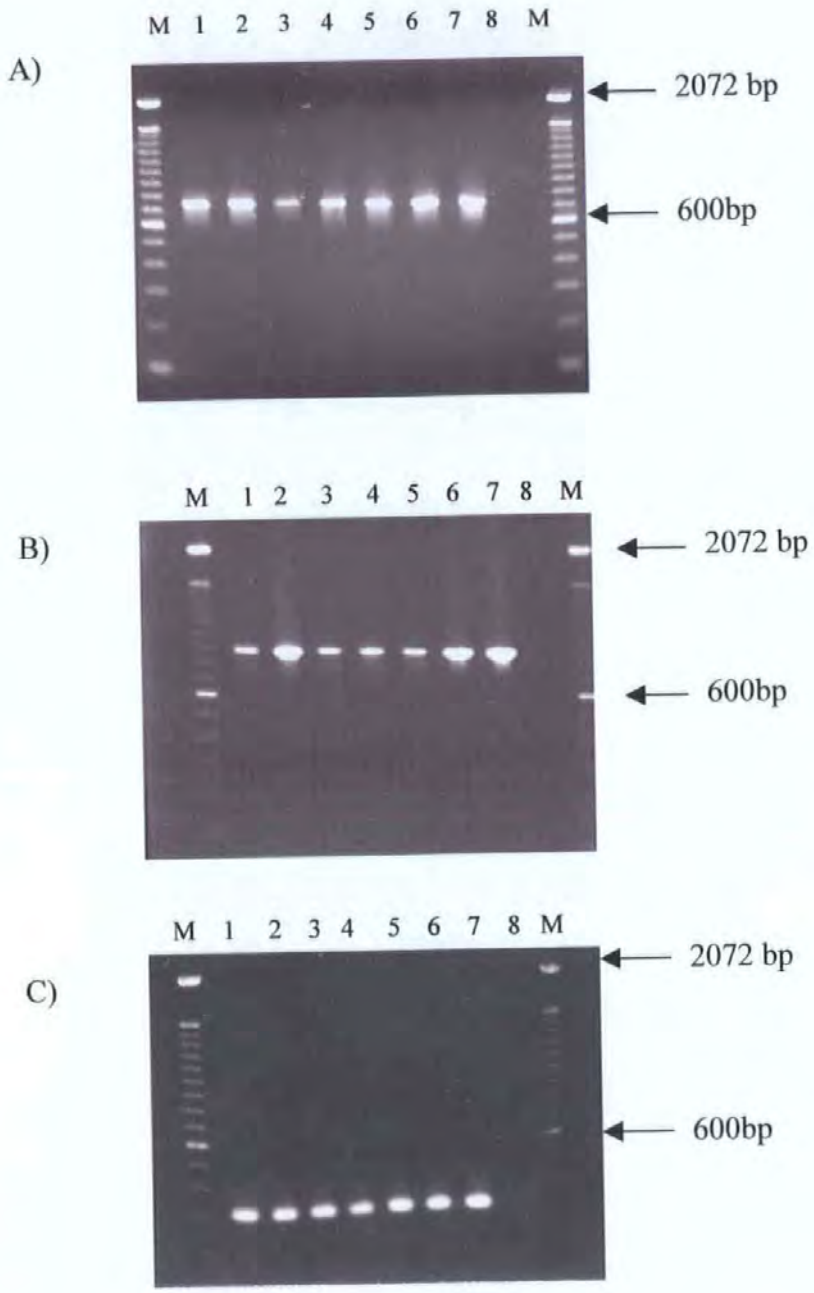


Figure III.7: PCR amplification of the rDNA ITS regions of *R. salmoninarum*. PCR reactions using primers RS+1002 & ML-1339 (panel A: ITS1), RS+1002 & ML-1469 (panel B: ITS1), R5+118 & R5-433. (panel C: ITS2). Lanes 1-8 corresponds to the following 980297#97, 980109F95, 980109F85, 980109F82, 980109F60, 980109F47, 980109F3 and negative control, respectively. Lane M: 100 bp Ladders (0.3µg), the size of selected markers are indicated by arrows on the right.

III.4 Additional gel images relevant to Chapter 7

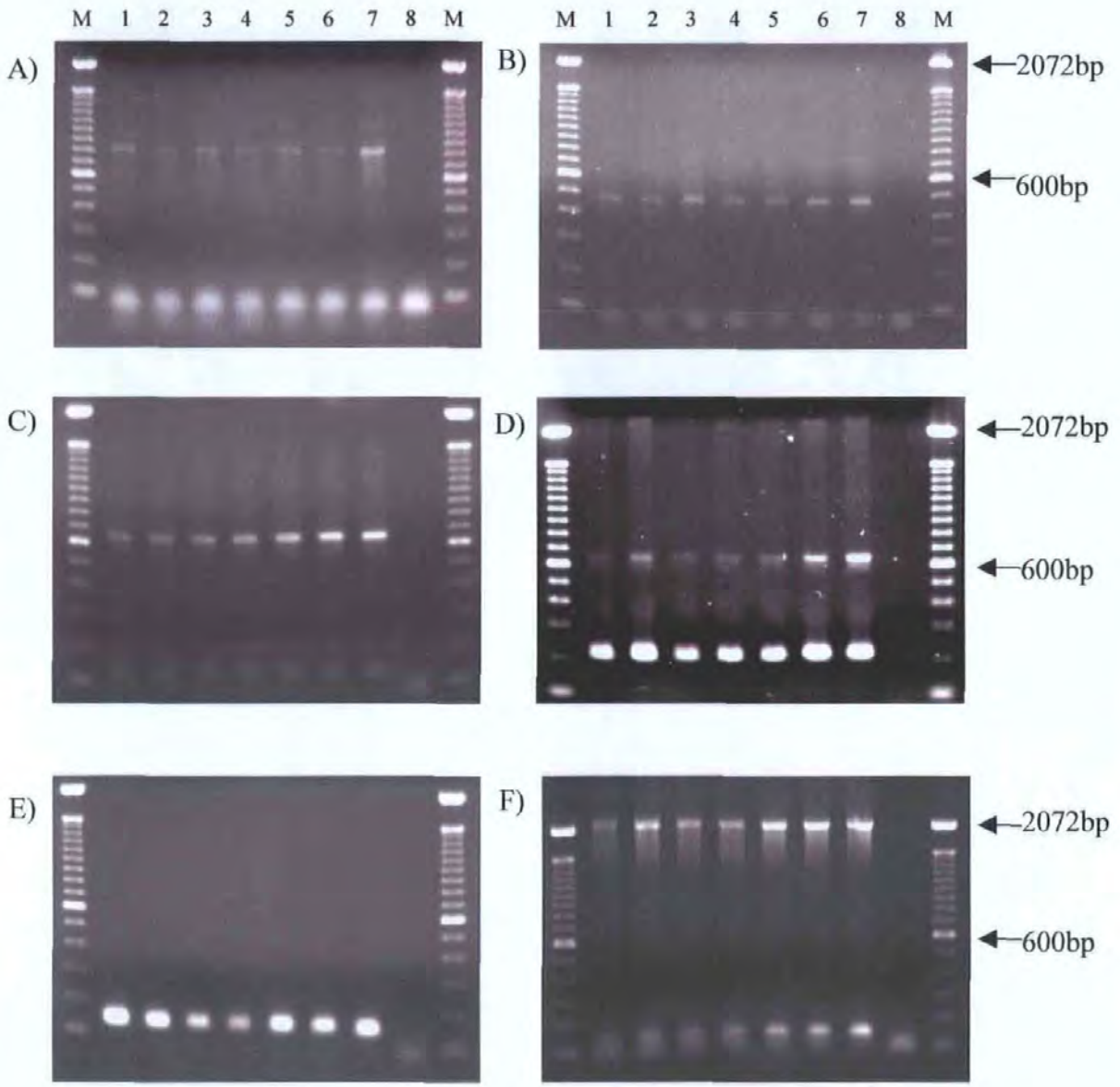


Figure III.8: tDNA-PCR amplification of *R. salmoninarum* DNA. PCR reactions using primers T25D+115 & T17C-135 (panel A) T25D+115 & A35K+760 (panel B), A35K+754 & T17C-135 (panel C), A35K+754 & T25D-120 (panel D), A35K+754 & T3C-123 (panel E), T3C+42 & T25D-120 (panel F). Lanes 1-7 corresponds to the following isolates: 980297#97, 980109F95, 980109F85, 980109F82, 980109F60, 980109F47 and 980109F3. Lane 8: negative control. Lane M: 100 bp ladders (0.3µg), the size of selected markers are indicated by arrows on the right.

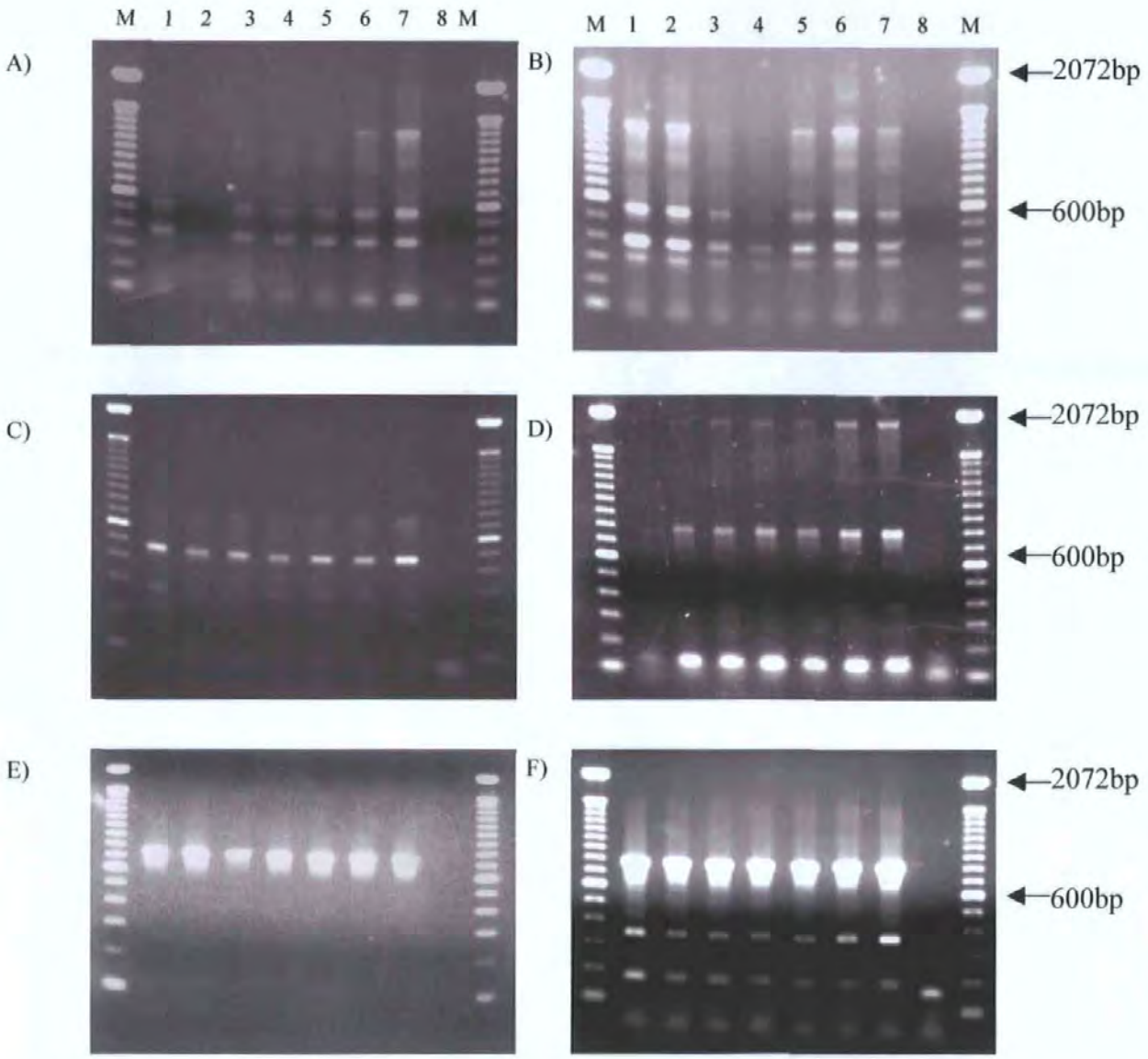


Figure III.9: tDNA-PCR amplification of *R. salmoninarum* DNA. PCR reactions using primers T35E+128 & A35K-760 (panel A), T35E+128 & T3C-123 (panel B), T17C+80 & A35K-760 (panel C), T17C+80 & T25D-120 (panel D), T17C+80 & T25E-128 (panel E) and T17C+80 & T3C-123 (panel F). Lanes 1-7 correspond to the following isolates: 980297#97, 980109F95, 980109F85, 980109F82, 980109F60, 980109F47 and 980109F3. Lane 8: negative control. Lane M: 100 bp ladders (0.3µg), the size of selected markers are indicated by arrows on the right.

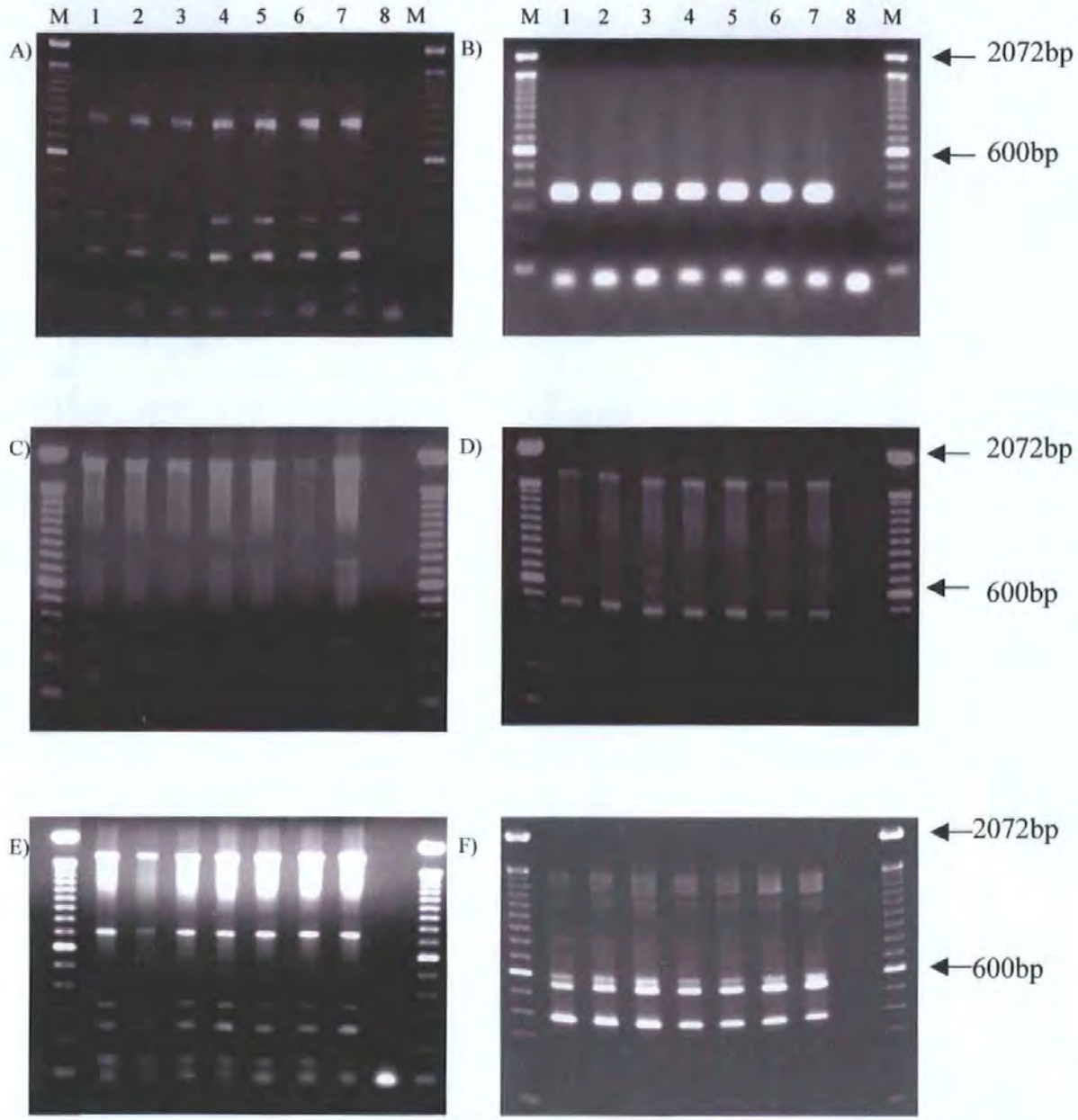


Figure III.10: tDNA-PCR amplification of *R. salmoninarum* DNA. PCR reactions using primers T3C+42 & T25-128 (panel A), T25D+19 & T17C-135 (panel B), T25E+19 & T25D-120 (panel C), T25E+19 & T25D-120 (panel D), T25E+19 & T3C-123 (panel E) and T35E+128 alone (panel F). Lanes 1-7 correspond to the following isolates: 980297#97, 980109F95, 980109F85, 980109F82, 980109F60, 980109F47 and 980109F3. Lane 8: negative control. Lane M: 100 bp ladders (0.3µg), the size of selected markers are indicated by arrows on the right.

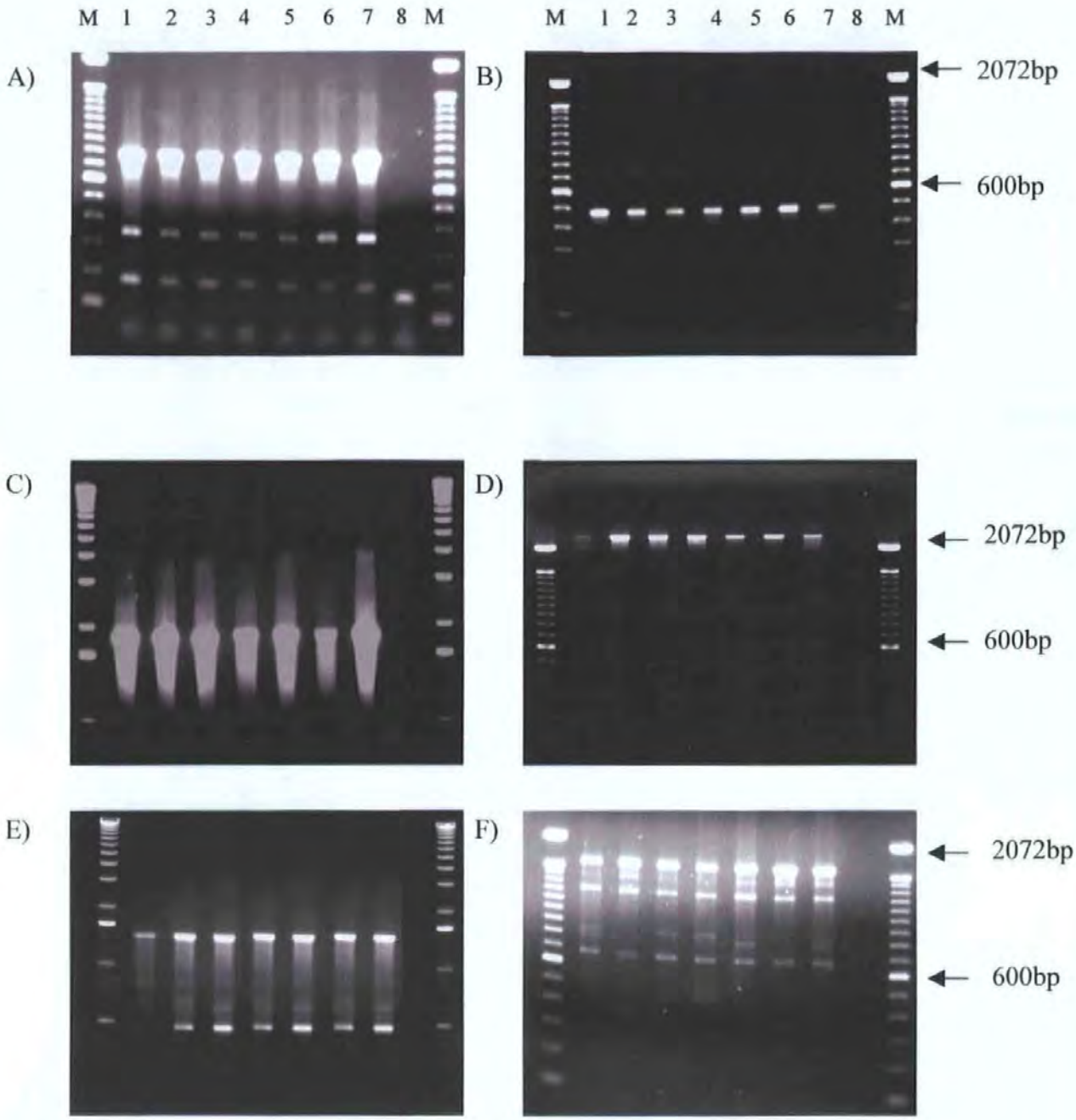


Figure III.11: tDNA-PCR amplification of *R. salmoninarum* DNA. PCR reactions using primers T17C+80 only (panel A), A35K-760 (panel B), T25D-120 (panel C), T3C+42 (panel D), T17C-135 (panel E) and T25E-128 alone (panel F). Lanes 1-7 correspond to the following isolates: 980297#97, 980109F95, 980109F85, 980109F82, 980109F60, 980109F47 and 980109F3. Lane 8: negative control. Lane M: 100 bp ladders (0.3µg), the size of selected markers are indicated by arrows on the right.

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Table III.2: tDNA-PCR primer combinations that failed to amplify visible products.

1	T35E+128 & T17C-135
2	T35E+128 & T25E-128
3	T35E+128 & T25D-120
4	T17C+80 & T35E-94
5	T17C+80 & T3C-123
6	T25D+115 & T3C-123
7	T25D+115 & T25E-128
8	A35K+754 & T35E-94
9	A35K+754 & T25E-128
10	T3C+42 & T35E-94
11	T3C+42 & T17C-135
12	T3C+42 & A35K-760
13	A7A+46 & A7A-309
14	A25A+4 & A25A-247
15	T25E+19 & T35E-94
16	T25D+115
17	A35K+754
18	T25E+19
19	T35E-94
20	T3C-123

REFERENCE LIST

Adair, D.M., Worsham, P.L., Hill, K.K., Klevytska, A.M., Jackson, P.J., Friedlander, A.M., and Keim, P. 2000. Diversity in a variable-number tandem repeat from *Yersina pestis*. Journal of Clinical Microbiology 38, 1516-1519.

Aded, Y., Davin, A., Charrel, R., Bollet, C., and De Micco, P. 1995. Variation of RAPD fingerprint patterns using different DNA extraction methods with gram positive bacteria. World Journal of Microbiology and Biotechnology 11, 238-239.

Akopyanz, N., Bukanov, N.O., Westblom, T.U., Kresovich, S., and Berg, D.E. 1992. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. Nucleic Acids Research 20, 5137-5142.

Alexander, S.M., Grayson, T.H., Chambers, E.M., Cooper, L.F., Barker, G.A., and Gilpin, M.L. 2001. Variation in the spacer regions separating tRNA genes in *Renibacterium salmoninarum* distinguishes recent clinical isolates from the same location. Journal of Clinical Microbiology 39, 119-128.

Allardet-Servent, A., Michaux-Charachon, S., Jamas-Bilak, E., Karayan, L., and Ramuz, M. 1993. Presence of one linear and one circular chromosome in the A*grobacterium tumefaciens* C58 genome. Journal of Bacteriology. 175, 7869-7874.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389-3402.

Amikam, D., Glaser, G., and Razin, S. 1984. Mycoplasmas (Mollicutes) have a low number of ribosomal RNA genes. Journal of Bacteriology 158, 376-378.

Appuhamy, S., Coote, J.G., Low, J.C., and Parton, R. 1998. PCR methods for rapid identification and characterisation of *Actinobacillus seminis* strains. Journal of Clinical Microbiology 36, 814-817.

Arakawa, E., Murase, T., Matshshita, S., Shimada, T., Yamai, S., Ito, T., and Watanabe, H. 2000. Pulsed field gel electrophoresis-based molecular comparison of *Vibrio cholerae* 01 isolates from domestic and imported cases of cholera in Japan. Journal of Clinical Microbiology 38, 424-426.

Arias, C., Pujalte, M.J., Garay, E., and Aznar, R. 1998. Genetic relatedness among environmental, clinical, and diseased eel *Vibrio vulnificus* isolates from different geographical regions by ribotyping and randomly amplified polymorphic DNA PCR. Applied and Environmental Microbiology 64, 3403-3410.

Atienzar, F., Child, P., Evenden, A., Jha, A., Savva, D., Walker, C., and Depledge, M. 1998. Application of the arbitrarily primed polymerase chain reaction for the detection of DNA damage. Marine Environmental Research 46, 331-335.

Austin, B. 1985. Evaluation of antimicrobial compounds for the control of bacterial kidney disease in rainbow trout, *Salmo gairdneri* Richardson. Journal of Fish Diseases 8, 209-220.

Austin, B., Bucke, D., Feist, S., and Rayment, J.N. 1985. A false positive reaction in the indirect fluorescent antibody test for *Renibacterium salmoninarum* with a coryneform organism. Bulletin of the European Association of Fish Pathologists 5, 8-9.

Austin, B., Embley, T.M., and Goodfellow, M. 1983. Selective isolation of *Renibacterium salmoninarum*. FEMS Microbiology Letters 17, 111-114.

Austin, B. and Rayment, J.N. 1985. Epizootiology of *Renibacterium salmoninarum*, the causal agent of bacterial kidney disease in salmonid fish. Journal of Fish Diseases 8, 505-509.

Austin, B., and Rodgers, C.J. 1980. Diversity among strains causing bacterial kidney disease in salmonid fish. Current Microbiology 3, 231-235.

Austin, B. and Stobie, M. 1992. Recovery of *Micrococcus luteus* and presumptive Planococcus Sp from moribund fish during an outbreak of rainbow trout, *Oncorhynchus mykiss* (Walbaum), fry syndrome in England. Journal of Fish Diseases 15, 203-206.

Baele, M., Baele, P., Vaneechoutte, M., Storms, V., Butaye, P., Devriese, L.A., Varschraegen, G., Gillis, M., and Haesebrouck, F. 2000. Application of tRNA intergenic spacer PCR for identification of *Enterococcus* species. Journal of Clinical Microbiology 38, 4201-4207.

Balfry, S.K., Albright, L.J., and Evelyn, T.P.T. 1996. Horizontal transfer of *Renibacterium salmoninarum* among farmed salmonids via the faecal-oral route. Diseases of Aquatic Organisms 25, 63-69.

Bandin, I, Ellis, A.E., Barja, J.L., and Secombes, C.J. 1993. Interaction between rainbow trout macrophages and *Renibacterium salmoninarum in vitro*. Fish & Shellfish Immunology 3, 5-33.

Bandin, I, Santos, Y., Bruno, D.W., Raynard, R.S., Toranzo, A.E., and Barja, J.L. 1991. Lack of biological activities in the extracellular products of *Renibacterium salmoninarum*. Canadian Journal of Fisheries and Aquatic Sciences 48, 421-425.

Banks, J.L. 1994. Raceway density and water-flow as factors affecting spring chinook salmon (*Oncorhynchus tshawytscha*) during rearing and after release. Aquaculture 119, 201-217.

Banner, C.R., Rohovec, J.S., and Fryer, J.L. 1991. A new value for mol percent guanine + cytosine of DNA for the salmonid fish pathogen *Renibacterium salmoninarum*. FEMS Microbiology Letters 79, 57-59.

Baril, C., Richaud, C., Baranton, G., and Saint Girons, I.S. 1989. Linear chromosome of *Borrelia burgdorferi*. Research in Microbiology 140, 507- 516.

Barlow, R.E.L., Gascoyne-Binzi, D.M., Gillespie, S.H., Dickens, A., Qamer, S., and Hawkey, P.M. 2001. Comparison of variable number tandem repeat and IS*6110* restriction fragment length polymorphism analyses for discrimination of high and low copy number IS*6110* *Mycobacterium tuberculosis* isolates. Journal of Clinical Microbiology 39, 2453-2457.

Barry, T., Colleran, G., Glennon, M., Dunican, L. K., and Gannon, F. 1991. The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. PCR Methods and Applications 1, 51-56.

Bassam, B.J., Caetano-Anolles, G., and Gresshoff, P.M. 1992. DNA amplification fingerprinting of bacteria. Applied Microbiology and Biotechnology 38, 70-76.

Beacham, T.D. and Evelyn, T.P.T. 1992. Genetic variation in disease resistance and growth of chinook, coho, and chum salmon with respect to vibriosis, furunculosis, and bacterial kidney disease. Transactions of the American Fisheries Society 121, 456-485.

Belding, D.L. and Merril, B. 1935. A preliminary report upon a hatchery disease of the *Salmonidae*. Transactions of the American Fisheries Research Board of Canada 18, 559-562.

Bell, G.R. 1961. Two epidemics of apparent kidney disease in cultured pink salmon (*Oncorhynchus gorbuscha*). Journal of Fisheries Research Board of Canada 18, 559-562.

Bell, G.R., Higgs, D.A., and Traxler, G.S. 1984. The effect of dietary ascorbate, zinc, and manganese on the development of experimentally induced bacterial kidney disease in sockeye salmon (*Oncorhynchus nerka*). Aquaculture 36, 293-311.

Bell, G.R., Hoffmann, R.W., and Brown, L.L. 1990. Pathology of experimental infections of the sablefish, *Anoplopoma fimbria* (Pallas), with *Renibacterium salmoninarum*, the agent of bacterial kidney disease in salmonids. Journal of Fish Diseases 13, 355-367,

Bell, G.R. and Traxler, G.S. 1986. Resistance of the Pacific lamprey, *Lampetra tridentata* (Gairdner), to challenge by *Renibacterium salmoninarum*, the causative agent of kidney disease in salmonids. Journal of Fish Diseases 9, 277-279.

Bell, G.R., Traxler, G.S., and Dworschak, C. 1988. Development *in vitro* and pathogenicity of an erythromycin resistant strain of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in salmonids. Diseases of Aquatic Organisms 4, 19-25.

Benediktsdottir, E., Helgason, S., and Gudmundsdottir, S. 1991. Incubation time for the cultivation of *Renibacterium Salmoninarum* from Atlantic salmon, *Salmo salar* L, broodfish. Journal of Fish Diseases 14, 97-102.

Bercovier, H., Kafri, O., and Sela, S. 1986. *Mycobacterium* possess a surprisingly small number of ribosomal RNA genes in relation to the size of their genome. Biochemical and Biophysical Research Communications 136, 1136-1141.

Bereswill, S., Hinkelmann, S., Kist, M., and Sander, A. 1999. Molecular analysis of riboflavin synthesis genes in *Bartonella henselae* and use of the *rib*C gene for differentiation of *Bartonella* species by PCR. Journal of Clinical Microbiology 37, 3159-3166.

Berg, D.E., Akopyants, N., and Kersulyte, D. 1994. Fingerprinting microbial genomes using the RAPD or AP-PCR method. Methods in Molecular and Cellular Biology 5, 13-24.

Beyazova, M., Brodsky, B.C., Shearer, M.C., and Horan, A.C. 1995. Preparation of actinomycete DNA for pulsed-field gel electrophoresis. International Journal of Systematic Bacteriology 45, 852-854.

BioRad. 1993. CHEF-DR II pulsed field electrophoresis systems instruction manual and applications guide. pp. 12 – 13.

Birren, B. and Lai, E. 1993. Pulsed field gel electrophoresis: a practical guide. pp. 118-156. Academic Press.

Brown, L.I., Evelyn, T.P.T., Iwama, G.K., Nelson, W.S., and Levine, R.P. 1995. Bacterial species other than *Renibacterium salmoninarum* cross react with antisera against *Renibacterium salmoninarum* but are negative for the p57 gene of *Renibacterium. salmoninarum* as detected by the polymerase chain reaction (PCR). Diseases of Aquatic Organisms 21, 227-231.

Brown, L.L., Iwama, G.K., Evelyn, T.P.T., Nelson, W.S., and Levine, R.P. 1994. Use of the polymerase chain reaction (PCR) to detect DNA from *Renibacterium salmoninarum* within individual salmonid eggs. Diseases of Aquatic Organisms 18, 165-171.

Bruno, D.W. 1988. The relationship between auto-agglutination, cell surface hydrophobicity and virulence of the fish pathogen *Renibacterium salmoninarum*. FEMS Microbiology Letters 51, 135-139.

Bruno, D.W. 1986. Histopathology of bacterial kidney disease in laboratory infected rainbow trout, *Salmo gairdneri* (Richardson) and Atlantic salmon, *Salmo salar L.*, with reference to naturally infected fish. Journal of Fish Diseases 9, 523-537.

Bruno, D.W. and Munro, A.L.S. 1986. Uniformity in the biochemical properties of *Renibacterium salmoninarum* isolates obtained from several sources. FEMS Microbiology Letters 33, 247-250.

Bruno, D.W. and Munro, A.L.S. 1982. Detection of the causative agent of bacterial kidney disease. Bulletin of the European Association of Fish Pathologists 2, 10-12.

Bullock, G.L., Griffin, B.R., and Stuckey, H.W. 1980. Detection of *Corynebacterium salmonis* by direct fluoresent antibody test. Canadian Journal of Fisheries and Aquatic Sciences 37, 719-721.

Bullock, G.L., Stuckey, H.W., and Mulcahy, D. 1978. Corynebacterial kidney disease: egg transmission following iodophore disinfection. Fish Health News 7, 51-52.

Bullock, G.L. and Stuckey, H.W. 1975. Fluorescent antibody identification and detection of the *Corynebacterium* causing kidney disease of salmonids. Journal of the Fisheries Research Board of Canada 32, 2224-2227.

Bullock, G.L., Stuckey, H.W., and Chen, P.K. 1974. Corynebacterial kidney disease of salmonids: growth and serological studies on the causative bacterium. Applied Microbiology 28, 811-814.

Burr, M.D., Josephson, K.L., and Pepper, I.L. 1998. An evaluation of DNA based methodologies for subtyping *Salmonella*. Critical Reviews in Environmental Science and Technology 28, 283-323.

Butendieck, B., Morales, P., Figueroa, J., Concha, M., and Leon, G. 1995. Specific gene amplification as a means to detect *Renibacterium salmoninarum*. Archivos De Medicina Veterinaria 27, 47-54.

Campbell, A.M. 1992. Chromosomal insertion sites for phages and plasmids. Journal of Bacteriology 174, 7494-7499.

Carlson, C.R., and Kolsto, A.B. 1993. A complete physical map of a *Bacillus thuringiensis* chromosome. Journal of Bacteriology 175, 1053-1060.

Chachaty, E., Saulnier, P., Martin, A., Mario, N., and Andremont, A. 1994. Comparison of ribotyping, pulsed field gel electrophoresis and random amplified polymorphic DNA for yyping *Clostridium difficile* strains. FEMS Microbiology Letters 122, 61-68.

Chakroun, C., Urdaci, M., Faure, D., Grimont, F., and Bernardet, J.F. 1997. Random amplified polymorphic DNA analysis provides rapid differentiation among isolates of the fish pathogen *Flavobacterium psychrophilum* and among *Flavobacterium* species. Diseases of Aquatic Organisms 31, 187-196.

Chase, D.M., and Pascho, R.J. 1998. Development of a nested polymerase chain reaction for amplification of a sequence of the p57 gene of *Renibacterium salmoninarum* that provides a highly sensitive method for detection of the bacterium in salmonid kidney. Diseases of Aquatic Organisms 34, 223-229.

Chen, P.K., Bullock, G.L., Stuckey, H.W., and Bullock, A.C. 1974. Serological diagnosis of corynebacterial kidney disease of salmonids. Journal of the Fisheries Research Board of Canada 31, 1939-1940.

Chen, W.H., Kuspa, A., Keseler, I.M., and Shimkets, L.J. 1991. Physical map of the *Myxococcus xanthus* chromosome. Journal of Bacteriology 173, 2109-2115.

Chien, M.S., Gilbert, T.L., Huang, C., Landolt, M.L., Ohara, P.J., and Winton, J.R. 1992. Molecular cloning and sequence analysis of the gene coding for the 57 kDa major soluble

antigen of the salmonid fish pathogen *Renibacterium salmoninarum*. FEMS Microbiology Letters 96, 259-266.

Claros, M.C., Gerardo, S.H., Citron, D.M., Goldstein, E.J.C., Schonian, G., and Rodloff, A.C. 1997. Use of polymerase chain reaction fingerprinting to compare clinical isolates of *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* from Germany and the United States. Clinical Infectious Diseases 25, S295-S298.

Cobben, N.A.M., Drent, M., Jonkers, M., Wouters, E.F.M., Vaneechoutte, M., and Stobberingh, E.E. 1996. Outbreak of severe *Pseudomonas aeruginosa* respiratory infections due to contaminated nebulizers. Journal of Hospital Infection 33, 63-70.

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Sequares, S., Sulston, J.E., Taylor, K., Whitehead, S., and Barrell, B.G. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393, 537-544.

Cole, S.T. and Girons, I. 1994. Bacterial genomics. FEMS Microbiology Reviews. 14. 139-160.

Colman, S.D., Hu, P.C., Litaker, W., and Bott, K.F. 1990. A physical map of the *Mycoplasma genitalium* genome. Molecular Microbiology 4, 683-687.

Concha, M.I. and Leon, G. 2000. Cloning, functional expression and partial characterisation of the glucose kinase from *Renibacterium salmoninarum*. FEMS Microbiology Letters 186, 97-101.

Cook, M. and Lynch, W.H. 1999. A sensitive nested reverse transcriptase PCR assay to detect viable cells of the fish pathogen *Renibacterium salmoninarum* in Atlantic salmon (*Salmo salar* L.). Applied and Environmental Microbiology 65, 3042-3047.

Crespi, M., Messens, E., Caplan, A.B., van Montagu, M., and Desomer, J. 1992. Fasciation induction by the phytopathogen *Rhodococcus fascians* depends upon a linear plasmid encoding a cytokinin synthase gene. EMBO Journal. 11. 795-804.

Daffonchio, D., Borin, S., Frova, G., Manachini, L., and Sorlini, C. 1998. PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. International Journal of Systematic Bacteriology 48, 107-116.

Dale, O.B., Gutenberger, S.K., and Rohovec, J.S. 1997. Estimation of variation of virulence of *Renibacterium salmoninarum* by survival analysis of experimental infection of salmonid fish. Journal of Fish Diseases 20, 177-183.

Daly, J.G. and Stevenson, R.M.W. 1990. Characterisation of the *Renibacterium salmoninarum* hemagglutinin. Journal of General Microbiology 136, 949-953.

Daly, J.G. and Stevenson, R.M.W. 1989. Agglutinating of salmonid spermatozoa by *Renibacterium salmoninarum*. Journal of Aquatic Animal Health 1, 163-164.

Dams, E., Yamdam, T., De Baere, R., Huysmans, E., Vandenberghe, A., and De Wachter, R. 1987. Structure of 5S rRNA in actinomycetes and relatives and evolution of eubacteria. Journal of Molecular Evolution 180, 4804-4813.

Das, I., Lambert, P., Hill, D., Noy, M., Bion, J., and Elliot, T. 2002. Carbapenem-resistant *Acinetobacter* and role of curtains in an outbreak in intensive care units. Journal of Hospital Infection 50, 110-114.

de Bruijn, F.J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Applied and Environmental Microbiology 58, 2180-2187.

Dempsey, J.A., Litaker, W., Madhure, A., Snodgrass, T.L., and Cannon, J.G. 1991. Physical map of the chromosome of *Neisseria gonorrhoeae* FA*1090* with locations of genetic markers, including *opa* and *pil* genes. Journal of Bacteriology 173, 5476-5486.

Dennis, P.P., Ziesche, S., and Mylvaganam, S.1998. Transcription analysis of two disparate rRNA operons in the halophilic archaeon *Haloarcula marismortui.* Journal of Bacteriology 180, 4804-4813.

Department for Food, Environment and Rural Affairs. 2001. A guide to importing fish; an explanation of the controls governing fish: the movement of live and dead fish, fish eggs and gametes into and from Great Britain. Appendix III.

Department for Food, Environment and Rural Affairs. 2002. Combating fish disease: an explanation of the controls on notifiable fish diseases in Great Britain and advice on steps to prevent the spread of disease. pp. 4-6.

Dingle, K.E., Colles, F.M., Wareing, D.R., Ure, R., Fox, A.J., Bolton, F.E., Bootsma, H.J., Willems, R.J., Urwin, R., and Maiden, M.C. 2001. Multilocus sequence typing system for *Camplyobacter jejuni.* Journal of Clinical Microbiology 39, 14-23.

Dixon, P.F. 1987. Detection of *Renibacterium salmoninarum* by the enzyme linked immunosorbent assay (ELISA). Journal of Applied Ichthyology Zeitschrift Fur Angewandte Ichthyologie 3, 77-82.

Dombek, P.E., Johnson, L.K., Zimmerley, S.T., and Sadowsky, M.J. 2000. Use of repetitive DNA sequence and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. Applied and Environmental Microbiology 66, 2572-2577.

Drake, M.A., Small, C.L., Spence, K.D., and Swanson, B.G. 1996. Differentiation of *Lactobacillus helveticus* strains using molecular typing methods. Food Research International. 29, 451-455.

Dubreuil, D, Lallier, R., and Jacques, M. 1990. Immunoelectron microscopic demonstration that *Renibacterium salmoninarum* is encapsulated. FEMS Microbiology Letters 66, 313-315.

Dundon, W., Marshall, D.G., Morain, C., and Smyth, C.J. 1999. A novel tRNA-associated locus (trl) from *Helicobacter pylori* is co-transcribed with $tRNA^{gly}$ and reveals genetic diversity. Microbiology 145, 1289-1298.

Earp, B.J., Ellis, C.H., and Ordal, E.J. 1953. Kidney disease in young salmon. Washington State, Department of Fisheries, Special Report Series No.1.

Ehrenstein, B., Bernards, A.T., Dijkshoorn, L., GernerSmidt, P., Towner, K.J., Bouvet, P.J.M., Daschner, F.D., and Grundmann, H. 1996. *Acinetobacter* species identification by using tRNA spacer fingerprinting. Journal of Clinical Microbiology 34, 2414-2420.

Elliott, D.G. and Barila, T.Y. 1987. Membrane filtration fluorescent-antibody staining procedure for detecting and quantifying *Renibacterium salmoninarum* in celomic fluid of chinook salmon (*Oncorhynchus tshawytscha*). Canadian Journal of Fisheries and Aquatic Sciences 44, 206-210.

Elliott, D.G., Pascho, R.J., Jackson, L.M., Matthews, G.M., and Harmon, J.R. 1997. *Renibacterium salmoninarum* in spring-summer chinook salmon smelts at dams on the Columbia and Snake rivers. Journal of Aquatic Animal Health 9, 114-126.

Elliott, D.G., Pascho, R.J., and Palmisano, A.N. 1995. Brood stock segregation for the control of bacterial kidney disease can affect mortality of progeny chinook salmon (*Oncorhynchus tshawytscha*) in seawater. Aquaculture 132, 133-144.

Ellsworth, D. L., Rittenhouse, K. D., and Honeycutt, R. J. 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. Biotechniques 14, 214-217.

Embley, T.M., Goodfellow, M., and Austin, B. 1982. A semi-defined growth medium for *Renibacterium salmoninarum*. FEMS Microbiology Letters 14, 299-301.

Embley, T.M., Goodfellow, M., Minnikin, D.E., and Austin, B. 1983. Fatty acid, isoprenoid quinone and polar lipid composition in the classification of *Renibacterium salmoninarum*. Journal of Applied Bacteriology 55, 31-37.

Engels, W.R. 1993. Contributing software to the Internet: the Amplify program. Trends in Biochemical Science 18, 448-450.

Etchegaray, J.P., Martinez, M.A., Krauskopf, M., and Leon, G. 1991. Molecular cloning of *Renibacterium salmoninarum* DNA fragments. FEMS Microbiology Letters 79, 61-64.

Evelyn, T.P.T. 1993. Bacterial kidney disease. In: Bacterial Diseases of Fish, edited by Inglis, V., Roberts, R.J., and Bromage, N.R., pp. 177-195. Blackwell Scientific Publications.

Evelyn, T.P.T. 1988. Bacterial kidney disease in British Columbia, Canada: comments on its epizootiology and methods for its control on fish farms. In: AQUA NOR 87 Trondheim International Conference, Norske Fiskeoppdretteres Porening-Fiskeoppdretternes Sagslag A/L. Trondheim, Norway, pp. 51-57.

Evelyn, T.P.T. 1977. An improved growth medium for the kidney bacterium and some notes in using the medium. Bulletin de l'Office International des Epizootiologies 87, 511-513.

Evelyn, T.P.T., Bell, G.R., Prosperi-Porta, L., and Ketcheson, J.E. 1989. A simple technique for accelerating the growth of the kidney disease bacterium *Renibacterium salmoninarum* on a commonly used culture-medium (KDM2). Diseases of Aquatic Organisms 7, 231-234.

Evelyn, T.P.T., Ketcheson, J.E., and Prosperi-Porta, L. 1986a. Use of erythromycin as a means of preventing vertical transmission of *Renibacterium salmoninarum*. Diseases of Aquatic Organisms 2, 7-11.

Evelyn, T.P.T., Ketcheson, J.E., and Prosperi-Porta, L. 1984. Further evidence for the presence of *Renibacterium salmoninarum* in salmonid eggs and for the failure of povidone iodine to reduce the intra-ovum infection rate in water hardened eggs. Journal of Fish Diseases 7, 173-182.

Evelyn, T.P.T., Prosperi-Porta, L., and Ketcheson, J.E. 1990. Two new techniques for obtaining consistent results when growing *Renibacterium salmoninarum* on KDM2 culture medium. Diseases of Aquatic Organisms 9, 209-212.

Evelyn, T.P.T., Prosperi-Porta, L., and Ketcheson, J.E. 1986b. Experimental intraovum infection of salmonid eggs with *Renibacterium salmoninarum* and vertical transmission of the pathogen with such eggs despite their treatment with erythromycin. Diseases of Aquatic Organisms 1, 197-202.

Evenden, A. 1993. The use of gene cloning techniques in the study of the fish pathogen *Renibacterium salmoninarum*. PhD Thesis. University of Plymouth.

Evenden, A.J., Gilpin, M.L., and Munn, C.B. 1990. The cloning and expression of a gene encoding hemolytic activity from the fish pathogen *Renibacterium salmoninarum*. FEMS Microbiology Letters 71, 31-34.

Evenden, A.J., Grayson, T.H., Gilpin, M.L., and Munn, C.B. 1993. *Renibacterium salmoninarum* and bacterial kidney disease - the unfinished jigsaw. Annual Review of Fish Diseases 3, 87-104.

Falla, T.J., Crook, D.W., Brophy, L.N., Maskell, D., Kroll, J.S., and Moxon, E.R. 1994. PCR for capsular typing of *Haemophilus influenzae*. Journal of Clinical Microbiology 32, 2382-2386.

Ferdows, M.S. and Barbour, A.G. 1992. Mega-based sized linear DNA in the bacterium *Borrelia burgdorferi* the Lyme disease agent. Proceedings of the National Academy of Sciences of the United States of America. 86. 5969-5972.

Ferrus, M.A., Hernandez, M., and Hernandez Haba, J. 1998. Ribotyping of *Pseudomonas aeruginosa* from infected patients: evidence of common strain types. APMIS 106, 456-462.

Fevolden, S.E., Nordmo, R., Refstie, T., and Roed, K.H. 1993. Disease resistance in Atlantic salmon (*Salmo salar*) selected for high or low responses to stress. Aquaculture 109, 215-224.

Fiedler, F. and Draxl, R. 1986. Biochemical and immunochemical properties of the cell surface of *Renibacterium salmoninarum*. Journal of Bacteriology 168, 799-804.

Fichant, G.A. and Burks, C. 1991. Identifying potential tRNA genes in genomic DNA sequences. Journal of Molecular Biology 220, 659-671.

Foissac, X., Danet, J., Zreik, L., Gandar, J., Nourrisseau, J., Bove, J., and Garnier, M. 2000. Cloning of the *spo*T gene of "*Candidatus* Phlomobacter fragariae" and development of a PCR restriction fragment length polymorphism assay for detection of the bacterium in insects. Applied and Environmental Microbiology 66, 3474-3480.

Folkesson, A., Advani, A., Sukupolvi, S., Pfeifer, J.D., Normark, S., and Lofdahl, S. 1999. Multiple insertions of fimbrial operons correlate with the evolution of *Salmonella* serovars responsible for human disease. Molecular Microbiology 33, 612-622.

Fonstein, M. and Haselkorn, R. 1995. Physical mapping of bacterial genomes. Journal of Bacteriology 177, 3361-3369.

Food and Drug Administration, US Department of Agriculture US Environmental Protection Agency and Centres for Disease Control and Prevention. 1997. Food safety from farm to table: a National Food Safety Initiative Report to the President. pp. 3.

Fraser, C.M., Gocayne, J.D., White, O., Adams, M.D., Clayton, R.A., Fleischmann, R.D., Bult, C.J., Kerlavage, A.R., Sutton, G., Kelley, J.M., Fritchman, J.L., Weidman, J.F., Small, K., Sandusky, M., Fuhrmann, J., Nguyen, D., Utterback, T.R., Saudek, D.M., Phillips, C.A., Merrick, J.M., Tomb, J., Doughery, B.A., Bott, K.F., Hu, P.C., Lucier, T.S., Peterson, S.N., Smith, H.O., Hutchison, C.A., and Venter, J.C. 1995. The minimal gene complement of *Mycoplasma genitalium*. Science. 270. 397-403.

Frothingham, R. and Meeker-O'Connell, W.A. 1998. Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. Microbiology 144, 1189-1196.

Frothingham, R. and Wilson, K. M. 1993. Sequence based differentiation of strains in the *Mycobacterium avium* complex. Journal of Bacteriology 175, 2818-2825.

Fryer, J.L. and Lannan, C.N. 1993. The history and current status of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in Pacific salmon. Fisheries Research 17, 15-33.

Fryer, J.L. and Sanders, J.E. 1981. Bacterial kidney disease of salmonid fish. Annual Reviews of Microbiology 35, 273-298.

Gasc, A., Kauc, L., Barraille, P., Sicard, M., and Goodgal, S. 1991. Gene localisation, size, and physical map of the chromosome of *Streptococcus pneumoniae*. Journal of Bacteriology 173, 7361-7367.

Gaydos, C.A., Bobo, L., Welsh, L., Hook, E.W., Viscidi, R., and Quinn, T.C. 1992. Gene typing of *Chlamydia trachomatis* by polymerase chain reaction and restriction endonuclease digestion. Sexually Transmitted Diseases [19],303-308.

Getchell, R.G., Rohovec, J.S., and Fryer, J.L. 1985. Comparison of *Renibacterium salmoninarum* isolates by antigenic analysis. Fish Pathology 20, 149-159.

Gibson, J.R., Sutherland, K., and Owen, R.J. 1994. Inhibition of DNase activity in PFGE analysis of DNA from *Campylobacter jejuni*. Letters In Applied Microbiology. 19. 357-358.

Gillings, M. and Holley, M. 1997. Amplification of anonymous DNA fragments using pairs of long primers generates reproducible DNA fingerprints that are sensitive to genetic variation. Electrophoresis 18, 1512-1518.

Gilson, E., Clement, J.M., Brutlag, D., and Hofnung, M. 1984. A family of dispersed repetitive extragenic palindromic DNA sequences in *Escherichia coli*. EMBO Journal. 3, 1417-1421.

Gilson, E., Clement, J.M., Perrin, D., and Hofnung, M. 1987. Palindromic units - a case of highly repetitive DNA sequences in bacteria. Trends in Genetics. 3, 226-230.

Gilson, E., Rousset, J.P., Clement, J.M., and Hofnung, M. 1986. A subfamily of *Escherichia coli* palindromic units implicated in transcripiton termination? Annales de l' Institut Pasteur Microbiologie .137B, 259-270.

Glover, D.J. and Harris, W.J. 1998. The use of alkaline phosphatase-labelled oligonucleotide probes as culture confirmation reagents for the identification of commercially important bacteria. Letters in Applied Microbiology 27, 116-120.

Goering, R.V. and Duensing, T.D. 1990. Rapid field inversion gel electrophoresis in combination with an rRNA gene probe in the epidemiological evaluation of Staphylococci. Journal of Clinical Microbiology 28, 2059-2063.

Gomez-De-Leon, P., Santos, J.I., Caballero, J., Gomez, D., Espinosa, L.E., Moreno, I, Pinero, D., and Cravioto, A. 2000. Genomic variability of *Haemophilus influenzae* isolated from Mexican children determined by using enterobacterial repetitive intergenic consensus sequences and PCR. Applied and Environmental Microbiology 38, 2504-2511.

Gomez Zavaglia, A., de Urraza, P., and DeAntoni, G. 2000. Characterisation of *Bifidobacterium* strains using BOX primers. Anaerobe 6, 169-177.

Goodfellow, M., Embley, T.M., and Austin, B. 1985. Numerical taxonomy and emended description of *Renibacterium salmoninarum*. Journal of General Microbiology 131, 2739-2752.

Grayson, T.H., Alexander, S.M., Cooper, L.F., and Gilpin, M.L. 2000a. *Renibacterium salmoninarum* isolates from different sources possess two highly conserved copies of the rRNA operon. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 78, 51-61.

Grayson, T.H., Atienzar, F.A., Alexander, S.M., Cooper, L.F., and Gilpin, M.L. 2000b. Molecular diversity of *Renibacterium salmoninarum* isolates determined by randomly amplified polymorphic DNA analysis. Applied and Environmental Microbiology 66, 435-438.

Grayson, T.H., Bruno, D.W., Evenden, A.J., Gilpin, M.L., and Munn, C.B. 1995a. Iron acquisition by *Renibacterium salmoninarum* - contribution of iron reductase. Diseases of Aquatic Organisms 22, 157-162.

Grayson, T.H., Cooper, L.F., Atienzar, F.A., Knowles, M.R., and Gilpin, M.L. 1999. Molecular differentiation of *Renibacterium salmoninarum* isolates from worldwide locations. Applied and Environmental Microbiology 65, 961-968.

Grayson, T.H., Evenden, A.J., Gilpin, M.L., Martin, K.L., and Munn, C. B. 1995b. A gene from *Renibacterium salmoninarum* encoding a product which shows homology to bacterial zinc-metalloproteases. Microbiology 141, 1331-1341.

Grayson, T.H., Evenden, A.J., Gilpin, M.L., and Munn, C.B. 1995c. Production of the major soluble antigen of *Renibacterium salmoninarum* in *Escherichia coli* K12. Diseases of Aquatic Organisms 22, 227-231.

Grayson, T.H., Evenden, A.J., Gilpin, M.L., and Munn, C.B. 1995d. Production of a *Renibacterium salmoninarum* hemolysin fusion protein in *Escherichia coli* K12. Diseases of Aquatic Organisms 22, 153-156.

Green, C.J. and Vold, B. 1993. *Staphylococcus aureus* has clustered tRNA genes. Journal of Bacteriology 175, 5091-5096.

Griffiths, S.G Melville, K.J., and Salonius, K. 1998. Reduction of *Renibacterium salmoninarum* culture activity in Atlantic salmon following vaccination with avirulent strains. Fish and Shellfish Immunology 8, 607-619.

Grimont, F. and Grimont, P.A.D. 1986. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. Annales de l' Institut Pasteur [137B], -166.

Groman, D.B. and Klontz, G.W. 1983. Chemotherapy and prophylaxis of bacterial kidney disease with erythromycin. Journal of the World Mariculture Society 14, 226-253.

Grundmann, H., Schneider, D., Hartung, F.D., and Pitt, T. 1995. Discriminatory power of three DNA based typing techniques for *Pseudomonas aeurginosa*. Journal of Clinical Microbiology 33, 528-534.

Gudmundsdottir, S., Helgason, S., and Benediktsdottir, E. 1991. Comparison of the effectiveness of three different growth media for primary isolation of *Renibacterium salmoninarum* from Atlantic salmon, *Salmo salar* L., broodfish. Journal of Fish Diseases 14, 89-96.

Gupta, V.S., Rajebhosale, M.D., Sodhi, M, Singh, S., Gnanamanickam, S.S., Dhaliwal, H.S., and Ranjekar, P.K. 2001. Assessment of genetic variability and strain identification of *Xanthomonas oryzae* pv. *oryzae* using RAPD-PCR and IS*1112* based PCR. Current Science 80, 1043-1049.

Gurtler, V and Mayall, B.C. 2001. New approaches to typing, taxonomy and evolution of bacterial isolates. International Journal of Systematic Bacteriology 51, 3-16.

Gurtler, V. and Stanisich, V.A. 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. Microbiology 142, 3-16.

Gutenberger, S., Giovannoni, J.G., Field, K.G., Fryer, J.L., and Rohovec, J.S. 1991. A phylogenetic comparison of the 16S rRNA sequence of the fish pathogen, *Renibacterium salmoninarum*, to gram positive bacteria. FEMS Microbiology Letters 77, 151-156.

Hariharan, H., Qian, B., Despres, B., Kibenge, F.S., Heaney, S.B., and Rainnie, D.J. 1995. Development of a specific biotinylated DNA-probe for the detection of *Renibacterium salmoninarum*. Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire 59, 306-310.

Harrington, C and On, S.L. 1999. Extensive 16S rRNA gene sequence diversity in *Campylobacter hyointestinalis* strains: taxonomic and applied implications. International Journal of Systematic and Evolutionary Microbiology 49, 1171-1175.

Harris, L.J. and Griffiths, M.W. 1992. The detection of foodborne pathogens by the polymerase chain reaction (PCR). Food Research International 25, 457-469.

Hendson, M., Purcell, A.H., Chen, D.C., Smart, C., Guilhabert, M., and Kirkpatrick, B. 2001. Genetic diversity of Pierce's disease strains and other pathotypes of *Xylella sastidiosa*. Applied and Environmental Microbiology 67, 895-903.

Hermans, P.W.M., Sluijter, M., Hoogenboezem, T., Heersma, H., van Belkum, A., and de Groot, R. 1995. Comparative study of five different DNA fingerprinting techniques for molecular typing of *Streptococcus pneumoniae* strains. Journal of Clinical Microbiology 33, 1606-1612.

Higgins, C.F., Ames, G.F., Barnes, W.M., Clement, J.M., and Hofnung, M. 1982. A novel intercistronic regulatory element of prokaryotic operons. Nature 298, 760-762.

Higgins, C.F., McLaren, R.S., and Newbury, S.F. 1988. Repetitive extragenic palindromic sequences, messenger RNA stability and gene expression, evolution by gene conversion, a review. Gene. 72, 3-14.

Hilton, A.C. and Penn, C.W. 1998. Comparison of ribotyping and arbitrarily primed PCR for molecular typing of *Salmonella enterica* and relationships between strains on the basis of these molecular markers. Journal of Applied Microbiology 85, 933-940.

Hinnebusch, J. and Tilly, K. 1993. Linear plasmids and chromosomes in bacteria. Molecular Microbiology. 10, 917-922.

Hori, H. and Osawa, S. 1979. Evolutionary change in 5S RNA secondary structure and a phylogenic tree of 54 5S RNA species. Proceedings of the National Academy of Sciences of the United States of America. 76, 381-385.

Honeycutt, R.J., Sobral, B.W.S., and McClelland, M. 1995. tRNA intergenic spacers reveal polymorphisms diagnostic for *Xanthomonas albilineans*. Microbiology 141, 3229-3239.

Hulton, C.S.J., Higgins, C.F., and Sharp, P.M. 1991. ERIC sequences - a novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria. Molecular Microbiology 5, 825-834.

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M., Kersters, K. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology 142, 1881-1893.

Jensen, M., Webster, J., and Straus, N. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction amplified ribosomal DNA spacer polymorphisms. Applied and Environmental Microbiology 59, 945-952.

Jersek, B., Gilot, P., Gubina, M., Klun, N., Mehle, J., Tcherneva, E., Rijpens, N., and Herman, L. 1999. Typing of *Listeria monocytogenes* strains by repetitive element sequence based-PCR. Journal of Clinical Microbiology 37, 103-109.

Ji, K.E. and Kempsell, K.E. 1994. Nucleotide sequences of the spacer 1, spacer 2 and trailer regions of the rrn operons and secondary structures of precursor 23S ribosomal RNA and precursor 5S ribosomal RNAs of slow growing mycobacteria. Microbiology 140, 1763-1773.

Johansson, M.L., Quednau, M., Molin, G., Ahrne, S. 1995. Randomly Amplified Polymorphic DNA (RAPD) for rapid typing of *Lactobacillus plantarum* Strains. Letters in Applied Microbiology 21, 155-159.

Jonsdottir, H., Malmquist, H.J., Snorrason, S.S., Gudbergsson, G., and Gudmundsdottir, S. 1998. Epidemiology of *Renibacterium salmoninarum* in wild Arctic charr and brown trout in Iceland. Journal of Fish Biology 53, 322-339.

Kaattari, S.L. and Piganelli, J.D. 1997. Immunisation with bacterial antigens: bacterial kidney disease. Karger, London.

Kariyama, R., Mitsuhata, R., Chow, J.W., Clewell, D.B., and Kumon, M.H. 2000. Simple and reliable multiplex PCR assay for surveillance of isolates of vancomycin resistant enterococci. Journal Of Clinical Microbiology 38, 3092-3095.

Kato, H., Kato, N., Watanabe, H., Ueno, K., Sakata, Y., and Fujita, K. 1996. Relapses or reinfections: analysis of a case of *Clostridium difficile* associated colitis by two typing systems. Current Microbiology 33, 220-223.

Kaufmann, M.E. and Pitt, T. 1994. Pulsed field gel electrophoresis of bacterial DNA. In: Methods in Practical Laboratory Bacteriology, Edited by Chart, H. pp. 83-92. CRC Press, London.

Kent, M.L., Traxler, G.S., Kieser, D., Richard, J., Dawe, S.C., Shaw, R.W., Prosperi-Porta, G., Ketcheson, J., and Evelyn, T.P.T. 1998. Survey of salmonid pathogens in ocean caught fishes in British Columbia, Canada. Journal of Aquatic Animal Health 10, 211-219.

Kersulyte, D., Levine, R.P., and Berg, D.E. 1994. Unpublished work as cited in: Berg, D. *et al* (1994). Fingerprinting microbial genomes using the RAPD or AP-PCR method. Methods in Molecular and Cellular Biology, 5: 13-24.

Kimura, T. and Awakura, T. 1977. Bacterial kidney disease of salmonids: first observation in Japan. Bulletin of the Japanese Society of Scientific Fisheries 43, 143-150.

Kimura, T., Ezura, Y., Tajima, K., and Yoshimizu, M. 1978. Serological diagnosis of bacterial kidney disease of salmonids (BKD): immunodiffusion test by heat stable antigen extracted from infected kidney. Fish Pathology 13, 103-108.

Koch, C., Rainey, F.A., and Stackebrandt, E. 1994. 16S rDNA studies on members of *Arthrobacter* and *Micrococcus* - an aid for their future taxonomic restructuring. FEMS Microbiology Letters 123, 167-171.

Kolsto, A.B. 1990. Physical map of the *Bacillus cereus* chromosome. Journal of Bacteriology 172, 3821-3825.

Kolsto, A.B. 1997. Dynamic bacterial genome organization. Molecular Microbiology 24, 241-248.

Koeuth, T., Versalovic, J., and Lupski, J.R. 1995. Differential subsequence conservation of interspersed repetitive *Streptococcus pneumoniae* Box elements in diverse bacteria. Genome Research 5, 408-418.

Krawiec, S. and Riley, M. 1990. Organisation of the bacterial chromosome. Microbiological Reviews 54, 502-539.

Kunze, Z.M., Wall, S., AppelBerg, R., Silva, M.T., Portaels, F., and McFadden, J.J. 1991. IS*901*, a new member of a widespread class of atypical insertion sequence, is associated with pathogenicity in *Mycobacterium avium*. Molecular Microbiology 5, 2265-2272.

Kusser, W. and Fiedler, F. 1983. Murein type and polysaccharide composition of cell walls from *Renibacterium salmoninarum*. FEMS Microbiology Letters 20, 391-394.

Leblond-Bourget, N., Philippe, H., Mangin, I., and Decaris, B. 1996. 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bididobacterium* phylogeny. International Journal of Systematic Bacteriology 46, 102-111.

Leblond, P., Demuyter, P., Simonet, J.M., and Decaris, B. 1991. Genetic instability and associated genome plasticity in *Streptomyces ambofaciens* pulsed field gel electrophoresis evidence for large DNA alterations in a limited genomic region. Journal of Bacteriology. 173, 4229-4233.

Leon, G., Martinez, M. A., Etchegaray, J.P., Vera, M.I., Figueroa, J., and Krauskopf, M. 1994a. Specific DNA probes for the identification of the fish pathogen, *Renibacterium salmoninarum*. World Journal of Microbiology and Biotechnology 10, 149-153.

Leon, G., Maulen, N., Figueroa, J., Villanueva, J., Rodriguez, C., Vera, M.I., and Krauskopf, M. 1994b. A PCR based assay for the identification of the fish pathogen *Renibacterium salmoninarum*. FEMS Microbiology Letters 115, 131-136.

Lezhava, A., Mizukami, T., Kajitani, T., Kameoka, D., Redenbach, M., Shinkawa, H., Nimi, O., and Kinashi, H. 1995. Physical map of the linear chromosome of *Streptomyces griseus*. Journal of Bacteriology 177, 6492-6498.

Lin, Y.S., Kieser, H.M., Hopwood, D.A., and Chen, C. 1993. The chromosomal DNA of *Streptomyces lividans* is linear. Molecular Microbiology 10, 923-933.

Ling, M.L., Goh, K.T., Wang, G., Neo, K.S., and Chua, A. 2002. An outbreak of multidrug-resistant *Salmonella enterica* subsp *enterica* serotype typhimurium, DT104L linked to dried anchovy in Singapore. Epidemiology and Infection 128, 1-5.

Linton, C.J., Jalal, H., Leeming, J.P., and Millar, M.R. 1994. Rapid discrimination of *Mycobacterium tuberculosis* strains by random amplified polymorphic DNA analysis. Journal of Clinical Microbiology 32, 2169-2174.

LiPuma, J.J. 1998. Molecular tools for epidemiologic study of infectious diseases. Pediatric Infectious Disease Journal 17, 667-675.

Lobato, M.J., Landeras, E., Gonzalez Hevia, M.A., and Mendoza, M.C. 1998. Genetic heterogeneity of clinical strains of *Yersinia enterocolitica* traced by ribotyping and relationships between ribotypes, serotypes and biotypes. Journal of Clinical Microbiology 36, 3297-3302.

Loutit, J.S. and Tompkins, L.S. 1991. Restriction enzyme and Southern hybridisation analyses of *Pseudomonas aeruginosa* strains from patients with cystic fibrosis. Journal of Clinical Microbiology 29, 2897-2900.

Lowe, T.M. and Eddy, S.R. 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Research 25, 955-964.

Luna, V.A., Jernigan, D.B., Tice, A., Kellner, J.D., and Roberts, M.C. 2000. A novel multiresistant *Streptococcus pneumoniae* serogroup 19 clone from Washington state identified by pulsed field gel electrophoresis and restriction fragment length patterns. Journal of Clinical Microbiology 38, 1575-1580.

Ludwig, W. and Schleifer, H. 2001. Bacterial phylogeny based on 16S and 23S sequence analysis. FEMS Microbiology Reviews 15, 155-173.

Mackie, T.J., Arkwright, J.A., Pryce-Tannatt, T.E., Mottram, J., Johnson, W.D., Menzies, W.J.M., and Martin, W. 1933. Second interim report of the furunculosis committee. 1-5.

Maes, N., DeGheldre, Y., DeRyck, R., Vaneechoutte, M., Meugnier, H., Etienne, J., and Struelens, M.J. 1997. Rapid and accurate identification of *Staphylococcus* species by tRNA intergenic spacer length polymorphism analysis. Journal of Clinical Microbiology 35, 2477-2481.

Magnusson, H.B., Fridjonsson, O.H., Andresson, O.S., Benediktsdottir, E., Gudmundsdottir, S., and Andresdottir, V. 1994. *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in salmonid fish, detected by nested reverse transcription PCR of 16S ribosomal RNA sequences. Applied and Environmental Microbiology 60, 4580-4583.

Mahillon, J. and Chandler, M. 1998. Insertion sequences. Microbiology and Molecular Biology Reviews 62, 725-774.

Mahony, J.B., Luinstra, K.E., Tyndall, M., Sellors, J.W., Krepel, J., and Chernesky, M. 1995. Multiplex PCR for detection of *Chlamydia trachomatis* and *Nesseria gonorrhoeae* in genitourinary specimens. Journal of Clinical Microbiology 33, 3049-3053.

Maiden, M.C., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J.J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M., and Spratt, B.G. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proceedings of the National Academy of Sciences of the United States of America 95, 3140-3145.

Maiden, M.C.J. 1993. Population genetics of a transformable bacterium: the influence of horizontal genetic exchange on the biology of *Neisseria meningitidis*. FEMS Microbiology Letters 112, 243-250.

Malathum, K., Singh, K., Weinstock, G., and Murray, B. 1998. Repetitive sequence-based PCR versus pulsed field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. Journal of Clinical Microbiology 36, 211-215.

Martin-Didonet, C.G., Chubatsu, L.S., Souza, E.M., Kleina, M., Rego, F.G.M., Rigo, L.U., Yates, M.G, and Pedrosa, F.O. 2000. Genome structure of the genus *Azospirillum*. Journal of Clinical Microbiology 182, 4113-4116.

Martinez-Murcia, A.J., Anton, A.I., and Rodriguez-Valera, F. 1999. Patterns of sequence variation in two regions of the 16S rRNA multi gene family of *Escherichia coli.* International Journal of Systematic and Evolutionary Microbiology 49, 601-610.

Maslow, J. and Mulligan, M.E. 1996. Epidemiologic typing systems. Infection Control and Hospital Epidemiology 17, 595-604.

Matsuoka, M., Maeda, S., Kai, M., Nakata, N., Chae, G.T., Gillis, T.P., Kobayashi, K., Izumi, S., and Kashiwabara, Y. 2000. *Mycobacterium leprae* typing genomic diversity and global distribution of genotypes. International Journal of Leprosy and other Mycobacterium Diseases 68, 121-128.

Mattsson, J.G., Gersdorf, H., Jansson, E., Hongslo, T., Göbel, U.B., and Johansson, K.E. 1993. Rapid identification of *Renibacterium salmoninarum* using an oligonucleotide probe complementary to 16S ribosomal RNA. Molecular and Cellular Probes 7, 25-33.

Maule, J. 1998. Pulse-field gel electrophoresis. Molecular Biotechnology 9, 107-126.

Maulen, N.P., Morales, P.J., Aruti, D., Figueroa, J.E., Concha, M.I., Krauskopf, M., and Leon, G. 1996. Identification of a *Renibacterium salmoninarum* DNA fragment associated with bacterial internalisation into CHSE cultured cells. FEMS Microbiology Letters 135, 37-43.

Mazur, C.F., Tillapaugh, D., and Iwama, G.K. 1993. The effects of feeding level and rearing density on the prevalence of *Renibacterium salmoninarum* in chinook salmon (*Oncorhynchus tshawytscha*) reared in salt-water. Aquaculture 117, 141-147.

McClelland, M., Petersen, C., and Welsh, J. 1992. Length polymorphisms in transfer RNA intergenic spacers detected by using the polymerase chain reaction can distinguish streptococcal strains and species. Journal of Clinical Microbiology 30, 1499-1504.

McClelland, M., Jones, R., Patel, Y., and Nelson, M. 1994. Restriction endonucleases for pulsed field mapping of bacterial genomes. Nucleic Acid Research. 15, 5985-6005.

McIntosh, D., Flaño, E., Grayson, T.H., Gilpin, M.L., Austin, B., and Villena, A.J. 1997. Production of putative virulence factors by *Renibacterium salmoninarum* grown in cell culture. Microbiology 143, 3349-3356.

McIntosh, D., Meaden, P.G., and Austin, B. 1996. A simplified PCR-based method for the detection of *Renibacterium salmoninarum* utilising preparations of rainbow trout (*Oncorhynchus mykiss*, Walbaum) lymphocytes. Applied and Environmental Microbiology 62[11], 3929-3932.

Meunier, J.R. and Grimont, P.A.D. 1993. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. Research in Microbiology 144, 373-379.

Mesa, M.G., Maule, A.G., Poe, T.P., and Schreck, C.B. 1999. Influence of bacterial kidney disease on smoltification in salmonids: is it a case of double jeopardy? Aquaculture 174, 25-41.

Mesa, M.G., Maule, A.G., and Schreck, C.B. 2000. Interaction of infection with *Renibacterium salmoninarum* and physical stress in juvenile chinook salmon: physiological responses, disease progression, and mortality. Transactions of the American Fisheries Society 129, 158-173.

Mesa, M.G., Poe, T.P., Maule, A.G., and Schreck, C.B. 1998. Vulnerability to predation and physiological stress responses in juvenile chinook salmon (*Oncorhynchus tshawytscha*) experimentally infected with *Renibacterium salmoninarum*. Canadian Journal of Fisheries and Aquatic Sciences 55, 1599-1606.

Meyers, T.R., Short, S., Farrington, C., Lipson, K., Geiger, H.J., and Gates, R. 1993. Comparison of the enzyme linked immunosorbent assay (ELISA) and the fluorescent antibody test (FAT) for measuring the prevalence and levels of *Renibacterium salmoninarum* in wild and hatchery stocks of salmonid fishes in Alaska, USA. Diseases of Aquatic Organisms 16, 181-189.

Miller, M. P. 1997. Tools for population genetic analysis (TFPGA) 3.1. A Windows computer program for the analysis of allozyme and molecular population genetic data. Software distributed by author: http://bioweb.usu.edu/mpmbio/tfpga.htm.

Miriam, A., Griffiths, S.G., Lovely, J.E., and Lynch, W.H. 1997. PCR and probe PCR assays to monitor broodstock Atlantic salmon (*Salmo Salar* L.) ovarian fluid and kidney tissue for presence of DNA of the fish pathogen *Renibacterium salmoninarum*. Journal of Clinical Microbiology 35, 1322-1326.

Mitchum, D.L. and Sherman, L.E. 1981. Transmission of bacterial kidney disease from wild to stocked hatchery trout. Canadian Journal of Fisheries and Aquatic Sciences 38, 547-551.

Mitchum, D.L., Sherman, L.E., and Baxter, G.T. 1979. Bacterial kidney disease in feral populations of brook trout (*Salveinus fontinalis*), brown trout (*Salmo trutta*), and rainbow trout (*Salmo gairdneri*). Journal of Fisheries Research Board of Canada 36, 1370-1376.

Moffitt, C.M. and Kiryu, Y. 1999. Toxicity, teratogenesis, and efficacy of injectable erythromycin (Erythro-200) administered repeatedly to adult spring chinook salmon. Journal of Aquatic Animal Health 11, 1-9.

Moss, J.E., Cardozo, T.J., Zychlinsky, A., and Groisman, E.A. 1999. The *sel*C-associated SHI-2 pathogencitiy island of *Shigella flexneri*. Molecular Microbiology 33, 74-83.

Navarrow, E., Simonet, P., Normand, P., and Bardin, R. 1992. Characterisation of natural populations of *Nitrobacter* spp. using PCR/RFLP analysis of the ribosomal intergenic spacer. Archives of Microbiology 157, 107-115.

Nei, M. 1972. Genetic distance between populations. American Naturalist 106, 283-292.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89, 583-590.

Newbury, S.F. and Higgins, C.F. 1987. Stabilisation of messenger RNA by prokaryotic REP sequences and their effect on gene expression. Journal of Cellular Biochemistry, 56-56 Suppl. 11C

Newbury, S.F., Smith, N.H., Robinson, E.C., Hiles, I.D., and Higgins, C.F. 1987. Stabilisation of translationally active messenger RNA by prokaryotic REP sequences. Cell 48, 297-310.

Nigatu, A., Ahrne, S., and Molin, G. 2001. Randomly amplified polymorphic DNA (RAPD) profiles for the distinction of *Lactobacillus* species. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 79, 1-6.

Nishikawa, Y., Hase, A., Ogasawara, K., Cheasty, T., Willshaw, G.A., Smith, H.R., Tatsumi, Y., and Yasukawa, A. 2001. Phage typing and DNA based comparison of strains of enterohemorrhagic *Escherichia coli* 0157 from apparently sporadic infection in Osaka City, Japan, 1996. Japanese Journal of Infectious Diseases 54, 140-143.

Nour, M. 1998. 16S-23S and 23S-5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. Research in Microbiology 149, 433-448.

Norton, R., Roberts, B., Freeman, M., Wilson, M., Ashhurst-Smith, C., Lock, W., Brookes, D., and La Brooy, J. 1998. Characterisation and molecular typing of *Burkholderia pseudomallei*: are disease presentations of melioidosis clonally related? FEMS Immunology and Medical Microbiology 20, 37-44.

O'Farrell, C.L. and Strom, M.S. 1999. Differential expression of the virulence associated protein p57 and characterisation of its duplicated gene *msa* in virulent and attenuated strains of *Renibacterium salmoninarum*. Diseases of Aquatic Organisms 38, 115-123.

Oakey, H., Gibson, L.F., and George, A.M. 1998. Co-migration of RAPD-PCR amplicons from *Aeromonas hydrophila*. FEMS microbiological Letters 164, 35-38.

Olive, D.M. and Bean, P. 1999. Principles and applications of methods for DNA based typing of microbial organisms. Journal of Clinical Microbiology 37, 1661-1669.

Olsen, A.B., Hopp, P., Binde, M., and Gronstol, H. 1992. Practical aspects of bacterial culture for the diagnosis of bacterial kidney disease (BKD). Diseases of Aquatic Organisms 14, 207-212.

Olsen, G.J., Larsen, N., and Woese, C. 1991. The ribosomal RNA database project. Nucleic Acids Research, 19(suppl.), 2017-2021.

Ordal, E.J. and Earp, B.J. 1956. Cultivation and transmission of etiological agent of kidney disease in salmonid fishes. Proceedings of the Society for Experimental Biology and Medicine. 92, 85-88.

Park, Y., Yim, D., Kim, E., Kho, Y., Mheen, T., Lonsdale, J., and Goodfellow, M. 1991. Classification of acidophilic, neutrotolerant and neutrophilic streptomycetes by nucleotide sequencing of 5S ribosomal RNA. Journal of General Microbiology 137, 2265-2269.

Pascho, R.J., Chase, D., and McKibben, C.L. 1998. Comparison of the membrane filtration fluorescent antibody test, the enzyme-linked immunosorbent assay, and the polymerase chain reaction to detect *Renibacterium salmoninarum* in salmonid ovarian fluid. Journal of Veterinary Diagnostic Investigation 10, 60-66.

Pascho, R.G., Elliott, D.G., and Achord, S. 1993. Monitoring of in-river migration of smolts from h1two groups of spring chinook salmon *Oncorhynchus tshawytscha* (Walbaum) with different profiles of *Renibacterium salmoninarum* infection. Aquatic and Fisheries Management 24, 157-163.

Pascho, R.J., Elliott, D.G., Mallett, R.W., and Mulcahy, D. 1987. Comparison of 5 techniques for the detection of *Renibacterium salmoninarum* in adult coho salmon. Transactions of the American Fisheries Society 116, 882-890.

Pascho, R.J., Landolt, M.L., and Ongerth, J.E. 1995. Inactivation of *Renibacterium salmoninarum* by free chlorine. Aquaculture 131, 165-175.

Pascho, R.J. and Mulcahy, D. 1987. Enzyme linked immunosorbent assay for a soluble antigen of *Renibacterium salmoninarum*, the causative agent of salmonid bacterial kidney disease. Canadian Journal of Fisheries and Aquatic Sciences 44, 183-191.

Paterson, W.D., Lall, S.P, Airdrie, D., Greer, P., Greenham, G., and Poy, M. 1985. Prevention of disease in salmonids by vaccination and dietary modification. Fish Pathology 20, 427-434.

Pestel-Caron, M., Graff, G., Berthelot, G., Pons, J.L., Lemeland,J.F. 1999. Molecular analysis of *Mycobacterium avium* isolates by using pulsed-field gel electrophoresis and PCR. Journal of Clinical Microbiology 37, 2450-455.

Peters, K.K. and Moffitt, C.M. 1996. Optimal dosage of erythromycin thiocyanate in a new feed additive to control bacterial kidney disease. Journal of Aquatic Animal Health 8, 229-240.

Picardeau, M., Varnerot, A., Lecompte, T., Brel, F., May, T., and Vincent, V. 1997. Use of different molecular typing techniques for bacteriological follow-up in a clinical trial with AIDS patients with *Mycobacterium avium* bacteremia. Journal of Clinical Microbiology 35, 2503-2510.

Pirhonen, J., Schreck, C.B., and Gannam, A. 2000. Appetite of chinook salmon (*Oncorhynchus tshawytscha*) naturally infected with bacterial kidney disease. Aquaculture 189, 1-10.

Pisabarro, A., Correia, A., and Martin, J.F. 1998. Pulsed field gel electrophoresis analysis of the genome of *Rhodococcus fascians*: genome size and linear and circular replicon composition in virulent and avirulent strains. Current Microbiology 36, 302-308.

Pooler, M.R., Ritchie, D.F., and Hartung, J.S. 1996. Genetic relationships among strains of *Xanthomonas fragariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR, and enterobacterial repetitive intergenic consensus PCR data and generation of multiplexed PCR primers useful for the identification of this phytopathogen. Applied and Environmental Microbiology 62, 3121-3127.

Poussier, S., Trigalet-Demery, D., Vandewalle, P., Goffinet, B., Luisettis, J., and Trigalet, A. 2000. Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the *hrp* gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision. Microbiology 146, 1679-1692.

Power, E.G. 1996. RAPD typing in microbiology - a technical review. Journal of Hospital Infection 34, 247-265.

Ramasoota, P., Chansiriponchai, N., Kallenius, G., Hoffner, S.E., and Stevenson, S.B. 2001. Comparison of *Mycobacterium avium* complex (MAC) strains from pigs and humans in Sweden by random amplified polymorphic DNA (RAPD) using standardised reagents. Veterinary Microbiology 78, 251-259.

Reeves, A.R., Post, D.A., and Vanden Boom, T.J. 1998. Physical genetic map of the erythromycin producing organsim *Saccharopolyspora erythraea*. Microbiology 144, 2151-2159.

Renesto, P., Gouvernet, J., Drancourt, M., Roux, V., and Raoult, D. 2001. Use of *rpo*B gene analysis for detection and identification of *Bartonella* species. Journal of Clinical Microbiology 39, 430-437.

Rhodes, L.D., Grayson, T.H., Alexander, S.M., and Strom, M.S. 2000. Description and characterization of IS*994*, a putative IS3 family insertion sequence from the salmon pathogen, *Renibacterium salmoninarum*. Gene 244, 97-107.

Rhodes, L.D., Nilsson, W.B., and Strom, M.S. 1998. Sensitive detection of *Renibacterium salmoninarum* in whole fry, blood, and other tissues of Pacific salmon by reverse transcription polymerase chain reaction. Molecular Marine Biology and Biotechnology 7, 270-279.

Rhodes, L.D. and Strom, M.S. 1998. Identification and characterisation of a transposable element in the fish pathogen *Renibacterium salmoninarum*. Poster Session for 1998 International Symposium on Aquatic Animal Health.

Rivera, I., Chowdhury, A., Huq, A., Jacobs, D., Martins, M.T., and Colwell, R.R. 1995. Enterobacterial repetitive intergenic consensus sequences and the PCR to generate fingerprints of genomic DNAs from *Vibrio cholerae* 01, 0139, and non-01 strains. Applied and Environmental Microbiology 61, 2898-2904.

Rockey, D.D., Gilkey, L.L., Wiens, G.D., and Kaattari, S.L. 1991a. Monoclonal antibody based analysis of the *Renibacterium salmoninarum* p57 protein in spawning chinook and coho salmon. Journal of Aquatic Animal Health 3, 23-30.

Rockey, D.D., Turaga, P.S.D., Wiens, G.D., Cook, B.A., and Kaattari, S.L. 1991b. Serine proteinase of *Renibacterium salmoninarum* digests a major autologous extracellular and letedCcell surface protein. Canadian Journal of Microbiology 37, 758-763.

Rodriguez, J.M. 1997. Detection of animal pathogens by using the polymerase chain reaction (PCR). Veterinary Journal 153, 287-305.

Roller, C., Ludwig, W., and Schleifer, H. 1992. Gram positive bacteria with a high DNA G+C content are characterised by a common insertion within their 23S rRNA genes. Journal of General Microbiology 138, 1167-1175.

Romling, U., Fislage, R., and Tummler, B. 1996. Macroresriction mapping and analysis of bacterial genomes. In: Non mammalian genomic analysis: a practical guide, edited by Birren, B. & Lai, E. pp. 165-179. Academic Press, San Diego.

Ruiz-Ordaz, N., Hernandez, M., Ruiz-Lagunez, J.C., Cristiani-Urbina, E., and Galindez-Mayer, J. 1998. Growth kinetic model that describes the inhibitory and lytic effects of phenol on *Candida tropicalis* yeast. Biotechnology Progress 14, 966-969.

Rupnik, M., Avesani, V., Janc, M., von Eichel-Streiber, C., and Delmee, M. 1998. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. Journal of Clinical Microbiology 36, 2240-2247.

Rydkina, E., Roux, V., and Raoult, D. 1999. Determination of the genome size of *Ehrlichia* spp., using pulsed field gel electrophoresis. FEMS Microbiology Letters 176, 73-78.

Sadowsky, M., Kinkel, L., Bowers, J., and Schottel, J. 1996. Use of repetitive intergenic DNA sequences to classify pathogenic and disease suppressive *Streptomyces* strains. Applied and Environmental Microbiology 62, 3489-3493.

Sakai, M., Atsuta, S., and Kobayashi, M. 1992. Detection of *Renibacterium salmoninarum* antigen in migrating adult chum salmon (*Oncorhynchus keta*) in Japan. Journal of Wildlife Diseases 28, 110-112.

Sakai, M. and Kobayashi, M. 1992. Detection of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in salmonid fish, from pen cultured coho salmon. Applied and Environmental Microbiology 58, 1061-1063.

Sakai, M., Koyama, G., Atsuta, S., and Kobayashi, M. 1987. Detection of *Renibacterium salmoninarum* by a modified peroxidase antiperoxidase (PAP) procedure. Fish Pathology 22, 1-5.

Sakai, M., Ogasawara, K., Atsuta, S., and Kobayashi, M. 1989. Comparative sensitivity of carp, *Cyprinus carpio* L. and rainbow trout, *Salmo gairdneri* Richardson, to *Renibacterium salmoninarum.* Journal of Fish Diseases 12, 367-372.

Sakai, M., Soliman, M.K., Yoshida, T., and Kobayashi, M. 1993. Identification of pathogenic fish bacteria using the API ZYM system. Canadian Journal of Fisheries and Aquatic Sciences 50, 1137-1141.

Sanders, J.E. and Fryer, J.L. 1980. *Renibacterium salmoninarum* gen. nov., sp. nov., the causative agent of bacterial kidney disease in salmonid fishes. International Journal of Systematic Bacteriology 30, 496-502.

Sanders, J.E., Long, J.J., Arakawa, C.K., Bartholomew, J.L., and Rohovec, J.S. 1992. Prevalence of *Renibacterium salmoninarum* among downstream migrating salmonids in the Columbia river. Journal of Aquatic Animal Health 4, 72-75.

Schad, P. 1995. Preparation and restriction digestion of *Escherichia coli* chromsomal DNA in agarose plugs for use in PFGE. BioRad Bulletin.

Schwartz, D.C. and Cantor, C.R. 1984. Separation of yeast chromosome size DNAs by pulsed field gradient gel electrophoresis. Cell 37, 67-75.

Seal, S.E., Jackson, L.A., and Daniels, M.J. 1992. Use of transfer RNA consensus primers to indicate subgroups of *Pseudomonas solanacearum* by polymerase chain reaction amplification. Applied and Environmental Microbiology 58, 3759-3761.

Sechi, L.A., Zanetti, S., Dupre, I., and Delogu, G. 1998. Enterobacterial repetitive intergenic consensus sequences as molecular targets for typing of *Mycobacterium tuberculosis* strains. Journal of Clinical Microbiology 36, 128-132.

Sela, S., Clark-Curtiss, J.E., and Bercovier, H. 1989. Characterisation and taxonomic implications of the rRNA genes of *Mycobacterium leprae*. Journal of Bacteriology 171, 70-73.

Senson, P.R. and Stevenson, R.M. W. 1999. Production of the 57 kDa major surface antigen by a non agglutinating strain of the fish pathogen *Renibacterium salmoninarum*. Diseases of Aquatic Organisms 38, 23-31.

Shyamala, V, Schneider, E., and Ames, G.F.L. 1990. Tandem chromosomal duplications - role of REP sequences in the recombination event at the join point. EMBO Journal 9, 939-946.

Slutsky, A.M., Arbeit, R.D., Barber, T.W., Rich, J., Vonreyn, C.F., Pieciak, W., Barlow, M.A., and Maslow, J.N. 1994. Polyclonal infections due to *Mycobacterium avium* complex in patients with AIDS detected by pulsed field gel electrophoresis of sequential clinical isolates. Journal of Clinical Microbiology 32, 1773-1778.

Smith, C. and Cantor, C.R. 1987. Purification, specific fragmentation and separation of large DNA molecules. Methods in Enzymology 155, 449-476.

Smith, I.W. 1964. The occurrence and pathology of Dee disease. Freshwater Salmon Reseach 34,1-12.

Sneath, P.H.A. and Sokal, R.R. 1973. Numerical taxonomy: the principles and practice of numerical classification. W.H. Freeman, San Francisco. California.

Snieszko, S.F. and Griffin, P.J. 1955. Kidney disease in brook trout and its treatment. Progressive Fish Culturist 17, 3-13.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 98, 503-517.

Spratt, B.G. 1999. Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the Internet. Current Opinion in Microbiology 2, 312-316.

Sreevatsan, S., Pan, X., Stockbauer, K.E., Connel, N.D., Kreiswirth, B.N., Whittam, T.S., and Musser, J.M. 1997. Restricted structural gene polymorphsim in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proceedings of the National Academy of Sciences of the United States of America. 94, 9869-9874.

Stackebrandt, E., Koch, C., Gvozdiak, O., and Schumann, P. 1995. Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *dermacoccus* gen. nov., and Micrococcus Cohn 1872 gen. emend. International Journal of Systematic Bacteriology 45, 682-692.

Stackebrandt, E. and Schumann, P. 2000. Description of *Bogoriellaceae* fam. nov., *Dermacoccaceae* fam. nov., *Rarobacteraceae* fam. nov and *Sanguibacteraceae* fam. nov and emendation of some families of the suborder *Micrococcineae*. International Journal of Systematic and Evolutionary Microbiology 50, 1279-1285.

Stackebrandt, E., Wehmeyer, U., Nader, H., and Fiedler, F. 1988. Phylogenetic relationship of the fish pathogenic *Renibacterium salmoninarum* to *Arthrobacter*, *Micrococcus* and related taxa. FEMS Microbiology Letters 50, 117-120.

Stanley, J. and Saunders, N. 1996. DNA insertion sequences and the molecular epidemiology of *Salmonella* and *Mycobacterium*. Journal of Medical Microbiology 45, 236-251.

Starliper, C.E. 1996. Genetic diversity of North American isolates of *Renibacterium salmoninarum*. Diseases of Aquatic Organisms 27, 207-213.

Starliper, C.E. and Morrison, P. 2000. Bacterial pathogen contagion studies among freshwater bivalves and salmonid fishes. Journal of Shellfish Research 19, 251-258.

Starliper, C.E., Schill, W.B., and Mathias, J. 1998. Performance of serum free broth media for growth of *Renibacterium salmoninarum*. Diseases of Aquatic Organisms 34, 21-26.

Starliper, C.E., Smith, D.R., and Shatzer, T. 1997. Virulence of *Renibacterium salmoninarum* to salmonids. Journal of Aquatic Animal Health 9, 1-7.

Stern, M.J., Ames, G.F.L., Smith, N.H., Robinson, E.C., and Higgins, C.F. 1984. Repetitive extragenic palindromic sequences - a major component of the bacterial genome. Cell 37, 1015-1026.

Stern, M.J., Prossnitz, E., and Ames, G.F.L. 1988. Role of the intercistronic region in post transcriptional control of gene-expression in the histidine transport operon of *Salmonella typhimurium* - involvement of REP sequences. Molecular Microbiology 2, 141-152.

Struelens, M.J. 1998. Molecular epidemiological typing systems of bacterial pathogens: current issues and perpectives. Memorias do Instituto Oswaldo Cruz, Rio de Janeiro 93, 581-585.

Stull, T.L., LiPuma, J.J., and Edlind, T.D. 1988. A broad spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. Journal of Infectious Disease 157, 280-286.

Sun, L.V., Foster, J.M., Tzertzinis, G., Ono, M., Bandi, C., Slatko, B. E., and O'Neil, S. 2001. Determination of *Wolbachia* genome size by pulsed field gel electrophoresis. Journal of Bacteriology 183, 2219-2225.

Syzmanski, M., Specht, T., Barciszewska, M.Z., Barciszewski, J., and Erdmann, V.A. 1998. 5S rRNA data bank. Nucleic Acids Research 26, 156-159.

Tang, W.Y., Procrop, G.W., and Persing, D.H. 1997. Molecular diagnostics of infectious diseases. Clinical Chemistry 43, 2021-2038.

Tatusova, T.A. and Madden, T.L. 1999. BLAST 2 sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiology Letters 174, 247-250.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D., and Swaminathan, B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. Journal of Clinical Microbiology 33, 2233-2239.

Thal, L.A., Silverman, J., Donabedian, S., and Zervos, M.J. 1997. The effect of Tn916 insertions on contour clamped homogeneous electrophoresis patterns of *Enterococcus faecalis*. Journal of Clinical Microbiology 35, 969-972.

Thorarinsson, R., Landolt, M.L., Elliott, D.G., Pascho, R.J., and Hardy, R.W. 1994. Effect of dietary vitamin E and selenium on growth, survival and the prevalence of *Renibacterium salmoninarum* infection in chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture 121, 343-358.

Timmins, E.M., Howell, S.A., Alsberg, B.K., Noble, W.C., and Goodacre, R. 1998. Rapid differentiation of closely related *Candida* species and strains by pyrolysis mass spectrometry and Fourier transform infrared spectroscopy. Journal of Clinical Microbiology 36. 367-374.

Towner, K.J. and Cockayne, A. 1994. Molecular methods for microbial identification and typing. pp. 64-190. Chapman & Hall, London.

Toze, S. 1999. PCR and the detection of microbial pathogens in water and waste water. Water Research 33, 3545-3556.

Trakulsomboon, S., Dance, D.A.B., Smith, M.D., White, N.J., and Pitt, T.L. 1997. Ribotype differences between clinical and environmental isolates of *Burkholderia pseudomallei*. Journal of Medical Microbiology 46, 565-570.

Tran, C., Simonet, M., Nicolas, M., Offredo, C., Grimont, F., Lefevre, M., Ageron, E., Debure, A., and Grimont, P.A.D. 1991. Molecular typing of nosocomial isolates of *Legionella pneumophila* serogroup 3. Journal of Clinical Microbiology 28, 242-245.

Tsen, H.Y., Hu, H.H., Lin, J.S., Huang, C., and Wand, T.K. 2000. Analysis of the *Salmonella typhimurium* isolates a from food poisoning cases by molecular subtyping methods. Food Microbiology 17, 143-152.

Vaivelu, J., Puthucheary, S.D., Mifsud, A., Drasar, B.S., Dance, D.A.B. and Pitt, T.L. 1997. Ribotyping and DNA macrorestriction analysis of isolates of *Burkholderia pseudomallei* from cases of melioidosis in Malaysia. Transactions of the Royal Society of Tropical Medicine and Hygiene 91, 358-360.

van Belkum, A. 1999. The role of short sequence repeats in epidemiologic typing. Current Opinion in Microbiology 2, 306-311.

van Belkum, A., Hermans, P.W.M., Licciardello, L., Stefani, S., Grubb, W., van Leeuwen, W., and Goessens, W.H.F. 1998a. Polymerase chain reaction mediated typing of microorganisms: tracking dissemination of genes and genomes. Electrophoresis 19, 602-607.

van Belkum, A., Scherer, S., van Alphen, L., and Verbrugh, H. 1998b. Short sequence DNA repeats in prokaryotic genomes. Microbiology and Molecular Biology Reviews 62, 275-293.

van Belkum, A., van Leeuwen, W., Kaufmann, M.E., Cookson, B.C., Forey, F., Etienne, J., Goering, R.V., Tenover, F.C., Steward, C., O'Brain, F., Grubb, W., Tassios, P.T., Legakis, N.J., Morvan, A., El Solh, N., de Ryck, R., Struelens, M.J., Salmenlinna, S., Vuopio-varkila, J., Kooistra, M., Talens, A., Witte, W., and Verbrugh, H. 1998c. Assessment of resolution and intercenter reproducibility of results of genotyping *Staphylococcus aureus* by pulsed field gel electrophoresis of *Sma*I macrorestriction fragments: a multicenter study. Journal of Clinical Microbiology 36, 1653-1659.

van der Giessen, J.W.B., Haring, R.M., and van der Zeijst, B.A.M. 1994. Comparison of the 23S ribosomal RNA genes and the spacer region between the 16S and 23S ribosomal RNA genes of the closely related *Mycobacterium avium* and *Mycobacterium paratuberculosis* and the fast growing *Mycobacterium phlei.* Microbiology 140, 1103-1108.

van der Zee,A., Mooi, F., van Embden, J., and Musser, J. 1997. Molecular evolution and host adaptation of *Bordetella* spp.: phylogenetic analysis using multilocus enzyme

electrophoresis and typing with three insertion sequences. Journal of Bacteriology 179, 6609-6617.

Vaneechoutte, M., Boerlin, P., Tichy, H.V., Bannerman, E., Jager, B., and Bille, J. 1998. Comparison of PCR-based DNA fingerprinting techniques for the identification of *Listeria* species and their use for atypical *Listeria* isolates. International Journal of Systematic Bacteriology 48, 127-139.

Versalovic, J., Koeuth, T., Britton, R., Geszvain, K., and Lupski, J.R. 1993. Conservation and evolution of the *rpsU dnaG rpoD* macromolecular-synthesis operon in bacteria. Molecular Microbiology 8, 343-355.

Versalovic, J., Koeuth, T., and Lupski, J.R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Research 19, 6823-6831.

Vold, B. 1985. Structure and organisation of genes for transfer ribonucleic acid in *Bacillus subtilis*. Microbiological Reviews 49, 71-80.

Wain, J., Hien, T.T., Connerton, P., Ali, T., Parry, C.M., Chinh, N.T.T., Vinh, H., Phuong, C.X., Ho, V.A., Diep, T.S., Farrar, J.J., White, N.J., and Dougan, G. 1999. Molecular typing of multiple antibiotic resistant *Salmonella enterica* serovar typhi from Vietnam: application to acute and relapse cases of Typhoid Fever. Journal of Clinical Microbiology 37, 2466-2472.

Walker, R.A., Saunders, N., Lawson, A.J., Dassama, M., Ward, L.R., Woodward, M.J., Davies, R.H., Liebana, E., and Threlfall, E.J. 2001. Use of lightcycler *gyrA* mutation assay for rapid indentification of mutations conferring decreased susceptibility to ciprofloxacin in multiresistant *Salmonella enterica* serotype *typhimurium* DT104 isolates. Journal of Clinical Microbiology 39, 1443-1448.

Wang, G., van Dam, A.P., Spanjaarm, L., and Dankert, J. 1998. Molecular typing of *Borrelia burgdorferi* sensu lato by randomly amplified polymorphic DNA fingerprint analysis. Journal of Clinical Microbiology 36, 768-776.

Wang, Y., Zhang, Z., and Ramanan, N. 1997. The actinomycete *Termobispora bispora* contains two distinct types of transcriptionally active 16S rRNA genes. Journal of Bacteriology 79, 3270-3276.

Wei, H.L. and Chiou, C.S. 2002. Molecular subtyping of *Staphylococcus aureus* from an outbreak associated with a food handler. Epidemiology and Infection 128, 15-20.

Weisburg, W. 1989. Polyphyletic origin of bacteria. In: Intracellular parasitism, edited by J.W.Moulder, pp. 1-15. CRC Press, Boca Raton, F.L.

Welsh, J. and McClelland, M. 1992. PCR amplified length polymorphisms in transfer RNA intergenic spacers for categorising staphylococci. Molecular Microbiology 6, 1673-1680.

Welsh, J. and McClelland, M. 1991. Genomic fingerprints produced by PCR with consensus tRNA gene primers. Nucleic Acid Research, 19, 861-866.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18, 7213-7218.

Wiens, G.D. and Kaattari, S.L. 1991. Monoclonal antibody characterisation of a leukoagglutinin produced by *Renibacterium salmoninarum*. Infection and Immunity 59, 631-637.

Wiens, G.D. and Kaattari, S.L. 1989. Monoclonal-antibody analysis of common surface protein(s) of *Renibacterium salmoninarum*. Fish Pathology 24, 1-7.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. 1990. DNA polymorphisms eletedAamplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18, 6531-6535.

Withler, R.E. and Evelyn, T.P.T. 1990. Genetic variation in resistance to bacterial kidney disease within and between two strains of coho salmon from British- Columbia. Transactions of the American Fisheries Society 119, 1003-1009.

White, T., Madej, R., and Persing, D. 1992. The polymerase chain reaction: clinical applications. Advances in Clinical Chemistry 29, 161-196.

Woese, C. 1987. Bacterial evolution. Microbiological Reviews 51, 221-271.

Wolf, K. and Dunbar, C.E. 1959. Test of 34 therapeutic agents for control of kidney disease in trout. Transactions of the American Fisheries Society 88, 117-124.

Wood, J.W. and Wallis, J. 1955. Kidney disease in adult chinook salmon and its transmission by feeding to young chinook salmon. Res.Briefs, Fish.Commission of Oregon 6, 32-40.

Yakubu, D.E., Abadi, F., and Pennington, T.H. 1999. Molecular typing methods for *Neisseria meningitidis*. Journal of Medical Microbiology 48, 1055-1064.

Yap, W.H., Zhang, Z.S., and Wang, Y. 1999. Distinct types of rRNA operons exist in the genome of the actinomycete *Thermomonospora chromogena* and evidence for horizontal transfer of an entire rRNA operon. Journal of Bacteriology 181, 5201-5209.

Yoo, H.S., Lee, S.U., Park, K.Y., and Park, Y.H. 1997. Molecular typing and epidemiological survey of prevalence of *Clostridium perfringens* types by multiplex PCR. Journal of Clinical Microbiology 35, 228-232.

Yoon, J.H., Lee, S.T., Kim, S.B., Goodfellow, M., and Park, H.A. 1997. Inter- and intraspecific genetic analysis of the genus *Saccharomonospora* with 16S to 23S ribosomal DNA (rDNA) and 23S to 5S rDNA internally transcribed spacer sequences. International Journal of Systematic Bacteriology 47, 661-669.

Yoshimizu, M., Nomura, T., and Kimura, T. 1987. A false positive reaction in the indirect fluorescent antibody test for *Renibacterium salmoninarum* ATCC33209 caused by a *Pseudomonas sp.* Scientific Report of the Hokkaido Salmon Hatchery 41, 121-127.

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Variation in the Spacer Regions Separating tRNA Genes in *Renibacterium salmoninarum* Distinguishes Recent Clinical Isolates from the Same Location

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A means for distinguishing between clinical isolates of *Renibacterium salmoninarum* that is based on the PCR amplification of length polymorphisms in the tRNA intergenic spacer regions (tDNA-ILPs) was investigated. The method used primers specific to nucleotide sequences of *R. salmoninarum* tRNA genes and tRNA intergenic spacer regions that had been generated by using consensus tRNA gene primers. Twenty-one PCR products were sequenced from five isolates of *R. salmoninarum* from the United States, England, and Scotland, and four complete tRNA genes and spacer regions were identified. Sixteen specific PCR primers were designed and tested singly and in all possible pairwise combinations for their potential to discriminate between isolates from recent clinical outbreaks of bacterial kidney disease (BKD) in the United Kingdom. Fourteen of the isolates were cultured from kidney samples taken from fish displaying clinical signs of BKD on five farms, and some of the isolates came from the same farm and at the same time. The tDNA-ILP profiles separated 22 clinical isolates into nine groups and highlighted that some farms may have had more than one source of infection. The grouping of isolates improved on the discriminatory power of previously reported typing methods based on randomly amplified polymorphic DNA analysis and restriction fragment length profiles developed using insertion sequence IS*994*. Our method enabled us to make divisions between closely related clinical isolates of *R. salmoninarum* that have identical exact tandem repeat (ETR-A) loci, rRNA intergenic spacer sequences, and IS*994* profiles.

Renibacterium salmoninarum is an obligate pathogen and the causative agent of bacterial kidney disease (BKD), a chronic systemic infection of salmonid fish (15). The pathogen is a gram-positive bacterium that represents a genospecies placed within the high-G+C subgroup of the actinomycetes (4, 24, 27, 36). *R. salmoninarum* survives intracellularly and can be transmitted both vertically inside the ova and horizontally between cohabiting fish. Although BKD is geographically widespread and is responsible for significant losses in farmed and wild salmonids, knowledge of the epizootiology of the disease has been hampered due to a remarkable degree of uniformity among isolates of the pathogen (5, 19, 37).

We have examined the rRNA genes of *R. salmoninarum* for evidence of variation and have shown that the bacterium possesses two copies of the rRNA operon, which are identical or nearly identical and which are highly conserved among a wide variety of isolates (20). The spacer regions between the rRNA genes often vary in size and nucleotide sequence and can be useful for typing bacterial species (10, 18), but this is not the case in *R. salmoninarum*. Analysis of the 16S-23S rRNA intergenic spacer (ITS1) of *R. salmoninarum* has shown that the spacers of all isolates are identical in length, and, although four sequence variants (SV) have been described, most isolates from a wide variety of sources belong to a single sequevar, SV1 (21, 22). An exact tandem repeat locus, ETR-A, has been identified and has been shown to be a specific marker for SV1 isolates (21). The three remaining ITS1 sequence variants, which have from 1 to 3 base substitutions, are confined to isolates with temporal and spatial origins that set them apart from the mainstream of salmonid fisheries. Variation in the 23S-5S rRNA intergenic spacer (ITS2) was less obvious, and two sequence variants, SV21 and SV22, were identified (20).

Examining variation throughout the whole *R. salmoninarum* genome using randomly amplified polymorphic DNA (RAPD) and recently characterized insertion element IS*994* has provided up to 21 arbitrary groupings on the basis of banding patterns (21, 22, 34). Nevertheless, many isolates from obviously unrelated sources are still indistinguishable, and there is a need to identify more specific markers of variation that will facilitate a better understanding of the relationships between isolates that share the same spatial and temporal origins.

Use of length polymorphisms of the spacer regions that separate tRNA genes is a PCR-based strategy for exploring the degree of relatedness between bacteria. The arrangement of tRNA gene clusters in multiple tandem repeating units on the bacterial genome (23, 40) allows the amplification of intergenic length polymorphisms (tDNA-ILPs) by PCR employing consensus primers that are annealed at low stringency. Welsh and McClelland developed four "universal" tRNA gene primers designed to face outwards from the end of the tRNA genes, which have been shown to amplify a tDNA-ILP fingerprint that is determined by the arrangement of tRNA genes on the bacterial genome (42, 43). The order and arrangement of tRNA genes are highly conserved, and the fingerprints generated by the consensus primers are often characteristic of a particular

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species (12, 30), although in some cases consensus tRNA gene primers have been used to generate divisions below species level (7, 9, 35). Furthermore, specific tRNA gene primers can be developed from the DNA sequences of PCR products generated using consensus primers, and this approach was used to distinguish between streptococci on the basis of tRNA gene spacer length polymorphisms (31).

The tRNA genes and their flanking regions in a wide range of bacteria have been reported to be prone to disruption by mobile genetic elements including insertion sequences, tandem repeats, pathogenicity islands, prophage, and plasmids (6, 8, 11, 17, 32). An area of the genome prone to such a high degree of genetic change may have the potential to differentiate between isolates in a highly conserved organism such as *R. salmoninarum*. Here we report the development of a tool for epizootiological studies of *R. salmoninarum* based on tDNA-ILPs which uses PCR primers specific to tRNA genes and spacer sequences. We show that the method can discriminate between isolates of *R. salmoninarum* from the United Kingdom that possess identical ITS1 and ITS2 nucleotide sequences, IS*994* patterns, and ETR-A profiles.

MATERIALS AND METHODS

Bacterial isolates and DNA extraction. A total of 67 isolates of *R. salmoninarum* were used in this study, and their designation codes, countries of origin, and sources of isolation are listed in Tables 1 and 2. The isolates were cultured for 6 to 8 weeks in selective kidney disease medium supplemented with 5% spent culture broth at 15°C (3, 14) and then freeze-dried and checked for purity. The names and addresses of colleagues who have kindly provided isolates, derived from confirmed clinical outbreaks of BKD, have been previously published (22, 34). The isolates are maintained in a culture collection at the University of Plymouth, in collaboration with the Centre for Environmental, Fisheries and Aquacultural Sciences, Weymouth, United Kingdom.

DNA extraction and analysis. Genomic DNA was extracted and analyzed as described previously (22) using the Puregene D-6000 DNA isolation kit according to the manufacturer's instructions (Gentra Systems Inc.). The DNA concentration was determined for each isolate by Kodak digital imaging following agarose gel electrophoresis, and the identity of *R. salmoninarum* DNA was confirmed for all isolates by PCR using six sets of primers specific for *R. salmoninarum* genes *msa*, *hly*, and *rsh* as previously described (22).

Amplification of tRNA genes and intergenic spacer regions using consensus primers. PCRs were performed on DNA extracted from 60 isolates of *R. salmoninarum* (Table 1) using consensus tRNA gene primers T5A (5'-AGTCCGGT GCTCTAACCAACTGAG-3'), T5B (5'-AATGCTCTACCAACTGAACT-3'), T3A (5'-GGGGGTTCGAATTCCCGCCGGCCCCA-3'), and T3B (5'-AGGTC GCGGGTTCGAATCC-3') in each of the six possible paired combinations in accordance with the published protocols (42, 43). The 50-μl reaction mixtures consisted of 1 U of *Taq* polymerase and reaction buffer containing 1.5 mM $MgCl_2$ (Roche), 24 pmol of each primer (Genosys), 0.2 mM deoxynucleoside triphosphates, and 10 ng of bacterial DNA. PCR amplification was performed in a DNA thermal cycler (Perkin-Elmer). The reaction mixture was overlaid with mineral oil (Sigma), incubated at 94°C for 2 min, and then subjected to 44 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 90 s and a final cycle of 94°C for 30 s, 45°C for 30 s, and 72°C for 10 min. The amplification products were visualized after electrophoresis at 80 V in 1.2% agarose gels.

Cloning of tRNA genes and intergenic spacers from *R. salmoninarum*. Products which were generated in PCRs using consensus primers were purified using the Prep-a-Gene DNA purification kit (Bio-Rad), and were blunted, kinased, and ligated into pUC18 using the Sureclone kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Host *Escherichia coli* DH5α cells were transformed with the ligation mixture, and recombinants were recovered on Luria-Bertani agar containing 50 μg of ampicillin/ml with a 5-ml overlay containing 240 μg of IPTG (isopropyl-β-D-thiogalactopyranoside/ml and 250 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/ml.

DNA sequencing and analysis of cloned products. Plasmid DNA containing cloned inserts was isolated using the Quantum Prep plasmid miniprep kit (Bio-Rad) according to the manufacturer's instructions. Both strands of the cloned insert DNA were sequenced by MWG-Biotech Ltd. (Milton Keynes, United Kingdom). The DNA sequences obtained in this way were compared with sequences from other organisms obtained from the GenBank database (1). Pairwise and multiple alignments were performed using the BLAST 2 sequence program (38) available at GenBank and the GeneBee multiple-alignment program available at http://www.genebee.msu.su/services/malign_reduced.html. The tRNA genes were identified using the tRNA-scan program available at http://www.genetics.wustl.edu/eddy/tRNAscan-SE/ (16, 29).

TABLE 1. *R. salmoninarum* isolates which were used for the PCR amplification of tRNA genes and intergenic spacer regions using consensus tRNA primers

Isolate	Geographic origin and yr	Biological source[a]
980036-150	Wales, 1998	Rainbow trout (f)
980036-87	Wales, 1998	Rainbow trout (f)
970083-88	England, 1997	Rainbow trout (f)
970083-102	England, 1997	Rainbow trout (f)
980106#1.1.5	England, 1998	Rainbow trout (f)
970419-1.2.3	England, 1997	Atlantic salmon (w)
970153-19	England, 1997	Grayling (w)
A6	England, 1998	Rainbow trout (f)
A80	England, 1998	Rainbow trout (f)
MT409	Scotland, 1987	Unknown
MT417	Scotland, 1988	Atlantic salmon (f)
MT239	Scotland, 1986	Atlantic salmon (f)
MT426	Scotland, 1988	Unknown
NCIMB1111	Scotland, 1962	Atlantic salmon (w)[b]
NCIMB1112	Scotland, 1962	Atlantic salmon (w)[b]
NCIMB1113	Scotland, 1962	Atlantic salmon (w)[b]
NCIMB1114	Scotland, 1962	Atlantic salmon (w)
NCIMB1115	Scotland, 1962	Atlantic salmon (w)
NCIMB1116	Scotland, 1962	Atlantic salmon (w)
MT420	Scotland, 1988	Atlantic salmon (f)
MT452	Scotland, 1988	Rainbow trout (f)
MT1363	Scotland, 1993	Rainbow trout (f)
MT410	Scotland, 1987	Unknown
FT-10	Scotland	Atlantic salmon
DR143	Alberta, Canada, 1972	Brook trout (f)
DR384	British Columbia, Canada, 1979	Coho salmon (f)
960023	British Columbia, Canada, 1996	Coho salmon (f)
960046	British Columbia, Canada, 1996	Coho salmon (f)
980002	British Columbia, Canada, 1998	Chinook salmon (f)
RS-TSA	Nova Scotia, Canada	Atlantic salmon (f)
AcF6-1	Northwest Territories, Canada, 1985	Arctic char (w)
F-120-87(P-2)	Iceland, 1987	Atlantic salmon (f)
F-130-87(P-4)	Iceland, 1987	Rainbow trout (f)
F-138-87(O-78)	Iceland, 1987	Atlantic salmon (f)
F-273-87(P-19)	Iceland, 1987	Atlantic salmon (f)
F-283-87(P-10)	Iceland, 1987	Atlantic salmon (f)
F-358-87(P-13)	Iceland, 1987	Atlantic salmon (w)
S-182-90(P-7)	Iceland, 1990	Atlantic salmon (f)
Siletz	Oregon, 1976	Coho salmon (f)
Marion Forks	Oregon, 1987	Chinook salmon (f)
Little Goose	Washington, 1994	Chinook salmon (f)
CCM6205	Washington, 1975	Coho salmon (f)
84-019-OC	Washington, 1984	Chinook salmon (w)
SS-ChS-94-1	Oregon, 1994	Chinook salmon
Cow ChS94 P22	Washington, 1994	Chinook salmon (f)
Idaho 91-126	Idaho, 1993	Sockeye salmon (f)
RFL-643.94#1	Washington, 1994	Sockeye salmon (f)
CCM6206	Oregon, 1978	Chinook salmon (f)
Round Butte	Oregon, 1973	Chinook salmon (f)
NCIMB2235	Oregon, 1981	Chinook salmon (f)
BY1996	Alaska, 1998	Chinook salmon (f)
Rs 9	Sweden, 1985	Rainbow trout
Rs 19	Sweden, 1987	Atlantic salmon
Rs 61	Sweden, 1989	Arctic char
Rs 116	Sweden, 1993	Grayling
Rs 122	Sweden, 1994	Rainbow trout (f)
Rs 125	Sweden	Rainbow trout
Rs 126	Sweden	Rainbow trout
3015-86	Norway	Atlantic salmon
4451-86	Norway	Atlantic salmon

[a] Isolates were obtained from wild fish (w) or farm-raised fish (f). The full histories of some isolates are not known.

[b] The host species is uncertain but probably Atlantic salmon.

TABLE 2. Isolates of *R. salmoninarum* which were used for the PCR amplification of tDNA-ILPs with specific tRNA gene and intergenic spacer primers

No.	Isolate	Geographic origin	Biological source[b]	Details of isolation (date)
1	970083-88[c]	England	Rainbow trout (f)	Farm A, tank A (February 1997)
2	970083-102[c,d]	England	Rainbow trout (f)	Farm A, tank A (February 1997)
3[f]	980106#1.1.5	England	Rainbow trout (f)	Farm B, raceway (March 1998)
4[f]	980036-150[c]	Wales	Rainbow trout (f)	Farm C, pond (February 1998)
5[f]	980036-87[c,d]	Wales	Rainbow trout (f)	Farm C, raceway (February 1998)
6	970419-1.2.3[d]	England	Atlantic salmon (w)	Unknown (1997)
7	970153-19	England	Grayling (w)	Unknown (1997)
8	A6[c,d]	England	Rainbow trout (f)	Farm D (March 1998)
9	A80[c,d]	England	Rainbow trout (f)	Farm D (March 1998)
10	980297#97[c,d,e]	England	Rainbow trout (f)	Hatchery E, raceway (1998)
11[g]	F95[c,d]	England	Rainbow trout (f)	Farm A, tank B (1998)
12[g]	F85[c,d]	England	Rainbow trout (f)	Farm A, tank B (1998)
13[g]	F82[c,d,e]	England	Rainbow trout (f)	Farm A, tank C (1998)
14[g]	F60[c,d]	England	Rainbow trout (f)	Farm A, tank D (1998)
15[g]	F47[c,d]	England	Rainbow trout (f)	Farm A, tank E (1998)
16[g]	F3[c,d]	England	Rainbow trout (f)	Farm A, raceway (1998)
17	NCIMB1111[c,d]	Scotland	Atlantic salmon (w)[a]	River Dee (1962)
18	NCIMB1112[c,d]	Scotland	Atlantic salmon (w)[a]	River Dee (1962)
19	NCIMB1113[c,d]	Scotland	Atlantic salmon (w)[a]	River Dee (1962)
20	NCIMB1114	Scotland	Atlantic salmon (w)	River Dee (1962)
21	NCIMB1115[c,d]	Scotland	Atlantic salmon (w)	River Dee (1962)
22	NCIMB1116[d]	Scotland	Atlantic salmon (w)	River Dee (1962)

[a] The host species is uncertain but probably Atlantic salmon.
[b] Isolates were obtained from wild fish (w) or farm-raised fish (f). The full histories of some isolates are not known.
[c] The ITS1 region was sequenced (sequevar 1). GenBank accession no., AF239179 to AF239195.
[d] The ITS2 region was sequenced (sequevar 21). GenBank accession no., AF239890 to AF239906.
[e] The ETR-A locus was sequenced. GenBank accession no., AF242882 and AF242883.
[f] The ponds and raceways contained fish from the same hatchery but not from hatchery E.
[g] The fish came from various unlisted origins, probably not the same as that of no. 3 to 5.

Amplification of tRNA intergenic spacer regions using specific primers. The isolates that were used for tDNA-ILP analysis are listed in Table 2 and represent 22 isolates of *R. salmoninarum* from England, Wales, and Scotland, 14 of which were cultured from the kidneys of farmed fish displaying clinical symptoms of BKD and 8 of which were from wild fish. Sixteen PCR primers (Table 3) were designed using Amplify software (13) on the basis of the nucleotide sequences of amplicons derived from PCRs using consensus tRNA primers. The primers were tested singly and in all possible pairwise combinations in PCRs for their ability to amplify reproducible tDNA-ILPs. The PCR mixtures were prepared as described for amplification using consensus primers. Each reaction mixture was incubated at 96°C for 2 min and then subjected to 40 cycles consisting of 96°C for 30 s, 50°C for 30 s, and 72°C for 90 s.

RAPD analysis of isolates from the same outbreak of BKD. RAPD analysis was carried out on isolates 980297#97, F3, F47, F60, F82, F85, and F95, which were isolated from the kidneys of rainbow trout showing clinical signs of BKD, and six of these isolates were obtained at the same time from a single farm in England (Table 2). Two methods of RAPD analysis were used as previously described (22). The first method employed Ready-to-go RAPD analysis beads (Amersham Pharmacia Biotech), which were used according to the manufacturer's instructions. Briefly, six distinct random 10-mer primers, namely, P1 (GGT GCGGGAA), P2 (GTTTCGCTCC), P3 (GTAGACCCGT), P4 (AAGAGCCC GT), P5 (AACGCGCAAC), and P6 (CCCGTCAGCA), were used at a concentration of 25 pmol with 10 ng of template DNA in a 25-μl volume. The PCR conditions consisted of 1 cycle of 95°C for 4 min and then 45 cycles of 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min.

For the second method, described by Atienzar et al. (2), we used primers OPA9 (GGGTAACGCC) and OPB1 (GTTTCGCTCC) (Operon Technologies Inc.). The 25-μl reaction mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5.11 mM $MgCl_2$, 0.1% Triton X-100, 0.1% gelatin, each deoxynucleoside triphosphate at a concentration of 0.33 mM, 2 μM primer, 2.5 μg of bovine serum albumin, 2.8 U of *Taq* DNA polymerase (Immunogen International), and 10 ng of bacterial DNA. The PCR conditions consisted of 1 cycle of 95°C for 4 min, 39 cycles of 95°C for 1 min, 50°C for 1 min, and 74°C for 1 min, and a final cycle of 95°C for 1 min, 50°C for 1 min, and 74°C for 10 min.

ITS1 sequence analysis. The analysis of ITS1 was performed in accordance with the published protocol (22). Briefly, PCR mixtures were prepared using primers RS+1002 and ML−1329, which span ITS1 and which have previously been shown to amplify a single unambiguous 751-bp product of known sequence from *R. salmoninarum* DNA (22). Each 50-μl reaction mixture was prepared as described for tRNA gene and intergenic spacer amplification, incubated at 96°C for 2 min, and then subjected to 24 cycles of 96°C for 30 s, 65°C for 30 s, and 72°C for 90 s and a final cycle of 96°C for 30 s, 65°C for 30 s, and 72°C for 5 min. The reaction products were analyzed in 1.0% agarose gels.

ITS2 sequence analysis. The analysis of ITS2 was performed by PCR amplification using primers R5+118 (5′-CTGACCGGTACTAATAGGCCAACA-3′) and R5-433 (5′-GTCTTAGCTTCCGGGTTCGAGATG-3′) under the same reaction conditions used for ITS1 analysis. Primers R5+118 and R5−433 are specific to sequences in the 3′ end of the 23S rRNA gene and the 5′ end of the 5S rRNA gene, respectively, thereby flanking the ITS2 region of the rRNA operon of *R. salmoninarum*. The primers amplify a single 318-bp reaction product with a nucleotide sequence which corresponds to the ITS2 regions of a variety of isolates of *R. salmoninarum* from many sources (20).

TABLE 3. PCR primers specific to *R. salmoninarum* DNA sequences that were used to amplify tRNA intergenic spacer regions

Designation	Sequence (5′–3′)	Source[a]
A35K+754	TTGGTAAGAACGAGGTCACCGGAT	tRNA gene Thr (GGT)
A35K−760	TTACCAAGAACGCGCTCTACCACT	tRNA gene Thr (GGT)
T17C+80	GACAGGATTCGAACCTGTGACCAT	tRNA gene Arg (TCT)
T17C−135	TCAGTTGGATAGAGCATCCGCCTT	tRNA gene Arg (TCT)
T25D+115	CCTTAGTTCGTAGCCAAGTGCTCT	tRNA gene Arg (ACG)
T25D−120	TGGATAGAGCACTTGGCTACGAAC	tRNA gene Arg (ACG)
T35E+128	ACCAACTGCGCTACAGGGCCTTGC	tRNA gene Asp (GTC)
T35E−94	GTTCAAGTCCCGTCAGGGTCGCTA	tRNA gene Asp (GTC)
A7A+46	GCGTGAAAGATCTTAACCGGTGAG	tRNA ITS region
A7A−309	ATGATCGACGTCAGCTCCATCAAG	tRNA ITS region
A25A+4	CCGGTGCTCTAACCAACTGAGCTA	tRNA ITS region
A25A−247	GGTTGTTGCGTTTAGCTCAAGACG	tRNA ITS region
T3C+42	GGCTCGTGTCAAGACGGTTTTTGA	tRNA ITS region
T3C−123	TGCTCTAACCAACTGAGCTACACC	tRNA ITS region
T25E+19	AACTGAGCTAAGCGCCCTTGAGAA	tRNA ITS region
T25E−128	CGCCATTTTCTAGATCCCTTGTGC	tRNA ITS region

[a] tRNA gene anticodons are in parentheses.

ETR-A locus profile of *R. salmoninarum* isolates. The analysis of the ETR-A locus was performed using a previously described protocol (21). PCR amplification was carried out using primers 17D+95 and 17D−344, which previously have been shown to amplify a single fragment spanning the ETR-A locus of *R. salmoninarum* (21). The PCR conditions were the same as those described for the amplification of ITS1 and ITS2. The identities of the PCR products were confirmed by nucleotide sequencing for isolates 980297#97 and F82.

DNA sequencing and sequence analysis of PCR products. Both strands of all PCR products were sequenced using a cycle sequencing method by MWG-Biotech Ltd. Pairwise and multiple alignments of the nucleotide sequences were performed using the BLAST 2 sequence program (38) and the GeneBee multiple-alignment program, respectively.

Nucleotide sequence accession numbers. The nucleotide sequences reported here have been deposited in GenBank with accession no. AF239179 to AF239195 (ITS1 regions), AF239890 to AF239906 (ITS2 regions), AF242882 to AF242889, AF245384 to AF245386 (tRNA genes and tRNA intergenic spacer regions), and AF242882 and AF242883 (ETR-A locus).

RESULTS

After confirmation of culture purity and DNA extraction, the identity of the template DNA was confirmed using six sets of primers that are believed to be specific for *R. salmoninarum* DNA. Each of the PCRs produced a single distinct band of the expected size (22).

The ITS1 sequences and the ITS2 sequences are conserved among recent clinical isolates of *R. salmoninarum*. ITS1 and ITS2 were amplified by PCR from the genomic DNA of 17 isolates of *R. salmoninarum* including recent clinical isolates from five fish farms in England and Wales. Many of the isolates had been cultured from the kidneys of fish showing clinical signs of BKD which were held on the same farm and sampled at the same time (Table 2). The ITS1 and ITS2 sequences of the remaining five isolates listed in Table 2 have been previously published (21, 22). All of the isolates examined in this way were found to have ITS1 regions of identical size and nucleotide sequence that matched that of previously designated sequevar SV1 (22). In addition, the analysis of ITS2 sequences from these isolates (Table 2) showed that all isolates had regions identical in size and nucleotide sequence, which exactly matched that of previously described ITS2 sequevar SV21 (20).

Among recent isolates from the same farm the ETR-A locus has two copies of a 51-bp tandem repeat. The ETR-A loci of seven isolates were amplified by PCR using primers specific to sequences that are known to flank this region of the *R. salmoninarum* genome. The seven isolates, 980297#97, F3, F47, F60, F82, F85, and F95, were isolated from the kidneys of rainbow trout showing clinical signs of BKD, and six of these were obtained at the same time from a single farm in England (Table 2). The ETR-A loci of the remaining 15 isolates listed in Table 2 have been previously examined (21). All of the isolates were found to possess a single amplicon of 301 bp, which corresponds to the expected size of a PCR product containing two copies of the 51-bp tandem repeat unit at ETR-A. This was confirmed by the nucleotide sequence analysis of the PCR products from two selected isolates, 980297#97 and F82.

The RAPD profiles of recent isolates from the same farm are almost identical. RAPD analysis was carried out on 7 isolates, 980297#97, F3, F47, F60, F82, F85, and F95, which are listed in Table 2. The remaining 15 isolates listed in Table 2 have been previously characterized by RAPD analysis (21). RAPD analysis revealed that all seven isolates produced identical RAPD profiles using primers P1, P2, P4, P5, and OPA9 (Fig. 1). However, the RAPD patterns obtained using primers P3, P6, and OPB1 did reveal differences in the intensities of some bands between some of the isolates. When primer P3 was used, isolates F3, F60, F82, and F85 possessed stronger bands of 600 and 750 bp than isolates 980297#97, F47, and F95 (Fig. 1C). Primer P6 generated a strong band of 1.25 kb in isolate F3; this band was weak or absent in the other isolates (Fig. 1F). Furthermore, the RAPD patterns generated using primer OPB1 showed that a 1.15-kb band, which was strongly present in isolates 980297#97, F3, F60, and F82, was indistinct in isolates F47, F85, and F95 (Fig. 1G).

PCR amplification using consensus tRNA gene primers. A series of PCRs were carried out using four consensus tRNA gene primers in each of the six possible paired combinations. The primers are located within the tRNA consensus sequence and are designed to amplify the intergenic spacer region from the 5′ and 3′ ends of tRNA genes (31, 42). PCR amplification of DNA templates from 60 *R. salmoninarum* isolates (Table 1) resulted in profiles that were identical in appearance for many of the isolates regardless of their origin (Fig. 2). For example, with consensus primer set T3B-T5A all of the isolates showed identical tDNA PCR profiles consisting of a major band of 230 bp (Fig. 2A). Similarly, the use of primer set T3B-T5B showed that a fragment of 230 bp was present in all of the isolates examined (data not shown). However, using other primer combinations revealed that some isolates, particularly Marion Forks, 970153-19, and AcF6-1, consistently showed distinct differences. In the majority of isolates primer set T5A-T5B produced two major bands of approximately 1.1 and 2.4 kb, but 970153-19 and Marion Forks showed only single main bands of 490 and 300 bp, respectively (Fig. 2B). The patterns produced by primer set T3A-T3B were identical for most of the isolates and consisted of three main bands of approximately 120, 790, and 840 bp. Three isolates differed from this pattern. Cow ChS94 P22 showed an additional band of 180 bp, 970153-19 possessed two additional bands of 620 bp and 1.2 kb, and AcF6-1 possessed an additional band of 900 bp (Fig. 2C). The use of primer set T3A-T5A (Fig. 2D to F) or T3A-T5B (data not shown) produced the same fingerprints, which consisted of a faint band of 290 bp and a major band of 830 bp in all except three isolates. The three isolates which differed from this pattern were 970153-19 (major band of 200 bp), Marion Forks (major band of 230 bp), and AcF6-1 (major band of 870 bp).

Sequencing *R. salmoninarum* tRNA genes and intergenic spacers. A variety of products that had been amplified in tDNA PCRs using consensus tRNA gene primers were purified and cloned into pUC18. The products were chosen from *R. salmoninarum* isolates NCIMB2235, Marion Forks, 970153-19, 980106#1.1.5, and NCIMB1114, which represented some of the variations that were found in the tRNA intergenic spacer regions. Sequencing 21 clones revealed that many of the products spanned part of the $tRNA^{Ile}$ gene and all of the $tRNA^{Asp}$ gene, anticodon GTC. In a single clone, the $tRNA^{Thr}$ gene, anticodon GGT, preceded a partial $tRNA^{Met}$ gene sequence. Two distinct and complete $tRNA^{Arg}$ genes, anticodons TCT and ACG, were identified, and these were flanked by partial $tRNA^{Ala}$ and $tRNA^{Val}$ sequences, respectively. Other partial tRNA gene sequences were found in the primer sites but could not be fully identified.

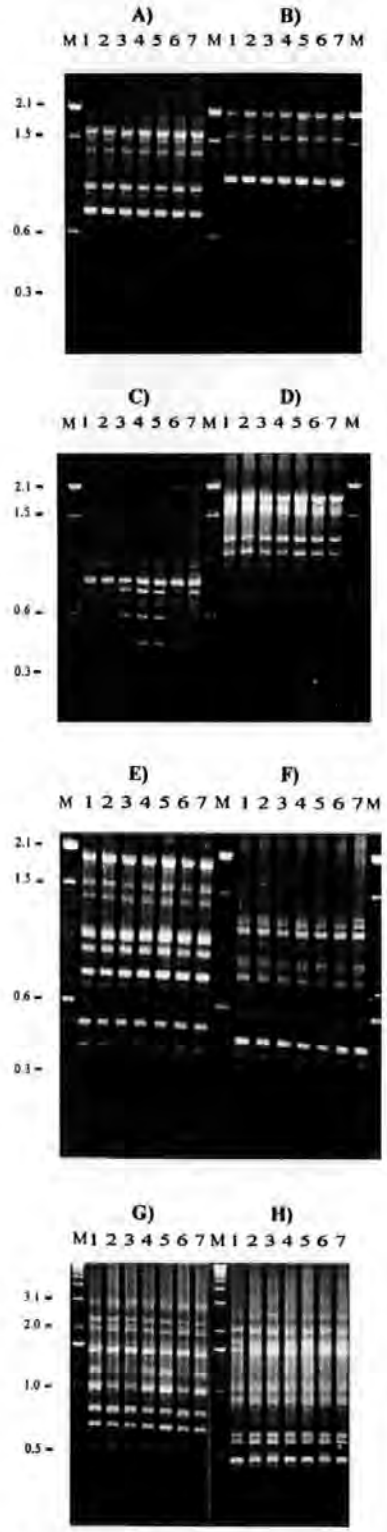

FIG. 1. The RAPD patterns of seven isolates of *R. salmoninarum*. The patterns were generated using primers P1 (A), P2 (B), P3 (C), P4 (D), P5 (E), P6 (F), OPB1 (G), and OPA9 (H). Lanes 1 to 7 correspond to isolates 980297#97, F95, F85, F82, F60, F47, and F3, respectively. Lanes M, 100-bp (A to F) or 1-kb (G and H) DNA ladder (Gibco BRL).

PCR amplification using primers specific to *R. salmoninarum* tRNA genes and intergenic spacer sequences. Sixteen specific primers were designed based on the sequences of the tRNA genes and intergenic spacer regions that had been obtained from amplicons generated using consensus tRNA gene primers (Table 3). The primers were specific for tRNA gene sequences encoding threonine, aspartic acid, and two forms of arginine and for intergenic spacer sequences. The primers were tested singly and in paired combinations in a series of PCRs in order to determine their potential to discriminate between 22 *R. salmoninarum* isolates from sources in England, Wales, and Scotland (Table 2). The results of this initial screening showed that most of the isolates possessed identical banding patterns (Fig. 3). For example, primers A35K+754 and T25D−120 span the intergenic region between the $tRNA^{Thr}$ and $tRNA^{Arg}$ (ACG) genes and amplified two bands of 210 and 650 bp that were present in all of the 22 isolates examined (Fig. 3A). Similarly, PCRs using primers T17C+80 and T25E−128 amplified a single band of 780 bp that was present in each isolate (Fig. 3B).

In contrast, some primers did reveal differences. Primers A35K+754 and T17C−135 amplify the spacer region between the $tRNA^{Thr}$ and $tRNA^{Arg}$ (TCT) genes. In these reactions all isolates showed two bands of 110 and 680 bp but isolate 970153-19 also possessed an additional band of 1.2 kb (Fig. 3C). Furthermore, the profiles produced using primers T3C+42 and T25E−128 showed two major bands of 200 and 870 bp in all of the isolates, while an additional 300-bp fragment was found to be present in 970419-1.2.3, 970153-19, A6, 980297#97, F3, F47, F60, F82, and F95 (Fig. 3D). Using primer T25E-128 alone produced a banding pattern of up to six amplicons that separated the isolates into nine groups (Fig. 3E). The groupings of the isolates in relation to the observed band sizes are shown in Table 4. Some isolates from the same source were placed into separate groups, for example, isolates A6 and A80 from farm D were placed into groups 2 and 4, respectively, and isolates from farm E were placed into groups 2, 5, 8, and 9. Group 1 contained five isolates from farms A, B, and C in England and Wales and four isolates from the River Dee, Scotland. Three of these isolates were obtained in 1998 from two farms, one in England and one in Wales, and are known to have a common hatchery source.

DISCUSSION

In this study tDNA PCR profiling was used to separate 22 isolates of *R. salmoninarum* from various sources in the United Kingdom into nine groups on the basis of the sizes of the amplicons which were generated using primers specific to tRNA genes and intergenic spacer regions. Many of these isolates were cultured from samples which had been gathered from the same farm and at the same time in England and Wales and from wild fish sampled in 1962 from the River Dee. Using other methods of analysis we have shown that 20 of the isolates possessed identical ITS1 and ITS2 nucleotide sequences, SV1 and SV21, respectively, and two copies of a 51-bp repeat unit at the ETR-A locus. We have previously shown that two of the isolates used here, NCIMB1114 and NCIMB1116, from the River Dee possess a different ITS1 nucleotide sequence, SV4, and have a single copy of a 51-bp sequence at ETR-A (21). In addition, many of the isolates have

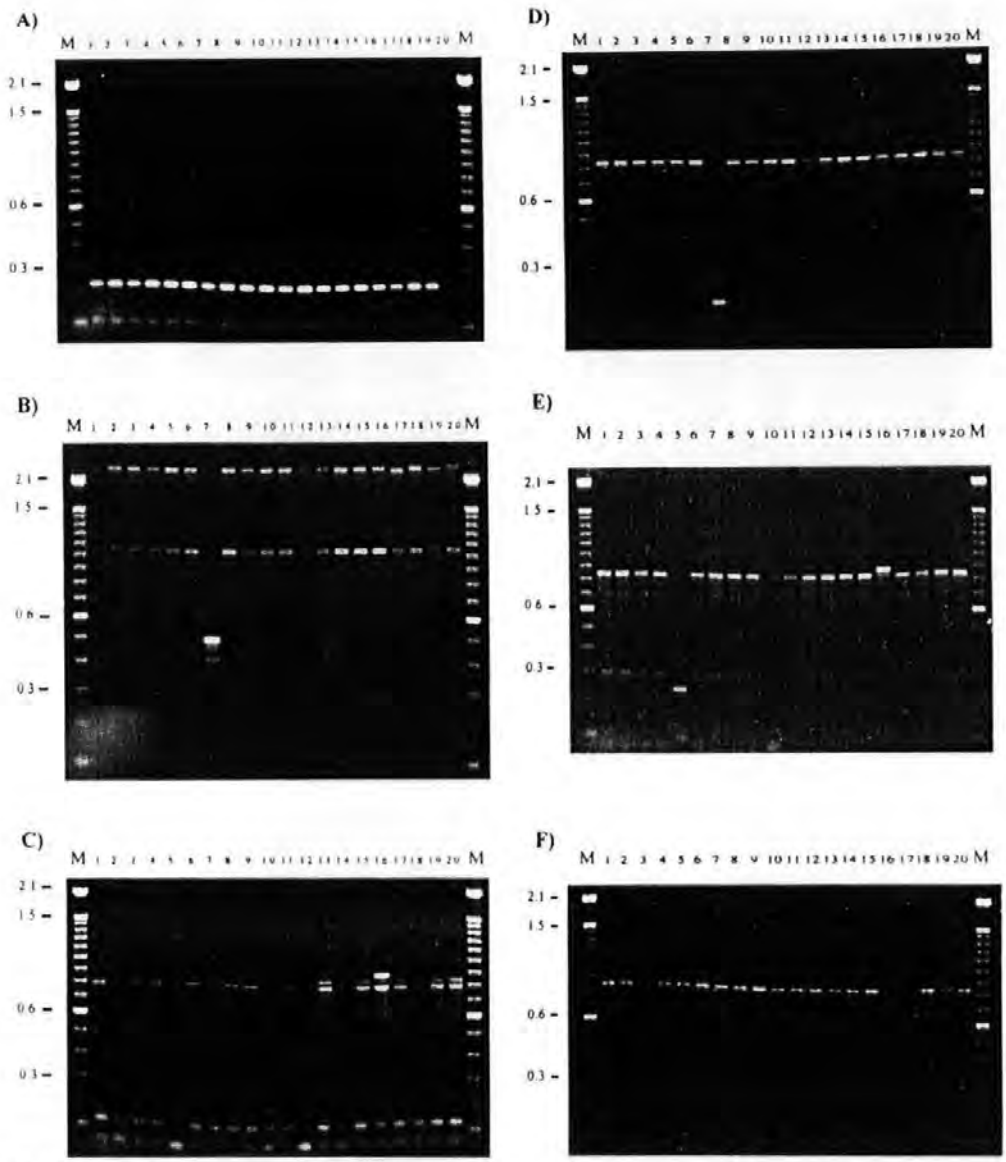

FIG. 2. The tDNA PCR profiles of 60 isolates of *R. salmoninarum*. The patterns were generated using consensus primer sets T3B-T5A (A), T5A-T5B (B), T3A-T3B (C), and T3A-T5A (D to F). (A and F) Lanes 1 to 20, isolates 960046, F-120-87(P-2), F-130-87(P-4), F-138-87(O-78), F-273-87(P-19), F-283-87(P-10), F-358-87(P-13), S-182-90(P-7), Rs 9, Rs 19, Rs 61, Rs 116, Rs 122, Rs 125, Rs 126, 3015-86, 4451-86, RS-TSA, FT10, and BY1996, respectively. (C and E) Lanes 1 to 20, isolates MT452, MT1363, MT410, Siletz, Marion Forks, Little Goose, CCM6205, 84-019-OC, SS-ChS-94-1, Cow ChS94 P22, Idaho 91-126, RFL-643.94#1, CCM6206, Round Butte, NCIMB2235, AcF6-1, DR143, DR384, 980002, and 960023, respectively. (B and D) Lanes 1 to 20, isolates 970083-88, 970083-102, 980106#1.1.5, 980036-150, 980036-87, 970419-1.2.3, 970153-19, A6, A80, MT409, MT417, MT239, MT426, NCIMB1111, NCIMB1112, NCIMB1113, NCIMB1114, NCIMB1115, NCIMB1116, and MT420, respectively. Lanes M, 100-bp DNA ladder (Gibco BRL).

been shown to be identical using IS*994* restriction fragment length polymorphism (RFLP) and RAPD analysis (21, 34).

The ITS1 and ITS2 sequence analyses support the findings of our previous work showing that all *R. salmoninarum* isolates from England and Wales which have been examined in this way are SV1 (ITS1) and SV21 (ITS2). These are the most commonly occurring sequence variants among *R. salmoninarum* isolates, particularly in regions of the world with a history of intensive salmonid aquaculture. In the United Kingdom, only isolates which have been gathered from Scotland show any variation in the ITS1 region, and these date back to 1962, before the substantial development of salmonid culture in that region (21). We have previously found that SV1 isolates are distinguished by the possession of two copies of a 51-bp tandem repeat at ETR-A, an exact tandem repeat locus on the *R. salmoninarum* genome (21). The present work supports this finding.

Consensus tRNA primers were used in each of the possible paired combinations to generate tDNA PCR profiles of 60 isolates of *R. salmoninarum*. Welsh and McClelland speculated

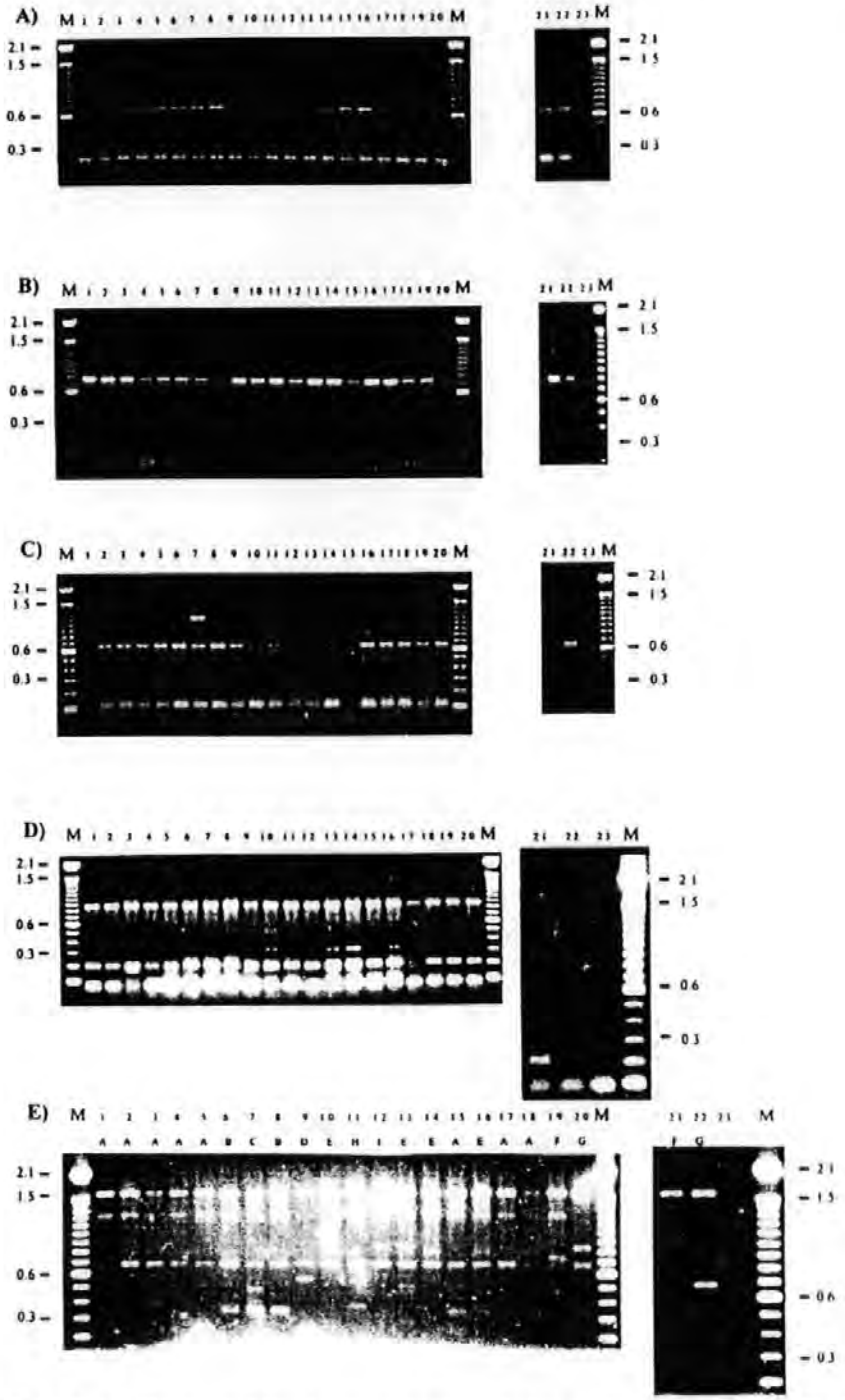

FIG. 3. The tDNA PCR profiles of 22 isolates of *R. salmoninarum* generated when using specific primers A35K+754 and T25D−120 (A), T17C+80 and T25E−128 (B), A35K+754 and T17C−135 (C), T3C+42 and T25E−128 (D), and T25E−128 (E). Lanes 1 to 22 correspond to the isolate numbers in Table 2. Lanes 23, negative control; lanes M, 100-bp DNA ladder (Gibco BRL).

that because of the highly conserved nature of tRNA genes and their arrangement on the genome tDNA-ILPs are often characteristic of a species or even a genus, and this has been apparent in a number of other studies (42, 43). However, our work has shown that isolates 970153-19, Marion Forks, Cow CHs94 P22, and AcF6-1 produced different tDNA-ILPs when consensus primers T3A-T5B, T3A-T5A, T3A-T3B and T5A-T5B were used. We previously showed that isolates 970153-19, Marion Forks, and AcF6-1 differed in their RAPD profiles (21). Furthermore, isolate AcF6-1 has unique ITS1 and ITS2 sequences, SV3 and SV22, respectively, a single copy of the 51-bp repeat unit at the ETR-A locus, and a unique IS*994*

TABLE 4. Groupings of 22 isolates of *R. salmoninarum* from United Kingdom sources based on band sizes generated using primer T25E−128

Group	Bands sizes (bp)	Isolates[a]
1	690, 1,250, 1,600	970083-88, 970083-102, 980106#1.1.5, 980036-150, 980036-87, NCIMB1111, NCIMB1112, NCIMB1115, NCIMB1116
2	320, 690, 1,250, 1,600	970419-1.2.3, A6, F47
3	320, 480, 690, 1,250, 1,600	970153-19
4	550, 690, 1,250, 1,600	A80
5	690, 790, 850, 1,250, 1,600	980297#97, F3, F60, F82
6	690, 780, 1,250, 1,600	NCIMB1113
7	690, 850, 1,250, 1,600	NCIMB1114
8	350, 690, 1,250, 1,600	F95
9	480, 690, 790, 850, 1,250, 1,600	F85

[a] Isolates 980106#1.1.5, 980036-150, and 980036-87 were derived from fish sampled from ponds and raceways that contained fish from the same hatchery.

RFLP pattern (20, 21, 22, 34). Interestingly, while RAPDistance analysis grouped Cow Chs94 P22 together with another 28 isolates of *R. salmoninarum* from different geographical origins (21), the IS*994* RFLP profile of this isolate was unique (34).

We have shown that several sets of consensus primers were capable of amplifying more than one major PCR product for each isolate. This probably occurred because the products are derived from multiple tRNA genes from the same tRNA gene cluster or from different clusters (25). Sequence analysis of a selection of PCR products in our study provided evidence that this was indeed the case. It is also possible that some of the amplicons that were generated using consensus primers were not from tRNA genes but rather represented fragments produced by the arbitrary amplification of unrelated loci. However, the lack of tRNA or tRNA-like genes in the sequenced amplicons occurred in only two cases.

Primers with perfect homology to specific tRNA genes were designed in order to discriminate, and assist in determining the epizootiological relationships, among a selection of United Kingdom isolates of *R. salmoninarum*. Twenty-two isolates were examined; of these 7 isolates had been cultured from a single outbreak of BKD at a fish farm in England, while a further 7 isolates were derived from single outbreaks on four separate farms. The remaining eight isolates were cultured from wild fish including six isolates from the River Dee in 1962. Most of the PCRs using specific primers showed that there were no differences between these isolates. However, the use of primers A35K+754 and T17C−135 to amplify the spacer region between the $tRNA^{Thr}$ and $tRNA^{Arg}$ genes showed that most isolates produced two bands of 110 and 630 bp but that 970153-19 possessed an additional band. This suggests there is some fundamental difference either in the sequence or the order of tRNA genes and the associated intergenic spacer regions in this isolate. Generating two or more products from primers that correspond to specific tRNA genes is possible because bacterial genomes can contain multiple copies of tRNA genes and because the organization of the genes within a cluster is often the same (23, 25, 35). The tDNA PCR profiles produced using primers T3C+42 and T25E−128 divided the 22 isolates into two groups. Interestingly, one of these groups contained six clinical isolates from fish held on the same farm but three other isolates with which they were grouped were from different host species and from different areas of the United Kingdom. These three isolates have previously been grouped together by RAPDistance analysis, suggesting that they may be derived from the same clone (21). When T25E-128 was used as a single primer, banding patterns of up to six amplicons, which enabled the differentiation of the 22 isolates into nine groups, were produced. Of particular interest was the finding that some of the isolates that had been cultured from fish held on the same farm and sampled at the same time possessed different tDNA PCR fingerprints. We can only speculate as to whether the groups emerged on the farm over the course of time from an initial isolate, in which case the past use of antibiotic and chemical treatments may have played some role in the selection of genetic variants. Alternatively, there may have been successive introductions from external sources which are related to differences in the hatchery supply of eggs, the cohorts of fish held on the farm, and the identity of the brood stock. Interestingly, 980106#1.1.5, 980036-87, and 980036-150 were grouped together and, although they were isolated from fish held on two farms, one in Wales and one in England, they had a common hatchery source. While it is possible to draw links between some isolates, it is unfortunate that the full details of the precise origins of all of the fish stocks from which *R. salmoninarum* was isolated are unavailable.

In some cases, the groups based on tDNA-ILPs corresponded with previous divisions that had been created using either IS*994* or RAPDistance analyses (21, 34). However, some United Kingdom isolates that were grouped together by either IS*994* RFLP or RAPDistance analysis were found to be different using tDNA-ILP profiling and vice versa. For example, it was possible to differentiate between isolates NCIMB1114 and NCIMB1116 by using tDNA-ILPs even though the isolates were identical using RAPDistance analysis (21). On the other hand, isolates 970419-1.2.3 and A6 were found to be different using RAPDistance software but were identical using tDNA PCR. The methods measure molecular variation in different ways; RAPD and IS*994* analyses measure variation throughout the genome, while tDNA-ILP analyses are restricted to variation within the tRNA intergenic spacer regions, and so this outcome is not surprising. Our previous studies have shown that there is molecular variation in the *R. salmoninarum* genome that cannot be attributed to variation within the rRNA operon (20). Recent work by Rhodes et al. (34) has shown that the pattern of insertion of putative IS*3* family insertion element IS*994* throughout the genome helps to explain some of this variation. We propose that polymorphisms in both the tRNA genes and the tRNA intergenic spacer regions may also

help to explain some of this variation. tRNA genes have consistently been reported to be sites for the integration of pathogenicity islands, bacteriophage, repeat elements, transposons, and plasmid DNA. Due to their highly conserved and repeated nature tRNA genes are prone to the insertion of mobile genetic elements and indeed may play a key role in the evolution of microbial pathogens. The variations that exist between and within tRNA genes on the *R. salmoninarum* genome may also be a reflection of this process.

One of the major shortcomings of the objective analysis of RAPD profiles is that the binary matrix that is used to input data into RAPDistance software necessitates that bands be scored as either present or absent. Consequently, there is no account taken of variation in the brightness of bands. For example, for primer P6, compare the brightness of the 1.25-kb band in isolate F3 with that of the faint bands in the same position in some of the other isolates (Fig. 1F). RAPDistance analysis necessitates that either the bands be determined to be the same, that is, present no matter how faint or bright, or else the band at that position is not used in the analysis. In addition, it is often necessary to reject the use of some bands because of ambiguities between the isolates, for example, bands that are amplified in RAPD reactions to which a present or absent score cannot be definitively made for every isolate, either because of their proximity to other bands, e.g., multiple bands, or because of background or smeared amplification. In such cases there is a loss of discriminatory power, and these are the main reasons why 28 isolates of *R. salmoninarum* were grouped into a single cluster in our previous work using RAPDistance analysis (21).

When comparing tDNA PCR with other methods for determining the relationships between isolates of *R. salmoninarum* it would be advisable to use a multifactorial approach. Each of the techniques developed so far for this purpose seems to complement the information that can be gained from the use of just a single technique. Therefore, it would be better to use a number of techniques in order to attach more confidence to the outcome. In conclusion, tDNA PCR profiling provides another method for discriminating between clinical isolates of *R. salmoninarum* which show identical rRNA ITS sequences, ETR-A loci, and IS*994* patterns. The tDNA PCR profiling was in good agreement with previous divisions made using RAPD profiles and in some cases improved on the discriminatory power of RAPD. When used in combination with other molecular typing methods, tDNA-ILPs will be a useful tool for epizootiological studies of BKD outbreaks in populations of both wild and farmed fish.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipmann.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389–3402.
2. **Atienzar, F., P. Child, A. Evenden, A. Jha, D. Savva, C. Walker, and M. Depledge.** 1998. Application of the arbitrarily primed polymerase chain reaction for the detection of DNA damage. Mar. Environ. Res. **46**:331–335.
3. **Austin, B., T. M. Embley, and M. Goodfellow.** 1983. Selective isolation of *Renibacterium salmoninarum*. FEMS Microbiol. Lett. **17**:111–114.
4. **Banner, C. R., J. S. Rohovec, and J. L. Fryer.** 1991. A new value for mol percent guanine + cytosine of DNA for the salmonid fish pathogen *Renibacterium salmoninarum*. FEMS Microbiol. Lett. **63**:57–59.
5. **Bruno, D. W., and A. L. S. Munro.** 1986. Uniformity in the biochemical properties of *Renibacterium salmoninarum* isolates obtained from several sources. FEMS Microbiol. Lett. **33**:247–250.
6. **Campbell, A. M.** 1992. Chromosomal insertion sites from phages and plasmids. J. Bacteriol. **174**:7495–7499.
7. **Claros, M. C., G. Haase, G. Schonlau, D. M. Citron, S. H. Gerardo, E. J. C. Goldstein, U. Schumacher, and A. C. Rodloff.** 1997. Differentiation of clinical *Bacteroides ovatus* isolates using RNA gene size polymorphisms. Rev. Med. Microbiol. **8**(Suppl. 1):S103–S104.
8. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Conner, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature (London) **393**:537–544.
9. **Daffonchio, D., S. Borin, G. Frova, L. Manachini, and C. Sorlini.** 1998. PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. Int. J. Syst. Bacteriol. **48**:107–116.
10. **Dolzani, L., E. Tonin, C. Lagatolla, and C. Monti-Bragadin.** 1994. Typing of *Staphylococcus aureus* by amplification of the 16S–23S rRNA intergenic spacer sequences. FEMS Microbiol. Lett. **119**:167–174.
11. **Dundon, W. G., D. G. Marshall, C. A. Morain, and C. J. Smyth.** 1999. A novel tRNA-associated locus (*trl*) from *Helicobacter pylori* is co-transcribed with $tRNA^{Gly}$ and reveals genetic diversity. Microbiology **145**:1289–1298.
12. **Ehrenstein, B., A. T. Bernards, L. Dijkshoorn, P. Gerner-Smidt, K. J. Towner, P. J. M. Bouvet, F. D. Daschner, and H. Grundmann.** 1996. *Acinetobacter* species identification by using tRNA spacer fingerprinting. J. Clin. Microbiol. **34**:2414–2420.
13. **Engels, W. R.** 1993. Contributing software to the Internet: the Amplify program. Trends Biochem. Sci. **18**:448–450.
14. **Evelyn, T. P. T., L. Prosperi-Porta, and J. E. Ketcheson.** 1990. Two new techniques for obtaining consistent results when growing *Renibacterium salmoninarum* on KDM2 culture medium. Dis. Aquat. Org. **9**:209–212.
15. **Evenden, A. J., T. H. Grayson, M. L. Gilpin, and C. B. Munn.** 1993. *Renibacterium salmoninarum* and bacterial kidney disease—the unfinished jigsaw. Annu. Rev. Fish Dis. **3**:87–104.
16. **Fichant, G. A., and C. Burks.** 1991. Identifying potential tRNA genes in genomic DNA sequences. J. Mol. Biol. **220**:659–671.
17. **Folkesson, A., A. Advani, S. Sukupolvi, J. Pfeifer, S. Normark, and S. Lofdahl.** 1999. Multiple insertions of *Salmonella* serovars responsible for human disease. Mol. Microbiol. **33**:612–622.
18. **Forsman, P., A. Tilsala-Timisjarvi, and T. Alatossava.** 1997. Identification of staphylococcal and streptococcal causes of bovine mastitis using 16S–23S rRNA spacer regions. Microbiology **143**:3491–3500.
19. **Goodfellow, M., T. M. Embley, and B. Austin.** 1985. Numerical taxonomy and emended description of *Renibacterium salmoninarum*. J. Gen. Microbiol. **131**:2739–2752.
20. **Grayson, T. H., S. M. Alexander, L. F. Cooper, and M. L. Gilpin.** 2000. *Renibacterium salmoninarum* isolates from different sources possess two highly conserved copies of the rRNA operon. Antonie Leeuwenhoek **78**:51–61.
21. **Grayson, T. H., F. A. Atienzar, S. M. Alexander, L. F. Cooper, and M. L. Gilpin.** 2000. Molecular diversity of *Renibacterium salmoninarum* isolates determined by randomly amplified polymorphic DNA. Appl. Environ. Microbiol. **66**:435–438.
22. **Grayson, T. H., L. F. Cooper, F. A. Atienzar, M. R. Knowles, and M. L. Gilpin.** 1999. Molecular differentiation of *Renibacterium salmoninarum* isolates from world-wide locations. Appl. Environ. Microbiol. **65**:961–968.
23. **Green, C. J., and B. Vold.** 1993. *Staphylococcus aureus* has clustered tRNA genes. J. Bacteriol. **175**:5091–5096.
24. **Gutenberger, S. K., S. J. Giovannoni, K. G. Field, J. L. Fryer, and J. S. Rohovec.** 1991. A phylogenetic comparison of the 16S rRNA sequence of the fish pathogen, *Renibacterium salmoninarum*, to gram-positive bacteria. FEMS Microbiol. Lett. **77**:151–156.
25. **Honeycutt, R. J., B. W. Sobral, and M. McClelland.** 1995. tRNA intergenic spacers reveal polymorphisms diagnostic for *Xanthomonas albilineas*. Microbiology **141**:3229–3239.
26. **Kersulyte, D., J. P. Woods, E. J. Keath, W. E. Goldmann, and D. E. Berg.** 1992. Diversity among clinical isolates of *Histoplasma capsulatum* detected by polymerase chain reaction with arbitrary primers. J. Bacteriol. **174**:7075–7095.
27. **Koch, C. F., F. A. Rainey, and E. Stackebrandt.** 1994. 16S rDNA studies on

members of *Arthrobacter* and *Micrococcus*: and aid for their future taxonomic restructuring. FEMS Microbiol. Lett. **123**:167–172.
28. **Kunze, Z. M., S. Wall, R. Appelberg, M. T. Silva, F. Portaels, and J. J. McFadden.** 1991. IS*901*, a new member of a widespread class of atypical insertion sequences, is associated with pathogenicity in *Mycobacterium avium*. Mol. Microbiol. **5**:2265–2272.
29. **Lowe, T. M., and S. R. Eddy.** 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. **25**:955–964.
30. **Maes, N., and Y. Degheldre.** 1997. Rapid and accurate identification of *Staphylococcus* species by tRNA intergenic spacer length polymorphism analysis. J. Clin. Microbiol. **35**:2477–2481.
31. **McClelland, M., C. Petersen, and J. Welsh.** 1992. Length polymorphisms in tRNA intergenic spacers detected by using the polymerase chain reaction can distinguish streptococcal strains and species. J. Clin. Microbiol. **30**:1499–1504.
32. **Moss, F. E., T. J. Cardozo, A. Zychlinsky, and E. A. Groisman.** 1999. The *selC*-associated SHI-2 pathogenicity isolate of *Shigella flexneri*. Mol. Microbiol. **33**:74–83.
33. **Norton, R., B. Roberts, M. Freeman, M. Wilson, C. Ashurst-Smith, W. Lock, D. Brooks, and J. La Brooy.** 1998. Characterisation and molecular typing of *Burkholderia pseudomallei*: are disease presentations of melioidosis clonally related? FEMS Immunol. Med. Microbiol. **20**:37–44.
34. **Rhodes, L. D., T. H. Grayson, S. M. Alexander, and M. S. Strom.** 2000. Description and characterisation of IS*994*, a putative IS*3* family insertion sequence from the salmon pathogen, *Renibacterium salmoninarum*. Gene **244**:97–107.
35. **Seal, S. E., L. A. Jackson, and M. J. Daniels.** 1992. Use of tRNA consensus primers to indicate subgroups of *Pseudomonas solanacearum* by polymerase chain reaction amplification. Appl. Environ. Microbiol. **58**:3759–3761.
36. **Stackebrandt, E., U. Wehmeyer, H. Nader, and F. Fiedler.** 1988. Phylogenetic relationship of the fish pathogen *Renibacterium salmoninarum* to *Arthrobacter*, *Micrococcus* and related taxa. FEMS Microbiol. Lett. **50**:117–120.
37. **Starliper, C. E.** 1996. Genetic diversity of North American isolates of *Renibacterium salmoninarum*. Dis. Aquat. Org. **27**:207–213.
38. **Tatusova, T. A., and T. L. Madden.** 1999. BLAST 2 sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol. Lett. **174**: 247–250.
39. **van der Zee, A., F. Mooi, J. van Emben, and J. Musser.** 1997. Molecular evolution and host adaptation of *Bordetella* sp.: phylogenetic analysis using multilocus enzyme electrophoresis and typing with three insertion sequences. J. Bacteriol. **179**:6609–6617.
40. **Vold, B.** 1985. Structure and organization of genes for transfer ribonucleic acid in *Bacillus subtilis*. Microbiol. Rev. **49**:71–80.
41. **Wang, G., A. P. van Dam, L. Spanjaard, and J. Dankert.** 1998. Molecular typing of *Borrelia burgdorferi* sensu lato by randomly amplified polymorphic DNA fingerprinting analysis. J. Clin. Microbiol. **36**:768–776.
42. **Welsh, J., and M. McClelland.** 1991. Genomic fingerprints produced by PCR with consensus tRNA gene primers. Nucleic Acids Res. **19**:861–866.
43. **Welsh, J., and M. McClelland.** 1992. PCR-amplified length polymorphisms in tRNA intergenic spacers for categorizing *Staphylococci*. Mol. Microbiol. **6**:1673–1680.

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Renibacterium salmoninarum isolates from different sources possess two highly conserved copies of the rRNA operon *

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Key words: actinobacteria, phylogeny, rRNA operon, 23S–5S rRNA intergenic spacer

Abstract

The nucleotide sequences of the rRNA genes and the 5′ flanking region were determined for *R. salmoninarum* ATCC 33209^T from overlapping products generated by PCR amplification from the genomic DNA. Comparison of the sequences with rRNA genes from a variety of bacteria demonstrated the close relatedness between *R. salmoninarum* and the high G+C group of the actinobacteria, in particular, *Arthrobacter* species. A regulatory element within the 5′ leader of the rRNA operon was identical to an element, CL2, described for mycobacteria. PCR, DNA sequence analysis, and DNA hybridisation were performed to examine variation between isolates from diverse sources which represented the four 16S–23S rRNA intergenic spacer sequevars previously described for *R. salmoninarum*. Two 23S–5S rRNA intergenic spacer sequevars of identical length were found. DNA hybridisation using probes complementary to 23S rDNA and 16S rDNA identified two rRNA operons which were identical or nearly identical amongst 40 isolates sourced from a variety of countries.

Abbreviations: ITS1, 16S–23S rRNA – intergenic spacer; ITS2, 23S–5S rRNA – intergenic spacer; SV – sequevar, sequence variant

Introduction

Renibacterium salmoninarum is a slowly growing, gram-positive bacterium taxonomically placed within the *Micrococcus-Arthrobacter* subdivision of the actinomycetes (Stackebrandt et al. 1988; Banner et al. 1991; Gutenberger et al. 1991; Koch et al. 1994). It is an obligate pathogen, capable of intracellular survival, which causes bacterial kidney disease (BKD) of salmonid fish (Evelyn 1993). *R. salmoninarum* possesses a number of unique features including cell wall polysaccharide composition (Kusser & Fiedler 1983; Fiedler & Draxl 1986), lipid and menaquinone composition (Embley et al. 1983), growth characteristics and pathogenic status (Evenden et al. 1993).

There is no effective treatment for BKD of salmonid fish and control strategies rely on the detection and eradication of infected stock. To facilitate contact tracing of the pathogen a means of differentiating between some isolates of *R. salmoninarum* using randomly amplified polymorphic DNA has been developed (Grayson et al. 1999, 2000). However, a more specific means of distinguishing between isolates is needed.

The characterisation of the ribosomal RNA genes of bacteria has provided a powerful tool for the classification, identification and typing of isolates (Ludwig & Schleifer 1994). In particular, the 16S rRNA (Olsen et al. 1991) and the 5S rRNA genes (Dams et al. 1987; Park et al. 1991; Syzmanski et al. 1998) have been used to establish phylogenetic relationships between a wide variety of organisms. Although 23S rRNA genes are larger and more variable than either 16S or 5S rRNA genes, and therefore potentially more useful for phylogenetic studies, relatively few 23S rRNA gene sequences are available for this purpose. Incomplete 16S rRNA gene sequences are known for 2 isolates

* The GenBank accession numbers of the sequences reported in this paper are AF143477-AF143486, AF180950.

of *R. salmoninarum* (Gutenberger et al. 1991; Magnusson et al. 1994), and a 112 bp common insertion fragment of the 23S rRNA gene has been sequenced (Roller et al. 1992).

Most organisms have multiple copies of rRNA genes and sequence uniformity is usual, although among procaryotes there are at least three exceptions, the archaeon *Haloarcula marismortui* (Dennis et al. 1998) and the actinomycetes *Thermobispora bispora* (Wang et al. 1997) and *Thermomonospora chromogena* (Yap et al. 1999). In eubacteria, the intergenic spacer regions of the rRNA operon show the most variation, in sequence and length, and disparity between copies of the operon would be most likely to occur here (Dennis et al. 1998; Gurtler & Stanisich 1996; Leblond-Bourget et al. 1996; Wang et al. 1997).

Sequence analysis of the 16S–23S rRNA intergenic spacer (ITS1) has proven to be useful for distinguishing between members of closely related bacterial species and, sometimes, between strains (Barry et al. 1991; Jensen et al. 1993; Gurtler & Stanisich 1996; Leblond-Bourget et al. 1996). Recent analyses of the *R. salmoninarum* ITS1 have shown that among isolates sourced from different regions of the world ITS1 is of identical length, 534 bp, although four ITS1 sequence variants containing from one to three nucleotide substitutions have been identified (Grayson et al. 1999, 2000). There have been few published works examining variation in bacterial 23S–5S rRNA intergenic spacer regions (ITS2) although there is some evidence that ITS2 may prove useful for designing species-specific probes and establishing relationships between closely related species (Nour 1998; Ji et al. 1994b).

We report here the characterisation of the rRNA operon of *R. salmoninarum* and examine the rRNA operon for the presence of variation in a representative set of isolates.

Materials and methods

Bacterial isolates

The sources of the tested isolates of *R. salmoninarum* are shown in Table 1. Isolates were cultured for 6–8 weeks in selective kidney disease medium (SKDM) broth supplemented with 5% spent broth culture at 15°C (Austin et al. 1983; Evelyn et al. 1990), then freeze-dried and checked for purity. The names and addresses of those who have provided these isolates, derived from confirmed clinical outbreaks of bacterial kidney disease, have been published (Grayson et al. 1999). The isolates are maintained in a culture collection at the University of Plymouth in collaboration with CEFAS Weymouth, U.K.

Sequencing of the rRNA genes of R. salmoninarum ATCC33209

Genomic DNA was isolated using the Puregene D-6000 DNA isolation kit according to the manufacturer's instructions (Gentra Systems Inc.) and quantified using digital images taken after agarose gel electrophoresis as previously described (Grayson *et al.* 1999). The identity of *R. salmoninarum* DNA was confirmed for all isolates by PCR using sets of primers specific for *R. salmoninarum* genes (Grayson et al. 1999).

PCR amplification of the 16S rRNA gene and 5′ flanking region, the 23S rRNA gene, and the 5S rRNA gene of *R. salmoninarum* ATCC33209^T (T = type strain) was performed in a Perkin Elmer thermal cycler; all primers were chosen using Amplify software (Engels 1993). Eleven sets of PCR primer pairs were designed to amplify overlapping fragments for amplification of the 23S rRNA gene, 5S rRNA gene, and ITS2 (Figure 1). Almost the entire DNA sequence spanning the 23S rRNA gene, the 5S rRNA gene and the ITS2, was derived from both strands of two different templates. The primers were selected from highly conserved regions which were identified following alignments of rDNA sequences from bacteria known to be closely related to *R. salmoninarum*. The sequences (and their GenBank accession numbers) included those for *Micrococcus luteus* (X06484), *Rhodococcus erythropolis* (AF001265), *Rhodococcus fascians* (Y11196), *Streptomyces griseus* (X55435; X61478), *Streptomyces rimosus* (X62884) and *Frankia* sp. (M88466). Templates for sequencing of the 16S rRNA gene of *R. salmoninarum* ATCC33209^T were generated using PCR primers based on the incomplete sequences of the *R. salmoninarum* 16S rRNA gene available on the GenBank database (accession nos. X51601, X76977). The complete sequence of the 16S rRNA gene, including the 5′ leader sequence, was derived from both strands of four overlapping amplicons (Figure 1). The reaction mixture (50 μl) contained 1 unit *Taq* polymerase in reaction buffer (Boehringer Mannheim), 1.5 mM $MgCl_2$, 24 pmol of each primer, 0.2 mM of each dNTP and 10 ng bacterial DNA. The reaction mixtures were

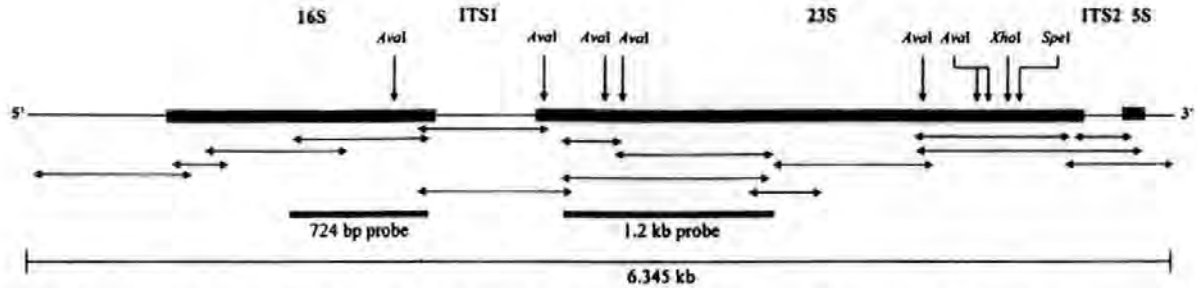

Figure 1. Strategy for sequencing and restriction map of the rRNA operon of *R. salmoninarum* ATCC33209^T. The sequence spanning the 5′ leader of the 16S rRNA gene and the 3′ tail of the 5S rRNA gene was obtained from both strands of PCR amplicons. The sequence of the 16S–23S ITS (534 nucleotides) has been previously reported (GenBank accession no. AF093461). The positions of rDNA probes (with sizes) used for hybridisation studies are indicated as bold lines.

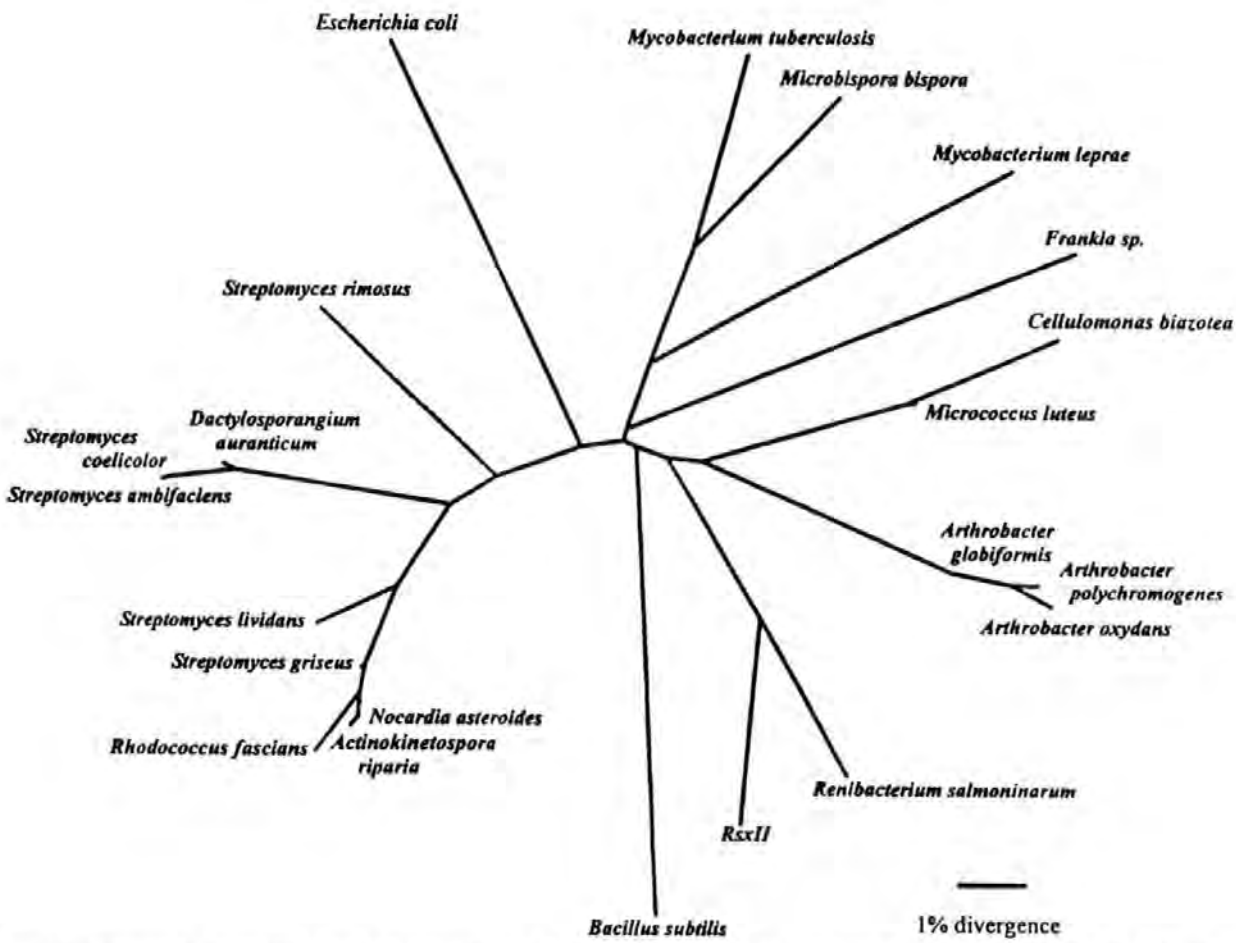

Figure 2. Phylogenetic relationships among representatives of the actinomycetes and selected taxa based on 16S rDNA sequence data obtained from the GenBank database. The tree was constructed by using the neighbour-joining method of Nei & Saitou (1987) from an evolutionary distance matrix that provides a maximum likelihood estimate of the divergence time (total branch length) between the sequences. The analysis was performed on sequence data for positions that were present in members of all the species analysed (1441 nucleotides).

Table 1. R. salmoninarum isolates used in this study

Isolate	Geographic origin	Biological source[a]	Isolate	Geographic origin	Biological source[a]
980036-150[f]	Wales	Rainbow trout(f)	960046	British Columbia, Canada	Coho salmon(f)
980036-87	Wales	Rainbow trout(f)	GC96-1	British Columbia, Canada	Sockeye salmon(w)
980297#97	England	Rainbow trout(f)	DR-128	British Columbia, Canada	Rainbow trout(w)
F3	England	Rainbow trout(f)	RS-TSA	Nova Scotia, Canada	Atlantic salmon(f)
F47	England	Rainbow trout(f)	AcF6-1[d,g]	Northwest Territories, Canada	Arctic charr(w)
F60	England	Rainbow trout(f)	F-120-87(P-2)	Iceland	Atlantic salmon(f)
F82	England	Rainbow trout(f)	F-130-87(P-4)	Iceland	Rainbow trout(f)
F85	England	Rainbow trout(f)	F-138-87(0-78)	Iceland	Atlantic salmon(f)
F95	England	Rainbow trout(f)	F-260-87(P-16)	Iceland	Atlantic salmon(f)
970083-88[f]	Southern England	Rainbow trout(f)	F-273-87(P-19)	Iceland	Atlantic salmon(f)
970083-102	Southern England	Rainbow trout(f)	F-283-87(P-10)[c]	Iceland	Atlantic salmon(f)
980106 #1.1.5[b,f]	Southern England	Rainbow trout(f)	F-358-87(P-13)[c]	Iceland	Atlantic salmon(w)
970419-1.2.3[b]	Southern England	Atlantic salmon(w)	S-182-90(P-7)[c,f]	Iceland	Atlantic salmon(f)
970153-19[b,f]	Southern England	Grayling(w)	Siletz	Oregon, USA	Coho salmon(f)
A6	Southern England	Rainbow trout(f)	Marion Forks[b,f]	Oregon, USA	Chinook salmon(f)
A80	Southern England	Rainbow trout(f)	Little Goose	Washington, USA	Chinook salmon(f)
W2	Northern England	Rainbow trout(f)	CCM6205	Washington, USA	Coho salmon(f)
W6[b]	Northern England	Rainbow trout(f)	84-019-OC	Washington, USA	Chinook salmon(w)
WMV1	Southern England	Rainbow trout(f)	SS-ChS-94-1	Oregon, USA	Chinook salmon
MT409	Scotland	Unknown	Cow ChS94 P22	Washington, USA	Chinook salmon(f)
MT417[b]	Scotland	Atlantic salmon(f)	Idaho 91-126	Idaho, USA	Sockeye salmon(f)
MT239	Scotland	Atlantic salmon(f)	RFL-643.94 #1	Washington, USA	Sockeye salmon(f)
MT426	Scotland	Unknown	CCM6206	Oregon, USA	Chinook salmon(f)
NCIMB1111	Scotland	Atlantic salmon(w)	Round Butte	Oregon, USA	Chinook salmon(f)
NCIMB1112	Scotland	Atlantic salmon(w)	NCIMB2196	Wyoming, USA	Brook trout(f)
NCIMB1113	Scotland	Atlantic salmon(w)	ATCC33209[b,f]	Oregon, USA	Chinook salmon(f)
NCIMB1114[e,f]	Scotland	Atlantic salmon(w)	D-6	Oregon, USA	Coho salmon
NCIMB1115	Scotland	Atlantic salmon(w)	Cole River	Oregon, USA	Unknown
NCIMB1116[e]	Scotland	Atlantic salmon(w)	Looking Glass	Oregon, USA	Unknown
MT420	Scotland	Atlantic salmon(f)	BY1996[b]	Alaska	Chinook salmon(f)
MT452	Scotland	Rainbow trout(f)	Rs 9[b]	Sweden	Rainbow trout
MT1363	Scotland	Rainbow trout(f)	Rs 19	Sweden	Atlantic salmon
MT410	Scotland	Unknown	Rs 61	Sweden	Arctic char
FT-10	Scotland	Atlantic salmon	Rs 116	Sweden	Grayling
BA99	Scotland	Atlantic salmon	Rs 122	Sweden	Rainbow trout(f)
DR143[b]	Alberta, Canada	Brook trout(f)	Rs 125	Sweden	Rainbow trout
DR384	British Columbia, Canada	Coho salmon(f)	Rs 126	Sweden	Rainbow trout
3784	British Columbia, Canada	Sockeye salmon(f)	3015-86[b]	Norway	Atlantic salmon
980002	British Columbia, Canada	Chinook salmon(f)	4451-86[e]	Norway	Atlantic salmon
960023	British Columbia, Canada	Coho salmon(f)	Iwate[c,f]	Japan	Coho salmon

[a] Isolates were obtained from wild fish (w) or farm (captive reared) fish (f) sources. The complete histories of some isolates are not known.

[b] ITS1 sequevar 1 isolates. GenBank accession nos. AF093461, AF093462, AF093465, AF093467, AF093470, AF093473.

[c] ITS1 sequevar 2 isolates. GenBank accession nos. AF093463, AF093474, AF179001, and AF179002.

[d] ITS1 sequevar 3 isolates. GenBank accession no. AF093464.

[e] ITS1 sequevar 4 isolates. Gen Bank accession nos. AF178998, AF178999, and AF179000.

[f] ITS2 sequevar 21 isolates. Gen Bank accession nos. AF143477-AF143485.

[g] ITS2 sequevar 22 isolate. Gen Bank accession no. AF143486.

```
SV21  ACCTCACCGATAAACCCACCCTTCAAAAGGGGTTTATCACCAAAGGTAAGTGTAAAAACCTGCATGCACGCGTCCACTCTACGGTTCCCAACCAACAAAC 100
SV22  ----------------------------------T-------------------------------------------------------------

SV21  CCACACCTAAAAAGGGTAGCAACGGTTCGTTGACACGGAAACCACATAACTACATACACGCAACACCAAACACTTCACACCACCACACCCCACAACAGGG 200
SV22  -------C-------------------------------------------------------------------------------------------

SV21  TGCGGACCGGTAAAAAAGA                                                                                  219
SV22  --------------------
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Figure 3. Nucleotide sequences of two sequevars, SV21 and SV22, of the 23S–5S rRNA ITS of *R. salmoninarum*. The isolates with each sequevar are identified in Table 1. The sequence of the region was determined for 10 isolates by using PCR-amplified products obtained with primers R5+118 and R5-433.

overlaid with mineral oil (Sigma), incubated at 96 °C for 2 min, then subjected to 35 cycles of 96 °C for 30s, 65 °C for 30s, 72 °C for 90s. Amplification products were analysed in 1.5% agarose gels. Using a cycle sequencing method, both strands of all PCR products spanning the 5′-flanking region of the 16S rRNA gene and the 3′ tail of the 5S rRNA gene were sequenced directly by MWG-Biotech Ltd; Milton Keynes, UK.

Sequence variation within the 23S–5S rRNA intergenic spacer

The ITS2 was amplified using the *R. salmoninarum* sequence specific primers R5+118 (5′-CTGACCGGTACTAATAGGCCAACA-3′) and R5-433 (5′-GTCTTAGCTTCCGGGTTCGAGATG-3′) in order to determine the variation within the 23S–5S rRNA intergenic region of the tested strains. The reaction mixture (50 μl) contained 1 unit *Taq* polymerase (Boehringer Mannheim), reaction buffer (Boehringer Mannheim), 1.5 mM $MgCl_2$, 24 pmol of each primer, 0.2 mM of each dNTP and 10 ng bacterial DNA. The reaction mixtures were overlaid with mineral oil (Sigma), incubated at 96 °C for 2 min, then subjected to 35 cycles of 96 °C for 30s, 65 °C for 30s, 72 °C for 90s. Amplification products were analysed on 1.5% agarose gels. The ITS2 sequences of ten *R. salmoninarum* isolates were determined by MWG-Biotech Ltd, Milton Keynes, UK by directly sequencing both strands of PCR-amplified products.

DNA restriction and hybridisation

Restriction enzymes (Boehringer Mannheim) were used, as recommended by the manufacturer. Genomic DNA digests were separated by electrophoresis in 0.8% agarose gels and transferred to positively charged nylon membranes (Boehringer Mannheim) by capillary blotting. Two rDNA probes were used in the hybridisation experiments (Figure 1). Firstly, a DNA probe spanning 1.2 kb of the 5′ end of the *R. salmoninarum* 23S rRNA gene was generated by PCR using primer RsF1+184 (5′-GAACGTGGGGAACTGAAACATCTC-3′) and primer RsR3-1358 (5′-CTTTCGCTACTCATGCCTGC-ATTC-3′). Secondly, a DNA probe spanning 724 bp of the *R. salmoninarum* 16S rRNA gene was prepared using primer R16+774 (5′-CAGGATTAGATACCCTG-GTAGTCC-3′) and primer R16-1491 (5′-TACGACT-TAGTCCCAATCGCCAGT-3′). Both probes were labeled with digoxigenin dUTP. Hybridisation was performed with a digoxigenin kit (Boehringer Mannheim) following the manufacturer's instructions.

Sequence analysis

R. salmoninarum sequences were compared with those from other organisms on the GenBank database using the gapped BLAST program (Altschul et al. 1997). The 16S and 5S rRNA gene sequences of a selection of closely related bacteria were compared using the ALIGNn program (Myers & Miller 1988) available at: http://www.infobiogen.fr/services/analyseq/cgi-bin/alignn_in.pl (InfoBiogen, France). The sequences of 16S rRNA genes from representatives of a range of actinomycete species most similar to *R. salmoninarum* were obtained from the GenBank database and aligned using the PHYLIP phylogenetic inference package version 3.5c (Felsenstein 1989) available at: http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html. The alignments were constructed from sequence data for positions that were present in all of the strains analysed, covering 1441 nucleotides. The maximum likelihood method was used to compute an evolutionary distance matrix that provided a maximum likelihood estimate of the divergence time (total branch length) between the sequences. The topologies of the trees were evaluated using bootstrapping by resampling the data 100 times. An unrooted tree was constructed using the neighbour-joining method (Saitou & Nei 1987). The tree was plotted using the Drawtree program.

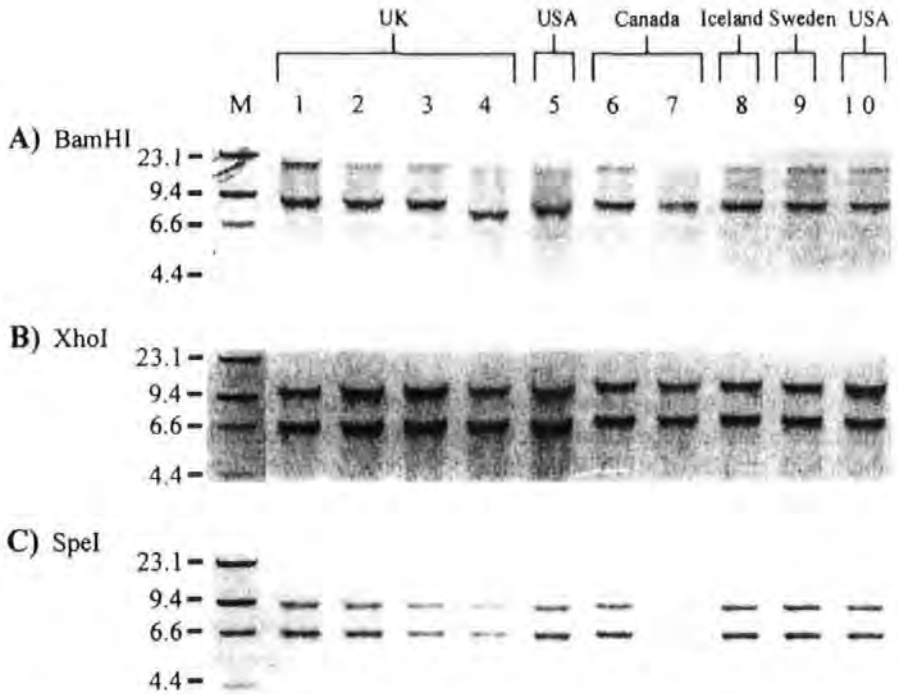

Figure 4. Southern blot hybridisations of total DNA from different strains of *R. salmoninarum* isolated from a variety of geographic origins and host species with a 1.2 kb 23S rDNA probe. Genomic DNA was digested with A) *Bam*HI, B) *Xho*I, and C) *Spe*I. Lanes 1 to 10, isolates 1) 970083-88, 2) 980036-150, 3) 980036-87, 4) NCIMB1114, 5) ATCC33209, 6) AcF6-1, 7) DR143, 8) F-273-87 (P-19), 9) RS61, 10) BY1996. Lane M, markers (Boehringer Mannheim). The molecular sizes (in kilobases) are indicated on the left. The geographic origins of the isolates are indicated at the top.

Results and discussion

Sequencing of the 16S, 23S and 5S rRNA genes and sequence analysis

The complete 16S rDNA, 23S rDNA, 5S rDNA and 23S–5S rDNA spacer region was sequenced for *R. salmoninarum* ATCC33209^T by directly sequencing PCR-amplified products. Fifteen sets of primer pairs were used to amplify a series of overlapping PCR products that completely covered the full length sequence (Figure 1). The presence of DNA encoding rRNA genes was confirmed by comparison of the *R. salmoninarum* sequences with nucleotide sequences from the GenBank database. The 5′ and 3′ ends of the *R. salmoninarum* rRNA genes were assigned on the basis of alignments with the rRNA genes from the most similar organisms and the ambiguities present in the previous incomplete sequence for the 16S rRNA gene of strain ATCC33209^T were resolved. The analysis confirmed the close relationship between *R. salmoninarum* and *Arthrobacter* species and showed that the rRNA genes of *R. salmoninarum* are arranged in the order 16S-ITS1-23S-ITS2-5S (GenBank accession nos. AF143477, AF180950).

The *R. salmoninarum* 16S rRNA gene is composed of 1540 nucleotides (G+C content 55.2 mol%). When ambiguous nucleotides were removed from the previously determined incomplete sequence for the gene (Gutenberger et al. 1991) an exact match was obtained in the regions where overlap occurred. The total length of the 23S rRNA gene was determined to be 3135 nucleotides (G+C content 54.1 mol%), separated by a 219 bp intergenic spacer (G+C content 48.9 mol%) from the 5S rRNA gene of 120 nucleotides (G+C content 65.0 mol%). An exact match with the nucleotide sequence of a common insertion fragment of 112 bp, which was previously identified within the 23S rRNA genes of many gram-positive bacteria with a high G+C content, including *R. salmoninarum* (Roller *et al.* 1992), was located from nucleotide 1537 to 1648. No tRNA genes or pseudogenes were identified either within the ITS2 or within the 176 nucleotide sequence following the 3′ end of the 5S rRNA gene. An inver-

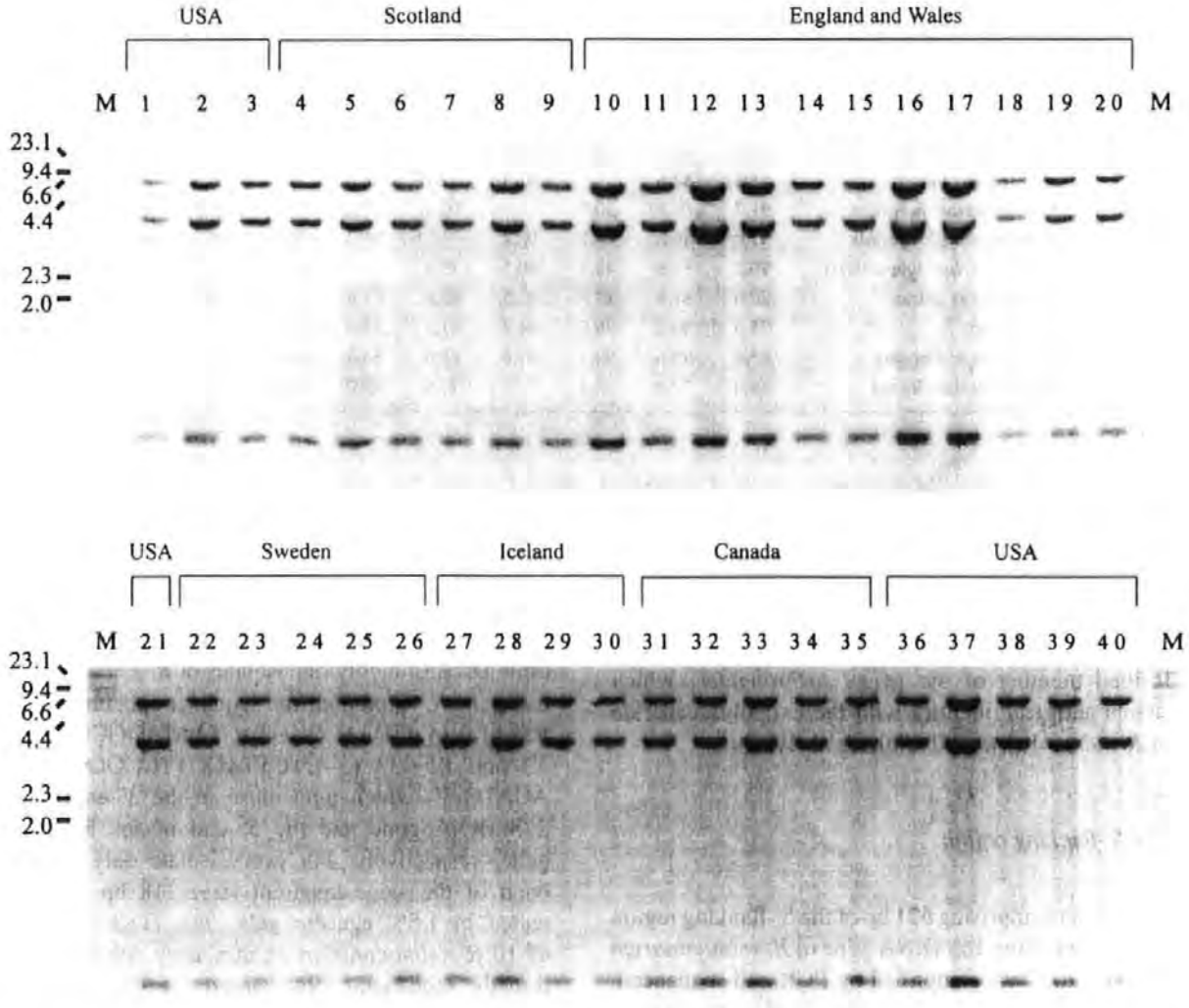

Figure 5. Southern blot hybridisations of total DNA from different strains of *R. salmoninarum* isolated from a variety of geographic origins and host species with a 724 bp 16S rDNA probe. Genomic DNA was digested with *Aval*. Lanes 1 to 20, isolates 1) SS-ChS-94-1, 2) CCM6205, 3) Siletz, 4) NCIMB1116, 5) NCIMB1115, 6) NCIMB1114, 7) NCIMB1113, 8) NCIMB1112, 9) NCIMB1111, 10) F3, 11) F47, 12) F60, 13) F82, 14) F85, 15) F95, 16) 980297#97, 17) A80, 18) A6, 19) 970083-102, 20) 970083-88. Lanes 21-40, isolates 21) BY1996, 22) RS125, 23) RS122, 24) RS116, 25) RS61, 26) RS19, 27) S-182-90, 28) F-283-87, 29) F-273-87, 30) F-120-87, 31) 960046, 32) 960023, 33) 980002, 34) DR143, 35) AcF6-1, 36) Round Butte, 37) CCM6206, 38) RFL-643.94#1, 39) Idaho 91-126, 40) Cow ChS 94 P-22. Lane M, markers (Boehringer Mannheim). The molecular sizes (in kilobases) are indicated on the left. The geographic origins of the isolates are indicated above each blot.

ted repeat (5′-*aataaaggtcccac*aacgac*gtgggacctttatt*-3′) was located 9 bp downstream from the 3′ end of the 5S rRNA gene. This region, which could form a hairpin structure consisting of a perfectly matched stem of 14 bp and a loop of 6 bp, was followed by a U-rich region that is typical of a transcriptional termination sequence.

Pairwise alignment of the *R. salmoninarum* rRNA gene sequences with the nucleotide sequences of either 16S or 5S rRNA genes from other bacteria available on the GenBank database and the construction of phylogenetic trees confirmed the close relationship with the high G+C group of actinobacteria, particularly *Arthrobacter* species and *Micrococcus luteus* (Table 2, Figure 2). The close genetic relationship between *R. salmoninarum* and *Arthrobacter* species is reflected in other important shared features, notably obligate aerobic metabolism and the possession

Table 2. Percentage identity calculated from pairwise alignments between the 16S and 5S rRNA genes of *R. salmoninarum* and other taxa obtained from the GenBank database: 16S rRNA gene (upper right) and 5S rRNA gene (lower left)

	Species	1	2	3	4	5	6	7	8	9	10
1.	*Renibacterium salmoninarum*	-	85.3	89.9	87.3	91.1	83.3	85.6	84.2	88.5	81.3
2.	*Nocardia asteroides*	87.6	-	88.2	87.4	87.0	90.9	88.5	87.7	91.9	87.8
3.	*Micrococcus luteus*	88.3	82.6	-	91.3	92.7	86.5	88.8	86.5	86.0	83.7
4.	*Brevibacterium helvolum*	91.7	89.3	90.0	-	90.1	85.3	88.7	86.7	85.7	83.1
5.	*Arthrobacter globiformis*	91.8	89.3	90.2	96.7	-	85.0	87.8	88.8	85.3	83.6
6.	*Mycobacterium tuberculosis*	79.2	81.8	78.5	80.3	79.8	-	85.5	85.3	87.6	94.8
7.	*Streptomyces griseus*	80.0	81.8	82.5	82.5	82.8	75.8	-	86.7	86.5	83.0
8.	*Frankia* sp.	78.3	80.2	79.8	81.7	81.1	73.0	86.7	-	86.5	84.6
9.	*Rhodococcus fascians*	88.4	92.6	84.3	87.6	88.6	84.3	86.7	81.8	-	85.4
10.	*Mycobacterium leprae*	76.0	77.9	75.2	77.7	77.9	89.0	78.9	74.0	80.3	-

of cell walls containing A3α peptidoglycan, lysine as the diamino acid, but lacking mycolic acids (Kusser & Fiedler 1983; Goodfellow et al. 1985). Of particular interest is the close relationship that exists between *R. salmoninarum* and RsxII, a recently described member of the genus *Arthrobacter*, which shares antigenic identity with the exopolysaccharide of *R. salmoninarum* (Griffiths et al. 1998).

The 5′-flanking region

The region comprising 621 bp of the 5′-flanking region and 135 bp of the 16S rRNA gene of *R. salmoninarum* ATCC33209T was amplified by PCR and sequenced. Interestingly, an element was identified upstream from the 16S rRNA coding region which shares 100% identity with an element (CL2; 5′ TGT TGT TTG AGA ACT CAA TAG TGT GT 3′) comprising Box A and Box C, involved in antitermination, and an RNAse III cleavage site in members of *Mycobacterium* species (Ji et al. 1994a, b; Gonzalez-y-Merchand et al. 1996, 1997). CL2 is considered to be confined to members of the genus *Mycobacterium* (Gonzalez-y-Merchand et al. 1997) and its presence suggests that *R. salmoninarum* shares a similar mechanism for the control of transcription. However, unlike mycobacteria, the *R. salmoninarum* 5′ leader region did not contain CL1 or Box B motifs and whilst putative -35 and -10 promoter regions were identified there was no evidence for replicate or 'additional' promoters.

Sequence variation within the 23S–5S rRNA intergenic spacer

ITS2 was amplified in a PCR reaction from the genomic DNA of eighty one isolates of *R. salmoninarum* using the *R. salmoninarum* sequence specific primers R5+118 (5′-CTGACCGGTACTAATAGGCCAACA-3′) and R5-433 (5′-GTCTTAGCTTCCGGGTTCG-AGATG-3′) which bind close to the 3′ end of the 23S rRNA gene and the 5′ end of the 5S rRNA gene, respectively. For every isolate only a single band of the same apparent size, 318 bp, was detected in 1.5% agarose gels. The ITS2 sequences of 10 *R. salmoninarum* isolates were determined by directly sequencing both strands of PCR-amplified products and were found to be the same length, 219 bp (Figure 3). Furthermore, nine isolates possessed an identical nucleotide sequence, which was designated sequevar 21 (SV21; GenBank accession nos. AF143477–AF143485). These nine isolates were sourced from England, Scotland, Wales, Iceland, Japan, and the United States, and from various host species, including rainbow trout, Atlantic salmon, chinook salmon, and grayling.

A single isolate, AcF6-1, sourced from Arctic char from the Northwest Territories of Canada, was found to differ at two positions and the ITS2 sequence of this isolate was designated sequevar 22 (SV22; GenBank accession no. AF143486). This result supports the finding of previous work showing that the ITS1 sequence of *R. salmoninarum* is highly conserved though some small variation exists in isolates derived from areas of the world which are relatively isolated from mainstream intensive salmonid cultiva-

tion (Grayson et al. 1999, 2000). Four ITS1 sequence variants that are of identical length, SV1, SV2, SV3, and SV4, have been previously identified from 19 *R. salmoninarum* isolates and members of each of these sequevars were included in this study. Only the single isolate from Northwest Territories of Canada (SV3) showed sequence variation in ITS2 and this is probably a reflection of the smaller size of ITS2, 219 bp, compared with ITS1, 534 bp. Following an exhaustive search of the GenBank database, the ITS2 sequence appears to be unique and is highly conserved among *R. salmoninarum* isolates from different countries and salmonid host species, therefore it is potentially useful for the development of a specific oligonucleotide probe for the identification of *R. salmoninarum*.

Determination of the number of rRNA genes

The full length of the *R. salmoninarum* rRNA operon, 6345 bp, was mapped using both the sequence data generated above and previous sequence data for the ITS1 (Grayson et al. 1999). The 6345 bp DNA sequence that covered the rRNA operon of *R. salmoninarum* contains neither *Stu*I nor *Bam*HI sites (Figure 1). Therefore, we hybridised blots of either *Stu*I or *Bam*HI-digested genomic DNA from ten isolates of *R. salmoninarum*, which had a broad geographic derivation, with the 23S rDNA probe. Digestion of *R. salmoninarum* genomic DNA with *Stu*I revealed a single band in all isolates of approximately 25 kb (data not shown) whilst two positive *Bam*HI bands were obtained with the DNA from all ten isolates (Figure 4A). The largest of the *Bam*HI bands was estimated to be 19.7 kb in all the isolates and the estimated size of the smaller band was 8.3 kb in seven isolates, 7.2 kb in ATCC33209^T, and 7.0 kb in isolate NCIMB1114. We assessed whether a different restriction pattern was produced using enzymes *Xho*I and *Spe*I which cut the *R. salmoninarum* rRNA operon at single positions close to the 3′ end of the 23S rRNA gene. Two positive bands were obtained with *Xho*I in all isolates, which varied within the range 10.9–11.1 kb and 6.8–7.0 kb (Figure 4B). Similarly, two bands were obtained with *Spe*I and the sizes of these were estimated to be 9.2 kb and 6.6 kb in all isolates (Figure 4C). These findings suggest that the *R. salmoninarum* genome contains two copies of the *rrn* operon. In order to confirm this we used a 16S rDNA probe hybridised with genomic DNA from 40 isolates obtained from a variety of countries in Europe and North America which had been digested with *Ava*I. *Ava*I cleaves the *R. salmoninarum* operon at seven points, one of these lies 118 bp within the 3′ end of the region to which the 16S rDNA probe hybridises (Figure 1). In every case three identical bands were obtained for each isolate. The bands represent two fragments of 6.5 kb and 3.5 kb corresponding to the two operons and the third fragment of 775 bp includes the 3′ end of the 16S rRNA gene and the complete ITS1, which must be of an identical size within each operon (Figure 5). This proves conclusively that *R. salmoninarum* strains possess two copies of the rRNA operon and confirms previous work which showed that *R. salmoninarum* possesses one size of ITS1, 534 bp (Grayson et al. 1999).

The rRNA operons were designated *rrnA* and *rrnB* on the basis of the descending sizes of the *Bam*HI bands. Many other slowly growing pathogens have one or two copies of the rRNA operon (Amikam et al. 1984; Bercovier et al. 1986; Frothingham & Wilson 1993; Sela et al. 1989; van der Giessen et al. 1994). The remarkable phenotypic and genotypic uniformity amongst *R. salmoninarum* isolates from diverse sources reflects the slowly growing and intracellular existence of this fastidious organism (Fryer & Sanders, 1981).

Acknowledgements

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References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402.

Amikam D, Glaser G & Razin S (1984) Mycoplasmas (*Mollicutes*) have a low number of rRNA genes. J Bacteriol 158: 376–378.

Austin B, Embley TM & Goodfellow M (1983) Selective isolation of *Renibacterium salmoninarum*. FEMS Microbiol Lett 17: 111–114.

Banner CR, Rohovec JS & Fryer JL (1991) A new value for mol percent guanine + cytosine of DNA for the salmonid fish pathogen *Renibacterium salmoninarum*. FEMS Microbiol Lett 79: 57–60.

Barry T, Colleran G, Glennon M, Dunican LK & Gannon F (1991) The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. PCR Methods Appl 1: 51–56.

Bercovier H, Kafri O & Sela S (1986) Mycobacteria possess a surprisingly small number of ribosomal RNA genes in relation to the size of their genome. Biochem Biophys Res Commun 136: 1136–1141.

Dams E, Yamada T, De Baere R, Huysmans E, Vandenberghe A & De Wachter R (1987) Structure of 5S rRNA in actinomycetes and relatives and evolution of eubacteria. J Mol Evol 25: 255–260.

Dennis PP, Ziesche S & Mylvaganam S (1998) Transcription analysis of two disparate rRNA operons in the halophilic archaeon *Haloarcula marismortui*. J Bacteriol 180: 4804–4813.

Embley TM, Goodfellow M, Minnikin DE & Austin B (1983) Fatty acid, isoprenoid quinone and polar lipid composition in the classification of *Renibacterium salmoninarum*. J Appl Bacteriol 55: 31–37.

Engels WR (1993) Contributing software to the Internet: the Amplify program. TIBS 18: 448–450.

Evelyn TPT (1993) Bacterial kidney disease-BKD. In: Inglis V, Roberts RJ & Bromage NR (Ed) Bacterial diseases of fish, pp.177–195. Blackwell, Oxford.

Evelyn TPT, Prosperi-Porta L & Ketcheson JE (1990) Two new techniques for obtaining consistent results when growing *Renibacterium salmoninarum* on KDM2 culture medium. Dis Aquat Org 9: 209–212.

Evenden AJ, Grayson TH, Gilpin ML & Munn CB (1993) *Renibacterium salmoninarum* and bacterial kidney disease – the unfinished jigsaw. Ann Rev Fish Dis 3: 87–104.

Felsenstein J (1989) PHYLIP-phylogeny inference package (version 3.5). Cladistics 5: 164–166.

Fiedler F & Draxl R (1986) Biochemical and immunochemical properties of the cell surface of *Renibacterium salmoninarum*. J Bacteriol 168: 799–804.

Frothingham R & Wilson KH (1993) Sequence-based differentiation of strains in the *Mycobacterium avium* complex. J Bacteriol 175: 2818–2825.

Fryer JL & Sanders JE (1981) Bacterial kidney disease of salmonid fish. Annu Rev Microbiol 35: 273–298.

Gonzalez-y-Merchand JA, Colston MJ & Cox RA (1996) The rRNA operons of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*: comparison of promoter elements and of neighbouring upstream genes. Microbiology-UK 142: 667–674.

Gonzalez-y-Merchand JA, Garcia MJ, Gonzalez-Rico S, Colston MJ & Cox RA (1997) Strategies used by pathogenic and non-pathogenic mycobacteria to synthesize rRNA. J Bacteriol 179: 6949–6958.

Goodfellow M, Embley TM & Austin B (1985) Numerical taxonomy and emended description of *Renibacterium salmoninarum*. J Gen Microbiol 131: 2739–2752.

Grayson TH, Atienzar FA, Alexander SA, Cooper LF & Gilpin ML (2000) Molecular diversity of *Renibacterium salmoninarum* isolates determined by randomly amplified polymorphic DNA. Appl Environ Microbiol 66: 435–438.

Grayson TH, Cooper LF, Atienzar FA, Knowles MR & Gilpin ML (1999) Molecular differentiation of *Renibacterium salmoninarum* isolates from worldwide locations. Appl Environ Microbiol 65: 961–968.

Griffiths SG, Melville KJ & Salonius K (1998) Reduction of *Renibacterium salmoninarum* culture activity in Atlantic salmon following vaccination with avirulent strains. Fish Shellfish Immunol 8: 607–619.

Gurtler V & Stanisich VA (1996) New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. Microbiology-UK 142: 3–16.

Gutenberger SK, Giovannoni SJ, Field KG, Fryer JL & Rohovec JS (1991) A phylogenetic comparison of the 16S rRNA sequence of the fish pathogen, *Renibacterium salmoninarum*, to Gram-positive bacteria. FEMS Microbiol Lett 77: 151–156.

Jensen MA, Webster JA & Straus N (1993) Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl Environ Microbiol 59: 945–952.

Ji Y, Colston MJ & Cox RA (1994a) Nucleotide sequence and secondary structures of precursor 16S rRNA of slow-growing mycobacteria. Microbiology-UK 140: 123–132.

Ji Y, Kempsell KE, Colston MJ & Cox RA (1994b) Nucleotide sequences of the spacer-1, spacer-2 and trailer regions of the *rrn* operons and secondary structures of the precursor 23S rRNAs and precursor 5S rRNAs of slow-growing mycobacteria. Microbiology-UK 140: 1763–1773.

Koch C, Rainey FA & Stackebrandt E (1994) 16S rDNA studies on members of *Arthrobacter* and *Micrococcus*: an aid for their future taxonomic restructuring. FEMS Microbiol Lett 123: 167–172.

Kusser W & Fiedler F (1983) Murein type and polysaccharide composition of cell walls from *Renibacterium salmoninarum*. FEMS Microbiol Lett 20: 391–394.

Leblond-Bourget N, Philippe H, Mangin I & Decaris B (1996) 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium* phylogeny. Int J Syst Bacteriol 46: 102–111.

Ludwig W & Schleifer KH (1994) Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. FEMS Microbiol Rev 15: 155–173.

Magnusson HB, Fridjonsson OH, Andresson OS, Benediktsdottir E, Gudmundsdottir S & Andresdottir V (1994) *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in salmonid fish, detected by nested reverse transcription-PCR of 16S rRNA sequences. Appl Environ Microbiol 60: 4580–4583.

Myers E & Miller W (1988) Optimal alignments in linear space. Comput Appl Biosci 4: 11–17.

Nour M (1998) 16S–23S and 23S–5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. Res Microbiol 149: 433–448.

Olsen GJ, Larsen N & Woese CR (1991) The ribosomal RNA database project. Nucleic Acids Res 19 (suppl.): 2017–2021.

Park Y-H, Yim D-G, Yim E, Kho Y-H, Mheen T-I, Lonsdale J & Goodfellow M (1991) Classification of acidophilic, neutrotolerant and neutrophilic streptomycetes by nucleotide sequencing of 5S ribosomal RNA. J Gen Microbiol 137: 2265–2269.

Roller C, Ludwig W & Schleifer KH (1992) Gram-positive bacteria with a high DNA G+C content are characterized by a common insertion sequence within their 23S rRNA genes. J Gen Microbiol 138: 1167–1175.

Saitou N & Nei M (1987) The neighbor-joining method – a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425.

Sela S, Clark-Curtiss JE & Bercovier H (1989) Characterization and taxonomic implications of the rRNA genes of *Mycobacterium leprae*. J Bacteriol 171: 70–73.

Stackebrandt E, Wehmeyer U, Nader H & Fiedler F (1988) Phylogenetic relationship of the fish pathogenic *Renibacterium salmoninarum* to *Arthrobacter*, *Micrococcus* and related taxa. FEMS Microbiol Lett 50: 117–120.

Starliper CE (1996) Genetic diversity of North American isolates of *Renibacterium salmoninarum*. Dis Aquat Org 27: 207–213.

Syzmanski M, Specht T, Barciszewska MZ, Barciszewski J & Erdmann VA (1998) 5S rRNA data bank. Nucleic Acids Res 26: 156–159.

Van der Giessen JWB, Haring RM & van der Zeijst BAM (1994) Comparison of the 23S ribosomal RNA genes and the spacer region between the 16S and 23S rRNA genes of the closely related *Mycobacterium avium* and *Mycobacterium paratuberculosis* and the fast-growing *Mycobacterium phlei*. Microbiology-UK 140: 1103–1108.

Wang Y, Zhang Z & Ramanan N (1997) The actinomycete *Thermobispora bispora* contains two distinct types of transcriptionally active 16S rRNA genes. J Bacteriol 179: 3270–3276.

Yap WH, Zhang Z & Wang Y (1999) Distinct types of rRNA operons exist in the genome of the actinomycete *Thermomonospora chromogena* and evidence for horizontal transfer of an entire rRNA operon. J Bacteriol 181: 5201–5209.

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Description and characterization of IS*994*, a putative IS*3* family insertion sequence from the salmon pathogen, *Renibacterium salmoninarum*

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Abstract

Renibacterium salmoninarum, a slowly growing, Gram-positive bacterium, is responsible for bacterial kidney disease in salmonid fishes world-wide. To date, no mobile genetic elements have been reported for this pathogen. Here, we describe the first insertion sequence (IS) identified from *R. salmoninarum*. This element, IS*994*, has a significant predicted amino acid sequence homology (64.8 and 71.9%) to the two open reading frames encoding the transposase of IS*6110* of *Mycobacterium tuberculosis*. Protein parsimony and protein distance matrix analyses show that IS*994* is a member of group IS*51* of the IS*3* family. From a conservative estimate, there are at least 17 chromosomal insertions of IS*994* or closely related elements. Sequence analysis of seven of these loci reveals single nucleotide polymorphisms throughout the element (including the terminal inverted repeats), a 15 bp insertion in three of the seven loci, and an absence of flanking direct repeats or conserved insertion site. Restriction fragment length polymorphism analysis of *Xba*I-digested chromosomal DNA shows variations among European and North American isolates, indicating that IS*994* may be a useful molecular marker for epizootiological studies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Bacteria; Fish; Polymorphisms; RFLP; Teleost

1. Introduction

Bacterial kidney disease (BKD) is a debilitating disease of salmonid fishes that is caused by the Gram-positive diplococcobacillus, *Renibacterium salmoninarum*. Although this pathogen has been isolated from salmonids world-wide (Fryer and Sanders, 1981), its fastidious requirements and very slow growth in culture have hindered efforts to uncover the molecular mechanisms of virulence and pathogenesis. The manifestation of BKD can be acute or chronic, and the disease can be transmitted either horizontally or vertically (Evelyn et al., 1984; Balfry et al., 1996). Furthermore, infected fish can be asymptomatic, resulting in carrier fish that can serve as a potential source of *R. salmoninarum*, in both captive and free-ranging populations. Studies on the epizootiology of *R. salmoninarum* have been hampered by genetic homogeneity among isolates (Bruno and Munro, 1986; Starliper, 1996). Randomly amplified polymorphic DNA (RAPD) analyses have been developed to differentiate isolates of *R. salmoninarum* (Grayson et al., 1999), but additional useful markers, such as insertion sequences (ISs), have not yet been reported.

Although bacterial ISs are the most simple of mobile genetic elements, they are capable of effecting significant changes in the host genome. These changes, such as insertional mutagenesis, alteration of transcriptional activity, and genomic rearrangements, result from transpositional activity of the IS. The IS*3* family is the largest family of bacterial ISs [approximately one-tenth of the non-isoform bacterial elements belong to the IS*3* family (Mahillon and Chandler, 1998)], and IS*3* family elements have been found in both Gram-negative and Gram-positive bacterial species. Here, we report a description of the first IS element isolated from *R. salmoninarum*. We present evidence that it is a member

Abbreviations: bp, base pair(s); IS, insertion sequence; kb, kilobase; MSA, major soluble antigen; ORF, open reading frame; PCR, polymerase chain reaction; RAPD, randomly amplified polymorphic DNA; RFLP, restriction fragment length polymorphism.

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of the IS*3* family and demonstrate its utility as a marker for isolate differentiation.

2. Methods and materials

During an unrelated screen of *R. salmoninarum* chromosomal DNA for sequences related to thiol-activated cytolysins, candidate bands that hybridized to a listeriolysin O probe were identified. These candidate bands were directly cloned from size-selected *Eco*RI or *Hin*dIII fragments of *R. salmoninarum* (ATCC type 33209) chromosomal DNA. Fragments were ligated into *Eco*RI- or *Hin*dIII-digested pZErO-2.1 (InVitrogen, Carlsbad, CA), and *E. coli* Top10F′ cells (InVitrogen) were transformed with the ligation products. More than 2000 colonies were screened by hybridization of colony lifts on Hybond-N nylon membrane (Amersham, Arlington Heights, IL) with a 374 bp, digoxygenin-labeled PCR probe encompassing nt 1454–1827 of the wild-type listeriolysin O gene (Domann and Chakraborty, 1989). (This region includes codons for the undecapeptide that is conserved among thiol-activated cytolysins.) Probe synthesis, hybridization, washes, and visualization were carried out according to the manufacturer's instructions for the DIG/Genius system (Boehringer Mannheim, Indianapolis, IN). Plasmid DNA from candidate clones was prepared by alkaline lysis miniprep (Sambrook et al., 1989) and screened by Southern hybridization using the probe and method described above. Clones containing highly related sequences were identified by serial Southern hybridization using digoxygenin-labeled, random primed probes synthesized from inserts of the candidate clones according to the manufacturer's instructions for high-stringency hybridization [hybridize at 42°C in 50% (v/v) formamide, 5× SSC, 0.1% (w/v) sarkosyl, 0.02% (w/v) SDS, 1% (w/v) BMB blocking reagent; wash at 65°C in 0.5× SSC containing 0.1% SDS] by the DIG/Genius system (Boehringer Mannheim). Among 19 candidate clones, nine were found to contain a common, highly related sequence. From seven of these nine clones, the entire IS*994* was analyzed by cycle sequencing double-stranded templates with the FS sequencing kit (Applied Biosystems, Foster City, CA) and resolving the labeled strands on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) at the Western Fisheries Research Center (Seattle, WA). These sequences have been deposited in the GenBank database under Accession Nos AF163688 through AF163694

Database searches were performed using the BLAST algorithms (Altschul et al., 1997), and optimized multiple alignments were generated (Genetics Computer Group, 1999). For subsequent analyses, predicted translations of the following IS elements were used (GenBank Accession No. in parentheses): IS*51* (M14365), IS*401* (L09108), IS*629* (X51586, AF074613), IS*868* (X55075), IS*911* (X17613), IS*1069* (X78469), IS*1137* (X70913), IS*1203* (U06468), IS*1206* (X69104), IS*1372* (U50076), IS*1601* (AF060182), IS*3411* (M19532), IS*6110* (X57835). Input protein sequences were bootstrapped 100 times. Protein parsimony analyses were performed using the PROTPARS algorithm, and distance matrices were computed using the Dayhoff PAM matrix (Dayhoff, 1979) method of the PROTDIST program of PHYLIP (Felsenstein, 1993). Trees were constructed by neighbor-joining (Saitou and Nei, 1987) and Fitch–Margoliash (Fitch and Margoliash, 1967) methods, using the respective programs of PHYLIP (Felsenstein, 1993). When possible, input data were randomized 10 times, and global branch-swapping was employed.

Isolates of *R. salmoninarum* used in this study are described in Table 1. The isolates are maintained in a culture collection at the University of Plymouth, in collaboration with CEFAS Weymouth, UK. Isolates were cultured in selective kidney disease medium (SKDM) broth supplemented with 5% spent broth culture at 15°C (Austin et al., 1983), then freeze-dried and checked for purity. The names and addresses of those who have provided these isolates, derived from clinical outbreaks of bacterial kidney disease, are provided in Table 1.

Genomic DNA was isolated using the Puregene D-6000 DNA isolation kit according to the manufacturer's instructions (Gentra Systems, Plymouth, MN) or by the alkaline lysis method (Sambrook et al., 1989). Extracted DNA was quantified using digital images taken after agarose gel electrophoresis as previously described (Grayson et al., 1999) or by fluorometry (Hoefer Scientific Instruments, San Francisco, CA). The identity of *R. salmoninarum* DNA was confirmed for all isolates by PCR using sets of primers specific for *R. salmoninarum* genes as previously described (Grayson et al., 1999). Restriction enzymes (Boehringer Mannheim; New England Biolabs, Beverly, MA) were used as recommended by the manufacturer. Digests of 0.5–1.0 mg of genomic DNA were separated by electrophoresis in 0.8% agarose gels and transferred to positively charged nylon membranes (Boehringer Mannheim) by capillary blotting. Membranes were exposed to short-wavelength UV light, then prehybridized and probed, following the manufacturer's instructions (Boehringer Mannheim), with a digoxygenin-labeled 1.2 kb *Xba*I–*Eco*RI fragment excised from a subclone of IS*994* (pMNT159-3-2). The probe was labeled with alkali-labile digoxygenin dUTP by the random prime method, and the labeled fragment was purified before use (Prep-a-Gene, BioRad, Hercules, CA). Membranes were hybridized at high stringency

Table 1
Isolates of *R. salmoninarum* used in this study

Isolate	Geographic origin	Host fish (year isolated) [a,b]	Source[c]
33209	USA	Chinook salmon (1974)	ATCC
MT239	Scotland	Atlantic salmon (1986)	D. Elliott
Rs 125	Sweden	Rainbow trout	E. Jansson
Rs 122	Sweden	Rainbow trout (1994)	E. Jansson
Rs 116	Sweden	Grayling (1993)	E. Jansson
Rs 61	Sweden	Arctic char (1989)	E. Jansson
Rs 19	Sweden	Atlantic salmon (1987)	E. Jansson
S-182-90	Iceland	Atlantic salmon (1990)	S. Gudmundsdottir
F-283-87	Iceland	Atlantic salmon (1987)	S. Gudmundsdottir
F-273-87	Iceland	Atlantic salmon (1987)	S. Gudmundsdottir
F-102-87	Iceland	Atlantic salmon (1987)	S. Gudmundsdottir
960046	Canada	Coho salmon (1996)	D. Kieser
960023	Canada	Coho salmon (1996)	D. Kieser
980002	Canada	Chinook salmon (1998)	D. Kieser
DR143	Canada	Brook trout (1972)	T. Evelyn
AcF6-1	Canada	Arctic char (1985)	B. Souter
BY1996	USA	Chinook salmon (1998)	T. Meyers, S. Short
Round Butte	USA	Chinook salmon (1973)	C. Banner
CCM6206	USA	Chinook salmon (1978)	CCM
RFL-643.94 #1	USA	Sockeye salmon (1994)	C. Banner
Idaho 91-126	USA	Sockeye salmon (1993)	C. Banner
Cow ChS 94	USA	Chinook salmon (1994)	C. Banner
SS-ChS-94-1	USA	Chinook salmon (1994)	C. Banner
CCM6205	USA	Coho salmon (1975)	CCM
Siletz	USA	Coho salmon (1976)	C. Banner
NCIMB1116	Scotland	Atlantic salmon (1962)	NCIMB
NCIMB1115	Scotland	Atlantic salmon (1962)	NCIMB
NCIMB1114	Scotland	Atlantic salmon (1962)	NCIMB
NCIMB1113	Scotland	Atlantic salmon (1962)[b]	NCIMB
NCIMB1112	Scotland	Atlantic salmon (1962)[b]	NCIMB
NCIMB1111	Scotland	Atlantic salmon (1962)[b]	NCIMB
F3	UK	Rainbow trout (1998)	E. Chambers
F47	UK	Rainbow trout (1998)	E. Chambers
F60	UK	Rainbow trout (1998)	E. Chambers
F82	UK	Rainbow trout (1998)	E. Chambers
F95	UK	Rainbow trout (1998)	E. Chambers
F85	UK	Rainbow trout (1998)	E. Chambers
980297#97	UK	Rainbow trout (1998)	E. Chambers
A-80	UK	Rainbow trout (1998)	CEFAS
A-6	UK	Rainbow trout (1998)	CEFAS
970083-102	UK	Rainbow trout (1997)	CEFAS
970083-88	UK	Rainbow trout (1997)	CEFAS

[a] If the date of isolation is uncertain, no date is shown.
[b] The species of the host fish is not definite, but the most likely species is shown.
[c] Explanation of collection source abbreviations: ATCC, American Type Culture Collection, Manassas, Virginia, USA; D. Elliott, Western Fisheries Research Center, US Geological Survey, Seattle, Washington, USA; E. Jansson, Uppsala University, Uppsala, Sweden; S. Gudmundsdottir, University of Iceland, Reykjavik, Iceland; D. Kieser, T. Evelyn, Pacific Biological Station, Department of Fisheries and Oceans, Nanaimo, BC, Canada; B. Souter, Freshwater Institute, Winnipeg, Manitoba, Canada; T. Meyers, S. Short, Alaska Department of Fish and Game, Juneau, Alaska, USA; C. Banner, Oregon State University, Corvallis, Oregon, USA; CCM, Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic; NCIMB, National Collections of Industrial, Food and Marine Bacteria, University of Aberdeen, Aberdeen, Scotland, UK; E. Chambers, CEFAS Laboratory, Weymouth, Dorset, UK; CEFAS, Centre for Environment, Fisheries and Aquaculture Science, Weymouth, Dorset, UK.

(68°C) overnight, and bands were visualized according to the manufacturer's instructions (Boehringer Mannheim). In order to confirm the identity of the genomic DNA digests, membranes were stripped according to the protocol supplied by the manufacturer (Boehringer Mannheim), then reprobed with a 16S rDNA probe specific to 724 bp of the 3′ region of the *R. salmoninarum* 16S rRNA gene. All isolates showed the same pattern of bands when examined in this manner.

3. Results

3.1. The element from R. salmoninarum is a member of the IS3 family

During an unrelated screen of the *R. salmoninarum* genome, nine clones were found to be related by nucleic acid hybridization, suggesting that these clones shared sequence identity. Sequence analysis of seven of the nine clones (Table 2) revealed that six clones contained a common element that was either 1330 or 1345 bp in size. The difference in size was due to an additional 15 bp sequence that was present in three of the clones (Fig. 1). A seventh clone (#138; Table 2) included an element that contained a single base insertion, resulting in an element of 1331 bp. The element in all of the clones contained 24 bp terminal inverted repeats that exhibited high sequence identity to the terminal repeats of IS*986*, IS*987*, and IS*6110*, members of the IS*3* family of bacterial insertion sequences.

Table 2
Seven clones used for sequence analysis

Clone No.	GenBank Accession No.	Size of IS*994* element (bp)
61	AF163694	1345
70	AF163693	1330
110	AF163688	1330
123	AF163689	1345
138	AF163690	1331
150	AF163691	1345
159	AF163692	1330

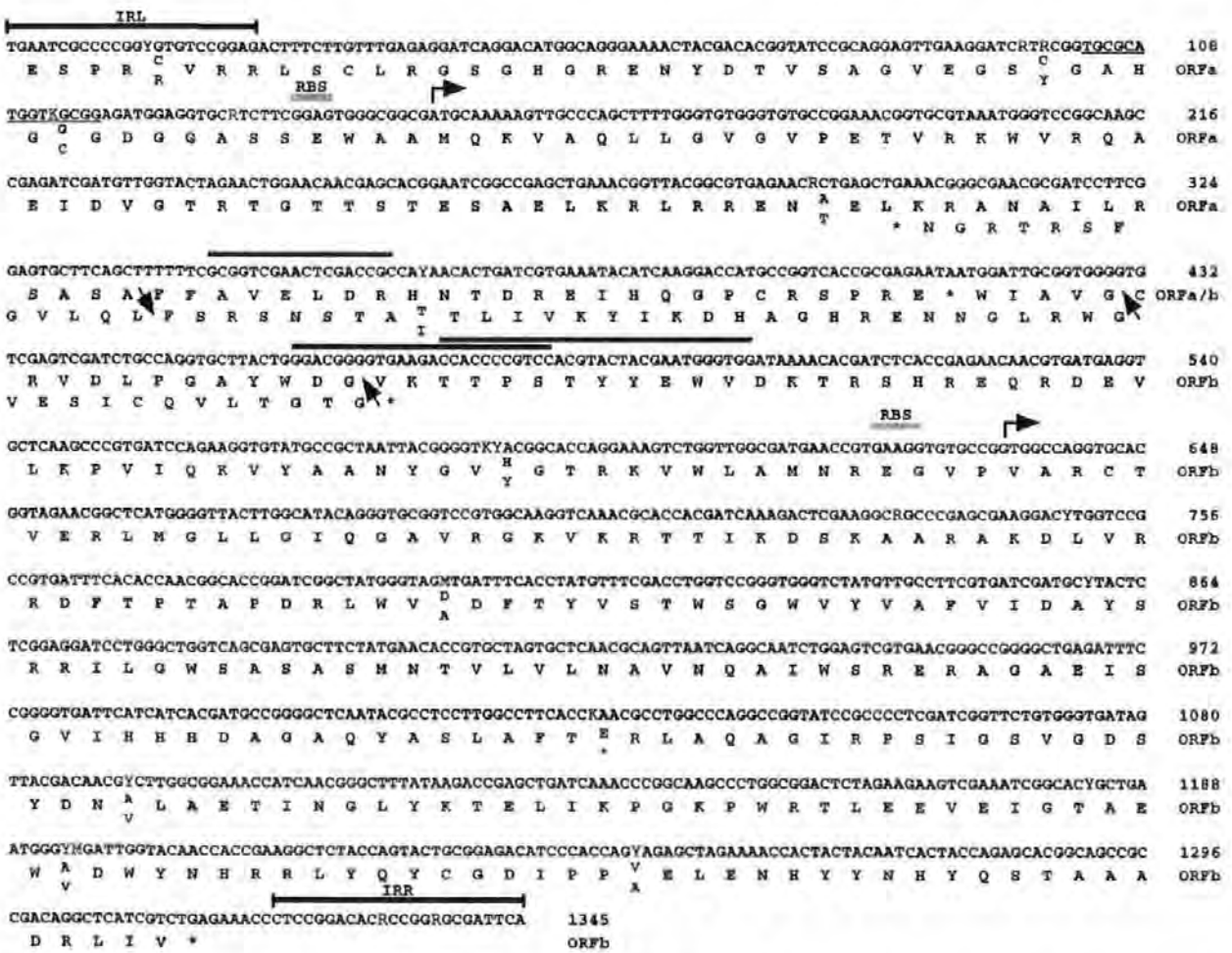

Fig. 1. Consensus nucleotide sequence and predicted open reading frames of IS*994* from *R. salmoninarum*. Terminal inverted repeats are marked by heavy overlines and labeled 'IRL' and 'IRR' for left and right repeats, respectively. The best initiation codons and ribosome binding sites (RBS) are indicated by a bent arrow (↱) and light overline, respectively. Stop codons are marked by asterisks. Potential frameshift sites are marked by diagonal arrows, with the arrowhead indicating the proposed direction of phase change. Sequences that may be involved in potential stem-loop structures are marked with medium overlines. The additional 15 bp present in three of the loci is noted by an underline. Polymorphic nucleotides are written as outlined letters; standard IUPAC symbols are used. When nucleotide polymorphisms result in different encoded residues, alternative translations are shown.

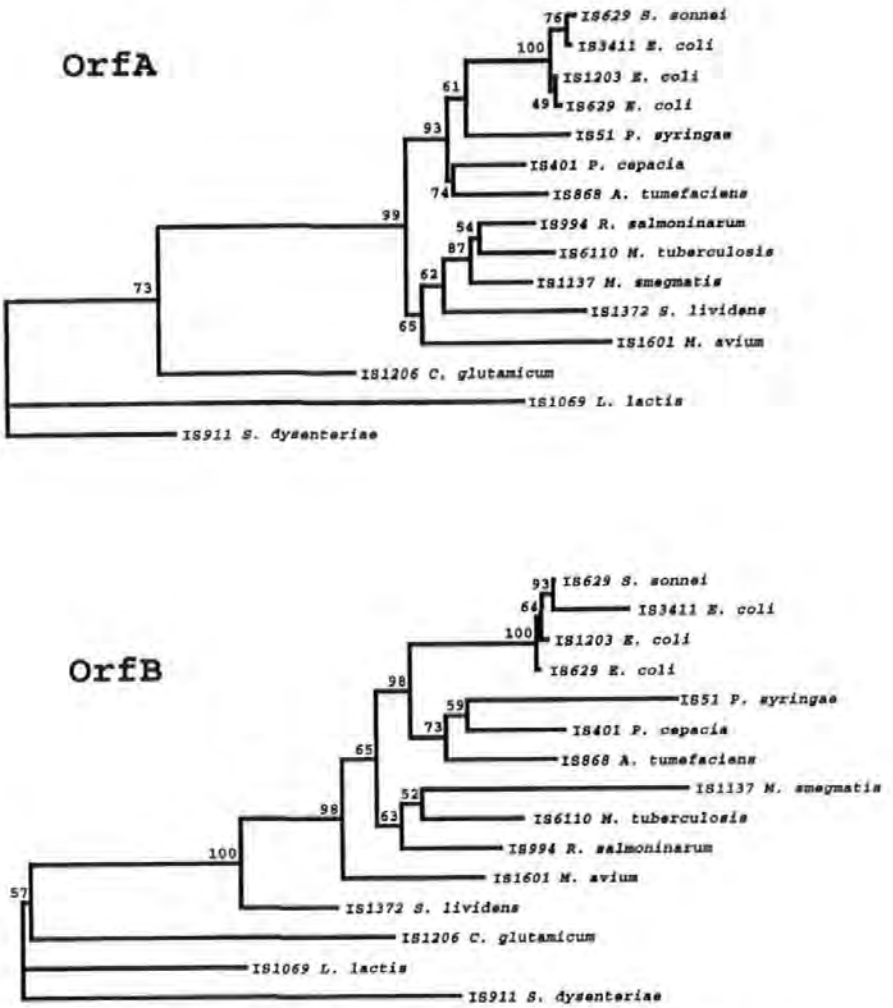

Fig. 2. Relationship of OrfA and OrfB of IS*994* with homologous transposase ORFs of other IS*3* elements. Trees shown are constructed from the Dayhoff PAM distance matrix (Dayhoff, 1979) using the method of Fitch and Margoliash (1967), setting IS*911* (IS*3* family, group IS*3*) as the outgroup. For OrfA, identical consensus trees were constructed by protein parsimony (Felsenstein, 1993) and neighbor-joining (Saitou and Nei, 1987) methods. For OrfB, very similar consensus trees were constructed by these alternative analyses. Horizontal branch lengths are drawn to scale, and the number at each node is the percentage of bootstrapped trees in which the species to the right of the node were clustered.

Surprisingly, polymorphisms in single nucleotides were found throughout the element among the seven clones (Fig. 1); as a result, no two clones contained identical sequences for the common element. A total of 21 polymorphic positions were found: three in the terminal inverted repeats, one within the additional 15 bp insertion found in three of the clones, and 17 in the remainder of the element. A polymorphism in clone #138 resulted in a stop codon at nt 1028–1030 (Fig. 1); this clone also contained a single nucleotide insertion 3′ to this polymorphism, resulting in additional premature stop codons. When the inverted repeats for each clone were matched for complementarity, only two clones (#110 and #150) possessed perfect inverted repeats; the remaining five clones were mismatched at one of two positions (Fig. 1).

Conceptual translation revealed two large ORFs that encompassed almost the entire element (Fig. 1). These ORFs are arranged in the same reading frame in direct tandem order, separated by a single stop codon. The predicted amino acid sequence of each ORF was most similar to IS*3* family elements found in *Mycobacterium* complex organisms: IS*986*/IS*6110* of *Mycobacterium tuberculosis* and IS*987* of *M. bovis*. The larger ORF, designated *orfB*, encodes a hypothetical translation product of 299 residues and possesses 58.9% identity (176 of 299) and 71.9% similarity (215 of 299) with ORFb of the IS*986*/IS*6110* transposase. The second ORF, designated *orfA*, begins at the 5′ end of one of the terminal inverted repeats, and the predicted translation product contains 137 residues. Among the potential initiation codons identified in *orfA*, the codon located

at nt 149–151 is preceded by the strongest ribosome binding site (Fig. 1). Among the 88 residues from this initiation codon to the stop codon located at nt 413–415, there is a 59.0% identity (52 of 88) and 64.8% similarity (57 of 88) to ORF2a of IS*986*/IS*987*/IS*6110*.

BLASTP comparisons (Altschul et al., 1997) of these ORFs against protein database sequences found similarity to a number of IS*3* elements with translation products encoding putative transposases. Protein parsimony and protein distance matrix analyses (Fitch and Margoliash, 1967; Saitou and Nei, 1987; Felsenstein, 1993) of the predicted product of *orfA* with potentially related IS*3* transposases resulted in identical trees. OrfA of the *Renibacterium* element clustered with the *Mycobacterium* elements and was most closely related to IS*6110* and IS*1137* (Fig. 2). The same analyses of the predicted *orfB* product produced similar, but not identical, trees. However, for all methods, the *Renibacterium* element clustered with IS*6110* and IS*1137*(Fig. 2). These results indicate that the *Renibacterium* element is a member the IS*51* group of the IS*3* family of insertion elements (Fig. 2), and we have designated this element as IS*994*.

In addition to a strong identity to the transposases of other IS*3* elements, the predicted amino acid sequence of *orfB* appears to contain the 'DDE motif', a primary sequence feature observed in IS*3* family transposases and retroviral integrases (Fig. 3; Polard and Chandler, 1995). The DDE motif consists of a triad of acidic residues (aspartic acid and glutamic acid) with conserved spacing and associated conserved amino acids. In IS*994*, the aspartic acids are separated by 63 residues, and the second aspartic acid and glutamic acid are 35 residues apart (Fig. 3); this spacing is consistent with DDE motifs described in IS*3* transposases. Further, 75% (21 of 28) of the consensus residues identified with the DDE motif of IS*3* family transposases are found within the putative DDE motif of IS*994* (Fig. 3).

3.2. Some features of IS994 differ from members of the IS3 family

One feature of certain IS*3* family elements is the production of a fusion transposase protein by translational frameshifting. In the case of IS*911*, the fusion protein is encoded by two overlapping ORFs and results from −1 frameshifting (Polard et al., 1991). The conceptual translation product of *orfA* of IS*994* has 23.9% identity (21 of 88) and 35.2% similarity (31 of 88) to the ORFA protein of IS*911*, suggesting that *orfA* may encode the amino portion of a transposase for IS*994*. However, since *orfA* and *orfB* of IS*994* are in the same reading frame, at least two frameshifts would be required to generate a fusion protein. Examination of nucleotide sequences and predicted protein sequences in all three frames proximate to the junction of *orfA* and *orfB* identified three sites that are candidates for frameshifting (Fig. 1). The first site, located at nt 337–343, consists of UUUUUUC, and in all reading frames, this sequence encodes phenylalanine. The second and third sites, located at nt 427–431 and nt 464–468, respectively, contain GGGGT, which encodes glycine in all reading frames. A +1 frameshift at the first site from frame 2 (*orfA*) to frame 3 and a −1 frameshift at the second or third site from frame 3 back to frame 2 (*orfB*) would 'bridge' the stop codon separating the ORFs (Fig. 1).

Among the seven loci sequenced, between 225 and 970 nt of sequence flanking IS*994* was determined. Examination of these sequences failed to uncover a conserved insertion site. A flanking dinucleotide repeat was found in only in one clone (#138), and no pattern of similarity was apparent among the 5′ or 3′ sequences (Fig. 4). Flanking sequences were also analyzed for repetitive patterns up to 35 nt in length, but none was found.

Flanking sequences were also examined for coding regions with a similarity to previously reported genes. In clone #159, IS*994* was positioned 200 nt 5′ to an ORF with a very high amino acid similarity to DNA ligase from a range of bacteria ($P<10^{-51}$, BLASTX, Altschul et al., 1997). In clone #70, IS*994* appeared to have disrupted a gene with a strong similarity to *fadD*, a long-chain fatty acid CoA ligase found in diverse microorganisms including *E. coli*, *Bacillus subtilis*, *M. tuberculosis*, and *Archaeoglobus fulgidus* ($P<10^{-10}$,BLASTX, Altschul et al., 1997). Based on the amino acid similarity to those enzymes, IS*994* is

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IS911       WCG□VTY....54 residues.....HS□QGSHYTSRQFRQLLWRYQIRQSMSRRGNCWDNSPM□RFFRSLKNE
IS629       WVA□FTY....53 residues.....HS□KGSQYVSLAYTQRLKEAGLLASTGSTGDSYDNAMA□SINGLYKAK
IS6110      WVA□LTY....59 residues.....HT□RGSQYTSIRFSERLAEAGIQPSVGAVGSSYDNALA□TINGLYKTE
IS994       WV(D/A)□FTY....58 residues.....HH□AGAQYASLAFTERLAQAGIRPSIGSVGDSYDN(A/V)LA□TINGLYKTE

consensus W--□-TY...52-61 residues...HS□QGSQYTS--F---L---GI--S-S--G-S-DNA--□TF-G-LK-E
                                     T N  F    Y   A    L    T    C   V   S     R
                                                  I    V         P
```

Fig. 3. Alignment of the DDE motif from several IS*3* family elements (IS*911*, IS*629*, IS*6110*) and IS*994* from *R. salmoninarum* with a consensus DDE motif sequence for IS*3* transposase compiled by Polard and Chandler (1995). The critical acidic residues (aspartic acid and glutamic acid) are shown as outlined letters. Alternative residues encoded by the polymorphic sites in IS*994* are shown at a reduced size. A variable-sized region with no specified residues is indicated by the number of residues contained. In the consensus sequence, a dash indicates no preferred amino acid, and when more than one amino acid is preferred at a position, the additional residues are aligned at their respective positions. The consensus DDE motif sequence is from Polard and Chandler (1995).

clone #	5' flanking sequence	3' flanking sequence
61	CTTTCAAGACGATTTTGGGGCCGCTCCGGC	IS*994* GAGCGGGGCGATGCGGCGCGCAATCGAGAG
70	TCTCTGGTGGGGCTGCGCTTCCGCTCGGTG	IS*994* TATCTACACAGGGGACCTGGCTCGCCAAGA
110	GGACACTGCCTATTCAACGCAGAATGTGCG	IS*994* AAGTGCTGCTCACGGTGATGAATGCGGGCA
123	TGGGCCGTTGACTTTCCAGTCTCTCATTCA	IS*994* CCTCGACTTTGAATCCTCGATTCGCTCAGC
138	TCCACGATCTTCAGGTCGTCCGCTTTGAAG	IS*994* AGATGCATGAAAACGGTGATCTTCGAAATC
150	CTGCAACTCCAACTCACCAGATTCGCCAAC	IS*994* GTGCCGCTTTAGCAGAGACCTTGCCTTTGA
159	GCTCCAGATCGGTGACGATCTTGCTCAGGG	IS*994* GTTTGTGAGCGCCCGGCTAAAGCATCAGGT

Fig. 4. Thirty nucleotides of 5' and 3' flanking sequences for the seven IS*994* loci analyzed. The IS element is denoted as '–IS*994*–'. The sole flanking repeat is underlined (clone #138).

inserted into the central portion of the gene, resulting in a deletion of approximately 300 bp (data not shown).

3.3. *Variations in IS994 RFLPs allow differentiation of* R. salmoninarum *isolates*

Since the IS*994* elements sequenced from seven clones were clearly inserted at different loci, we reasoned that restriction fragment length polymorphism (RFLP) analysis might be useful for distinguishing isolates of *R. salmoninarum*. First, we attempted to estimate the number of insertion loci in the *R. salmoninarum* chromosome. Southern analysis of restriction digestions of genomic DNA from ATCC culture type 33209 and isolate MT239 (Bruno and Munro, 1986; Bruno, 1988) was performed using 14 individual enzymes and 20 double enzyme combinations. The combination of *Hin*cII and *Pst*I, which should not cut within the element, produced the largest number of bands. Bands were counted, using only easily visualized bands and considering broader bands as single bands. By this method, we estimate that there are at least 17 chromosomal insertions of IS*994*, or elements closely related to IS*994*, in the *Renibacterium* chromosome (Fig. 5).

Next, we examined 42 isolates of *R. salmoninarum* for differences by RFLP analysis. Table 1 shows that the isolates were derived from a variety of salmonid hosts over a wide range of geographic locations, spanning more than 30 years. We chose three restriction endonucleases for this analysis: *Eco*RV, which does not cut within the element; *Ava*I, which cuts at nt 735, near the middle of the element and the probe; and *Xba*I, which cuts at nt 1159, near the 3' end of the element, and at the end of the probe. As expected, the *Eco*RV digestion yielded the fewest number of bands (~13). Only two variations were observed with this digestion: a prominent 1.8 kb band was missing from Canadian isolate AcF6-1, and a 1.1 kb band was missing from American isolate Round Butte (data not shown). The greatest number of bands appeared with the *Ava*I digestion (≥25 bands), but a variation was observed only in a single ~4 kb band. This band was present in the Swedish and English isolates, absent in the Icelandic isolates, and variable among the Scottish, Canadian, and American isolates (data not shown). RFLP analysis of the *Xba*I digestions yielded the greatest number of variations among the isolates: eight distinct patterns were observed among bands < 2 kb (Fig. 6). The most common pattern (pattern A) was found among all five isolates from Sweden, all four isolates from Iceland, and nine of the 10 isolates from the USA (includes ATCC type 33209, which is not shown in Fig. 6). In contrast, only one of 11 isolates from the UK exhibited pattern A. Instead, six English isolates had an additional 1.2 kb band (pattern B; lanes 30–35), and the four remaining English isolates lacked a 1.3 kb band present in patterns A and B (pattern C; lanes 37–40). Among the five isolates from Canada, three displayed pattern A, one (DR143) displayed pattern C (lane 14), and one (AcF6-1) lacked a 1.5 kb band (pattern D; lane 15).

The greatest variation in the *Xba*I RFLP analysis

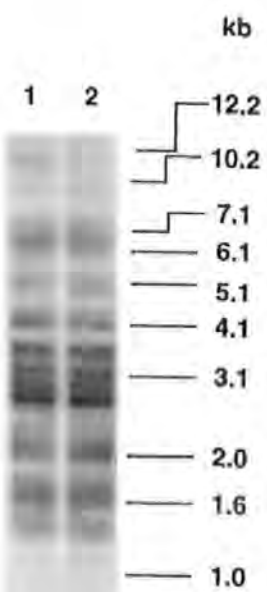

Fig. 5. Southern analysis for estimated number of IS*994* loci. One microgram of genomic DNA was extracted from isolates 33209 (lane 1) and MT239 (lane 2), digested with *Hin*cII and *Pst*I, resolved on a 0.8% agarose gel, and transferred to a nylon membrane. The membrane was hybridized to a digoxygenin-labeled, random-primed probe generated from a 1.2 kb *Xba*I fragment encompassing 1159 nt of a subclone of IS*994* clone #159. The blot was washed at high stringency, and the hybridized probe was visualized by chemiluminescence. Molecular weights are indicated to the right. To estimate the number of loci, only easily visible bands were counted, and broad bands were considered to be single bands.

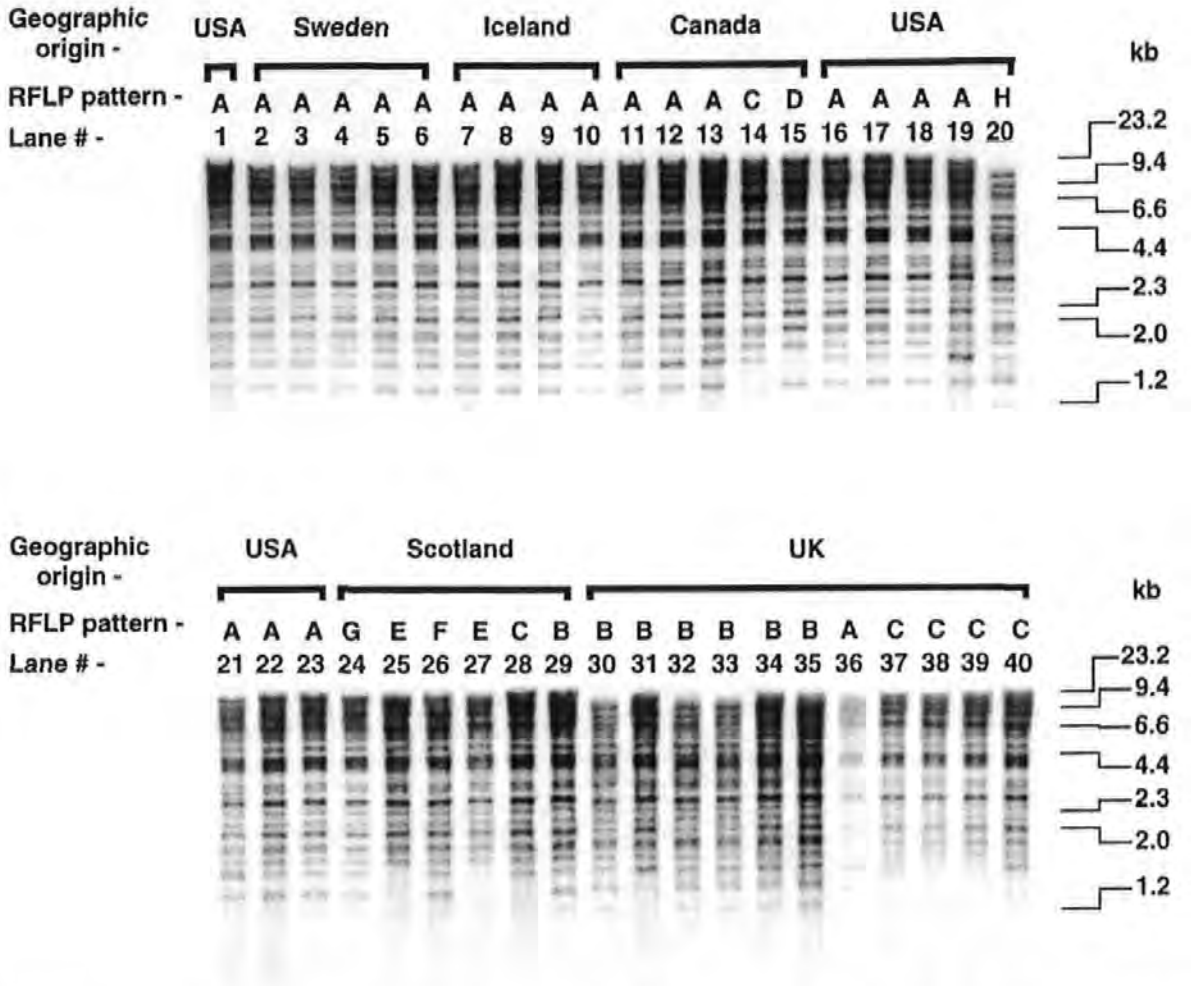

Fig. 6. RFLP analysis of *Xba*I-digested genomic DNA from forty isolates of *R. salmoninarum*. Genomic DNA (0.5 μg) was digested with *Xba*I, resolved on 0.8% agarose, transferred to nylon membrane, and hybridized to a digoxygenin-labeled, random-primed probe generated from a 1.2 kb *Xba*I fragment encompassing 1159 nt of a subclone of IS*994* clone #159. Blots were washed at high stringency, and the hybridized probe was visualized by phosphatase color development . Molecular weights are indicated to the right. Lanes: 1, BY1996; 2, Rs 125; 3, Rs 122; 4, Rs 116; 5, Rs 61; 6, Rs 19; 7, S-182-90; 8, F-283-87; 9, F-273-87; 10, F-120-87; 11, 960046; 12, 960023; 13, 980002; 14, DR143; 15, AcF6-1; 16, Round Butte; 17, CCM6206; 18, RFL-643.94#1; 19, Idaho 91-126; 20, Cow ChS 94; 21, SS-ChS-94-1; 22, CCM6205; 23, Siletz; 24, NCIMB1116; 25, NCIMB1115; 26, NCIMB1114; 27, NCIMB1113; 28, NCIMB1112; 29, NCIMB1111; 30, F3; 31, F47; 32, F60; 33, F82; 34, F85; 35, F95; 36, 980297#97; 37, A-80; 38, A-6; 39, 970083-102; 40, 970083-88. Additional information about these isolates is listed in Table 1.

was found among the isolates from Scotland: five different patterns were observed among the seven isolates examined. One isolate (MT239, not shown) displayed pattern A, one isolate (NCIMB1111) exhibited pattern B (lane 29), and one isolate (NCIMB1112) displayed pattern C (lane 28). The remaining four isolates exhibited patterns found only in the Scottish isolates. Two isolates (NCIMB1115 and NCIMB1113) lacked both the 1.3 and 1.5 kb bands (pattern E; lanes 25 and 27), one isolate (NCIMB1114) possessed an additional ~0.5 kb band (pattern F; lane 26), and one isolate (NCIMB1116) possessed both an additional ~0.5 kb band and an additional 1.2 kb band (pattern G; lane 24).

The single US isolate that did not display pattern A exhibited a pattern unique from all others (pattern H; lane 20). Pattern H possessed many bands (≥24), with only a few bands appearing to correspond to bands observed in the other seven patterns. Furthermore, no high-molecular-weight (≥20 kb) bands were observed in pattern H.

Since the IS*3* family of insertion elements is the largest family of insertion sequences, we wished to determine whether IS*994*-related elements may be present in other commonly occurring pathogens of salmonid fishes. A high-stringency Southern blot analysis of genomic DNA from *Aeromonas salmonicida*, *A. hydrophila*,

Vibrio anguillarum, *Flexibacterium columnare*, and *F. psychrophilum* failed to detect any evidence of insertion sequences closely related to IS*994* (data not shown).

4. Discussion

Molecular studies of the salmon pathogen, *Renibacterium salmoninarum*, have expanded considerably in recent years, giving rise to hopes that probes useful for studying this difficult organism may be developed. Here, we report the first insertion sequence (IS) identified in the *R. salmoninarum* genome and describe features that lead us to classify this element, IS*994*, as a member of the IS*3* family, group IS*51*.

Both protein parsimony and pairwise distance analysis of the two largest ORFs of IS*994* with ORFs encoding the putative transposase of members of the IS*51* group of the IS*3* family clearly show that the IS*994* is a member of this group and family (Fig. 2). OrfA and OrfB of IS*994* exhibit a striking similarity (64.8 and 71.9% homology, respectively) to the predicted amino acid sequences of two ORFs encoding a putative transposase of IS*6110* (previously designated as IS*986*, IS*987*, and IS*6110*) found in *M. tuberculosis* and *M. bovis*, and parsimony analysis places IS*994* very closely to this *Mycobacterium* IS.

Other features of IS*994* are consistent with its classification as a member of the IS*3* family. For example, a DDE motif with a close similarity to motifs found in IS*3* transposases and retroviral integrases (Fayet et al., 1990) is present in OrfB of IS*994* (Fig 3). Detailed analyses of retroviral integrases have shown the DDE motif to be a catalytic site for cleavage of the phosphodiester backbone (Baker and Luo, 1994), suggesting a common transposition mechanism between transposases and integrases (Polard and Chandler, 1995). Another feature found in most IS*3* elements and retroviral integrases is the conserved terminal dinucleotides 5′ TG–CA 3′, which are also found in IS*994* (Fig. 1). The G+C content of IS*994* ranged from 56.6 to 57.1% (depending upon the locus that was sequenced), and was similar to the reported genomic G+C content of 55.5% for *R. salmoninarum* (Banner et al., 1991). Similarity between the G+C content of the IS element and that of the host genome has been observed in many IS*3* family members, in spite of the conservation of amino acid residues in their putative transposases (Mahillon and Chandler, 1998).

Although IS*994* is clearly a group IS*51*, IS*3* family member, it displays features that are not typical of this family. Most notable is the organization of the reading frames of the putative transposase. Typically, IS*3* transposases have two partially overlapping ORFs, with the second or downstream ORF (*orfB*) at phase −1 relative to the first or upstream ORF (*orfA*). The functional transposase is a fusion protein resulting from a −1 frameshift from *orfA* to *orfB*. However, variants to this arrangement have been reported. Some IS*3* transposases exhibit a +1, rather than a −1, frameshift (Mahillon and Chandler, 1998), whereas other transposases may be encoded by a single ORF (Huang et al., 1991). In IS*994*, *orfA* and *orfB* are tandem and in the same phase, separated by a single stop codon. This arrangement suggests several possibilities. A non-universal genetic code may be employed, resulting in suppression of termination, but there is no evidence that *R. salmoninarum* uses an alternative code. The IS*994* transposase could consist of two separate polypeptides, since structure–function analyses of IS*3* transposases reveal that OrfA and OrfB appear to contain a DNA-binding domain and a catalytic domain (the DDE motif), respectively (Fayet et al., 1990; Polard and Chandler, 1995). However, transposases composed of separate polypeptides have not been reported for any bacterial insertion sequence. Alternatively, two frameshifts, rather than one, may be needed to produce a functional transposase. Although the requirement for a double frameshift is unique among transposases, several features support this possibility. First, there are potential frameshift sites bearing 'slippery codons' (Fig. 1). Although these mostly homopolymeric runs of nucleotides (UUUUUUC or GGGGT) differ from well-characterized frameshift sequences (e.g. AAAAAAC, AAAAAAG, reviewed by Chandler and Fayet, 1993), they do encode the same residue (phenylalanine or glycine) in all three frames. Second, all three candidate frameshift sites are followed by sequences capable of forming step-loop structures (Fig. 1). Such secondary structures are postulated to play a role in inducing ribosomal pausing and enhancing frameshifting. Finally a comparison of the predicted amino acid sequence of the third reading frame region that could 'bridge' the stop codon between *orfA* and *orfB* of IS*994* has significant alignment with both ORFA and ORFB of IS*6110* and other IS*3* transposases (data not shown).

Surprisingly, characteristic direct repeats or duplicated sequences were not commonly observed adjacent to IS*994*: only one locus of the seven that were analyzed was found to contain a flanking, direct repeat duplication. Short, direct repeats of target DNA flanking the IS element are thought to result from staggered breaks during transpositional insertion (Sekine et al., 1994, 1996). An absence of direct repeats could result from recombination between different IS elements with different target sites, but this is unlikely since there was wide variation in the flanking di- and trinucleotides among the seven analyzed loci (Fig. 4). Alternatively, the dissimilarities in adjacent sequences may have resulted from IS-mediated deletion. IS-mediated adjacent deletion is considered to be a relatively low-frequency event during non-replicative transposition

(Roberts et al., 1991) but may occur more readily with duplicative intramolecular transposition (Galas and Chandler, 1989; Turlan and Chandler, 1995; Ton-Hoang et al., 1998). Minicircle intermediates of IS*3* transposition exhibit adjacent deletions of varying length (Sekine et al., 1994), and IS*4* deletions appear to remove sequences from both sides of the element (Habermann and Starlinger, 1982). The occurrence of a possible deletion within an ORF with homology to *fadD* (clone #70) suggests that adjacent deletion may be associated with IS*994* transposition. A third possibility is that the sequences flanking the analyzed IS*994* loci have mutated away from the direct repeats. This would indicate that *R. salmoninarum* undergoes a rapid sequence variation or that the elements at these loci have been in place for a long time. Analysis of more extensive stretches of flanking sequence found no evidence of preferential insertion sites, suggesting that IS*994* may insert randomly.

Since IS*994* was most similar to IS*6110*, an element that has been used for epidemiological studies of *M. tuberculosis* and *M. bovis*, we were interested in determining whether IS*994* might be useful for molecular typing of *R. salmoninarum* isolates. When we probed a wide variety of *R. salmoninarum* isolates collected from disparate geographic locations, we observed complex RFLP patterns with extensive homogeneity, but sufficient differences were found to permit differentiation among isolates. IS*994* RFLP analysis was most useful in distinguishing differences among isolates from Scotland, including several isolates with unique patterns (NCIMB1115 and NCIMB1116). USA isolate Cow ChS94, which also presented a unique pattern by IS*994* RFLP analysis, was not distinguished from most other USA isolates by RAPD analysis (Grayson et al., 2000). Conversely, IS*994* RFLP analysis identified a difference between the ATCC type strain 33209 and the Icelandic isolates S-182-90 and F-273-87, confirming conclusions from RAPD analysis (Grayson et al., 1999). Thus, IS*994* RFLP analysis can be useful as a complementary and confirming method for molecular differentiation of *R. salmoninarum* isolates.

The degree of homogeneity of RFLP banding patterns among the examined isolates was also striking. This result may indicate that the divergence of *R. salmoninarum* is recent. An alternative, and not mutually exclusive, explanation is that IS*994* has extremely low transpositional activity. Several characteristics of IS*994* support this possibility. First, the occurrence of multiple nucleotide polymorphisms and the presence of a 15 bp insertion (which itself contains a single base polymorphism) suggest that these loci have been resident for a sufficient length of time to permit sequence divergence. These variations are not likely to be due simply to sequencing errors since care was taken to minimize errors and since the variant bases were found at more than one locus for nearly half (10 of 21) of the polymorphisms. Second, the requirement for two frameshift events to produce a putative transframe protein is likely to lower the probability of transposition, compared to the probability of a single frameshift event. The frequency of single event frameshifting can be as low as 0.2–0.3% in IS*1* (Ohtsubo and Sekine, 1996), and low-frequency frameshifting can be a mechanism to maintain low-level expression of transposase (e.g. IS*3*; Sekine et al., 1997).

The multiple insertions of IS*994* appear to have had interactions with coding regions of the *R. salmoninarum* genome. For example, IS*994* may have disrupted a gene encoding an enzyme involved in fatty acid metabolism, a *fadD* homologue. Based on the predicted amino acid homology to *fadD* from *Bacillus subtilis*, *Archaeoglobus fulgidus*, and *Mycobacterium tuberculosis*, IS*994* appears to have deleted approximately 300 bp of coding DNA upon insertion, and this insertion was conserved in eight different isolates of *R. salmoninarum* that were examined by PCR (our unpublished observations). IS*994* may have had a role in the duplication of a gene encoding an immunodominant surface protein, major soluble antigen or MSA (Chien et al., 1992). Two identical ORFs for MSA have been identified (O'Farrell and Strom, 1999), and we have found IS*994* flanking on both sides of one copy of MSA and a single IS*994* insertion flanking on the 3′ side of the second copy of MSA (our unpublished observations). This tantalizing finding leads us to speculate that IS*994* may have been involved in this duplication, perhaps through recombination.

In summary, we have isolated and analyzed the first IS reported for the significant fish pathogen, *R. salmoninarum*. This IS element, or very closely related elements, is inserted at least 17 times in the *R. salmoninarum* genome. In spite of these multiple loci, RFLP typing of this element was relatively homogeneous among a large number of isolates. This relative homogeneity of the restriction fragment patterns, the substantial number of nucleotide sequence polymorphisms within IS*994*, and the possible requirement for double frameshifting for a full length transposase indicate that IS*994* may be a low activity IS element. None the less, sufficient differences in RFLP patterns are present to allow differentiation of isolates, suggesting the IS*994* will be a useful molecular tag for epizootiological studies.

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References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Austin, B., Embley, T.M., Goodfellow, M., 1983. Selective isolation of *Renibacterium salmoninarum*. FEMS Microbiol. Lett. 17, 111–114.

Baker, T.A., Luo, L., 1994. Identification of residues in the Mu transposase essential for catalysis. Proc. Natl. Acad. Sci. USA 91, 6654–6658.

Balfry, S.K., Albright, L.J., Evelyn, T.P.T., 1996. Horizontal transfer of *Renibacterium salmoninarum* among farmed salmonids via the fecal–oral route. Dis. Aquat. Org. 25, 1–2.

Banner, C.R., Rohovec, J.S., Fryer, J.L., 1991. A new value for mol percent guanine+cytosine of DNA for the salmonid fish pathogen *Renibacterium salmoninarum*. FEMS Microbiol. Lett. 79, 57–60.

Bruno, D.W., Munro, A.L.S., 1986. Uniformity in the biochemical properties of *Renibacterium salmoninarum* isolates obtained from several sources. FEMS Microbiol. Lett. 33, 247–250.

Bruno, D.W., 1988. The relationship between auto-agglutination, cell surface hydrophobicity and virulence of the fish pathogen *Renibacterium salmoninarum*. FEMS Microbiol. Lett. 51, 135–140.

Chandler, M., Fayet, O., 1993. Translational frameshifting in the control of transposition in bacteria. Mol. Microbiol. 7, 497–503.

Chien, M.S., Gilbert, T.L., Huang, C., Landolt, M.L., O'Hara, P.J., Winton, J.R., 1992. Molecular cloning and sequence analysis of the gene coding for the 57-kDa major soluble antigen of the salmonid fish pathogen *Renibacterium salmoninarum*. FEMS Microbiol. Lett. 75, 259–265.

Dayhoff, M.O., 1979. Atlas of Protein Sequence and Structure Vol. 5. National Biomedical Research Foundation, Silver Spring, MD. Suppl. 3

Domann, E., Chakraborty, T., 1989. Nucleotide sequence of the listeriolysin gene from a *Listeria monocytogenes* serotype 1/2a strain. Nucleic Acids Res. 17, 6406

Evelyn, T.P.T., Ketcheson, J.E., Prosperi-Porta, L., 1984. Further evidence for the presence of *Renibacterium salmoninarum* in salmonid eggs and for the failure of povidine-iodine to reduce the intra-ovum infection in water-hardened eggs. J. Fish Dis. 7, 173–182.

Fayet, O., Ramond, P., Polard, P., Prère, M.F., Chandler, M., 1990. Functional similarities between retroviruses and the IS*3* family of bacterial insertion sequences? Mol. Microbiol. 4, 1771–1777.

Felsenstein, J., 1993. PHYLIP (Phylogeny Inference Package). Department of Genetics, University of Washington, Seattle, WA.

Fitch, W.M., Margoliash, E., 1967. Construction of phylogenetic trees. Science 155, 279–284.

Fryer, J.L., Sanders, J.E., 1981. Bacterial kidney disease of salmonid fish. Ann. Rev. Micro. 35, 273–298.

Galas, D.J., Chandler, M., 1989. Bacterial insertion sequences. In: Berg, D.E., Howe, M.M (Eds.), Mobile DNA. American Society for Microbiology, Washington, DC, pp. 109–162.

Genetics Computer Group, I., 1999. Wisconsin Package. Genetics Computer Group, Madison, WI.

Grayson, T.H., Cooper, L.F., Atienzar, F.A., Knowles, M.R., Gilpin, M.L., 1999. Molecular differentiation of *Renibacterium salmoninarum* isolates from worldwide locations. Appl. Environ. Microbiol. 65, 961–968.

Grayson, T.H., Atienzar, F.A., Alexander, S.M., Cooper, L.F., Gilpin, M.L., 2000. Molecular diversity of *Renibacterium salmoninarum* isolates determined by randomly amplified polymorphic DNA. Appl. Environ. Microbiol. 66, 435–438 in press.

Habermann, P., Starlinger, P., 1982. Bidirectional deletions associated with IS4. Mol. Gen. Genet. 185, 216–222.

Huang, D.C., Novel, M., Novel, G., 1991. A transposon-like element on the lactose plasmid of *Lactococcus lactis* subsp. *lactis* Z270. FEMS Microbiol. Lett. 61, 101–106.

Mahillon, J., Chandler, M., 1998. Insertion sequences. Microbiol. Mol. Biol. Rev. 62, 725–774.

O'Farrell, C.L., Strom, M.S., 1999. Differential expression of the virulence-associated protein p57 and characterization of its duplicated gene *msa* in virulent and attenuated strains of *Renibacterium salmoninarum*. Dis. Aquat. Org. 38, 115–123.

Ohtsubo, F., Sekine, Y., 1996. Bacterial insertion sequences. Curr. Top. Microbiol. Immunol. 204, 1–26.

Polard, P., Prère, M.F., Chandler, M., Fayet, O., 1991. Programmed translational frameshifting and initiation at an AUU codon in gene expression of bacterial insertion sequence IS*911*. J. Mol. Biol. 222, 465–477.

Polard, P., Chandler, M., 1995. Bacterial transposases and retroviral integrases. Mol. Microbiol. 15, 13–23.

Roberts, D.E., Ascherman, D., Kleckner, N., 1991. IS*10* promotes adjacent deletions at low frequency. Genetics 128, 37–43.

Saitou, N., Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. second ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sekine, Y., Eisaki, N., Ohtsubo, E., 1994. Translational control in production of transposase and in transposition of insertion sequence IS*3*. J. Mol. Biol. 235, 1406–1420.

Sekine, Y., Eisaki, N., Ohtsubo, E., 1996. Identification and characterization of the linear IS*3* molecules generated by staggered breaks. J. Biol. Chem. 271, 197–202.

Sekine, Y., Izumi, K., Mizuno, T., Ohtsubo, E., 1997. Inhibition of transpositional recombination by OrfA and OrfB proteins encoded by insertion sequence IS*3*. Genes Cells 2, 547–557.

Starliper, C.E., 1996. Genetic diversity of North American isolates of *Renibacterium salmoninarum*. Dis. Aquat. Org. 27, 207–213.

Ton-Hoang, B., Polard, P., Chandler, M., 1998. Efficient transposition of IS*911* circles in vitro. EMBO J. 17, 1169–1181.

Turlan, C., Chandler, M., 1995. IS*1*-mediated intramolecular rearrangements: formation of excised transposon circles and replicative deletions. EMBO J. 14, 5410–5421.

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Molecular Diversity of *Renibacterium salmoninarum* Isolates Determined by Randomly Amplified Polymorphic DNA Analysis

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The molecular diversity among 60 isolates of *Renibacterium salmoninarum* which differ in place and date of isolation was investigated by using randomly amplified polymorphic DNA (RAPD) analysis. Isolates were grouped into 21 banding patterns which did not reflect the biological source. Four 16S-23S rRNA intergenic spacer (ITS1) sequence variations and two alleles of an exact tandem repeat locus, ETR-A, were the bases for formation of distinct groups within the RAPD clusters. This study provides evidence that the most common ITS1 sequence variant, SV1, possesses two copies of a 51-bp repeat unit at ETR-A and has been widely dispersed among countries which are associated with mainstream intensive salmonid culture.

Renibacterium salmoninarum is an important cause of clinical and subclinical infections among farmed and wild salmonid populations in North and South America, Europe, and Japan (5). The organism causes a chronic, systemic, and granulomatous infection, bacterial kidney disease (BKD), that is often fatal under conditions which are stressful to the host (11). There is no effective vaccine or chemotherapy, and the presence of subclinical infections complicates attempts to control the disease through programs of eradication. An improved understanding of the transmission and spread of BKD is of considerable importance in policy management issues relating to aquaculture and wildfisheries. There have been a number of studies investigating the presence, prevalence, and means of transmission of BKD within and between fish populations. This work has shown that *R. salmoninarum* is endemic within many wild salmonid populations as a low-level, subclinical infection; it has been isolated in up to 100% of samples (9, 12, 15). However, the epidemiology of BKD remains unclear, mainly because of the difficulty of differentiating isolates of *R. salmoninarum* by biochemical, serological, and multilocus enzyme electrophoresis techniques (1, 6, 16).

We used two approaches to assess the extent of molecular variation among *R. salmoninarum* isolates from different geographic locations. First, we investigated possible polymorphisms in specific regions within the genome, genes *msa* (3), *rsh* (4), and *hly* (8), and the rRNA genes, including the intergenic spacer (ITS) regions. PCR and DNA sequencing studies have shown that *R. salmoninarum* has only limited variation in these regions (7). Identifying specific markers of variation in the *R. salmoninarum* genome, such as insertion sequences or variable numbers of tandem repeats (TR), has been constrained by a paucity of sequence information. Second, we analyzed differences throughout the genome using randomly amplified polymorphic DNA (RAPD) analysis. RAPD analysis is a PCR-based alternative method to the use of species-specific DNA sequences for isolate or strain differentiation. The method uses short random primers for rapidly detecting genomic polymorphisms under low-stringency conditions (18, 19). RAPD analysis is widely used for differentiating bacterial isolates (2, 10, 17) and relies on small quantities of genomic DNA, making it ideal for the study of slowly growing and fastidious organisms, such as *R. salmoninarum*. Previous studies show that, compared with other techniques, RAPD analysis is a reliable and reproducible means for differentiating isolates of *R. salmoninarum* (7). In the present study we used RAPDistance software to produce an objective analysis of RAPD profiles which were generated from the genomes of 60 *R. salmoninarum* isolates from a variety of sources in order to identify clusters of the isolates and determine whether there is any correlation with geographic or biological source. Furthermore, we identified the locus of a TR and showed that variation within this locus and within another specific region of the *R. salmoninarum* genome, the nucleotide sequence of the 16S-23S rRNA ITS region, is reflected in the RAPD analysis.

Generating RAPD profiles of *R. salmoninarum*. Sixty isolates of *R. salmoninarum* obtained from a variety of countries in Europe and North America, including the type strain, NCIMB2235 (ATCC 33209), were cultured in selective kidney disease medium (SKDM) broth supplemented with 5% spent broth culture at 15°C for 6 to 10 weeks. A description of the isolates, sources, and the positive identification of each as *R. salmoninarum* has been previously published (7). Genomic DNA was isolated by using the Puregene D-6000 DNA isolation kit according to the manufacturer's instructions (Gentra Systems Inc.). PCR amplification was performed in a DNA thermal cycler (Perkin-Elmer), and we used two RAPD protocols and eight random 10-mer primers which have been described elsewhere (7). PCR products were analyzed on 1.2% agarose gels in Tris-borate-EDTA buffer. The RAPD patterns were visualized by UV illumination, images of each gel were captured with a Kodak DC40 digital camera, and the DNA profile was analyzed by using the RAPDistance software package (http://life.anu.edu.au/molecular/solfware/rapd.html). The patterns were normalized with the bands that were uniformly present in all patterns, and the presence or absence of major bands was recorded in a binary matrix. Very faint bands were excluded from the analysis. A band was scored as absent only

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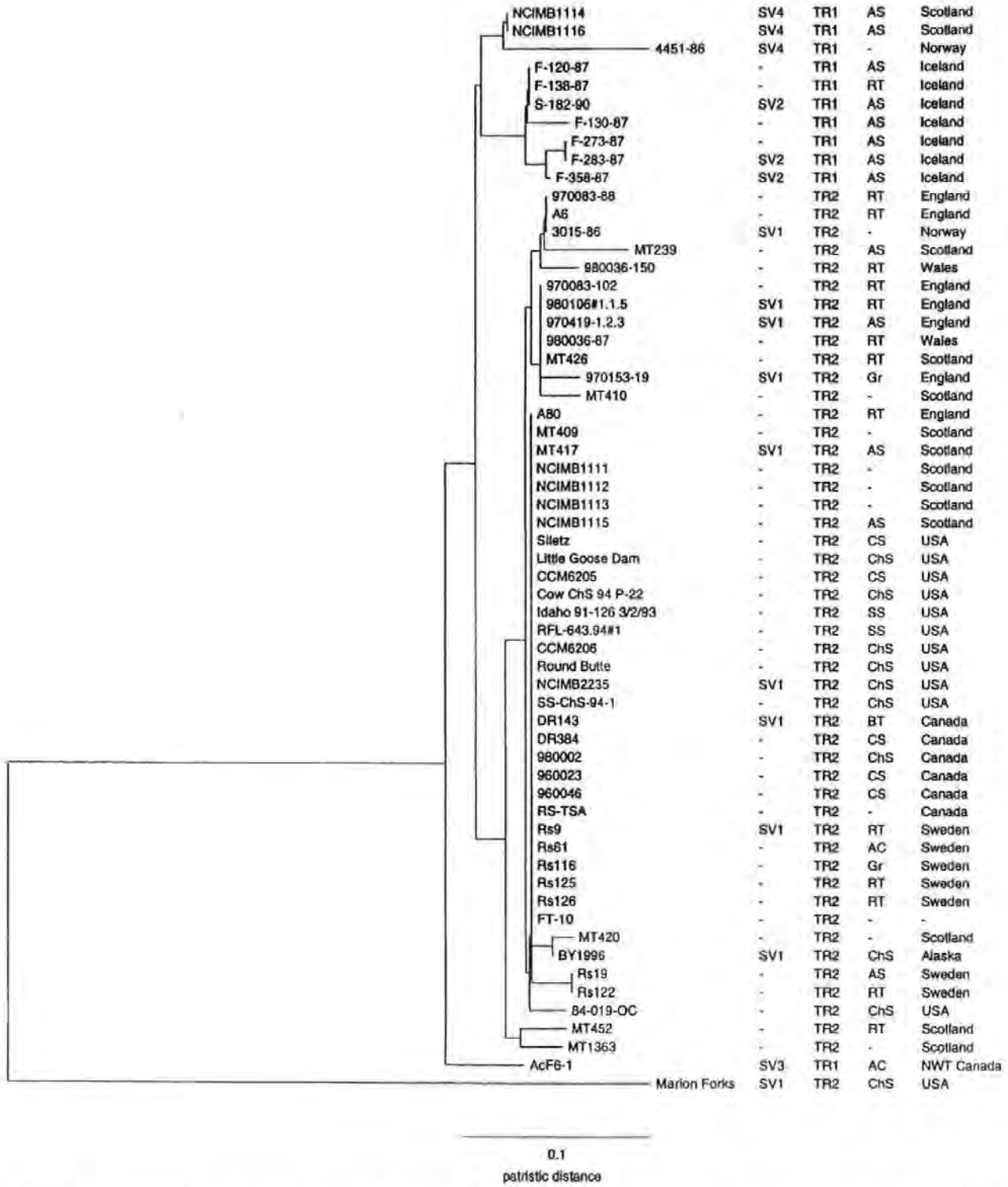

FIG. 1. Unrooted dendrogram, generated by the neighbor-joining method (15), of RAPD patterns for *R. salmoninarum* isolates (n = 60). Isolate designations and the respective ITS1 sequevar, number of TR at the ETR-A locus, biological source, and geographical origin are indicated. RT, rainbow trout; BT, brook trout; AS, Atlantic salmon; CS, coho salmon; ChS, chinook salmon; SS, sockeye salmon; Gr, grayling; AC, Arctic char.

if no visible band was present within a 2% size range. The patterns generated with each of the primers were combined for each isolate, and the pairwise distances for the combined band patterns were calculated by using the Dice algorithm described by Nei and Li (13). An unrooted tree was constructed based on the neighbor-joining method of Saitou and Nei (14), using NJTREE and TDRAW software (L. Jin and J. W. H. Ferguson, University of Texas Health Science Centre, Houston).

Differentiating *R. salmoninarum* isolates by RAPDistance analysis. The data for each primer were combined, and for each isolate a total of 86 bands were used to generate a distance matrix, of which 11 bands were invariant, i.e., present in all 60 isolates. By using RAPDistance software, isolates were placed in 21 clusters; 1 of these was a single major cluster which contained 29 of the 60 isolates studied (Fig. 1). The patristic distance between most paired groups was less than 0.1, reflecting the close relatedness of most isolates. Only a single isolate, Marion Forks (from the United States), was sufficiently different to exceed this value. There was no correlation of banding pattern with biological source. All Icelandic isolates were grouped in four closely associated clusters, and most of the isolates from England and Wales were grouped in four adjoining clusters. However, no strong correlation with the geographic origin of isolates was found; the single major cluster of 29 isolates contained the bulk of isolates from the United States, Canada, and Sweden and half of the isolates from Scotland.

TR allele profile of *R. salmoninarum* isolates. We identified an exact TR repeat locus (ETR-A) in the *R. salmoninarum* genome during routine sequencing of DNA fragments cloned from a number of different isolates. The repeat unit, with a length of 51 bp, was located in an open reading frame; we used PCR to examine variation in this region of the genomes of 60 *R. salmoninarum* isolates which differ in place and date of isolation. The isolate numbers are listed in Fig. 1, and the isolates are more fully described elsewhere (7). We amplified this locus using a set of specific PCR primers, 17D+95 (5′-TCGCGAATAGCTTGGCCATTTTGC-3′) and 17D-344 (5′-CGTAGCACCGAAGTCAGATAAGAG-3′), complementary to flanking DNA. Both strands of selected PCR amplicons were sequenced to confirm that our PCR products corresponded to the expected region and number of TR copies. PCR amplification and sequencing were performed under conditions exactly as described for the amplification of specific *R. salmoninarum* genes (7). Most isolates yielded PCR products of an identical size, 301 bp, which contained two copies of the TR. Interestingly, all Icelandic isolates examined, as well as NCIMB1114 and NCIMB1116 (from Scotland), 4451-86 (from Norway), and AcF6-1 (from the Canadian northwest territories), yielded PCR products of 250 bp which contained only a single copy of the repeat. Furthermore, all of these isolates were clustered separately from the majority of *R. salmoninarum* isolates by RAPDistance analysis (Fig. 1).

***R. salmoninarum* isolates with a single TR unit are not SV1.** Members of our group have previously shown that although the *R. salmoninarum* 16S-23S rRNA ITS (ITS1) is highly conserved three sequence variants which reflect the geographic origin of isolates exist (7). A majority of *R. salmoninarum* isolates from a wide variety of sources appear to belong to SV1. The other ITS1 sequence variants SV2 and SV3, are more restricted in their distribution. The DNA sequences of ITS1 are already known for isolates S-182-90 (from Iceland) and AcF6-1, and they correspond to SV2 and SV3, respectively. In order to investigate whether any relationship between ITS1 sequence variation and ETR-A exists, we sequenced ITS1 for five isolates, NCIMB1114, NCIMB1116, 4451-86, F-283-87, and F-358-87, which possess a single copy of the TR at the ETR-A locus. The ITS1 was amplified and sequenced by the protocol previously described for PCR amplification and double-stranded sequencing of this region (7). The DNA sequences obtained in this way were found to belong to SV2 (F-283-87 and F-358-87) and a previously unknown ITS1 sequevar, SV4 (NCIMB1114, NCIMB1116, and 4451-86) (Gen Bank accession no. AF178998 to AF179002). DNA sequence data for the ITS1 region of selected isolates, including 3015-86 (from Norway) and MT417 (from Scotland), which possess two copies of the TR show that these belong to SV1 (Fig. 1). Therefore, ETR-A has a potential use as a specific marker for rapidly distinguishing ITS1 sequence variants.

The purpose of this study was to examine the molecular diversity of isolates of *R. salmoninarum* from the United Kingdom, other European countries, and North America and from a variety of salmonid host species. Previous research has shown that *R. salmoninarum* is a highly conserved genospecies with a remarkable degree of biochemical, serological, and genetic uniformity among isolates (1, 6, 16). Furthermore, studies of the *R. salmoninarum* genome have shown that isolates from diverse sources possess only limited sequence variation in the ITS of the 16S and 23S rRNA genes (7). Members of our group have previously (7) identified three ITS1 sequevars (SV1, SV2, and SV3). We found that isolates from Iceland (SV2), Japan (SV2), and the Canadian northwest territories (SV3) possessed three single-base substitutions in the ITS1 and showed some divergence from the highly conserved SV1, which was present in isolates from the United States, the United Kingdom, mainland Europe, and Canada. We proposed that in areas of the world which could be regarded as relatively isolated from the mainstream intensive salmonid culture of North America and Europe, the bacterium shows genetic divergence. The results presented here broadly support this hypothesis, although some isolates, most notably Marion Forks, vary from this pattern.

This study used an objective method based on RAPDistance software to examine the extent of molecular diversity among *R. salmoninarum* isolates from different countries around the world and related this information to specific regions of variation within the genome. We have identified four 16S-23S rRNA ITS1 sequevars and an exact TR locus (ETR-A) which are specific markers of variation within the genome of the bacterium, and furthermore, we have shown that an objective method of analysis of RAPD profiles, which can be used to differentiate *R. salmoninarum* isolates, reflects these specific markers.

Nucleotide sequence accession numbers. Sequences for DNA fragments and for ITS1 regions of isolates have been deposited in GenBank with accession numbers AF178991 to AF178997 and AF178998 to AF179002, respectively.

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REFERENCES

1. **Bruno, D. W., and A. L. S. Munro.** 1986. Uniformity in the biochemical properties of *Renibacterium salmoninarum* isolates obtained from several sources. FEMS Microbiol. Lett. 33:247–250.
2. **Chakroun, C., M. C. Urdaci, D. Faure, F. Grimont, and J.-F. Bernadet.** 1997. Random amplified polymorphic DNA analysis provides rapid differentiation among isolates of the fish pathogen *Flavobacterium psychrophilum* and among *Flavobacterium* species. Dis. Aquat. Org. 31:187–196.
3. **Chien, M. S., T. L. Gilbert, C. Huang, M. L. Landolt, P. J. O'Hara, and J. R. Winton.** 1992. Molecular cloning and sequence analysis of the gene coding for the 57-kDa major soluble antigen of the salmonid fish pathogen *Renibacterium salmoninarum*. FEMS Microbiol. Lett. 75:259–265.

4. **Evenden, A. J.** 1993. Gene cloning techniques in the study of the fish pathogen *Renibacterium salmoninarum*. Ph.D. thesis. University of Plymouth, Plymouth, United Kingdom.
5. **Evenden, A. J., T. H. Grayson, M. L. Gilpin, and C. B. Munn.** 1993. *Renibacterium salmoninarum* and bacterial kidney disease—the unfinished jigsaw. Annu. Rev. Fish Dis. 3:87–104.
6. **Goodfellow, M., T. M. Embley, and B. Austin.** 1985. Numerical taxonomy and emended description of *Renibacterium salmoninarum*. J. Gen. Microbiol. 131:2739–2752.
7. **Grayson, T. H., L. F. Cooper, F. A. Atienzar, M. R. Knowles, and M. L. Gilpin.** 1999. Molecular differentiation of *Renibacterium salmoninarum* isolates from worldwide locations. Appl. Environ. Microbiol. 65:961–968.
8. **Grayson, T. H., A. J. Evenden, M. L. Gilpin, K. L. Martin, and C. B. Munn.** 1995. A gene from *Renibacterium salmoninarum* encoding a product which shows homology to bacterial zinc-metalloproteases. Microbiology 141:1331–1341.
9. **Jonsdottir, H., H. J. Malmquist, S. S. Snorrason, G. Gudbergsson, and S. Gudmundsdottir.** 1998. Epidemiology of *Renibacterium salmoninarum* in wild Arctic charr and brown trout in Iceland. J. Fish Biol. 53:322–339.
10. **Kersulyte, D., J. P. Woods, E. J. Keath, W. E. Goldman, and D. E. Berg.** 1992. Diversity among clinical isolates of *Histoplasma capsulatum* detected by polymerase chain reaction with arbitrary primers. J. Bacteriol. 174:7075–7079.
11. **Mesa, M. G., A. G. Maule, T. P. Poe, and C. B. Schreck.** 1999. Influence of bacterial kidney disease on smoltification in salmonids: is it a case of double jeopardy? Aquaculture 174:25–41.
12. **Meyers, T. R., S. Short, C. Farrington, K. Lipson, H. J. Geiger, and R. Gates.** 1993. Comparison of the enzyme-linked immunosorbent assay (ELISA) and the fluorescent antibody test (FAT) for measuring the prevalences and levels of *Renibacterium salmoninarum* in wild and hatchery stocks of salmonid fishes in Alaska, USA. Dis. Aquat. Org. 16:181–189.
13. **Nei, M., and W. H. Li.** 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76:5269–5273.
14. **Saitou, N., and M. Nei.** 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.
15. **Sanders, J. E., J. J. Long, C. K. Arakawa, J. L. Bartholomew, and J. S. Rohovec.** 1992. Prevalence of *Renibacterium salmoninarum* among downstream-migrating salmonids in the Columbia River. J. Aquat. Anim. Health 4:72–75.
16. **Starliper, C. E.** 1996. Genetic diversity of North American isolates of *Renibacterium salmoninarum*. Dis. Aquat. Org. 27:207–213.
17. **Wang, G., W. T. S. Wittam, C. M. Berg, and D. M. Berg.** 1993. RAPD (arbitrary primer) PCR is more sensitive than multilocus enzyme electrophoresis for distinguishing related bacterial strains. Nucleic Acids Res. 21:5930–5933.
18. **Welsh, J., and M. McClelland.** 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18:7213–7218.
19. **Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingley.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531–6535.