

1993

MOLECULAR CLONING AND CHARACTERIZATION OF POTENTIAL VACCINE ANTIGENS F R O M *Renibacterium salmoninarum*

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<http://hdl.handle.net/10026.1/11118>

<http://dx.doi.org/10.24382/3313>

University of Plymouth

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MOLECULAR CLONING AND
CHARACTERIZATION OF
POTENTIAL VACCINE ANTIGENS
FROM *Renibacterium salmoninarum*

T. H. GRAYSON

Ph.D. 1993

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**MOLECULAR CLONING AND CHARACTERIZATION OF POTENTIAL VACCINE
ANTIGENS FROM *Renibacterium salmoninarum***

by

THOMAS HILTON GRAYSON B.Sc., M.Sc.

A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

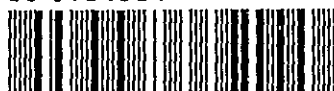
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DECLARATION

I hereby declare that this thesis has been composed by myself, that it has not been accepted in any previous application for a higher degree, that the work of which it is a record has been performed by myself, and that all sources of information have been specifically acknowledged.

Signed T.H. Grayson

T.H. Grayson

Signed Colin Blunn

Dr C.B. Munn
(Supervisor)

MOLECULAR CLONING AND CHARACTERIZATION OF POTENTIAL VACCINE ANTIGENS FROM *Renibacterium salmoninarum*

Thomas Hilton Grayson

ABSTRACT

A number of *R. salmoninarum* gene libraries were constructed in *E. coli* host-vector systems and screened for the production of molecules which may provide material for the immunisation of salmonid fish. One immunopositive clone was isolated from a *EcoRI* gene bank constructed in the plasmid vector pUC18 and another clone, which possessed membrane-active properties, was isolated from a *HindIII* gene bank constructed in the plasmid vector pBR328. The immunopositive clone was found to contain the major portion of gene *msa* encoding the major secretory antigen of *R. salmoninarum*, known as P57.

The membrane-active clone possessed a broad activity against erythrocytes from a number of mammalian species and rainbow trout, but not rabbits. No lecithinase, caseinase or gelatin degrading activities were detected in active clones. The haemolytic product was not identifiable on either SDS-PAGE gels or Western blots of cells taken from the active clone. However, minicell analysis revealed that the membrane-active protein was about 65K, and the promoter region of the gene encoding this protein was identified. Southern blotting showed that the gene was present in seven strains of *R. salmoninarum* of differing virulence. The gene was sequenced and the sequence analysed by computer. After comparison with the contents of the Protein Identification Resource database, the gene was found to encode a protein which bears strong similarities with a family of secreted zinc-dependent metalloendopeptidases and on this basis the gene was tentatively named *mpr* and the product, MPR. Aspects of the predicted structure and possible function of MPR are discussed.

In order to simplify the purification of material for further studies, gene fusions were constructed, from *msa*, *mpr* and *hly*, a gene encoding another membrane-active product from *R. salmoninarum*, in either pMAL or pAX5 vectors. The resultant fusion proteins were produced in a mainly soluble form and purified by affinity chromatography. The fusion proteins were found to be immunogenic in rats but only poorly immunogenic in rainbow trout. Epitopes of each of the fusion proteins were identified on Western blots by serum derived from rainbow trout undergoing a clinical outbreak of BKD and it is proposed that this provides circumstantial evidence that the native molecules are of immunological importance to BKD and may be useful candidates for future immunisation studies. Seven strains of *R. salmoninarum* were cultured *in vitro* under conditions of either iron-restriction or iron-sufficiency. Epitopes of each of the fusion proteins were identified in *R. salmoninarum* cell extracts and, in the case of *msa*, in the ECP. There is preliminary evidence that the production of MPR and the processing of P57 may be regulated by the availability of iron or other metals. No evidence for the production of siderophores by *R. salmoninarum* was found, although culture supernatants did possess some ability to inhibit the binding of iron by transferrin. A strong iron reducing activity was found to be associated with *R. salmoninarum* cells and the production of reducing sugars accompanied iron-restricted culture conditions. Comparative discussion of the pathogenicity of *R. salmoninarum* and of other Gram positive intracellular pathogens is provided.

PUBLICATIONS

Parts of this research have been published or presented at conferences:

Evenden, A.J., Grayson, T.H., Gilpin, M.L., Munn, C.B. (1993). *Renibacterium salmoninarum* and bacterial kidney disease- the unfinished jigsaw. *Ann. Rev. Fish Dis.* 3: 87-104.

Grayson, T.H., Bruno, D.W., Evenden, A.J., Gilpin, M.L., Munn, C.B. (1991). Growth of *Renibacterium salmoninarum* in iron-restricted medium. Proceedings of the V EAFP Diseases of Fish and Shellfish International Conference, Budapest, Hungary.

Grayson, T.H., Evenden, A.J., Gilpin, M.L., Munn, C.B. (1991). Partial characterisation of a second cloned haemolytic toxin from *R.salmoninarum*. Proceedings of the V EAFP Diseases of Fish and Shellfish International Conference, Budapest, Hungary.

CONFERENCES ATTENDED

First Bacterial Diseases of Fish International Conference, Stirling, Scotland, 26-29 June, 1990.

Fourth International Colloquium on Pathology in Marine Aquaculture, Vigo, Spain, 17-21 September, 1990.

119th Ordinary meeting of the Society for General Microbiology, Edinburgh, Scotland, 9-12 April, 1991.

Fifth EAFP Diseases of Fish and Shellfish International Conference, Budapest, Hungary, 25-29 August, 1991.

123rd Ordinary meeting of the Society for General Microbiology, Dublin, Eire, 2-4 September, 1992.

ACKNOWLEDGEMENTS

I wish to acknowledge the contributions of the following people, without whom none of this work would have been possible:

Dr L.Jervis and Professor C.Hawkes for providing me with the opportunity to carry out this research project in the Department of Biological Sciences at University of Plymouth.

Dr C.B.Munn, for his help, advice, support and supervision.

Dr M.L.Gilpin, for his expert guidance, advice, assistance and good humour throughout the course of this project.

Dr A.B.Wrathmell, for her expert assistance and advice.

Drs D.W.Bruno and A.E.Ellis, and all the staff at the Marine Laboratory, Aberdeen for providing bacterial strains, collaborative assistance and discussion.

Dr G.Rae of the Scottish Salmon Growers Association for support and useful discussions.

Stan McMahon and A.Gough for expertly caring for all experimental animals used in this work.

Dr P.J.Glynn for help and advice with protein separation.

Dr C.J.Rodgers of MAFF, Weymouth for fish serum, bacterial strains and helpful advice.

Dr M.O'Hare of the Rheumatology Unit, Addenbrooks Hospital, Cambridge for providing the pAX5+ vector.

Drs R.W.Titball and K.Martin of the Chemical Defence Establishment, Porton Down for providing the opportunity and expertise required to analyse gene and protein sequence data.

J.Brown of the University library for obtaining literature.

A.J.Evenden for providing genetic material and whose knowledge of pathogenicity and sense of humour extended far beyond the bounds of reasonable expectations.

All staff and researchers who have contributed to this work, particularly Sara McMahon, L.Cooper, J.Hone, Drs J.E.Harris and P.Burgess, E.Lavelle, P.Jenkins, R.Barton, C.Palmer, A.Bell, N.Crocker, D.Crocker, J.Carter, M.Pill and M.Noor. Thanks are especially due to S.M.Jones and S.Kingston for invaluable assistance with computing, to B.Fox for photography, C.Wilton for typing and to B.Griffin, M.Waters, W.Locock and M.Pawley for long hours spent cleaning glassware.

Jill, whose love, friendship and patience made all this worthwhile.

This research was financed with the aid of a studentship from the University of Plymouth, and the financial support of the Scottish Salmon Growers Association Ltd.

All experimental work using animals was carried out under Personal Home Office Licence number 30/00072, Project Licence number 30/00765.

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ABBREVIATIONS

A	adenine
A _{nm}	absorbance at the indicated wavelength
mA	milliampere
APTG	p-aminophenyl β -D-thiogalactopyranoside
ATP	adenosine triphosphate
dATP	deoxyadenosine triphosphate
ddATP	dideoxyadenosine triphosphate
BHIB	brain heart infusion broth
BKD	bacterial kidney disease
bp	base pairs
C	cytosine
°C	degrees centigrade
C3	complement component C3
CBB	coomassie brilliant blue R250
CMI	cell-mediated immunity
dCTP	deoxycytidine triphosphate
ddCTP	dideoxycytidine triphosphate
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPD	α, α' -dipyridyl
DTH	delayed-type hypersensitivity
ECP	extracellular products
EDDA	ethylenediamine di(o-hydroxyphenylacetic acid)
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
F _c	crystallisable fragment of immunoglobulin
FAT	fluorescent antibody test
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
G	guanine
g	gram
kg	kilogram
mg	milligram
μ g	microgram
dGTP	deoxyguanosine triphosphate
ddGTP	dideoxyguanosine triphosphate
x g	acceleration due to gravity
h	hour
HI	hyperosmotic infiltration
HLY	putative <i>R. salmoninarum</i> haemolysin
i.p.	intraperitoneal
IFAT	indirect fluorescent antibody test
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl- β ,D-thiogalactopyranoside

K	thousand
kbp	kilobase pairs
kDa	kilodaltons
LB	Luria-Bertani medium
LBA	LB agar
LBB	LB broth
ml	millilitre
μ l	microlitre
cm	centimetre
mm	millimetre
μ m	micrometre
M	molar
mM	millimolar
μ M	micromolar
pM	picomolar
MBP	maltose-binding protein
MHCA	Mueller-Hinton cysteine agar
MHCB	Mueller-Hinton cysteine broth
min	minute
MPR	putative <i>R.salmoninarum</i> metalloprotease
NADH	reduced β nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
dNTP	deoxynucleoside triphosphate
ddNTP	dideoxynucleoside triphosphate
p	statistical probability
p.f.u.	plaque forming units
P57	<i>R.salmoninarum</i> major secretory antigen
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid-Schiff's reagent
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFC	plaque forming cells
PHA	phytohaemagglutinin
PIR	Protein Identification Resource
PMA	phorbol myristate acetate
PMSF	phenylmethylsulphonylfluoride
%	percent
RNA	ribonucleic acid
mRNA	messenger RNA
tRNA	transfer RNA
RNase	ribonuclease
RNI	reactive nitrogen intermediates
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second
T	thymine
TBE	tris borate EDTA buffer
TE	tris EDTA buffer
TNP-LPS	trinitrophenylated lipopolysaccharide
Tris	tris(hydroxymethyl)aminomethane
dTTP	deoxythymidine triphosphate

ddTTG	dideoxythymidine triphosphate
U	uracil
UV	ultraviolet
v	volt
v/v	volume/volume
w/v	weight/volume
Xgal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

CHAPTER 1.

INTRODUCTION

Renibacterium salmoninarum is a Gram positive, intracellular pathogen of salmonid fish and is the aetiological agent of bacterial kidney disease (BKD). This is an important research area because salmonid fish have a worldwide distribution and are widely exploited for aquacultural, commercial wildfishing and sportfishing purposes. Indeed, *R. salmoninarum* has become a major cause of mortality amongst wild and farmed salmonids around the world (Evelyn, 1993). Fish experiencing a clinical outbreak of BKD do not respond well to treatment with antibiotics; to date no effective vaccine exists and current methods for the control of the disease rely upon tedious screening, segregation and quarantine procedures for a small proportion of breeding populations and their offspring.

The overall aim of this work was the application of recombinant DNA technology to facilitate the isolation of bacterial components which may be useful in the development of an effective vaccine against this disease. These methods represent a novel approach to the study of *R. salmoninarum*, although such techniques have been applied to the study of many other pathogens.

Because of the paucity of information available about the molecular basis of pathogenicity of *R. salmoninarum*, the direction of the study was guided to a large extent by comparative examples drawn from research into mammalian bacterial pathogens. Although an experimental study of pathogenicity was beyond the scope of this investigation it is hoped that the information presented will lead to an improved understanding of the mechanisms by which *R. salmoninarum* has evolved to become such a successful intracellular pathogen and the possible means of disrupting this cosy relationship with the salmonid host. This aspect of the research was guided, principally, by the concept of common themes which underlie microbial pathogenicity and are represented in all host/pathogen interactions (Finlay & Falkow, 1989; Falkow, Isberg & Portnoy, 1992). One of the main aims of the work detailed in this thesis was to attempt to identify and isolate from *R. salmoninarum* some of the components which have been shown to play an important role in pathogenicity in relation to other microorganisms. This was accomplished by using modern molecular techniques in order to bypass the difficulties faced by past researchers, who were forced to rely on less than ideal conventional methods. There was one limitation in planning this

investigation and that was the need to balance purely molecular studies with the more classical microbiological or immunological approach to these problems.

The initial aims of the work were:

- (i) construction of gene libraries and molecular cloning of genes from *R.salmoninarum*
- (ii) identification of a range of immunologically or biochemically relevant molecules encoded by these genes
- (iii) isolation and characterisation of selected candidate antigens
- (iv) expression of these antigens in *E.coli* host-vector systems
- (v) demonstration of the potential immunological importance of the recombinant proteins.

The central finding of this study has been the identification of three strong candidates for future BKD vaccine studies. During the course of the work it was decided to focus attention on those areas which it was felt would prove most promising in contributing to the eventual control of BKD. Consequently, much of this thesis is devoted to the molecular characterisation of one protein.

CHAPTER 2

LITERATURE REVIEW

2.1 A BRIEF HISTORY OF BACTERIAL KIDNEY DISEASE (BKD)

According to Smith (1964) the first record of bacterial kidney disease (BKD, also called Dee disease, corynebacterial kidney disease and salmonid kidney disease at various times) appears in the Second Interim Report of the Furunculosis Committee (1933) when wild Atlantic salmon, *Salmo salar* L., from two Scottish rivers, the Aberdeenshire Dee and the Spey, were found to have small necrotic lesions on the spleen. Despite unsuccessful attempts to culture small Gram positive diplobacilli from the lesions, the disease was transmitted to brown trout, *Salmo trutta* L., injected with emulsified spleen from diseased fish. A short time later, Belding & Merrill (1935) reported similar findings in diseased brook trout, *Salvelinus fontinalis* (Mitchill), and rainbow trout, *Oncorhynchus mykiss* (Walbaum), from a hatchery in Massachusetts, U.S.A.

Since then, cases of BKD have been reported from many countries in North America and Europe as well as Chile, Iceland, Japan and Turkey, affecting *Oncorhynchus* sp., *Salmo* sp., *Salvelinus* sp., *Hucho hucho* L., and probably also *Thymallus thymallus* L., all salmonid fish species (Fryer & Sanders, 1981; Klontz, 1983; Pfeil-Putzien, Hoffman & Popp, 1985; Kettler, Pfeil-Putzien & Hoffman, 1986; Austin & Austin, 1987; Souter, Dwilow & Knight, 1987; Sakai, Atsuta & Kobayashi, 1992a; Evelyn, 1988; 1993). The expansion of salmonid culture has assisted the spread of BKD and in many areas it is now recognised as one of the most prevalent and persistent diseases causing up to 80% losses amongst stocks of infected Pacific salmon (*Oncorhynchus* sp.) and 40% losses amongst stocks of infected Atlantic salmon (Bruno, 1988a; Elliot, Pascho & Bullock, 1989)...

Whilst the disease has been regularly found in both fresh and seawater salmonid culture it has been shown to be a potentially more serious threat to wild salmonid stocks, particularly after migration from fresh to salt water (Banner, Long, Fryer & Rohovec, 1986; Sanders, Long, Arakawa, Bartholomew & Rohovec, 1992). There are no reports of non-salmonids showing clinical symptoms of BKD following a natural outbreak of the disease. However, experimental infections have been established in sablefish, *Anoplopoma fimbria* Pallas (Bell, Hoffman & Brown, 1990), Pacific herring, *Clupea harengus pallasii* L. (Traxler & Bell, 1988), fathead minnow, *Pimephales promelas* (Rafinesque), and common shiner, *Notropis cornutus* (Mitchill), (Hicks, Daly & Ostland,

1986) although such attempts have failed to cause disease in either Pacific lamprey, *Lampetra tridentata* Gairdner (Bell & Traxler, 1986) or common carp, *Cyprinus carpio* L. (Sakai, Ogasawara, Atsuta & Kobayashi, 1989a). It is therefore possible that susceptible non-salmonid fishes living near BKD infected salmonid farms may consequently become carriers of the disease. The possible involvement of vectors other than fish, such as ectoparasites (Nylund, Bjoorknes & Wallace, 1991), molluscs (Sakai & Kobayashi, 1992) or protozoans, has received little attention.

2.2 CHARACTERISTICS OF *Renibacterium salmoninarum*

2.2.1 NATURE AND TAXONOMY

Renibacterium salmoninarum, the causative agent of BKD, is a Gram-positive, non-motile, non-acid-fast, non-sporulating, aerobic rod about 0.1-1.0 μm x 0.3-1.5 μm in size and often occurs in pairs or short chains (Sanders & Fryer, 1980). The organism has recently been shown to possess a capsule (Dubreuil, Lallier & Jacques, 1990a) and fimbriae or fibrils (Dubreuil, Jacques, Graham & Lallier, 1990b; Graham & Daly, 1990). *R. salmoninarum* is an obligate pathogen of salmonid fish found both intracellularly, in 'professional' and 'non-professional' phagocytes, and extracellularly (Young & Chapman, 1978; Bruno, 1986a).

The description and classification of the causative agent of BKD has been a convoluted process reviewed by Fryer & Sanders (1981) and Austin & Austin (1987). Earlier workers experienced considerable difficulties in culturing this slow growing, fastidious pathogen and relied heavily on a few morphological features. Consequently, the bacterium has been uneasily associated with *Rickettsia* (Sniesko & Griffin, 1955), *Corynebacterium* (Ordal & Earp, 1956; Smith, 1964), *Listeria* (Bullock, Stuckey & Wolf, 1975), *Brevibacterium* (Smith, 1964) and *Lactobacillus* (Vladik, Vitovec & Cervinka, 1974). Finally, after consideration of a variety of factors including guanine plus cytosine content (53 \pm 0.46 mol %) and a unique cell wall sugar and peptidoglycan composition, Sanders & Fryer (1980) proposed the establishment of a new genospecies *Renibacterium salmoninarum*. The mol % G+C has been recently corrected to 55.5% (Banner, Rohovec & Fryer, 1991).

Subsequent studies of the fatty acid composition (Goodfellow, Collins & Minnikin, 1976; Collins, 1982; Embley, Goodfellow, Minnikin & Austin, 1983), cell wall polysaccharides and peptidoglycan (Kusser & Fiedler, 1983; Fiedler & Draxl, 1986) have supported the validity of *R. salmoninarum* as distinctive from other taxa. In fact, the numerical phenetic study of Goodfellow, Embley & Austin (1985) showed that *R. salmoninarum* was distinct from most other

Gram-positive organisms and recent comparisons of the bacterium with more than 165 Gram-positive representatives from 50 genera by analysing genes encoding 16S ribosomal RNA placed *R. salmoninarum* as a member of the morphologically and chemotaxonomically diverse subgroup of the actinomycetes which includes *Arthrobacter*, *Micrococcus*, *Cellulomonas*, *Jonesia*, *Promicromonospora*, *Stomatococcus* and *Brevibacterium* (Stackebrandt, Wehmeyer, Nader & Fiedler, 1988; Gutenberger, Giovannoni, Field, Fryer & Rohovec, 1991).

2.2.2 CULTURE OF *R. salmoninarum*

Cultivation of *R. salmoninarum* has been the subject of considerable study and was not successfully achieved until Earp, Ellis & Ordal (1953) improved upon an initial medium containing fish extract, glucose, yeast extract and either beef serum or meat infusion. Even on the improved media of either minced chick embryos in 1% agar or Dorset's egg medium growth was slow requiring at least two weeks incubation at 15-20°C. Further work by Ordal & Earp (1956) demonstrated an absolute requirement for L-cysteine and led to the formulation of cysteine blood agar. This work also led to the fulfilment of Koch's postulates, establishing *R. salmoninarum* as the aetiological agent of BKD.

Since then, two media have emerged for the routine culture of *R. salmoninarum*, the commercial preparation Mueller-Hinton medium (Wolf & Dunbar, 1959) and the serum based KDM2 (Evelyn, Hoskins & Bell, 1973; Evelyn, 1977). Other notable developments have included the replacement of serum with charcoal (Daly & Stevenson, 1985), a semi-defined medium (Embley, Goodfellow & Austin, 1982) and a selective isolation medium, SKDM, developed by Austin, Embley & Goodfellow (1983). All media require the addition of 0.1% w/v L-cysteine hydrochloride. The superiority of SKDM over other media for the isolation of *R. salmoninarum* from infected fish tissue has been conclusively demonstrated by Gudmundsdottir, Helgason & Benediktsdottir (1991).

Despite these developments, the *in vitro* culture of *R. salmoninarum* remains time consuming and prone to contamination. Currently, there is no defined or minimal medium available; nor is there a medium which allows the consistent, rapid isolation of single colonies in pure culture. Growth is usually recorded as a shiny, smooth, raised, sticky, creamy-white mass on agar plates and any discrete colonies present are round, entire, convex and highly variable in size. In addition, a recent study by Benediktsdottir, Helgason & Gudmundsdottir (1991) has shown that incubation times of up to 19 weeks may be required for growth of the bacterium on agar. This prolonged period allows faster growing organisms to rapidly outcompete *R. salmoninarum* on rich media and is

possibly the major reason why an understanding of the pathogen has progressed so slowly. Various theories have been proposed for this slow growth, including the failure of sub-optimal media to meet complex nutritional or growth requirements (Embley, 1983; Evelyn, Bell, Prosperi-Porta & Ketcheson, 1989), the presence of inhibitory or toxic factors either produced by the pathogen or present in the medium (Evelyn *et al.*, 1989; Daly & Stevenson, 1990; Benediktsdottir *et al.*, 1991), the inhibitory effects of host tissues on clinical isolates (Evelyn, Ketcheson & Prosperi-Porta, 1981; Daly & Stevenson, 1988) and the need for an undefined endogenously produced and stored growth factor (Evelyn *et al.*, 1989). Evelyn & Prosperi-Porta (1989) have recently correlated inconsistencies in the growth of *R.salmoninarum* on KDM2 with variations in the quality of batches of peptone ingredient and suggested using single production lots which have been tested and found to be suitable. The use of peptone (0.1% w/v)-saline (0.85% w/v) as a diluent to remove any inhibiting factors in tissue isolations (Evelyn *et al.*, 1981) and the use of 'nurse' cultures for accelerating the growth of the pathogen have also been recommended (Evelyn *et al.*, 1989). The recent identification of a low molecular mass (< 10 kDa), heat stable growth promoting metabolite which is produced by *R.salmoninarum* (Evelyn, Prosperi-Porta & Ketcheson, 1990) provides some hope for the development of improved culture techniques.

2.2.3 BIOCHEMICAL PROPERTIES OF *R. salmoninarum*

Preliminary observations by Ordal & Earp (1956) mentioned protease and catalase activities and reported good growth at 17°C on cysteine blood agar. Further observations by Smith (1964) noted that growth was best at 15°C, slow at 5°C or 22°C and absent at 37°C. Smith (1964) also found that the bacterium failed to liquefy gelatin and possessed slow proteolytic activity without any apparent pH change in litmus milk made up with 25% Ordal and Earp's basal broth, i.e. without blood. Both Bell (1961) and Fryer & Sanders (1981) reported the failure to grow *R.salmoninarum* anaerobically although culture under microaerophilic conditions was not investigated.

Embley (1983) examined 44 isolates for biochemical characteristics and found that optimal growth occurred between pH 5.8 and 7.8 at 15-18°C. He found that growth of *R.salmoninarum* was enhanced by the addition of either albumin, blood, charcoal, Tween 60 or the low molecular weight fraction of foetal calf serum which included proteins of molecular weight less than 12000. The addition of either Tween 20 or Tween 80 has been shown to inhibit the growth of the bacterium (Daly & Stevenson, 1990).

Recent studies by Goodfellow *et al.* (1985) and Bruno & Munro (1986a) covering 70 isolates from Europe, Canada, America and Japan have found a remarkable uniformity in their biochemical properties and results obtained using the commercial API-ZYM kit give a characteristic diagnostic profile. Positive reactions include acid and alkaline phosphatase, periodic acid-Schiff's reagent (PAS), caprylate esterase, caseinase, catalase, DNase, degradation of tributyrin, Tween 40 and Tween 60 and utilisation of a number of 4-methylumbelliferyl fluorogenic esterase substrates. Some of the more notable negative reactions include β -galactosidase, cytochrome oxidase, degradation of lecithin and starch and acid production from sugars.

Bruno & Munro (1986a) reported the presence of previously unrecorded properties including β -haemolysis, gelatin liquefaction and glycogen in the cells, but failed to find phosphatase activity. They also noted that haemolytic activity declined with repeated sub-culturing and reported difficulty in establishing clinical disease in rainbow trout injected with such cultures. Bruno & Munro (1982) noted that whilst glycogen was present in the cells during all phases of growth it was depleted within 24 hours when cells were maintained in 0.1% peptone and 0.9% saline solution. They suggested this polysaccharide served as a food reserve.

2.2.4 CHEMICAL COMPOSITION OF *R.salmoninarum*

Sanders & Fryer (1980) undertook the first serious attempt to gather basic chemical data regarding the guanine plus cytosine content of the DNA, cell wall sugar composition and amino acid composition of the peptidoglycan cell wall layer of three strains of *R.salmoninarum* including the type strain. This study reported glucose to be the principal cell wall sugar with rhamnose, mannose and arabinose detected in smaller quantities.

Fiedler & Draxl (1986) analysed 13 strains of the pathogen isolated from different salmonid fish species in the United States, Canada and Europe. They found a marked similarity in both peptidoglycan and the covalently linked cell wall polysaccharide with those recorded for the type strain by Kusser & Fiedler (1983). The cell wall peptidoglycan contained alanine, glutamic acid, glycine and lysine as the diamino acids and is of the unique A3 α type according to the classification of Schleifer & Kandler (1972). In contrast to Sanders & Fryer (1980) the findings of Fiedler & Draxl (1986) were that galactose was the principal cell wall sugar accompanied by rhamnose, N-acetylglucosamine and N-acetylglucosamine. The polysaccharide amounted to 60% of the dry weight of the cell wall and was found to contain antigenic determinants.

The lipid composition of *R.salmoninarum* is also a distinguishing feature. An examination of 21 strains by Embley *et al.* (1983) revealed that methyl-branched fatty acids formed over 92% of the total fatty acid component and that the mycolic acids, phosphatidylinositol and related dimannosides and diaminobutyric acid found in *Corynebacterium* and coryneform bacteria were lacking. Straight chain fatty acids accounted for less than 1% of total fatty acids and unsaturated fatty acids were not detected. Over 81% of total fatty acids were composed of the low melting point *anteiso* acids which probably contribute to membrane fluidity at low temperatures. All strains possessed very characteristic polar lipid patterns consisting of diphosphatidylglycerol, two major and six or seven minor glycolipids and two unclassified minor phospholipids. In all cases the major menaquinone components consisted of unsaturated menaquinones with nine isoprene units. The results of Embley *et al.* (1983) confirm the findings of other preliminary studies (Goodfellow *et al.*, 1976; Sanders & Fryer, 1980; Collins, 1982).

2.3 PATHOLOGY AND EPIZOOTIOLOGY OF BKD

2.3.1 EXTERNAL AND INTERNAL SIGNS

While BKD infections can exhibit either an acute or chronic pathology and the infection may be localised, the disease is most usually a chronic, systemic, granulomatous and often fatal infection of juvenile and adult fish. A variety of factors have been reported that may affect the pathology and epizootiology of BKD infection, such as water temperature (Smith, 1964; Sanders, Pilcher & Fryer, 1978; Munro & Bruno, 1988), salinity (Banner *et al.*, 1983; Bruno, 1986b), host species (Bell, 1961; Sanders *et al.*, 1978; Bell, Higgs & Traxler, 1984; Bruno, 1988b), host genotype (Suzumoto, Schreck & McIntyre, 1977; Winter, Schreck & McIntyre, 1980; Withler & Evelyn, 1990; McGreer, Baranyi & Iwama, 1991; Beacham & Evelyn, 1992a; b), water chemistry (Warren, 1963; Fryer & Sanders, 1981), diet (Lall, Paterson, Hines & Adams, 1985; Landolt, 1989) and probably also the strain of the pathogen (Hoffman, Popp & van der Graff, 1984), although differentiation between strains of *R.salmoninarum* is still in its infancy (Bandin, Santos, Magarinos, Barja & Toranzo, 1992). However, these studies often relate either to outbreaks of clinical disease in intensively cultured salmonids or to acute laboratory induced infections and have relied upon methods of detection and diagnosis which have been shown to yield inconsistent results (Evelyn, 1988). Additionally, some work has been complicated by other factors such as the differing physiological responses of different salmonid species or stocks to stress (Wedemeyer, 1976; McGreer *et al.*, 1991) and the presence of other pathogens whose role has often been undefined e.g. *Aeromonas salmonicida* (Bell *et al.*, 1984), infectious pancreatic necrosis (IPN, Yamamoto, 1975), *Aeromonas hydrophila* (Mitchum, Sherman & Baxter, 1979; Winter *et al.*,

1980), *Acanthocephala* and *Cystidicola* sp. (MacLean & Yoder, 1970) and salmon fluke, *Nanophyetus salmincola* (Wood & Yasutake, 1956).

Generally, gross external symptoms may include darkening of the fish, exophthalmia, distended abdomen (ascites), ocular lesions, blebs, ulcers and abscesses on the skin (Hendricks & Leek, 1975; Fryer & Sanders, 1981; Hoffman *et al.*, 1984). A number of histopathological studies have described the similarities between BKD and other granulomatous inflammatory reactions such as mammalian proteinuria, glomerulonephritis and nephrotic syndrome (Wolke, 1975; Young & Chapman, 1978; Bruno, 1986a). Internally, the pathology is typified by a swollen, grey-white kidney with granular lesions and often lesions in the liver, spleen, heart and eyes. The central nervous system is often severely affected by a pyogranulomatous meningoencephalitis (Speare, Ostland & Ferguson, 1991). The spleen is often enlarged and darker red than usual and the liver pale. Histologically, affected organs, especially the kidney, have foci of caseous necrosis and evidence of chronic inflammation with granulomatous tissue infiltrated by macrophages and leucocytes. In all lesions, *R.salmoninarum* may be found both extra- and intracellularly (within macrophages, neutrophils, thrombocytes, monocytes and 'non-professional' phagocytes) along with leucocytes and cellular debris (Lester & Budd, 1979; Fryer & Sanders, 1981). Variable quantities of ascitic fluid may collect in the abdominal cavity and petechial haemorrhaging of the abdominal muscles may also occur (Smith, 1964). A whitish pseudo-membrane may cover but not penetrate the vital organs (Sniesko & Griffin, 1955). Smith (1964) described this membrane as consisting of three distinct layers of (a) fibroblasts and histiocytes innermost, (b) degenerating leucocytes and histiocytes between and (c) fibrin and nucleated cells outermost. It has been suggested that the membrane represents an unsuccessful host attempt to encapsulate the pathogen (Bruno, 1986a). The destruction of tissue is thought to be a function of the release of hydrolytic and catabolic enzymes by ruptured host cells, particularly phagocytes, although the liberation of toxic or lytic agents from bacteria or autolysis of bacteria may also contribute. Undoubtedly, the release of aggressive agents by cells of the cell-mediated immune system into areas of infection will also exacerbate the reaction and *R.salmoninarum* has been shown to induce the hyperproduction of reactive oxygen intermediates by phagocytes (Kaattari, Holland, Turaga & Weins, 1987; Kaattari, Chen, Turaga & Weins, 1988; Bandin, Ellis, Barja & Secombes, 1993). Recent studies shows that this pathology is part of a granulomatous inflammatory reaction caused by the deposition of immune complexes in the lympho-haemopoietic tissues of the kidney, spleen and liver, proliferation of melanomacrophages, macrophage recruitment and activation and a consequent type-III hypersensitivity response (Villena, Razquin, Turaga & Kaattari, 1989; Sami, Fischer-

Scherl, Hoffman & Pfeil-Putzien, 1992). Damage to renal tubules and glomeruli is inevitably extensive.

A number of changes in blood parameters accompany this pathology and are consistent with damage to the lymphopoietic and haemopoietic tissues of the kidney, liver and spleen (Table 2.1). The depletion of erythrocytes is a result of their retention by the spleen, hence the splenic enlargement, and it has been suggested that this may be due either to liver damage affecting sterol composition of the membranes or associated with the production of a toxin by the pathogen (Turaga, Wiens & Kaattari, 1987a). Presumably death is a result of either one or a combination of vital organ dysfunction, renal failure, osmoregulatory failure or loss of blood and body fluids. Whilst the pathology of BKD is characteristic of clinically diseased fish an asymptomatic carrier state has been found to exist amongst wild fish stocks, cultured salmonids surviving a clinical outbreak of BKD or following antibiotic treatment and even amongst stocks with no prior history of clinical BKD (Evelyn *et al.*, 1981; Paterson, Lall & Desautels, 1981a; Austin, 1985). Subjecting carrier fish to stressors such as handling or crowding (Wedemeyer, 1976) or low oxygen (MacLean & Yoder, 1970) may be sufficient to initiate clinical disease.

Table 2.1: Changes in Blood Parameters Observed in Bacterial Kidney Disease

Parameter	Observation	References
Erythrocyte depletion		
Hematocrit; erythrocyte count; erythrocyte diameter; haemoglobin content; ratio of mature:immature cells	Decrease	Suzomoto <i>et al.</i> 1977, Aldrin <i>et al.</i> 1978, Bruno & Munro 1986b, Iwama <i>et al.</i> 1986
Lymphocytosis		
Circulating monocytes as a proportion of total leucocytes	Increase	Bruno & Munro 1986b
Blood chemistry		
Protein	Decrease	Suzomoto <i>et al.</i> 1977, Aldrin <i>et al.</i> 1977, Hunn 1964, Kimura 1978, Bruno 1986c
Cholesterol	Decrease	Aldrin <i>et al.</i> 1977, Bruno 1986c
Serum sodium	Decrease	Bruno 1986c
Vitamin C	Decrease	Wedemeyer & Ross 1973
Vitamin A	Decrease	Paterson <i>et al.</i> 1981a, Suzomoto <i>et al.</i> 1977
Zinc	Decrease	Paterson <i>et al.</i> 1981a, Suzomoto <i>et al.</i> 1977
Iron	Decrease	Paterson <i>et al.</i> 1981a, Suzomoto <i>et al.</i> 1977
Urea nitrogen	Increase	Bruno 1986c, Wedemeyer & Ross 1973
Bilirubin	Increase	Bruno 1986c
Potassium	Increase	Bruno 1986c

2.3.2 TRANSMISSION OF THE DISEASE

R. salmoninarum is one of the few fish pathogens which has been shown to be transmitted both 'horizontally' by infected fish and fish tissue and 'vertically' in the yolk of eggs from infected female fish. In addition, subclinically infected or asymptomatic carrier fish have provided a reservoir of infection. Early pathogenicity experiments succeeded in transmitting BKD by subcutaneous, intramuscular and intraperitoneal injection of either infected tissue or pure culture (Belding & Merrill, 1935; Earp, 1950; Smith, 1964). Successful transmission of the disease to naive fish has also been achieved by feeding infected flesh and viscera (Wood & Wallis, 1955), skin abrasion followed by immersion in a suspension of the bacterium (Wolf & Dunbar, 1959) and cohabitation (Bell *et al.*, 1984; Evelyn, 1988). Transmission from cultured to wild fish has been reported (Pippy, 1969; Mitchum *et al.*, 1979) and *vice versa* (Mitchum & Sherman, 1981).

R. salmoninarum is therefore capable of survival outside of the host for sufficient time to ensure 'horizontal' transmission to cohabiting salmonids in both fresh and seawater. However, Austin & Rayment (1985) found no evidence to suggest that the pathogen occurs free-living in the environment and showed that *Renibacterium* was capable of surviving for only four days in non-sterile freshwater at 15°C although it was found to survive in fish tank sediment/faecal matter for up to 21 days in the absence of fish. They suggested that the organism has an affinity for organic matter and this is supported by reports of the recovery of *R. salmoninarum* from the faeces of wild and cultured salmonids (Mitchum & Sherman, 1981). Evelyn (1988) reported the survival of *Renibacterium* to be limited to a maximum of 12-16 days in filter sterilised samples of 'hard' freshwater and seawater and 7-12 days in 'soft' freshwater at 15°C.

Allison (1958) first reported the apparent transmission of BKD by infected eggs and Bullock, Stuckey & Mulcahy (1978) found that transmission occurred despite disinfection of the egg surface. The suspicions of these and other researchers (MacLean & Yoder, 1970; Mitchum *et al.*, 1979) were confirmed by Evelyn, Ketcheson & Prosperi-Porta (1984a) and Evelyn, Prosperi-Porta & Ketcheson (1984b; 1986a) who firmly established the intra-ovum location of the pathogen as within the yolk rather than the perivitelline space and demonstrated the ineffectiveness of egg surface disinfection procedures for reducing 'vertical' transmission of the disease (Brown, Ricks, Evelyn & Albright, 1990a). Surface disinfection of eggs with iodine or water hardening eggs in an erythromycin solution effectively reduced the numbers of viable bacteria on the external egg surface but failed to prevent intra-ovum transmission because of the short exposure time to the antimicrobials and the failure of the drugs to reach the egg yolk. The source of infection was

shown to be the coelomic or ovarian fluid which surrounds the egg following ovulation (Evelyn, Ketcheson & Prosperi-Porta, 1986b) and which in infected females may contain sufficiently high numbers of the bacterium to ensure intra-ovum infection (Lee & Evelyn, 1989). The male salmonid appears to play a minor role in the egg infection process despite evidence that *R.salmoninarum* agglutinates salmonid spermatozoa by binding exclusively to the sperm tail (Daly & Stevenson, 1989). Evelyn *et al.* (1986b) concluded that when present in sufficiently high numbers *R.salmoninarum* was small enough to enter the egg via the micropyle, resided in the yolk and did not produce particularly lethal toxins, thus affected fish were allowed to survive and spawn and 'vertical' transmission was accomplished. However, Bruno & Munro (1986c) observed the pathogen entering oogonia early in oogenesis, prior to ovulation, and noted that the prolonged lag phase for growth of the bacterium may contribute to survival within or on ova without damaging the host.

2.3.3 EFFECT OF HOST SPECIES

Munro & Bruno (1988) in reviewing the pathogenesis of BKD concluded that on the basis of studies by Bell (1961), Sanders *et al.* (1978) and Bell *et al.* (1984) and in their own experience all species of the genus *Oncorhynchus* are more susceptible to BKD than *Salmo* species. Within the genus *Oncorhynchus*, Bell (1961), Sanders *et al.* (1978) and Bell *et al.* (1984) consider pink salmon, *O.gorbuscha* (Walbaum), the most susceptible followed by sockeye, *O.nerka* (Walbaum), chinook, *O.tshawytscha* (Walbaum), chum, *O.keta* (Walbaum) and finally coho, *O.kisutch* (Walbaum) the least susceptible. These findings have been confirmed by Sakai, Atsuta & Kobayashi (1991a). In contrast, Kaattari, Chen, Turaga & Weins (1988) reported that coho were more resistant to BKD than rainbow trout (*O. mykiss*, Walbaum) and chinook the most susceptible of the three species. According to Munro & Bruno (1988) Atlantic salmon, *S.salar*, and European strains of *O.mykiss* have some modest resistance to BKD and Mitchum *et al.* (1979) reported higher mortalities and incidence of the disease among *Salvelinus fontinalis* followed by *Salmo trutta* and *O.mykiss* in wild fish populations. It is interesting to note that a study of ocean caught salmonids by Banner *et al.* (1986) detected a much higher incidence of *R.salmoninarum* infection (11%) in *O.tshawytscha* as compared to other species of *Oncorhynchus* (1-4%). In support of this finding Hsu, Bowser & Schachte (1991) utilised a sensitive monoclonal antibody based immunoassay and showed that amongst samples taken from spawning Great Lakes salmonid populations 80% of *O.tshawytscha*, 20% of *O.kisutch* and 11.7% of *O.mykiss* were BKD infected. The presence of such a high incidence of infection amongst wild populations gives some indication of the scale of the problem (Sakai, Atsuta & Kobayashi, 1992a; Sanders *et al.*, 1992).

Differential susceptibility to BKD was found to exist between strains of coho salmon by Suzumoto *et al.*, (1977) and Winter *et al.* (1980) and was associated with possession of the 'C'-transferrin allele (Utter, Ames & Hodgins, 1970). Suzumoto *et al.* (1977) assessed the survival of coho salmon representing three transferrin genotypes in an injected challenge of BKD. They used limited numbers of fish for the tests and consequently relied upon pooled data from different treatments for statistical analysis. Nevertheless, they concluded that the addition of exogenous iron did not enhance the pathogenicity of *R. salmoninarum* and that the 'C'-transferrin allele conferred some resistance to the disease. After further experimentation Winter *et al.* (1980) suggested that the importance of transferrin genotype of coho salmon in the resistance to BKD was stock specific. However, this is by no means clear since resistance to BKD may be correlated to a particular transferrin genotype without being related to the protein (Winter *et al.*, 1980). The presence of stock specific resistance to BKD among coho salmon was demonstrated by McGreer *et al.* (1991) but was not correlated with transferrin genotype. In a recent study by Withler & Evelyn (1990) it was demonstrated that a substantial additive genetic variation for BKD resistance existed within coho salmon strains which may not be accounted for by transferrin polymorphism and may be more closely related to other selective pressures. It has been demonstrated that substantial improvements in resistance to BKD amongst Pacific salmon can be made by judicious selection for the strain or population which is to be used for broodstock (Beacham & Evelyn, 1992a; b). Bruno & Johnstone (1990) found that there were no consistent differences in the level of susceptibility to BKD following intraperitoneal challenge between an all female triploid population of Atlantic salmon and an all female diploid population raised for aquacultural purposes.

2.3.4 EFFECTS OF TEMPERATURE

Belding & Merrill (1935) first described the seasonal nature of BKD outbreaks. Their initial findings were that generally most epizootics occurred in autumn and winter as water temperatures declined although most mortalities occurred in spring as water temperatures rose. Similar findings have been reported by other researchers (Earp, 1950; Earp *et al.*, 1953; Sniesko & Griffin, 1955; Smith, 1964; Austin, 1985; Bruno, 1986b).

Experimental infections show that as water temperatures rise above 10-12°C BKD infections shift from chronic to acute with a reduction in the mean time to death (Kimura, 1978; Sanders *et al.*, 1978). In contrast to many other fish pathogens such as *Aeromonas salmonicida* (Hastings, 1988), temperatures above 15°C exert a suppressive effect on the disease process and evidence of BKD may disappear entirely from the affected stock (Earp, 1950; Bruno, 1988b), although this will

depend on host susceptibility (Sanders *et al.*, 1978) and perhaps other complicating factors such as crowding and handling stress (Wedemeyer & Ross, 1973), low oxygen levels (MacLean & Yoder, 1970) and chemical stress (Iwama & Greer, 1980).

Fryer & Sanders (1981) concluded that observations of natural epizootics have generally shown agreement with laboratory studies indicating that BKD is a slowly developing infection causing death over a wide range of water temperatures. Depending on the challenge dosage, mortalities from BKD usually occur from about 17-54 days post-exposure at temperatures above 11°C and from 60-90 days at water temperatures of 4-10°C (Evelyn, Hoskins & Bell, 1973; Iwama, 1980; Fryer & Sanders, 1981).

2.3.5 EFFECTS OF DIET

Woodall & LaRoche (1964) whilst researching the iodine requirements of young chinook salmon found the incidence of BKD in their test groups varied according to the level of iodine in the diet. Whether variation in dietary iodine directly affected the course of the disease was not established because the outbreak of disease was fortuitous, the source of infection was not identified and the principal cause of death was not ascertained.

Since then a variety of studies have sought to establish whether a nutritional basis for the outcome of BKD exists. In 1973 Wedemeyer & Ross fed Abernathy dry diet containing cottonseed meal to one group of coho salmon and a similar diet containing corn gluten to another group. After one month, each group was fed viscera from BKD infected fish and maintained for a further six months on the test diets whilst a variety of blood parameters were monitored. Infected fish were found to comprise about 20% of each dietary group as identified by Gram-staining kidney smears, a relatively insensitive technique for identifying *R.salmoninarum*, and whilst the incidence of infection did not differ significantly between dietary groups, the non-specific stress of infection as indicated by increased interrenal vitamin C depletion was more severe in the corn gluten group. The possibility that the low incidence of infection in each group reflected either unsuccessful transmission of the disease or failure to successfully diagnose infected fish cannot be excluded. Whatever the reason, Wedemeyer & Ross (1973) stated that the Abernathy diet prepared with corn gluten has been shown to be poorer for the growth of salmon as compared to the equivalent cottonseed formulation (Fowler & Burrows, 1971) and this alone may have affected the outcome.

The effect of dietary components on the course of natural BKD outbreaks in Atlantic salmon were investigated in a series of related studies by Paterson *et al.* (1981a), Paterson, Lall, Airdrie, Greer, Greenham & Poy (1985) and Lall *et al.* (1985). Paterson *et al.* (1981a) noted that infected fish had lower serum, renal and hepatic levels of vitamin A, zinc and iron and conducted an 11 month trial utilising six different diets containing various mineral and vitamin additives, with a commercial diet for comparison. No mortality figures were reported for any of the diets but the incidence of BKD, as determined by the presence of internal lesions and serological identification of *R.salmoninarum*, was highest amongst fish fed the commercial diet and lowest amongst fish fed a diet supplemented either with high levels of iron, copper, manganese, cobalt, iodine and fluorine or with low levels of calcium. Diets enriched with vitamins A and C showed a small improvement over the commercial diet although a later study by Sakai, Nagata, Iwami, Koide, Tamiya, Ito & Atoda (1986) using masu salmon, *Oncorhynchus masou* (Brevoort), showed no difference in BKD mortalities between fish fed a commercial diet and those fed food supplemented with vitamins A, C, D, E, B₁, B₂, B₆, K₃, calcium pantothenate, folic acid and inositol. Subsequent studies by Paterson *et al.* (1985) and Lall *et al.* (1985) over the course of two years largely supported the findings of Paterson *et al.* (1981a) although this time the low calcium diet was found not to be effective in reducing the incidence of BKD whilst fish fed with a high fluorine and iodine supplement showed the lowest incidence of the disease. It is interesting to note that Lall *et al.* (1985) reported that BKD mortalities for each dietary group were similar and quite low varying from 8-17%. The interpretation of these results is complicated by the marked variation in the prevalence of the disease from year to year. Whether this variation reflected different environmental influences which may be expected to affect the nutritional requirements of the fish was not made clear (Landolt, 1989). Bowser, Landy & Wooster (1988) have also reported on the efficacy of fluoride for preventing BKD mortality in rainbow trout fed a fluoride supplemented diet for three weeks prior to intraperitoneal challenge at 15°C.

Bell *et al.* (1984) evaluated the effect of dietary ascorbate, zinc and manganese on the resistance of sockeye salmon to experimentally induced BKD. Fish were fed test diets containing either of three levels of Na-L-ascorbate-2-sulfate combined with either high levels of zinc and manganese or low levels of manganese alone. After 231 days groups of fish were challenged intraperitoneally with either of three bacterial doses. Survival time was found to be inversely related to the level of ascorbate when diets contained low levels of manganese but there was no such correlation when high levels of zinc and manganese were present in the diet although interpretation of the results was complicated by the 'horizontal' transmission of BKD to control fish and a cross-infection of

furunculosis. In addition, recent studies of ascorbate-2-sulfate as a dietary vitamin C source for Atlantic salmon show that it does not provide the tissues of the fish with adequate supplies of vitamin C to secure optimal physiological functions (Sandnes, Hansen, Killie & Waagbo, 1990) and is similarly poorly utilised in rainbow trout (Dabrowski & Kock, 1989). Hence it may have been unwise for Bell *et al.* (1984) to correlate differences in susceptibility to BKD with dietary levels of ascorbate-2-sulfate particularly as no mention was made of other vitamin C sources in the diets.

The role of vitamin C supplied as ascorbic acid may warrant further investigation particularly as studies of other fish pathogens have shown its importance in relation to the disease process and immune function (Landolt, 1989; Navarre & Halver, 1989; Blazer, 1991) and a recent report by Enriquez, Schafer & Monras (1989) showed a dramatic reduction in BKD-associated mortalities amongst coho salmon fed a diet containing high doses of ascorbic acid when stocking densities were reduced. Indeed much of the pathology of BKD resembles that found in vitamin C deficient (scurbutic) salmonids e.g. reduced haematocrit, haemoglobin, iron levels in the serum, liver and spleen and ascorbate levels in the kidney and liver (Hilton, Cho & Slinger, 1978; Maage, Waagbo, Olsson, Julshamn & Sandnes, 1990; Sandnes *et al.*, 1990).

2.3.6 EFFECTS OF WATER CHEMISTRY

Fryer & Sanders (1981), Banner *et al.* (1983) and Sakai, Goma, Seto, Atsuta & Kobayashi (1992b) found that the severity of BKD particularly in Pacific salmon may increase in seawater and Paterson, Gallant, Desautels & Marshall (1979) suggested that the same is probably true of Atlantic salmon. Bruno (1986b) on the other hand observed that when Atlantic salmon are introduced into seawater the progress of the disease may be halted and the infection eliminated or reduced to undetectable levels. Warren (1963) reported an apparent correlation between water hardness and BKD mortalities i.e. the 'softer' the water the more severe the disease. However, Lall *et al.* (1985) reported that salmonids from Margaree Hatchery, Nova Scotia, Canada where water chemistry shows wide seasonal fluctuations suffer more severely from BKD mortalities than stocks from 'soft' water areas in the same region. Research by Mitchum *et al.* (1979) on the incidence of BKD in a stream system in Wyoming, U.S.A. found that environmental factors including water flow rates, seasonal variation in water levels, water chemistry and even the topography of the river were important in the seasonal occurrence of the disease. As Lall *et al.* (1985) suggested, it may be necessary to consider a variety of dietary, water quality, environmental, physiological and genetic factors affecting the prevalence and severity of BKD

rather than simply one or two in isolation. It is important to remember that epizootiological studies of BKD have relied heavily upon the sensitivity and specificity of the available diagnostic tests.

2.4 DETECTION AND DIAGNOSIS

For many years presumptive diagnosis of BKD relied upon the presence of typical clinical signs and small Gram-positive diplobacilli in infected tissues. However, the presence of melanin granules or other bacteria can complicate diagnosis and Gram-staining will reliably detect only very high numbers of the bacterium (Fryer & Sanders, 1981). Another histochemical stain, Lillie's allochrome (Bruno & Munro, 1982; 1986c), has been used to detect glycogen in *R.salmoninarum* cells within infected tissues but may encounter similar difficulties if other glycogen containing bacteria are present.

Definitive diagnosis of BKD has relied upon the isolation of *R.salmoninarum* which remains very time consuming despite the development of a selective medium (Benediktsdottir *et al.*, 1991). Hence a variety of serological tests initially utilising polyclonal antisera were devised in attempts to find a rapid and specific means of diagnosis. Chen, Bullock, Stuckey & Bullock (1974) described an Ouchterlony immunodiffusion test to identify the presence of *R.salmoninarum* antigens in the internal tissues of salmonids showing clinical symptoms. Bullock, Stuckey & Chen (1974) used this method as well as the serum agglutination test on 10 isolates of the pathogen to conclude that *R.salmoninarum* was antigenically homogeneous. The results of Kimura, Ezura, Tajima & Yoshimizu (1978) using a heat stable antigen extract from clinically diseased fish would seem to support this finding. The immunodiffusion test takes no more than 24 hours for the precipitation reaction to occur but is relatively insensitive compared to other immunological tests.

The search for greater speed and sensitivity led firstly to the development of a coagglutination test using staphylococcal cells coated with anti-*Renibacterium* antibody which were reacted with heated tissue extracts (Kimura, 1978; Kimura & Yoshimizu, 1981). Agglutination of the staphylococcal cells occurred in the presence of specific antigen. However, the test proved insufficient for the detection of subclinically infected or asymptomatic carrier fish.

Development of the indirect fluorescent antibody technique (IFAT, Bullock & Stuckey, 1975; Mitchum *et al.*, 1979; Paterson *et al.*, 1979; Laidler, 1980) and the direct fluorescent antibody technique (FAT, Bullock, Griffin & Stuckey, 1980) provided a rapid and specific alternative for screening large numbers of samples, taking only two hours to perform IFAT and 10 minutes for

FAT. In addition, it was claimed to be 100-1000 times more sensitive than Gram-stain and 10 times more sensitive than culture. However, Evelyn (1981) and Evelyn *et al.* (1981) voiced concern about the possible cross-reactivity of antisera with other organisms and reported that in certain situations, particularly when attempting to detect low numbers of cells, culturing was more sensitive than fluorescent antibody methods (Armstrong, Martin, Evelyn, Hicks, Dorward & Ferguson, 1988). Indeed, studies by Shortt, Olivier & Elner (1988) and Paclibare, Evelyn & Albright (1988) showed that culture was 10^3 - 10^5 fold more sensitive than fluorescent antibody techniques for the detection of low numbers of cells in diseased tissues. Although the sensitivity of FAT and IFAT can be improved by using membrane filtration to concentrate bacteria on the surface of a filter (Elliot & Barila, 1987; Lee, 1989) the problem of cross-reactive antigens remained. Bullock *et al.* (1980) observed large FAT-positive bacteria in faecal samples of brook trout which clearly were not *R.salmoninarum* and after an extensive study Austin & Rayment (1985) reaffirmed the sensitivity of the culture method and found false positive IFAT reactions with the coryneform bacteria *Mycobacterium* sp., *Rothia dentocariosa* and an unidentified isolate (Austin, Bucke, Feist & Rayment, 1985; Austin & Austin, 1987). Yoshimizu, Ji, Nomura & Kimura (1987) also reported a false positive IFAT reaction caused by a *Pseudomonas* sp. although the use of partially purified IgG removed the cross-reaction. Austin & Austin (1987) recommended the use of preabsorbed polyclonal antisera or monoclonal antibodies to overcome problems of antigenic cross-reaction in serological tests and suggested that whenever possible observations should be confirmed by more than one method and ideally including culture. An interesting development of the fluorescent antibody technique called QFAT is claimed to allow the quantitative assessment of the prevalence, severity and outcome of BKD infections (Cvitanich, 1987; 1988). The method relies upon the enumeration of 'bar forms' which appear to be dead or dying *R.salmoninarum* cells in the kidney and spleen of infected fish.

The use of the enzyme-linked immunosorbent assay (ELISA) has been investigated for the detection of *R.salmoninarum* antigens using polyclonal antisera and found to be a more sensitive method than FAT or IFAT although apparently not as sensitive as culture (Dixon, 1987a; b; Pascho, Elliot, Mallett & Mulcahy, 1987; Pascho & Mulcahy, 1987; Pascho, Elliot & Streufert, 1991). By preabsorbing the IgG fraction of the antiserum with the main cross-reacting bacterial species, the specificity of the assay was greatly improved (Dixon, 1987a). ELISA also compared very favourably in sensitivity with the dot blot and Western blot assays for the detection of *R.salmoninarum* antigen described by Sakai, Amaaki, Atsuta & Kobayashi (1987a), Sakai, Atsuta & Kobayashi (1989b) and Sakai, Sugawara, Atsuta & Kobayashi (1990).

More recently, the identification of monoclonal antibodies (Mabs) directed against heat stable epitopes of *R.salmoninarum* have offered diagnosticians some hope of bypassing the problems associated with antigenic cross-reactivity. Arakawa, Sanders & Fryer (1987) developed 13 different Mabs against *R.salmoninarum* cells which demonstrated the existence of antigenic diversity among nine isolates of the bacterium. Wiens & Kaattari (1989) reported the identification of two different Mabs which recognised different epitopes of the major surface component known as P57, haemagglutinin or antigen F, of *R.salmoninarum* and did not cross-react with three other Gram-positive and two other Gram-negative species of bacteria. Research involving both polyclonal and monoclonal antibodies directed against P57 epitopes indicated that the proteins are antigenically similar in isolates from a wide geographic area (Getchell, Rohovec & Fryer, 1985; Wiens & Kaattari, 1989; 1991). In addition, P57 is produced in sufficiently high concentrations in the kidney, spleen, blood and reproductive fluids of diseased fish to enable accurate detection and quantitation by an ELISA using either monoclonal or polyclonal antibodies (Turaga, Wiens & Kaattari, 1987b; Hsu, Bowser & Schachte, 1991; Rockey, Gilkey, Wiens & Kaattari, 1991a). Such an approach could allow an assessment of the disease status of fish stocks since progression of the disease is accompanied by a gradual increase in the quantity of soluble antigen present in the infected host (Turaga, Wiens & Kaattari, 1987a). The uses of monoclonal and specific polyclonal antibodies have also been extended to dot blot (Sakai, Atsuta & Kobayashi (1991b) and also to immunohistochemical methods for the detection of *R.salmoninarum* antigen which despite being time consuming have proved to be effective diagnostic tests (Jansson, Honglo, Lindberg, Ljungberg & Svensson, 1991; Evensen & Dale, 1991).

An alternative to the detection of *R.salmoninarum* antigen has been the detection of specific antibody from infected host fish for diagnostic purposes. Being a non-destructive method this would have an advantage over other diagnostic tests that require samples to be killed. Generally, low serum agglutination titres have been detected in wild and hatchery reared salmonids during outbreaks of clinical disease although when disease signs are absent agglutinating titres may decline (Weber & Zwicker, 1979; Evelyn *et al.*, 1981; Bruno, 1987). Although some of these problems may be overcome through the use of a more sensitive assay, such as ELISA (Sakai, Atsuta & Kobayashi, 1991c) or electroimmunotransfer (Olivier, Griffiths, Fildes & Lynch, 1992), the widespread occurrence of low agglutination titres in the sera of non-BKD infected fish (Weber & Zwicker, 1979; Evelyn *et al.*, 1981; Paterson, Desautels & Weber, 1981b; Bruno, 1987) and the lack of correlation between antibody titres and BKD antigen levels (Hsu *et al.*, 1991) suggests that the presence of antibody may be an unreliable indication of the presence of BKD.

Armstrong *et al.* (1988) first noted the presence of *R.salmoninarum* agglutinating activity in ovarian fluid samples from chinook salmon and Griffiths & Lynch (1990) subsequently utilised a sensitive Western blotting technique to show that the *R.salmoninarum*-specific antibody present in the ovarian fluid (or the serum) of infected Atlantic salmon could be used as a non-lethal means of detecting female carriers and interrupting 'vertical' transmission. Griffiths, Olivier, Fildes & Lynch (1991) and Olivier *et al.* (1992) have also compared the Western blot, fluorescent antibody and culture methods of detection of *R.salmoninarum* in Atlantic salmon and found Western blotting to be closely correlated with the culture technique and a more sensitive and reliable method than FAT.

The most recent proposals for diagnostic methods involve the use of gene probes. These methods, which involve the use of DNA, RNA or oligonucleotide sequences which hybridise with 'target' sequences of the bacterial genome offer considerable advantages in sensitivity and specificity (Macario & de Macario, 1990). As well as their use in detection of the pathogen in tissue samples for the diagnosis of disease, the use of amplification techniques such as PCR (Steffan & Atlas, 1991) may permit detection of the bacterium in the carrier state and in environmental samples. Recombinant DNA technology and the polymerase chain reaction (PCR) may therefore be applied to the development of a highly specific and sensitive DNA or RNA probe. Initial successes with DNA probes specific for *R.salmoninarum* have already been reported (Etchegaray, Martinez, Krauskopf & Leon, 1991; Fridjonsson, Andresdottir, Benediktsdottir & Gudmundsdottir, 1991). In addition, Mattson, Gersdorf, Jansson, Hongso, Gobel & Johansson (1993) reported synthesis of a 30 base oligonucleotide DNA probe complementary to a partial sequence of the gene for 16S ribosomal RNA from *R.salmoninarum* which also shows some promise. Considerable development will be required before such probes become acceptable for routine diagnosis and environmental monitoring in non-specialist laboratories. One of the major difficulties concerns the extraction of nucleic acids from clinical and environmental samples, and loss of specificity and sensitivity due to interfering substances such as humic acids.

2.5 CONTROL OF BKD

2.5.1 CHEMOTHERAPY AND CHEMOPROPHYLLAXIS

BKD has been one of the most difficult bacteraemias to treat possibly because the intracellular location of *R.salmoninarum* places it beyond the reach of many antimicrobials. Initial attempts to control the disease centred on the oral administration of sulphonamides which successfully reduced mortalities although the disease recurred when therapy stopped (Rucker, Bernier, Whipple &

Burrows, 1951; Earp *et al.*, 1953; Rucker, Earp & Ordal, 1953; Sniesko & Griffin, 1955; Wood & Wallis, 1955; Allison, 1958). Subsequently, Wolf & Dunbar (1959) screened 34 therapeutic agents against 16 isolates of *R.salmoninarum* and concluded that erythromycin was the most effective of those tested and this has been confirmed by others (Kawamura, Awakura, Watanabe & Matsumoto, 1977). Some success has also been reported with furazolidone (Millan, 1977) and penicillin, streptomycin and oxytetracycline combined (DeCew, 1972).

Although a tremendous range of chemicals have been tested relatively few have shown promise (Bandin, Santos, Toranzo & Barja, 1991a; Rohovec, 1991). Austin (1985) tested 79 compounds against 40 isolates of *R.salmoninarum* and showed by oral administration to infected fish that penicillin G, erythromycin phosphate, erythromycin thiocyanate, clindamycin and spiramycin were the best choices for chemotherapy. In addition, for chemoprophylaxis, cephradine, kitasamycin and rifampicin were recommended. An interesting finding by Austin (1985) was that the efficacy of antibiotic therapy may be abolished if lipids such as phosphatidylcholine or Tween 20, 40, 60 or 80 were incorporated into the medicated diet. More recently, Gutenberger, Dale & Rohovec (1989) tested *in vitro* 116 mostly non-macrolide antibiotics against four isolates of *R.salmoninarum* and identified 19 which were inhibitory at concentrations of 1 mg/l or less and at least as effective as erythromycin. The usefulness of these drugs *in vivo* awaits further evaluation. Interestingly, the fluoroquinolones tested in the study were not effective against *R.salmoninarum* even though such drugs penetrate phagocytes and are active intracellularly against other Gram-positive bacteria (Neer, 1988).

For many researchers, erythromycin has been the drug of choice for oral, immersion and injection chemotherapy and chemoprophylaxis with no detectable damage to the progeny of treated adults at therapeutic dosages (Groman & Klontz, 1983; Moffitt, 1991). Wolf & Dunbar (1959) recommended a daily oral dosage of 100 mg/kg body weight for 21 days and Austin (1985) found a 10 day treatment to be nearly as effective. This may be useful not only in reducing the cost of treatment but also in avoiding the impairment of kidney function associated with prolonged erythromycin treatment (Hicks & Geraci, 1984) and thus aiding the recovery of the host. Moffitt & Schreck (1988) studied the accumulation and depletion of erythromycin thiocyanate in the tissues of chinook salmon fed the drug for 21 days and found that *in vitro* minimum inhibitory concentrations for *R.salmoninarum* were reached within 3 days of starting treatment and were maintained for at least 19 days after treatment ended. This correlates well with the results of Moffitt & Bjornn (1989) who reported significant protection of fish challenged either 1 day before

or 1 day after a 21 day erythromycin treatment. However, fish challenged either 11 days or 29 days after the treatment ended were not significantly protected. Hence the antibiotic must be used either in the early stages of a BKD outbreak or prophylactically during periods of stress. Nevertheless, these drugs cannot be relied upon to eliminate carrier status and recurrences of BKD can and do occur when treatment is withdrawn (Austin, 1985; Evelyn 1988). In addition, the fish must be feeding and willing to accept medicated food (Moffitt, 1992).

The need to reduce both BKD-associated prespawning mortality in adult salmonids and 'vertical' transmission of the disease has led to the widespread use of antibiotic injections for broodstock prior to spawning (Petersen, 1982; Groman & Klontz, 1983; Bullock & Leek, 1986; Sakai *et al.*, 1986). This procedure is particularly important for mature female fish as it reduces the numbers of viable *R.salmoninarum* cells present in the fish and therefore reduces the chance that egg infection will occur. In addition, therapeutic levels of non-leachable erythromycin will accumulate and persist in the egg yolk and sac fry and help to destroy any viable bacteria present (Bullock & Leek, 1986; Evelyn *et al.*, 1986c; Armstrong, Evelyn, Martin, Dorward & Ferguson, 1989). This has proven to be quite effective for reducing BKD transmission and mortality particularly when coupled with egg surface disinfection and prophylactic feeding of fry with erythromycin (Evelyn, 1988). The timing of the injection is important and 14-56 days prior to spawning has been found to be suitable (Evelyn *et al.*, 1986c; Brown, Albright & Evelyn, 1990b). Brown *et al.* (1990b) also reported that erythromycin phosphate, penicillin G, oxytetracycline, cephadrine and rifampicin were all effective at significantly reducing the prevalence of BKD infection within alevins at hatch.

Antibiotic injection of broodstock offers considerable hope for preventing 'vertical' transmission and prespawning mortality but relies upon the fish being easily accessible at the appropriate time. Additionally, large scale use of antibiotics may increase the possibility of selecting for antibiotic resistant strains. Austin & Rodgers (1980) identified several erythromycin resistant isolates of *R.salmoninarum* from various sources and Bell, Traxler & Dworschak (1988) generated an erythromycin resistant strain of *R.salmoninarum in vitro* by serial passage on culture media containing the drug. Interestingly, this strain was much reduced in virulence which was shown to be partly a consequence of the serial passage. At best, chemotherapy and chemoprophylaxis offer means of reducing BKD transmission and mortalities but not of eliminating the pathogen. Therefore, strategies aimed at controlling BKD have usually included a variety of means of avoidance in conjunction with chemical treatments.

2.5.2 AVOIDANCE MEASURES

Probably the most widely used method of controlling BKD has been the implementation of avoidance measures. Means of avoiding BKD have tended to concentrate on the implementation of good husbandry practices for the convenience of meeting local conditions and have therefore included a wide variety of measures, including quarantine restrictions. Broodstock culling or segregation has been a widely practiced procedure whereby broodstock are screened, most recently by ELISA (Kaattari, Rockey, Weins, Gilkey, Winton, Bartholomew, Lehner-Fournier & Diehm, 1989; Pascho *et al.*, 1991), and until the results of the tests are known either the gametes are held separately under refrigeration or fertilised eggs are kept in separate incubation systems. Once carriers are identified the eggs or gametes are either destroyed or segregated from groups with a lower level of infection. Bruno (1988b), Evelyn (1988) and Warren (1991) have mentioned various other avoidance measures available in the fish culture situation such as reducing stocking densities; keeping stressors such as handling, net changing and grading to a minimum; rapid and effective disposal of infected carcasses, offal and processing effluent; purchase of BKD free stocks; minimum farm separation distances to reduce the spread of infection; stocking only one age group of fish at any given time or segregation of different age groups in such a way as to reduce the likelihood of 'horizontal' transmission and provision of a high quality diet. If the situation demands it may be possible to consider filtration or disinfection of hatchery water using ozone (Austin, 1983) or ultraviolet light. However, such systems are expensive and given the affinity of *R.salmoninarum* for organic matter (Austin & Rayment, 1985) and the extent of hatchery water requirements, probably of little benefit.

2.5.3 VACCINATION STUDIES

The vaccination of fish against a variety of bacterial, viral and parasitic infections has been the subject of considerable research and in some cases has proven to be commercially successful (reviewed by Ellis, 1988). However, despite many attempts the vaccination of salmonids against BKD has thus far not been widely accepted as an effective means for controlling the disease (Goering, Bell, Pfeil-Putzien & Hoffman, 1989).

The first report of vaccination is that of Evelyn (1971) who intraperitoneally (i.p.) injected 1-3 year old sockeye salmon held in freshwater at 12-15°C with heat-killed *R.salmoninarum* cells emulsified in mineral oil plus Arlacel C adjuvant. Despite very high doses of antigen of up to 450 mg/kg of fish the agglutinin response was slow. However, 10% of the fish responded within 30 days, 50% within 60 days and 80% within 90 days of inoculation. Evelyn (1971) also repeated

the procedure substituting Freund's complete adjuvant (FCA) for mineral oil/Arlacel and detected agglutinating antibodies for 16 months after a single injection. A second injection given 13 months after the first elicited an anamnestic response. The ability of vaccinated fish to resist BKD was not tested in these studies. By comparison, Groman (1981) using counter-immunoelectrophoresis failed to detect precipitating antibodies in the serum of chinook salmon injected i.p. or subcutaneously with either heat-killed *R.salmoninarum* cells in FCA or live *R.salmoninarum* cells.

Resistance to BKD was assessed by Evelyn, Ketcheson & Prosperi-Porta (1988) in an extensive study to evaluate various BKD vaccine preparations using both sockeye and coho salmon. The vaccines tested were derived from formalin-killed *R.salmoninarum* cultures and included cell-associated and extracellular antigens from whole cultures, intact and fractured cells, extracellular products (ECP) and cell wall fractions. In two trials, each BKD vaccine was administered as part of a polyvalent vaccine containing similar preparations from *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. In two other trials, each BKD vaccine was tested in monovalent form. Each vaccine was administered by either i.p. injection (with or without FCA), feeding, spraying or the two-step hyperosmotic infiltration method and the efficacy of the vaccines was tested by comparing mortalities in vaccinated and unvaccinated groups following either natural or experimental challenge with the live pathogen. Serum agglutinating antibody production was at best feeble, even for the injected vaccines, and vaccinated fish showed no evidence of protection. In fact, some of the vaccinated groups showed a much shorter mean time to death which Evelyn (personal communication) associated with the possible presence of immunosuppressive components and these sentiments are echoed in the extensive series of vaccination studies conducted by Kaattari, Getchell, Turaga & Irwin (1985), Kaattari, Blaustein, Turaga, Irwin & Weins (1986), Kaattari, Holland, Turaga & Weins (1987) and Kaattari, Chen, Turaga & Weins (1988). Smaller studies of the vaccination of coho salmon against BKD have also reported unpromising results (Baudin-Laurencin, Vigneulle & Mevel, 1977).

Paterson *et al.* (1981a,b) and Paterson *et al.* (1985) reported similar results during studies to evaluate vaccination of Atlantic salmon against BKD. Atlantic salmon parr of 0.3-0.6 g were vaccinated at water temperatures over 12°C either by i.p. injection with 2.5×10^8 heat-killed *R.salmoninarum* cells (with or without FCA) or by two-step hyperosmotic infiltration (HI) with 10^{10} *R.salmoninarum* cells/ml, heat-killed and sonicated for 5 minutes, with a repeat exposure two weeks later. Serum agglutinating antibody titres were detected after 5 weeks, peaked after 13-26 weeks and diminished slowly thereafter in parr injected with the vaccine containing FCA. No

serum agglutinin response was detected in parr vaccinated i.p. without FCA and a low serum agglutinin titre was recorded for HI vaccinated parr. No agglutinins were detected in the intestinal mucus but low titres were detected in the cutaneous mucus of parr injected with vaccine with FCA. Following natural exposure to BKD, the prevalence of the disease as determined by the presence of lesions and IFAT appeared to be higher in vaccinated groups, particularly amongst those fish administered the vaccine i.p. with FCA. Interpretation of these results has been complicated by the use of 0.3-0.6 g fish, given that the vaccination of salmonids of less than about 2.5 g has been shown to produce equivocal results (Johnson, Flynn & Amend, 1982a; b). In a separate study, Paterson *et al.* (1981b) vaccinated 16-20 g Atlantic salmon with 1.2×10^9 heat-killed or formalin-killed *R. salmoninarum* cells with FCA by i.p. injection. A strong serum agglutinin response was recorded up to 53 weeks post-vaccination and after a natural challenge it was observed that compared to control fish, vaccinated fish showed a much reduced prevalence of lesions but, unfortunately, infection rates as determined by IFAT were almost the same.

McCarthy, Croy & Amend (1984) reported on the vaccination of rainbow trout with several preparations of formalin-killed *R. salmoninarum* cells administered without adjuvants. Four vaccines consisting of either whole cells in the culture medium, whole cells double concentrated in saline or pH-lysed suspensions of each of these were delivered by i.p. injection, direct immersion or two-step hyperosmotic infiltration (HI). Fish were held for at least 34 days at 11°C before challenge by i.p. injection. In accordance with Paterson *et al.* (1981b) the results showed an absence of protection in both HI and immersion vaccinated fish. However, compared to other groups, i.p. vaccinated fish not only experienced lower levels of mortality but also possessed fewer kidney lesions and positively Gram-stained kidney smears amongst the survivors. This was interpreted by McCarthy *et al.* (1984) as evidence of protection, however as the experiments were monitored for only 40 days post-challenge some caution is recommended. Indeed, a more recent study by Sakai, Atsuta & Kobayashi (1989c) of rainbow trout vaccinated by i.p. injection with formalin-killed *R. salmoninarum* both with and without FCA shows that monitoring is required for at least 60 days post-challenge as the onset of mortalities amongst vaccinated fish may be delayed compared to control fish. Sakai *et al.* (1989c) reported that no protection was conferred by vaccination despite the presence of low serum agglutinating antibody titres and the enhanced activity of kidney phagocytes when the fish were challenged, about 5 weeks post-vaccination. In addition, 35-50% of vaccinated fish surviving challenge were found to be carriers by IFAT. They suggested that challenging fish when agglutinin titres were at a peak, 90-150 days post-vaccination, may provide more information about acquired immunity.

2.6 IMMUNOLOGICAL AND PATHOGENIC ASPECTS OF BKD

2.6.1 IMMUNE RESPONSE

Munro & Bruno (1988) reviewed studies of vaccination against BKD in the light of evidence gathered from a cultured population of Atlantic salmon experiencing a natural outbreak of the disease. Salmonids have been shown to be capable of producing specific antibodies both during the course of a natural infection and in response to experimental vaccination (Weber & Zwicker, 1979; Paterson *et al.*, 1981a,b; 1985; Bruno, 1987; Sakai *et al.*, 1991c). Serum agglutination titres under these circumstances have been reported to be low and at a relatively consistent level during either successive outbreaks of the disease or recurring exposure to the pathogen and the presence of circulating antibody has so far not been correlated with protection against BKD. However, under certain conditions and with increasing water temperature infected fish were capable of recovering from the disease and resolving all evidence of the pathogen (Earp, 1950; Sanders *et al.*, 1978; Bruno, 1986b). Munro & Bruno (1988) suggested that the resolution of granulomatous lesions and loss of all evidence of *R.salmoninarum* in recovered fish resulted from cell-mediated immune mechanisms although it is questionable as to whether the infection is eliminated or the presence of the pathogen is simply undetectable or confined to specific tissues. Furthermore, the 'bar forms' of *R.salmoninarum* found by Cvitanich (1987; 1988) in the kidney, spleen and skin blisters of infected fish representing all three genera of the Salmonidae appear to represent a successful host response to the pathogen thus yielding higher numbers of dead or dying bacteria in recovering fish and very few (< 0.1%) 'bar forms' in dying fish. Whether the 'bar forms' result from an intracellular or extracellular immunological event has yet to be determined. Therefore Munro & Bruno (1988) concluded that the recovery of salmonids from BKD and the clearance of intracellular bacteria is to some extent dependent upon warmer water temperatures (Earp, 1950; Sanders *et al.*, 1978) and cell-mediated immunity. This offers a plausible explanation as to why humoral immunity has not been correlated with the resolution of the disease.

In a series of studies designed to isolate and identify antigenic components of *R.salmoninarum* and enhance their ability to activate host defences, particularly cell-mediated immunity, Kaattari and fellow researchers in Oregon, U.S.A. undertook some of the first steps toward an understanding of the pathogenesis of BKD at the molecular level. They noted that aspects of the pathology of BKD bore some resemblance to corynebacterial, mycobacterial and listerial infections of mammals; i.e. chronic, inflammatory, granulomatous reactions with extensive tissue necrosis caused by the ability of the pathogen to survive and multiply inside phagocytes. They reasoned that the intracellular location of *R.salmoninarum* provided a protected environment for the pathogen and

a constant stimulus for the immune system of the host which was capable of responding but in an inappropriate manner (Young & Chapman, 1978). Like Munro & Bruno (1988), they noted that the lack of protection conferred by agglutinating antibody and the importance of temperature in the resistance of fish to the disease appeared to provide evidence of cell-mediated immunity acting as in listerial, mycobacterial and corynebacterial diseases to eliminate infection. They also noted that immunological tolerance may be important in the apparent inability of some fish to overcome infections of *R.salmoninarum*. 'Vertical' transmission of the bacterium means that the immune system of the infected developing fry may be exposed to and thus tolerant of any antigens expressed by the bacterium during this phase of the life cycle, thereby disrupting an effective immune response when the animal is older.

Kaattari *et al.* (1985) showed that the type strain of *R.salmoninarum* (ATCC 33209) produced a major antigen(s) of approximately 57/58 kilodaltons (kDa) molecular mass with an isoelectric point (pI) of about 4.5. The protein doublet known as P57 was both cell associated as well as present in the extracellular products (ECP's) along with two other proteins of 34 and 26K and the soluble proteins were shown to bind to both salmon and rabbit erythrocytes (Kaattari *et al.*, 1986). Subsequent studies using monoclonal antibodies demonstrated the presence of a variety of antigenically related proteins less than 58K and showed that these smaller proteins represented breakdown products of the major soluble antigen and were produced in concentrations of up to 1mg/ml in the tissues of infected fish (Kaattari *et al.*, 1987; Turaga *et al.*, 1987a). Importantly, Western blots probed with serum taken from experimentally infected coho salmon at intervals of 5, 10, 15, 20 and 25 days post-injection and from naturally infected Atlantic salmon not only recognised the major antigen of 58K and other breakdown products but also identified both a 67K protein, which was only detected in the ECP of *R.salmoninarum* cultures, and a 100-105K band which appeared 5-10 days following experimental infection (Kaattari *et al.*, 1987; Griffiths *et al.*, 1991). In a study of the specificity of the antibody responses of Pacific salmon naturally infected or immunised with *R.salmoninarum*, it was found that P57 was the main antigenic component (Bartholomew, Arkoosh & Rohovec, 1991). Coho salmon injected with the soluble antigens alone showed evidence of *in vivo* suppression when humoral and cellular responses to mitogens were tested *in vitro* although the inclusion of an adjuvant during immunisation reduced the suppressive effect (Kaattari *et al.*, 1986). Additionally, the soluble antigens were collectively shown to suppress the *in vitro* plaque-forming cell (PFC) responses of coho salmon lymphocytes to TNP-LPS (trinitrophenylated-lipopolysaccharide) and were associated with decreasing haematocrit values (Turaga, Wiens & Kaattari, 1987a), a 64-90% reduction in lymphocyte mitogenic responses to

either PHA (phytohaemagglutinin) or *Vibrio anguillarum* extract and a 35% reduction in plaque-forming cell responses to TNP-LPS in BKD infected coho salmon (Kaattari *et al.*, 1987). Kaattari *et al.* (1987) reported that salmon leucocytes cultured *in vitro* with different concentrations of the same soluble proteins showed reduced viability as assessed by trypan blue dye exclusion after one to three days although after seven days this effect was no longer apparent. When added to cultured salmon leucocytes soluble protein antigens from *R.salmoninarum* were observed to cause a decrease in the numbers of adherent cells e.g. macrophages, polymorphonuclear leucocytes (Turaga *et al.*, 1987a). Adherent cell function has been shown to be required for eliciting salmon lymphocyte antibody responses to TNP-LPS (Tripp, Maule, Schreck & Kaattari, 1987). Similarly, the chemiluminescence responses of coho salmon leucocytes to PMA (phorbol myristate acetate) were suppressed by up to 97.5% by the addition of soluble antigens and the chemiluminescence responses of leucocytes derived from a BKD-free coho salmon stock were 100 times higher than the responses of leucocytes derived from a coho stock with a history of BKD (Kaattari *et al.*, 1988). Other workers have confirmed these findings (Sakai, Konishi, Atsuta & Kobayashi, 1991d). Kaattari *et al.* (1987) concluded that the soluble proteins of *R.salmoninarum* have an important suppressive action on both humoral and cellular immune functions *in vitro* and possibly *in vivo*.

Despite the evidence of immunosuppression, fish immunised against the soluble proteins of *R.salmoninarum* are capable not only of producing specific antibody but also of generating PFC and lymphocyte proliferation responses to the soluble antigens *in vitro* (Kaattari *et al.*, 1986; 1987; 1988) and are also apparently capable of responding positively to the antigens of other bacteria delivered in the same vaccine (Amend & Johnson, 1984; Nikl, Albright & Evelyn, 1991). Additionally, chemiluminescence studies showed firstly, that *R.salmoninarum* soluble protein is slightly stimulatory for phagocytes and secondly, that live *R.salmoninarum* cells elicit a dramatic response from phagocytes lasting for over two hours as compared to 30 minutes for live *Vibrio anguillarum* cells (Kaattari *et al.*, 1987). Kaattari *et al.* (1987) observed that *R.salmoninarum* produced unique biological effects on salmonid phagocytes which responded well to the pathogen but were apparently unable to destroy it. They concluded that *R.salmoninarum* was resistant to lysosomal killing and felt that this may explain the lack of vaccination success since the stimulation of phagocytic activity may exacerbate the pathology of the disease. In this respect, Bruno (1988c) reported unpublished observations of *R.salmoninarum* multiplying within the phagolysosome of phagocytic cells. Kaattari *et al.* (1987) suggested that future vaccine research should be directed toward producing antibodies to block any adherence factors of the bacterium in order to prevent

colonisation of fish tissues, especially phagocytes, as well as antibodies to counter the immunosuppressive components.

In a series of vaccination and challenge experiments carried out concurrently with the molecular studies, Kaattari *et al.* (1985; 1986; 1987; 1988) tested the feasibility of improving the immune response of salmonids to both *R.salmoninarum* soluble proteins and whole cells by either mixing with, or chemical conjugation to, antigens derived from other bacteria. Conjugation methods included the use of glutaraldehyde, cyanuric acid, ethyl carbodiimide and tannic acid and accessory antigens utilised included muramyl dipeptide, whole *V.anguillarum* cells, *V.anguillarum* extract, *E.coli* LPS and FCA. Whilst vaccinated groups of fish possessed variable degrees of humoral and cellular stimulation, no consistent protective effect could be attributed to any of the vaccine preparations and often vaccinated groups demonstrated a shorter mean time to death as compared to controls. Kaattari *et al.* (1987) concluded that lymphocyte proliferation and PFC responses to any of the antigens employed were not a measure of protection against BKD, that mortalities may be exacerbated by these inappropriate responses and that the pathology may be at least in part immunologically derived. Nevertheless, coho salmon challenged with live *R.salmoninarum* cells mixed with coho salmon serum with high agglutination and ELISA titres against *R.salmoninarum* soluble proteins took 25% longer to die compared to control fish challenged with the live pathogen in saline suggesting that humoral responses are not entirely without effect. Because one group of fish received 'normal' coho salmon serum which was negative by an ELISA employing a monoclonal anti-*O.mykiss* IgM for detection but positive by serum agglutination test, Kaattari *et al.* (1988) considered the possibility that another antibody isotype may have been involved in the observed resistance to the disease. In a separate experiment, i.p. challenge results showed higher mortalities in fish injected with live *R.salmoninarum* cells in the original culture medium rather than live cells in either fresh KDM2 (with or without serum) or with 100 times concentrated soluble proteins in saline. Overall, the results of the research by Kaattari *et al.* (1985-88) showed that the major soluble proteins present in the ECPs of *in vitro* cultures of *R.salmoninarum* have an important role in the pathogenesis of BKD probably related to the suppression of the humoral and cellular responses of the host during infection as well as certain aspects of the pathology, rather than any acute toxic effect. However, evidence of the usefulness of such components in vaccines aimed at eliminating intracellular bacteria and inducing protection against BKD was not forthcoming. The possibility that other components of *R.salmoninarum* expressed either *in vitro* or *in vivo* may also be of importance to both the progress of disease and effective host resistance seems likely.

2.6.2 PATHOGENICITY FACTORS

Following the initial identification of P57 by Getchell *et al.* (1985) and subsequent studies confirming the antigens as the predominant cell surface proteins and constituents of ECP both *in vitro* and *in vivo* (Turaga *et al.*, 1987b; Wiens & Kaattari, 1989; Griffiths *et al.*, 1991; Rockey *et al.*, 1991a), a number of researchers sought to further characterise the proteins. Kaattari *et al.* (1985-1988) inferred immunosuppressive functions on P57 on the basis of *in vitro* studies. However, this conclusion may be an over-simplification since a variety of other *in vitro* activities of P57 have been described. P57 is loosely bound to the surface of *R.salmoninarum* and is easily removed by washing cells in distilled water or saline (Daly & Stevenson, 1987; 1989). Other properties of P57 include binding to salmonid erythrocytes (Kaattari *et al.*, 1986), haemagglutination of the erythrocytes of certain mammalian species but not usually those of fish species (Daly & Stevenson, 1987; Bandin, Santos, Barja & Toranzo, 1989), agglutination of salmonid sperm (Daly & Stevenson, 1989), hydrophobicity of the cell surface of *R.salmoninarum* (Daly & Stevenson, 1990) and agglutination of the leucocytes of various salmonid fish species (Weins & Kaattari, 1989; 1991). Degradation of P57 has recently been shown to be the result of an endogenous proteinase activity which increases with time and temperature, generates most, if not all, immunoreactive bands in the ECP of *R.salmoninarum* and can be inhibited by PMSF (phenylmethylsulphonyl fluoride), a serine proteinase inhibitor (Griffiths & Lynch, 1991). Rockey, Turaga, Weins, Cook & Kaattari (1991b) have recently isolated and characterised a 105K serine proteinase with a pI 6.5-7.5 from the ECP which is believed to be involved in the digestion of P57 *in vivo*. The proteinase was inactivated rapidly at temperatures over 65°C and was shown to possess an activity specific for P57 *in vitro* as it failed to digest other substrates, including rainbow trout IgM, unless the proteins were partially denatured or reduced first. In addition, the proteolytic degradation of P57 *in vitro* was accompanied by the loss of immunosuppressive activity. Rockey *et al.* (1991b) detected the presence of another enzyme, probably a serine proteinase, in the ECP which possessed a molecular mass of about 12-15 kDa and performed an unknown function.

Weins & Kaattari (1991) used a panel of monoclonal antibodies (Mabs) to characterise P57 and the associated breakdown products. They showed that a group of Mabs specific for the amino terminal part of P57, which it was proposed represented the exposed portion of the protein on the cell surface, inhibited either or both of the leucocyte agglutinating and haemagglutinating activities of the protein. This suggests that the hydrophobic and haemagglutinating properties of P57 are not conferred by the same epitopes of the protein and supports earlier work showing that heating *R.salmoninarum* at 80°C for 15 minutes removes hydrophobicity but not haemagglutinating activity

(Bandin *et al.*, 1989). Another group of Mabs were found to recognise a different epitope of P57 which Weins & Kaattari (1991) considered of importance for the attachment and *in vitro* reassembly of the protein to the bacterial cell surface (Daly & Stevenson, 1990). They also noted that certain characteristics of P57 such as acidic pI, hydrophobicity and haemagglutination bear resemblance to a number of other bacterial adhesins and they suggested that P57 may bind to host cellular receptors and thus initiate internalisation. Indeed, Dubreuil *et al.* (1990b) have recently shown that *R.salmoninarum* possesses peritrichous fimbriae composed of an immunodominant, hydrophobic, 57K cell surface protein of pI 4.8, which was found to possess haemagglutinating activity. Also, one mechanism of attachment of *R.salmoninarum* to phagocytes in the absence of specific antibody is a consequence of opsonisation by complement component C3b and subsequent binding to the CR1 complement receptors (Rose & Levine, 1992). Therefore, evidence accumulated to date suggests that P57 has an involvement in the adhesion of *R.salmoninarum* to host leucocytes, thus aiding internalisation, and serves an immunosuppressive function, perhaps of more importance in the early stages of infection, as well as a role in the sequestration of erythrocytes by the spleen and the type III hypersensitivity response observed in the organs of infected fish. The N-terminal sequence of P57 has been determined (Radacovici & Dubreuil, 1991) and the gene encoding this protein has recently been cloned and sequenced (Chien, Gilbert, Huang, Landolt, O'Hara & Winton, 1992).

In an investigation of the biological functions of the ECP derived from 10 strains of *R.salmoninarum*, Bandin, Santos, Bruno, Raynard, Toranzo & Barja (1991b) reported a low overall production of extracellular proteins and only two strains were found to produce proteolytic enzymes as determined by digestion of casein, gelatin or azocoll. No haemolytic activity or capacity to alter the osmotic integrity of fish erythrocytes was attributable to ECP which also displayed no cytotoxicity for six fish and two mammalian cell lines after seven days incubation and fish injected with 10-20 μ g ECP protein/g fish showed no mortalities. Bandin *et al.* (1991b) concluded that the ECP of *R.salmoninarum* were not a determinative factor in fish mortalities from BKD although the virulence of each bacterial isolate for salmonid fish was not confirmed in this study. In contrast to the ECP, live *R.salmoninarum* cells did display strong enzymatic activities and Bandin *et al.* (1991b) suggested that these are properties likely to be associated with the cell envelope. The possibility that storage (or subculture) of bacterial strains may lead to a loss of detectable biological activity was also mentioned. Despite the body of research effort which has so far failed to attribute a toxic effect to *R.salmoninarum* ECP, Shieh (1988) demonstrated the presence of an uncharacterised toxic effect in the ECP of the type strain, ATCC 33209, when 9-12

g Atlantic salmon were administered a massive dose of 320 μg ECP protein/g fish by intramuscular injection. Heating the ECP at 100°C for 20 minutes completely eliminated the toxic effect. Shieh (1989) also reported that 15-18 g Atlantic salmon administered four intramuscular injections of low dosage (6 μg ECP protein/g fish) at weekly intervals were protected when challenged intramuscularly with the live pathogen four weeks after the last immunisation. No details of tests confirming the cause of death, duration of the challenge experiment or examination of survivors to determine carrier status were given. Further details of the production and nature of the toxin and interaction with the host are required before it is possible to interpret the results in the context of current knowledge. However, it is possible that a massive dose of ECP protein may have such a toxic effect in 9-12 g Atlantic salmon.

Cell surface hydrophobicity has been correlated with virulence by Bruno (1988c). By challenging rainbow trout i.p. with the live cells of a number of biochemically indistinguishable isolates he showed that strains of *R.salmoninarum* which were non-autoagglutinating and non-hydrophobic were only weakly virulent when compared to those strains which possessed a strongly autoagglutinating, hydrophobic phenotype. Mortalities amongst fish injected with hydrophobic strains ranged from 73-81% and occurred from 9-25 days post-challenge compared with 8-18% mortalities occurring up to 38 days post-challenge amongst fish injected with non-hydrophobic strains. No evidence of *R.salmoninarum* was found in Gram-stained kidney and spleen smears from fish surviving at 50 days after challenge. Furthermore, Bruno (1990) found that the isolates of low virulence did not possess the 57K saline extractable cellular protein which had been associated with cell surface hydrophobicity by Daly & Stevenson (1987; 1989). Bruno (1988c; 1990) postulated that increased hydrophobicity may contribute to the serum resistance, intracellular survival and host cell adhesion of the pathogen whereas strains lacking a hydrophobic cell surface may be more readily phagocytosed and susceptible to host defences. However, Bandin *et al.* (1989) could not correlate hydrophobicity, haemagglutination and adhesion to fish cell lines for different strains of *R.salmoninarum* and before a hydrophobic portion of P57 or other cell surface protein can be conclusively associated with virulence the genetic integrity, uniformity and stability of non-hydrophobic strains must be demonstrated.

Bandin *et al.* (1989) and Bruno (1988c) observed that the hydrophobic character of *R.salmoninarum* was not a stable trait because hydrophobicity was lost after routine passage on various types of culture media. Serial passage of one strain of *R.salmoninarum* on KDM2 with or without the addition of erythromycin has been shown to induce a considerable reduction in the

virulence of the isolate for sockeye salmon as compared to the parent strain and attenuation on media containing erythromycin was particularly marked (Bell *et al.*, 1988). Whether loss of hydrophobicity accompanied the reduction in virulence was not mentioned. However, Bruno & Munro (1986a) noted that the β -haemolytic activity of *R.salmoninarum* for rabbit erythrocytes also declined following routine subculture on Mueller-Hinton cysteine agar. They stated that although no role had been attributed to a haemolysin *in vivo*, as the haemolytic activity declined clinical disease in rainbow trout became difficult to establish following i.p. injection of live *R.salmoninarum*. Nevertheless, Bandin, Ellis, Barja & Secombes (1993) utilised an *in vitro* colorimetric assay (Graham, Jeffries & Secombes, 1988) in an attempt to resolve differences in the ability of rainbow trout phagocytes to kill virulent, hydrophobic strains of *R.salmoninarum* as compared to non-hydrophobic strains of low virulence. The infection of phagocytes with live *R.salmoninarum* was found to stimulate the respiratory burst although from 24 hours post-infection the phagocytes were refractory to further stimulation with PMA confirming the earlier work of Kaattari *et al.* (1987). The inhibition of O_2^- production could not be attributed to the production of superoxide dismutase and it was found that both hydrophobic and non-hydrophobic strains were able to survive within trout phagocytes for three to four days but thereafter the numbers of viable bacteria decreased. This was interpreted as evidence of the resistance of *R.salmoninarum* to O_2^- -mediated killing although the possibility exists that the bacteria either rapidly escaped from or were not directly exposed to the oxidative metabolites. Recent electron microscopical studies by Gutenberger (1993) have shown that live *R.salmoninarum* cells are capable of escaping from the phagosome into the cytoplasm of trout phagocytes within two hours of infection. Although no evidence of bacterial replication was observed the numbers of intracellular bacteria remained high after 10 days incubation. Formalin-killed *R.salmoninarum* cells were also able to escape from the phagosome. Whether the slow decline in bacterial numbers recorded by Bandin *et al.* (1993) represented macrophage killing by means other than O_2^- , competition with macrophages for nutrients or that the bacteria retained viability but failed to multiply was unresolved. The numbers of viable bacteria in controls also declined to some extent over the course of the assay particularly when a lower initial inoculum was used and would suggest that the establishment of chronic, intracellular infection by *R.salmoninarum* is not well reproduced by an assay that for reasons of cellular viability is restricted to a maximum of about seven to ten days incubation and requires a high initial bacterial inoculum. Similar difficulties have been experienced with *in vitro* assays of macrophage killing of mycobacteria and are related to the variable loss of viability of adherent cells and bacterial cells over the incubation period and their consequent removal during washing and medium changing and also to variation in the efficiency of phagocytosis of bacteria as opposed

to cell surface association (Collins, 1990). The possibility exists that a factor important for the intracellular survival of the bacterium has not been expressed within the limits of the assay. Additionally, the importance of host-derived factors which are absent from such a model must be considered. The presence of a particular hormone, growth factor, antibody, lymphokine or even neighbouring cell type may be necessary to reproduce the response of an infected cell (Moulder, 1985) and the ability of hydrophobic strains of *R.salmoninarum* to survive within phagocytes may depend upon one or more of these factors. For example, the survival of *Corynebacterium pseudotuberculosis* and *Pasteurella haemolytica* within ovine pulmonary alveolar macrophages may be enhanced by the ability of the pathogens to induce the hyperproduction of tumor necrosis factor alpha (Ellis, Lairmore, O'Toole & Campos, 1991) and the growth of *Listeria monocytogenes* in murine macrophages pretreated with interleukin-6 (IL-6) or IL-4 was also enhanced (Denis, 1990).

2.7 OTHER MODELS OF PATHOGENICITY

Despite over 40 years of research into *R.salmoninarum*, surprisingly little is known about the pathogenesis of BKD and the complex series of interactions between the host and pathogen which determine the outcome of infection. Whilst routinely applied to the study of many host-pathogen interactions, animal and cell culture models have been largely absent from studies of BKD. The lack of a reliable means of routinely reproducing the infection *in vitro*, particularly the crucial intracellular phase, and reliance on crude i.p. challenge procedures have restrained studies of the virulence and pathogenicity determinants of *R.salmoninarum* although an immersion and cohabitation challenge method has been recently tested (Murray, Evelyn, Beacham, Barner, Ketcheson & Prosperi-Porta, 1992). However, considerable research has been devoted to understanding the mechanisms by which a variety of other organisms, particularly intracellular pathogens, interact with a potential host and recent reviews by Moulder (1985; 1989), Horwitz (1988), Finlay & Falkow (1989), Smith (1990), Falkow, Isberg & Portnoy (1992), Penn (1992) and Kaufmann & Flesch (1992) cover some of the wealth of literature which documents both the popularity of the subject and the important implications for the control of infection and disease. Finlay & Falkow (1989) have observed that whilst different microorganisms have evolved separate and distinct mechanisms for overcoming host or environmental barriers to infection, several common themes are repeatedly used by pathogenic agents for infection. For this reason, an examination of these aspects, particularly as they relate to intracellular and Gram-positive pathogens, is likely to provide useful clues in understanding the molecular basis of pathogenicity of *R.salmoninarum*. Because of obvious similarities in the disease process of BKD with listeriosis,

legionellosis and mycobacterial infections, a detailed description of the current state of knowledge is provided for these examples.

2.7.1 ATTACHMENT TO HOST CELLS

Many microorganisms utilise one or more means of host cell attachment and these alternative mechanisms may be expressed under different environmental and host conditions or at different host cell surfaces (Finlay & Falkow, 1989; Evans & Evans, 1990; Finlay, 1990). Many species of the Enterobacteriaceae, for example, possess one or more types of fimbrial adhesin which enables them to bind to D-mannose or other carbohydrate receptors and thus colonise the mucosae of the mammalian gastrointestinal and urinogenital tracts. Adhesins play a vital role in the pathogenesis of disease and the colonisation of host mucosae can be inhibited by the presence of anti-fimbrial antibody. For example, the K88, K99 and 987P fimbrial adhesins of enterotoxigenic *E.coli* have for many years formed the basis for vaccines used in veterinary medicine (Morgan, Isaacson, Moon, Brinton & To, 1978; Nagy, Moon, Isaacson, To & Brinton, 1978; Nagy, Walker, Bhogel & Mackenzie, 1978). Various other fimbrial adhesins have been described e.g. N-methylphenylalanine pili of *Pseudomonas*, *Neisseria* and *Vibrio* (Finlay & Falkow, 1989), as well as several non-fimbrial adhesins e.g. filamentous haemagglutinin from *Bordetella pertussis* (Weiss & Hewlett, 1986), mannose-resistant haemagglutinin from *Salmonella typhimurium* (Jones & Richardson, 1981). Bacterial adherence to fibronectin, a glycoprotein associated with intracellular cohesion and the mucosae, has been found to provide the specific substrate to which two well known pathogens, *Staphylococcus aureus* and *Streptococcus pyogenes*, adhere on epithelial cells (Finlay, 1990).

The initial means of colonisation of host fish has yet to be demonstrated for *R.salmoninarum*; however it seems possible that the hydrophobic and haemagglutinating cell surface properties would be of importance in the 'horizontal' transmission of the pathogen. Like *Legionella pneumophila* and *Mycobacterium tuberculosis* (Horwitz, 1988; Schlesinger, Bellinger-Kawahara, Payne & Horwitz, 1990), *R.salmoninarum* can use complement receptors to mediate uptake into phagocytic cells by fixing complement component C3 to the bacterial cell surface (Rose & Levine, 1992). However, the presence of two functionally distinct portions of the P57 fimbrial adhesin of *R.salmoninarum* suggests that more than one means of host cell attachment may exist (Weins & Kaattari, 1991). Indeed, reports of the presence of actively dividing *R.salmoninarum* cells within a variety of host cell types would appear to support this view. In this case, the presence of anti-colonising factor antibody in the mucosae of exposed fish may be of little use in preventing the

spread on infection in fish already carrying the bacterium intracellularly or may simply extend the means by which *R.salmoninarum* gains access to phagocytes expressing Fc and C3 receptors. Recent evidence shows that *L.pneumophila* requires the presence of specific antibody, which may be present at very low levels in non-immune serum, for opsonised adherence to Fc and C3 receptors (Husmann & Johnson, 1992).

2.7.2 ENTRY AND INTRACELLULAR SURVIVAL

In order to pursue an intracellular lifestyle an organism must not only adhere to the host cell but must also gain entry. This may involve energy-dependent or energy-independent pathogen induced endocytosis (e.g. *Shigella*, *Salmonella*; Finlay & Falkow, 1989), or host-specified endocytosis either with or without specialised ligands (e.g. *L.pneumophila*; Cianciotto, Eisenstein, Engleberg & Shuman, 1989). Once ingested by 'professional' phagocytes the pathogen must survive the lethal consequences of phagosome-lysosome fusion which includes the generation of reactive oxygen intermediates (O_2^- , H_2O_2 , 1O_2 , OH^-) and myeloperoxidase, reactive nitrogen intermediates (NO^- , NO_2^- , NO_3^-), acid hydrolases (glycosidases, proteases, lipases), lysozyme, lactoferrin and cationic proteins. Successful intracellular pathogens have evolved some means of either escaping from the phagosome (e.g. *Listeria*, *Shigella*, *Rickettsia*, *Trypanosoma cruzi*; Moulder, 1985), resistance to lysosomal enzymes (e.g. *Leishmania*, *Salmonella*, *Yersinia*, *Coxiella burnetti*; Akporiaye, Rowatt, Aragon & Bacca, 1983; Finlay & Falkow, 1989; Maurin, Benoliel, Bongrand & Raoult, 1992) or prevention of phagosome-lysosome fusion (e.g. *M.tuberculosis*, *L.pneumophila*, *S. typhimurium*; Moulder, 1985; Buchmeier & Heffron, 1991).

Toxins may play a vital role in the disease process and have often been associated with the perpetuation of infection, host cell damage, immune suppression and intracellular survival. Alouf & Freer (1991) have compiled an extensive reference work on bacterial protein toxins, their nature and production and interaction with the host.

Stress or heat shock proteins represent a considerable proportion of all living cells and although their significance has yet to be precisely determined they are thought to be important factors in the intracellular survival of many pathogens (Murray & Young, 1992). By virtue of their polypeptide chain binding activity, the primary function of these proteins is considered to be as mediators of the folding and translocation of cellular proteins (Ellis & van der Vies, 1991; Gething & Sambrook, 1992), and the term molecular chaperonin has been used to describe this activity (Dorman & Bhriain, 1993). Sequence analysis of several major protein antigens from a wide

variety of organisms including *Mycobacterium*, *Salmonella*, *L.monocytogenes*, *L.pneumophila*, *Plasmodium falciparum*, *Schistosoma mansoni* and *Coxiella* has resulted in the identification of highly conserved heat shock protein families related to the *E.coli* proteins DnaK, GroEL and GroES (Hoffman, Butler & Quinn, 1989; Young, 1990; Young & Garbe, 1991; Kaufmann, 1992). Characteristically, the synthesis of stress proteins is subject to regulation in response to various environmental stimuli such as heat shock or oxidative stress. For example, the induction of listeriolysin and 15 other stress proteins of unknown function occurs under conditions of heat or H₂O₂ stress in *L.monocytogenes* (Sokolovic, Fuchs & Goebel, 1990). Regulation of the heat shock response in *E.coli* has been extensively studied and shown to be mediated by changes in the level of specific RNA polymerase σ subunits (Gross, Strauss, Erickson & Yura, 1990). The importance of stress proteins to intracellular survival is exemplified by two recent studies of *Salmonella typhimurium* showing the selective induction of 30 proteins including DnaK and GroEL during *in vitro* infection of macrophages with the pathogen (Buchmeier & Heffron, 1990) and identifying a gene, *htrA*, encoding an *E.coli* heat shock protein homolog which conferred resistance to oxidative stress *in vitro* (Johnson, Charles, Dougan, Pickard, O'Gaora, Costa, Ali, Miller & Hormaeche, 1991). Inactivation of *htrA* by transposon mutagenesis generated mutants which were avirulent *in vivo*. Kaufmann (1992) provides further discussion on the role of stress proteins in the immune response and the progress of infection.

2.7.3 PATHOGENICITY OF *Legionella pneumophila*

L. pneumophila is a fastidious, aerobic Gram-negative bacillus capable of surviving intracellularly and of causing severe pneumonia in certain mammals including humans. According to Horwitz (1988), in studies of the comparative intracellular biology of *L.pneumophila*, the complement receptor pathway may be a preferred route of entry for intracellular parasites because ligation to C3 receptors does not result in the release and the consequent exposure of the pathogen to superoxide, hydrogen peroxide or arachidonic acid metabolites. However, *L.pneumophila* does not depend solely on this for intracellular survival and in the presence of immune antibody is able to enter via the Fc receptor and survive but not multiply (Horwitz, 1984). In addition, the infectivity of *L.pneumophila* has recently been shown to be enhanced by a 24K 'macrophage infectivity potentiating' protein (*mip*) which has analogues in pathogens from other genera (Cianciotto, Eisenstein, Mody, Toews & Engleberg, 1989; Bangsberg, Cianciotto & Hindersson, 1991). Once engulfed, live *L.pneumophila* cells induce the formation of a novel ribosome-studded phagosome (Horwitz, 1983a) and inhibit both phagosomal acidification (Horwitz and Maxfield, 1984) and phagosome-lysosome fusion (Horwitz, 1983b), although the bacterium is capable of surviving

within fused phagolysosomes (Horwitz, 1988). *L.pneumophila* multiplies within the phagosome, eventually lysing the host cell and releasing numerous progeny (Horwitz & Silverstein, 1980). This process of adaptation to growth within the macrophage is complex and involves the induction of at least 35 proteins and the repression of a further 32 proteins (Kwaik, Eisenstein & Engleberg, 1993). This phenotypic modulation appears to be the result of a globally regulated stress response which may often be associated with the expression of virulence determinants by pathogens (Dorman & Bhriain, 1992). Evidence to date suggests that *L.pneumophila* resists killing by human monocytes, macrophages and polymorphonuclear leucocytes partly by evasion and partly by producing superoxide dismutase to inhibit superoxide generation (Horwitz, 1989) although a number of enzymes which are implicated in the pathogenesis of the disease have been identified in the ECP and include proteases (Conlan, Baskerville & Ashworth, 1986), cytotoxins (Friedman, Iglewski & Miller, 1980) and haemolysins (Baine, 1985). An excreted 38K protein, a zinc metalloprotease, also called 'major secretory protease' (MSP), possessing cytotoxic and haemolytic activities plays an important role in the development of cellular immunity to *L.pneumophila* infection (Blander & Horwitz, 1989). The gene encoding this enzyme, *mspA*, has been cloned and the sequence analysed (Quinn & Tompkins, 1989) and by using transposon mutagenesis to generate isogenic mutants, in which the expression of the gene was abolished, it was demonstrated that the protease was neither a virulence factor nor required for intracellular growth or resistance to host cell killing (Blander, Szeto, Shuman & Horwitz, 1990; Szeto & Shuman, 1990). Preliminary studies by Clemens & Horwitz (1990) have demonstrated that the zinc metalloprotease is produced by *L.pneumophila* in infected human monocytes and that epitopes of the protein are displayed on the monocyte surface. Of more recent interest is the cloning and identification of a 39K haemolysin (legiolysin) by Rdest, Wintermeyer, Ludwig & Hacker (1991) which is coded for by a different gene, *lly*, and which has a previously unknown role in infection being both present and expressed in all *L.pneumophila* isolates so far examined (Bender, Ott, Debes, Rdest, Heeseman & Hacker, 1991; Wintermeyer, Rdest, Ludwig, Debes & Hacker, 1991). The possibility that the control or the regulation of expression of *lly* or activation of legiolysin is in some way linked to *mspA* has yet to be investigated.

Precisely which factors determine the ability of *L.pneumophila* to enter, survive and replicate within host cells are not well defined. Interferon-gamma (IFN- γ) has been shown to activate monocytes and prevent intracellular replication of the bacterium (Bhardwaj, Nash & Horwitz, 1986) by reducing the number of transferrin receptors on the monocyte surface and thus decreasing the availability of intracellular iron (Byrd & Horwitz, 1989; 1991) although other

mechanisms of resistance are also involved (Skerrett & Martin, 1991). The growth limitation imposed by IFN- γ treatment of monocytes can be removed by the addition of exogenous transferrin iron (Byrd & Horwitz, 1989; 1991). IFN- γ may also act to downregulate complement receptors on activated monocytes thus limiting the access of the bacterium to the intracellular environment (Horwitz & Silverstein, 1981a; Esparza, Fox & Schrieber, 1986; Firestein & Zvaifler, 1987). Cell-mediated immunity, in contrast to humoral immunity, appears to play the major defensive role against *L.pneumophila*. Sub-lethally infected humans and guinea pigs develop a strong humoral response to *L.pneumophila* which fails to promote complement lysis or intracellular killing (Horwitz & Silverstein, 1981b; c). However, lymphocytes from infected individuals respond strongly to *L.pneumophila* antigens with proliferation and lymphokine production such that intracellular multiplication is limited and bacteria eventually cleared (Horwitz, 1983c; Breiman & Horwitz, 1987).

2.7.4 PATHOGENICITY OF *Listeria monocytogenes*

L. monocytogenes is an opportunistic, Gram-positive, β -haemolytic, intracellular aerobe which is motile at room temperature but only slightly at 37°C (Gellin & Broome, 1989). Human infections follow transplacental or transintestinal routes and consequently the pathogen expresses its virulence by growing within macrophages (Mackaness, 1962) and rapidly spreading from cell to cell using the host cell actin assembly mechanism (Sanger, Sanger & Southwick, 1992). Molecular aspects of the pathogenesis of listerial infections have been recently reviewed (Cossart & Mengaud, 1989; Portnoy, Chakraborty, Goebel & Cossart, 1992) and reveal that most of the major virulence determinants are located together on the genome. The major virulence factor, listeriolysin O, is a secreted sulfhydryl-activated 58K protein (Geoffroy, Gaillard, Alouf & Berche, 1987) which is inducible under conditions of heat shock or oxidative stress (Sokolovic & Goebel, 1989). The gene encoding listeriolysin, *hly* (also called *hlyA* or *lisA*) has been cloned and sequenced for three strains representing three serotypes of *L.monocytogenes* (Mengaud, Vicente, Chenevert, Pereira, Geoffroy, Gicquel-Sanzey, Baquero, Perez-Diaz & Cossart, 1988; Domann & Chakraborty, 1989; Rasmussen, Beck, Olsen, Dons & Rossen, 1991) and found to share considerable sequence homology with streptolysin O from *Streptococcus pyogenes* (Kehoe, Miller, Walker & Boulnois, 1987), pneumolysin from *Streptococcus pneumoniae* (Walker, Allen, Falmagne, Johnson & Boulnois, 1987), perfringolysin from *Clostridium perfringens* (Tweten, 1988) and alveolysin from *Bacillus alvei* (Geoffroy, Mengaud, Alouf & Cossart, 1990). In addition, listeriolysin has been found to share similar properties and modes of action with a class of poreforming bacterial toxins produced by other Gram-positives, notably bacilli and clostridii (Alouf & Geoffroy, 1991). Some

of the functions ascribed to these toxins include inhibition of the respiratory burst, bactericidal activity, chemotaxis and random migration of polymorphonuclear leucocytes (Paton & Ferrante, 1983) and activation of the classical complement pathway (Mitchell, Andrew, Saunders, Smith & Boulnois, 1991). Listeriolysin (LLO) is invariably present in clinical isolates of *L.monocytogenes* and transposon mutagenesis studies have confirmed the status of LLO as essential for virulence (Cossart, 1988), escape from the phagosome (Gaillard, Berche, Mounier, Richard & Sansonetti, 1987) and intracellular growth (Gaillard, Berche & Sansonetti, 1986; Cossart & Mengaud, 1989) but not entry to host cells (Kuhn, Kathariou & Goebel, 1988). Cossart, Vicente, Mengaud, Baquero, Perez-Diaz & Berche (1989) demonstrated conclusively that non-haemolytic, isogenic mutants of *L.monocytogenes* are avirulent and virulence can be restored by complementation of the inactivated gene and recovery of the haemolytic phenotype. Acquisition of the *hly* gene was found to be sufficient to convert the non-pathogenic soil bacterium *Bacillus subtilis* into an intracellular parasite (Bielecki, Youngman, Connelly & Portnoy, 1990). The addition of exogenous iron has been shown to exacerbate listerial infection (Sword, 1966) although the presence of iron downregulates LLO expression. It seems possible that this is related to the saturation of serum transferrin affecting the availability of intracellular iron to the pathogen rather than any repression of iron-regulated promoters in the promoter regions located near *hly* (Cowart & Foster, 1985; Mengaud, Vicente & Cossart, 1989). Cellular adhesion is associated with P60, a major secretory protein (Kuhn & Goebel, 1989) but studies indicate that invasion of 'non-professional' phagocytes is mediated by internalin, a surface protein which bears some similarity to streptococcal M protein (Gaillard, Berche, Frehel, Gouin & Cossart, 1991). Internalisation allows the bacterium a phase of active multiplication which may be crucial in the infectious process (Berche, Gaillard & Richard, 1988). The invasion of host cells may also partly be a consequence of secreted phospholipase C, encoded by the *plcA* gene, acting to cleave phosphatidylinositol and glycosylphosphatidylinositol plasma membrane anchors for eucaryotic cell surface proteins (Mengaud, Braun-Breton & Cossart, 1991). Although the precise role of the molecule awaits further investigation, it has been shown to play some role in escape from the phagocytic vacuole (Camilli, Tilney & Portnoy, 1993). Phosphatidylcholine-phospholipase C, encoded by the *plcB* gene, is part of an operon which is co-ordinately expressed with *L.monocytogenes* haemolysin (listeriolysin O) and may also be involved in escape from the phagolysosome (Leimeister-Wachter, Domann & Chakraborty, 1991) and cell to cell spread (Vazquez-Boland, Kocks, Dramsi, Ohayon, Geoffroy, Mengaud & Cossart, 1992). Phospholipase C activity has also been identified in a variety of pathogens and is often associated with damage and entry to eucaryotic cells e.g. *Plasmodium falciparum* (Braun-Breton, Rosenberry & da Silva, 1988), *Bacillus cereus* (Little,

1981), *Bacillus thuringiensis* (Low, 1981), *Staphylococcus aureus* (Ikezawa & Taguchi, 1981), *Clostridium perfringens* (Titball, Hunter, Martin, Morris, Shuttleworth, Rubidge, Anderson & Kelly, 1989) and *Rickettsia rickettsi* (Silverman, Santucci, Meters & Sekeyova, 1992). Two other genes which form part of the lecithinase operon, *actA* and *mpl*, are associated with actin assembly (Kocks, Gouin, Tabouret, Ohayon, Berche & Cossart, 1992) and maturation of the lecithinase (Poyart, Abâchin, Razafimanantsoa & Berche, 1993), respectively. Lesions in the *mpl* gene have been shown to reduce the virulence of *L.monocytogenes* (Raveneau, Geoffroy, Beretti, Gaillard, Alouf & Berche, 1992; Poyart *et al.*, 1993).

Recovery from listerial infection is a function of the activation and proliferation of anti-*Listeria* specific T-cells in order to recruit and activate monocytes (Cossart & Mengaud, 1989). In particular, CD4⁺ T-cells (T-helper cells) which are involved in the expression of delayed type hypersensitivity and cytotoxic CD8⁺ T-cells are critical for protection (De Libero & Kaufmann, 1986; Mielke, Ehlers & Hahn, 1988; Mielke, Niedobitek, Stein & Hahn, 1989; Brunt, Portnoy & Unanue, 1990; Munk, Gatrill & Kaufmann, 1990). It has been shown that listeriolysin is the dominant target antigen during the immune response (Bouwer, Nelson, Gibbons, Portnoy & Hinricks, 1992). Both IFN- γ and IFN- β have been shown to confer increased resistance of mice to listerial infection *in vivo* (Buchmeier & Schrieber, 1985; Fujiki & Tanaka, 1988) but not *in vitro* (Campbell, Canono & Cook, 1988; Fujiki & Tanaka, 1988). This may be explained by the required presence of tumor necrosis factor α (Desiderio, Kiener, Lin & Warr, 1989; Havell, 1989) which is critical for the production of IFN- γ by natural killer cells (Bancroft, Sheehan, Schrieber & Unanue, 1989) and therefore for the synergistic activation of macrophages which can kill *L.monocytogenes* in the early stages of the infection (Nakane, Minagawa, Kohanawa, Chen, Sato, Moriyama & Tsuruoka, 1989; Dunn & North, 1991). It is thought that macrophage killing follows lysis of infected cells (Conlan & North, 1992). Specific T-cell mediated responses are acquired later in the course of infection. Other cytokines and growth factors, such as granulocyte and macrophage colony stimulating factors, (Kayashima, Tsuru, Shinomiya, Katsura, Motoyoshi, Rokutanda & Nagata, 1991; Shinomiya, Tsuru, Katsura, Kayashima & Nomoto, 1991; Iizawa, Brown & Czuprynski, 1992), also play a role in resistance to listerial infection and interleukins IL-6 and IL-4 have been shown to induce listericidal activity in macrophages already infected with the pathogen (Denis, 1990). The exact means by which macrophages kill *L.monocytogenes* is unclear. Whilst some researchers have suggested a role for reactive oxygen or nitrogen intermediates other work has emphasised the importance of lysosomal enzymes and peptides (Higginbotham, Lin & Pruett, 1992).

The induction of protective immunity against *L.monocytogenes* has been shown to require living organisms which are capable of growing intracellularly (Mackaness, 1962; von Koenig, Finger & Hof, 1982; Berche, Gaillard & Sansonetti, 1987a). However, this is not due to the expression of specific protective T-cell epitopes only by live bacteria. Brocke & Hahn (1991) have shown that killed *L.monocytogenes* cells or nonviable antigen are capable of inducing specific protective CD4⁺ T-cells when emulsified in Freund's complete adjuvant. Furthermore the protective function of these T-cells could not be correlated with their ability to produce IFN- γ , IL-2 or IL-4 but may be related instead to the stimulation of IL-1 production by macrophages. Killed *Listeria* are therefore sufficient as T-cell epitopes but in the absence of a suitable adjuvant are unable to elicit cytokines such as IL-1 which are needed for the amplification of a T-cell response (Mitsuyama, Igarashi, Kawamura, Ohmori & Nomoto, 1990). Whilst the nature of protective antigens is still under investigation, listeriolysin has been found to be a potent T-cell activator and this immunogenicity requires the integrity of tertiary conformation (Berche, Gaillard, Geoffroy & Alouf, 1987b). Site-directed mutagenesis of the *hly* gene has been shown to provide both a means of inactivating LLO whilst retaining immunogenicity and hope of developing a live vaccine (Michel, Reich, Favier, Berche & Cossart, 1990).

2.7.5 PATHOGENICITY OF *Mycobacterium* spp.

The mycobacteria are acid fast, Gram positive rods which are responsible for some of the world's most devastating diseases and include a wide range of species which are pathogenic for both humans and animals. Information regarding the mechanisms of host-pathogen interaction in mycobacterial infections has been recently reviewed by Jacobs (1992) and Kaufmann & Young (1992). Rastogi (1990) reports that mycobacteria possess a complex trilaminar cell wall composed of peptidoglycan, a matrix of mycolic acid residues and lipid moieties and a proteinaceous outer layer. Like *L.pneumophila*, mycobacteria are also capable of avoiding both phagosomal acidification and lysosomal fusion (e.g. *Mycobacterium tuberculosis*; Crowle, Dahl, Ross & May, 1991) and can interfere with the normal mechanism of endo- and exocytosis of macrophages, although coating the bacilli with specific antibody can partly reverse the inhibition of phagosome-lysosome fusion. Nevertheless, many pathogenic mycobacteria are relatively resistant to oxidative killing systems and the hydrophobic, capsule-like cell wall acts, in conjunction with superoxide dismutase, to inhibit the diffusion and degrading action of lysosomal enzymes despite phagosome-lysosome fusion. The presence of capsular lipids which are relatively resistant to degradation also serves to protect the bacilli intracellularly as well as suppressing lymphoproliferative responses (Sibley & Krahenbuhl, 1988; Chan, Fujiwara, Brennan, McNeil, Turco, Sibille, Snapper, Aisen

& Bloom, 1989; Tassell, Pourshafie, Wright, Richmond & Barrow, 1992). The combination of a resistant capsule and interference with macrophage endocytosis is thought to account for the failure of chemotherapeutic drugs to be deposited inside affected phagosomes in quantities sufficient to kill the pathogen. Mycobacterial infections are controlled when specific T-cells activate macrophages to limit the spread of infection, which leads to a chronic carrier state, although the infection may disappear entirely (Collins, 1990). However, whether infection is ever completely eliminated may depend on the genetic predisposition of the host (Crowle, 1990) and although T-cell activation of phagocytes has been pursued as a means of controlling mycobacterial infections, evidence for the involvement of cytokines produced by either T-cell dependent or T-cell independent means has yet to be conclusively demonstrated (Crowle, 1990; van Furth, 1990; Rastogi, 1990; Rook, 1990). Of interest is a study by Flesch & Kaufman (1991) which has shown that reactive nitrogen intermediates (RNI) are of greater importance than reactive oxygen intermediates to the inhibition of *M.bovis* growth in murine macrophages *in vitro*. Similarly, Denis (1991) has demonstrated that whilst superoxide dismutase acts to protect *M.avium* from macrophage superoxide anion, tumour necrosis factor- α and granulocyte macrophage colony stimulating factor either alone or in combination increase human macrophage effector functions against *M.avium* and that RNI are involved. Many mycobacterial antigens have been shown to stimulate both cell-mediated (CMI) and delayed type hypersensitivity (DTH) responses and are not only capable of activating phagocyte killing mechanisms but also of inducing a chronic inflammatory response in which lymphocytes and phagocytes act to cause caseous necrosis and tissue liquefaction (Kaufmann & Young, 1992). This situation is reminiscent of attempts to induce protective immunity against BKD in which *R.salmoninarum* antigens have exacerbated the pathology of the disease. Dannenberg (1990) has suggested that mycobacterial vaccines may have to be a balance between eliciting a cell-mediated response of sufficient strength to limit the spread of infection rather than eradicate the pathogen, whilst minimising the tissue damage caused by DTH reaction. Nevertheless, recent studies have shown that, at least in animal models of infection, immunisation with fractions of soluble or extracellular mycobacterial proteins can provide some measure of protection (Gelber, Murray, Siu & Tsang, 1992; Kaufmann, Gulle, Daugelat & Schoel, 1992; Pal & Horwitz, 1992). Reports of the cloning of genes encoding immunologically reactive proteins from *M.leprae* (Sathish, Esser, Thole & Clark-Curtiss, 1990; Sela, Thole, Ottenhoff & Clark-Curtiss, 1991) and complex cell wall glycopeptidolipids from *M.avium* (Belisle, Pascopella, Inamine, Brennan & Jacobs, 1991) provide hope of a means of studying the role of these highly antigenic structures in the disease process and perhaps of overcoming some of the problems associated with strong DTH reactions.

2.8 MOLECULAR APPROACHES TO VACCINE DEVELOPMENT

Dougan (1989) argues for a molecular approach to the identification and manipulation of virulence and pathogenicity determinants which will allow the investigation of host immune interactions and lead to the development of vaccines, chemotherapeutics and diagnostic tools. The cloning, sequencing and analysis of genes encoding such factors may yield vital information about the structure, function and regulation of these and other potentially useful components e.g. T-cell activating epitopes (Flynn, Weiss, Norris, Seifert, Kumar & So, 1990). This information is important not only for identifying potential antigens but also for the development of immunogenic rationally attenuated strains either for direct application as live vaccines or as carriers of heterologous antigens. Dougan (1989) also documents the successful development of recombinant vaccines against a variety of diseases and the likely success in producing many more such as tetanus (Fairweather, Chatfield, Makoff, Strugnell, Bester, Maskell & Dougan, 1990), cholera (Kaper, Lockman, Baldini & Levine, 1984), typhoid (Clements & El-Morshidy, 1984; Levine, Ferrecio, Black & Germanier, 1987), whooping cough (Gross, Arico & Rappuoli, 1989; Molina & Parker, 1990), malaria (Flynn *et al.*, 1990) and anthrax (Iacono-Connors, Schmaljohn & Dalrymple, 1990).

In addition to the successful application of genetic manipulation to mammalian vaccination there have been recent successes in applying molecular techniques to the development of fish vaccines (Leong & Munn, 1991). Most fish vaccine research has been based on the use of killed whole cells for reasons of cost and simplicity (Ellis, 1988). However, other possibilities exist in the context of recombinant technology. The rational attenuation of virulent strains by identifying and inactivating virulence determinants to produce avirulent vaccine strains is one approach. An alternative approach to attenuation has been the construction of mutant strains which are deficient in the biosynthesis of crucial components, such as UDP-galactose-4-epimerase (*galE*) mutants of *Salmonella*. This mutation affects the conditions under which the lipopolysaccharide virulence factor is expressed in *Salmonella* causing autolysis of the *galE* mutants (Nnalue & Stocker, 1987). Mutants which lack one or more of the genes encoding enzymes forming either the pre-chorismic acid pathway for the synthesis of aromatic compounds (*aro* mutants) and the purine pathway (*pur* mutants) have also been isolated. The *aro* pathway has been a popular target because it is present only in bacteria and certain plants and *aro* mutants can only survive *in vivo* for a limited period of time or *in vitro* if the aromatic deficiency is redressed. This approach has led to the recent successful development of an *aroA* deficient strain of *Aeromonas salmonicida* as a live vaccine against furunculosis of fish (Vaughan, Smith & Foster, 1993). Another approach to vaccination

involves the identification of subunits e.g. toxins, cell wall antigens, adhesins, enzymes which can either be administered in a purified form or else the genes encoding for the components can be modified to remove any undesirable features whilst retaining immunogenicity i.e. protein engineering. Such inactivated genes may be introduced into attenuated carriers such as *Salmonella aroA* or *htrA* mutants without the risk of disseminating undesirable genetic material. In this manner multivalent vaccines can be constructed and delivered in an appropriate way in order to induce the required response (Brown, Hormaeche, Demarco de Hormaeche, Winther, Dougan, Maskell & Stocker, 1987; Dougan, 1989). An example would be the recent development of a recombinant subunit vaccine for infectious haematopoietic necrosis (IHN) developed from the cloned gene for the IHN viral glycoprotein expressed in *E.coli*. Fish are immersion vaccinated in a crude bacterial lysate and consequently protected against IHN challenge (Gilmore, Engelking, Manning & Leong, 1988).

Until now, a molecular approach to BKD vaccine development has not been attempted. Further research into the pathogenicity factors of *R.salmoninarum* and the application of recombinant DNA technology may provide a means to overcome some of the difficulties that researchers have encountered in the past.

CHAPTER 3.

CLONING, ISOLATION AND CHARACTERISATION OF A GENE ENCODING A HAEMOLYTIC PRODUCT FROM *R.salmoninarum*

3.1 INTRODUCTION

The application of recombinant DNA technology to the study of *R.salmoninarum* and the isolation of cloned products for further immunological studies represents a new approach to BKD vaccine research. In relation to many other bacterial pathogens, the development of molecular techniques for genetic manipulation has allowed the rapid identification and characterisation of a wide variety of components. The information gained has provided insight into the relationship between host and pathogen and greatly facilitated the investigation of host immune responses to individual antigens, of particular relevance to vaccine design (Dougan, 1989). Genetic manipulation enables the movement of DNA sequences conferring a phenotype of interest from one organism to another in order to simplify and amplify the production of both the gene and gene product. Using this approach, it is possible to investigate the structure and control of a gene, search for related genes in other organisms and manipulate the gene to produce a gene product to facilitate further biochemical and immunological research. The development of a wide variety of well characterised *E.coli* host-vector systems has accompanied the expansion in genetic manipulation and the development of the necessary tools and techniques is well documented in the literature. Excellent coverage of these and other host-vector systems is given by Neidhardt (1987), Old & Primrose (1989), Sambrook, Fritsch & Maniatis (1989) and Singer & Berg (1991). Gram positive host-vector systems are also available, such as those used successfully with *Bacillus subtilis* (Glover, 1985; 1987). These may prove to be more compatible for the cloning of DNA from other Gram positive organisms but considerable development is still required before they could approach the range and flexibility offered by currently available *E.coli* host-vector systems.

There are a number of basic considerations involved in any cloning research. The replication of DNA requires an origin of replication and DNA molecules which carry such an origin are termed replicons. Hence, the introduction of foreign DNA into a host cell requires the presence of a replicon compatible with the host cell replication system in order to ensure the efficient replication, survival and perhaps also expression of the foreign DNA. Small replicons can be used as cloning vectors and there are three main types used to clone fragments of foreign DNA

and propagate them within *E.coli*: (i) plasmids, (ii) bacteriophages and (iii) cosmids. As well as replicating autonomously in *E.coli*, even when joined covalently to foreign DNA, these vectors can be readily isolated and purified and possess non-essential regions into which foreign DNA can be inserted and subsequently replicated with the vector. Plasmids, phages and cosmids differ in their sizes and structures and each possesses particular features which influences the circumstances under which they are used. A variety of general texts such as Sambrook *et al.* (1989) as well as commercial catalogues and guides are available to aid in the choice of the most suitable vector for each circumstance. Publications from Promega Corporation, New England BioLabs Incorporated, Boehringer-Mannheim, United States Biochemical Corporation, BRL Gibco Ltd., Stratagene Ltd. and Pharmacia Biosystems Ltd. are especially useful.

Plasmids are extrachromosomal genetic elements which consist of double-stranded, closed circular DNA molecules ranging in size from 1-200 kilobase pairs (kbp). Plasmids used as cloning vectors are usually 2-7 kbp in size and can accept up to about 10 kbp of foreign DNA. In addition, plasmid vectors usually possess one or more selectable markers such as genes encoding antibiotic resistance or enzymatic activity to allow the identification of host cells carrying either the vector alone or the vector with a foreign DNA insert. Markers also serve to maintain the plasmid within the bacterial population. Different plasmids also vary in their copy number which refers to the number of copies of the plasmid present in each host cell and is also an indication of how closely plasmid replication is linked to the host cell replication mechanism. The degree of expression of plasmid-borne genes is partly a function of copy number, host cell replication and the strength of the promoter that directs the binding of RNA polymerase and the initiation of messenger RNA (mRNA) transcription. Many other factors may also affect transcription and subsequent translation or gene expression, including the efficiency of ribosome binding, the presence of untranslated leader sequences and variation in the host chromosomal genes which regulate plasmid replication (*pcn* in *E.coli*). Plasmids are perhaps the most popular cloning vectors because they are small, easy to handle, resistant to mechanical damage and readily isolated from host cells. In addition, a wide variety of plasmids are commercially available which confer a range of readily selectable phenotypes on host cells and possess a variety of restriction sites, copy numbers, promoters to add flexibility to cloning strategies.

Bacteriophage vectors fall broadly into two categories: (i) double-stranded DNA phage such as λ and (ii) single-stranded DNA phage such as M13. Single-stranded phage vectors contain a single-stranded DNA molecule and are most commonly used for applications where single-

stranded DNA confers some advantage such as DNA sequencing, oligonucleotide mutagenesis and gene probe work. However, for most cloning applications the popular choice has been phage λ . This well characterised *E. coli* virus possesses a linear duplex genome of about 49 kbp. At each end are short single-stranded cohesive termini which are complementary in sequence and which associate to form the *cos* site and consequently a circular DNA structure following injection into a host cell. A large portion of the central region of the λ genome is non-essential and can be replaced by up to 23 kbp of foreign DNA without impairing its infectious growth cycle. Using the technique of *in vitro* packaging, λ phage provides a simple means of obtaining a high level of both gene expression and recovery of recombinant phage DNA; a process which is carefully described by Old & Primrose (1989). Vectors based on λ phage either have a single target site at which foreign DNA can be inserted (insertional vectors such as λ gt11) or have a pair of sites defining a fragment that can be removed and replaced by foreign DNA (replacement vectors such as λ L47.1). There are a range of derivatives of λ phage with a variety of deletions and mutations which have extended the number of cloning opportunities (reviewed by Chauthaiwale, Therwath & Deshpande, 1992).

Cosmids are plasmids which contain a fragment of λ DNA which includes the *cos* site thus allowing cosmids to be packaged *in vitro* into phage coats and then used to infect a suitable host. After infection the cosmid behaves as a normal plasmid without the expression of phage functions. For efficient packaging, there is a requirement that, as with λ DNA, the *cos* sites must be 37-52 kbp apart. Thus, cosmids are usually 4-6 kbp in size and therefore provide a means of cloning 32-48 kbp of foreign DNA. Cosmids were developed for cloning large fragments of eucaryotic DNA but have been largely superseded by the development of eucaryotic yeast host-vector systems.

Any cloning experiment has four essential parts: (i) the generation of foreign DNA fragments and cut vector DNA using restriction endonucleases which cleave DNA at specific sites, (ii) the ligation of foreign DNA with vector DNA fragments, (iii) the introduction or transformation of the consequent recombinant DNA molecules into a host cell within which replication can occur and (iv) the detection of clones by screening for those expressing a desired phenotype or possessing a desired genotype.

Because of the paucity of information available concerning the molecular genetics of *R. salmoninarum* the work presented in this study utilised a number of different vectors and

techniques in attempting to clone and isolate genes encoding products for potential use in BKD vaccine research. This represented a novel approach to the study of this fastidious and poorly understood organism and the general lack of information about pathogenicity factors necessitated screening for products considered to be of importance in other pathogens. For example, the production of phosphatases has been found to block phagocyte superoxide generation and contribute to the intracellular survival of *Legionella pneumophila* (Sahney, Lambe, Summersgill & Miller, 1990), *Legionella micdadei* (Saha, Dowling, LaMarco, Das, Remaley, Olomu, Pope & Glew, 1985) and *Leishmania donovani* (Glew, Saha, Das & Remaley, 1988). Kagaya, Miyakawa, Watanabe & Fukazawa (1992) have recently shown that the catalase II produced by *Salmonella typhimurium* in phagocytes is a dominant, stress-induced antigen that is largely involved in the induction of protective cell-mediated immunity in mice. As shown in Table 3.1, *R. salmoninarum* exhibits a number of biochemical characteristics of particular interest including phosphatase, catalase, gelatinase, caseinase, esterase and haemolytic activities. The contribution of proteolytic and haemolytic activities to the pathology and pathogenesis of BKD has been reviewed in Chapter 2. It seems possible that given the unique lipid composition of *R. salmoninarum*, esterase activities may also be important in the disease process. In addition to these biochemical activities, screening was carried out on the basis of immunologically recognised products in the extracellular products, cell wall and whole cells of *R. salmoninarum*.

3.2 MATERIALS AND METHODS

3.2.1 CHEMICALS AND REAGENTS

Unless otherwise stated, all chemicals and reagents were purchased from either Sigma Chemical Company, BDH Ltd., Oxoid or LabM and were of analytical or molecular biology grade if possible. Restriction endonucleases, T4 ligase, DNA modifying enzymes, incubation buffers and plasmid and bacteriophage DNA were purchased from either Northumbria Biologicals Ltd., Stratagene Ltd., Boehringer-Mannheim, Promega Corporation, New England Biolabs Incorporated or United States Biochemical Corporation.

3.2.2 BACTERIOLOGY

3.2.2.1 Bacterial strains

Seven isolates of *R. salmoninarum* including the type strain (ATCC33209, Table 3.2) were used. Three isolates were non-autoagglutinating and non-hydrophobic and have been shown to lack the 57kDa saline extractable cell-associated protein which has been associated with virulence (Bruno & Munro, 1986a; Bruno, 1988c, 1990). The remaining four isolates were autoagglutinating and

hydrophobic. Tests for autoagglutination, hydrophobicity and biochemical properties were carried out at the Marine Laboratory, Scottish Office Agriculture and Fisheries Department, Aberdeen, Scotland as described by Bruno & Munro (1986a) and Bruno (1988c). All strains of *R.salmoninarum* were supplied by Dr Bruno except isolate 910019 from an outbreak of BKD in 1991, which was provided by Dr C.J.Rodgers, Ministry of Agriculture, Food and Fisheries, Fish Disease Laboratory, Weymouth, Dorset, England. The strains of *E.coli* used in this investigation are given in Table 3.3 along with information relevant to their use.

Table 3.1 Biochemical properties of *R.salmoninarum*
(Bruno & Munro, 1986a; Austin & Austin, 1987).

Gram's stain	+	Guanine	-
PAS stain	+	Hyaluronic acid	-
Albert's stain	-	Hypoxanthine	-
Ziehl Nielsen	-	Lecithin	-
Capsule	-	RNA	-
Nitrate reduction	-	Starch	-
Haemolytic activity	β haemolysis	Testosterone	-
Production of:		Tributylin	+
Acid phosphatase	+	Tween 40	+
Alkaline phosphatase	+	Tween 60	+
Butyrate esterase	-	Tween 80	-
Caprylate esterase	+	Tyrosine	-
Catalase	+	Xanthine	-
Chymotrypsinase	-	Utilisation of 4-methylumbelliferyl substrates:	
Cystine arylamidase	-	Acetate	+
α -fucosidase	-	Butyrate	+
α -galactosidase	-	β -D-cellobiopyranoside	-
β -galactosidase	-	Elaidate	-
β -glucosaminidase	-	α -L-arabinopyranoside	-
α -glucosidase	+	2-acetamido-2-deoxy- β -D-galactopyranoside	-
β -glucosidase	-	β -L-fucopyranoside	-
β -glucuronidase	-	Heptanoate	+
Leucine arylamidase	+	Laurate	+
α -mannosidase	+	Nonanoate	+
Myristate esterase	-	Oleate	+
Oxidase	-	Palmitate	-
Trypsinase	+	Propionate	+
Urease	-	Carbohydrate fermentation:	
Valine arylamidase	-	Arabinose	-
Degradation of:		Dextrin	-
Adenine	-	Fructose	-
Aesculin	-	Galactose	-
Agar	-	Glucose	-
Arbutin	-	Lactose	-
Arginine	-	Maltose	-
Casein	+	Mannitol	-
Chitin	-	Raffinose	-
Chondroitin	-	Salicin	-
DNA	+	Sorbitol	-
Elastin	-	Sucrose	-
Gelatin	+	Xylose	-

Table 3.2 Source and origin of *R.salmoninarum* strains used in this study.

STRAIN	OTHER STRAIN NUMBERS	HYDROPHOBICITY/ AUTOAGGLUTINATION	FISH SOURCE	ORIGIN
ATCC 33209	MT 251	+	<i>O.tshawytscha</i>	USA
MT 414	MT 239	-	<i>S.salar</i>	Scotland
MT 417	MT 241	-	<i>O.kisutch</i>	France
MT 420	n.a.	+	n.a.	n.a.
MT 425	n.a.	+	<i>O.mykiss</i>	Scotland
MT 452	n.a.	-	<i>O.mykiss</i>	Scotland
910019	n.a.	+	<i>O.mykiss</i>	England

Note: n.a.= not available.

Table 3.3 Strains of *E.coli* K12 used in this investigation:

STRAIN	GENOTYPE	SOURCE
C600	F ⁻ <i>mcrA</i> ⁻ <i>mcrB</i> ⁺ <i>supE44</i> <i>thi-1</i> <i>thr-1</i> <i>leuB6</i> <i>lacY1</i> <i>tonA21</i> <i>hsdR</i> <i>hsdM</i> ⁺	M.L.Gilpin
DH1	F ⁻ <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>hsdR17</i> (<i>r_k</i> ⁻ , <i>m_k</i> ⁺) <i>supE44</i> <i>relA1</i>	M.L.Gilpin
DS410	F ⁻ <i>lac</i> <i>rpsL</i> <i>minA</i> <i>minB</i>	M.L.Gilpin
E15	<i>Hfr</i> <i>phoA</i> <i>relA</i> <i>tonR</i> <i>T₂R</i> <i>fadL</i>	M.L.Gilpin
Q359	F ⁻ P2 ⁺ <i>hsdR</i> (<i>r_k</i> ⁻ , <i>m_k</i> ⁺) <i>supE</i> <i>fhuA</i> ($\phi 80'$)	M.L.Gilpin
XL1-Blue	<i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>hsdR17</i> <i>supE44</i> <i>relA1</i> <i>lac</i> [F' <i>proAB</i> <i>lacI^q</i> Δ M15 <i>Tn10</i> (<i>tet'</i>)]	Stratagene
Y1089	F' Δ (<i>lac</i>)U169 <i>proA</i> ⁺ Δ (<i>lon</i>)? <i>araD139</i> <i>strA</i> <i>hflA150::</i> <i>Tn10</i> (<i>tet'</i>) [pMC9 <i>amp^r</i> <i>tet'</i>]	M.L.Gilpin
Y1090	F' Δ (<i>lac</i>)U169 <i>proA</i> ⁺ Δ (<i>lon</i>)? <i>araD139</i> <i>strA</i> <i>supF</i> <i>mcrA</i> <i>trpC22::</i> <i>Tn10</i> (<i>tet'</i>) [pMC9 <i>amp^r</i> <i>tet'</i>]	M.L.Gilpin

Strain information: C600 A useful strain for the assay and propagation of λ phage.

DH1 A recombination-deficient strain for propagating plasmids and cosmids.

DS410 A minicell producing strain.

E15 A phosphatase deficient strain useful for screening for phosphatase activity.

Q359 A P2 lysogen for the selection of Spi⁻ recombinants.

XL1-Blue A recombination-deficient strain which is particularly useful for blue-white screening for recombinants since an uncharacterised mutation enhances the vector encoded α -peptide β -galactosidase activity to give a more intense blue colour on plates containing an appropriate inducer and substrate.

Y1090 This is the strain of choice for immunological screening of expression libraries and the propagation of λ gt11. Expression of the foreign protein is controlled by high levels of *lac* repressor made by the resident plasmid pMC9 which carries *lacI^q*. The *supF* tyrosine inserted amber suppressor is required for lytic growth of λ gt11 and allows for cell lysis facilitating antibody screening. It is also *lon* protease deficient which may reduce the degradation of foreign protein. pMC9 is pBR322 with *lacI^q* inserted.

3.2.2.2 Culture media

For routine culture *R. salmoninarum* was grown on Mueller-Hinton agar supplemented with 0.1% w/v L-cysteine hydrochloride (MHCA) at 15°C and subcultured at 3 week intervals. All strains were removed from liquid nitrogen storage, grown at 15°C for 10 days and then maintained at 4°C on master plates of MHCA. Broth cultures (MHCB) were prepared from master plates and grown statically at 15°C. MHCA and MHCB were routinely used to obtain bacterial inocula for experimental work. Every manipulation involved two checks for contaminating organisms; (i) Gram's stain and (ii) spread plating on MHCA and incubation at 15°C followed by Gram's stain of the subsequent growth.

Unless otherwise stated, strains of *E. coli* were incubated aerobically at 37°C. Stock cultures were maintained at -20°C in 50% glycerol. Cultures in regular use were streaked on the appropriate medium, stored at 4°C and subcultured every 2-3 months. For routine culture, *E. coli* was grown on Luria-Bertani (LB) agar (LBA) or in LB broth (LBB). Brain Heart Infusion broth (BHIB, Difco) was used for minicell analysis (section 3.2.11). When required, antibiotic stock solutions were prepared, filter sterilised (0.45 µm, Minisart, Sartorius) and added to media as follows: ampicillin sodium salt, 50 mg ml⁻¹ stock solution in double-distilled water, working concentration 100 µg ml⁻¹; chloramphenicol, 25 mg ml⁻¹ stock solution in 100% ethanol, working concentration 25 µg ml⁻¹; kanamycin monosulphate, 25 mg ml⁻¹ stock solution in distilled water, working concentration 25 µg ml⁻¹; streptomycin sulphate, 50 mg ml⁻¹ stock solution in double-distilled water, working concentration 200 µg ml⁻¹; tetracycline hydrochloride, 15 mg ml⁻¹ stock solution in 50% ethanol, working concentration 15 µg ml⁻¹. In some cases, isopropyl-β-D-thiogalactopyranoside (IPTG, 24 mg ml⁻¹ stock solution in distilled water) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 20 mg ml⁻¹ stock solution in dimethylformamide, DMF) were added to media. When necessary, strains were passaged on glucose M9 minimal agar prepared as follows: to 80 ml of water agar containing 1.5 g bacto-agar (Difco), autoclaved and cooled to 50°C, was added the following sterile ingredients: 10 ml of 10 x concentrated M9 salts, 10 ml of 10% glucose, 1 ml 0.01 M CaCl₂, 1 ml 1 M MgSO₄ and 1 ml 0.1M thiamine-HCl. Stock 10 x concentrated M9 salts consisted of 24 g Na₂HPO₄ (29 g for the hydrated form), 12 g KH₂PO₄, 2 g NaCl and 4 g NH₄Cl prepared in a final volume of 400 ml with double distilled water and autoclaved. In cases where phosphate-free minimal agar was required, 4 x concentrated M9 salts were prepared as follows: to 1 litre of 0.1 M Tris-HCl, pH 7.4 was added 2 g NaCl, 4 g NH₄Cl and the solution autoclaved. To 70 ml of water agar containing 1.5 g bacto-agar (Difco), autoclaved and cooled to 50°C was added 25 ml of 4 x concentrated

phosphate-free M9 salts, 5 ml filter sterilised 10% glucose and 1 ml of autoclaved 0.1 M thiamine-HCl.

3.2.3 PREPARATION OF GENOMIC DNA

Broth cultures (500 ml) of *R. salmoninarum* prepared as described (section 3.2.2.1) were centrifuged (4000 x g, 4°C, 20 min) and the pellet resuspended in 27 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Following the addition of 3 ml of 10% SDS and protease K (50 µg ml⁻¹) the solution was heated at 56°C. An aliquot was removed every 30 min, cooled and checked for increased viscosity as an indication of cell lysis. After about 3 h the solution was centrifuged (9000 x g, 20°C, 20 min), 0.1 volumes of 3 M sodium acetate and 0.6 volumes of isopropanol were added to the decanted supernatant, mixed and left at room temperature for 20 min. Following centrifugation (4000 x g, 20°C, 20 min) the pellet was washed in 70% ethanol, dried at 37°C and resuspended in 28 ml of TE buffer. To this solution was added 31 g of caesium chloride which was dissolved prior to the addition of 3 ml of ethidium bromide solution (10 mg ml⁻¹). After thorough mixing, the sample was left in the dark for 24-48 h prior to centrifugation (9000 x g, 20°C, 30 min) to remove contaminating protein. The supernatant was sealed into Beckman Quickseal centrifuge tubes and centrifuged at 40000 rpm (40 h, 20°C) in a Beckman 70.1Ti rotor in a Beckman L7 ultracentrifuge. The consequent genomic DNA band was visualised under ultra-violet radiation and removed by puncturing the tube with a needle and syringe. Ethidium bromide was removed by extraction with isopropanol saturated with water and CsCl. CsCl was removed by dialysis against 3 x 1 litre changes of TE buffer at 4°C over 12-24 h. After dialysis, the DNA was precipitated at -70°C for 30 min following the addition of 0.1 volumes of 3 M sodium acetate and 3 volumes of 100% ethanol. After centrifugation (9000 x g, 10 min) the DNA pellet was washed with 70% ethanol, dried at 37°C, then resuspended in an appropriate volume of TE buffer. The DNA concentration was estimated by agarose gel electrophoresis (section 3.2.5) in comparison with known concentrations of λ wild type DNA; the volume of TE buffer was adjusted such that the DNA concentration was no greater than 1 µg µl⁻¹.

3.2.4 PURIFICATION OF PLASMID DNA

3.2.4.1 Small scale preparations (minipreps)

For small scale purification of plasmid DNA, a variation of the method of Birnboim & Doly (1979) was used. Small volumes (1-5 ml) of overnight broth cultures were centrifuged (9000 x g, 3 min) and the pellets resuspended in 100 µl of B&D I (25 mM Tris-HCl, 50 mM glucose,

10 mM EDTA, pH 8.0, autoclaved). After 5 min incubation at room temperature, cells were lysed by adding 200 μ l of B&D II (0.5 M NaOH, 1% w/v SDS), mixed well and placed on ice for 5 min. Protein was precipitated on ice for 5 min after adding and mixing 150 μ l of cold (4°C) B&D III (3 M sodium acetate, pH 4.8 with glacial acetic acid). After centrifugation (9000 x g, 5 min), the supernatant was transferred to a new microfuge tube and nucleic acids were precipitated by filling the tube with cold (-20°C) ethanol and then incubating on ice for 10 min. Nucleic acids were pelleted (9000 x g, 5 min), the supernatant discarded and the pellet dried at 37°C. The pellet was resuspended in 50 μ l of RNase solution (1 mg ml⁻¹ in 50 mM Tris-HCl, pH 8.0, heated at 95°C for 10 min, then stored at 4°C) for 1 h at 37°C. Plasmid DNA was then purified by the addition of 50 μ l of phenol:chloroform (equal parts of phenol equilibrated with 0.1 M Tris-HCl, pH 8.0 and a water-saturated mixture of chloroform:isoamyl alcohol, 24:1). The contents of the tube were well mixed until emulsified and then centrifuged (9000 x g, 5 min). The aqueous (upper) phase was removed, mixed well with 50 μ l of water-saturated chloroform:isoamyl alcohol (24:1) and centrifuged (9000 x g, 5 min). This step was repeated and plasmid DNA in the aqueous phase was precipitated by adding 10% v/v 3 M sodium acetate, mixing well and then adding 3 volumes of cold (-20°C) ethanol and incubating on ice for 10 min. Pure plasmid DNA was pelleted (9000 x g, 5 min), washed in 70% ethanol and resuspended in an appropriate volume of TE buffer or Analar water. In some cases, plasmid DNA was extracted and purified from bacterial cultures by using the Magic minipreps DNA purification system (Promega) according to the manufacturer's instructions.

3.2.4.2 Large scale preparations

Cultures (500 ml) were centrifuged (4000 x g, 4°C, 20 min), resuspended in 18 ml of B&D I then 2 ml of lysozyme solution (10 mg ml⁻¹ in 10 mM Tris-HCl, pH 8.0) was added and the mixture incubated at room temperature for 5 min. The cells were lysed by adding 40 ml of B&D II with mixing and incubation on ice for 10 min. Proteins were precipitated on ice for 10 min by the addition with mixing of 20 ml of B&D III. After centrifugation (4000 x g, 4°C, 20 min) the supernatant was decanted, warmed to room temperature, 0.6 volumes of isopropanol added and then left at room temperature for 20 min. Nucleic acids were pelleted (4000 x g, 20°C, 30 min), the pellet washed in 70% ethanol, dried at 37°C and resuspended in 4.62 ml of TE buffer to which 5.12 g of CsCl was dissolved, and finally 0.25 ml of ethidium bromide (10 mg ml⁻¹) was added. After thorough mixing, the sample was left in the dark for 24-48 h prior to centrifuging (9000 x g, 20°C, 30 min) to remove contaminating protein. The supernatant was sealed into Beckman Quickseal centrifuge tubes and centrifuged at 80000 rpm (24 h, 20°C) using

a Beckman TLA100.3 rotor in a Beckman TL100 tabletop ultracentrifuge. Plasmid DNA was recovered as described in section 3.2.3. Occasionally, CsCl was removed from the recovered DNA by ethanol precipitation, instead of dialysis, followed by several washes in 70% ethanol.

3.2.5 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was carried out using TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M Na₂EDTA pH 8.0). The buffer was prepared at 10 x strength, autoclaved and diluted when required. Agarose (0.8% w/v) was dissolved in TBE buffer at 100°C, cooled to 50°C and 5 µl of ethidium bromide (10 mg ml⁻¹) was added per 100 ml of gel solution. The gel was poured into UV-transparent gel moulds (Pharmacia) and samples were usually run at 150 V in a horizontal agarose gel electrophoresis tank (G100 or G200, Pharmacia) until DNA bands were resolved (30 min-2 h). DNA molecular size standards (1 kb ladders, BRL Gibco) were included on gels to assist in the estimation of the sizes of DNA fragments. DNA bands were visualised by examining gels under UV light. Gels were photographed using a Polaroid camera, a Kodak Wratten orange filter (22A) and Polaroid type 667 black and white film. To resolve small DNA fragments (< 1 kb) the percentage of agarose in the gel was increased to 1%. Sample loading buffer contained 0.25% bromophenol blue and 15% Ficoll 400 in distilled water.

3.2.6 TRANSFORMATION OF *E.coli*

Bacteria were grown in 10 ml volumes of LBB overnight at 37°C. A 1.5 ml volume was centrifuged (3000 x g, 2 min), the pellet resuspended in 200 µl of cold (4°C) 50 mM CaCl₂ and kept on ice for 20 min. After centrifugation (2000 x g, 1 min), the competent cells were resuspended in 100 µl of cold (4°C) 50 mM CaCl₂ and either kept on ice or at 4°C for 20 min to 24 h. Competent *E.coli* cells were transformed with plasmid DNA as follows. From 0.2-0.5 µg of plasmid DNA in a maximum volume of 20 µl was added to 100 µl of competent cells, mixed and placed on ice for 10 min. The mixture was then incubated at room temperature for 5 min, on ice for 15 min, heat shocked for 2 min at 37°C prior to the addition of 900 µl of pre-warmed (37°C) LBB and then incubated at 37°C for 30-90 min without shaking. Transformants were selected by plating on the appropriate selective medium which usually contained one or more antibiotics (section 3.2.2.2).

3.2.7 DIGESTING DNA WITH RESTRICTION ENDONUCLEASES

Restriction endonucleases are enzymes which cleave double-stranded DNA on the basis of a specific recognition sequence. Type II restriction enzymes, which are particularly useful for their

ability to cut DNA at or near to their recognition sequence, are available from a number of commercial sources and were utilised in this study. Restriction enzyme digestions typically contained 0.5-5 μg of DNA in a volume of 30 μl or less. The DNA to be digested was precipitated, washed (section 3.2.4.1) and then resuspended in sterile Analar water containing an appropriate dilution of 10 x strength restriction enzyme incubation buffer. The amount of restriction enzyme to be used depended on the amount of DNA to be cut, the number of times the enzyme was expected to cut the DNA and the specific activity of the enzyme. One unit of enzyme activity is defined as the amount required to cleave 1 μg of λ wild-type DNA to completion in 1 h. The specific activity of each enzyme and the number of times each enzyme cleaves λ DNA is specified by suppliers and it is possible to then calculate the amount required for each digestion. For quicker reactions a two- to three-fold excess of the desired enzyme was used. Restriction enzyme constituted no more than 10% of the final reaction mixture to avoid inhibition by the glycerol used to stabilise commercially supplied enzymes. Digests were usually carried out at 37°C in sterile microfuge tubes which were sealed with Parafilm. Reactions were stopped either by phenol:chloroform extraction (section 3.2.4.1) or heat inactivation at 65°C for 20 min depending upon the properties of the enzyme.

3.2.8 LIGATION OF DNA

The specific cleavage of DNA by restriction enzymes yields fragments which may possess cohesive (sticky) ends or blunt ends depending on the enzyme used. Cohesive termini may be rejoined or ligated to compatible ends whereas blunt termini can be ligated to any other blunt-ended fragment. Ligation of cohesive plasmid termini usually involved the use of a two- to three-fold excess of cleaved insert DNA over cut vector DNA in volumes of 20 μl or more. The total amounts of DNA used depended on the planned subsequent manipulations. Previously digested vector and insert DNA were precipitated and washed (section 3.2.4.1), dried, pooled and resuspended in sterile Analar water in a sterile microfuge tube. To this mixture was added 10% final volume of 10 x strength T4 DNA ligase buffer, 0.5 mM ATP, 1 μM hexammine cobalt chloride and usually 0.5-2 units of ligase. Microfuge tubes were sealed with Parafilm and the reactions were incubated for 16-18 h at 15°C. ATP was prepared at 10 x strength, pH 7.4, snap frozen in liquid nitrogen and stored at -70°C. Hexammine cobalt chloride was prepared at 100 x strength and stored at 4°C.

Ligation of blunt-ended DNA was performed as for cohesive DNA but with higher concentrations of DNA and T4 ligase, and the reactions were incubated at 22°C for 24 h.

Bacteriophage ligations were carried out under slightly different conditions which favoured the formation of concatamers of phage and insert DNA. Concatamers tend to form in the presence of a two- to three-fold excess of vector DNA and are required for efficient *in vitro* packaging of the recombinant phage. Thus, phage ligations were carried out in small volumes (5 μ l) with an excess of λ DNA.

In some cases, cleaved plasmid DNA was treated with calf intestinal alkaline phosphatase in order to suppress self-ligation and recircularisation of the plasmid. During ligation, T4 ligase catalyses the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide contains a 5'-phosphate group and the other contains a 3'-hydroxyl group. If 5'-phosphates are removed by treatment with alkaline phosphatase then recircularisation of plasmid DNA is minimised whilst insert DNA with 5'-terminal phosphates can be efficiently ligated. Digested vector DNA was precipitated, washed (section 3.2.4.1), dried and resuspended in 10 mM Tris-HCl, pH 8.3. A 10% final volume of 10 x strength dephosphorylation buffer was added and for protruding 5'-termini, 1 unit of alkaline phosphatase was added for each 100 pmol of 5'-terminal phosphate residues and incubated at 37°C for 30 min. For blunt or recessed termini, 1 unit of alkaline phosphatase was used for each 2 pmol of 5'-terminal phosphate residues, incubated at 37°C for 15 min, then more enzyme added and incubation continued at 55°C for 45 min. The reaction was stopped by the addition, with mixing, of 10 mM final concentration of EGTA, pH 8.0 and heating at 65°C for 45 min. Vector DNA was recovered by phenol:chloroform extraction and ethanol precipitation, washing in 70% ethanol and resuspension in TE buffer (section 3.2.4.1).

3.2.8 CONSTRUCTION OF GENE BANKS

3.2.8.1 Bacteriophage λ gene banks

Bacteriophage λ gene banks were constructed in collaboration with Dr M.L. Gilpin using λ gt11 and λ L47.1. λ L47.1 is Spi^+ (sensitive to P2 interference) so recombinants can be positively selected because growth of λ wild type is restricted in lysogens carrying prophage P2, such as *E. coli* Q359. Strains of λ which possess two genes, *red* and *gam*, display a Spi^+ phenotype and can not grow in P2 lysogens. However, cloning in λ L47.1 involves the replacement of a *Bam*HI "stuffer" fragment, which contains *red* and *gam*, with up to 18 kbp of foreign DNA and thus recombinant phages will be Spi^- and will grow in P2 lysogens of *E. coli* K12. λ gt11 is an insertion vector with a unique *Eco*RI site located in *lacZ* allowing expression of a fusion protein if the foreign DNA is in-frame with *lacZ*. A high level of recovery of recombinant phage is

ensured by using λ gt11 which has been digested with *EcoRI* and then treated with alkaline phosphatase prior to the insertion of up to 7 kbp of foreign DNA. The presence of *lacZ* allows the efficiency of recovery of recombinants to be checked by exposing *E.coli* infected with recombinant phage to both IPTG, $192 \mu\text{g ml}^{-1}$, an inducer of β -galactosidase activity, and X-gal, $160 \mu\text{g ml}^{-1}$, a chromogenic substrate for β -galactosidase. In addition, λ gt11 possesses the *cIts857* temperature sensitive repressor which allows the control of phage replication and production of recombinant proteins. Recombinants will form lysogens at $<35^\circ\text{C}$ whilst at 42°C *cIts857* is inactive and λ gt11 will initiate host cell lysis.

The size of the *R. salmoninarum* genome is not yet known but comparison with other intracellular bacterial pathogens would suggest that an estimate of about $2\text{--}3 \times 10^6$ bp is not unreasonable. Assuming that a restriction digest of *R. salmoninarum* will yield a random representation of base sequences and a uniform size of DNA fragments, then it is possible to estimate the number of recombinants that must be recovered to have a high probability of recovering any particular DNA sequence. Although the assumptions are unfounded this estimate provides a working approximation using the following relationship derived by Clarke & Carbon (1976):

$$N = \ln(1-p) \times (\ln(1-a/b))^{-1}$$
 where *a* is the insert size, *b* is the haploid genome size and *N* is the number of recombinants which have a probability *p* of possessing any given DNA sequence. For λ gt11, assuming 5 kbp inserts and making $p=0.99$ we require a gene bank size $N \approx 2000$ individual recombinants. For λ L47.1, assuming 10 kbp inserts, for $p=0.99$ $N \approx 1000$.

R. salmoninarum strain MT444 genomic DNA was prepared by Evenden (1993). λ L47.1 DNA was obtained from Dr M.L.Gilpin. λ gt11 DNA which had been pre-cut with restriction endonuclease *EcoRI* and pre-treated with alkaline phosphatase was obtained from Promega Corporation. About $4 \mu\text{g}$ of MT444 genomic DNA was dissolved in restriction endonuclease *Sau3A* incubation buffer and partially digested with *Sau3A* at 37°C . Control reactions which contained varying amounts of enzyme were monitored by agarose gel electrophoresis (section 3.2.5) to determine the conditions required to reach the desired fragment size. About $1 \mu\text{g}$ of λ L47.1 DNA was dissolved in restriction endonuclease *BamHI* incubation buffer and digested to completion with *BamHI* at 37°C . The DNA digestion reactions were stopped either by phenol:chloroform extraction (section 3.2.4.1) for *BamHI* reactions or heat inactivation at 65°C for 20 min for *Sau3A* digests. The DNA was recovered by ethanol precipitation, washed (section

3.2.4.1) and 0.5 μg of *Sau3A* digested MT444 DNA was resuspended in 5 μl of ligation buffer containing 1 μg of *Bam*HI digested λ L47.1 DNA, 2 units of T4 DNA ligase and 0.5 mM ATP. The ligation mixture was incubated for 18 h at 15°C.

For λ gt11 gene banks, about 4 μg of MT444 genomic DNA was dissolved in restriction endonuclease *Eco*RI incubation buffer and partially digested with *Eco*RI at 37°C until the desired fragment size was reached. The digestion reaction was stopped by heat inactivation at 65°C for 20 min, the DNA precipitated with ethanol, washed (section 3.2.4.1) and then 0.5 μg was resuspended in 5 μl of ligation buffer containing 1 μg of pre-cut and alkaline phosphatase treated λ gt11 DNA, 2 units of T4 ligase and 0.5 mM ATP. The ligation mixture was incubated for 18 h at 15°C.

3.2.8.2 Infection

Ligation mixtures were packaged *in vitro* using λ phage heads, tails and assembly and packaging proteins purchased as packaging mixes (Promega) and used according to the manufacturer's instructions. Recombinant packaged phage were pooled and then titred by adding 100 μl of serially diluted (1:1000-1:10000) recombinant packaged phage to 100 μl of *E. coli* culture ($A_{600} \approx 0.6$) grown in LBB supplemented with 0.2% maltose. Phage were adsorbed for 30 min at 37°C prior to the addition of 3 ml of molten (45°C) 0.75% LBA. The mixture was vortexed and immediately poured as a "top" agar onto LBA plates. The plates were incubated at 37°C overnight, plaques were counted and the numbers of plaque forming units (pfu) ml^{-1} packaged phage was calculated prior to screening the unamplified gene bank. In some cases IPTG was added to the top agar for λ gt11 gene banks in order to induce the *lac* promoter.

3.2.8.3 Amplification of the λ gene banks

Following titration, the recombinant λ phage were amplified prior to screening. For λ gt11, *E. coli* strain Y1090 was used and for λ L47, *E. coli* Q359 was used. Q359 is a P2 lysogen and hence only recombinant λ phages (Spi⁻) give plaques on this strain. The efficiency of recovery of recombinants for λ gt11 gene banks was checked by including IPTG and X-gal in the top agar and counting the numbers of blue (non-recombinant) and white (recombinant) phage plaques. The efficiency was found to be >95%.

Aliquots of the packaged phage containing 2×10^4 pfu in a volume <50 μl were mixed with 0.2 ml of the appropriate strain of *E. coli* grown to $A_{600} \approx 0.6$ in LBB supplemented with 0.2%

maltose at 37°C. The mixture was plated as described (section 3.2.8.2) and incubated at 37°C until readily distinguishable clear plaques appeared, usually after 8-12 h. Immediately before confluent lysis, the plates were overlaid with 12 ml of sterile SM buffer (20 mM Tris-HCl pH 7.4 100 mM NaCl 10 mM MgSO₄ containing 2% w/v gelatin) and stored at 4°C overnight. The buffer was subsequently removed and transferred to sterile polypropylene tubes and plates were rinsed with 4 ml of fresh SM buffer. After the addition of a few drops of chloroform the bacteriophage suspension was incubated at room temperature for 20 min with occasional shaking. Cell and agar debris was removed by centrifugation (4000 x g, 4°C, 20 min) and the supernatant stored at 4°C. Following amplification, the λ gene banks were titred (section 3.2.8.2) prior to screening.

3.2.8.4 Plasmid gene banks

Plasmid gene banks were constructed in collaboration with Dr M.L.Gilpin using the vectors pBR328, pGD103 and pUC18. Plasmid vector pBR328 is a general cloning vector of about 4.9 kbp with considerable flexibility and possesses three genes conferring antibiotic resistance to ampicillin, chloramphenicol and tetracycline. The presence of three markers allows for selection of recombinants on the basis of insertional inactivation of one of the antibiotic resistance genes. In addition, pBR328 possesses a copy number of about 16-32 copies per cell, which may facilitate the cloning of genes encoding products that *E.coli* can not easily handle. pUC18 on the other hand, is a high copy number vector (up to 700 copies per cell) of about 2.7 kbp which possesses a gene encoding ampicillin resistance and also the *lacZ* gene. This allows for blue/white screening for recombinants (section 3.2.8.3) because pUC18 has a multiple cloning site (MCS) located within the *lacZ* and inserted foreign DNA will inactivate the β -galactosidase activity. In addition, the MCS contains a variety of useful restriction sites for insertion of DNA fragments adding considerable flexibility to cloning strategies and which may allow for a high level of expression under the control of the inducible *lac* promoter. pGD103 is a low copy vector (1-2 copies per cell) of about 5.6 kbp which possesses a kanamycin resistance gene and is especially useful for the cloning of genes encoding toxic products. pGD103 also possesses the *lacZ* gene with MCS derived from pUC18 thus providing considerable opportunity as discussed above.

Separate lots of 5 μ g of *R. salmoninarum* strain MT444 DNA were cut to completion with either *Pst*I, *Bam*HI, *Eco*RI, *Hind*III or *Sal*I at 37°C. About 1 μ g of pBR328 was cleaved with restriction endonuclease *Hind*III, 1 μ g of pUC18 was cleaved with *Eco*RI and 4 x 1 μ g lots of

pGD103 were cut with either *Pst*I, *Eco*RI, *Bam*HI or *Sa*II. The digested plasmids were treated with alkaline phosphatase to minimise recircularisation, and plasmids and inserts possessing compatible termini were ligated and transformed into *E.coli* DH1 or in some cases *E.coli* E15 (sections 3.2.6 and 3.2.8). Transformants were recovered on LBA supplemented with either kanamycin (25 μ g ml⁻¹) for pGD103 gene banks, ampicillin (100 μ g ml⁻¹) for pUC18 gene banks or chloramphenicol (25 μ g ml⁻¹) and ampicillin (100 μ g ml⁻¹) for pBR328 gene banks.

3.2.8.5 Replica plating

This technique was used to allow each plasmid gene bank to be screened a number of times for the expression of different biochemical and immunological products. After bacterial colonies had grown on the master plates they were transferred by using a piece of sterile velvet about 12-15 cm square stretched flat over the end of a metal cylinder 8 cm in diameter and held taut by a metal band. The master plate was inverted, placed onto the velvet and very gently pressed into contact taking care to avoid smearing or spreading the colonies. The agar surfaces of up to 3 replica plates were then pressed gently onto the velvet and bacteria from each colony transferred. The replica plates were then incubated until suitably sized colonies were available for screening.

3.2.9 SCREENING

3.2.9.1 Immunological screening of bacteriophage λ and plasmid gene banks

Phage plaques or bacterial colonies were overlaid with gridded nitrocellulose filter discs (0.45 μ m, Schleicher & Schuell) which had been marked for orientation. Care was taken to avoid trapping air bubbles under the filters which would prevent the binding of proteins. After 30 min to 1 h at 37°C the filters were removed from the plates. For plasmid gene banks, adherent bacterial colonies were lysed by exposing the filters to chloroform-saturated vapours within a lidded glass dish. Nitrocellulose filters were then washed and probed with rabbit antisera raised against *R.salmoninarum* whole cells, extracellular products and cell walls as described for Western blotting (section 3.2.15). In addition, bacterial colonies of abnormal appearance were picked from plates and grown overnight in LBB supplemented with the appropriate antibiotic(s) at 22°C with shaking. An aliquot of the culture was harvested, resuspended in SDS-PAGE sample buffer, electrophoresed, blotted onto nitrocellulose and probed with *R.salmoninarum* specific antisera which had been preabsorbed with acetone powder extracts of *E.coli* K12 prepared by Evenden (1993).

3.2.9.2 Biochemical screening of bacteriophage λ and plasmid gene banks

Phage plaques and bacterial colonies were overlaid with either gridded nitrocellulose discs (Schleicher & Schuell) or sterile Whatman number 1 filter paper discs which had been marked for orientation and pre-soaked in enzyme substrate. The plates were incubated at room temperature and examined every few minutes for evidence of enzyme activity. Fluorogenic substrates were viewed under UV light. 4-Methylumbelliferyl (MU) substrates were dissolved in dimethylsulphoxide (DMSO) to give 0.02 M solutions of acyl ester. Working solutions were prepared by diluting 100 μ l in 5 ml of medium. The substrates utilised in this way included the following derivatives of MU: for esterase activities; acetate, butyrate, heptanoate, laurate, nonanoate, oleate, propionate; for protease activity guanidino benzoate hydrochloride and for phosphatase activity phosphate lithium salt. The chromogenic substrate N α -benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA) was utilised as follows: a 5 mg ml⁻¹ solution in dimethylformamide (DMF) was diluted 1:9 in 0.05 M Na₂HPO₄, pH 8.6 as a working solution.

Phage and plasmid gene banks were also screened with a series of 5 ml overlays consisting of 0.75% agarose dissolved in PBS, cooled to 50°C prior to the incorporation of enzyme substrates and overlaying on either phage plaques or discrete bacterial colonies. Plates were incubated at room temperature. Plates containing fluorogenic substrates were monitored constantly for evidence of enzyme activity. All of the above fluorogenic and chromogenic substrates were tested in this way as well as the following substrates: 1% w/v casein (plates were developed with 3% v/v acetic acid to precipitate undigested protein); 0.1% w/v gelatin (plates were developed with 5% w/v tannic acid); 20% v/v washed horse erythrocytes and 25% v/v washed rainbow trout erythrocytes. Plates were incubated at room temperature for up to 4 days. Phosphatase activity was screened by incorporating 45 μ l of nitroblue tetrazolium (75 mg ml⁻¹ in 70% DMF) and 35 μ l of 5-bromo-4-chloro-3-indolyl phosphate (50 mg ml⁻¹ in DMF) in 10 ml of 0.75% w/v agarose dissolved in 0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M MgCl₂, pH 9.5 cooled to 50°C and overlaid on colonies of E15 transformants. In order to screen for catalase activity a 3% solution of hydrogen peroxide in sterile distilled water was used to overlay bacterial colonies and colonies were checked every 5-10 min for the production of bubbles of oxygen. In addition, bacterial colonies of abnormal appearance were picked from plates and grown overnight in LBB supplemented with the appropriate antibiotic(s) at 20°C with shaking. An aliquot was centrifuged (9000 x g, 5 min) and the pellet resuspended in SDS-PAGE sample buffer and electrophoresed in 10% SDS-PAGE gels containing either 0.1% w/v porcine gelatin or 0.2% v/v teleost fish skin gelatin as co-polymerised substrates (Heussen & Dowdle, 1980).

3.2.10 SOUTHERN BLOTTING OF DNA

DNA fragments separated according to size by agarose gel electrophoresis were denatured, transferred to nitrocellulose filters and probed for a specific complementary sequence using a non-radioactive labelled DNA probe according to the method of Southern (1975). Genomic DNA (5 μ g) obtained as described (section 3.2.3) from the following bacterial strains and recombinant plasmid DNA (0.1 μ g) was digested to completion with *Hind*III (section 3.2.7) and separated by agarose gel electrophoresis: *R.salmoninarum* ATCC33209, MT414, MT417, MT420, MT425, MT444, MT452; *Aeromonas salmonicida* CM30 and *Yersinia ruckeri* 12/6. Genomic DNA from *A.salmonicida* CM30 and *Yersinia ruckeri* 12/6 were obtained from Dr M.L.Gilpin. Following electrophoresis, the gel was depurinated by soaking in 0.2 M HCl for 10 min and then denatured by soaking in 100 ml of 1.5 M NaCl, 0.5 M NaOH for 1 h at room temperature with constant shaking. The gel was then neutralised by soaking in 200 ml of 1 M Tris-HCl, pH 8.0, 1.5 M NaCl for 1 h at room temperature with constant shaking. A capillary transfer apparatus was assembled by wrapping a piece of Whatman 3MM paper around a polystyrene support to serve as a wick. The support and wick were placed in a large dish which was half filled with 10 x SSC buffer. SSC (20 x strength) was prepared as follows: 175.3 g NaCl and 88.2 g sodium citrate were dissolved in 800 ml of Analar water, the pH adjusted to 7.0 with 5 M NaOH and the volume adjusted to 1 litre prior to autoclaving. A template was cut in aluminium baking foil and placed on the support in order to prevent a "short circuit" during transfer and ensuring that only the agarose gel would contact the wick. The gel was placed on the template and any air bubbles were gently smoothed out. A piece of nitrocellulose (0.45 μ m pore size) was cut to fit the gel, soaked in 2 x SSC until saturated, carefully placed on top of the gel and air bubbles were removed. Two pieces of Whatman 3MM paper were saturated in 2 x SSC, placed on top of the nitrocellulose and any air bubbles were removed. A stack of paper hand towels about 20 cm high were placed on top of the assembled apparatus and held down with a 500 g weight. Transfer was allowed to proceed for 16 h after which the nitrocellulose filter was peeled away from the gel, marked for orientation and soaked in 6 x SSC at room temperature for 5 min. After drying at room temperature, the filter was sandwiched between two sheets of Whatman 3MM paper and baked for 2 h at 80°C in a vacuum oven (Townson & Mercer Ltd, Runcorn, England). A commercial nonradioactive DNA, labelling and detection kit (Boehringer-Mannheim) was used according to the manufacturers instructions to detect the presence of complementary DNA sequences on nitrocellulose filters. The method relies upon the incorporation of digoxigenin-labelled deoxyuridine triphosphate into a random primed DNA probe using Klenow fragment of DNA polymerase which generates a range of digoxigenin-labelled fragments from the template

DNA. Filters were prehybridised at 68°C for 2 h, hybridised with 0.5 µg of digoxigenin-labelled probe at 68°C for 24 h, washed and dried. The presence of the probe was detected with anti-digoxigenin phosphatase conjugated antiserum and visualised by enzyme-substrate colour reaction.

3.2.11 MINICELL ANALYSIS

Minicell-producing strains of *E. coli* provide a means of clearly distinguishing specifically labelled plasmid encoded products from the tremendous number of products encoded by the host cell's chromosome (Dogan & Keyhoe, 1984). Minicells are small (0.1 µm) spheres which bud off the ends of the cells of certain mutant strains of bacteria such as *E. coli* DS410. The mutant strains are also capable of normal cell division hence minicells can be produced in bulk and purified. Minicells contain little or no genomic DNA and do not divide but remain metabolically active for long periods and can transcribe and translate any DNA which they may contain. If the minicell strain is transformed with plasmid DNA then the minicells will contain plasmid DNA and express plasmid-encoded products. By purifying the minicells and specifically radiolabelling the products encoded by plasmid DNA containing a foreign DNA insert it is possible to distinguish any additional products by comparison with minicells containing only the parental plasmid DNA.

Strain DS410 was grown in BHIB (18 h, 37°C) and checked microscopically under oil immersion for the presence of minicells. Recombinant plasmid DNA was introduced into DS410 by transformation (section 3.2.6), transformants were selected on the appropriate selective medium after growth at room temperature and the phenotype was checked by comparison with DS410 transformed with the plasmid vector alone as a control. Transformants containing either recombinant plasmid DNA or plasmid vector DNA were purified by streaking and checked microscopically for minicell production. Plasmid DNA was extracted from recombinant clones and control clones, checked by restriction digestion and agarose gel electrophoresis. All clones, including controls containing only plasmid vector DNA, were grown separately overnight in 200 ml volumes of BHIB containing ampicillin (100 µg ml⁻¹) and chloramphenicol (25 µg ml⁻¹). Each culture was centrifuged (2000 x g, 5 min, 4°C), the supernatant transferred to another centrifuge tube and again centrifuged (15000 x g, 10 min, 4°C) to pellet the minicells and the remaining normally sized cells. All of the following steps were performed at 4°C. The pellets were resuspended in 2 ml of sterile PGS (0.85% w/v NaCl, 0.03% w/v KH₂PO₄, 0.06% w/v Na₂HPO₄, 100 µg ml⁻¹ gelatine) and mixed vigorously to disrupt cell aggregates. The

resuspended minicells were carefully layered onto 25 ml sucrose gradients and centrifuged (4000 x g, 20 min, 4°C). Sucrose gradients were prepared by dispensing 25 ml of 20% w/v sucrose solution in PGS into 30 ml sterile, plastic universal bottles, freezing at -20°C and thawing slowly overnight at 4°C. The minicells formed a broad band in the top half of the gradient, with a broad band of normal cells below and a pellet of larger whole cells at the bottom. The minicell band was removed and transferred to a centrifuge tube and pelleted (15000 x g, 10 min, 4°C). The pellet was resuspended in 2 ml of PGS and layered onto another sucrose gradient. The procedure was repeated three or four times until only a pure band of minicells was visible. Afterwards, the minicells were washed with a 10 ml volume of sterile PGS and counted microscopically using a haemocytometer. Minicells were resuspended to a concentration of about $2-4 \times 10^9$ minicells ml⁻¹ in methionine assay medium (Difco) supplemented with thiamine (0.5 µg ml⁻¹) and incubated at 37°C with shaking for 20 min to allow them to reach maximum metabolic activity. Then 20 µCi of trans-³⁵S-label methionine and cysteine (ICN Flow) was added to specifically radiolabel the plasmid-encoded polypeptides and incubation was continued for 30 min. The labelled minicells were pelleted by centrifugation (15000 x g, 10 min, 4°C), resuspended in 100 µl of SDS-PAGE sample buffer and boiled for 2 min. Samples of 20 µl were subjected to SDS-PAGE, blotted onto nitrocellulose and blots stained for total protein using colloidal gold (section 3.2.15). The dried stained blots were autoradiographed using Kodak X-OMAT film and the autoradiographs developed to reveal only the plasmid encoded proteins.

3.2.12 PREPARATION OF CELL COMPONENTS AND EXTRACELLULAR PRODUCTS FROM *R.salmoninarum*

3.2.12.1 Sonicated cell extracts

Bacterial cultures were centrifuged (4000 x g, 4°C, 20 min) and resuspended in 5% of the original volume in 10 mM Tris-HCl, pH 7.4. Bacterial suspensions were disrupted by sonication (W-385 Ultrasonic Processor, Heat Systems Ultrasonics) on ice (5 s cycle, 50% duty cycle, 20 min). Unbroken cells and debris were removed by centrifugation (9000 x g, 4°C, 15 min) and the protein concentration of the resultant supernatant was estimated by the method of Bradford (1976) using a commercial assay kit following the manufacturer's instructions (BioRad). Supernatants and pellets were stored frozen at -70°C.

3.2.12.2 Solubilisable proteins from whole cells

Soluble proteins from *R.salmoninarum* cells were prepared as described by Daly & Stevenson (1987). Bacterial cultures were centrifuged (4000 x g, 4°C, 20 min), resuspended in 5% of the

original volume in sterile distilled water and washed twice before pelleting cells (4000 x g, 4°C, 20 min). The pooled supernatants were removed and stored frozen at -70°C after the protein concentration had been estimated.

3.2.12.3 Extracellular products

Bacterial cultures were centrifuged (4000 x g, 4°C, 20 min) and supernatants were precipitated, either on ice stirred with an equal volume of cold acetone (-20°C) or by the addition of 720 g litre⁻¹ ammonium sulphate with stirring at 4°C overnight. Following centrifugation (9000 x g, 4°C, 30 min), acetone precipitated pellets were dried at 37°C, resuspended in 10 mM Tris-HCl, pH 7.4 and stored frozen at -70°C after estimation of the protein concentration. Ammonium sulphate precipitates were centrifuged (9000 x g, 4°C, 30 min), the supernatant was checked for protein and the pellet was resuspended in 20 mM Tris-HCl, 1 mM EDTA, pH 7.2 and dialysed for 48 h against four changes of buffer. The protein concentration of the dialysate was determined and the samples stored frozen at -70°C.

3.2.12.4 Purification of cell walls

Cell wall fractions from *R.salmoninarum* were prepared according to the method of Russell, Peach, Colman & Colman (1983). Cells were pelleted (4000 x g, 4°C, 20 min), resuspended in 0.15 M NaCl and heated at 60°C for 30 min, washed twice in 0.15 M NaCl, sonicated on ice, washed twice in distilled water and twice in 1 M NaCl, resuspended in 1% SDS, centrifuged to remove intact cells (3000 x g, 4°C, 20 min) and cell walls pelleted (11000 x g, 4°C, 20 min). The last two steps were repeated before washing the pellet three times in distilled water and protein concentrations were estimated prior to freeze drying for 12 h at -70°C.

3.2.13 PREPARATION OF RABBIT ANTISERA

Blood was collected as outlined below, clotted in glass at room temperature and then overnight at 4°C. Serum was withdrawn and stored at -70°C. Dutch rabbits were injected subcutaneously (s.c.) with 500 µg of the appropriate protein in Freund's complete adjuvant (FCA), boosted 4 and 8 weeks later with 500 µg of the same protein in Freund's incomplete adjuvant (FIA), and then bled 4 weeks after the last injection. Control sera were gathered prior to immunisation.

3.2.14 ELECTROPHORESIS

Preparations were separated using either a Mini Protean II system (BioRad), LKB 2001 system or Phastsystem according to the manufacturer's instructions. Usually, the method of Laemmli

(1970) for discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE) was utilised. All gel mixtures and buffers were either purchased ready made or prepared following the manufacturer's instructions. Polyacrylamide gels were electrophoresed according to the manufacturer's recommendations for constant voltage or current and run durations. Standard molecular weight markers were run simultaneously with all preparations (Sigma SDS 6H, 7). Pre-stained molecular weight markers (Sigma SDS Blue) were used in gels for Western blotting.

3.2.14.1 Staining of polyacrylamide gels

For routine protein staining, gels were immersed for 2-4 h at room temperature with constant shaking in 0.2% w/v Coomassie Brilliant Blue R250 (CBB) in 30% methanol and 10% acetic acid. Gels were destained in 30% v/v methanol, 10% acetic acid solution.

3.2.15 WESTERN BLOTTING

Samples separated by PAGE or SDS-PAGE were transferred electrophoretically (Transblot, BioRad) onto nitrocellulose membranes (pore size either 0.45 or 0.2 μm) in 0.25 M Tris, 0.192 M glycine, 20% methanol, pH 8.3 for 12-16 h at a constant current of 30 mA according to the method of Towbin, Staehelin & Gordon (1979). Following transfer, gels were stained to confirm protein transfer and the nitrocellulose membranes (blots) were stained for total protein using either colloidal gold (Aurodyne; Janssen) or Ponceau S solution (Sigma) according to the manufacturer's instructions. Alternatively, nitrocellulose membranes were probed with specific antibody using a modification of the method described by Towbin *et al.* (1979). All steps were carried out at room temperature on an orbital shaker, unless otherwise stated.

3.2.15.1 Antisera from immunised rabbits

Blots were washed for 3 x 5 min in 0.05 M Tris containing 0.2 M NaCl (Tris-saline, pH 7.4) and subsequently for 3 x 5 min in Tris-saline containing 2% w/v skimmed milk (Tris-saline-milk, pH 7.4). The blots were incubated for 2 h in primary antiserum diluted 1:200 in Tris-saline-milk, then washed as above. Blots were then incubated for 1 h in swine anti-rabbit immunoglobulins peroxidase conjugate (SRPC, Dako Ltd., High Wycombe, U.K.), diluted 1:1000-1:3000 in Tris-saline-milk, and then washed 3 x 10 min in Tris-saline. The immunoblots were developed in Tris-saline containing 0.06% w/v 3,3'-diaminobenzidine tetrahydrochloride, 0.03% w/v NiCl_2 and 0.03% v/v hydrogen peroxide. Bands developed within 1-2 min and the reaction was stopped by rinsing with PBS. Identical blots were probed with control rabbit serum.

3.3 RESULTS

3.3.1 ISOLATION OF AN IMMUNOPOSITIVE CLONE

Screening of bacteriophage λ and plasmid gene banks resulted in the identification of a single immunopositive clone designated RSIP1 from the pUC18 *Eco*R1 gene bank. The clone was picked from plates which had been immunologically screened using nitrocellulose filter discs and was purified by streaking on LBA containing ampicillin ($100 \mu\text{g ml}^{-1}$). The phenotype was checked by Western blot probed with rabbit antisera raised against *R.salmoninarum* ECPs (Figure 3.1) which demonstrated the presence of several strongly immunoreactive bands of molecular masses from 20-58 kDa. The plasmid isolated from this clone was designated pMLG57 and further characterised by Dr M.L.Gilpin.

3.3.2 ISOLATION OF A HAEMOLYTIC CLONE

Screening of bacteriophage λ and plasmid gene banks resulted in the identification of a single clone designated H from the pBR328 *Hind*III gene bank which possessed a haemolytic phenotype on LBA plates overlaid with horse blood in 0.75% agarose. The clone was picked and purified by streaking. The phenotype was checked by comparison with *E.coli* DH1 containing pBR328 vector DNA only (Figure 3.2).

3.3.3 SUBCLONING OF A DNA FRAGMENT ENCODING A HAEMOLYTIC PRODUCT

Plasmid DNA extracted from clone H was designated pHY1 and was digested with *Hind*III and examined by agarose gel electrophoresis (Figure 3.3A). This revealed the presence of four DNA fragments of 3.1, 2.8, 1.2 and 0.8 kbp in size in addition to the 4.9 kbp fragment of pBR328 vector DNA. In order to isolate a single fragment of more manageable size which encoded the haemolytic activity, a *Hind*III digest of pHY1 was ligated and screened for activity with horse blood agarose overlays. Plasmid DNA was extracted from haemolytic colonies, digested with *Hind*III and analysed by agarose gel electrophoresis (Figure 3.3B). All clones possessing a haemolytic phenotype were found to possess a 3.1 kbp *Hind*III fragment of inserted DNA in addition to pBR328 vector DNA and any other *Hind*III fragments which may have been present. A haemolytic clone designated H3 which contained only a single 3.1 kbp insert was picked, purified by streaking, checked for haemolytic activity and the plasmid DNA (pRHLY'B') was extracted in bulk. The 3.1 kbp *Hind*III fragment was also successfully subcloned into the plasmid vector pUC18 but the phenotype proved to be too unstable for any further meaningful manipulation.

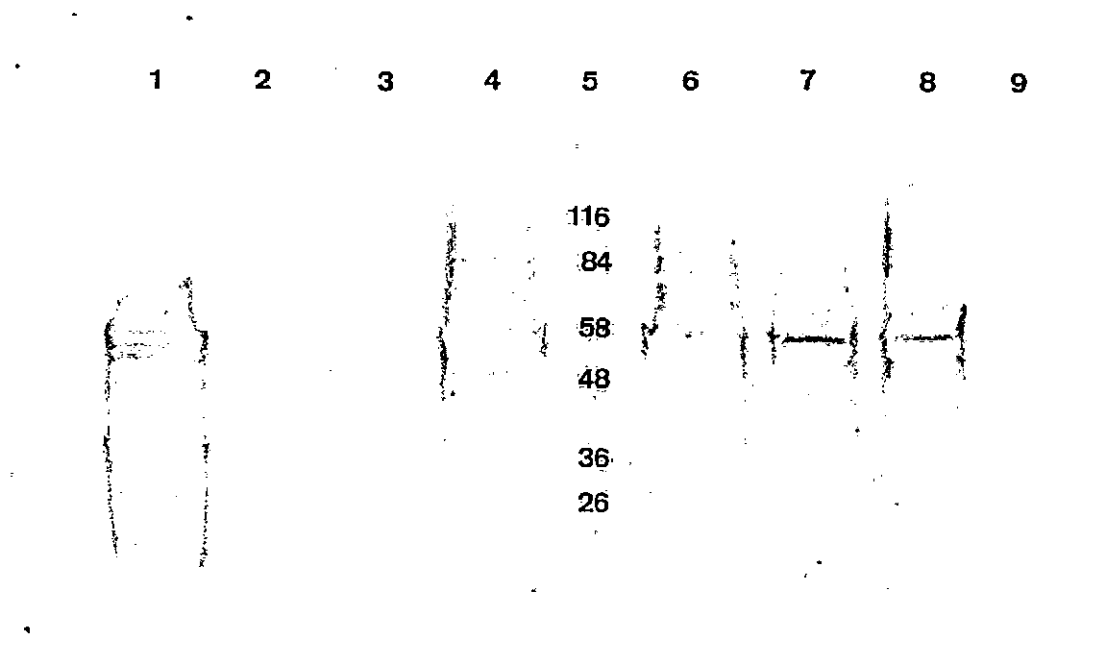


Figure 3.1 Western blot of immunopositive clone isolated from a pUC18 *Eco*R1 gene bank. Approximately 10 μ g of total cellular protein was separated by SDS-PAGE using a 10% polyacrylamide gel, transferred onto nitrocellulose, probed with a rabbit antiserum raised against *R.salmoninarum* ECPs and detected with a swine anti-rabbit immunoglobulins peroxidase conjugate. Tracks 1, 4, 6, 7, 8: RSIP1; track 2, 3, 9: *E.coli* XL1-BLUE containing pUC18; track 5: protein molecular weight standards in kilodaltons.

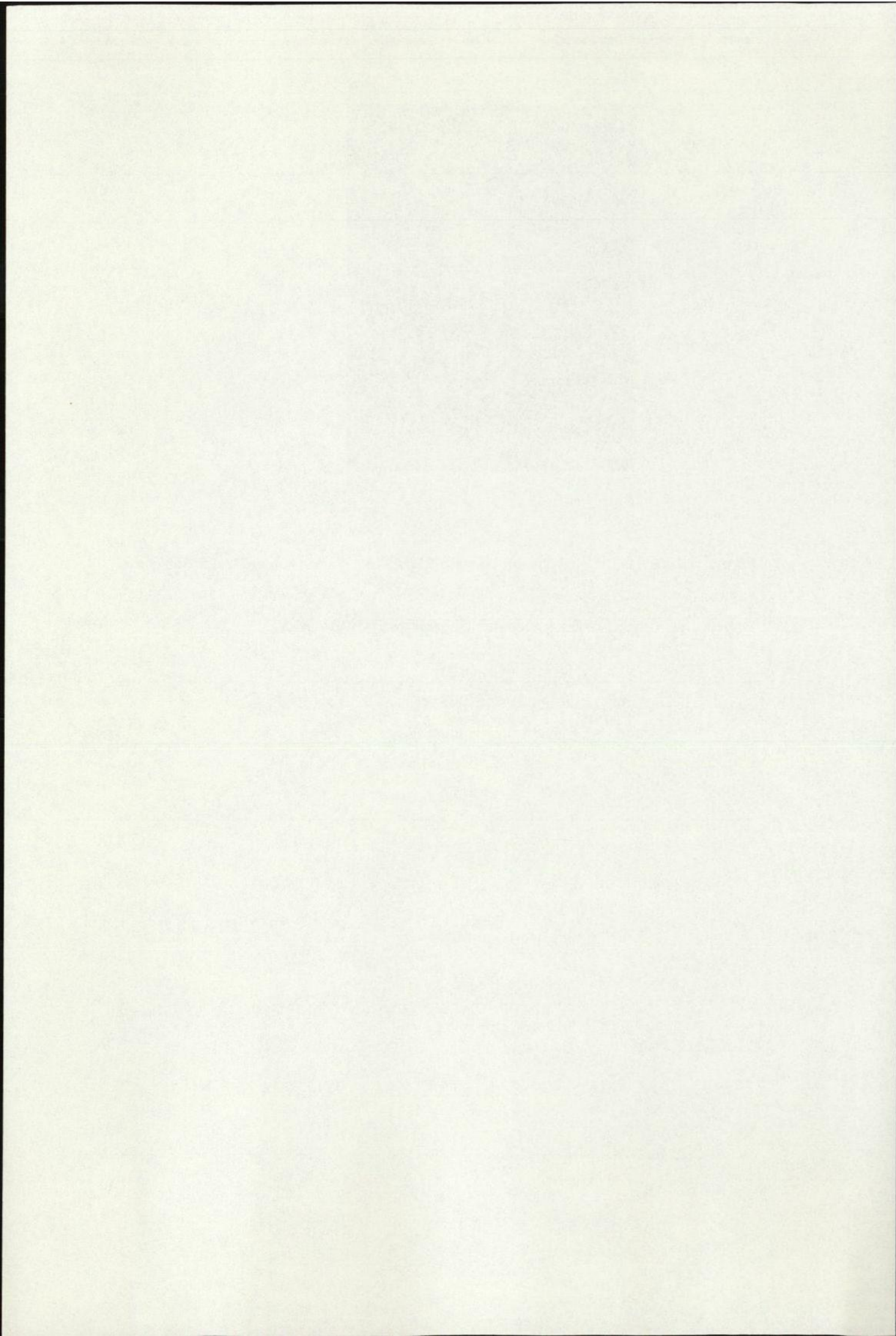
Figure 3.2 Identification of a haemolytic clone isolated from a pBR328 *Hind*III gene bank. The clone was isolated after growth at 25°C on LBA plates. Discrete colonies were overlaid with horse blood in 0.75% agarose. (a) Haemolytic phenotype of *E.coli* DH1 containing pRHLY'B'. (b) *E.coli* DH1 containing pBR328 only.

3.3.4 RESTRICTION MAPPING OF pRHLY'B'

Restriction mapping was performed to simplify further manipulation of the DNA insert encoding haemolytic activity. pRHLY'B' was cleaved with the following enzymes in a series of single digests: *Asp700* (*XmnI*), *AvaI*, *EcoRV*, *PstI*, *PvuI*, *PvuII* and *Sall*. Digests were separated by agarose gel electrophoresis (Figure 3.4) and the resulting fragment sizes allowed the restriction sites shown in Figure 3.5 to be accurately mapped on pRHLY'B'.

3.3.5 CHARACTERISATION OF THE HAEMOLYTIC ACTIVITY

Stable haemolytic clones of *E. coli* DH1 containing either pRHLY'B' or pBR328 as a control were streaked onto LBA containing ampicillin ($100 \mu\text{g ml}^{-1}$) and chloramphenicol ($25 \mu\text{g ml}^{-1}$), grown at room temperature and overlaid with 0.75% agarose in PBS containing 20% v/v fresh, washed erythrocytes from the following species: rabbit, sheep, horse, rat, mouse and rainbow trout. Duplicate plates were incubated at 6°C, 15°C, 20°C, 25°C, 30°C and 37°C for up to 4 days and compared at regular intervals with control plates. Haemolytic activity was observed from 6-37°C for erythrocytes from all species except rabbit, although only weak haemolysis was recorded against sheep erythrocytes. It was also observed that clones grown at 37°C were unstable and rapidly lost their haemolytic phenotype. Stable haemolytic clones were also streaked on LBA containing 1% L- α -phosphatidylcholine and grown for 4 days at 20°C. No lecithinase activity was evident. Stable clones grown on LBA and overlaid with either 1% casein or 0.1% gelatin displayed no evidence of degradation of either substrate when compared with identically treated controls. *E. coli* strains Y1089 and Y1090, both of which possess mutations preventing the production of the lon protease, were transformed with either pRHLY'B' or pBR328, stable clones were isolated and assayed for haemolytic activity as described above. The absence of the lon protease did not affect the haemolytic phenotype encoded by pRHLY'B'.



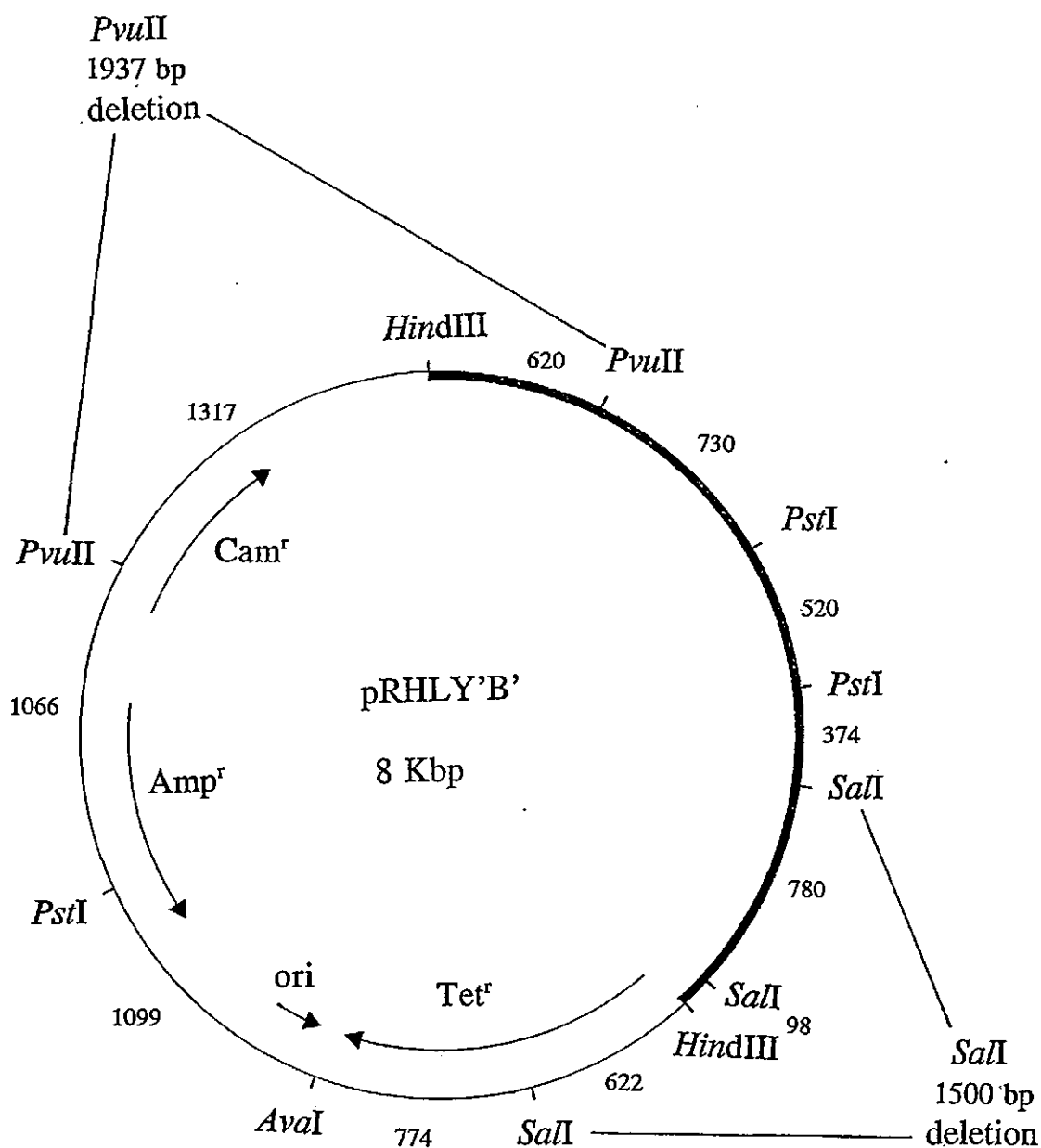


Figure 3.5 Restriction map of pRHLY'B' DNA deduced from single enzyme digests. The 3.1 kbp insert of *R.salmoninarum* DNA is represented by the heavy line. Also indicated are the *SalI* deletion and the *PvuII* deletion which were used in the minicell analysis of pRHLY'B' encoded proteins. Ori= origin of replication; *cam^r*= gene encoding chloramphenicol resistance; *amp^r*= gene encoding ampicillin resistance; *tet^r*= gene encoding tetracycline resistance. The numbers represent the number of base pairs of DNA between adjacent restriction sites.

3.3.6 IDENTIFICATION OF A HAEMOLYTIC PROTEIN

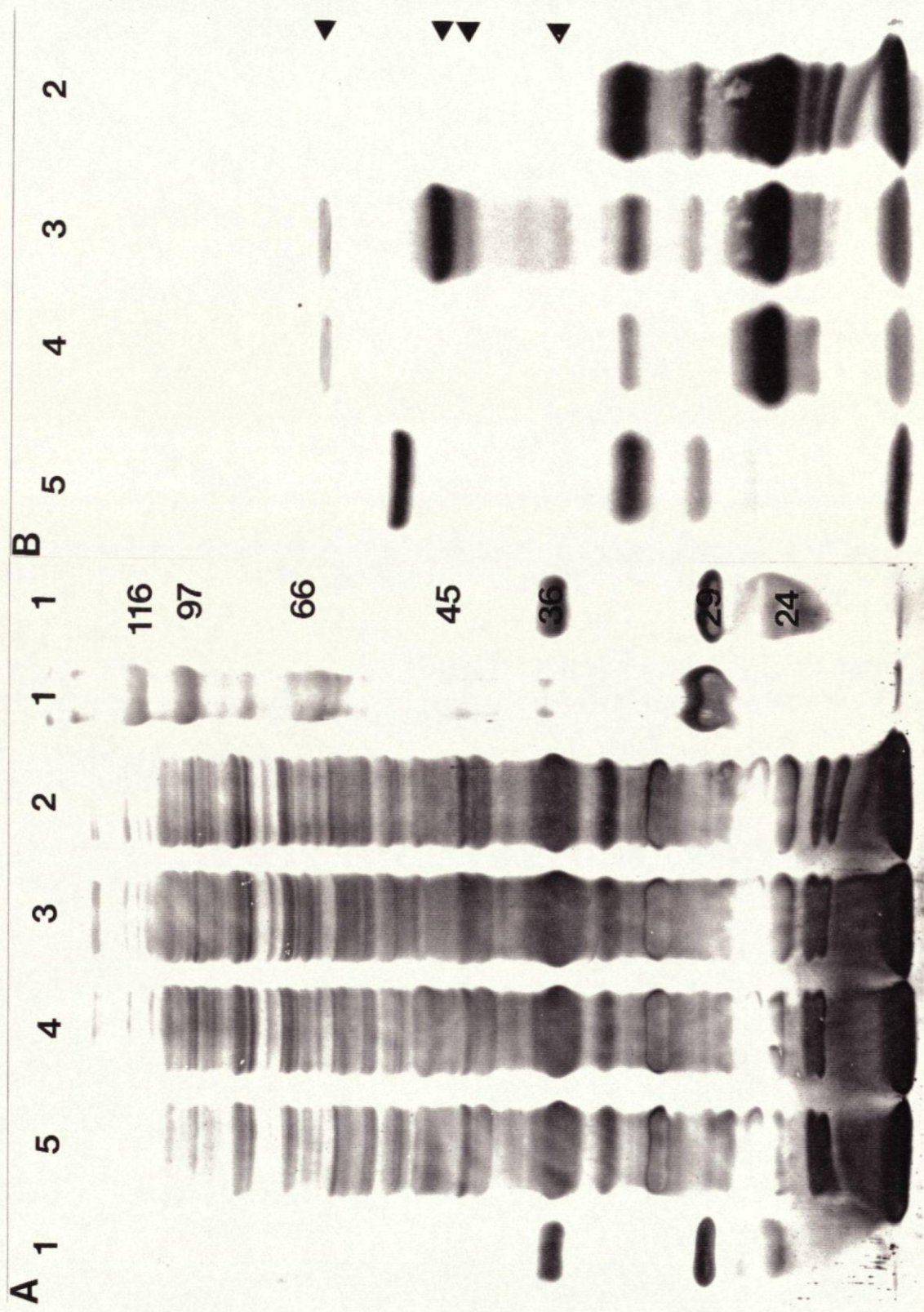
Stable haemolytic clones were also grown overnight in LBB at room temperature and the cells harvested, resuspended in SDS-PAGE buffer and electrophoresed in 11% SDS-PAGE gels. Gels were either stained for protein or transferred to nitrocellulose. Compared to control *E.coli* DH1 containing pBR328 alone, no additional proteins could be detected on SDS-PAGE gels or on Western blots probed with rabbit antisera raised against *R.salmoninarum* whole cells, ECPs or cell walls.

Two further manipulations of pRHLY'B' were performed in order to simplify the identification of the haemolytic protein. pRHLY'B' was cleaved with either *SalI* alone or *SalI/PvuII* and religated. Clones were then screened by multiple minipreps for the presence of pRHLY'B' from which either a 1.5 kbp *SalI* fragment or both a 1.5 kbp *SalI* fragment and a 2 kbp *PvuII* fragment had been deleted (Figure 3.5). This manipulation was performed in order to identify the portion of the 3.1 kbp *HindIII* insert which encoded the observed haemolytic phenotype. Clones containing a *SalI* deletion of pRHLY'B' (designated pRHS) were confirmed by digestion of miniprep DNA with both *HindIII* and *SalI* in order to release a single 2.25 kbp DNA fragment in addition to a 4.25 kbp vector derived DNA fragment (Figure 3.6). Clones containing pRHS were also checked for sensitivity to tetracycline. pRHLY'B' confers ampicillin, chloramphenicol and tetracycline resistance on host cells but the *SalI* deletion involved the removal of almost half of the *ter* gene and the consequent loss of tetracycline resistance. Similarly, clones containing both the *SalI* and *PvuII* deletions of pRHLY'B' (designated pRHPS) were confirmed by cleaving miniprep DNA with both *SalI* and *PvuII* to release two DNA fragments of 1.62 kbp and 2.94 kbp (vector derived, Figure 3.6). In addition, clones containing pRHPS were confirmed for sensitivity to chloramphenicol as the *PvuII* deletion involved the removal of most of the *cam* gene from the vector. *E.coli* DH1 containing pRHS possessed a haemolytic phenotype when streaked on LBA containing ampicillin (100 $\mu\text{g ml}^{-1}$) and chloramphenicol (25 $\mu\text{g ml}^{-1}$), overlaid with horse blood agarose and compared with control *E.coli* DH1 containing pBR328. However, *E.coli* DH1 containing pRHPS was non-haemolytic using the same assay.

Minicell analysis of the products encoded by each of the plasmid derivatives described above was used in order to overcome the difficulties encountered in identifying the haemolytic component. *E.coli* DS410 was transformed with either pBR328, pRHLY'B', pRHS or pRHPS and transformants recovered and the phenotype checked. Miniprep DNA was extracted and the presence of the desired inserts was confirmed. Separate cultures of DS410 containing each plasmid

were grown in BHIB at room temperature, the minicells harvested and purified. Following radiolabelling of the plasmid-encoded proteins, the minicells were harvested and the constituent proteins were separated in a 10% SDS-PAGE gel and then transferred onto nitrocellulose. The nitrocellulose was stained for total protein with colloidal gold (Figure 3.7A). Because minicells contain the full complement of cellular proteins it was difficult to discern any differences between minicells containing pBR328 alone and minicells containing either pRHLY'B', pRHS or pRHPS. However, the autoradiographed blot showed only plasmid-encoded proteins which included those conferring antibiotic resistance (Figure 3.7B). Compared to minicells containing pBR328 alone, minicells containing pRHLY'B' possessed at least four additional proteins of molecular masses 65, 46, 43 and 36. Minicells containing pRHS possessed only the 65K protein proving that the 46K molecule is transcribed from the *SalI* end of the 3.1 kbp *HindIII* DNA insert in pRHLY'B' and that the other protein bands probably represented breakdown products of this molecule. Minicells containing pRHPS possessed a 49K protein. On the basis of these results it was decided that haemolytic activity was conferred by the 65K protein and that the gene encoding this protein was located at the *PvuII* end of the 3.1 kbp *HindIII* DNA insert. pRHPS contained at most 1.2 kbp of insert DNA which would be available for transcription and which would be insufficient for the translation of a 49K truncated protein. The 49K protein encoded by pRHPS probably represented a serendipitous in-frame fusion of the remainder of the gene encoding the 65K protein with the remainder of the *cam*^r gene and under the control of the *cam* promoter. The *PvuII* site of the *cam*^r gene is located 114 bp downstream of the initiation codon. This would be sufficient to code for 38 amino acids of about 4000 molecular weight with the additional 45000 being encoded by insert DNA. This also suggested that the promoter of the gene encoding the 65K protein was located close to the *HindIII* site at the *PvuII* end of the 3.1 kbp DNA insert.

Figure 3.7 Analysis of minicells containing either pBR328, pRHLY'B', pRHS or pRHPS. Approximately 50 μ g of protein was separated by SDS-PAGE using a 10% polyacrylamide gel, transferred onto nitrocellulose and (A) stained for total protein using colloidal gold, then (B) autoradiographed to reveal which polypeptides were labelled with 35 S-cysteine and methionine. Track 1: protein molecular weight standards (kDa); track 2: pBR328; track 3: pRHLY'B'; track 4: pRHS; track 5: pRHPS. The four additional polypeptides encoded by pRHLY'B' are arrowed.



3.3.7 SOUTHERN BLOTTING

Southern hybridisation was carried out by digesting to completion with *Hind*III, chromosomal DNA from a number of strains of *R.salmoninarum* including the type strain (ATCC33209) as well as strains of *A.salmonicida* (CM30) and *Y.ruckeri* (12/6), two major fish pathogens. The plasmid pRHLY'B' was also cut to completion with *Hind*III. The cleaved DNA samples were separated by agarose gel electrophoresis, transferred to nitrocellulose and probed with pRHLY'B' which had been random primed and non-radioactively labelled. A 3.1 kbp fragment corresponding to the 3.1 kbp *Hind*III cloned insert was detected in chromosomal DNA from all strains of *R.salmoninarum* but not *A.salmonicida* or *Y.ruckeri* (Figure 3.8). This not only confirmed the derivation of the cloned fragment but also its presence in all strains examined in this study.

Southern Blot

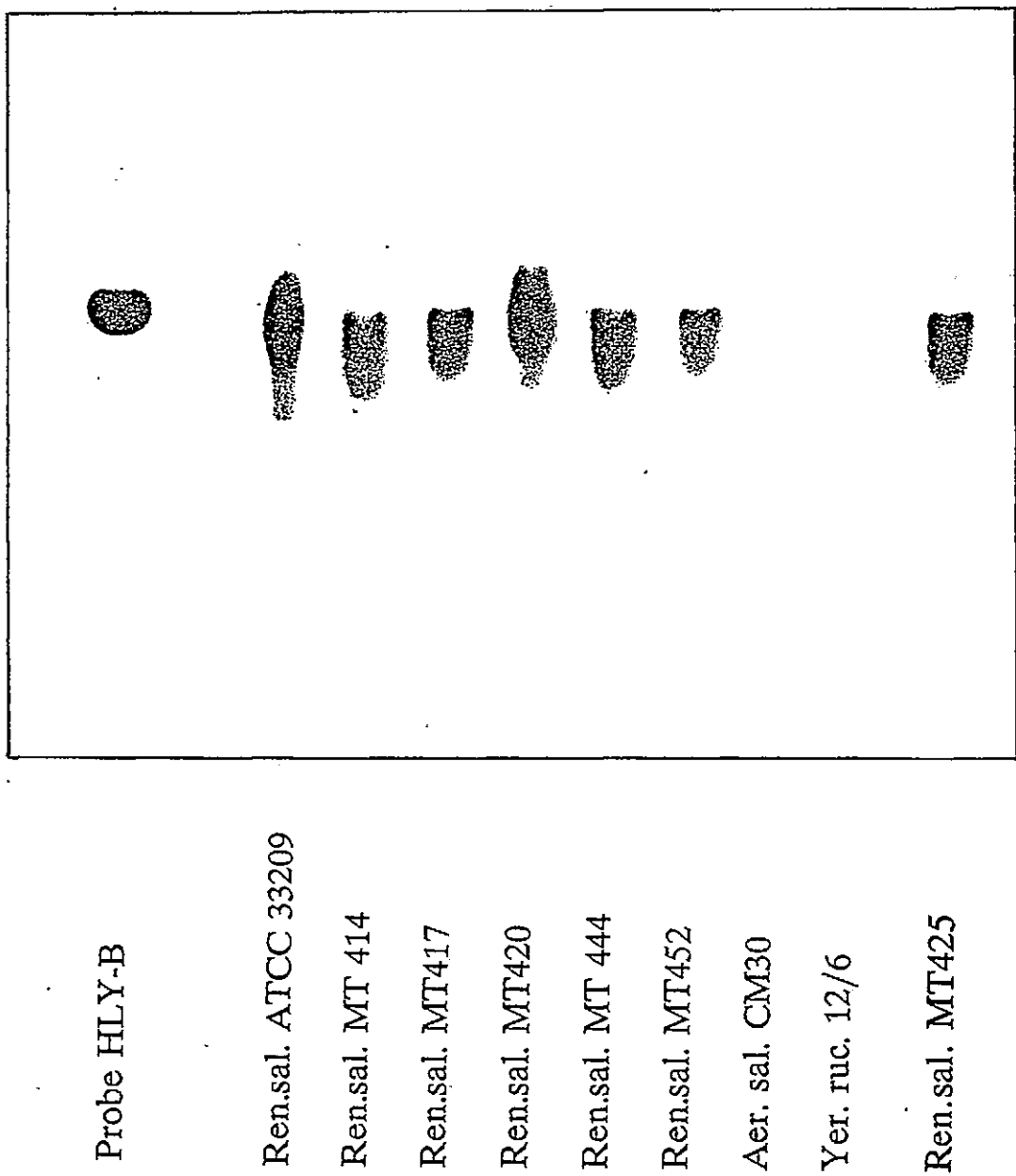


Figure 3.8 The detection of a 3.1 kbp *Hind*III *R.salmoninarum* DNA fragment in various bacterial strains by Southern hybridisation.

3.4 DISCUSSION

This study has identified a 3.1 kbp *Hind*III fragment of *R.salmoninarum* MT444 chromosomal DNA which confers a haemolytic phenotype on *E.coli* cells containing plasmid DNA with the fragment inserted (pRHLY'B'). The location of the promoter region of the gene encoding the membrane-active protein was identified with some certainty as being at one end of the 3.1 kbp *Hind*III fragment (Figure 3.5). The active component, as identified by minicell analysis, was found to have a molecular mass of 65 kDa and was active against erythrocytes from a number of animal species, including rainbow trout but not rabbit, from 6°C to 37°C. These results suggest that this protein possesses the features of a membrane-active toxin. Differences in the susceptibility to lysis of erythrocytes from the species tested may reflect differences in membrane lipid composition. For example, rabbit and sheep erythrocyte membranes contain a lower percentage of phosphatidylcholine and lysophosphatidylcholine than the other species tested (Fehrenbach & Jurgens, 1991; Lee, Raynard & Ellis, 1989). However, no evidence for lecithinase activity was detected in stable clones containing pRHLY'B'. In addition, pRHLY'B' conferred no detectable casein or gelatin degrading activity upon *E.coli* which had been transformed with the recombinant plasmid suggesting that a general proteolytic activity was also not involved in erythrocyte lysis.

Southern hybridisation using pRHLY'B' as a DNA probe identified the 3.1 kbp *Hind*III fragment in chromosomal DNA preparations from all of the strains of *R.salmoninarum* examined, including the type strain (Table 3.2). Despite this, there appeared to be no evidence for the immunological recognition of a 65K protein in cell extracts of stable clones which were probed by Western blotting with specific rabbit antisera raised against preparations derived from *in vitro* cultures of *R.salmoninarum*. The possible reasons for this are not clear. Whether the membrane-active protein is poorly immunogenic, rapidly degraded *in vitro*, expressed at a very low level or not expressed at all *in vitro* remains to be determined. The results of other researchers investigating the immunological and enzymic properties of ECPs or whole cells from *in vitro* cultures of *R.salmoninarum* have failed to identify any haemolytic, cytotoxic or strongly enzymatic components (Bandin, Santos, Bruno, Raynard, Toranzo & Barja, 1991b; Griffiths & Lynch, 1991; Rockey, Turaga, Weins, Cook & Kaattari, 1991b) although the possible contribution of an unidentified toxic component to the pathology of the disease has been suggested (Bruno & Munro, 1986b; Turaga, Weins & Kaattari, 1987a; Kaattari, Holland, Turaga & Weins, 1987). The instability of clones in which the gene is expressed at high levels, such as at 37°C or when inserted into the pUC18 vector, and the difficulty in maintaining cultures of

E. coli which contained pRHLY'B', either as glycerols at -20°C or on LBA plates at 4°C, provide an indication of the toxicity of the protein, at least to *E. coli* cells. It may be that in order to avoid inflicting self-injury the toxin may only be expressed by *R. salmoninarum* under certain conditions.

Evenden, Gilpin & Munn (1990) first reported the cloning of a haemolytic component from *R. salmoninarum*. The activity was encoded by a 1.6 kbp *SalI* fragment which had been initially isolated from a cosmid gene bank and then subcloned into pBR328 (designated pRHLY11). This haemolysin was also active against erythrocytes from a wide range of animal species, including both fish and rabbit, from 4°C to 37°C. Comparison of the restriction maps of pRHLY'B' and pRHLY11 reveals considerable differences between the inserted DNA fragments and Southern blotting by Evenden (1993) showed no evidence for DNA homology between the two fragments. It is interesting to note that Bruno & Munro (1986a) provide the only report of β haemolytic activity from *R. salmoninarum* against rabbit erythrocytes. It is therefore possible that this observation and the activity encoded by pRHLY11 (Evenden *et al.*, 1990) are the result of the same haemolysin. The membrane-active protein encoded by pRHLY'B' on the other hand, is not active against rabbit erythrocytes, does not degrade casein or gelatin and may therefore represent a previously uncharacterised component of *R. salmoninarum*. The cloning of a second membrane-active cytolysin from *R. salmoninarum* suggests that these toxins may have an as yet undefined role in the entry and survival of the bacterium intracellularly. It is likely that if the toxins are expressed *in vivo* they would make an important contribution to the pathology and pathogenesis of BKD. Damage to the erythrocyte and lymphocyte membranes of fish infected with *R. salmoninarum* has been proposed as the cause of much of the pathology of BKD (Bruno & Munro, 1986b) and both of these membrane damaging proteins are therefore worthy of further investigation as potential targets of the salmonid immune response. The importance of membrane-active cytolysins to the penetration of host cells, intracellular survival and multiplication, and contribution to both the pathology and pathogenesis of infection has been well documented for pathogens such as *Shigella flexneri* (Sansonetti, Ryter, Clerc, Maurelli & Mounier, 1986; Sansonetti, 1992), *Rickettsia rickettsii* (Silverman, Santucci, Meyers & Sekeyova, 1992), *Plasmodium falciparum* (Braun-Breton, Rosenberry & da Silva, 1988), *E. coli*, *Bordetella pertussis*, *Staphylococcus aureus*, *Legionella pneumophila* and *Listeria monocytogenes* (reviewed by Foster, 1992).

The term 'haemolysin' has been used by many authors to describe a membrane-active phenotype

which may be the result of proteolytic, phospholipolytic or toxic component(s) (Fehrenbach & Jurgens, 1991). It is also possible to distinguish between those single molecules capable of haemolysis, such as listeriolysin O (Geoffroy, Gaillard, Alouf & Berche, 1987) and *L.pneumophila* exoprotease (Keen & Hoffman, 1989), and bacterial enzymes or metabolites which may interact in a cooperative lytic process. A classic example of cooperative membrane-active processes is the interaction between *Staphylococcus aureus* sphingomyelinase and 'CAMP-factor' of group B streptococci (reviewed in Fehrenbach & Jurgens, 1991). It is unclear from the results obtained here as to whether the 65K protein identified is acting either as a true haemolysin alone or in concert with a component produced by *E.coli* K12, such as phospholipase A2, to lyse erythrocytes. In the case of the fish pathogen, *Aeromonas salmonicida*, the complete haemolysis of trout erythrocytes by the major lethal exotoxin glycerophospholipid:cholesterol acyltransferase (GCAT) was found to require the presence of an extracellular protease (Titball & Munn, 1985; Lee & Ellis, 1990). The GCAT toxin was also found to possess extremely high haemolytic activity for fish, but not rabbit, erythrocytes and it was suggested that this was a reflection of the substrate specificity of the enzyme (Buckley, 1982; Lee & Ellis, 1990).

The isolation of a strongly immunoreactive clone from a pUC18 *Eco*R1 gene bank underlines the success of a molecular approach to the study of *R.salmoninarum*. The failure to detect recombinants in bacteriophage λ gene banks has been reported by other researchers (Chien, Gilbert, Huang, Landolt, O'Hara & Winton, 1992). The antigenic profile of RSIP1 on Western blots looked very similar to that of P57, the major cell surface antigen of *R.salmoninarum*. Further characterisation including restriction mapping and DNA sequencing by Dr M.L.Gilpin confirmed that pMLG57 contained a 1.9 kbp *Eco*R1 fragment fused in-frame with and under the control of the *lac* promoter. This *Eco*R1 fragment of *R.salmoninarum* DNA contained all but the first 90 bases (30 amino acids) of the recently sequenced *msa* gene which encodes P57 (Chien *et al.*, 1992). P57 has been well characterised by previous workers and Chapter 2 provides a review of the nature of the protein and its role in BKD.

CHAPTER 4.

DNA SEQUENCING AND SEQUENCE ANALYSIS OF A GENE ENCODING A HAEMOLYTIC PRODUCT FROM *R.salmoninarum*

4.1 INTRODUCTION

Given the difficulties encountered in identifying a previously uncharacterised membrane-active protein cloned from *R.salmoninarum* (Chapter 3), which was not readily detectable on SDS-PAGE gels or Western blots, further characterisation of the active component was carried out by DNA sequencing and sequence analysis. Throughout the course of the last 20 years considerable improvements have been made in the molecular techniques used to determine the nucleotide sequence of DNA. DNA sequencing is now of fundamental importance to the analysis of gene structure, function and evolutionary history. The further manipulation of the DNA can also be greatly simplified. For example, computer analysis can provide a complete and precise restriction map which can greatly facilitate the production of fusion proteins (O'Hare, Clarke & Cawston, 1992).

Structural analysis of DNA sequences is invariably accomplished by computer in order to allow a large number of functions to be carried out with maximum speed. There are vast data bases of sequence information available for comparison, such as GenBank and EMBL. Most programs will calculate the base composition and identify the location and number of restriction sites for known enzymes thus providing confirmation of the sequence predictions by comparison with endonuclease cleavage patterns. Information relating to the structure and control of a gene may also be available. For example, the presence of direct repeats and inverted repeats of base sequences which may influence the secondary structure of the DNA and also the transcription and secondary structure of the mRNA (Singer & Berg, 1991); the presence of consensus regions which regulate transcription (Latchman, 1991) and the nature of the promoter, ribosome binding site, start and stop codons, termination loops and whether the sequence is polycistronic or monocistronic (Singer & Berg, 1991). Some programs are designed to predict the stable secondary structure of the DNA or RNA. Complete reviews of the methods available for DNA sequence analysis are provided by Doolittle (1990) and Langone (1991a; 1991b).

The major advantage of knowing a DNA sequence is that triplets of bases (codons) may be translated into amino acids and then compared with data banks of other protein sequences. This

comparison with better characterised proteins allows the determination of the predicted primary structure as well as yielding clues to the function of the gene product. Amino acid sequence identity searches may help identify groups of structurally and functionally similar proteins, such as the family of sulphhydryl-activated Gram positive cytolytic toxins (Alouf & Geoffroy, 1991). It may also be possible to identify functional domains on the basis of consensus sequences, internal repeated units, amino acid composition or hydropathy (Doolittle, 1986).

DNA sequencing techniques fall into two main categories: (i) the chemical degradation method of Maxam & Gilbert (1977) and (ii) the enzymatic chain-termination method of Sanger, Nicklen & Coulson (1977). Both methods rely on generating separate populations of radiolabelled oligonucleotides that represent a variety of chain lengths which have been randomly terminated at a specific residue. These populations of oligonucleotides can be separated according to chain length by electrophoresis through polyacrylamide gels under denaturing conditions which allows discrimination between DNA chains that differ by only one nucleotide. By comparing the autoradiographed images of gels containing adjacent lanes of populations of labelled oligonucleotides which have been terminated specifically at either A, T, C or G residues, the order of nucleotides can be directly determined.

The most popular and convenient method of DNA sequencing is that of Sanger *et al.* (1977) and a number of commercial suppliers provide kits, such as Sequenase® Version 2.0 (United States Biochemical Corporation), which are based on this method for the sequencing of either single or double stranded DNA. The method of Sanger *et al.* (1977) involves the *in vitro* synthesis of a DNA strand from a DNA template by DNA polymerase. This requires the use of a specific oligonucleotide primer which is annealed to the template DNA to allow for the initiation of extension by DNA polymerase. Base specific chain termination is achieved by the incorporation of 2',3'-dideoxynucleoside 5'-triphosphates (ddNTPs) which lack the 3'-OH group present in dNTPs and which are necessary for the formation of phosphodiester bonds and thus DNA chain elongation. When a small amount of one of the four ddNTPs is included with the four conventional dNTPs, enzymatic DNA synthesis will be terminated infrequently but specifically wherever the ddNTP has been incorporated. The products of the reaction are a series of oligonucleotide chains of varying lengths. By using the four different ddNTPs in four separate reactions and including a radiolabelled nucleotide, such as [α -³⁵S]-dATP, populations of labelled oligonucleotides are generated that terminate at positions occupied by every A, C, T or G in the template DNA. The labelled chains can be visualised by autoradiography after separation by gel electrophoresis. These

techniques are routinely utilised and are well documented in the literature (Sambrook, Fritsch & Maniatis, 1989; Singer & Berg, 1991).

The purpose of this study was to further characterise, through the use of DNA sequencing, the gene encoding a haemolytic product cloned from *R.salmoninarum* in the plasmid vector pBR328 (pRHLY'B', Chapter 3). Because good resolution of normal enzymatic sequencing reactions is limited to about 400-800 bases it was necessary to subclone specifically cleaved fragments of pRHLY'B' into the plasmid vector pUC18. pUC18 was chosen because as a high copy number vector a good yield of DNA for sequencing could be expected and, additionally, several companies market oligonucleotide primers which anneal to the sequence flanking either side of the pUC18 multiple cloning site (MCS). These primers allow synthesis on the double-stranded template DNA inserted into the MCS in both 'forward' and 'reverse' directions. Thus the DNA sequence can be read in both directions as a double check. Following the completion of sequencing, the DNA sequence was analysed, codons were translated into amino acids and the protein sequence was structurally and functionally characterised and compared to similar proteins from a variety of other species.

4.2 MATERIALS AND METHODS

Many of the materials and methods used in this study have been detailed in Chapter 3. Only those which are specific to the work described here are presented below.

4.2.1 BACTERIAL STRAINS, PLASMIDS AND MEDIA

E.coli XL1-BLUE was used as host and was grown either in LBB or on LBA at 37°C. When appropriate, 100 µg ml⁻¹ of ampicillin and 15 µg ml⁻¹ of tetracycline were added to the medium. Plasmid pUC18 was used as the vector for subcloning and nucleotide sequencing.

4.2.2 DNA MANIPULATION AND SUBCLONING

Fragments of pRHLY'B' were subcloned in pUC18 based on the knowledge that the gene to be sequenced was located at the *Pvu*II end of the DNA insert. From the restriction map of pRHLY'B' (Figure 3.5) it was decided to subclone the following fragments for sequencing:

(i) *Hind*III-*Pvu*II ≈ 620 bp, (ii) *Pvu*II-*Pst*I ≈ 730 bp and (iii) *Pst*I-*Pst*I ≈ 520 bp.

About 1 µg of pUC18 DNA was digested in separate reactions with either *Pst*I, *Hind*III/*Sma*I or *Pst*II/*Sma*I. Digests were checked by agarose gel electrophoresis, reactions stopped by either

heating at 65°C for 20 min (*Sma*I) or phenol:chloroform extraction and the DNA was precipitated, washed and stored at 4°C in TE buffer. About 10 µg pRHLY'B' was digested in separate reactions with either *Pst*I, *Hind*III/*Pvu*II or *Pst*I/*Pvu*II. Digests were checked by agarose gel electrophoresis and then the DNA fragments present in the entire reaction mixture were separated according to size by agarose gel electrophoresis. The gel was examined under UV light, the desired fragment of pRHLY'B' was located and excised, placed into sterile dialysis tubing (Medicell) with a minimum volume of TBE buffer and the ends of the tube were knotted. The DNA fragment was eluted electrophoretically from the agarose gel slice (100 v, 1 h) by aligning the dialysis tubing containing the gel slice crossways in a horizontal gel electrophoresis tank (G100, Pharmacia) filled with TBE buffer. After 1 h the current was reversed for 1 min to remove DNA from the sides of the dialysis tubing. Following elution, the buffer and DNA were removed from the dialysis tube, the gel slice was examined under UV light to confirm DNA transfer and the dialysis tube was washed out with fresh TE buffer. The solution containing the DNA fragment was centrifuged (9000 x g, 5 min) to remove agarose particles, the supernatant was placed in a fresh MCC tube and the DNA was precipitated with ethanol and then phenol:chloroform extracted. The purified DNA fragment was precipitated, washed and stored at 4°C in TE buffer.

Each fragment was ligated with the appropriately cleaved pUC18 vector DNA and then transformed into competent *E.coli* XL1-BLUE. Transformants were recovered on LBA plates supplemented with tetracycline (15 µg ml⁻¹) and ampicillin (100 µg ml⁻¹) and containing a 5 ml overlay of LBA with IPTG (192 µg ml⁻¹) and X-gal (160 µg ml⁻¹). White colonies, indicative of recombinant subclones, were selected for miniprep plasmid extraction. Plasmid DNA from recombinant subclones was digested with restriction enzymes and subjected to agarose gel electrophoresis in order to confirm the presence of the single desired insert without the presence of any undesired DNA fragments. Large scale extraction of plasmid DNA for DNA sequencing was carried out as described in section 3.2.4.2.

4.2.3 DNA SEQUENCING

DNA was sequenced by a modification of the dideoxy-chain termination method of Sanger *et al.* (1977) by using the Sequenase® Version 2.0 Kit (United States Biochemical Corporation) following the manufacturer's instructions. Double-stranded DNA templates were denatured in 0.2 M NaOH, 0.2 mM EDTA for 30 min at 37°C, the mixture was neutralised by adding 0.1 volumes of 3 M sodium acetate (pH 4.5-5.5) and the DNA was precipitated with 3 volumes of cold ethanol at -70°C for 15 min. After pelleting (9000 x g, 10 min), the DNA was washed in 70% ethanol and

either 'forward' or 'reverse' primers (Promega) were added and annealed to the template by heating to 65°C for 2 min, cooling slowly to <35°C and then chilling on ice. To a 10 µl volume of the annealed template-primer was added 1 µl of 0.1 M dithiothreitol (DTT), 2 µl of dNTP labelling mixture, 0.5 µl of [α -³⁵S]-dATP (> 1000 Ci mM⁻¹; Amersham) and 2 µl of Sequenase® Version 2.0 T7 DNA polymerase. The reactants were thoroughly mixed, avoiding air bubbles, and incubated at room temperature for 2-5 min. The dNTP labelling mixture consisted of dNTPs, namely dGTP, dCTP and dTTP. Occasionally, in order to resolve compressions in the DNA sequence, dITP, a nucleotide analog for dGTP, was used. The labelling reaction was chain-terminated by adding 3.5 µl of labelling reaction mixture to 2.5 µl of each ddNTP and incubating for 3-5 min at 37°C. Reactions were stopped by the addition of 4 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Samples were heated to 75°C for 2 min immediately prior to loading 3 µl samples onto a denaturing polyacrylamide gel.

4.2.4 DENATURING GEL ELECTROPHORESIS

Samples of radioactively labelled randomly terminated oligonucleotide mixtures were separated according to size by denaturing gel electrophoresis using a LKB 2010 MacroPhor Sequencing System (Pharmacia) according to the manufacturer's instructions. All chemicals and reagents were of either analytical or molecular grade. Polyacrylamide gels of either 4% or 6% acrylamide and 0.2 mm in thickness were prepared as follows. Acrylamide:bisacrylamide solution (19:1, Northumbria Biologicals Ltd.) was diluted to the appropriate volume with 10 x strength TBE buffer and Analar water. Urea (42% w/v) was dissolved in the mixture and the solution filtered (0.45 µm Minisart). The gel solution was degassed prior to the addition of TEMED and 10% ammonium persulphate. Gels were poured onto the thermostatic plate which had been pretreated with Repelcote (BDH) to permit the removal of the gel following electrophoresis. A notched glass plate which had been pretreated with γ -methacryloxypropyltrimethoxysilane to bind to the gel was slid into place such that the polymerised gel was sandwiched between the top plate and the thermostatic plate. Following polymerisation the gels were mounted in the MacroPhor apparatus, TBE buffer was added and the sample well at the top of the gel was thoroughly cleaned. The thermostatic plate was connected to a circulating water supply (50°C) and the gel was pre-electrophoresed for 1 h at 1500 v to remove any contaminants and allow the gel to reach the operating temperature of 50°C. This temperature helps to keep DNA samples denatured during electrophoresis. Samples (3 µl) were loaded into wells formed by a shark's-toothed comb using an adjustable sequencing pipette (Drummond Model 203). The samples were run into the gel at

1500 v for 5 min, then the comb was removed and the run was continued at 2500 v. The duration of the runs varied from 1.5-6 h according to the desired length of labelled fragments which were to be read. A series of gels were run for each DNA fragment inserted into pUC18 in order to ensure the complete 'forward' and 'reverse' sequencing of the DNA. Following electrophoresis, the top plate with the gel attached was detached from the thermostatic plate and washed for 15 min in 2.5 litre of 30% methanol, 10% v/v acetic acid to remove urea. The gel was then carefully dried in a fume cabinet with a hand-held hot air dryer. The dry gel was overlaid with a piece of Kodak X-OMAT film and another glass top plate was clamped in place to firmly hold the film in contact with the gel. Autoradiographs were exposed for 48 h at room temperature prior to development and reading of the sequence.

4.2.5 DNA SEQUENCE ANALYSIS

Initially, the DNA sequence was analysed by desktop computer using the Recombinant Toolkit Version 4.0 software for the analysis of DNA and amino acid sequences (Biosoft, Cambridge, U.K.). This program produced a complete restriction map of the sequenced fragment and translated the codons into amino acids in each of the six possible reading frames, three in the forward orientation and three in the reverse or complementary orientation. The reading frame of the gene was identified and codon usage, nucleotide composition and amino acid composition were calculated. Further DNA sequence analysis was carried out at the Chemical Defence Establishment, Porton Down, Wiltshire with the assistance of Dr Keith Martin and involved the use of the DNASTAR software package (DNASTAR Incorporated, Madison, Wisconsin, U.S.A.). The DNA sequence was analysed for the presence of start and stop codons, promoter region, secondary structure and the proposed strength of expression. The prediction of gene expression relies upon the strong correlation between the frequency of a codon and the abundance of the corresponding tRNA which has been reported in *E.coli* and *Salmonella* (Gribskov, Devereux & Burgess, 1984). Hence, genes that are highly expressed, such as ribosomal proteins, use codons corresponding to the most abundant tRNAs almost exclusively whilst genes expressed at a lower level show a reduced codon preference. The promoter region was further analysed by comparison with 100,000 DNA sequences contained in the Genetic Sequence Databank (GenBank) collected at Los Alamos National Laboratory.

4.2.6 PROTEIN SEQUENCE ANALYSIS

Protein sequence analysis was carried out at the Chemical Defence Establishment, Porton Down, Wiltshire with the assistance of Dr Keith Martin using the DNASTAR commercial software

package.

4.2.6.1 Sequence comparison

The translated protein was compared to 20,636 protein sequences contained in the Protein Identification Resource (PIR) data library collected at the National Biomedical Research Foundation, Washington, U.S.A. The best 200 scores of identity were obtained and the top 30 of these, as well as a variety of other selections, were compared by pairwise and multiple amino acid sequence alignments with the translated *R.salmoninarum* protein sequence. The percentage identity of matches was scored using a gap penalty of 3, deletion penalty of 6 and a standard probability of acceptable mutation (PAM).

4.2.6.2 Prediction of secondary structure

Elements of the secondary structure of proteins arise from the steric constraints imposed by the peptide bond and hydrogen bonding between residues. As a result, protein chains adopt two regular, compact structures which are termed α -helix or β -sheet. In addition, irregular motifs which expose the side-chain and backbone atoms to solvent are formed and are termed turns and loops or coils. The knowledge of the secondary structure of a protein is useful for predicting processing sites, such as the signal peptide cleavage point, and antigenicity. The prediction of α -helix, β -sheet, turn and coil regions was calculated by DNASTAR according to the methods of either Chou & Fasman (1974) or Garnier-Robson (Garnier, Osguthorpe & Robson, 1978) which are the two main algorithms for predicting secondary structure. Both of these methods represent a statistical approach to the correlation between amino acid composition and the secondary structure of proteins. The Chou-Fasman method estimates the propensity of each residue to adopt α -helix, β -sheet and turn conformation based on the observed versus the expected (chance) frequencies of a given residue type in a particular secondary structure. The Garnier-Robson method has a more sophisticated basis than that of Chou-Fasman and relies upon quantifying the contribution each central residue type in a 17 residue region makes towards the probability of α -helix, β -sheet, turn and coil.

4.2.6.3 Hydropathy

The hydropathy of amino acid side chains is one of the dominant factors influencing protein structure. Hydropathy is a measure of polarity and the underlying assumption is that when proteins fold under stable conditions the maximum number of polar, hydrophilic groups will be exposed on the surface of the protein whilst the maximum number of nonpolar hydrophobic groups will

be folded away from the surface of the molecule. Hydrophilic regions of charged, surface exposed residues are likely to be antigenic (Hopp & Woods, 1981). The hydrophilicity method of Kyte & Doolittle (1982) was used to predict the location of residues on the outside of the protein.

The correlation of amphipathy with secondary structure was performed according to the methods of Eisenberg, Weiss & Terwilliger (1984) and Eisenberg, Schwarz, Komaromy & Wall (1984). The methods rely on calculating a hydrophobic moment for α -helix and β -sheet regions which allows the prediction of structures which have a hydrophilic side, which is exposed to solvent, and a hydrophobic side, which is packed against the protein core. External α -helices are associated with maxima in the amphipathic moment plot.

4.2.6.4 Flexibility and accessibility

The neighbour-correlated chain flexibility was calculated as a weighted average over 7 residues according to the algorithm of Karplus & Schulz (1985). This method predicts the flexibility of protein segments on the basis of the atomic mobility of each amino acid residue of 31 well characterised proteins. Structural flexibility may influence the antigenicity and specificity profile of an enzyme (Pellequer, Westhof & van Regenmortel, 1991; Bone & Agard, 1991).

The probability that any group of residues will be exposed on the surface of a protein is termed accessibility. Accessibility scales have been constructed by measuring the accessible surface of all the residues in a number of well characterised proteins (Janin, Wodak, Levitt & Maigret, 1978). The surface probability profile is calculated for groups of seven residues using the formula of Emini, Hughes, Perlow & Boger (1985) and the surface probability values of Janin *et al.* (1978).

4.2.6.5 Antigenic index

In order to represent the antigenic aspect of protein architecture, Jameson & Wolf (1988) combined the parameters defining hydrophilicity, accessibility, flexibility and secondary structure into the 'antigenic index'. The five scales used for computing the index were weighted so that 40% of the antigenic index was derived from the secondary structure components of Chou-Fasman and Garnier-Robson, 30% was derived from the Kyte-Doolittle hydropathy plot, and the Karplus-Schulz flexibility and Emini accessibility values contributed 15% each.

4.3 RESULTS

4.3.1 SUBCLONING OF pRHLY'B'

Plasmid pRHLY'B' includes a 3.1 kbp *R.salmoninarum* MT444 chromosomal DNA insert which contains the structural gene that encodes a haemolytic product. This 3.1 kbp fragment was characterised by restriction enzyme analysis (Figure 3.5) and subcloned into the plasmid vector pUC18 as a series of three contiguous fragments. The sequencing templates, which were designated pURS1, pURS2 and pURS3 and constituted 1.87 kbp of pRHLY'B' insert DNA, are described in Figures 4.1 and 4.2. Haemolytic activity was not detected in *E.coli* XL1-BLUE transformants containing any one of these fragments inserted in pUC18.

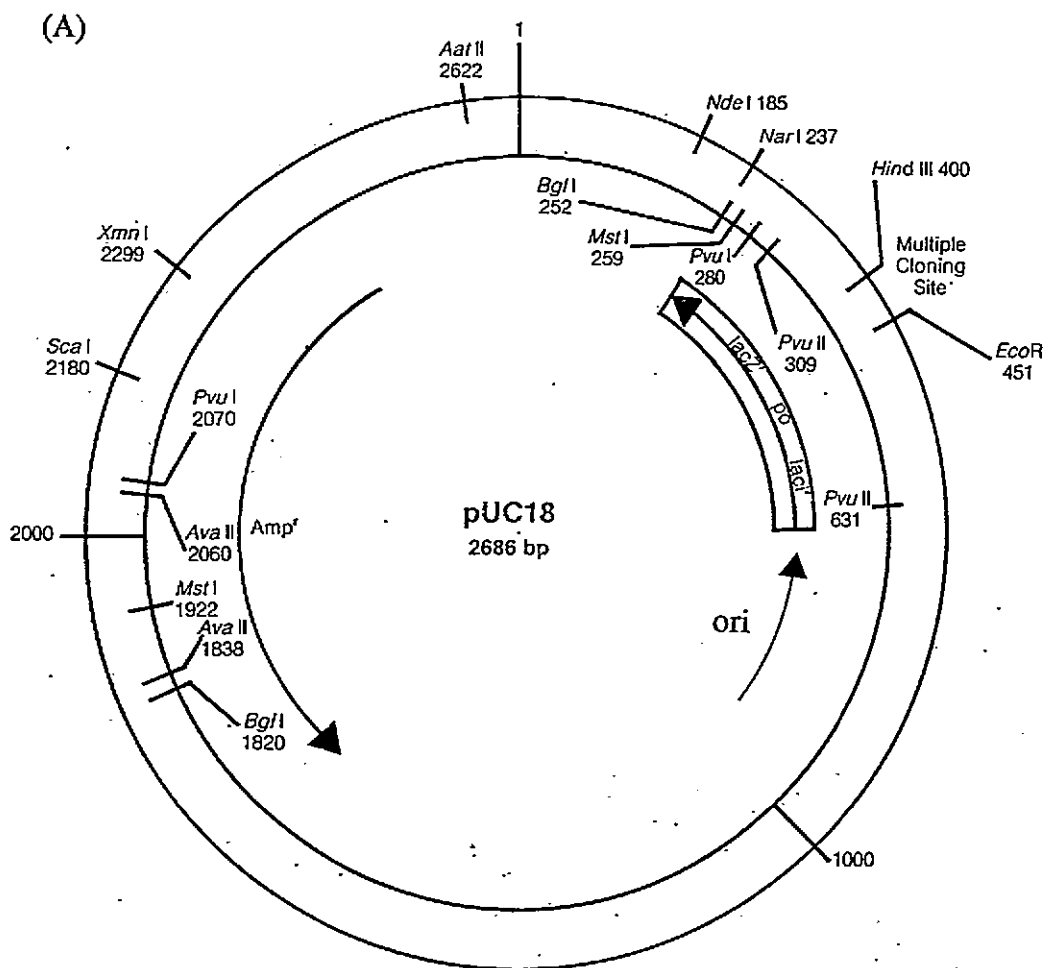
4.3.2 NUCLEOTIDE SEQUENCE OF THE 1.9 kbp *R.salmoninarum* MT444 DNA FRAGMENT

A complete restriction map of the DNA sequence (Appendix 1) showed that the predicted restriction sites matched the observed endonuclease cleavage pattern. The correct orientation of the contiguous DNA fragments which formed the sequencing templates was confirmed by digesting pRHLY'B' with *HincII* to release a 980 bp DNA fragment. The strength of expression in each of the three possible reading frames was calculated by comparison with the codon bias of highly expressed sequences from *E.coli* and *Salmonella* sp. (Gribskov *et al.*, 1984). Using this approach the nucleotide sequence (Figure 4.3) was found to contain an open reading frame (ORF) of 1647 base pairs starting at nucleotide 132 and ending at nucleotide 1778. Following translation, this open reading frame was found to code for a protein of 548 amino acid residues with a calculated molecular weight of 66,757 and pI of 5.57 (Figure 4.4). The predicted molecular weight differed by about 1,700 from the molecular weight of the gene product estimated by minicell analysis (section 3.3.6). The methionine encountered at base pair 132 was identified as the translation initiation codon and this was preceded by a putative ribosome-binding site (AGGAA) located seven nucleotides upstream from this ATG site. The putative -10 and -35 promoter regions are marked (Figure 4.3). There is a second predicted promoter sequence located downstream. The -35, -10 and ribosome-binding regions are also marked and show good agreement with *E.coli* promoter consensus sequences. However, the nearest ATG is located at nucleotide 324 which is 44 base pairs downstream from the ribosome-binding site (AAGGAAA). There is a possibility that an initiator other than ATG (Met-65), such as GTA (Val-51) or ATC (Iso-54), may be utilised. All of these codons are in-frame with the first initiation ATG (Met-1). Confirmation of the computer predictions of promoter region, transcriptional start site and initiation codon awaits further experimental evidence. There were four sequences of imperfect dyad symmetry which were found

to extend from nucleotides 10-80, 47-155, 189-298 and 1793-1830. The latter sequence, which is underlined in Figure 4.3, resembles a rho-independent termination sequence being rich in C and G and possessing seven T bases at one end (Platt, 1986). The presence of these stem and loop structures suggests that the mRNA transcribed by this open reading frame may not be part of a polycistronic message. In addition, the second and third of these structures fall between the two putative promoter regions and may therefore have some role in the regulation of transcription or possibly translation (Matthews, 1992). Alternatively, there may be a transcriptional regulatory element located between the two promoter regions. In an attempt to resolve this question the promoter region was compared with 100,000 entries in the GenBank data base. A search for transcription factors including heat-shock promoter elements, steroid hormones and metal-dependent inducible enhancers, which have been described by Serfling, Lubbe, Dorsch-Hasler & Schaffner (1985), Stuart, Searle & Palmiter (1985), Maniatis, Goodbourn & Fischer (1987) and Latchman (1991), failed to identify any DNA sequences within the region surrounding the two putative promoters that exactly matched the consensus sequences associated with inducible gene expression in eucaryotes. However, two short stretches of nucleotides, TCGCGGGCGCC (nucleotides 196-205) and TCCGCGCA (nucleotides 267-274), did bear some resemblance to the metal-regulatory elements TGCGCCCGCC (8 out of 11 bp matches) identified by Stuart *et al.* (1985) in eucaryotic metallothionein gene promoters and to the Sp1 binding site consensus sequence (C/T)CCGCCC(C/A) (7 out of 8 bp matches) (Stuart *et al.*, 1985; Latchman, 1991).

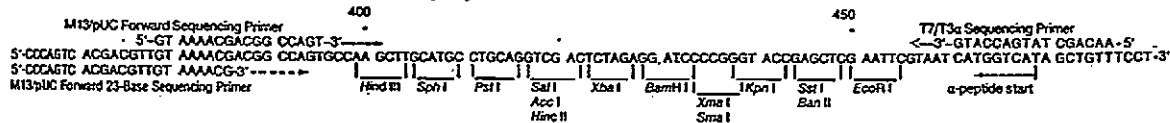
Analysis of the DNA base composition showed that the individual base content was as follows: A, 24.1%; C, 28.2%; G, 27.3% and T, 20.4%. As presented in Table 4.1, codons with a C or G at position 3 were preferentially used. The overall G+C content was 55.5%, and the G+C contents of positions 1, 2 and 3 were 53.3%, 48.0% and 64.6%, respectively.

Figure 4.1 (A) Restriction map of pUC18 DNA; *amp^r*= gene encoding ampicillin resistance; *ori*= origin of replication. (B) pUC18 multiple cloning site (MCS) with sequencing primer binding regions. (C) Sequencing strategy of the portion of *R.salmoninarum* DNA known to possess the gene encoding a haemolytic phenotype. Three contiguous fragments were subcloned separately into pUC18 to form sequencing substrates pURS1, pURS2 and pURS3. pURS1 contained a 620 bp *Hind*III-*Pvu*II fragment (1) inserted into the *Hind*III and *Sma*I sites of pUC18 MCS. pURS2 contained a 730 bp *Pvu*II-*Pst*I fragment (2) inserted into the *Sma*I and *Pst*I sites of pUC18 MCS. pURS3 contained a 520 bp *Pst*I-*Pst*I fragment (3) inserted into the *Pst*I site of pUC18 MCS.

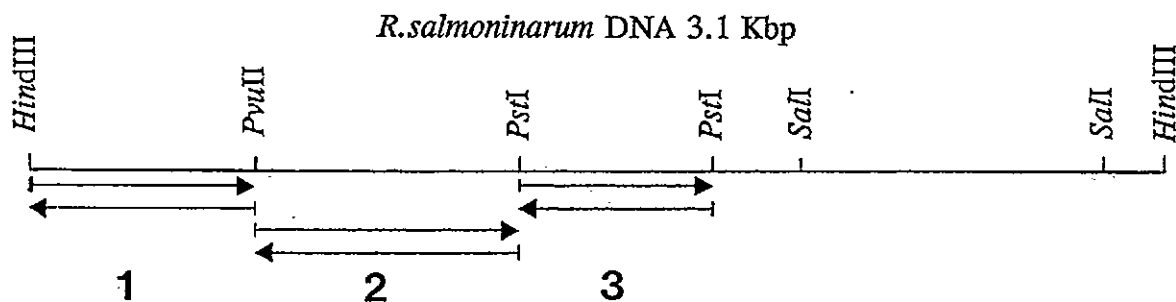


(B)

pUC18 multiple cloning site and primer binding regions: 364-480



(C)



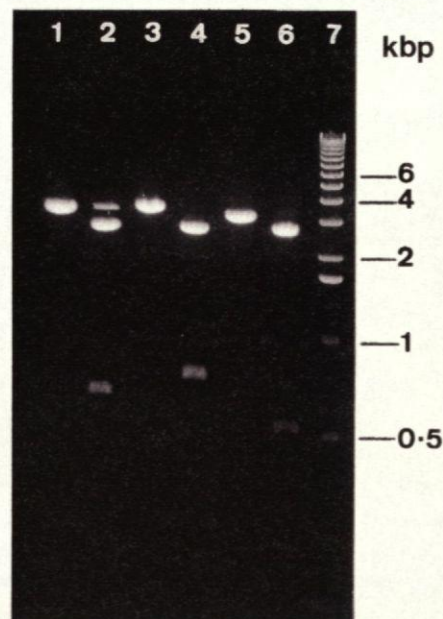


Figure 4.2 Identification of plasmid sequencing substrates by agarose gel electrophoresis. The molecular weights of DNA fragments were estimated by comparison with 1 kbp DNA ladders (track 7). Track 1: pURS1 plasmid DNA cut with *Hind*III produced a linear plasmid of 3.4 kbp. Track 2: pURS1 cut with *Hind*III and *Eco*RI (partial digest) released a 635 kbp fragment in addition to the vector. Track 3: pURS2 cut with *Eco*RI revealed a linear DNA band of 3.4 kbp. Track 4: pURS2 cut with *Eco*RI and *Pst*I released a 745 bp fragment in addition to vector DNA. Track 5: pURS3 cut with *Hind*III to linearise a 3.2 kbp plasmid. Track 6: pURS3 cut with *Pst*I released a 520 bp fragment in addition to vector DNA.

Figure 4.3 Nucleotide sequence of a 1870 base pairs fragment of *R.salmoninarum* MT444 chromosomal DNA. The putative -35 region, -10 region, transcriptional start site, ribosome-binding site (rbs) and initiation codon (Met) are marked for each of the two possible promoters. In addition, the putative stop codon (nucleotide 1778) and transcription termination sequence (nucleotides 1793-1830) are underlined.

10	20	30	40	50	60	70
AAGCTTCAAA	CATAGATTTT	TCACAGTCTT	TGAAAACCGC	AATAGGAATA	TTGCGGTTTT	AATTATGTGG
80	90	100	110	120	130	140
AAATCCTGTT	⁻³⁵ CAGTAATCAA	AGATGTTTAC	⁻¹⁰ TGATAGCTTT	⁺¹ CTCTAACTTA	rbs GGAATAGGTT	Met TATGAAAAAG
150	160	170	180	190	200	210
TACTACGCCG	TAACTGGCAT	AGCTTTGGCT	GTTGGCATGC	TGTGTACAAC	GCAGCTCGCG	GGCGCCACTC
220	230	240	250	260	270	280
AAGCCGCTGA	CCCCTCGGTG	GGCAGCCTTG	⁻³⁵ ACTCGTCTAA	⁻¹⁰ CGTCGTTACC	⁺¹ GAGTTCCTCG	rbs CGCAAGGAAA
290	300	310	320	330	340	350
TGTAGAGCAG	ATCACTTTCA	AATCTGCCAT	CAAGTCTGCG	Met CCGATGAGTT	CGGCACGATC	GGCTCAGACT
360	370	380	390	400	410	420
AGCGCCATAA	TCCCCGGCCT	TAAGAACTTG	TTTGTGAGCG	CGCCAGGTAG	CGATTCAGC	CTGAATGATT
430	440	450	460	470	480	490
CATCGAACAA	CTACATCAAG	CGGTTCACGC	AGAACATCGC	AGGCATCCCG	GTGCTGGGCT	CGTCAATTAC
500	510	520	530	540	550	560
TGAGGTCTTT	GATGGTCAGG	GCGCTGTTAC	CTCGGCAATT	GGTGCAGTCA	CCTCTGCTAC	TAAGGGTGCT
570	580	590	600	610	620	630
TTCCCGGCTG	ATCTGCCGCA	GGCCAAGCAG	CAGCCCTGGC	TTCGGCAACC	AAGATCGCCT	CAGCTGGCAA
640	650	660	670	680	690	700
AGACGCCAGC	CGCGATCTCG	CTTGTTGACC	AGAAAGCAAT	CTGGTTCGAC	GCCGTCTCTA	TCGGCAAGGG
710	720	730	740	750	760	770
AGCTACAGGC	TCCGTTGCAG	TCCCGGCGTA	CCAGTTCAGC	TTCACCACTG	GATTTCGCAGA	ATCGCGGGTG
780	790	800	810	820	830	840
CTCACGGTAG	CTGCCAACGA	CGGCGCTATC	TTGAATGACC	GCACCGATCG	CAAGGACATC	AACCGCGTGG
850	860	870	880	890	900	910
TCTGCGATGC	GAACTCGAAG	GTCATCGATC	TTGAAGCTAG	TAATGCAGAT	GCTCTGTTGA	AGTGTGGCAA
920	930	940	950	960	970	980
GACTCAGGCC	AATAAGCCGA	CGCGCATTGA	AGGCCAAGCT	GCATCGTCAG	TGGCCGATGT	GAACTCGGTT
990	1000	1010	1020	1030	1040	1050
TACAACTTTC	TGAATGACAC	TGCGTCGTTT	TACGGTGCAA	ACACTAAGGC	CAATGATCTG	ACCGCACTTA
1060	1070	1080	1090	1100	1110	1120
TTGGCAACGA	TGAAGGCGAC	GGTTTGGGCA	AGGCAATGCG	TGCCGTGGTC	CGGATCTGCG	TGACTGACAG
1130	1140	1150	1160	1170	1180	1190
CCAGAACGGC	GAGCAGTGCC	CGTTTCGCTAA	TGCTTTCTGG	TACAACGGCC	AGATGACCTA	CGGCCAAGGC
1200	1210	1220	1230	1240	1250	1260
GTGACCACCG	ACGACATTAC	CGGCCACGAA	CTGACGCACG	GCGTGACCGA	AAAGACCAAT	GGTTTGGTCT

1270	1280	1290	1300	1310	1320	1330
ATGCAAATGA	ATCTGGCGCA	ATCAACGAAT	CGATGTCCGA	TGTATTTCGGC	GAGTTCATCG	ACTTGAGCAA
1340	1350	1360	1370	1380	1390	1400
CGGCAGCTCG	GATGACACTG	CAGCTAACCG	CTGGGCAATC	GGCGAAGGCA	GCAGCCTTGG	TGTGATCCGG
1410	1420	1430	1440	1450	1460	1470
AGCATGAAAG	ATCCCGGAAA	GTACGGTGAA	CCGGCGATCT	ATAAGGGCTC	CAACTGGAAG	CCAACCGCTA
1480	1490	1500	1510	1520	1530	1540
CCAACCCGAA	CGACAACAAT	GACCAGGGTG	GAGTTCACTC	CAACAGCGGT	GTGGGCAACA	AGCTCGCATT
1550	1560	1570	1580	1590	1600	1610
CTTGATCACT	GACGGCCAGA	CTTTCAACGG	CCAGACCGTG	ACCGGAATTG	GCATTGCTAA	GGCCGCTCAG
1620	1630	1640	1650	1660	1670	1680
CTTTACTGGG	CCGCACAGCG	ACAGTTGACC	GCAAATGCGA	CTTACTCGAG	CTTGGGTAAG	GCTTTGAACT
1690	1700	1710	1720	1730	1740	1750
CTGCTTGCAG	TGCAAACGTT	TCTAACAACG	TTGCAGGAAC	CACCGCAGCA	AACTGCACCC	AGGTAGCCAA
1760	1770	1780	1790	1800	1810	1820
CGCCATCAAG	GCAGTCGGCA	TCAAATACC	TGAGATAGCT	AGAAAGCGCC	GGTTTTCTCG	CGAGAGGCAA
1830	1840	1850	1860	1870		
CCGGCGCTTT	GCTTTTAGAT	CGCTTAGCGA	GTGCTTTCTA	TGGCCTGCAG		

Figure 4.4 Amino acid sequence derived from the translated nucleotide sequence of a 1870 base pairs fragment of *R.salmoninarum* MT444 chromosomal DNA. The translation commenced from the first in-frame ATG starting at nucleotide 132.

Met	Lys	Lys	Tyr	Tyr	Ala	Val	Thr	Gly	Ile	Ala	Leu	Ala	Val	Gly	9	18	27	36	45
ATG	AAA	AAG	TAC	TAC	GCC	GTA	ACT	GGC	ATA	GCT	TTG	GCT	GTT	GGC					
Met	Leu	Cys	Thr	Thr	Gln	Leu	Ala	Gly	Ala	Thr	Gln	Ala	Ala	Asp	54	63	72	81	90
ATG	CTG	TGT	ACA	ACG	CAG	CTC	GCG	GGC	GCC	ACT	CAA	GCC	GCT	GAC					
Pro	Ser	Val	Gly	Ser	Leu	Asp	Ser	Ser	Asn	Val	Val	Thr	Glu	Phe	99	108	117	126	135
CCC	TCG	GTG	GGC	AGC	CTT	GAC	TCG	TCT	AAC	GTC	GTT	ACC	GAG	TTC					
Ser	Ala	Gln	Gly	Asn	Val	Glu	Gln	Ile	Thr	Phe	Lys	Ser	Ala	Ile	144	153	162	171	180
TCC	GCG	CAA	GGA	AAT	GTA	GAG	CAG	ATC	ACT	TTC	AAA	TCT	GCC	ATC					
Lys	Ser	Ala	Pro	Met	Ser	Ser	Ala	Arg	Ser	Ala	Gln	Thr	Ser	Ala	189	198	207	216	225
AAG	TCT	GCG	CCG	ATG	AGT	TCG	GCA	CGA	TCG	GCT	CAG	ACT	AGC	GCC					
Ile	Ile	Pro	Gly	Leu	Lys	Asn	Leu	Phe	Val	Ser	Ala	Pro	Gly	Ser	234	243	252	261	270
ATA	ATC	CCC	GGC	CTT	AAG	AAC	TTG	TTT	GTG	AGC	GCG	CCA	GGT	AGC					
Asp	Phe	Ser	Leu	Asn	Asp	Ser	Ser	Asn	Asn	Tyr	Ile	Lys	Arg	Phe	279	288	297	306	315
GAT	TTC	AGC	CTG	AAT	GAT	TCA	TCG	AAC	AAC	TAC	ATC	AAG	CGG	TTC					
Thr	Gln	Asn	Ile	Ala	Gly	Ile	Pro	Val	Leu	Gly	Ser	Ser	Ile	Thr	324	333	342	351	360
ACG	CAG	AAC	ATC	GCA	GGC	ATC	CCG	GTG	CTG	GGC	TCG	TCA	ATT	ACT					
Glu	Val	Leu	Asp	Gly	Gln	Gly	Ala	Val	Thr	Ser	Ala	Ile	Gly	Ala	369	378	387	396	405
GAG	GTC	CTT	GAT	GGT	CAG	GGC	GCT	GTT	ACC	TCG	GCA	ATT	GGT	GCA					
Val	Thr	Ser	Ala	Thr	Lys	Gly	Ala	Phe	Pro	Ala	Asp	Leu	Pro	Gln	414	423	432	441	450
GTC	ACC	TCT	GCT	ACT	AAG	GGT	GCT	TTC	CCG	GCT	GAT	CTG	CCG	CAG					
Ala	Lys	Gln	Gln	Pro	Trp	Leu	Arg	Gln	Pro	Arg	Ser	Pro	Gln	Leu	459	468	477	486	495
GCC	AAG	CAG	CAG	CCC	TGG	CTT	CGG	CAA	CCA	AGA	TCG	CCT	CAG	CTG					
Ala	Lys	Thr	Pro	Ala	Ala	Ile	Ser	Leu	Val	Asp	Gln	Lys	Ala	Ile	504	513	522	531	540
GCA	AAG	ACG	CCA	GCC	GCG	ATC	TCG	CTT	GTT	GAC	CAG	AAA	GCA	ATC					
Trp	Phe	Asp	Ala	Val	Leu	Ile	Gly	Lys	Gly	Ala	Thr	Gly	Ser	Val	549	558	567	576	585
TGG	TTC	GAC	GCC	GTT	CTT	ATC	GGC	AAG	GGA	GCT	ACA	GGC	TCC	GTT					
Ala	Val	Pro	Ala	Tyr	Gln	Phe	Ser	Phe	Thr	Thr	Gly	Phe	Ala	Glu	594	603	612	621	630
GCA	GTC	CCG	GCG	TAC	CAG	TTC	AGC	TTC	ACC	ACT	GGA	TTC	GCA	GAA					
Ser	Arg	Val	Leu	Thr	Val	Ala	Ala	Asn	Asp	Gly	Ala	Ile	Leu	Asn	639	648	657	666	675
TCG	CGG	GTG	CTC	ACG	GTA	GCT	GCC	AAC	GAC	GGC	GCT	ATC	TTG	AAT					
Asp	Arg	Thr	Asp	Arg	Lys	Asp	Ile	Asn	Arg	Val	Val	Cys	Asp	Ala	684	693	702	711	720
GAC	CGC	ACC	GAT	CGC	AAG	GAC	ATC	AAC	CGC	GTG	GTC	TGC	GAT	GCG					
Asn	Ser	Lys	Val	Ile	Asp	Leu	Glu	Ala	Ser	Asn	Ala	Asp	Ala	Leu	729	738	747	756	765
AAC	TCG	AAG	GTC	ATC	GAT	CTT	GAA	GCT	AGT	AAT	GCA	GAT	GCT	CTG					
Leu	Lys	Cys	Gly	Lys	Thr	Gln	Ala	Asn	Lys	Pro	Thr	Arg	Ile	Glu	774	783	792	801	810
TTG	AAG	TGT	GGC	AAG	ACT	CAG	GCC	AAT	AAG	CCG	ACG	CGC	ATT	GAA					
Gly	Gln	Ala	Ala	Ser	Ser	Val	Ala	Asp	Val	Asn	Ser	Val	Tyr	Asn	819	828	837	846	855
GGC	CAA	GCT	GCA	TCG	TCA	GTG	GCC	GAT	GTG	AAC	TCG	GTT	TAC	AAC					

Phe	Leu	864			873			882			891			900	
TTT	CTG	Asn	Asp	Thr	Ala	Ser	Phe	Tyr	Gly	Ala	Asn	Thr	Lys	Ala	
		AAT	GAC	ACT	GCG	TCG	TTT	TAC	GGT	GCA	AAC	ACT	AAG	GCC	
		909			918			927			936			945	
Asn	Asp	Leu	Thr	Ala	Leu	Ile	Gly	Asn	Asp	Glu	Gly	Asp	Gly	Leu	
AAT	GAT	CTG	ACC	GCA	CTT	ATT	GGC	AAC	GAT	GAA	GGC	GAC	GGT	TTG	
		954			963			972			981			990	
Gly	Lys	Ala	Met	Arg	Ala	Val	Val	Arg	Ile	Cys	Val	Thr	Asp	Ser	
GGC	AAG	GCA	ATG	CGT	GCC	GTG	GTC	CGG	ATC	TGC	GTG	ACT	GAC	AGC	
		999			1008			1017			1026			1035	
Gln	Asn	Gly	Glu	Gln	Cys	Pro	Phe	Ala	Asn	Ala	Phe	Trp	Tyr	Asn	
CAG	AAC	GGC	GAG	CAG	TGC	CCG	TTC	GCT	AAT	GCT	TTC	TGG	TAC	AAC	
		1044			1053			1062			1071			1080	
Gly	Gln	Met	Thr	Tyr	Gly	Gln	Gly	Val	Thr	Thr	Asp	Asp	Ile	Thr	
GGC	CAG	ATG	ACC	TAC	GGC	CAA	GGC	GTG	ACC	ACC	GAC	GAC	ATT	ACC	
		1089			1098			1107			1116			1125	
Gly	His	Glu	Leu	Thr	His	Gly	Val	Thr	Glu	Lys	Thr	Asn	Gly	Leu	
GGC	CAC	GAA	CTG	ACG	CAC	GGC	GTG	ACC	GAA	AAG	ACC	AAT	GGT	TTG	
		1134			1143			1152			1161			1170	
Val	Tyr	Ala	Asn	Glu	Ser	Gly	Ala	Ile	Asn	Glu	Ser	Met	Ser	Asp	
GTC	TAT	GCA	AAT	GAA	TCT	GGC	GCA	ATC	AAC	GAA	TCG	ATG	TCC	GAT	
		1179			1188			1197			1206			1215	
Val	Phe	Gly	Glu	Phe	Ile	Asp	Leu	Ser	Asn	Gly	Ser	Ser	Asp	Asp	
GTA	TTC	GGC	GAG	TTC	ATC	GAC	TTG	AGC	AAC	GGC	AGC	TCG	GAT	GAC	
		1224			1233			1242			1251			1260	
Thr	Ala	Ala	Asn	Arg	Trp	Ala	Ile	Gly	Glu	Gly	Ser	Ser	Leu	Gly	
ACT	GCA	GCT	AAC	CGC	TGG	GCA	ATC	GGC	GAA	GGC	AGC	AGC	CTT	GGT	
		1269			1278			1287			1296			1305	
Val	Ile	Arg	Ser	Met	Lys	Asp	Pro	Gly	Lys	Tyr	Gly	Glu	Pro	Ala	
GTG	ATC	CGG	AGC	ATG	AAA	GAT	CCC	GGA	AAG	TAC	GGT	GAA	CCG	GCG	
		1314			1323			1332			1341			1350	
Ile	Tyr	Lys	Gly	Ser	Asn	Trp	Lys	Pro	Thr	Ala	Thr	Asn	Pro	Asn	
ATC	TAT	AAG	GGC	TCC	AAC	TGG	AAG	CCA	ACC	GCT	ACC	AAC	CCG	AAC	
		1359			1368			1377			1386			1395	
Asp	Asn	Asn	Asp	Gln	Gly	Gly	Val	His	Ser	Asn	Ser	Gly	Val	Gly	
GAC	AAC	AAT	GAC	CAG	GGT	GGA	GTT	CAC	TCC	AAC	AGC	GGT	GTG	GGC	
		1404			1413			1422			1431			1440	
Asn	Lys	Leu	Ala	Phe	Leu	Ile	Thr	Asp	Gly	Gln	Thr	Phe	Asn	Gly	
AAC	AAG	CTC	GCA	TTC	TTG	ATC	ACT	GAC	GGC	CAG	ACT	TTC	AAC	GGC	
		1449			1458			1467			1476			1485	
Gln	Thr	Val	Thr	Gly	Ile	Gly	Ile	Ala	Lys	Ala	Ala	Gln	Leu	Tyr	
CAG	ACC	GTG	ACC	GGA	ATT	GGC	ATT	GCT	AAG	GCC	GCT	CAG	CTT	TAC	
		1494			1503			1512			1521			1530	
Trp	Ala	Ala	Gln	Arg	Gln	Leu	Thr	Ala	Asn	Ala	Thr	Tyr	Ser	Ser	
TGG	GCC	GCA	CAG	CGA	CAG	TTG	ACC	GCA	AAT	GCG	ACT	TAC	TCG	AGC	
		1539			1548			1557			1566			1575	
Leu	Gly	Lys	Ala	Leu	Asn	Ser	Ala	Cys	Ser	Ala	Asn	Val	Ser	Asn	
TTG	GGT	AAG	GCT	TTG	AAC	TCT	GCT	TGC	AGT	GCA	AAC	GTT	TCT	AAC	
		1584			1593			1602			1611			1620	
Asn	Val	Ala	Gly	Thr	Thr	Ala	Ala	Asn	Cys	Thr	Gln	Val	Ala	Asn	
AAC	GTT	GCA	GGA	ACC	ACC	GCA	GCA	AAC	TGC	ACC	CAG	GTA	GCC	AAC	
		1629			1638			1647							
Ala	Ile	Lys	Ala	Val	Gly	Ile	Lys	*							
GCC	ATC	AAG	GCA	GTC	GGC	ATC	AAA	TAA							

Table 4.1 Amino acid composition and codon usage deduced from an open reading frame of *R.salmoninarum* MT444 chromosomal DNA.

Alanine N=74 GCA=4.6% GCC=3.1% GCG=1.8% GCT=4.0% Total=13.5%	Arginine N=14 AGA=0.2% AGG=0.0% CGA=0.4% CGC=0.9% CGG=0.9% CGT=0.2% Total=2.6%	Asparagine N=42 AAC=5.5% AAT=2.2% Total=7.7%	Aspartic acid N=31 GAC=3.1% GAT=2.6% Total=5.7%
Cysteine N=7 TGC=0.9% TGT=0.4% Total=1.3%	Glutamine N=27 CAA=0.9% CAG=4.0% Total=4.9%	Glutamic acid N=15 GAA=1.8% GAG=0.9% Total=2.7%	Glycine N=52 GGA=1.3% GGC=6.0% GGG=0.0% GGT=2.2% Total=9.5%
Histidine N=3 CAC=0.5% CAT=0.0% Total=0.5%	Isoleucine N=30 ATA=0.4% ATC=3.8% ATT=1.3% Total=5.5%	Leucine N=33 CTA=0.0% CTC=0.5% CTG=1.6% CTT=1.8% TTA=0.0% TTG=2.0% Total=5.9%	Lysine N=28 AAA=0.9% AAG=4.2% Total=5.1%
Methionine N=7 ATG=1.3% Total=1.3%	Phenylalanine N=18 TTC=2.7% TTT=0.5% Total=3.2%	Proline N=18 CCA=0.7% CCC=0.7% CCG=1.6% CCT=0.2% Total=3.3%	Serine N=49 AGC=2.6% AGT=0.5% TCA=0.5% TCC=0.9% TCG=3.1% TCT=1.3% Total=8.9%
Threonine N=43 ACA=0.4% ACC=3.6% ACG=1.1% ACT=2.7% Total=7.8%	Tryptophan N=6 TGG=1.1% Total=1.1%	Tyrosine N=13 TAC=2.0% TAT=0.4% Total=2.4%	Valine N=38 GTA=0.9% GTC=1.6% GTG=2.6% GTT=1.8% Total=6.9%

4.3.3 PROTEIN SEQUENCE ANALYSIS

Table 4.1 also shows the amino acid composition as deduced from the translated nucleotide sequence of *mpr*. The translated protein was found to be rich in the polar uncharged residues of serine (8.9%) and asparagine (7.7%) as well as the hydrophobic nonpolar amino acid alanine (13.5%) but possessed low amounts of glutamic acid (2.7%) and arginine (2.6%). Positively charged residues formed only 8.2% of the total and this was reflected in the low predicted pI of 5.57. About 49% of all amino acid residues were found to be polar.

The complete deduced amino acid sequence is presented in single letter code in Figure 4.5. A polypeptide identity search of the PIR data base using the PROSCAN option of the DNASTAR program revealed that the deduced protein sequence possessed strong similarities to a range of secreted bacterial zinc metalloproteases, sometimes called neutral proteases (Table 4.2). All of these enzymes are members of a diverse family of zinc metalloendopeptidases which are recognised by a unique signature, His-Glu-X-X-His, and includes representatives from both procaryote and eucaryote groups (Jongeneel, Bouvier & Bairoch, 1989; Auld & Vallee, 1990). The similarities were substantial and highly conserved with respect to the residues forming the catalytic centre of these enzymes (Table 4.3; Figure 4.5). A complete alignment of all the sequences is presented in Appendix 2. Many of these proteases are preproenzymes which possess a putative signal peptide at the N-terminus. Mature protease is produced by cleavage of an N-terminal pro-sequence and comprises approximately 60% of the predicted full length protein. On the basis of this information the ORF encoding this polypeptide has been tentatively designated *mpr*, denoting metalloprotease of *R.salmoninarum*.

A number of nucleotide-binding enzymes and various protein kinases were also identified as sharing from 10% to 20% similarity to the *R.salmoninarum* metalloprotease by the PROSCAN search. Alignment of the sequences identified the presence of motifs within the first 340 amino acids which were not only identical to the nucleotide consensus binding site Gly-X-Gly-X-X-Gly, located between residues 188 and 193 and found in many nucleotide-binding proteins (Wierenga & Hol, 1983), but which also form part of the highly conserved catalytic domain of the diverse family of serine- and threonine-specific phosphotransferases (Hanks, Quinn & Hunter, 1988; Hanks & Quinn, 1991). The alignments of these conserved motifs are presented in Table 4.4.

The predicted structural properties of the protein are presented in Figure 4.6. The protein was found to be rich in β -sheet regions throughout the length of the molecule whilst α -helices occupied

the central 250 amino acids. The first 84 nucleotides of *mpr* encode a 28 amino acid peptide that bears all the features of a procaryotic signal peptide sequence (Watson, 1984; Oliver, 1985; Sarvas, 1986; Simonen & Palva, 1993). The amino terminus contained two positively charged lysine residues immediately following the methionine initiator. There was a hydrophobic core of 16 residues followed by more polar amino acids. The proposed processing site is between alanine residues 28 and 29, which are predicted to be surface exposed in a flexible, turn region (Figure 4.7).

The metalloprotease was predicted to possess many highly antigenic regions along its length, some of which are surface exposed (Figure 4.6). The metalloprotease zinc ligands, His-362, His-366 and Glu-386, and active centre, Glu-363, were spanned by two α -helices connected by a turn and were not surface exposed. However, regions surrounding these sites were expected to be highly antigenic and accessible on the surface of the molecule. The substrate binding site of Tyr-377, Asp-454 and His-459 were located in a highly antigenic, hydrophilic, surface exposed, turn region. In contrast, the consensus nucleotide-binding site of the metalloprotease stretches from the hydrophobic residue Trp-181 to Glu-210. This region corresponds to two β -sheets connected by a turn and is predicted to be amphipathic but not surface exposed. The reduced accessibility of this region is reflected in a low antigenic index.

Table 4.2 Sequence homology of the translated ORF to other metalloproteases in the PIR data base.

SPECIES	IDENTITY	REFERENCE
<i>Bacillus amyloliquifaciens</i>	25.4%	Vasanth, Thompson, Rhodes, Banner, Nagle & Filpula (1984)
<i>Bacillus caldolyticus</i>	27.1%	van den Burg, Enequist, van der Haar, Eijssink, Stulp & Venema (1991)
<i>Bacillus cereus</i>	31%	Sidler, Niederer, Suter & Zuber (1986)
<i>Bacillus megaterium</i>	23.5%	Kuhn & Fortnagel (1991)
<i>Bacillus polymyxa</i>	24.5%	Takekawa, Uozumi, Tsukagoshi & Udaka (1991)
<i>Bacillus stearothermophilus</i>	26.4%	Takagi, Imanaka & Aiba (1985)
<i>Bacillus stearothermophilus</i>	28.5%	Nishiya & Imanaka (1990)
<i>Bacillus subtilis</i>	25.4%	Yang, Ferrari & Henner (1984)
<i>Bacillus subtilis</i>	24.3%	Tran, Wu & Wong (1991)
<i>Bacillus thermoproteolyticus</i>	38.7%	Titani, Hermodson, Ericsson, Walsh & Neurath (1972)
<i>Erwinia carotova</i>	25.8%	Kyoestioe, Cramer & Lacy (1991)
<i>Legionella pneumophila</i>	20.4%	Black, Quinn & Tompkins (1990)
<i>Listeria monocytogenes</i>	39.6%	Domann, Leimeister-Wachter, Goebel & Chakraborty (1991)
<i>Pseudomonas aeruginosa</i>	21.8%	Bever & Iglewski (1988)
<i>Vibrio anguillarum</i>	ND	Milton, Norqvist & Wolf-Watz (1992)
<i>Vibrio cholerae</i>	20.4%	Hase & Finkelstein (1991)
<i>Vibrio proteolyticus</i>	19.1%	David, Deutch, Sloma, Pawlyk, Ally & Durham (1992)

ND = sequence not yet available on PIR database.

10	20	30	40	50	60	70
MKKYYAVTGI	ALAVGMLCTT	QLAGATQAAD	PSVGSLDSSN	VVTEFSAQGN	VEQITFKSAI	KSAPMSSARS
80	90	100	110	120	130	140
AQTSAILPGL	KNLFVSAPGS	DFSLNDSSNN	YIKRFTQNTA	GIPVLGSSIT	EVLDGQGAVT	SAIGAVTSAT
150	160	170	180	190	200	210
KGAFPADLPQ	AKQQPWL RQP	RSPQLAKTPA	AISLVDQKAI	WFDAVLIGKG	ATGSVAVPAY	QFSFTTGFAE
220	230	240	250	260	270	280
SRVLTVAAND	GAILNDRTDR	KDINRVVCDA	NSKVIDLEAS	NADALLKCGK	TQANKPTRIE	GQAASSVADV
290	300	310	320	330	340	350
NSVYNFLNDT	ASFYGANTKA	NDLTALIGND	EGDGLGKAMR	AVVRICVTDS	QNGEQCPFAN	AFWYNGQMTY
360	370	380	390	400	410	420
GQGVTTDDIT	GHEALTHGVTE	KTNGLVYANE	SGAINESMSD	VFGEFIDLSN	GSSDDTAANR	WAIGEGSSLG
430	440	450	460	470	480	490
VIRSMKDPGK	YGEPAIYKGS	NWKPTATNPN	DNNDQGGVHS	NSGVGNKLAF	LITDGQTFNG	QTVTIGIGIAK
500	510	520	530	540	550	
AAQLYWAAQR	QLTANATYSS	LGKALNSACS	ANVSNNVAGT	TAANCTQVAN	AIKAVGIK	

Figure 4.5 The deduced amino acid sequence in single letter code of the metalloprotease of *R.salmoninarum* which was translated from the open reading frame designated *mpr*. Symbols: ● = the highly conserved residues which form the catalytic centre of the protein; ▲ = the predicted signal peptide cleavage site. Single letter codes for amino acids are presented in Appendix III.

Protein	Coenzyme	Amino acid sequence																																
LDH	NAD	Lys #	Ile :	Thr :	Val :	Val :	Gly *	Val *	Gly *	Ala :	Val :	Gly *	Met :	Ala :	Cys :	Ala :	Ile :	Ser :	Ile :	Leu :	Met :	Lys :	Asp :	Leu :	Ala :	Asp :	Glu :	Val :	Ala :	Leu :	Val :	Asp x	Val :	Met :
ADH	NAD	Thr #	Cys :	Ala :	Val :	Phe :	Gly *	Leu *	Gly *	Gly :	Val :	Gly *	Leu :	Ser :	Val :	Ile :	Met :	Gly :	Cys :	Lys :	Ala :	Ala :	Gly :	Ala :	Ala :	-	Arg :	Ile :	Ile :	Gly :	Val :	Asp x	Ile :	Asn :
GPD	NAD	Lys #	Ile :	Gly :	Ile :	Asp :	Gly *	Phe *	Gly *	Arg :	Ile :	Gly *	Arg :	Leu :	Val :	Leu :	Arg :	Ala :	Ala :	Leu :	Ser :	Cys :	Gly :	Ala :	Gln -	-	Val :	Val :	Ala :	Val :	Asn x	Asp :	Pro :	Phe :
GR	FAD	Asp #	Tyr :	Leu :	Val :	Ile :	Gly *	Gly *	Gly *	Ser :	Gly :	Gly *	Leu :	Ala :	Ser :	Ala :	Arg :	Arg :	Ala :	Ala :	Glu :	Leu :	Gly :	Ala :	-	-	Arg :	Ala :	Ala :	Val :	Val :	Glu x	Ser :	His :
PHBH	FAD	Gln #	Val :	Ala :	Ile :	Ile :	Gly *	Ala *	Gly *	Pro :	Ser :	Gly *	Leu :	Leu :	Leu :	Gly :	Gln :	Leu :	Leu :	His :	Lys :	Ala :	Gly :	Ile -	-	Asp :	Asn :	Val :	Ile :	Leu :	Glu x	Arg :	Gln :	
MPR	?	Asp #	Ala :	Val :	Ile :	Ile :	Gly *	Lys *	Gly *	Ala :	Thr :	Gly *	Ser :	Val :	Ala :	Val :	Pro :	Ala :	Tyr :	Gln :	Phe :	Ser :	Phe -	-	-	Thr :	Thr :	Gly :	Phe :	Ala :	Glu x	Ser :	Arg :	

Secondary

[illegible]

The 'fingerprint' and secondary structure of the nucleotide-binding $\beta\alpha\beta$ unit as proposed by Wierenga & Hol (1983). A dash in the sequence indicates a deletion. LDH, porcine lactate dehydrogenase; ADH, equine liver alcohol dehydrogenase; GPD, lobster glyceraldehyde 3-phosphate dehydrogenase; GR, human erythrocyte glutathione reductase; PHBH, p-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*; MPR, *R. salmoninarum* metalloprotease; NAD, nicotinamide adenine dinucleotide; FAD, flavine adenine dinucleotide; #, invariant hydrophilic residue; :, neutral or hydrophobic groups which form the hydrophobic core of the $\beta\alpha\beta$ unit; *, invariant glycine residues; x, the invariant negatively charged residue involved in hydrogen bonding to a ribose hydroxyl group.

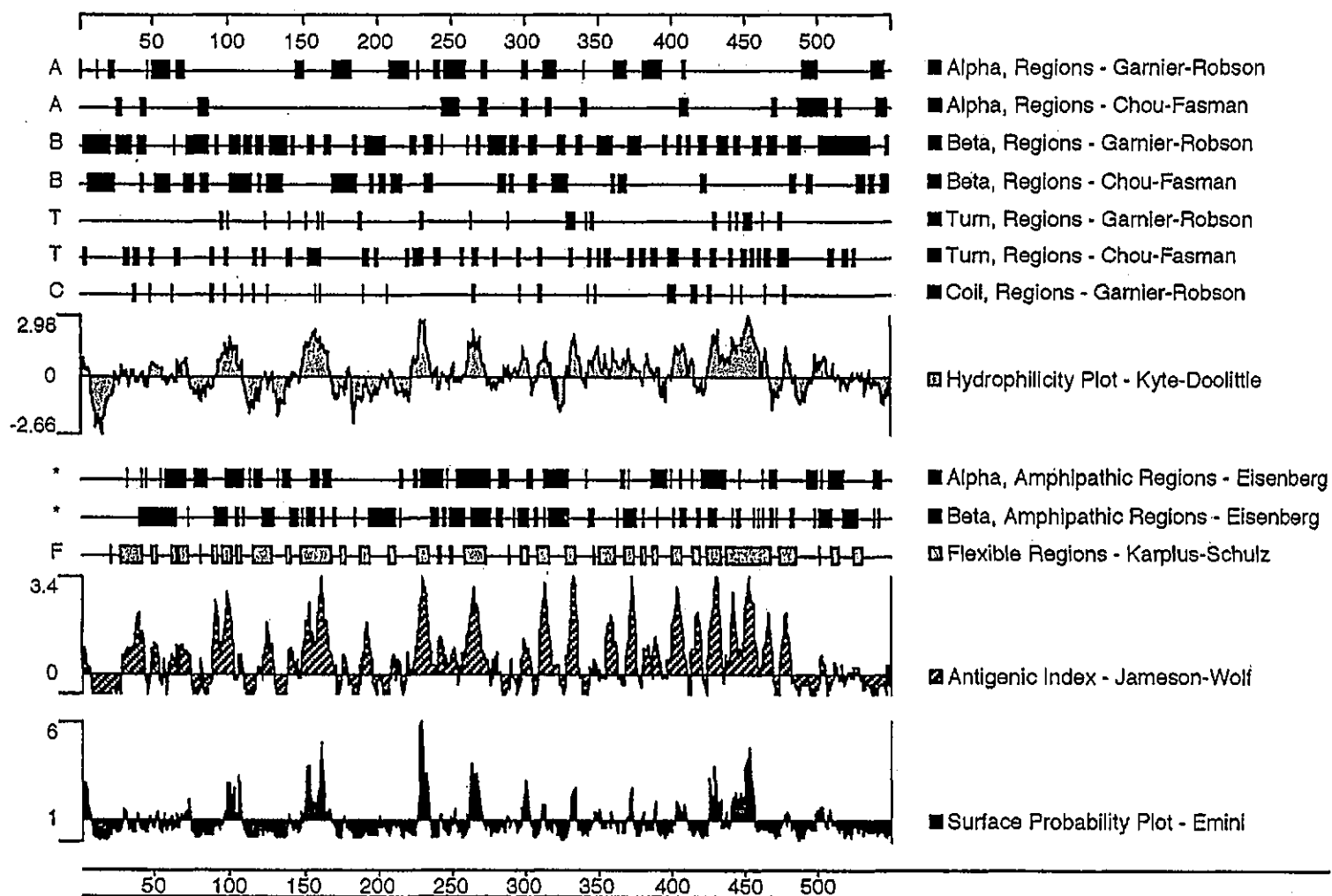


Figure 4.6 The predicted structural properties of *R. salmoninarum* metalloprotease. The numbers at the top and bottom of the figure refer to amino acid residues commencing from Met-1 and including the putative signal peptide.

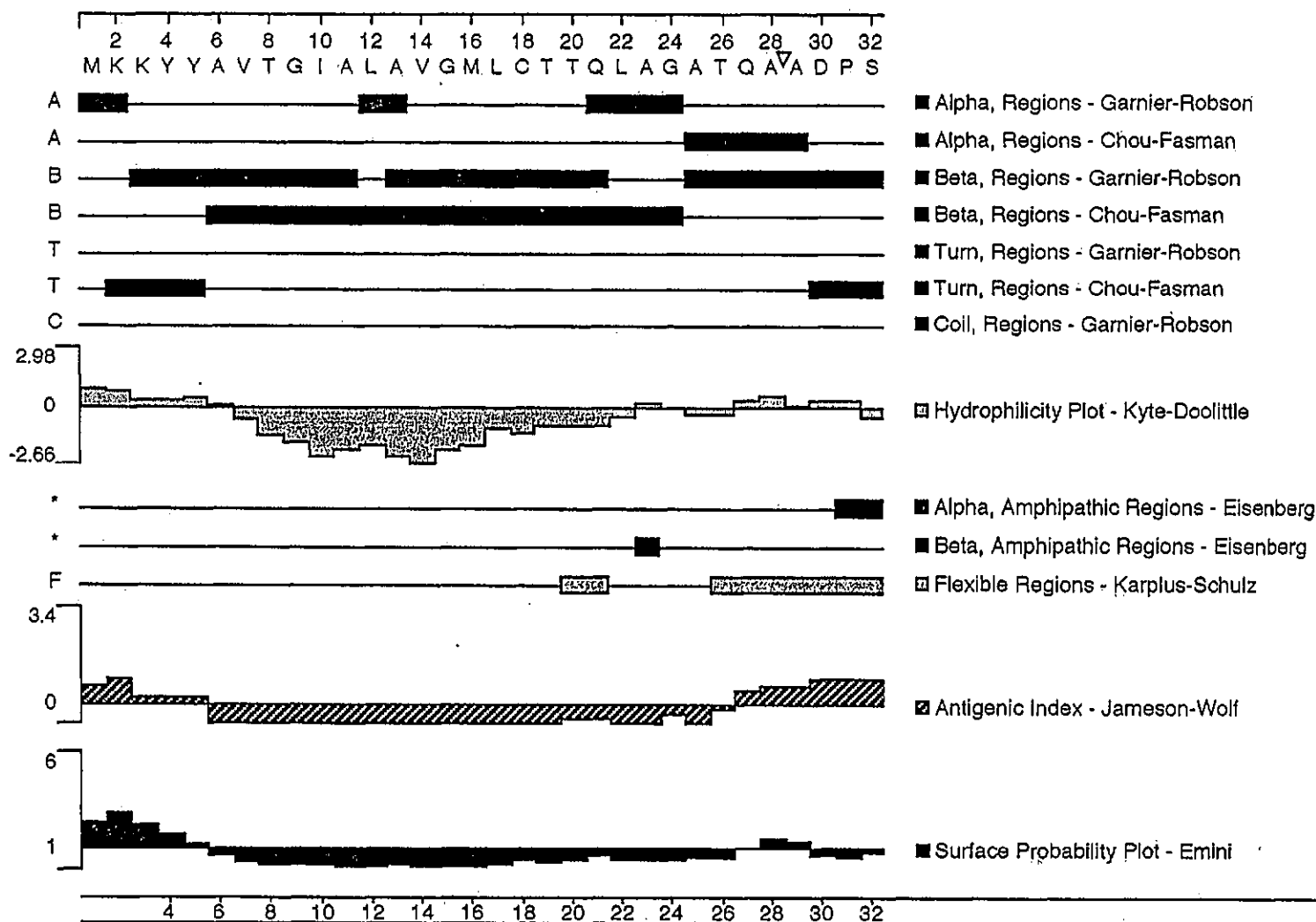


Figure 4.7 The structural properties of the N-terminal region of *R. salmoninarum* metalloprotease which displayed all the features of a procaryotic signal peptide. The arrow marks the predicted signal peptide cleavage site.

4.4 DISCUSSION

The *R. salmoninarum* gene cloned from strain MT444 in the plasmid vector pBR328, designated pRHLY'B' and encoding a haemolytic product, was characterised by nucleotide sequence analysis. The coding region was flanked by *Hind*III and *Pst*I restriction sites and contained one *Pvu*II site and one *Pst*I site. DNA encompassing this 1.9 kbp region was subcloned from pRHLY'B' into pUC18 as three separate fragments; *Hind*III-*Pvu*II, *Pvu*II-*Pst*I and *Pst*I-*Pst*I, to form sequencing substrates pURS1, pURS2 and pURS3, respectively. The sequence of the contiguous fragments was determined and an ORF of 1647 nucleotides was found to encode 548 amino acids with a predicted molecular weight of 66,757 and pI of 5.57. The amino acid sequence showed strong similarities to those of other secreted bacterial metalloproteases, especially those belonging to *Bacillus* sp., *Listeria monocytogenes*, *Legionella pneumophila*, *Pseudomonas aeruginosa* and *Erwinia carotovora* (Table 4.2). On this basis, the gene has been tentatively designated *mpr* denoting metalloprotease of *R. salmoninarum*. These results represent the first description from *R. salmoninarum* of a metalloprotease which does not possess a readily detectable caseinase or gelatin degrading activity. The gene has been found to be present in each of the seven strains of *R. salmoninarum* so far examined and the nucleotide content was 55.5% C+G, consistent with the overall 55.5% C+G of the *R. salmoninarum* chromosome (Banner, Rohovec & Fryer, 1991).

The predicted molecular weight of 66,757 for this metalloprotease is about 1,700 greater than the molecular weight of the protein estimated at 65000 by minicell analysis. The reasons for this are as yet unresolved but may be due to a rapid intrinsic or extrinsic processing immediately following translation, such as loss of the signal peptide. Alternatively, the transcription of *mpr* from the second promoter region would yield a protein of about 61000 molecular weight after translation and this may account for the observed difference. It is interesting to note the presence of two putative promoter regions at the start of gene *mpr* each of which possess in-frame initiation codons, characteristic ribosome-binding sites and possible -10 and -35 regions which exhibit good homology to the consensus sequences recognised by the σ^{70} RNA polymerase subunit for *E. coli* promoters (McClure, 1985). Translation of the mRNA derived from the first ATG would give rise to a protein of 66,757 kDa molecular mass possessing an N-terminal amino acid sequence which strongly resembles signal peptide sequences from other procaryotes, particularly those of Gram positive bacteria (Simonen & Palva, 1993). Translation of mRNA initiated from the second promoter would yield a protein lacking a signal peptide and of about 60,400 molecular weight. A similar situation has been reported for the expression in *E. coli* of

the gene encoding pneumolysin (*ply*), the sulphhydryl-activated toxin of *Streptococcus pneumoniae* (Walker, Allen, Falmagne, Johnson & Boulnois, 1987). Gene *ply* possesses two in-frame ATG codons 36 nucleotides apart, either of which may act to initiate translation in *E.coli*. Whether transcription or translation of *mpr* is regulated by *R.salmoninarum* such that either one of the two promoters or initiation codons are utilised under different environmental or physiological conditions, awaits further investigation. Studies of the control and regulation of gene expression in other pathogens have been recently reviewed (Dorman & Bhriain, 1992). Of interest are the recent reports that zinc, calcium and iron concentrations in culture media can affect the translation and processing of *P.aeruginosa* elastase (Brumlik & Storey, 1992; Olson & Ohman, 1992).

A unique amino acid sequence motif which identifies members of a family of zinc-dependent endopeptidases (Jongeneel *et al.*, 1989) has been shown to be preserved in the metalloprotease of *R.salmoninarum*. Many other bacterial species also produce proteases which share this motif as well as a number of other common features such as a signal peptide sequence, a propeptide which is cleaved to produce a mature protease, three zinc ligands, a glutamic acid active centre and conserved substrate-binding sites. As with other secreted proteins, zinc metalloproteases possess a signal peptide of between 23 and 34 amino acids at the N-terminus. The signal sequence is presumably required for secretion and is the only prominent feature which distinguishes between exported and cytoplasmic proteins. The general format of a signal peptide includes from one to three charged residues within the first five amino acids, a core of at least nine hydrophobic residues which are believed to be sufficient to span a membrane, and a carboxy-terminus containing polar amino acids (Watson, 1984; Oliver, 1985; Simonen & Palva, 1993). The majority of signal sequences end with either a glycine or alanine residue forming part of a consensus cleavage site, Ala-X-Ala (Oliver, 1985), where cleavage occurs after the carboxy-terminal alanine. Occasionally, either or both of the alanine residues are substituted by other amino acids with short side chains, such as serine, threonine, valine or cysteine. Frequently, a helix-breaking residue (Pro or Gly) or a large polar residue (Glu) occurs four to eight amino acids before the cleavage site (Watson, 1984). The proposed signal sequence of *R.salmoninarum* possesses all of these features and is remarkably similar to the signal sequences reported for metalloproteases from *Bacillus* sp. (Simonen & Palva, 1993), *L.monocytogenes* (Domann *et al.*, 1991), *L.pneumophila* and *P.aeruginosa* (Black *et al.*, 1990). It is probable that the metalloprotease of *R.salmoninarum* is also a secreted enzyme, although absolute proof of this will require the isolation of the native enzyme from *in vitro* culture supernatant.

The signal peptide of most of the characterised bacterial zinc metalloproteases is followed by a propeptide of approximately 150-200 amino acids which represents about 40% of the preproenzyme. Whilst the exact function of the propeptide is unknown there is some evidence to suggest that it may act to prevent enzyme activity during secretion, to temporarily anchor the protein in the cell membrane and to promote the correct folding and ensure activation of the mature enzyme after export (Vasanthan *et al.*, 1984; Ikemura, Takagi & Inouye, 1987; Wandersman, 1989; Zhu, Ohta, Jordan & Inouye, 1989). Much of the characterisation of propeptide sequences has been performed using *Bacillus* sp. proteases (reviewed by Simonen & Palva, 1993). More recent information gathered from a wide range of bacteria including *P.aeruginosa* (Bever & Iglewski, 1988; Kessler & Safrin, 1988), *L.pneumophila* (Black *et al.*, 1990), *L.monocytogenes* (Domann *et al.*, 1991), *V.anguillarum* (Milton *et al.*, 1992), *V.cholerae* (Hase & Finkelstein, 1991) and *Streptomyces cacaoi* (Chang & Lee, 1992), has led to the identification of propeptide regions which are cleaved either by autoproteolytic means (Power, Adams & Wells, 1986; Milton *et al.*, 1992) or by other cell-associated or extracellular proteases (Domann *et al.*, 1991). Because of the difficulties involved with the isolation of enzymatically active components from *in vitro* cultures of *R.salmoninarum* it is not possible to predict whether this will also occur in the case of the metalloprotease described by this study.

Zinc-dependent endopeptidases form a heterogeneous family, with widely differing specificities and sensitivity to inhibitors (Jongeneel *et al.*, 1989). It has been suggested that variation in the spacer regions between the catalytic zinc ligands (Vallee & Auld, 1989) and the residues forming the substrate-binding site (Milton *et al.*, 1992) could reflect differences in substrate specificity and the mechanism of catalysis. A better understanding of the structural requirements for enzyme activity has been constrained by the limited availability of protein sequence and tertiary structure information (Jongeneel *et al.*, 1989). In the absence of such information it is impossible to predict what degree of specificity the metalloprotease of *R.salmoninarum* may exhibit. However, many of the proteases with which it shares considerable similarity have been better characterised and found to possess broad substrate specificity. The elastase of *P.aeruginosa*, for example, has been shown to be active against elastin and collagen (Moriyama, 1964; Moriyama & Homma, 1985; Heck, Moriyama, McRae & Miller, 1986), human immunoglobulins A and G (Doring, Obernesser & Botzenhart, 1981), serum α_1 -proteinase inhibitor (Moriyama, Tsuzuki & Oda, 1979; Moriyama, Tsuzuki, Harada & Iwata, 1984), several complement components (Schultz & Miller, 1974; Hong & Ghebrehiwet, 1992) and human prekallikrein (Tanaka, Yamamoto, Shibuya, Nishino, Tanase, Miyauchi & Kambara, 1992). The crystal structures of both

B.thermoproteolyticus thermolysin and elastase have been determined by X-ray crystallography. Elastase shows about 49% identity with thermolysin, although the amino and carboxy termini of the two proteins are very different (Bever & Iglewski, 1988). Nevertheless, the identity and conformation of the amino acid residues constituting the active sites of both molecules are remarkably similar (Matthews, Weaver & Kester, 1974; Holmes & Matthews, 1982; Thayer, Flaherty & McKay, 1991). The similarities also extend to the secondary and tertiary structures such that the zinc ligands, active centre and substrate-binding sites (see Table 4.3) occur in helical regions of the protein. Analysis of the predicted secondary structure of *R.salmoninarum* metalloprotease indicates that the same catalytic sites also occur in helical regions of the molecule. Confirmation of this awaits analysis of the crystalline structure of the protein. The failure to detect proteolytic activity against casein and gelatin substrates suggests that the *R.salmoninarum* metalloprotease either requires some other factor for activity or differs from other zinc metalloproteases and is only active against specific substrates. Mengaud, Geoffroy & Cossart (1991) and Domann *et al.*(1991) reported similar difficulties in detecting any strong proteolytic activity in either *E.coli* clones expressing the *L.monocytogenes* metalloprotease or in *L.monocytogenes* culture medium and suggested that this may have been due to a lack of processing *in vitro*.

The most substantial similarities between *R.salmoninarum* metalloprotease and other zinc-dependent endopeptidases are in the amino acid residues that are directly involved in catalysing proteolysis and in residues that more generally determine the structural conformation of the active site. According to the structural analyses of thermolysin and elastase, the catalytic zinc moiety of *R.salmoninarum* metalloprotease would be expected to be stabilised by His-362, His-366 and Glu-386. The corresponding zinc ligands from other bacterial metalloproteases are identical and identically spaced (Table 4.3, Figure 4.5). The active site glutamic acid of *R.salmoninarum* metalloprotease, Glu-363, that acts to protonate the scissile bond in thermolysin and elastase (Thayer, Flaherty & McKay, 1991) is also conserved in all the other zinc metalloendopeptidases. Three residues of elastase and thermolysin that are implicated in the stabilisation of substrate carbonyls are also present in *R.salmoninarum* metalloprotease as Tyr-377, Asp-454 and His-459, although the spacer regions between these residues varies between members of the zinc-dependent endopeptidase family and may be related to substrate specificity.

The region of *R.salmoninarum* metalloprotease which is least similar to other zinc metalloproteases lies within the first 340 amino acids, the region which would form the

propeptide should this protein be found to possess one. It is within this region that a nucleotide-binding $\beta\alpha\beta$ "fingerprint" is located which is illustrated in Table 4.4. Wierenga & Hol (1983) have described this "fingerprint" unit as consisting of four essential parts:

- (1) The consensus sequence Gly-X-Gly-X-X-Gly, which is conserved in protein kinases as well as nucleotide-binding proteins (Hanks, Quinn & Hunter, 1988). The three glycines link the first β -sheet and the α -helix so as to allow the formation of hydrogen bonds and a favourable charge interaction between the protein and the ribose-pyrophosphate moiety of the nucleotide.
- (2) The hydrophobic core of this $\beta\alpha\beta$ unit requires the presence of several neutral and often hydrophobic residues.
- (3) The presence of a negatively charged residue at the distal end of the second β -sheet which forms strong hydrogen bonds with the 2'-hydroxyl group of the adenine-ribose.
- (4) The presence of a hydrophilic residue at the start of the first β -sheet.

Table 4.4 clearly shows that residues 183-210 of *R.salmoninarum* metalloprotease possesses these four characteristics. Wierenga & Hol (1983) examined the nucleotide-binding properties of human bladder p21 protein using interactive three-dimensional computer modelling and predicted that proteins possessing the $\beta\alpha\beta$ "fingerprint" would bind a nucleotide or dinucleotide, such as FAD, NAD, GTP, GDP, ATP or ADP, but not NADP. This was due to a predicted unfavourable charge interaction between the 2'-phosphate of NADP and the negatively charged residue at the distal end of the second β -sheet.

Whether the *R.salmoninarum* metalloprotease also functions as a nucleotide-binding protein and participates in electron transport remains to be determined. Whilst the extent of overall sequence identity between *R.salmoninarum* metalloprotease and other nucleotide-binding proteins varied from 10-20%, Wierenga & Hol (1983) concluded that the $\beta\alpha\beta$ 'fingerprint' provided a better guide in defining the essential features of nucleotide-binding proteins than sequence identity. It is interesting to note that a recently proposed model for the acquisition of ferrous and ferric iron by an extracellular reductant of *L.monocytogenes* which requires NADH, flavin mononucleotide and Mg^{2+} as co-factors probably involves the recycling of NAD^+ by a surface or perhaps secreted NADH dehydrogenase. Alternatively, there is the possibility that *R.salmoninarum* metalloprotease may be a functional kinase or phosphatase. The recently published sequence of *E.coli* isocitrate dehydrogenase kinase/phosphatase showed only partial sequence similarities with other mainly eucaryotic protein kinases (Klumpp, Plank, Bowdin, Stueland, Chung & LaPorte, 1988). The authors stated that extensive sequence homology is not a universal property of protein kinases.

The gene encoding *R.salmoninarum* metalloprotease has been shown to be present in a number of strains of *R.salmoninarum* which differ in their virulence (Chapter 3). The level of gene expression in each of these strains has yet to be determined and the role of the metalloprotease in the pathogenesis of BKD is open to conjecture. The contribution of zinc metalloproteases from other pathogens to the disease process has been better studied. *P.aeruginosa* elastase makes an important and well documented contribution to the establishment of opportunistic infections and ensuing tissue damage (Moriyama & Homma; 1985). In addition, the immunisation of animals with elastase has been shown to provide some measure of protection against *P.aeruginosa* infection (Kawamoto, Shibano, Fukushima, Ishii, Moriyama & Okuda, 1993). *L.pneumophila* zinc metalloprotease exhibits haemolytic and cytotoxic activities (Keen & Hoffman, 1989) and has been found to contribute to both the pathology and pathogenesis of Legionnaires' disease (reviewed in Dowling, Saha & Glew, 1992). Despite evidence that this molecule is inhibitory to phagocyte killing (Rechnitzer & Kharazmi, 1992) and induces protective immunity when administered as a vaccine to guinea pigs (Blander & Horwitz, 1989) it is neither a virulence factor (Blander, Szeto, Shuman & Horwitz, 1990) nor required for intracellular growth or host cell death (Szeto & Shuman, 1990). Similarly, the haemagglutinin/protease of *V.cholerae* has a role in cleaving mucin and fibronectin and in the activation of the A-subunit of cholera enterotoxin (Hase & Finkelstein, 1990) whilst not functioning as a primary virulence factor (Finkelstein, Boesman-Finkelstein, Chang & Hase, 1992). *L.monocytogenes* metalloprotease is encoded by the first gene of the lecithinase operon and apparently acts to proteolytically process phosphatidylcholine phospholipase C to its mature active form (Mengaud *et al.*, 1991; Poyart, Abachin, Razafimanantsoa & Berche, 1993). Whilst the precise role of the molecule is not yet understood, isogenic mutants of *L.monocytogenes* which have an inactivated metalloprotease gene show a significantly reduced level of virulence compared to the wild-type strain (Raveneau, Geoffroy, Beretti, Gaillard, Alouf & Berche, 1992). This may be at least partly due to a reduced phospholipase C activity in the metalloprotease deficient mutants. Phospholipase C has some role in the cell-to-cell spread of *L.monocytogenes* (Vasquez-Boland, Kocks, Dramsi, Ohayon, Geoffroy, Mengaud & Cossart, 1992).

The specific function and adaptive significance of the zinc metalloproteases of such a diversity of bacteria are perhaps unclear. For example, the neutral proteases of *Bacillus* may not be essential for growth or sporulation (Yang *et al.*, 1984). The primary role may be to serve a nutritional function (Black *et al.*, 1990) whilst making an undefined contribution to the pathogenesis of disease. The ubiquity and conservation of these proteases in the microbial world,

in both pathogenic and non-pathogenic species, suggests that they may provide some common survival advantage which is not necessarily essential for virulence but which, in the case of pathogenic species, may be associated with it (Hase & Finkelstein, 1990). Whilst the contribution of *R.salmoninarum* metalloprotease to the progress of BKD is unknown it is likely that the immunological disruption of this novel and potentially membrane-damaging molecule will provide material for future BKD vaccine research. The metalloprotease was predicted to be highly antigenic along its length, especially in the region of the proposed substrate-binding site. Therefore, it is possible that some form of immunological intervention may disrupt substrate binding and activity *in vivo*, given the constraints of the assumptions which underlie these structural predictions. The main problem associated with identifying antigenic sites lies in the fact that such sites are almost invariably conformational and rely on the folded structure of a protein to spatially arrange the antigenic configuration (Vajda, Kataoka, DeLisi, Margalit, Berzofsky & Cornette, 1990). In addition, in contrast to B cell antigenic sites, T cells recognise short (10-15 residues) peptide fragments which are characterised by an ability to form an amphipathic α -helix. Vajda *et al.* (1990) suggest that α -helical structures bind best to the major histocompatibility complex (MHC) antigens and therefore are stabilised for recognition by the T cell receptor in conjunction with MHC. Hence, such predictions must be viewed with some reservation particularly as many of the components of the fish immune system, such as T cell receptors, have yet to be fully characterised (Ellis, 1988).

CHAPTER 5.

THE PRODUCTION OF FUSION PROTEINS AND ANTIGENICITY OF HYBRID EPITOPES

5.1 INTRODUCTION

The identification and characterisation of a gene encoding a previously undescribed metalloprotease from *R.salmoninarum* has identified a product of interest for immunological investigation (Chapter 4). However, before research into the properties of the enzyme could proceed, a sufficient quantity of the purified protein was required. The expression of gene *mpr* in pRHLY'B' was not sufficient for this purpose. A number of factors may affect the level of expression of a gene at either the transcriptional or translational level including the secondary structure of the DNA and mRNA, the ability of the host RNA polymerase to bind to a foreign promoter, the ability of foreign mRNA to bind to the host ribosomes, post-translational modifications such as signal peptide or propeptide cleavage, and degradation of a foreign protein by the host cell. The level of expression may also be affected by codon preferences (Stormo, Schneider & Gold, 1982) and other as yet poorly understood aspects of the coding sequence (Riggs, 1990). However, recombinant DNA technology has been used to create a range of plasmid-based *E.coli* host-vector systems which will direct the synthesis of large amounts of the products of cloned genes in a controllable manner. This is often the logical next step for researchers who have isolated a gene and want to study the protein it encodes.

In many cases it has been found that problems associated with lack of or poor expression may be overcome by making fusions between genes. In addition, it often also simplifies purification procedures. In this way the cloned gene is introduced into an expression vector at the 3' end of a carrier sequence coding for the amino terminus of a highly expressed carrier protein. The carrier sequence may be from an *E.coli* gene, or indeed any gene which is strongly expressed in *E.coli*. The carrier sequence provides the necessary signals for good expression and the expressed fusion protein contains an N-terminal region encoded by the carrier. A wide variety of fusion vectors are available commercially which vary in the size and nature of the carrier protein. The portion of the fusion protein encoded by the carrier may be as small as six histidine residues (Qiaexpress, Qiagen) or may code for an entire functional moiety or protein, such as β galactosidase, maltose-binding protein (MBP), glutathione-S-transferase or protein A fusions. These carrier regions can often be exploited for purification purposes, either with antibodies or

an affinity purification specific for a particular carrier protein. Some carrier proteins, such as maltose-binding protein and protein A, may possess a signal peptide allowing export of the fusion protein into the periplasmic space or the growth medium. Export of the fusion protein may promote the proper folding of proteins that do not fold correctly in the cytoplasm (Takagi, Morinaga, Tsuchiya, Ikemura & Inouye, 1988). The correct folding of polypeptides is essential for conferring a biological function upon proteins and may require the assistance of either steric or non-steric factors known as molecular chaperones (Ellis & van der Vies, 1991; Gething & Sambrook, 1992). These factors are involved with the stress response of bacteria and may be associated with the regulation of expression of virulence factors (Dorman & Bhriain, 1992).

The main problems which have often been associated with the expression of fusion proteins are the solubility and stability of the expressed protein, and the presence of the fused carrier protein. The high level expression of many proteins can lead to the formation of dense aggregates of insoluble protein and RNA known as inclusion bodies. The formation of inclusion bodies may simplify protein purification and stabilise the expressed protein. However, recovery of soluble protein from inclusion bodies requires detergent extraction, denaturation and renaturation procedures which can prove troublesome and dramatically reduce yields (Hooey, 1990). There are a number of factors which have been found to affect the solubility of proteins. Generally, higher temperatures (37-42°C) promote inclusion body formation and lower temperatures (20-30°C) inhibit it (Bishai, Rappuoli & Murthy, 1987; Schein, 1989). Sometimes, lowering the expression level can increase the proportion of soluble protein and the choice of the host strain may also be important (Riggs, 1990). The size and nature of the carrier protein will also have some effect on the properties of the protein to which it is fused.

The stability of foreign proteins expressed in *E. coli* may also be problematical. A carrier protein may act to stabilise a fusion protein (Lee, Cozzicorto, Wainwright & Testa, 1984). However, this may not necessarily be the case, and either the expressed protein may be incorrectly folded and consequently degraded, the fusion may be cleaved at the fusion joint, or some part of the fusion may be sensitive to one or more *E. coli* proteases (Maurizi, 1992). Several approaches may be used to stabilise fusion proteins, such as expression of inclusion bodies, the use of protease-deficient mutants of *E. coli* or subcloning portions of a well characterised gene and gene product in order to yield smaller, more stable fusion proteins possessing a fragment of interest, such as specific B- or T-cell epitopes (Bennett, Whitby & Coleman, 1992).

Intact fusion proteins can be valuable for studying the regulation of gene expression as well as immunological and biochemical studies of protein structure and function, although it may be desirable to separate the carrier domain from the expressed protein (Silhavy & Beckwith, 1985). Chemical methods of site-specific protein cleavage include cyanogen bromide which cleaves at methionine residues (Szoka, Schreiber, Chan & Murthy, 1986) and oxidation at tryptophan residues by iodosobenzoic acid (Villa, De Fazio, Donini, Tarchi & Canosi, 1988). These methods present obvious difficulties for proteins with several methionine or tryptophan residues. An alternative approach to cleaving off the carrier protein is to use a fusion vector which encodes a specific protease cleavage site close to the fusion joint. Many commercial suppliers now offer this feature for use with site-specific proteases such as thrombin (Gearing, Nicola, Metcalfe, Foote, Willson, Gough & Williams, 1989), factor Xa (Nagai & Thogersen, 1984), renin (Haffey, Lehman & Boger, 1987), or collagenase (Germino & Bastia, 1984). The use of such enzymes may be complicated by the accessibility of the cleavage site, the presence of other cleavage sites within the protein of interest and reduced specificity of the proteases under some circumstances.

The purpose of this study was to produce fusion proteins which would be useful for further immunological characterisation. In the absence of any information regarding the stability, solubility and recovery of products cloned from *R.salmoninarum* and expressed in *E.coli*, it was necessary to select fusion vectors which conferred some degree of flexibility on the expression and purification of fusion proteins. The choice of fusion vector was limited by the paucity of suitable restriction sites on the *R.salmoninarum* genes which were available for the production of in-frame fusions; *msa* which encoded the major secretory antigen (P57) and was cloned and characterised by Dr M.L.Gilpin; *mpr* encoding the metalloprotease cloned and characterised in this study (Chapter 4) and *hly* which encoded a unique contact haemolysin and was cloned and characterised by Evenden (1993). The pMAL protein fusion and purification system from New England BioLabs offered a choice of two vectors for the expression of a maltose-binding protein (MBP) fusion protein. The method relies on the insertion of a cloned gene downstream from the *malE* gene of *E.coli*. The two vectors express the *malE* gene, either with (pMAL-p) or without (pMAL-c) its signal sequence, fused to the *lacZ* α gene. Insertion of a foreign gene into the multiple cloning site inactivates the β -galactosidase α -fragment activity and allows for blue/white screening for recombinants using IPTG and X-gal in an α complementing host such as XL1-BLUE. In addition, transcription is under the control of the strong *tac* promoter and the *lacI*^r repressor. If the signal sequence is present (pMAL-p vectors) MBP fusion proteins are directed to the periplasmic space should this be required for correct folding and disulphide bond

formation. The pMAL vectors also contain a factor Xa cleavage site close to the fusion joint and purification of MBP fusion proteins is greatly simplified by using an amylose resin affinity column to bind MBP followed by rapid elution of the fusion protein separate from other *E.coli* proteins. The pAX expression vector system described by Markmeyer, Ruhlman, Englisch & Cramer (1990), which is available from United States Biochemical Corporation, also offers considerable flexibility. There are currently eight pAX vectors available which differ in the reading frame of the multiple cloning site and the types of restriction sites available. The pAX vectors contain the *lacZ* gene encoding the full β -galactosidase protein of 116 kDa molecular mass and under the control of the inducible *lac* promoter. The multiple cloning site is joined to the carboxy terminus of the *lacZ* by a hinge region coding for a collagen fragment as well as a factor Xa site. The rigid hinge region separates the β -galactosidase and the foreign protein to allow both independent folding and free accessibility of the factor Xa cleavage site. One step purification of the fusion protein is achieved by affinity chromatography using p-aminophenyl β -D-thiogalactopyranoside (APTG) immobilised on an agarose column. APTG is a substrate analog for β -galactosidase which can specifically bind β -galactosidase fusion proteins. Using these methods, genetically engineered gene fusions were constructed and expressed in *E.coli* as a first step toward analysis of the immunological properties of products encoded by genes cloned from *R.salmoninarum*.

5.2 MATERIALS AND METHODS

Only materials and methods which were specific to this aspect of the work are described below. Those which have been used in previous studies are detailed elsewhere (Chapters 3 & 4).

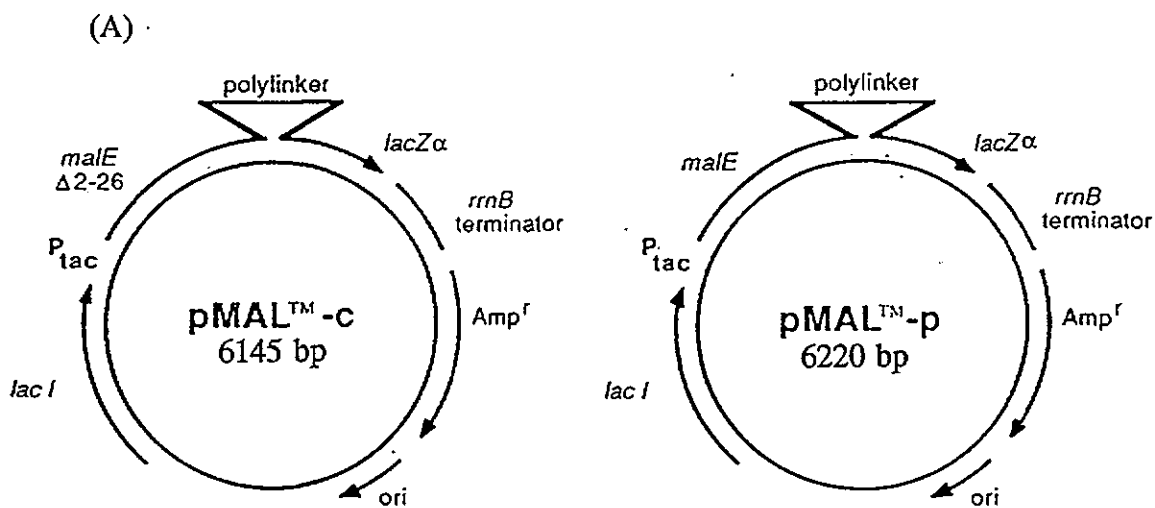
5.2.1 BACTERIAL STRAINS, PLASMIDS AND MEDIA

E.coli XL1-BLUE was used as host and was grown in LBB or on LBA at either 22°C or 37°C. When appropriate, 100 $\mu\text{g ml}^{-1}$ of ampicillin, 15 $\mu\text{g ml}^{-1}$ of tetracycline and 0.2% v/v glucose were added to the medium. Plasmid expression vectors pMAL-c (6.145 kbp), pMAL-p (6.22 kbp) (New England BioLabs) and pAX5+ (6.201 kbp) (U.S. Biochemical Corp.) were used for the construction of gene fusions.

5.2.2 DNA MANIPULATION AND THE CONSTRUCTION OF GENE FUSIONS

From the restriction maps available for the *R.salmoninarum* genes *msa*, *mpr* and *hly*, restriction sites close to the N-terminus were identified as suitable for the construction of in-frame gene fusions (Figures 5.1, 5.2 & 5.3).

Figure 5.1 Cloning strategy for the construction of a fusion of the *malE* gene with the *mpr* gene. (A) Diagrammatic representation of the pMAL vectors including a restriction map of the polylinker. Amp^r: gene encoding ampicillin resistance; P_{lac}: inducible promoter positioned to transcribe a *malE-lacZα* gene fusion; *lacI*: gene encoding the Lac repressor which turns off transcription from P_{lac} until an inducer such as IPTG is added; *rrnB* terminator: transcriptional termination sequence; ori: origin of replication (B) Restriction map of the fragment of *R.salmoninarum* DNA from which the 1.7 kbp *EheI* fragment of the *mpr* gene to be fused was excised. (C) Structure of the *malE-mpr* fusion in pMAL.

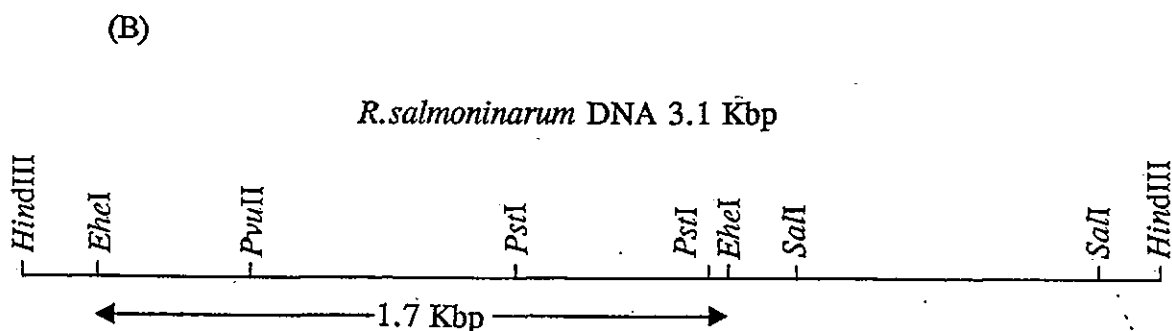


Polylinker:

malE... TCG AGC TCG GTA CCC GGC CGG GGA TCC ATC GAG GGT AGG CCT ... lacZα

Restriction sites: Sac I, Kpn I, Eag I, BamHI, Stu I, EcoRI, BamHI, Xba I, Sal I, Pst I, Hind III.

Factor Xa cleavage site: ATC GAG GGT AGG CCT (Ile Glu Gly Arg)

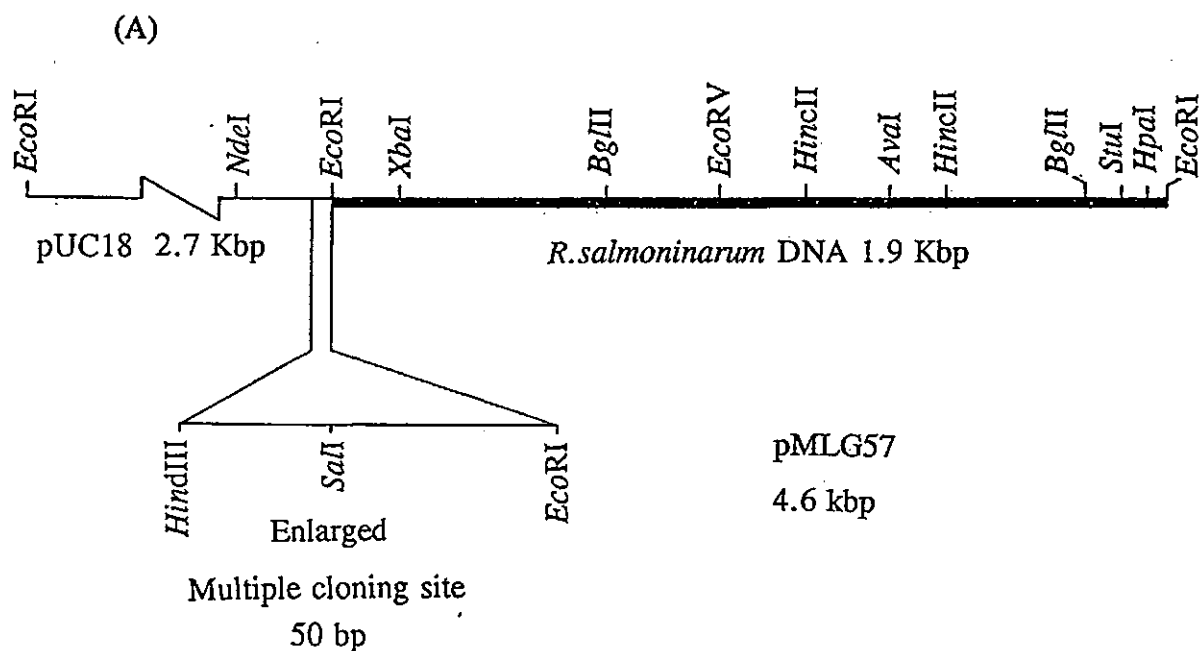


(C)

Factor Xa site *R. salmoninarum* MPR EheI fragment

malE.... ATC GAG GGT AGG GCC ACT ATC AAA TAA ...

I E G R A T I K STOP



(B)

	Factor Xa site	<i>R. salmoninarum</i> P57 <i>HpaI/SalI</i> fragment
<i>malE</i>	ATC GAG GGT AGG AAC ATT	TTT AAC TAA ...
	I E G R N I	F N STOP

(C)

	Factor Xa site	<i>R. salmoninarum</i> P57 <i>StuI/XbaI</i> fragment
<i>malE</i>	ATC GAG GGT AGG AAC ATT	TTT AAC TAA ...
	I E G R P D	F N STOP

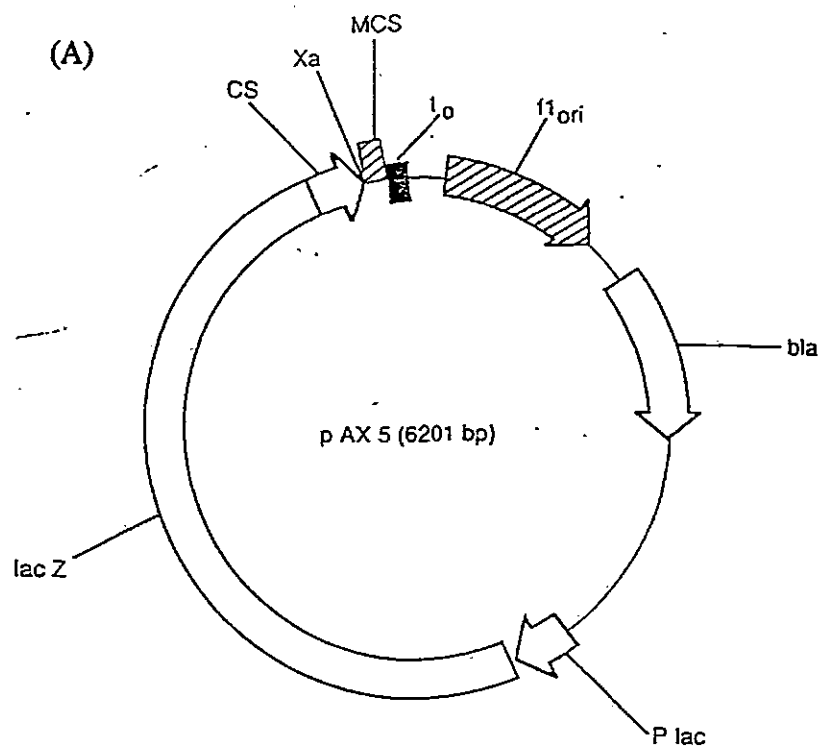
Figure 5.2 Cloning strategy for the construction of a fusion of the *malE* gene with the *msa* gene.

(A) Restriction map of the recombinant plasmid pMLG57 containing the fragment of *R. salmoninarum* DNA from which the portion of the *msa* gene to be fused was excised.

(B) Structure of the *malE*-*msa* fusion in pMAL using a 1.8 kbp *HpaI/SalI* inserted fragment.

(C) Structure of the *malE*-*mpr* fusion in pMAL using a 1.65 kbp *StuI/XbaI* inserted fragment.

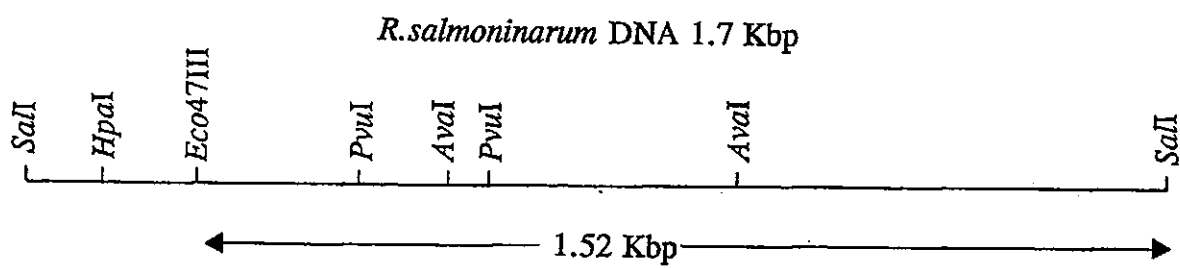
Figure 5.3 Cloning strategy for the construction of a fusion of the *lacZ* gene with the *hly* gene. (A) Diagrammatic representation of the pAX5+ vector including a restriction map of the multiple cloning site (MCS). *bla*: gene encoding ampicillin resistance; P_{lac}: inducible promoter positioned to transcribe a *lacZ* gene fusion; *lacZ*: gene encoding β -galactosidase; *t_o*: phage λ transcriptional termination sequence; f1 ori: phage f1 origin of replication; CS: sequence encoding a collagen fragment; Xa: factor Xa cleavage site. (B) Restriction map of the fragment of *R.salmoninarum* DNA from which the 1.52 kbp *Eco*47III/*Sa*I fragment of the *hly* gene to be fused was excised. (C) Structure of the *lacZ-hly* fusion in pAX5+.



5' -lacZ-CS-ATC GAG GGT CGC GAC GAC CAT GGA ATT CGG TAC CAG
 I E G R D D H G I R Y Q

..... XbaI SalI PstI SmaI
 ATC TAG AGT CGA CTG CAG CCC GGG TGATTGATTGA
 I STOP S R L Q P G STOP STOP
 STOP

(B)



(C)

Factor Xa site *R.salmoninarum* HLY Eco47III/SalI fragment

lacZ-CS- ATC GAG GGT CGG CTC GCT GCG CGT TAG ...

I E G R L A A R STOP

For gene *mpr*, a blunt-ended *EheI* site located at nucleotide 203 provided an in-frame fusion when ligated to the *StuI* site of the pMAL polylinker (Figure 5.1). This manipulation would yield 524 amino acids of metalloprotease with a molecular weight of 63,842 fused to MBP. Plasmid pRHLV'B' was cleaved with *EheI* overnight at 37°C in order to release a 1.7 kbp *EheI* fragment which was isolated and purified as described above. Plasmids pMAL-c and pMAL-p were cut with *StuI*, purified by Magic DNA Clean Ups and checked by agarose gel electrophoresis prior to ligation with the 1.7 kbp *EheI* fragment for 16 h at 20°C. The ligation was checked by agarose gel electrophoresis and then competent *E.coli* XL1-BLUE were transformed with the ligation mixture. Transformants were recovered after growth at 22°C on LBA plates supplemented with tetracycline (15 µg ml⁻¹), ampicillin (100 µg ml⁻¹) and 0.2% glucose and containing a 5 ml overlay of LBA with IPTG (192 µg ml⁻¹) and X-gal (160 µg ml⁻¹). Plasmid DNA was extracted from white colonies and digested with *PstI*, *BamHI* and *EcoRI* in separate reactions to confirm the orientation and identity of the fragment.

For gene *msa*, two blunt-ended restriction sites were chosen; *HpaI* located at nucleotide 180 and *StuI* located at nucleotide 228, which would provide in-frame fusions when ligated to the *StuI* site in the polylinker of the pMAL vectors. The *HpaI* truncated gene would encode a product of 513 amino acids and 49,795 molecular weight, whilst the *StuI* truncated gene would encode 497 amino acids of 47,613 molecular weight. Both of these truncated proteins would include the major epitopes of P57 which were identified by Chien, Gilbert, Huang, Landolt, O'Hara & Winton (1992). It was decided to directionally clone either a 1.88 kbp *HpaI/SalI* fragment or a 1.65 kbp *StuI/XbaI* fragment (Figure 5.2) which had been excised from pMLG57 and ligated into the polylinker of the pMAL vectors in order to enable the production of almost the full length of P57 fused to the carboxy-terminal end of maltose-binding protein (MBP) which has a molecular mass of 42,700 daltons. Plasmid pMLG57 containing the *msa* gene on a 1.9 kbp *EcoRI* insert was cleaved in separate reactions overnight at 37°C with either *HpaI* and *SalI* or *StuI* and *XbaI* (section 3.2.7). The desired fragments were separated by agarose gel electrophoresis (section 3.2.5), excised from the gel and electroeluted (section 4.2.2). The fragments were purified with Magic DNA Clean Ups (Promega) according to the manufacturer's instructions. Plasmids pMAL-c and pMAL-p were cut with either *StuI* and *SalI* or *StuI* and *XbaI* in separate reactions, purified with Magic DNA Clean Ups and ligated with the appropriate insert for 16 h at 20°C. Ligations were checked by agarose gel electrophoresis prior to the transformation of competent *E.coli* XL1-BLUE. Transformants were recovered as described above but were grown at 37°C. Plasmid DNA was extracted from white colonies (recombinant

subclones) and digested with the restriction endonucleases *Bam*HI, *Bgl*III, *Sal*I, *Stu*I and *Xba*I in separate reactions to confirm the orientation and identity of insert DNA.

A complete restriction map of pRHLY11, the plasmid bearing a 1.67 kbp *Sal*I fragment of *R.salmoninarum* DNA which included gene *hly*, was constructed by Evenden (1993). On the basis of this information the only suitable restriction site on the *hly* gene was a blunt-ended *Eco*47III site located at nucleotide 257 which provided an in-frame fusion when ligated to the *Nru*I site of the pAX5+ polylinker (Figure 5.3). This would yield a truncated gene product of 381 amino acids and 43,442 molecular weight fused to the β -galactosidase protein which possesses a molecular weight of 116,000. Plasmid pRHLY11 (Evenden, 1993) was cleaved with *Eco*47III and *Sal*I in separate reactions overnight at 37°C in order to release a 1.52 kbp *Eco*47III/*Sal*I fragment which was isolated and purified as described above. Plasmid pAX5+ was cleaved in separate reactions overnight at 37°C with *Nru*I and *Sal*I, purified by Magic DNA Clean Ups and checked by agarose gel electrophoresis prior to ligation with the 1.52 kbp *Eco*47III/*Sal*I fragment for 16 h at 20°C. Ligations were checked by agarose gel electrophoresis prior to the transformation of competent *E.coli* XL1-BLUE. Transformants were recovered at 37°C as described above. Blue/white screening for recombinants was not possible with the pAX5+ vector and plasmid DNA was extracted from a random selection of colonies, digested with *Ava*I, *Bam*HI, *Eco*RI and *Sal*I to confirm the presence and orientation of an insert.

5.2.3 IDENTIFICATION AND PURIFICATION OF FUSION PROTEINS

The procedures outlined below for the identification and purification of fusion proteins were performed according to the instructions provided by the manufacturers of the fusion vector systems used in this study, New England BioLabs and United States Biochemical Corporation, with some modifications. Following the isolation of pure clones containing plasmids with inserts of the correct size and orientation, 10 ml cultures were grown in LBB containing 100 μ g ml⁻¹ of ampicillin and 0.2% glucose at either 22°C or 37°C with good aeration until an absorbance at 620 nm of about 0.5 was reached ($A_{620} \approx 0.5$). A 1 ml sample was removed, centrifuged (9000 x g, 2 min) and the uninduced cell pellet was resuspended in 50 μ l of SDS-PAGE sample buffer (section 3.2.14). The remaining culture was then induced by adding 30 μ l of IPTG (24 mg ml⁻¹) and incubation was continued for a further 2 h at 37°C or 3 h at 22°C. A 0.5 ml sample was then removed, centrifuged (9000 x g, 2 min) and the cell pellet was resuspended in 100 μ l of SDS-PAGE sample buffer. Samples were boiled for 5 min and separated by SDS-PAGE on 10% gels with protein molecular weight standards and, in the case of MBP fusions, purified maltose-

binding protein (New England BioLabs). Following electrophoresis, the gels were either stained for protein using Coomassie Brilliant Blue R250 (section 3.2.14) or Western blotted onto nitrocellulose (section 3.2.15) and probed with either rabbit anti-MBP antiserum (New England BioLabs) or the IgG fraction of mouse anti- β -galactosidase antiserum (Sigma). The protocol has been described in section 3.2.15.1 but the following modifications were made here; rabbit anti-MBP was used at 1:10000 dilution and for the detection of β -galactosidase fusion proteins, mouse anti- β -galactosidase IgG fraction was used at 1:1000 followed by goat anti-mouse IgG peroxidase conjugate at 1:2000. This provided a means of confirming that an in-frame fusion protein was being produced by the clones.

A small scale pilot experiment was undertaken in order to determine the behaviour of each fusion protein; whether, for example, the fusion proteins were soluble or insoluble, bound effectively by the appropriate affinity resin and expressed at a sufficiently high level at different times and temperatures post-induction. Separate cultures of cells containing one of the fusion plasmids were grown overnight at either 22°C or 37°C in 10 ml of LBB containing 100 $\mu\text{g ml}^{-1}$ of ampicillin, 15 $\mu\text{g ml}^{-1}$ of tetracycline and 0.2% glucose to activate the *lacI^q* repressor. To 100 ml of prewarmed LBB containing 100 $\mu\text{g ml}^{-1}$ ampicillin and 0.2% glucose was added 0.8 ml of the overnight culture and incubation was continued at either 22°C or 37°C with good aeration until $A_{600} \approx 0.5$. A 1 ml sample of uninduced cells was then removed, centrifuged (9000 x g, 2 min) and the cell pellet was resuspended in 50 μl of SDS-PAGE sample buffer. To the remaining culture was added 350 μl of IPTG (24 mg ml^{-1}) and incubation was continued at either 22°C or 37°C. Samples (0.5 ml) of the IPTG induced cells were removed after 1, 2, 3 and 4 h, centrifuged (9000 x g, 2 min) and resuspended in an appropriate volume of SDS-PAGE sample buffer. All of the following steps were carried out either on ice or at 4°C to minimise protein degradation. At 2 h post-induction, 50 ml of the culture was harvested by centrifugation at 4000 x g, 4°C for 20 min. For pMAL-p fusion protein constructions, the cell pellet was resuspended in 10 ml of 30 mM Tris-HCl, 20% sucrose, pH 8.0 to which 20 μl of 0.5 M EDTA was added and the mixture was shaken at room temperature for 5 min. The cells were pelleted (8000 x g, 4°C, 10 min), the supernatant removed and the pellet was resuspended in 10 ml of ice-cold 5 mM MgSO_4 . The suspension was stirred for 10 min in an ice water bath before centrifuging at 8000 x g, 4°C for 10 min. The supernatant (cold osmotic shock fluid) was diluted 1:1 with SDS-PAGE sample buffer. For pMAL-c and pAX5+ constructions, where the fusion was expressed in the cytoplasm, the induced cell pellet was resuspended in 5 ml of column buffer which consisted of 20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4 for pMAL-c constructions

and 20 mM Tris-HCl, 10 mM MgCl₂, 0.5 M NaCl, 10 mM β -mercaptoethanol, pH 7.4 for pAX5+ constructions. Both 1 mM NaN₃ and 1 mM PMSF (phenylmethylsulphonylfluoride) were added to each column buffer to inhibit protein degradation. The pMAL-c and pAX5+ samples were frozen overnight at -20°C, thawed in cold water and sonicated on ice for 5 min using a 2 s cycle, 50% duty cycle (W-385 Ultrasonic Processor, Heat Systems Ultrasonics). The samples were centrifuged (9000 x g, 4°C, 20 min) and the supernatant (crude extract) removed and retained on ice. The pellets were resuspended in 5 ml of column buffer and retained as a suspension of insoluble material. Aliquots of the crude extract and insoluble matter were diluted 1:1 in SDS-PAGE sample buffer. A 50 μ l volume of either amylose resin (New England BioLabs) or APTG agarose resin (Sigma) was washed twice with 1.5 ml volumes of the appropriate column buffer and then mixed with 50 μ l of the appropriate crude extract. After incubating on ice for 15 min, the mixtures were centrifuged (9000 x g, 1 min), the supernatants were removed and the resin pellets were washed with 1 ml of column buffer. After centrifuging (9000 x g, 1 min), the resultant pellets were resuspended in 50 μ l of SDS-PAGE buffer. The samples of uninduced cells, induced cells (1,2,3 and 4 h post-induction), crude extracts, insoluble material, affinity resin and cold osmotic shock fluid which had been diluted in SDS-PAGE sample buffer, were boiled for 5 min and then separated on 10% SDS-PAGE gels. Following electrophoresis, the gels were either stained for protein with Coomassie Brilliant Blue (section 3.2.14) or Western blotted and developed with the appropriate antiserum as described above.

5.2.4 LARGE SCALE AFFINITY PURIFICATION OF FUSION PROTEINS

The purification of large quantities of the three fusion proteins involved scaling-up the pilot experiment on the basis of the observed behaviour of the fusions. Separate LBB cultures (1 litre) were inoculated with 10 ml of an overnight culture of cells containing the fusion plasmid and grown at 37°C in the presence of ampicillin (100 μ g ml⁻¹) and 0.2% glucose until $A_{600} \approx 0.5$. IPTG was added and incubation was continued either at 37°C for 3 h, in the case of *msa* and *hly* fusions, or at 22°C for 4 h for *mpr* fusions. All of the following steps were performed either on ice or at 4°C. Following induction, the cells were harvested quickly (4000 x g, 4°C, 20 min) and resuspended in 30 ml of the appropriate column buffer (section 5.2.3). The samples were then frozen overnight at -20°C prior to thawing in ice-cold water, sonication (section 5.2.3) and centrifuging for 30 min at 9000 x g and 4°C. The supernatant (crude extract) was retained on ice and diluted 1:5 with the appropriate column buffer. The pellet of insoluble matter was resuspended in 7 ml of column buffer, sonicated and pelleted as above and this supernatant was added to the first crude extract.

5.2.4.1 Fusions constructed in pMAL vectors

For the affinity isolation of MBP fusion proteins, 15 ml of amylose resin was poured into a 2 x 20 cm column (Pharmacia) and connected to a P1 peristaltic pump (Pharmacia), Uvicord II monitor set at 280 nm (Pharmacia), chart recorder (Pharmacia) and Ultrorac 7000 fraction collector (Pharmacia). The flow rate was adjusted to 1 ml min⁻¹ and the column was washed with 8 column volumes of column buffer. The diluted extract containing MBP fusion protein stored on ice was loaded onto the column and subsequently, the column was washed with 8 column volumes of column buffer. This process was monitored by tracing A₂₈₀ on the chart recorder to ensure that the amylose resin had been washed free of all protein other than the specifically bound MBP fusion protein. The fusion protein was eluted with column buffer containing 10 mM maltose, monitored by UV absorbance and collected in 3 ml fractions. The MBP fusion protein eluted in six fractions which were pooled, concentrated by centrifugation at 3000 x g, 4°C for 1.5 h using a Macrosep centrifugal concentrator (10K molecular weight cutoff, Flowgen Instruments Ltd., Kent, U.K.), aliquoted and stored at either 4°C or -20°C.

5.2.4.2 Fusions constructed in the pAX5+ vector

For the affinity isolation of β -galactosidase fusion proteins, 10 ml of APTG agarose resin (Sigma) was poured into a 2 x 20 cm column, connected and run as described above. The affinity resin was washed with 10 column volumes of column buffer (section 5.2.3) and then the diluted crude extract containing the β -galactosidase fusion protein stored on ice was loaded onto the column. After loading, the column was washed with 25 column volumes of column buffer until A₂₈₀ returned to baseline levels. The bound fusion protein was eluted with 0.1 M boric acid, pH 10.0, monitored by UV absorbance and collected in 3 ml fractions. The fusion protein eluted in five fractions which were pooled and mixed with an equal volume of 1 M Tris-HCl pH 7.0 in order to adjust the eluate to neutral pH. The pooled fractions were concentrated as described above, aliquoted and stored at either 4°C or -20°C. The purity of all fusions was checked by SDS-PAGE (section 3.2.14).

5.2.5 ANTISERA

Blood was collected as outlined below, clotted in glass at room temperature and then overnight at 4°C. Serum was withdrawn and stored at -70°C.

5.2.5.1 Immunisation of rats

Wistar rats were injected subcutaneously with 100 μ g of the appropriate protein emulsified in

Freund's complete adjuvant (FCA), boosted 3 weeks later with 100 μ g of the same protein emulsified in Freund's incomplete adjuvant (FIA), and then bled 3 weeks after the last injection. Control sera were gathered prior to immunisation.

5.2.5.2 Immunisation of rainbow trout

Rainbow trout with no prior history of BKD and of approximately 200 g in weight were obtained from Mill Leat Trout Farm, Ermington, Devon and maintained in a recirculating freshwater system at 12°C. Fish were injected intraperitoneally with 100 μ g of protein emulsified in FCA and boosted 3 weeks later with 100 μ g of the same protein emulsified in FIA. Fish were bled by dorsal aorta puncture 3 weeks after the last injection. Control sera were gathered prior to immunisation.

5.2.5.3 Rabbit anti-trout immunoglobulin M (IgM)

The preparation of rabbit anti-trout IgM occurred prior to this study and has been described by Grayson, Jenkins, Wrathmell & Harris (1991).

5.2.6 ELECTROPHORESIS AND WESTERN BLOTTING

All protocols have been described in section 3.2.14 and 3.2.15.

5.2.6.1 Antisera from immunised rats

The procedure used was as described in section 3.2.15.1 except that a rabbit anti-rat IgG peroxidase conjugate (Dako) was employed for detection.

5.2.6.2 Antisera from immunised rainbow trout

Blots were washed for 3 x 5 min in 0.02 M Tris-HCl containing 0.5 M NaCl (Tris-saline, pH 7.5) and then for 3 x 5 min in Tris-saline containing 0.5% skimmed milk (Tris-saline-milk). Blots were incubated for 3 h in primary antiserum diluted 1:100 in Tris-saline-milk, washed (3 x 5 min in Tris-saline), then incubated in secondary antibody (rabbit anti-trout IgM) diluted 1:500 in Tris-saline-milk, washed and then incubated for 1 h in swine anti-rabbit immunoglobulins peroxidase conjugate diluted 1:1000 in Tris-saline-milk. The blots were washed in Tris-saline (3 x 10 min) prior to development (section 3.2.15.1). Identical blots were probed with control sera.

5.3 RESULTS

5.3.1 CONSTRUCTION AND PURIFICATION OF THE MBP-MPR FUSION PROTEIN

The construction of a recombinant plasmid, designated pMMPR containing an in-frame fusion between *mpr* and *malE* is outlined in Figure 5.4 and described in section 5.2.2. The fusion protein was expressed cytoplasmically in *E.coli* by pMAL-c only, because of the difficulty in isolating clones expressing the fusion in the pMAL-p vector. The pMMPR recombinant plasmid allowed the production of a fusion protein composed of the entire maltose-binding protein molecule (42,700 molecular weight) fused at the carboxy terminus, with a short factor Xa cleavage site, to 524 amino acids (63,842 molecular weight) derived from *R.salmoninarum* metalloprotease (MPR). The small scale pilot experiment showed that the MBP-MPR fusion protein was expressed at a high level and mainly in a soluble form, from 2-4 h post-induction (Figure 5.5). Because considerable breakdown and instability was observed at 37°C, IPTG induction was carried out at 22°C. Inducing at 22°C in conjunction with a second sonication of the insoluble cell material greatly improved the stability and yield of MBP-MPR. The MBP-MPR protein was purified from the cytoplasm of *E.coli* XL1-BLUE/pMMPR by using a one-step affinity chromatography procedure. The purified MBP-MPR protein migrated as a single band on reduced SDS-PAGE with an apparent molecular mass of 105 kDa which was similar to the predicted molecular mass of 106,542 Da. The degradation of the MBP-MPR protein observed in the small scale pilot experiment was greatly reduced in the large scale purification method.

Figure 5.4 Physical map deduced for pMMPR using restriction enzyme digests analysed by agarose gel electrophoresis. Track 1: 1 kbp ladders; track 2: pRHLY'B' cleaved with *EheI* to release a 1.7 kbp fragment in addition to other vector derived fragments; track 3: pMMPR cleaved with *BamHI* released a single 1.742 kbp fragment in addition to 6.103 kbp of vector derived DNA; track 4: pMMPR cleaved with *PstI* released three fragments of 83, 517 and 7245 bp thereby confirming both the identity and orientation of the 1.7 kbp *EheI* DNA insert within pMAL-c; track 5: pMMPR cleaved with *EcoRI* produced a single linear band of 7845 bp.

Figure 5.5 Production of an MBP-MPR fusion protein in *E. coli* XL1-BLUE containing pMMPR. Cultures were grown at 22°C until $A_{600} \approx 0.5$ then induced with IPTG for 2-4 h. (A) 10% SDS-PAGE gel stained with Coomassie brilliant blue R. (B) Western blot of a 10% SDS-PAGE gel probed with rabbit anti-MBP antiserum. Track 1: protein molecular weight standards (kDa); track 2: uninduced cells; track 3: cells induced with IPTG for 2 h; track 4: cells induced for 3 h; track 5: cells induced for 4 h; track 6: insoluble cellular material; track 7: soluble cellular material; track 8: MBP-MPR purified from the soluble cellular extract by amylose resin affinity chromatography.

5.3.2 CONSTRUCTION AND PURIFICATION OF THE MBP-P57 FUSION PROTEIN

Recombinant plasmids, designated either pMC57HS or pMP57SX were isolated which possessed the *msa* gene inserted in-frame with *malE* as either a 1.88 kbp *HpaI/SalI* fragment or a 1.65 kbp *StuI/XbaI* fragment in either the pMAL-c or pMAL-p vectors, respectively (Figures 5.6 & 5.7). Despite this, the fusion protein isolated in the small scale pilot experiment showed evidence of severe degradation with a molecular weight on SDS-PAGE of 55,000 regardless of the vector (Figure 5.8). Only a tiny proportion of each of the fusion proteins was found to possess the expected molecular weights of about 90,313 (MBP-*StuI/XbaI* fusion) or 92,495 (MBP-*HpaI/SalI* fusion). Reducing the temperature during induction had no obvious effect on this breakdown process. Nevertheless, the MBP-P57 fusion still contained major epitopes which were recognised on Western blots by rabbit anti-*R.salmoninarum* ECP antiserum (Figure 5.9) and the fusion was therefore considered to be of use for further immunological study. In order to obtain a maximum yield, MBP-P57 fusion protein was purified from the cytoplasm of *E.coli* XL1-BLUE/pMC57HS by the one-step affinity chromatography procedure described in section 5.2.4.1 (Figure 5.9). P57 was fused to MBP starting from Asn-45 which is 18 amino acids downstream from the N-terminal serine which is exposed following cleavage of the signal peptide (Chien *et al.*, 1992).

5.3.3 CONSTRUCTION AND PURIFICATION OF THE β -GALACTOSIDASE-HLY FUSION PROTEIN

The construction of a recombinant plasmid, designated pGHLY, containing an in-frame fusion between *lacZ* and *hly* is outlined in Figure 5.10 and described in section 5.2.2. The small scale pilot experiment showed that the β -galactosidase-HLY fusion protein was expressed at a high level and mainly in a soluble form, from 1-4 h post-induction although considerable breakdown was observed (Figure 5.11). Inducing at 22°C served to reduce the yield of fusion protein without obviously reducing the breakdown of the protein. Hence, IPTG induction was carried out at 37°C. The β -galactosidase-HLY protein was purified from the cytoplasm of *E.coli* XL1-BLUE/pGHLY by using the one-step affinity chromatography method described in section 5.2.4.2. The purified β -galactosidase-HLY protein migrated as a single band on reduced SDS-PAGE with a calculated molecular weight of 160,000, which was close to the predicted size of 159,500. The degradation of the β -galactosidase-HLY protein which had been observed in the pilot experiment was greatly reduced in the large scale purification procedure. HLY was fused to β -galactosidase starting from Leu-36 which is close to the carboxy terminal end of the putative membrane-spanning portion of HLY located at Ala-37 (Evenden, 1993).

Figure 5.6 Physical map deduced for pMP57SX using restriction enzyme digests analysed by agarose gel electrophoresis. Track 1: pMLG57 cut with *StuI/XbaI* released a 1.65 kbp fragment in addition to 2.91 kbp of vector derived DNA. The band located at about 4.5 kbp represents pMLG57 which has cut only once; track 2: pMP57SX cleaved with *XbaI* produced a single linear band of 7.87 kbp; track 3: pMP57SX cleaved with *StuI/XbaI* released a single 1.65 kbp fragment in addition to 6.22 kbp of vector derived DNA; track 4: pMP57SX cleaved with *BglII* released three fragments of 905, 1089 and 5876 bp thereby confirming both the identity and orientation of the 1.65 kbp *StuI/XbaI* DNA insert within pMAL-p; track 5: 1 kbp ladders.

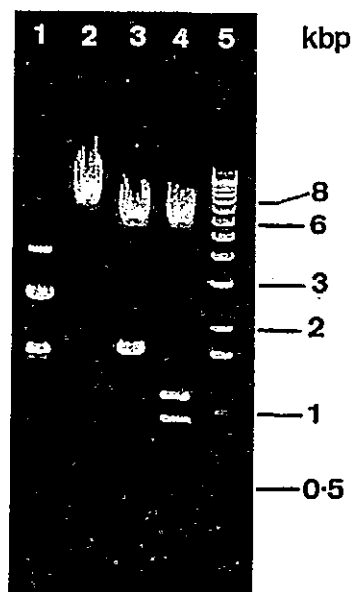
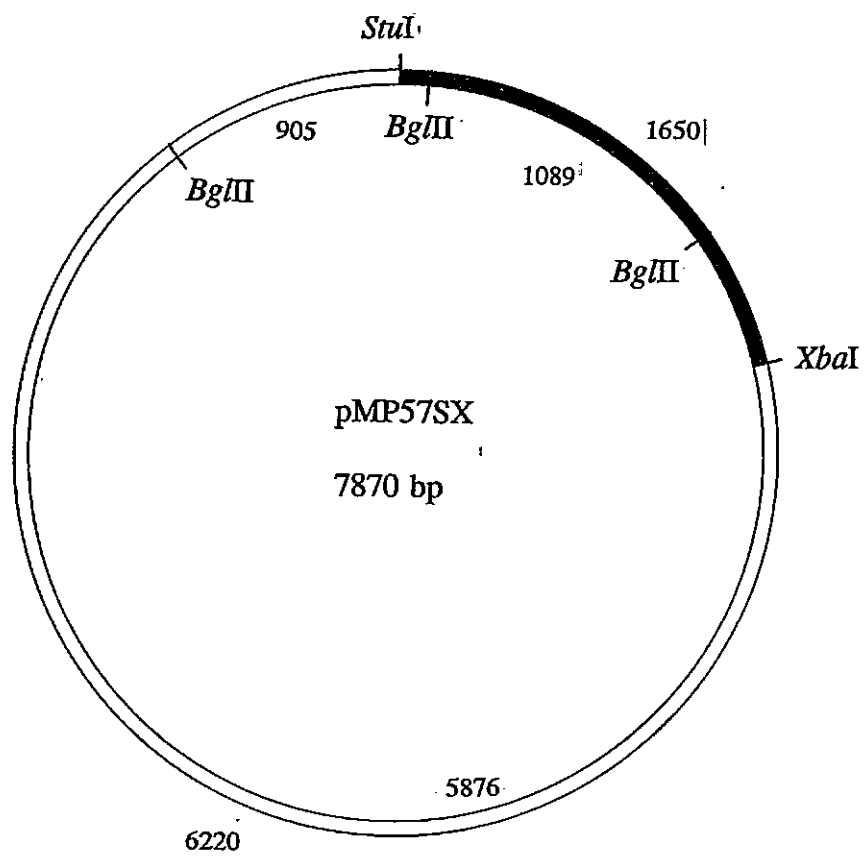
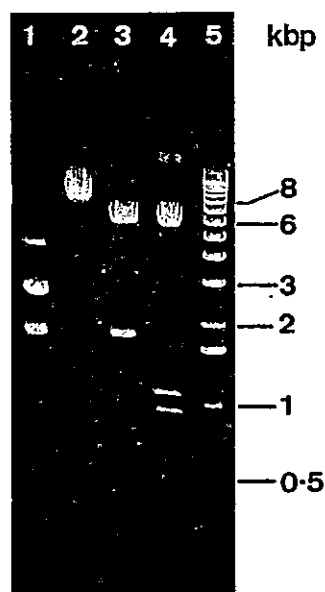
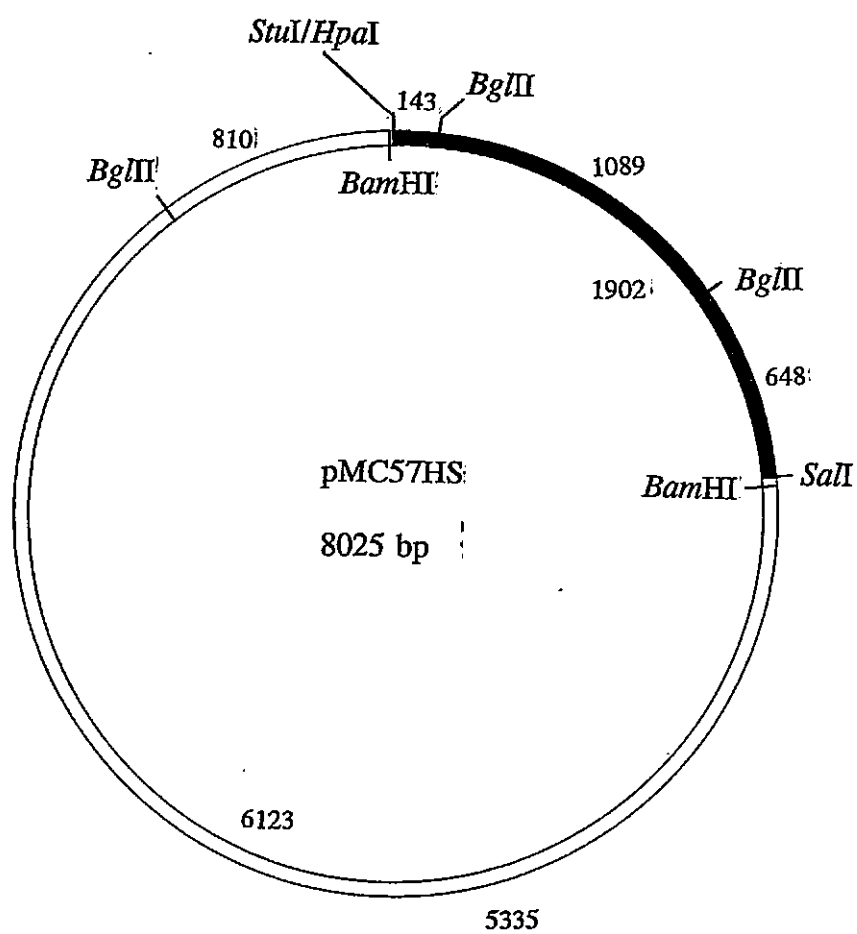


Figure 5.7 Physical map deduced for pMC57HS using restriction enzyme digests analysed by agarose gel electrophoresis. Track 1: pMLG57 cut with *HpaI/SalI* released a 1.88 kbp fragment in addition to 2.71 kbp of vector derived DNA. The band located at about 4.5 kbp represents pMLG57 which has cut only once; track 2: pMC57HS cleaved with *SalI* produced a single linear band of 8.025 kbp; track 3: pMC57HS cleaved with *BamHI* released a single 1.902 kbp fragment in addition to 6.123 kbp of vector derived DNA; track 4: pMC57HS cleaved with *BglII* released three fragments of 953, 1089 and 5983 bp thereby confirming both the identity and orientation of the 1.88 kbp *HpaI/SalI* DNA insert within pMAL-c; track 5: 1 kbp ladders.



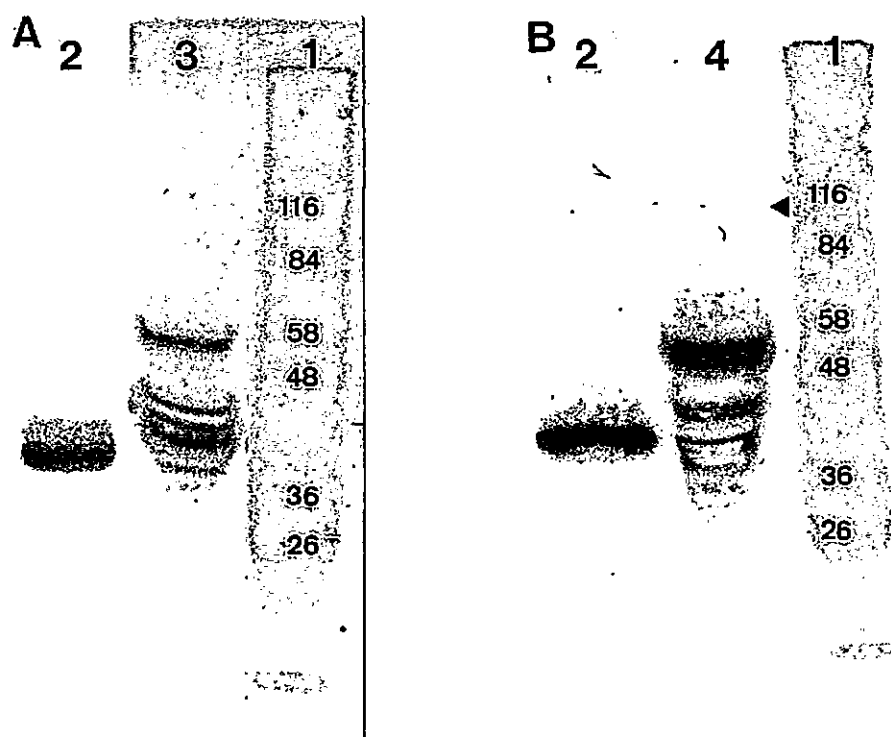
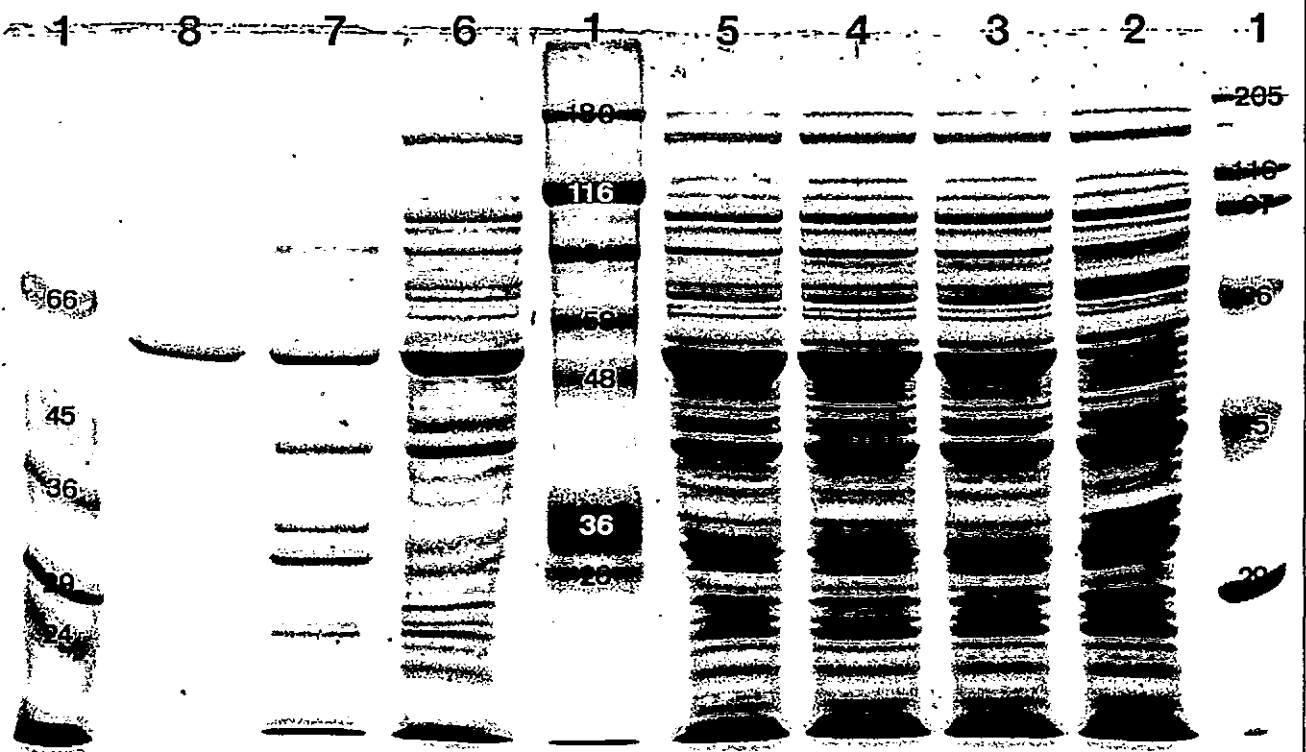


Figure 5.8 Production of an MBP-P57 fusion protein in *E. coli* XL1-BLUE containing either (A) pMP57SX or (B) pMC57HS. Cultures were grown at 37°C until $A_{600} \approx 0.5$ then induced with IPTG for 2 h. Western blots of a 10% SDS-PAGE gel were probed with rabbit anti-MBP antiserum. Track 1: protein molecular weight standards (kDa); track 2: pure maltose-binding protein; track 3: cells containing pMP57SX; track 4: cells containing pMC57HS. The arrow indicates the tiny amount of full length MBP-P57 fusion protein which remained intact.

Figure 5.9 Production of an MBP-P57 fusion protein in *E.coli* XL1-BLUE containing pMC57HS. Cultures were grown at 37°C until $A_{600} \approx 0.5$ then induced with IPTG for 1-3 h. (A) 10% SDS-PAGE gel stained with Coomassie brilliant blue R. (B) Western blot of a 10% SDS-PAGE gel probed with rabbit anti-*R.salmoninarum* ECP antiserum. Track 1: protein molecular weight standards (kDa); track 2: uninduced cells; track 3: cells induced with IPTG for 1 h; track 4: cells induced for 2 h; track 5: cells induced for 3 h; track 6: insoluble cellular material; track 7: soluble cellular material; track 8: MBP-P57 purified from the soluble cellular extract by amylose resin affinity chromatography. The arrow indicates the tiny proportion of full length MBP-P57 fusion protein which remained intact.

A



B 2 3 4 5 1 6 7 8

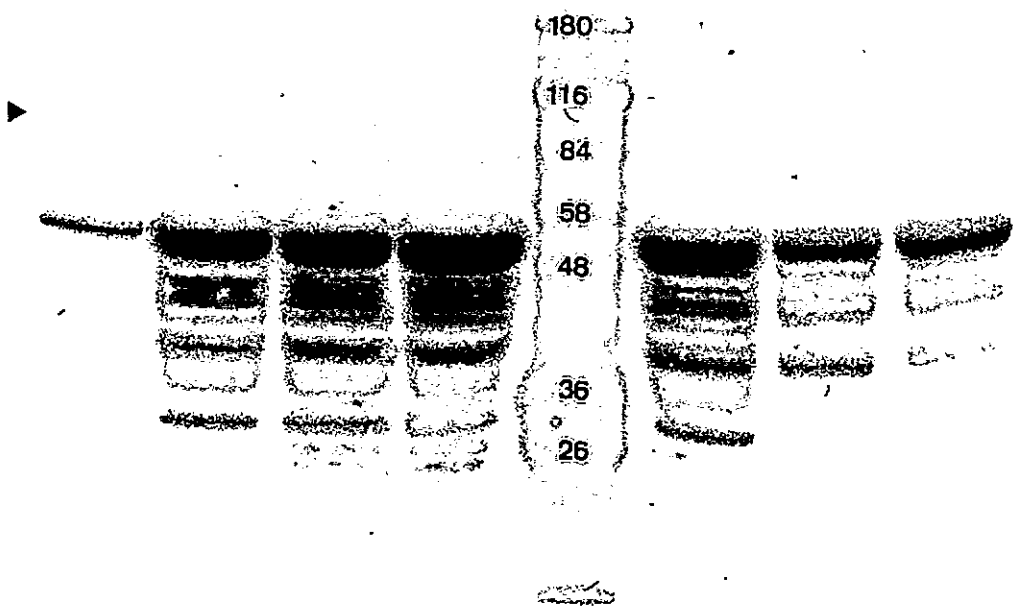


Figure 5.10 Physical map deduced for pGHLY using restriction enzyme digests analysed by agarose gel electrophoresis. Track 1: 1 kbp ladders; track 2: pRHL11 cleaved with *Eco47III/SalI* to release a 1.52 kbp fragment in addition to 5 kbp of pBR328 vector DNA. The other bands in this track are the result of a partial digest and include a 3 kbp fragment of supercoiled plasmid DNA, a 6.5 kbp linear fragment of pRHL11; track 3: pGHLY cleaved with *BamHI* released three fragments of 569, 1152 and 5899 bp which confirmed the orientation of the 1.52 kbp *Eco47III/SalI* fragment within pAX5+; track 4: pGHLY cleaved with *AvaI* released five fragments of 423, 623, 855, 1443 and 4267 bp thereby confirming the identity of the 1.52 kbp *Eco47III/SalI* DNA insert within pAX5+; track 5: pGHLY cleaved with *SalI* produced a single linear band of 7620 bp; track 6: pGHLY incubated with *EcoRI* remained uncut.

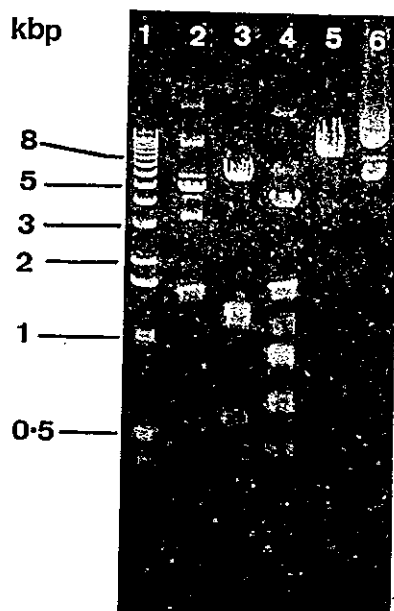
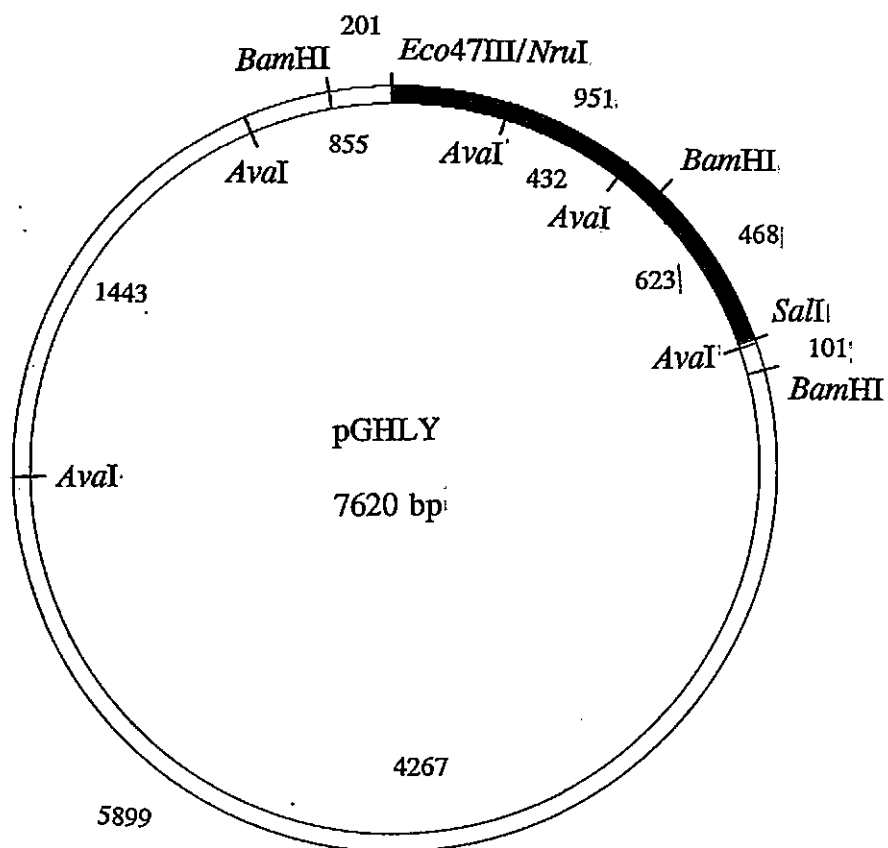
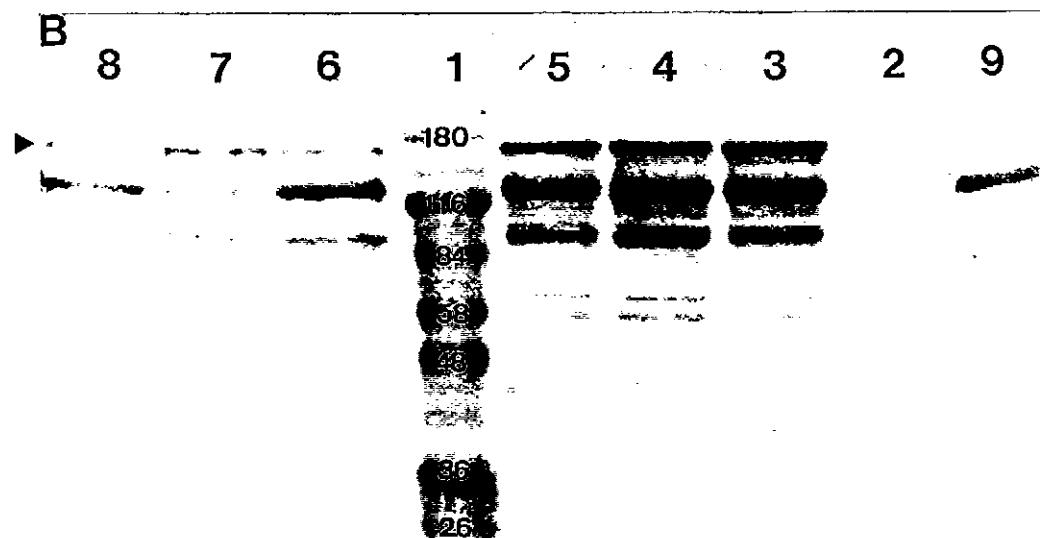
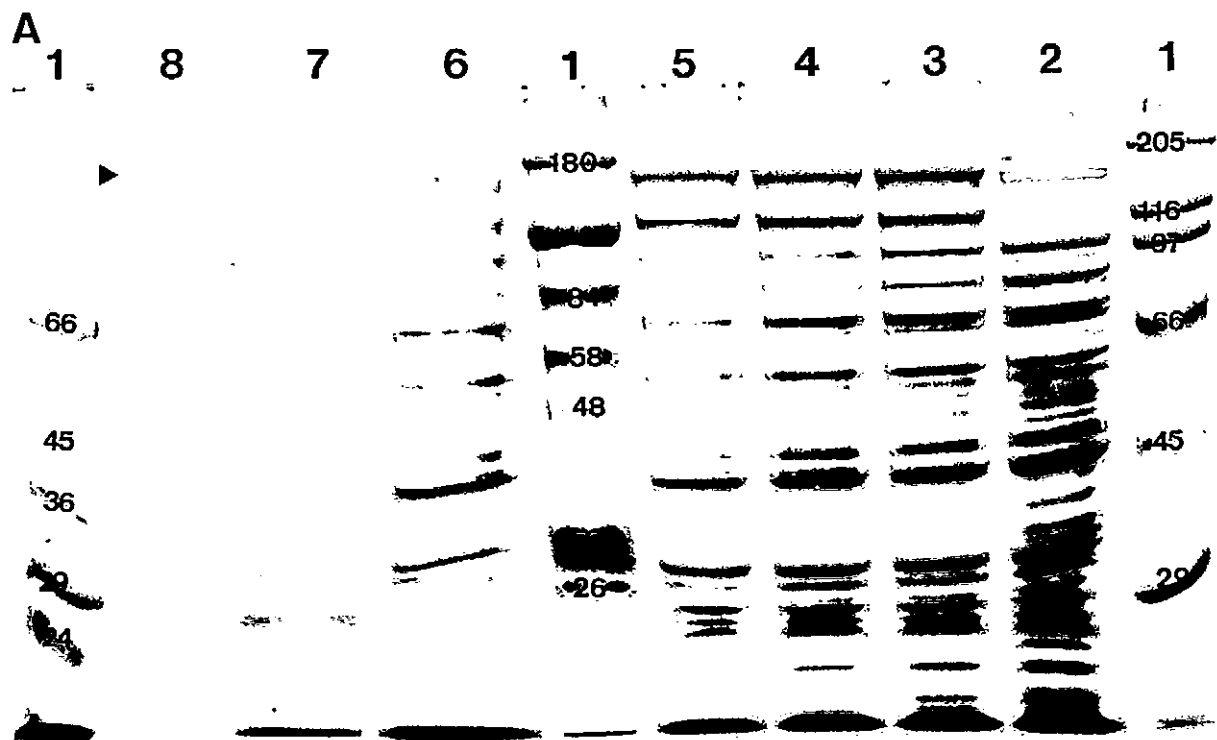


Figure 5.11 Production of an β -galactosidase-HLY fusion protein in *E.coli* XL1-BLUE containing pGHLY. Cultures were grown at 37°C until $A_{600} \approx 0.5$ then induced with IPTG for 1-3 h. (A) 10% SDS-PAGE gel stained with Coomassie brilliant blue R. (B) Western blot of a 10% SDS-PAGE gel probed with mouse anti- β -galactosidase antiserum. Track 1: protein molecular weight standards (kDa); track 2: uninduced cells; track 3: cells induced with IPTG for 1 h; track 4: cells induced for 2 h; track 5: cells induced for 3 h; track 6: insoluble cellular material; track 7: soluble cellular material; track 8: β -galactosidase-HLY purified from the soluble cellular extract by APTG-agarose affinity chromatography; track 9: pure β -galactosidase. The position of the full length β -galactosidase-HLY is arrowed.



5.3.4 ANTIGENICITY OF THE HYBRID EPITOPES IN RATS AND RAINBOW TROUT

Each of the purified fusion proteins was found to be antigenic in both rats (Figure 5.12A) and rainbow trout (Figure 5.12B) and the non-toxic nature of these products at the dosages used in this study was confirmed. Western blots of each fusion protein were probed with antiserum from the animal which had been immunised with the corresponding fusion protein. Specific antibodies raised in rats identified the corresponding fusion protein as a full length molecule as well as a variety of breakdown products (Figure 5.12A). MBP-P57 appeared as a full length fusion protein of estimated molecular weight 90,000 with the largest proportion of the protein degraded to 55K or less. MBP-MPR was detected as a predominant band with a calculated molecular weight of 105,000 with several smaller degradation products whilst β -galactosidase-HLY was mainly recognised as a full length 160K fusion protein. Compared to the antibody response in immunised rats, immunised rainbow trout showed a poorer antibody response to the fusion proteins. Western blots of the fusion proteins which were probed with the appropriate rainbow trout antiserum (Figure 5.12B) were much less intensively stained than blots probed with the corresponding rat antiserum (Figure 5.12A) even though the antigen concentrations were identical. Nevertheless, a weak specific antibody response in the trout to the major components of each fusion protein, as described for rat antisera, was observed. No immunological recognition of the fusion proteins was evident on blots probed with control sera from either rats or trout.

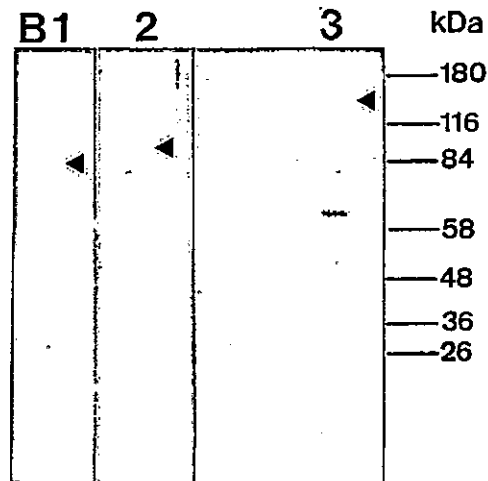
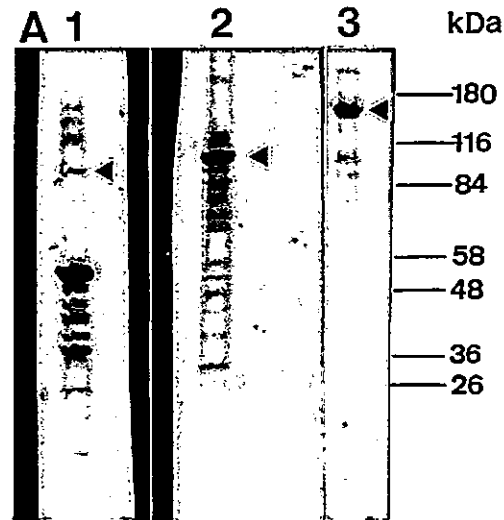


Figure 5.12 Immunological detection of purified fusion proteins by Western blotting. Approximately 1 μ g of each protein was separated by SDS-PAGE using a 10-15% gradient gel, transferred onto nitrocellulose and probed with either (A) a rat antiserum or (B) a trout antiserum raised against the corresponding protein. Track 1: MBP-P57; track 2: MBP-MPR; track 3: β -galactosidase-HLY. Protein molecular weight standards in kilodaltons are shown. The positions of the full length fusion proteins are marked with arrows. The remaining bands are considered to represent breakdown products of each fusion protein.

5.4 DISCUSSION

Three separate fusion proteins were constructed, successfully expressed within the cytoplasm of *E. coli* XL1-BLUE and sufficient quantities of each were purified to enable further immunological studies. Whilst each of the three fusion proteins was expressed in a mainly soluble form, their stability was found to vary. In the case of MBP fused to *R. salmoninarum* metalloprotease (MPR), the problem of degradation was largely overcome by inducing with IPTG at 22°C, suggesting that the breakdown observed at 37°C may have been due to the action of *E. coli* proteases. The MBP-MPR fusion protein was expressed cytoplasmically by pMAL-c because of the difficulty in isolating clones expressing the fusion in the pMAL-p vector. This was by no means a disadvantage since pMAL-c fusion proteins tend to be more stable and are expressed at a much higher level than fusion proteins which are exported to the periplasm (Riggs, 1990). MPR was fused to MBP starting from Ala-25, which is four amino acids upstream of the predicted signal peptide cleavage point at Ala-28 (Chapter 4). Hence, cleavage of MPR from MBP with factor Xa should yield a product with only four additional amino acids than would be expected in the full length MPR protein. It could be anticipated that the consequent interference with the correct folding, biochemical and immunological properties of MPR would be minimal.

Similarly, the construction of a β -galactosidase-HLY fusion protein in the plasmid vector pAX5+ allowed the production and purification of considerable quantities of stable, soluble full length fusion protein with an estimated molecular weight of 160,000. The fusion of HLY to the carboxy terminus of β -galactosidase starting from Leu-36 will allow the production of almost the full length protein based on the predictions made by Evenden (1993). Following cleavage from the β -galactosidase portion it is therefore likely that the recombinant HLY protein will retain most, if not all, of the characteristics of the native enzyme.

The production of MBP-P57, either within the cytoplasm or following export to the periplasmic space of *E. coli* XL1-BLUE, was accompanied by considerable degradation of the fusion protein regardless of the growth temperature and despite the care taken to carry out the purification of the fusion proteins as quickly as possible and at 4°C. The identity and orientation of the DNA inserts within each pMAL vector was confirmed by cleaving the recombinant plasmids with *Bam*HI, *Bgl*II, *Eco*RI, *Hpa*I, *Sal*I, *Stu*I and *Xba*I. No evidence could be found to show that the inserted DNA was oriented incorrectly or was a fragment of DNA other than that which contained the *msa* gene. In addition, an examination of the nucleotide sequence of *msa* showed that several out of frame stop codons were present within 120 bases of either the *Hpa*I or *Stu*I sites. In the event of

a frame shift, translation would be terminated within 20 amino acid residues of the MBP fusion joint and this would be insufficient to account for the observed molecular weight of 55,000 for the major portion of the MBP fusion protein. It is possible that the MBP-P57 fusion protein undergoes some form of autoproteolytic processing which predominantly yields a truncated fusion protein of molecular weight 55,000 and a variety of smaller breakdown products. Studies of native P57 derived from *in vitro* cultures of *R.salmoninarum* have shown that the P57 protein does possess an inherent instability (Griffiths & Lynch, 1991) although this has been associated with the presence of a serine protease of molecular mass about 105 kDa by other workers (Rockey, Turaga, Weins, Cook & Kaatari, 1991). Further examination of the amino acid sequence for P57 which was derived from *msa* by Chien *et al.*(1992) revealed the presence of a consensus serine protease motif, G-X-S-X-G (Aitken, 1990), located from Gly-406 to Gly-410. Whether P57 undergoes autoproteolytic processing has yet to be conclusively established but it seems that when gene *msa* is expressed in *E.coli* the P57 protein is rapidly processed. The availability of the cloned *msa* sequence which encodes P57 will greatly simplify any future work, such as site-directed mutagenesis, aimed at characterising the precise role of individual amino acid residues in the biochemical and immunological properties of the protein. One approach to controlling the proteolytic degradation of cloned proteins expressed in *E.coli* has been the use of mutant strains which are deficient in the production of one or more of the proteolytic enzymes of *E.coli*. There are at least 36 different *E.coli* proteases which have been identified and of these, two ATP-dependent proteases, *lon* and *clp*, are responsible for about 65-75% of the protein degradation occurring within the *E.coli* cytoplasm (Maurizi, 1992). The use of mutant strains which are deficient in the production of both *lon* and *clp* proteases may provide a means of reducing the degradation of fusion proteins expressed within *E.coli* if these proteases are responsible. However, attempts to obtain mutant strains of *E.coli* which were deficient in the *lon* and *clp* proteases for use in this study from the Coli Genetic Stock Centre, Yale University School of Medicine, New Haven, Connecticut, U.S.A., were unsuccessful. An alternative approach to the use of mutant strains of *E.coli* may be through the use of site-directed mutagenesis. Recent work by Kawamoto, Shibano, Fukushima, Ishii, Morihara & Okuda (1993) has shown that the proteolytic activity of *P.aeruginosa* elastase may be dramatically reduced by single amino acid substitutions in the catalytic centre of the molecule. The mutant elastases were found to retain their immunogenicity and provided an efficacious vaccine. Yet another possibility lies in the identification of smaller fragments of each protein which carry immunologically relevant information and can be subcloned to produce a more stable fusion. This approach has led to the successful development of an infectious haematopoietic necrosis virus (IHNV) vaccine based on the fusion of the *trpE* protein

of *E.coli* with a hydrophilic segment of 104 amino acids from the middle portion of the viral glycoprotein (Gilmore, Engelking, Manning & Leong, 1988).

Despite some difficulties with degradation, a sufficient quantity of each fusion protein was obtained to enable the production of specific antibodies in both rats and rainbow trout against the hybrid epitopes of the fusion proteins. This initial study will therefore enable further work to be undertaken aimed at biochemically and immunologically characterising the two previously undescribed membrane-active proteins, MPR and HLY, derived from *R.salmoninarum*. In particular, the investigation of some means of boosting the weak humoral responses to the fusion proteins which were observed in immunised rainbow trout. This may be resolved simply by altering the antigen dosage, adjuvant, number of boosters or the route of exposure (Ellis, 1988). Alternatively, the use of a live, attenuated vector as an antigen-delivery system may prove to be more effective (Chatfield, Li, Sydenham, Douce & Dougan, 1992). In addition, the identification of the number and minimum size of epitopes may be useful for eliciting cellular and humoral immune responses in immunised fish and in the evaluation of protective antigens. Epitope mapping has been applied to the study of many other immunologically important molecules, such as listeriolysin (Nato, Reich, Lhopital, Rouyre, Geoffroy, Mazie & Cossart, 1991) and elastase (Lagace & Frechette, 1991), and is especially useful for identifying epitopes which neutralise enzyme activity. This approach has led to the construction of fusion proteins which elicit a protective immune response when delivered as subunit vaccines against the major fish diseases IHNV (Gilmore *et al.*, 1988) and furunculosis (Bennett, Whitby & Coleman, 1992; Coleman, Bennett, Whitby & Bricknell, 1992). Whilst P57, the major secretory antigen of *R.salmoninarum*, has been characterised by previous researchers (reviewed in Chapter 2) the availability of the MBP-P57 fusion protein will allow an investigation of the controlled, targeted delivery of P57 epitopes to the salmonid immune system. In addition, it would be possible to construct chimaeric proteins which may provide protection against more than one disease, such as the diphtheria toxin A-pertussis toxin subunit vaccine (Barbieri, Armellini, Molkentin & Rappuoli, 1992).

Whilst the primary consideration would be to evaluate the efficacy of each fusion protein as an immunogen, it would also be possible to investigate the use of a live, attenuated bacterial carrier for the delivery of candidate antigens to the immune system (Cardenas & Clements, 1992). Live vectors have been used as a means of targeting the delivery and presentation of antigens to the immune system, thereby enhancing the appropriate humoral and cellular immune responses to delivered molecules (Dougan, 1989). In most instances, vaccinia virus (Perkins, Piccini, Lipinkas

& Paoletti, 1985; Hruby, Schneewind, Wilson & Fischetti, 1991), *Salmonella* species (Cardenas & Clements, 1992) or Gram positive bacteria such as mycobacteria (Stover, de la Cruz, Fuerst, Burlein, Benson, Bennett, Bansal, Young, Lee, Hatfull, Snapper, Barletta, Jacobs & Bloom, 1991) have been used successfully for this purpose. In the *Salmonella* vector, cloned antigens are often either expressed cytoplasmically or translocated to the periplasmic space and delivered to the immune system after cell lysis (Nakayama, Kelly & Curtiss, 1988). The resultant immune response may display both a strong humoral and cellular component (Brown, Hormaeche, de Hormaeche, Winther, Dougan, Maskell & Stocker, 1987; Flynn, Weiss, Norris, Seifert, Kumar & So, 1990). This approach has led to the successful development of an oral tetanus vaccine (Fairweather, Chatfield, Makoff, Strugnell, Bester, Maskell & Dougan, 1990). The use of such methods does not provide for the precise delivery of antigens and may prove insufficient for some applications (Stabel, Mayfield, Tabatabai & Wannamuehler, 1990). Consequently, other methods for the expression of immunogens on the exposed surface of bacterial carriers have been sought in order to improve the desired immune response (Newton, Jacob & Stocker, 1989; Pozzi, Contorni, Oggioni, Manganelli, Tommasino, Cavalieri & Fischetti, 1992). An ideal candidate for the construction of a BKD vaccine delivery system would be the recently developed non-revertant *aroA* mutant of *Aeromonas salmonicida* which has been shown to persist in the fish host for a period sufficient to induce a protective immune response against the pathogen which involves both humoral and cellular defences (Vaughan, Smith & Foster, 1993).

The *R.salmoninarum* fusion proteins produced in this work will allow a range of studies to be undertaken relating to their structure and function. Their ability to elicit an antibody response in rainbow trout shows the potential for the investigation of their usefulness in the future development of immunogens for the vaccination of salmonids against BKD.

CHAPTER 6.

IMMUNOLOGICAL DETECTION OF HYBRID EPITOPES OF CLONED FUSION PROTEINS IN *R.salmoninarum* CULTURED *in vitro* AND UNDER CONDITIONS OF IRON RESTRICTION

6.1 INTRODUCTION

The ability of an invading pathogen to multiply successfully within the host environment is a commonly shared and essential factor in all infections. Here, the complex interactions with the host may elicit physiological changes within the pathogen growing *in vivo* which may not be reflected during *in vitro* growth. In this respect the availability of iron may have dramatic effects because it is essential for bacterial growth and replication.

For many years, researchers in a variety of fields have firmly established not only the general importance of iron in cell physiology but also a correlation between iron assimilation and host defence against infection. This research has been comprehensively reviewed by Winkelmann, van der Helm & Neilands (1987) and Bullen & Griffiths (1987). The ability to obtain iron *in vivo* is an important virulence determinant for many microbes including intracellular bacterial pathogens such as *Listeria monocytogenes* (Sword, 1966; Alford, King & Campbell, 1991), *Legionella pneumophila* (Byrd & Horwitz, 1989; 1991) and *Shigella* species (Payne, 1989). Since iron is not normally freely available in body tissues and fluids, microorganisms have evolved diverse mechanisms for acquiring iron *in vivo*. These include:

- (i) the production of low molecular weight high-affinity iron chelators, known as siderophores, that can remove iron from transferrin or cell-derived sources as shown by enteropathogenic strains of *E.coli* (Brock, Williams, Liceaga & Wooldridge, 1991).
- (ii) the presence of specific receptors for transferrin and/or lactoferrin on the bacterial cell surface as shown by *Neisseria gonorrhoeae* (Blanton, Biswas, Tsai, Adams, Dyer, Davis, Koch, Sen & Sparling, 1990).
- (iii) the reduction of ferric iron to ferrous complex which may be readily bound and transported by the bacteria as shown by *L.monocytogenes* (Cowart & Foster, 1985; Adams, Vartivarian & Cowart, 1990), *L.pneumophila* (Johnson, Varner & Poch, 1991) and *Streptococcus mutans* (Evans, Arceneaux, Byers, Martin & Aranha, 1986).
- (iv) the proteolytic cleavage of transferrin and/or lactoferrin in order to release iron as shown by certain strains of *Aeromonas salmonicida* (I.Hirst, personal communication).

(v) the ability to bind and utilise haem compounds released as a result of haemolysis as shown by *Bacteroides fragilis* (Otto, Verweij-van Vught, van Doorn & Maclaren, 1988; Otto, Sparrius, Verweij-van Vught & Maclaren, 1990), *Bacteroides gingivalis* (Carman, Ramakrishnan & Harper, 1990) and *Vibrio vulnificus* (Nishina, Miyoshi, Nagase & Shinoda, 1992).

In most bacteria it has been noted that iron transport involves more than one of the above mechanisms. In *E.coli*, for example, at least five Fe^{3+} transport systems specific for siderophore chelating iron and one Fe^{2+} transport system have been described (Hantke, 1987). Many of the proteins and genes involved in these transport processes have been characterised (reviewed by Winkelmann *et al.*, 1987).

By restricting the availability of iron in culture media it is possible to imitate, at least with respect to iron, the *in vivo* condition and then to test for the production of molecules either associated with iron acquisition or in some way regulated by the availability of iron. For example, it has been shown that iron-repressible outer-membrane proteins (IROMPs) of *Pasteurella haemolytica* are recognised by convalescent sera from naturally infected hosts and are therefore expressed *in vivo* (Deneer & Potter, 1989; Lainson, Harkins, Wilson, Sutherland, Murray, Donachie & Baird, 1991). Similar results have been reported with a number of other pathogens including *E.coli* (Griffiths, Stevenson, Thorpe & Chart, 1985), *Neisseria* (Black, Dyer, Thompson & Sparling, 1986) and *Pseudomonas aeruginosa* (Brown, Anwar & Lambert, 1984). Such an approach has proved useful not only in extending an understanding of the virulence mechanisms of bacterial pathogens, but also in identifying candidate antigens for vaccine studies. The recent successful development of a vaccine against *A.salmonicida* based on IROMPs by Hirst and Ellis at the Marine Laboratory, Aberdeen is a topical example. Bacterial synthesis of virulence factors is often modulated by the concentration and availability of iron. Examples include the production of haemolysins by *L.monocytogenes* (Cewart & Foster, 1981), *Aeromonas hydrophila* (Riddle, Graham & Amborski, 1981), *E.coli* (Lebeck & Gruenig, 1985) and *Vibrio cholerae* (Stoebner & Payne, 1988) and the production of exotoxins by *Corynebacterium diphtheriae*, *Shigella dysenteriae* and *Clostridium* species (Crosa, 1987), *Pasteurella haemolytica* (Gentry, Confer, Weinberg & Homer, 1986) and *Pseudomonas aeruginosa* (Sokol, Cox & Iglewski, 1982).

The purpose of this study was to examine the effects of iron restriction on the growth *in vitro* of *R.salmoninarum* strains of differing virulence. Subsequently, the synthesis of proteins

possessing epitopes present in hybrid fusion proteins cloned from *R. salmoninarum* was evaluated by the comparison of cell components and extracellular products from iron-restricted and iron-sufficient cultures.

6.2 MATERIALS AND METHODS

Many of the materials and methods which relate to the following work are presented in Chapter 3. Only those specific to this study are presented here.

6.2.1 BACTERIOLOGY

Details of *R. salmoninarum* strains and routine culture media are presented in section 3.2.2.

6.2.1.1 Iron-restricted culture media

The availability of iron in MHCA and MHCB was restricted by the addition of either ethylene diamine di(o-hydroxyphenylacetic acid) (EDDA), α,α' -dipyridyl (DPD) or by the pretreatment of MHCB with Chelex 100 (BioRad). Chelex 100 resin is a chelating ion exchange resin which possesses a very high preference for metal cations, particularly divalent species such as Cu^{2+} , Zn^{2+} , Fe^{2+} , Mn^{2+} and also Fe^{3+} . Culture media which had been treated with Chelex 100 resin were therefore depleted of various metals including iron, although only the iron content of the media was assayed as described below. EDDA and DPD were prepared as 10 mM solutions and filter sterilised (0.45 μm , Minisart, Sartorius) prior to addition to autoclaved media. Preliminary experiments were carried out on MHCA using concentrations of 10-250 μM EDDA and 10-125 μM DPD in order to define minimum inhibitory concentrations of each chelator. On the basis of this work 200 μM EDDA and 100 μM DPD were chosen as suitable working concentrations. For treatment with Chelex 100, 5 g of resin was added for each 100 ml of MHCB and stirred gently for 1 h. The broth was filtered (Whatman number 3) to remove the resin and autoclaved. The iron content of the various media was determined by the ferrozine assay described by Stookey (1970) using a commercial assay kit (Aquaquant 14403, BDH) according to the manufacturer's instructions. Concentrations of 200 μM EDDA, 100 μM DPD or Chelex treatment reduced the free iron content of the media from in excess of 200 $\mu\text{g litre}^{-1}$ to less than 40 $\mu\text{g litre}^{-1}$. To minimise the storage of intracellular ferric iron, bacteria were passaged twice in iron-restricted MHCB (10 μM EDDA or DPD) for 10 days before use. It was observed that continuous culture of *R. salmoninarum* on iron-restricted media with higher chelator concentrations led to non-viability. All glassware was washed in 6 M HCl and rinsed 10 times in deionised-distilled water from a Milli Q purification system (Millipore) to remove

contaminating iron. Contaminating iron was removed from EDDA by the method of Rogers (1973) as follows. A solution of 5 g EDDA in 95 ml of 1 M HCl was boiled, cooled and then filtered in a Buchner funnel. Acetone was added (750 ml) and the pH raised to 6.0 with 1 M NaOH. After standing overnight at 4°C the precipitate was filtered off (Whatman number 1), washed with cold acetone (-20°C), filtered (Whatman number 1) and dried.

6.2.1.2 Growth measurements

The growth of *R.salmoninarum* was assessed in MHCB, MHCB containing 200 µM EDDA, MHCB containing 100 µM DPD and in MHCB pretreated with Chelex 100. After inoculation, 1 ml suspensions of *R.salmoninarum* were removed from flasks of the four cultivation media every two days for 3 weeks. Cells were pelleted by centrifugation (9000 x g, 5 min), the supernatant removed for siderophore assays and the pellet resuspended in 1 ml of boric acid-potassium chloride buffer pH 9.0 which was prepared by adding 20.8 ml of 0.1 M NaOH to 50 ml of a 0.1 M solution of both potassium chloride and boric acid then diluting the solution to 100 ml (Embley, 1983). The resuspended cell pellet was heated to 50°C for 2-3 min to dissolve cystine and recentrifuged (9000 x g, 5 min). The supernatant was discarded and the pellet was washed and resuspended in 1 ml of distilled water. Absorbance was read at 625 nm against a distilled water blank.

6.2.2 SIDEROPHORE AND IRON REDUCTASE ASSAYS

For all siderophore assays; controls containing MHCB were included, Milli Q water was used throughout, reactions were carried out in capped polystyrene bijoux bottles (Luckham), all reagents (other than stable acids and alkalis) were freshly prepared, and absorbances were read on a Philips spectrophotometer Model PU 8720 UV/VIS. Assays for catecholate and hydroxymate siderophore activity were carried out every two days up to three weeks post-inoculation and then weekly up to 13 weeks post-inoculation. Bovine apotransferrin was purchased from Sigma Chemical Company and contained less than 0.002% iron as determined by the manufacturer.

6.2.2.1 Catechols

The colorimetric assays of Arnow (1937) and Rioux, Jordan & Rattray (1983) were used to assess the possible production of catechol siderophores using 2,3-dihydroxybenzoic acid (2,3-DHBA) as standard. The Arnow (1937) method relies upon the colour change from yellow to intense orange-red that occurs when catechol is reacted with nitrous acid followed by a strong

base. The following were added in order with mixing to 1 ml of cell-free supernatant derived from 1 ml of whole culture centrifuged at $9000 \times g$ for 5 min; 1 ml of 0.5 M HCl, 1 ml of nitrite-molybdate reagent (10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml of water), 1 ml of 1 M NaOH and 1 ml of water. The method of Rioux *et al.* (1983) also involves the production of an orange-red ferrous chelate upon the reduction in acidic conditions of Fe^{3+} to Fe^{2+} by catechol. The following were added in order with mixing to 2.3 ml of water and 0.2 ml of 20% v/v H_2SO_4 ; 1 ml of cell-free supernatant, 0.1 ml of 1% w/v ferric ammonium citrate solution in 0.06 M H_2SO_4 , 0.4 ml of 2 M ammonium fluoride, 0.4 ml of 1% w/v 1,10-phenanthroline monochloride monohydrate and 0.6 ml of hexamethylenetetramine solution. The complete assay mixture (5 ml) was heated for 1 h in a water bath at 60°C then cooled to room temperature. Correction for Fe^{2+} contamination of reagents was made by preparing an assay mixture in which 1 ml water replaced the sample. Following the completion of reactions for the assays of both Arnow (1937) and Rioux *et al.* (1983), absorbances were read at 510 nm.

6.2.2.2 Hydroxamates

The colorimetric assays of Atkin, Neilands & Phaff (1970) and Arnold & Viswanatha (1983) were used to assess the possible production of hydroxamate siderophores using hydroxylamine monohydrochloride as standard. The method of Atkin *et al.* (1970) consisted of mixing 0.5 ml of cell-free culture supernatant with 2.5 ml of 5 mM ferric perchlorate in 0.1 M perchloric acid followed by measurements of optical density at 480 nm. For the assay of Arnold & Viswanatha (1983) the following procedure was employed. To 1.25 ml of an 80% solution of mercaptoacetic acid in water, 50 ml of water was added, followed by 2.2 ml of concentrated 5 M ammonium hydroxide and the volume adjusted to 100 ml with water. To 25.5 ml of this solution was added 0.44 ml of ferric nitrate solution (30 mg ml^{-1}). The mixture was then thoroughly shaken, left for 4 min in the dark, mixed again, transferred to a cuvette and the absorbance at 532 nm was recorded.

6.2.2.3 Iron binding activity of *R.salmoninarum* culture supernatants

The iron binding activity of *R.salmoninarum* cell-free culture supernatant was determined by the method of Norrod & Williams (1978). Either 0.2 ml of water, uninoculated culture medium (with or without chelator) or *R.salmoninarum* culture supernatant was added to 0.8 ml of iron-free bovine transferrin solution (2.5 mg ml^{-1} in 0.1 M Tris-HCl, pH 7.4) and incubated at room temperature (22°C) for 1 h. Absorbance at 470 nm was recorded as an indication of the binding of iron by transferrin after each addition of 5 μl of 1 mM ferric nitrilotriacetate; an iron

compound that reacts stoichiometrically with transferrin. To prepare 1 mM ferric nitrilotriacetate, 12.5 ml of 0.1 M Tris base was added to 40 ml of 10 mM disodium nitrilotriacetic acid and diluted to 80 ml with water. With rapid stirring, 1 ml of 100 mM ferric nitrate in 0.5 M HNO₃ was added dropwise and the pH adjusted to 7.4 with 0.5 M HNO₃ if necessary. The final volume was adjusted to 100 ml.

6.2.2.4 Iron reductase enzyme assay

Iron reductase activity was measured by the method of Dailey & Lascelles (1977) by using the ferrous iron chelator 3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine usually called ferrozine. The reaction mixture contained, in a final volume of 2 ml, 50 µg of sonicated cell extract protein (section 3.2.12.1), 5 µM of ferrozine in 0.01 M Tris-HCL, pH 7.6, 100 µg of ferric citrate (pH 7.0), 2 µM of either reduced β nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 0.01 M Tris-HCl, pH 7.6. Cuvettes were filled with nitrogen gas and sealed with Parafilm. The reactions were carried out in polystyrene cuvettes at room temperature and the change in optical density was monitored at 562 nm. The reference cuvettes contained all of the reaction components except reductant. The reductase activity was recorded as the change in optical density over time. Specific activity of the cell sonicates was calculated as the amount of product formed mg protein⁻¹ h⁻¹.

6.2.3 PREPARATION OF CELL COMPONENTS AND EXTRACELLULAR PRODUCTS

The preparation of these components has been described in section 3.2.12.

6.2.4 ANTISERA

Blood was collected as outlined below, clotted in glass at room temperature and then overnight at 4°C. Serum was withdrawn and stored at -70°C. The preparation of rat antisera and rabbit anti-trout IgM has been described in section 5.2.5.1 and section 5.2.5.3 respectively.

6.2.4.1 Serum from fish naturally infected with *R.salmoninarum*

Blood was obtained during clinical outbreaks of BKD from English freshwater farmed rainbow trout (approximately 500 g weight) by dorsal aorta puncture and from Scottish marine farmed Atlantic salmon (2-5 kg weight) by aortic severance. Serum was obtained from five rainbow trout and from four Atlantic salmon all of which possessed typical gross external symptoms of BKD as well as the diagnostic kidney lesions. Each outbreak was confirmed as BKD by culture isolations at MAFF Fish Diseases Laboratory, Weymouth, England and at SOAFD Marine

Laboratory, Aberdeen, Scotland.

6.2.4.2 Rabbit anti-salmon immunoglobulin M (IgM)

Atlantic salmon IgM was purified from Atlantic salmon serum by gel filtration chromatography using Ultrogel AcA22 (LKB) and 50 mM Tris-HCl buffer pH 7.4. Fractions were monitored at 280 nm (UV-1, Pharmacia), peak profiles traced by chart recorder and peaks collected (Frac-100, Pharmacia). Peak fractions were checked by SDS-PAGE on the Phastsystem (12.5% homogeneous Phastgel, Pharmacia) for the presence of protein bands of molecular mass 65 kDa and 23 kDa corresponding to the heavy and light chains respectively of salmon IgM. Fractions containing IgM were pooled and further purified by anion exchange chromatography using a 1 M NaCl stepped gradient and a Mono-Q HR5/5 column connected to a Fast Protein Liquid Chromatography system (FPLC, Pharmacia). The IgM containing fractions were pooled, concentrated by centrifugation (1000 x g, 4°C) in polysulphone membrane filtration tubes (Ultrafree, Millipore), and the protein concentration estimated by the method of Bradford using a commercial assay kit (BioRad). IgM (400 µg) was administered subcutaneously to Dutch rabbits in Freund's complete adjuvant, with a booster of 400 µg IgM in Freund's incomplete adjuvant given 6 weeks later. Animals were bled 3 weeks later. Specificity for Atlantic salmon IgM was checked by a standard method of immunoelectrophoresis (Hudson & Hay, 1989).

6.2.5 ELECTROPHORESIS

Protocols for SDS-PAGE are presented in section 3.2.14.

6.2.5.1 Detection of free aldehydes

For the detection of free aldehydes in SDS-PAGE gels of *R.salmoninarum* cell sonicates, gels were fixed in 7% v/v acetic acid (24 h, room temperature, constant shaking), oxidized in 0.2% w/v periodic acid (45 min, 4°C) and then immersed in Schiff's reagent (45 min, 4°C). Gels were destained in 10% v/v acetic acid until the red bands indicative of reducing sugar moieties were visible.

6.2.6 WESTERN BLOTTING

Protocols for Western blotting are presented in sections 3.2.15.

6.2.6.1 Antisera from immunised rats

The procedure followed has been described in section 5.2.7.1.

6.2.6.2 Serum from fish naturally infected with *R.salmoninarum*

Blots were washed and blocked as described in section 5.2.7.2 and then incubated individually in primary antiserum diluted 1:25 in Tris-saline (section 5.2.7.2) for 12 h. After washing 3 x 5 min in Tris-saline, blots were incubated with the secondary antibody; either rabbit anti-salmon IgM or rabbit anti-trout IgM, diluted 1:200 in Tris-saline for 2 h. Following 3 x 5 min washes in Tris-saline, blots were incubated for 1 h with swine anti-rabbit immunoglobulins peroxidase conjugate diluted 1:1000 in Tris-saline, then developed (section 3.2.15.1). Identical blots were probed with sera from three fish with no prior history of BKD.

6.2.7 STATISTICAL ANALYSIS

Comparison of paired groups or data sets was carried out using Student's t-test for the difference of means (paired samples) as described by Wardlaw (1987).

6.3 RESULTS

6.3.1 GROWTH MEASUREMENTS

In general, it was found that the restricted availability of iron reduced the growth of *R. salmoninarum*, although non-autoagglutinating strains (MT414, MT417, MT452) were more sensitive to iron-restricted conditions than autoagglutinating strains (ATCC33209, MT420, MT425; Figure 6.1). The presence of the ferrous iron chelator DPD was more effective in restricting the growth of autoagglutinating strains in MHCB than either Chelex treatment or EDDA. This may be a consequence of the accumulation of DPD within the bacterial cells which were observed to turn bright pink as the cells multiplied. It was also observed that continued culture of *R. salmoninarum* on iron restricted media led to non-viability.

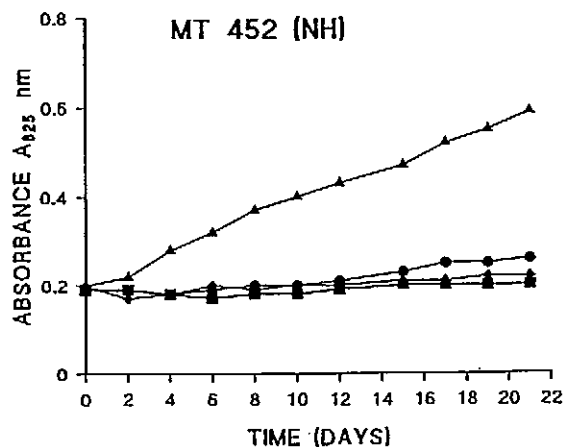
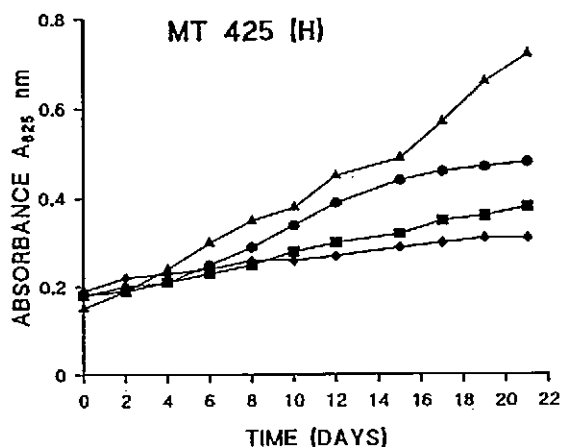
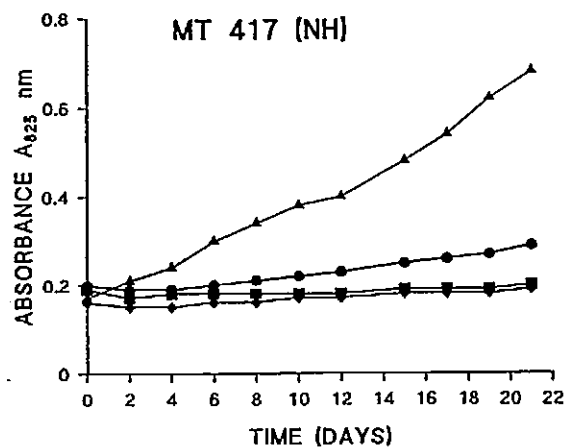
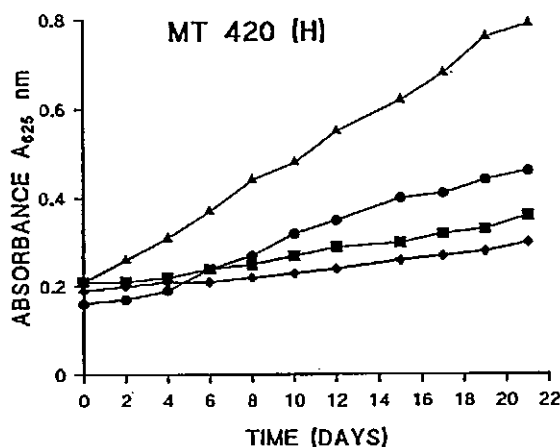
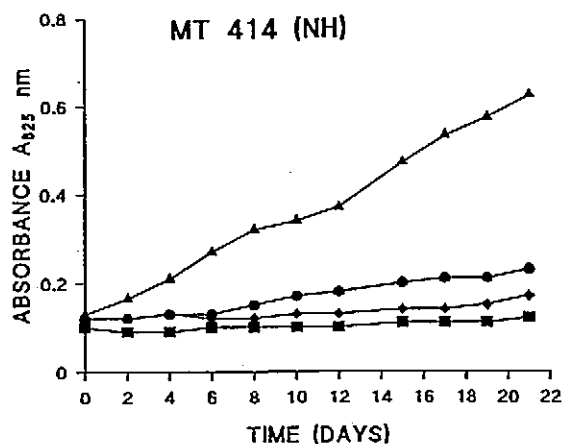
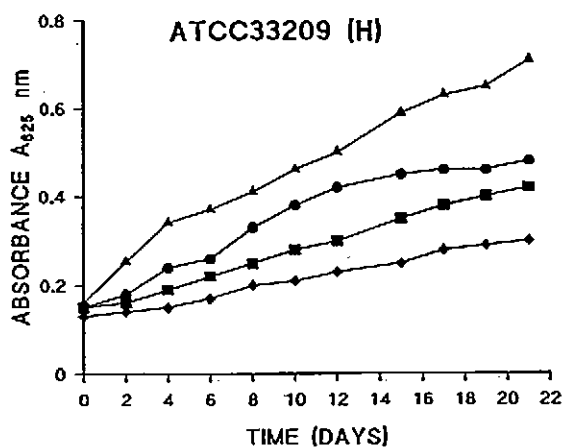
6.3.2 PRODUCTION OF SIDEROPHORES

Colorimetric assays for the presence of phenolate-catechol and hydroxamate siderophores in the culture supernatants of 3 non-autoagglutinating and 3 autoagglutinating strains of *R. salmoninarum* failed to provide any evidence for siderophore production. Supernatants from 13 week cultures were also assayed after 20-fold concentration by freeze drying, again with no success. Strain 910019 was not included in these assays. Standard curves for each assay are presented in Figure 6.2.

6.3.3 IRON BINDING ACTIVITY OF *R. salmoninarum* CULTURE SUPERNATANT

The ability of culture supernatant to inhibit the binding of ferric nitrilotriacetate to iron-free bovine transferrin was examined using spectrophotometry. When uninoculated culture medium, either with or without chelator, was present in the assay some inhibition of the binding of iron compared with samples containing only Milli Q water was observed. However, when compared with their respective controls, the culture supernatants of all strains did have some ability to prevent the binding of iron to iron-free transferrin (Figure 6.3). This inhibition, although present in iron-restricted culture supernatants ($p < 0.05$), was most pronounced in supernatants from iron-sufficient cultures ($p < 0.0001$).

Figure 6.1 Growth of six strains of *R.salmoninarum* in (▲) MHCB; (◆) MHCB + 100μM DPD; (●) MHCB + 200μM EDDA or (■) Chelex 100 treated MHCB. Hydrophobic/autoagglutinating properties are obtained from Table 3.1 (H=hydrophobic, autoagglutinating; NH=non-hydrophobic, non-autoagglutinating).



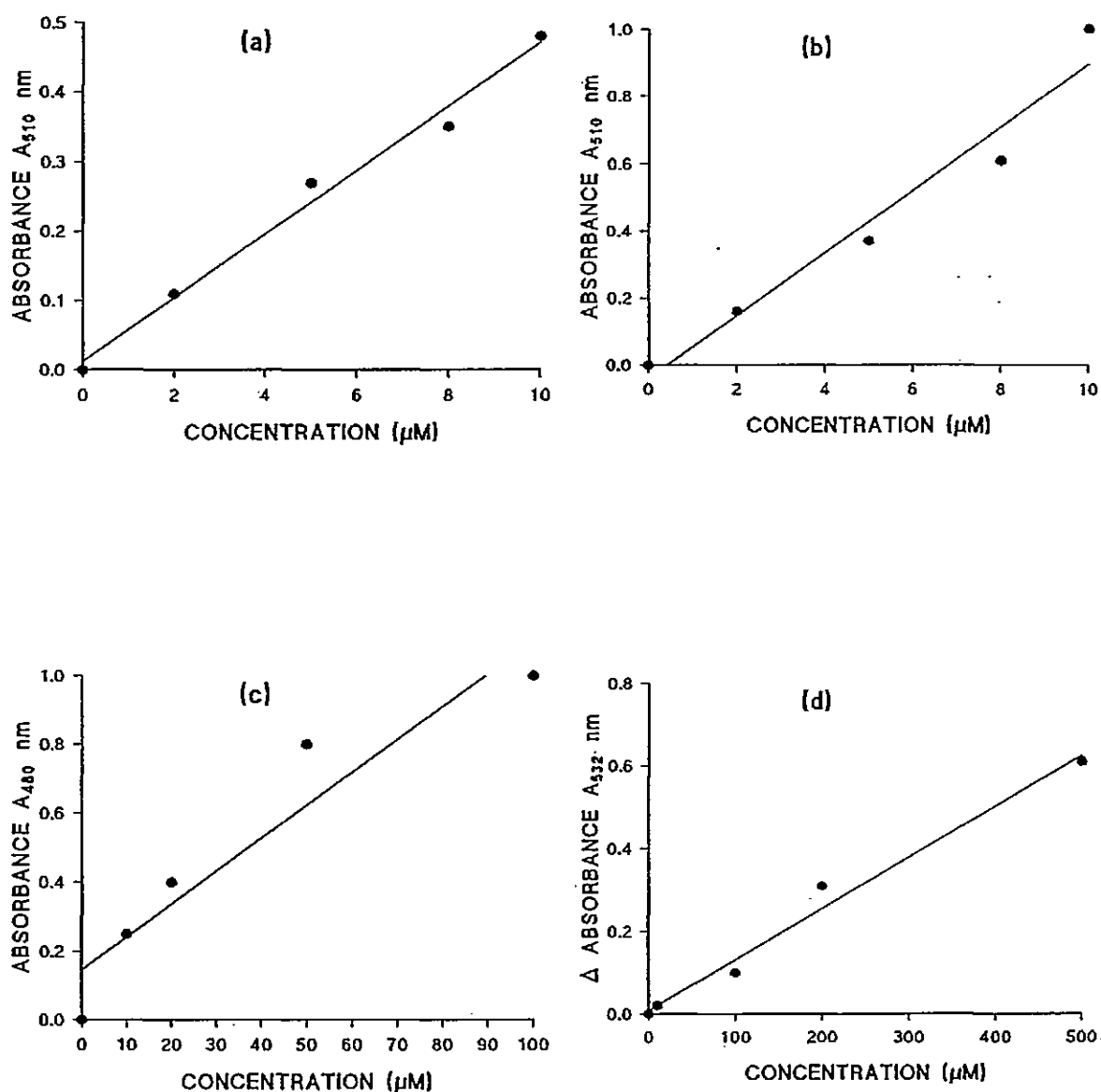
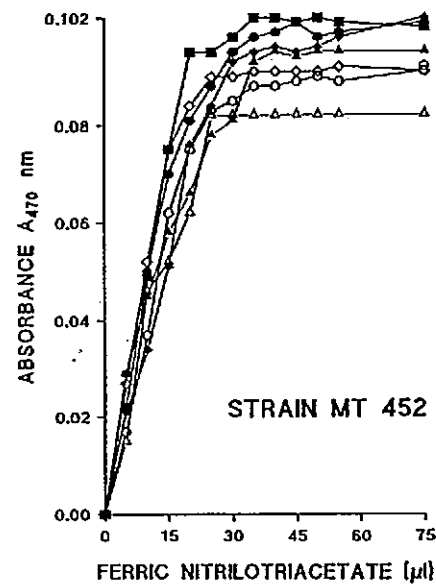
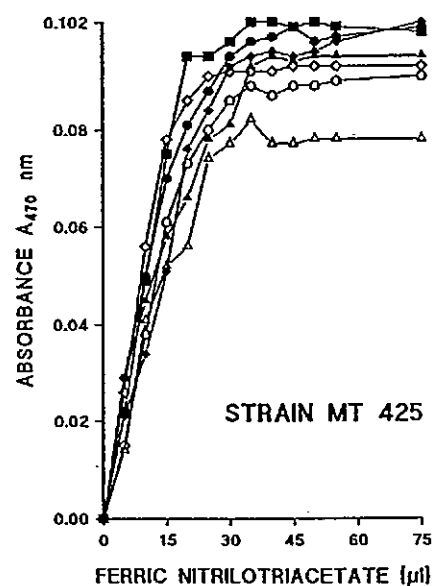
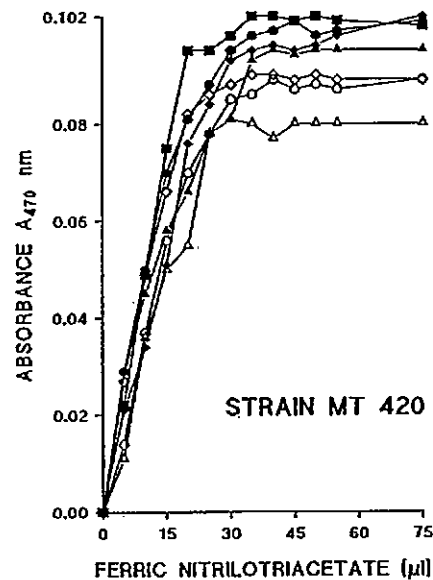
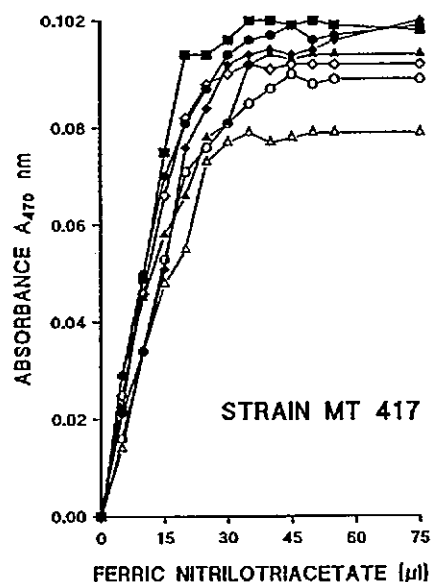
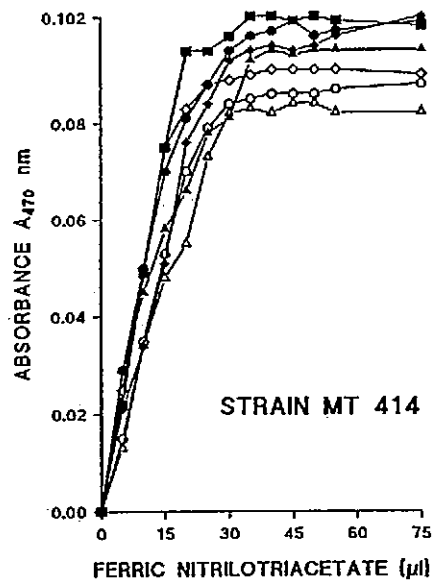
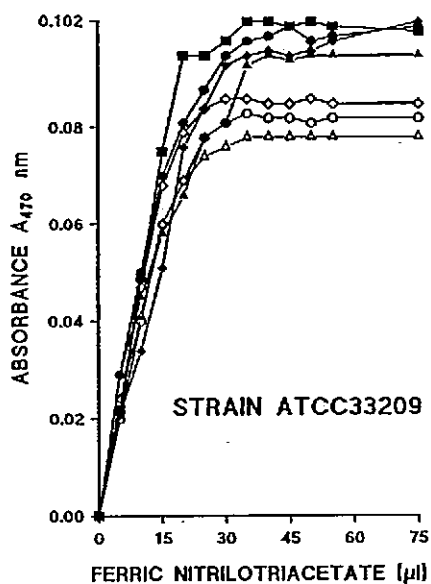


Figure 6.2 Optical density of siderophore assay standards for phenolate-catechols using the methods of (a) Arnow (1937) and (b) Rioux *et al.* (1983) with 2,3-dihydroxybenzoic acid as standard and for hydroxamates using the methods of (c) Atkin *et al.* (1970) and (d) Arnold & Viswanatha (1983) with hydroxylamine monohydrochloride as standard.

Figure 6.3 Effect of culture supernatant from six strains of *R. salmoninarum* on the binding of iron by transferrin. Either Milli Q water, uninoculated culture medium (with or without chelator) or culture supernatant were incubated with iron-free transferrin for 1 h at 22°C. The binding of iron was measured at A₄₇₀ nm after each addition of 5 µl ferric nitrilotriacetate. Symbols: ■, water; ▲, MHCB; ♦, MHCB + 100µM DPD; ●, MHCB + 200µM EDDA; △, iron-sufficient culture supernatant; ◇, 100µM DPD iron-restricted culture supernatant; ○, 200µM EDDA iron-restricted culture supernatant.



6.3.4 IRON REDUCTASE ACTIVITY OF *R.salmoninarum* CELL EXTRACTS AND CULTURE SUPERNATANTS FROM CHELEX TREATED MHCB

As presented in Table 6.1 both autoagglutinating and non-autoagglutinating strains of *R.salmoninarum* produce an iron reductase. Generally, regardless of the availability of iron, non-autoagglutinating strains possessed a higher level of enzyme activity than autoagglutinating strains, being an average of 20-64% greater ($p < 0.05$) using either NADH or NADPH as the reductant. For all strains, when NADH was used as the reductant the specific activities of sonicated cell extracts were from 12-174% higher than those recorded for NADPH ($p < 0.01$). Under iron-restricted culture conditions, all strains showed increased reductase activity which varied from 30-80% for NADH and 3.5-61% for NADPH ($p < 0.05$).

Table 6.1 Specific activity* of iron reductase in cell lysates of *R.salmoninarum* strains with NADH or NADPH as reductant.

STRAINS	NADH		NADPH	
	Iron restricted	Iron sufficient	Iron restricted	Iron sufficient
ATCC33209†	156.99 ± 3.42	92.34 ± 3.82	88.89 ± 3.00	82.29 ± 5.38
MT 414	208.36 ± 8.97	115.41 ± 6.53	106.09 ± 5.82	99.64 ± 2.49
MT 417	245.16 ± 10.98	164.87 ± 8.14	104.66 ± 4.80	69.54 ± 3.61
MT 420†	140.86 ± 7.87	90.86 ± 6.33	80.29 ± 3.46	77.60 ± 4.97
MT 425†	137.63 ± 7.76	105.77 ± 4.21	74.91 ± 4.27	57.17 ± 1.57
MT 452	258.78 ± 8.69	162.01 ± 10.31	122.43 ± 4.75	75.97 ± 3.57
910019†	169.17 ± 4.98	98.62 ± 7.05	61.65 ± 2.77	56.38 ± 3.25

* Specific activity is expressed as nM Fe^{2+} formed $\text{mg protein}^{-1} \text{ h}^{-1}$ calculated as $\Delta A \text{ h}^{-1} \times \epsilon^{-1} \times \text{vol (litres)} \times 10^9 \times \text{protein concentration (mg)}$. Figures represent the mean of three readings \pm standard error. † Hydrophobic, autoagglutinating strains; non-hydrophobic, non-autoagglutinating strains are unmarked.

6.3.5 SDS-PAGE OF *R.salmoninarum* CELL EXTRACTS

Electrophoresis of sonicated cell extracts by SDS-PAGE revealed no obvious additional cellular proteins in iron-restricted as compared to iron-sufficient cultures when gels were stained with CBB (Figures 6.4 & 6.5). However, SDS-PAGE gels stained with periodic acid-Schiff's reagent (PAS) consistently revealed the presence of reducing sugars associated with proteins of estimated molecular mass 140, 145, 180 and 200 kDa which were obvious only in cell extracts from iron-restricted cultures (Figure 6.6). In addition, cell extracts from iron-restricted cultures showed the presence of strongly PAS positive material at the top of 10% SDS-PAGE gels which was absent in cell extracts from iron-sufficient cultures.

6.3.6 WESTERN BLOTTING

6.3.6.1 Rat antisera raised against hybrid fusion epitopes

Cell extracts or ECPs were isolated from seven strains of *R.salmoninarum* (Table 3.2) which had been cultured under either iron-restricted or iron-sufficient conditions. The cell extracts were separated by SDS-PAGE and blotted onto nitrocellulose. The Western blots were probed with antisera from rats which had been immunised with the fusion proteins constructed as described in Chapter 5. Antiserum to maltose-binding protein fused to P57 (MBP-P57) recognised major epitopes in all strains of *R.salmoninarum* of molecular weights about 58, 45 and to a lesser extent 35K in sonicated *R.salmoninarum* cell extracts regardless of the availability of iron (Figure 6.7A & B). The ECPs derived from iron-sufficient cultures possessed major bands which were recognised by the antiserum at 25K and below although the type strain ATCC33209 and also strain 910019 were exceptions with the major band located at about 55K (Figure 6.7C & D). However, ECPs derived from iron-restricted cultures showed a dramatic reduction in the degradation of the major epitopes located at about 55K and strains MT417, MT420 and MT452 also showed evidence of considerably reduced production of P57.

Western blots of sonicated *R.salmoninarum* cell extracts derived from iron-sufficient cultures which were probed with rat antiserum to maltose-binding protein fused to *R.salmoninarum* metalloprotease (MBP-MPR) revealed the presence of immunoreactive bands at about 70, 50 and 25K in all strains (Figure 6.8A). However, the bands were barely discernible on Western blots of cell extracts from iron-restricted cultures (Figure 6.8B). No bands were detected by the MBP-MPR antiserum on Western blots of ECPs from either iron-restricted or iron-sufficient cultures.

Cell extracts of all strains of *R.salmoninarum* were found to possess epitopes recognised by rat

antiserum to β -galactosidase-HLY regardless of the availability of iron. Two major bands of 82 and 78K were found to be present together with a number of weaker bands between 80 and 56K and one very weak band at 25K (Figure 6.8C & D). No epitopes of β -galactosidase-HLY were detected on Western blots of ECPs.

6.3.6.2 Serum from Atlantic salmon and rainbow trout naturally infected with *R.salmoninarum*

Western blots containing each of the purified fusion proteins were probed individually with serum obtained from either Atlantic salmon or rainbow trout. Sera from naturally infected salmon, control salmon and control trout failed to identify any of the fusion proteins by immunoblotting. However, the sera from each of the five naturally infected trout consistently identified epitopes from each of the three fusion proteins. Both MBP-MPR and β -galactosidase-HLY were both strongly recognised, although MBP-P57 was only weakly detected. As presented in Figure 6.9, in all cases specific antibody bound to bands of the following estimated molecular masses: for MBP-MPR, 105, 94, 78, 65, 60, 26 and 15 kDa; for β -galactosidase-HLY, 160, 80, 63, 60 and 50 kDa; and for MBP-P57, 90, 78, 63, 60 and 24 kDa.

6.3.6.3 Rabbit antiserum raised against *R.salmoninarum* whole cells

In order to confirm the identity of the strains of bacteria used in this study Western blots were developed with a rabbit antiserum raised against whole cells of the type strain (ATCC33209) of *R.salmoninarum*. At the conclusion of the experiment (13 weeks post-inoculation) sonicated cell extracts were prepared, electrophoresed and blotted before being probed with the antiserum. All strains were recognised as bearing considerable antigenic identity with the type strain (Figure 6.10).

Figure 6.4 Cell extracts of seven *R.salmoninarum* strains which had been cultured under conditions of either iron restriction or iron sufficiency were separated by SDS-PAGE using a 10% gel then stained for protein with Coomassie brilliant blue R. Track M: protein molecular weight standards (kDa). C: cultures were grown in MHCB. D: cultures were grown in MHCB supplemented with 100 μ M DPD. E: cultures were grown in MHCB supplemented with 200 μ M EDDA. The relevant strain numbers appear above each set of samples (see Table 3.2).

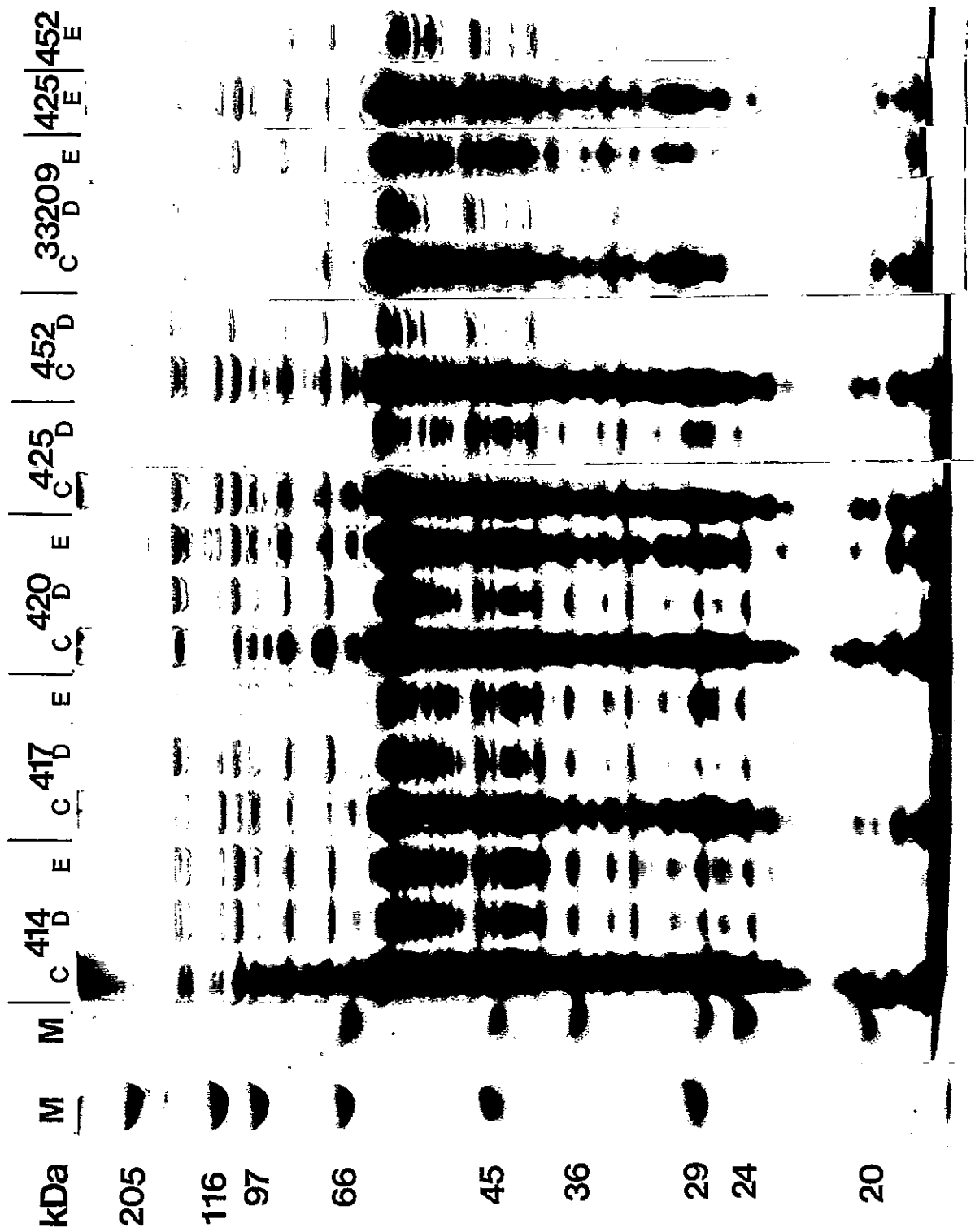


Figure 6.5 Cell extracts of seven *R. salmoninarum* strains which had been cultured in either (C) MHCB or (Q) MHCB treated with Chelex 100, were separated by SDS-PAGE using a 10% gel then stained for protein with Coomassie brilliant blue R. Track M: protein molecular weight standards (kDa). The relevant strain numbers appear above each set of samples (see Table 3.2).

33209 417
M C Q C Q

420
C Q
910019 C Q
452 C Q
M

425 C Q
414 C Q
M

M

kDa

205

116

97

66

45

36

29

24

20

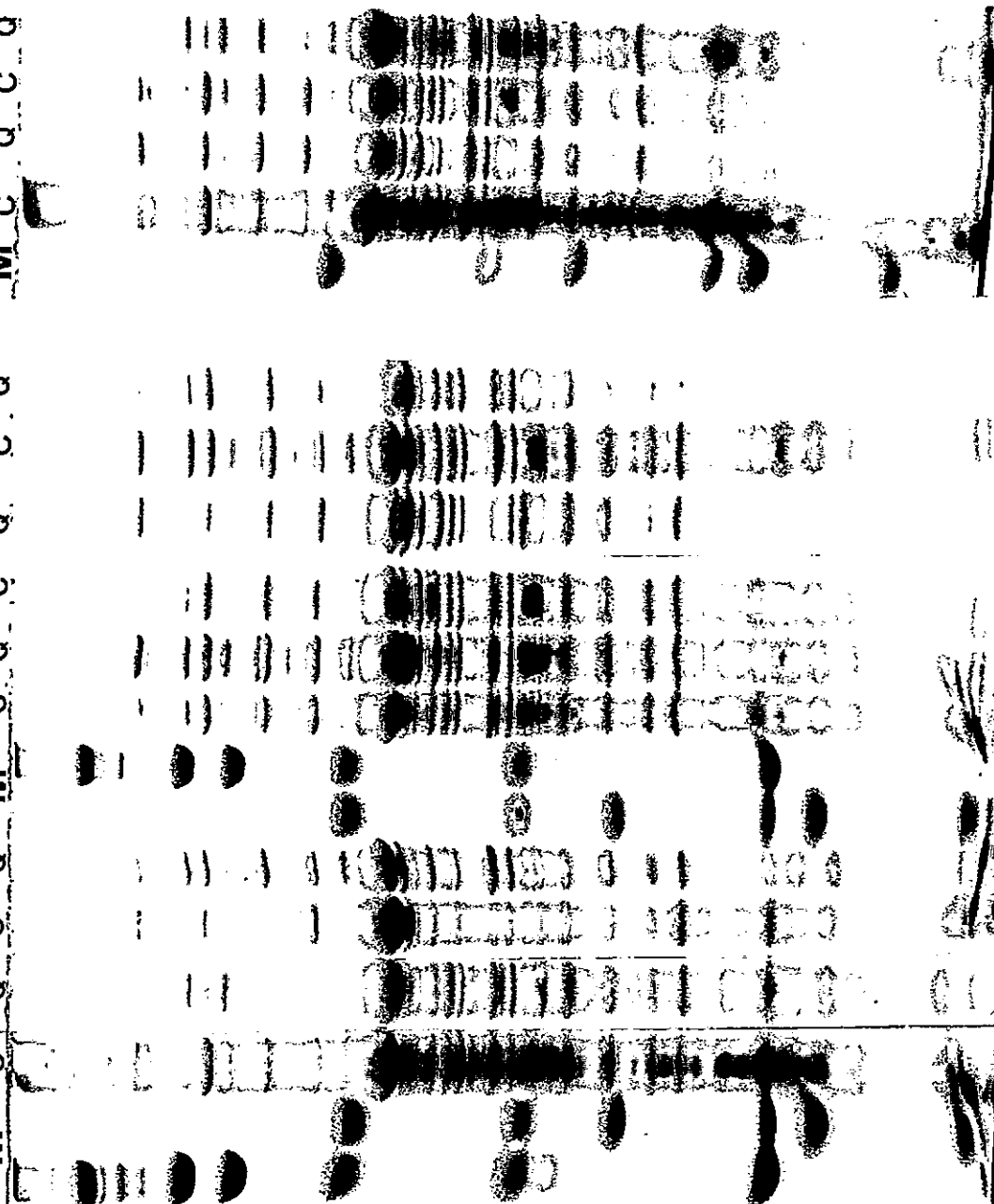


Figure 6.6 Cell extracts of seven *R.salmoninarum* strains which had been cultured under conditions of either iron restriction or iron sufficiency were separated by SDS-PAGE using a 10% gel then stained with Periodic Acid-Schiff's reagent in order to detect the presence of reducing sugars. Track M: protein molecular weight standards (kDa). C: cultures were grown in MHCB. D: cultures were grown in MHCB supplemented with 100 μ M DPD. E: cultures were grown in MHCB supplemented with 200 μ M EDDA. The relevant strain numbers appear above each set of samples (see Table 3.2). About 25 μ g protein was separated in each lane. The arrows mark the positions of the PAS positive molecules which were unique to strains grown under iron-restricted conditions.

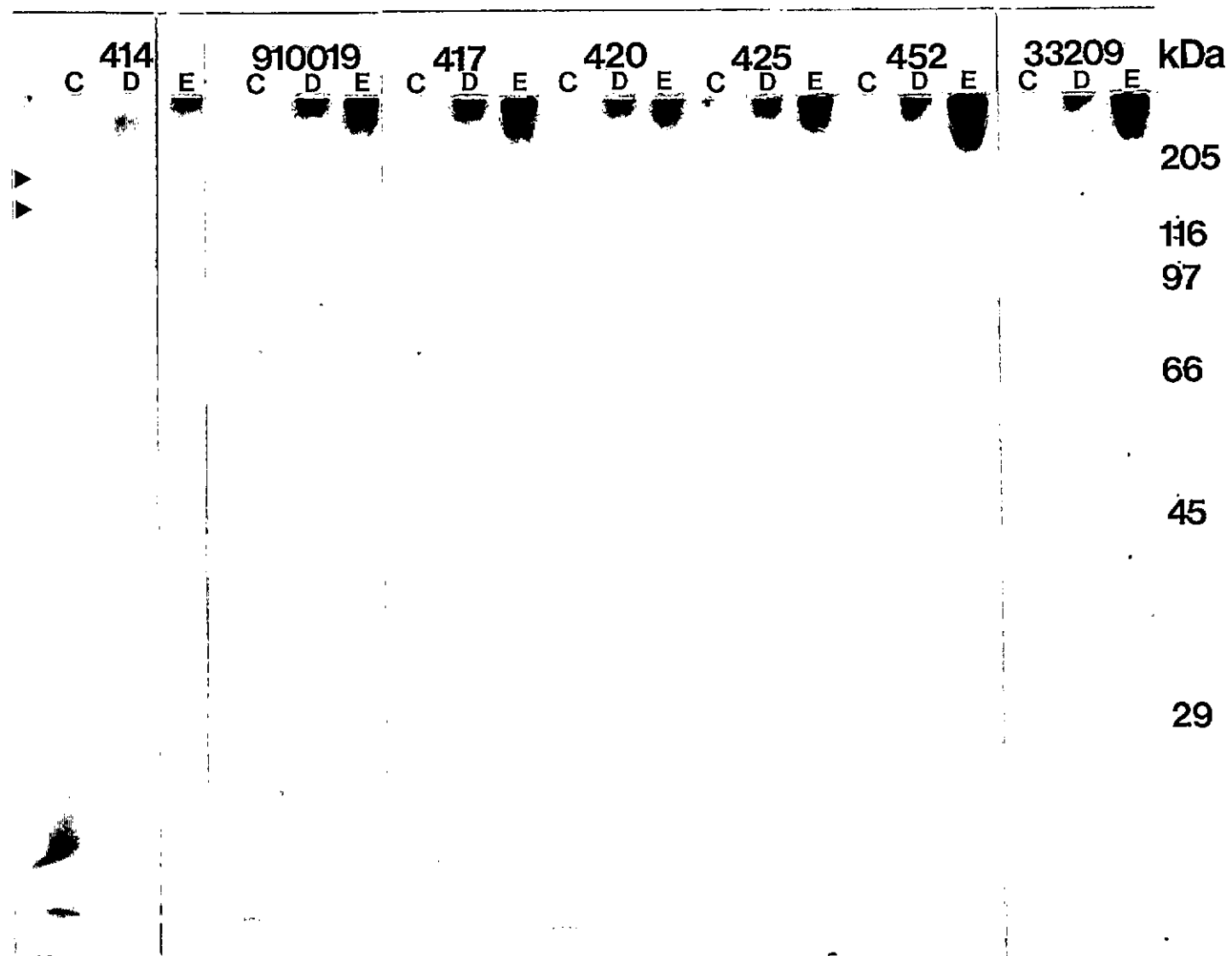


Figure 6.7 The detection of epitopes of MBP-P57 in cell extracts and ECPs of seven *R.salmoninarum* strains which had been cultured under conditions of either iron restriction or iron sufficiency. Preparations were separated by SDS-PAGE using a 10-15% gradient gel, transferred onto nitrocellulose and then probed with a rat antiserum raised against the MBP-P57 fusion protein. (A) and (B) are whole cell extracts of cultures grown in either MHCB or MHCB supplemented with 200 μ M EDDA, respectively. (C) and (D) are ECPs derived from cultures grown in either MHCB or MHCB supplemented with 200 μ M EDDA, respectively. Tracks were:(a) strain 910019; (b) strain MT452; (c) strain MT425; (d) strain MT420; (e) strain MT417; (f) strain MT414; (g) strain ATCC33209. Protein molecular weight standards are marked (kDa).

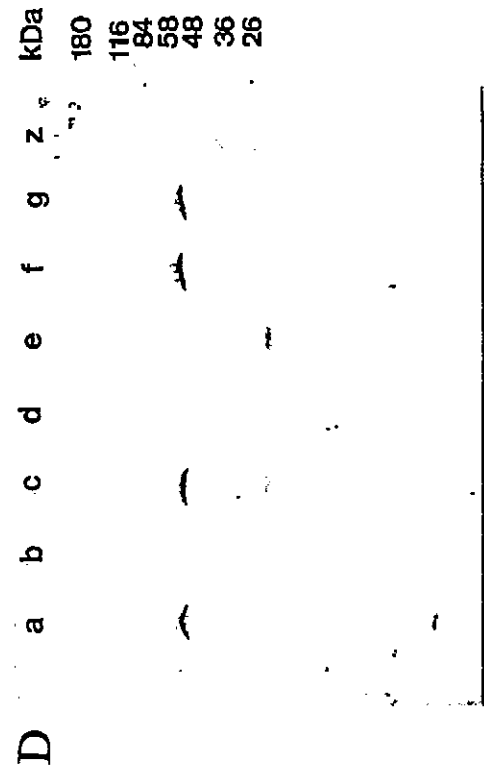
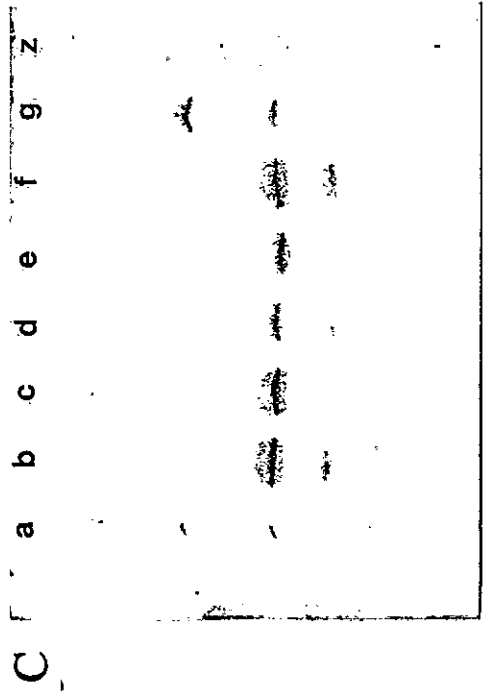
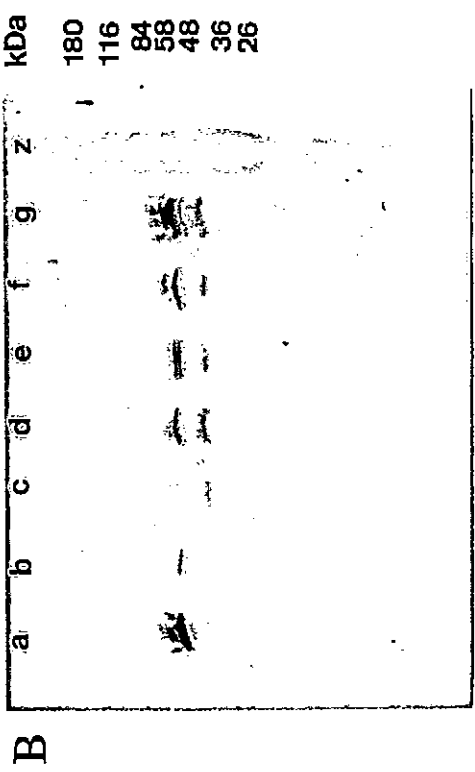
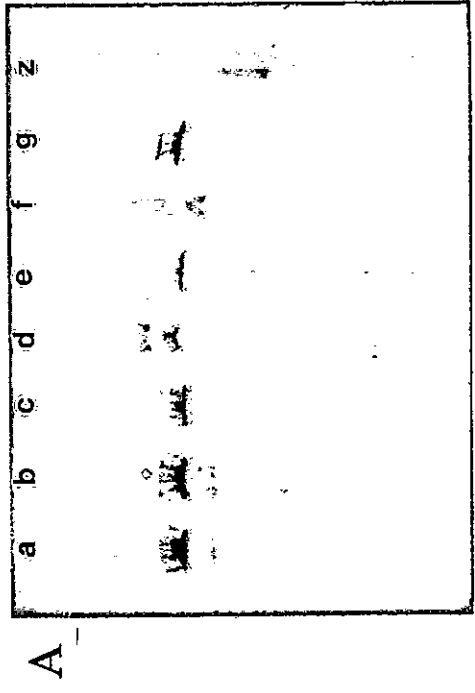


Figure 6.8 The detection of epitopes of MBP-MPR and β -galactosidase-HLY in cell extracts of seven *R.salmoninarum* strains which had been cultured under conditions of either iron restriction or iron sufficiency. Preparations were separated by SDS-PAGE using a 10-15% gradient gel, transferred onto nitrocellulose and then probed with a rat antiserum raised against either the MBP-MPR or β -galactosidase-HLY fusion proteins. (A) and (B) are whole cell extracts of cultures grown in either MHCB or MHCB supplemented with 200 μ M EDDA, respectively, which were probed with rat anti-MBP-MPR. (C) and (D) are whole cell extracts of cultures grown in either MHCB or MHCB supplemented with 200 μ M EDDA, respectively, which were probed with rat anti- β -galactosidase-HLY. Tracks were:(a) strain 910019; (b) strain MT452; (c) strain MT425; (d) strain MT420; (e) strain MT417; (f) strain MT414; (g) strain ATCC33209. Protein molecular weight standards are marked (kDa).

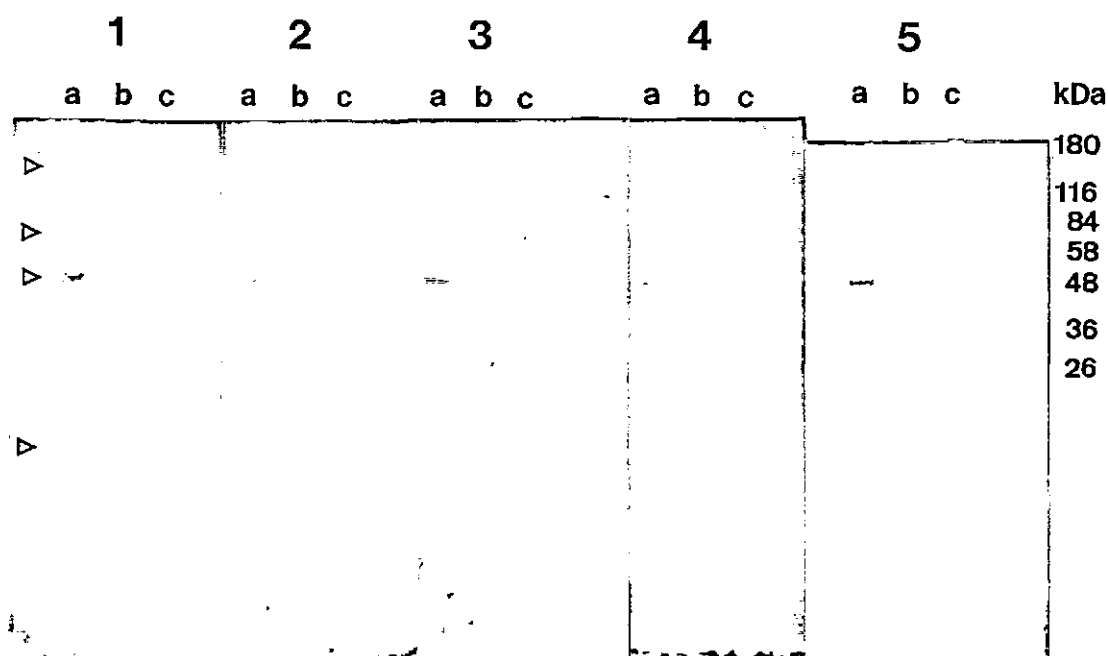
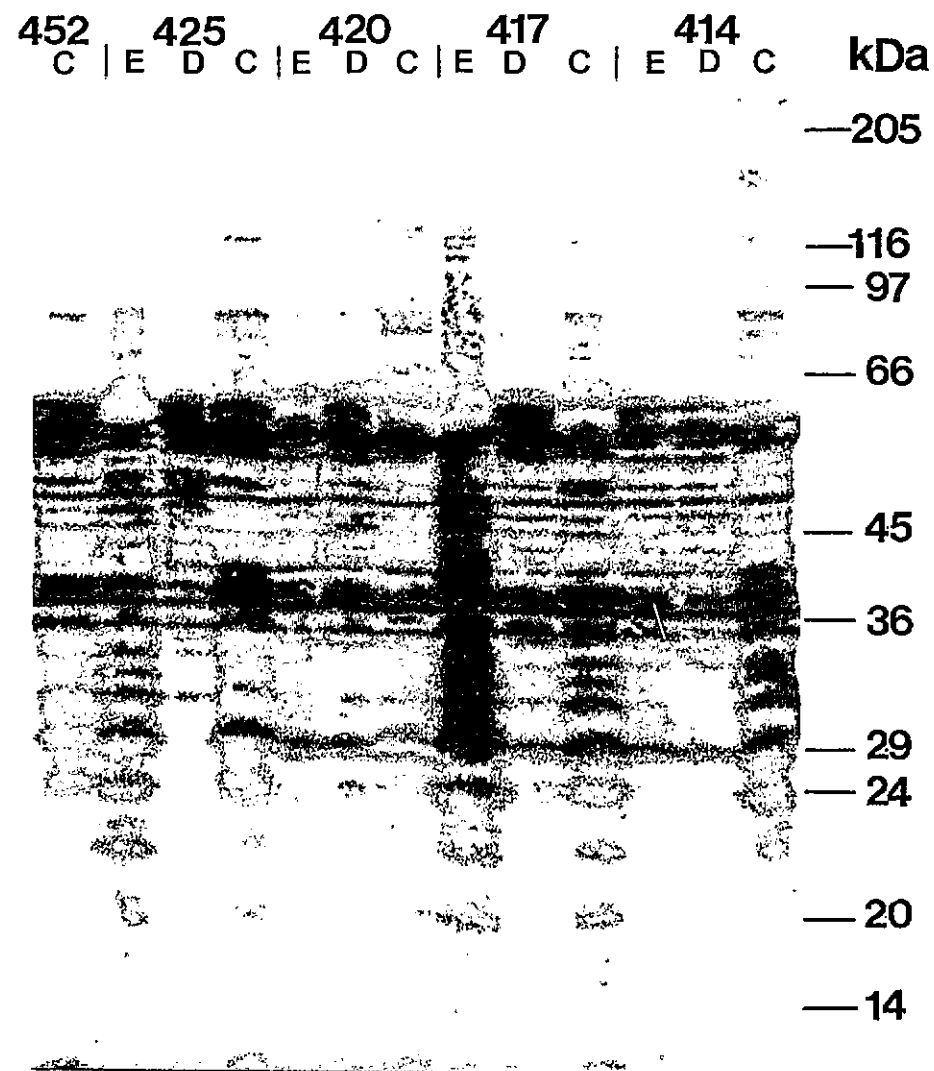
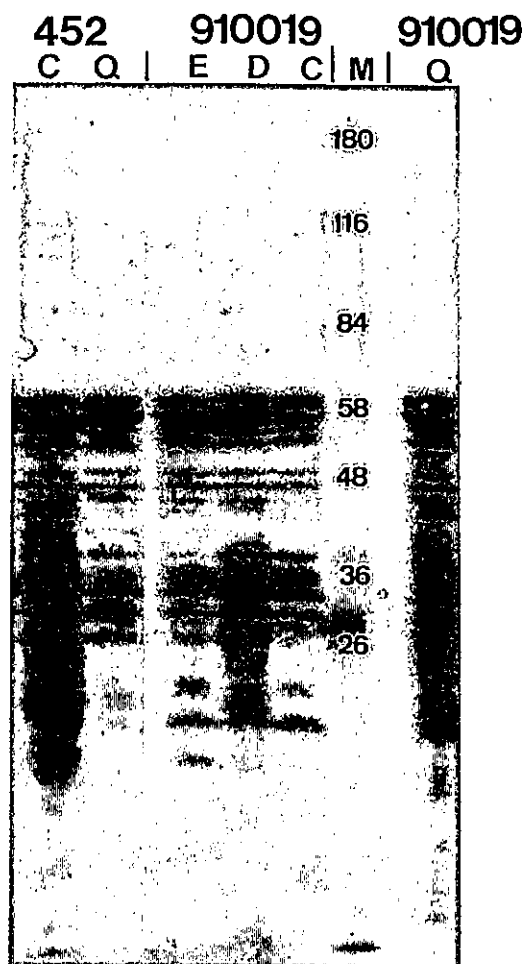


Figure 6.9 The detection of epitopes of (a) β -galactosidase-HLY, (b) MBP-P57 and (c) MBP-MPR by serum derived from rainbow trout which were undergoing a clinical outbreak of BKD. Preparations were separated by SDS-PAGE using a 10-15% gradient gel, transferred onto nitrocellulose and then probed individually with serum removed from five different infected fish (1-5). Protein molecular weight standards are marked (kDa). Arrows indicate the approximate positions of immunoreactive epitopes.

Figure 6.10 Cell extracts of seven *R.salmoninarum* strains which had been cultured under conditions of either iron restriction or iron sufficiency were separated by SDS-PAGE using a 10% gel, transferred electrophoretically onto nitrocellulose and then probed with a rabbit antiserum raised against whole cells of *R.salmoninarum* type strain ATCC33209. Track M: protein molecular weight standards (kDa). C: cultures were grown in MHCB. D: cultures were grown in MHCB supplemented with 100 μ M DPD. E: cultures were grown in MHCB supplemented with 200 μ M EDDA. Q: cultures were grown in MHCB which had been pretreated with Chelex 100. The relevant strain numbers appear above each set of samples (see Table 3.2).



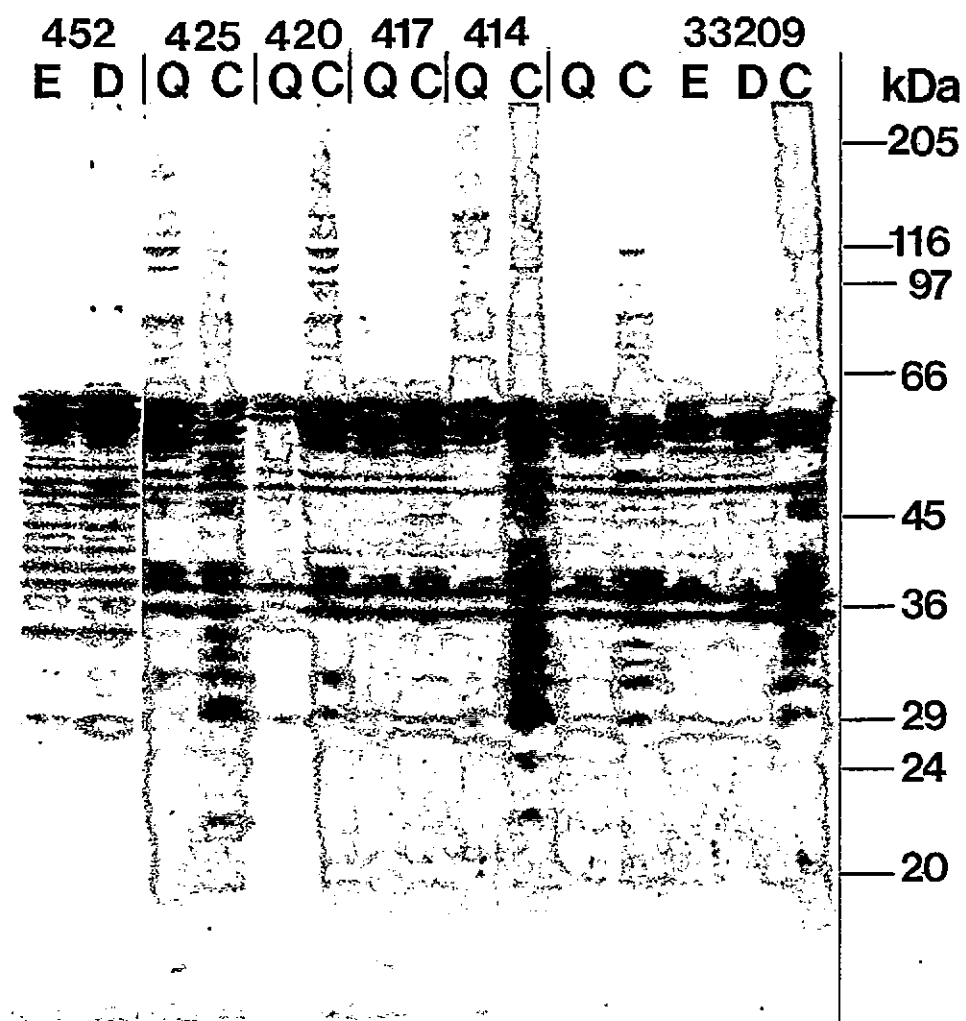


Figure 6.10 (continued). For legend see previous page.

6.4 DISCUSSION

The results of this study have shown that the growth of *R.salmoninarum*, particularly non-autoagglutinating strains, is considerably reduced under conditions of iron restriction. As discussed in Chapter 2, non-autoagglutinating strains have been shown to be non-hydrophobic and of low virulence. Nevertheless, no evidence for the synthesis of the common chemical types of siderophores in iron-restricted culture supernatant could be found. In fact, continued culture of *R.salmoninarum* on iron-restricted medium led to non-viability which further suggests that the bacterium does not possess a high-affinity iron uptake mechanism. Despite this, all of the strains tested did possess some ability to inhibit the binding of iron to iron-free transferrin. This may be due to the chelation of ferric iron by a siderophore not detected by the colorimetric tests employed here. Alternatively, the culture supernatants may have contained a protease capable of cleaving transferrin or possessed some other product that binds non-specifically to the iron binding site on the transferrin molecule. It is interesting to note that the inhibitory effect is decreased, rather than increased, under iron-restricted conditions. This is in contrast to the iron reductase enzyme activity which was greater in iron-restricted cultures and was accompanied by the production of a number of PAS positive proteins and unidentified PAS positive material on SDS-PAGE gels.

An absence of siderophores has been noted in a variety of bacteria including *Yersinia* species (Une & Brubaker, 1984), *S.mutans* (Evans *et al.*, 1986), *Neisseria* species (West, Michelsen, Dyer & Sparling, 1985), *L.pneumophila* (Reeves, Pine, Neilands & Balows, 1983) and *L.monocytogenes* (Cowart & Foster, 1985). Recent research into the iron acquisition mechanisms of *Neisseria gonorrhoeae* (Blanton *et al.*, 1990) and *Neisseria meningitidis* (Lee & Schryvers, 1988) has led to the identification of specific cell surface receptors for transferrin and lactoferrin. Such a mechanism has also been suggested for *Haemophilus influenzae*, *Bordetella* species and *Mycoplasma pneumoniae* (Tryon & Baseman, 1987; Schryvers, 1988; Menozzi, Gantiez & Loch, 1991). Whether *R.salmoninarum* possesses a similar means of obtaining iron has yet to be investigated.

Iron reductase enzymes have been shown to be important in the ability of bacteria such as *Mycobacterium smegmatis* (Brown & Ratledge, 1975; McReady & Ratledge, 1979), *Agrobacterium tumefaciens* (Lodge, Gaines, Arceneaux & Byers, 1982) and *Azotobacter vinelandii* (Huyer & Page, 1989) to acquire iron from siderophores. The reductase acts to reduce the iron in the ferrisiderophore chelate from Fe^{3+} to Fe^{2+} . Since Fe^{2+} has a lower affinity for

the siderophore, the Fe^{2+} is released and can be utilised by the bacterium. In addition, there is evidence that some iron reductase enzymes may be involved in the acquisition of iron from ferric complexes other than ferrisiderophore chelates (Cox, 1980; Huyer & Page, 1989; Johnson *et al.*, 1991). Recent studies of *L.monocytogenes* have identified a soluble protein of 8-10 kDa molecular mass which is present in culture supernatants and acts to rapidly reduce iron from transferrin (Cowart & Foster, 1985; Adams *et al.*, 1990). Johnson *et al.*(1991) have recently identified a cell-associated ferric citrate reductase which is important in the acquisition of iron by *L.pneumophila*. They found that compared to virulent *L.pneumophila* cells, avirulent cells showed a higher reductase activity which was maximal with NADPH as reductant. This finding compares favourably with the results of this study which have shown that low virulence, non-autoagglutinating strains of *R.salmoninarum* possess on average a 20-64% higher reductase activity compared to the more virulent autoagglutinating strains ($p < 0.05$), although maximum reductase activity for all *R.salmoninarum* strains occurred with NADH. Although reductase activity was found to increase under iron-restricted conditions the level of increase suggests that the activity is constitutive. This is particularly likely when comparison is made with the inducible reductases of *Saccharomyces cerevisiae* (Lesuisse, Raguzzi & Crichton, 1987) and *Neurospora crassa* (Ernst & Winkelmann, 1977) where up to a 7 fold increase in reductase activity may accompany iron-limited conditions. Both Cowart & Foster (1985) and Johnson *et al.* (1991) reported that avirulent strains of *L.monocytogenes* and *L.pneumophila* respectively have a higher iron requirement than virulent strains. They suggest that, given the limitations of iron availability in the intracellular environment and the documented effects of iron deprivation on the intracellular growth of both these pathogens (Byrd & Horwitz, 1989; Alford *et al.*, 1991), the inability of avirulent cells to survive within the phagocytic cell may be in part a result of the higher iron requirement of avirulent cells. Indeed, an early pathogenicity study by Sword (1966) documented the dramatic reduction in LD_{50} that occurred when mice which had been experimentally infected with *L.monocytogenes* were administered exogenous Fe^{2+} iron.

It seems likely that given the dramatic effects of iron restriction on the growth of *R.salmoninarum*, particularly ferrous iron restriction, the iron reductase enzyme activity detected here will be of considerable importance to the overall iron transport strategy and therefore viability of *R.salmoninarum*. It is of interest to note that an earlier study on BKD by Suzumoto, Schreck & McIntyre (1977) found that the addition of exogenous Fe^{3+} iron in the form of ferric nitrilotriacetate did not appear to increase the pathogenicity of *R.salmoninarum* when administered to experimentally infected coho salmon. However, the effect of exogenous Fe^{2+}

iron was not examined by Suzumoto *et al.* (1977). The importance and precise role of the iron reductase activity identified by this study in the virulence of *R. salmoninarum* and the pathogenesis of BKD required additional investigation which was beyond the scope of this study. The likely complexity of iron metabolism in *R. salmoninarum* and the lack of readily identifiable components for molecular characterisation provided justification for not pursuing this research further.

This study has demonstrated the immunological detection of epitopes of cloned fusion proteins in extracts derived from *R. salmoninarum* cultured *in vitro* and has therefore confirmed the conservation of B-cell epitopes on the hybrid fusion proteins constructed in Chapter 5. Rat antiserum raised against maltose-binding protein fused to P57 (MBP-P57) identified a series of bands on Western blots of whole cells and ECPs derived from all strains of *R. salmoninarum* used in this investigation. This series of bands strongly resembles the profile of breakdown products of native P57 which have been described by many researchers (Getchell, Rohovec & Fryer, 1985; Kaattari, Holland, Turaga & Weins, 1987; Weins & Kaattari, 1989; Griffiths & Lynch, 1991; Hsu, Bowser & Schachte, 1991; Rockey, Gilkey, Weins & Kaattari, 1991; Rockey, Turaga, Weins, Cook & Kaattari, 1991b). P57 is the major secretory antigen of *R. salmoninarum*, being associated with the cell wall and forming the main constituent of the ECPs. Interestingly, epitopes of MBP-P57 were detected in *R. salmoninarum* strains MT414 and MT417 which have been previously been shown to be non-autoagglutinating and non-hydrophobic and were described as lacking the 57K saline extractable cell-associated protein that has been associated with virulence (Bruno & Munro, 1986a; Bruno, 1988c; 1990). Nevertheless, this study has shown that these strains possess epitopes, both in cell extracts and ECPs, which are recognised by MBP-P57 antiserum. This finding suggests that the hydrophobicity of *R. salmoninarum* may be related to aspects of the cell surface other than simply the presence of P57. It is possible that the observed instability of the hydrophobic phenotype of *R. salmoninarum* (Bruno, 1988c; Bandin, Santos, Barja & Toranzo, 1989) represents either a deletion, mutation or frame-shift within the *msa* gene which affects the hydrophobicity of P57 without altering the major epitopes of the molecule. The growth of *R. salmoninarum* under iron-restricted conditions did not apparently alter the production of P57 in cell extracts. However, the ECPs from iron-restricted cultures did show a reduction in the breakdown and, in some cases, the production of P57 compared to the ECPs from iron-sufficient cultures. The reason for this is not yet known but it is possible that some form of proteolytic processing has been inhibited by the reduced availability of divalent metal cations.

Epitopes of the β -galactosidase-HLY fusion protein were found to be present in the cell extracts of *R.salmoninarum* cultured *in vitro* and their presence does not appear to be affected by the availability of metal ions. Evenden (1993) has predicted that the HLY protein is anchored to the cell wall and exposed on the surface of the bacterium. This would explain the absence of detectable quantities of the protein in the ECPs. In addition, sequence analysis by Evenden (1993) has shown that the *hly* gene encodes a protein of 47,600 molecular weight which is smaller than many of the bands identified on Western blots in this study. There are various possible explanations for this. Firstly, the native protein may possess carbohydrate or lipid moieties which alter its migration on SDS-PAGE. In this respect, Evenden (1993) has identified four possible carbohydrate attachment sites on the HLY protein and the molecule is rich in proline, which retards the migration of polypeptides on SDS-PAGE gels (Hames & Rickwood, 1990). Alternatively, other molecules produced by *R.salmoninarum* may share epitopes which are present on the β -galactosidase-HLY fusion protein. In relation to this possibility it is worth noting that *R.salmoninarum* has been reported to be negative for β -galactosidase activity and does not utilise maltose or starch (Table 3.1).

The immunological detection of epitopes of maltose-binding protein fused to *R.salmoninarum* metalloprotease (MBP-MPR) revealed a number of bands in the cell extracts of all seven strains of *R.salmoninarum* which were examined. No epitopes were detected in the ECPs. It seems likely that the various bands represent breakdown products of the 67K MPR protein. In addition, under iron-restricted conditions these bands were very weakly stained compared to iron-sufficient cultures suggesting that the production of MPR by *R.salmoninarum* is greatly reduced when divalent metal cations are no longer freely available. Evidence has recently been presented that the production and processing of *P.aeruginosa* elastase is enhanced by the addition of either or both zinc and calcium to the growth medium; the effect of iron availability is not quite so clear (Olson & Ohman, 1992). Brumlik & Storey (1992) reported that the addition of Fe^{3+} to a defined growth medium had a negative effect on the translation of elastase. Olson & Ohman (1992) using a Chelex treated complex medium concluded that Fe^{3+} had no major inhibitory role in the elastolytic potential of *P.aeruginosa*. The precise contribution of metal ions such as Fe^{3+} , Fe^{2+} , Zn^{2+} and Ca^{2+} to the regulation of *R.salmoninarum* growth and the production of specific components, such as MPR, P57 and HLY awaits the development of a defined growth medium and better elucidation of the metal ion requirements of *R.salmoninarum*. The lack of immunologically detectable MPR in the ECPs of *R.salmoninarum* cultures suggests that MPR was not secreted under the culture conditions employed in this study. However, the presence of

a signal peptide at the N-terminus of MPR which bears a strong resemblance to other procaryotic signal sequences (Chapter 4) suggests that this protease would be expected to be exported across the *R.salmoninarum* cell wall. It may be that MPR remains attached to or associated with the cell wall and thus does not form a readily detectable part of the ECPs. Alternatively, if MPR is secreted then secretion might occur only under conditions which favour the transcription and translation of the full length MPR protein including the signal peptide. It is clear from recent reviews (Mekalanos, 1992; Dorman & Bhriain, 1992; Olson, 1993) that the regulation of gene expression in bacterial pathogens is part of a complex network which can be better understood following detailed molecular analysis.

The presence of specific antibodies in the serum of rainbow trout undergoing a clinical outbreak of BKD demonstrated that *R. salmoninarum* must be expressing molecules *in vivo* which possess epitopes that are present on each of the fusion proteins constructed in Chapter 5. In addition, these molecules must be immunologically exposed during the course of the infection. This provides circumstantial evidence that HLY and MPR are either surface exposed or secreted *in vivo* by *R. salmoninarum*, although the possibility exists that immunological recognition occurs as a result of bacterial cell lysis. The finding that epitopes of MBP-P57 were also recognised by sera from naturally infected rainbow trout, albeit weakly, confirms the findings of others that the surface-associated 57K protein, P57, is a major antigen produced by *R. salmoninarum* in high concentrations during the course of BKD infections (Turaga, Weins & Kaattari, 1987a; Bartholomew, Arkoosh & Rohovec, 1991; Hsu *et al.*, 1991; Rockey *et al.*, 1991; Olivier, Griffiths, Fildes & Lynch, 1992). However, this study represents the first report of the host response to two previously uncharacterised products, namely the metalloprotease identified here and the haemolysin described by Evenden (1993). These have an as yet unknown role in the pathogenesis of BKD, but both appear to be of immunological relevance to the disease, at least in rainbow trout. The failure to detect specific antibodies in the sera of Atlantic salmon which were undergoing a clinical outbreak of BKD may reflect species differences in the ability to respond to antigens produced by *R. salmoninarum* and the consequent susceptibility to the disease (reviewed in Chapter 2). Alternatively, the different environmental conditions which were present at each farm site may provide an explanation. The Atlantic salmon were caged in the sea at Orkney and water temperatures were low (about 6°C) following winter at the time of the outbreak in April. These low water temperatures may have delayed and reduced the level of any immune response by the salmon. Low water temperatures have been found to slow down the immune responses of teleost fish and to depress the immune response to T-dependent antigens (reviewed by Ellis, 1988). The rainbow trout, on the other hand, were farmed on a freshwater

site in Cumbria. The outbreak occurred in May when water temperatures were about 12°C and at a sufficiently high level for a detectable immune response to occur. It is not yet known whether an immune response to these proteins is correlated with protection, and this is an obvious area for future study. Elucidation of this fact would require a detailed study of both humoral and cellular responses, together with protection experiments following laboratory challenge and field exposure to natural infection.

CHAPTER 7.

CONCLUSIONS

Renibacterium salmoninarum is a slow growing, fastidious intracellular pathogen of salmonid fish and these attributes have hampered previous investigations of individual bacterial components which may have some role in pathogenicity, virulence or host immunity. This study set out to identify components of *R. salmoninarum* which may prove to be useful candidates for inclusion in a BKD vaccine, by using recombinant DNA techniques. In this respect the work has proved to be successful and the novel approach of applying molecular techniques to the study of *R. salmoninarum* has circumvented the difficulties associated with the identification and isolation of macromolecules from *in vitro* cultures.

This thesis presents the first description of the membrane-active metalloprotease, MPR, which bears considerable resemblance to secreted enzymes that have been previously described for other intracellular pathogens. The mode of action of the enzyme either *in vitro* or *in vivo* is unknown but is quite clearly different to previously described activities. However, the results suggest that the expression of *mpr* may be regulated at either the transcriptional or translational level. Certainly, the production of MPR is greatly reduced when the availability of iron is restricted. Despite the presence of a signal peptide, MPR was not detected in the ECPs of *R. salmoninarum* cultures and it seems likely that either MPR is cell-associated or that the conditions which favour export of the protein await discovery.

In addition, fusion proteins were constructed from *R. salmoninarum* genes encoding P57, the major secretory antigen and HLY, a novel membrane-active enzyme described by Evenden (1993). The three proteins were produced in *E. coli* and found to possess epitopes recognised by serum taken from rainbow trout experiencing a clinical outbreak of BKD. Any or all of these components are therefore worthy candidates for inclusion in an experimental BKD vaccine. The feeble antibody responses of trout to each of the fusion proteins is unsurprising since many other researchers have reported similar findings in relation to *R. salmoninarum* products (Evelyn, 1993). There are a variety of options which may be pursued in order to improve the immunogenicity of these components including the use of immunomodulators, defined epitopes or a live, attenuated bacterial carrier as a means of targeting the delivery of the antigens. Whether these recombinant molecules will be of use in eliciting protective immunity requires

further investigations encompassing laboratory and field immunisation and challenge procedures.

The detection of epitopes of each of the three fusion proteins in seven strains of *R. salmoninarum* of differing virulence raises important questions regarding previous work which argued that P57 had a role in virulence (Bruno, 1988c; 1990). The presence of P57 epitopes in strains of both low and high virulence would appear to contradict this argument. However, the production of MPR, P57 and HLY proteins which possess immunologically recognised portions does not necessarily imply a functional property. The instability of haemolytic and hydrophobic phenotypes in *R. salmoninarum* strains cultured *in vitro* may be a consequence of single point mutations or a frame-shift affecting only part of the gene, rather than wholesale deletions. In addition, the involvement of other as yet unidentified factors which may be present can not be dismissed.

This study also represented an initial attempt to characterise the means by which *R. salmoninarum* sequesters iron. In the absence of freely available iron, *R. salmoninarum* grows more slowly, particularly strains of the bacterium which are known to be non-hydrophobic and of low virulence. Iron-restricted culture conditions were also found to lead to a reduction in the processing and, in some cases, the production of P57. No obvious effects on the production of HLY were observed although the possibility that carbohydrate moieties, which may be associated with the molecule, were in some way affected was not examined. In this respect it is interesting to note that one feature of iron-restricted cultures of *R. salmoninarum* is the appearance of reducing sugars associated with cellular proteins. The possession of a strong iron reducing activity and lack of detectable siderophores provides another example of the similarity between *R. salmoninarum* and other intracellular pathogens such as *Listeria monocytogenes* and *Legionella pneumophila*. As reported for *L. pneumophila*, more virulent strains of *R. salmoninarum* have a lower level of iron reductase activity compared to strains of lower virulence. Whether this represents a general correlation of virulence with iron requirement, as appears to be the case for *L. pneumophila*, can only be resolved by further investigation of a wide variety of *R. salmoninarum* strains. One of the main limitations of the overall project has been the use of only seven strains of *R. salmoninarum* and the reliance upon *in vitro* methods of analysis.

Whilst this project was not a study of pathogenicity or virulence mechanisms *per se*, it does provide some basis for future investigations in these areas. For example, utilising isogenic mutants and *in vitro* and *in vivo* models of infection may prove fruitful. The findings of this

study are also significant in drawing attention to the similarities between *R.salmoninarum* and other, particularly intracellular, pathogens. It would be wise for future researchers wishing to investigate aspects of *R.salmoninarum* virulence, pathogenicity and control to consider this before embarking on a programme of research. *R.salmoninarum* is first and foremost an intracellular parasite which has evolved in close association with the salmonid host and must therefore possess certain characteristics which are present in other organisms with a similarly evolved strategy for survival. Austin & Austin (1987) state that "careful thought is necessary to unravel many of the mysteries still surrounding the biology of this pathogen". It is hoped that the findings of this study will contribute to solving this problem.

APPENDIX I

Nucleotide sequence, deduced amino acid sequence and complete restriction map of a cloned DNA fragment from *R. salmoninarum* which includes the *mpr* gene.

Name-Near-Site:

10 20 30 40 50 60
AAGCTTCAAACATAGATTTTTCACAGTCTTTGAAAACCGCAATAGGAATATTGCGGTTT
AlaSerAsnIleAspPheSerGlnSerLeuLysThrAlaIleGlyIleLeuArgPhe
.....
HindIII SspI MseI
AluI
Tth1111IA

70 80 90 100 110 120
AATTATGTGGAAATCCTGTTTCAGTAATCAAAGATGTTTACTGATAGCTTCTCTAACTTA
* LeuCysGlyAsnProValGln * SerLysMetPheThrAspSerPheLeu * LeuA
.....
EcoRI AluI DdeI
EcoRIPA

130 140 150 160 170 180
GGAATAGGTTTATGAAAAGTACTACGCCGTAAGTGGCATAGCTTTGGCTGTTGGCATGC
rgAsnArgPheMetLysLysTyrTyrAlaValThrGlyIleAlaLeuAlaValGlyMetL
.....
ScaI MaeIII AluI NspHIGC
CviQI BsrI CviJIGT
BceFIxR NlaIII

EHE I

190 200 210 220 230 240
TGTGTACAACGCAGCTCGCGGGCGCCACTCAAGCCGCTGACCCCTCGGTGGGCAGCCTTG
euCysThrThrGlnLeuAlaGlyAlaThrGlnAlaAlaAspProSerValGlySerLeuA
.....
CviQI BstUI CviJIAC MnlI CviJIAC
BbvI Fnu4HI HhaI Fnu4HI NspBIICT SecI BbvI Fnu4HI
AluI AhaIIGC HinfI
NlaIV PleIR

250 260 270 280 290 300
ACTCGTCTAACGTCGTTACCGAGTTCTCCGCGCAAGGAAATGTAGAGCAGATCACTTTCA
spSerSerAsnValValThrGluPheSerAlaGlnGlyAsnValGluGlnIleThrPheL
.....
MaeII BstUI EcoBA
MaeIII HhaI DpnI

310 320 330 340 350 360
AATCTGCCATCAAGTCTGCGCCGATGAGTTCCGGCAGCATCGGCTCAGACTAGCGCCATAA
ysSerAlaIleLysSerAlaProMetSerSerAlaArgSerAlaGlnThrSerAlaIleI
.....
HhaI PvuI MaeI
DpnI HaeIIAC
CviJIGT HhaI
DdeI

370 380 390 400 410 420
TCCCCGGCCTTAAGAACTTGTTTGTGAGCGCGCCAGGTAGCGATTTTCAGCCTGAATGATT
leProGlyLeuLysAsnLeuPheValSerAlaProGlySerAspPheSerLeuAsnAspS
.....
SecI Tth1111IRT HhaI CviJIAC
NciIC BssHII HinfI
ScrFI HhaI
HpaII BstUI
CviJIGC BstNIA
AflII ScrFI
MseI

430 440 450 460 470 480
CATCGAACAACTACATCAAGCGGTTACGCGAGAACATCGCAGGCATCCCGGTGCTGGGCT
erSerAsnAsnTyrIleLysArgPheThrGlnAsnIleAlaGlyIleProValLeuGlyS
.....^.....
TaqI SfaNI BanIIGT
FokIR CviJIGT
NciIC
ScrFI
HpaII

490 500 510 520 530 540
CGTCAATTACTGAGGTCCTTGATGGTCAGGGCGCTGTTACCTCGGCAATTGGTGCAGTCA
erSerIleThrGluValLeuAspGlyGlnGlyAlaValThrSerAlaIleGlyAlaValT
.....^.....
EcoRIS HaeIIGT MfeI MaeIII
DdeI HhaI EcoRIS HphIR
MnlIR MaeIII Tsp45IC
DraIIAT MnlI
PpuMIATT SecI
AvaIIT
Sau96I

550 560 570 580 590 600
CCTCTGCTACTAAGGGTGCTTTCCCGGCTGATCTGCCGAGGCCAAGCAGCAGCCCTGGC
hrSerAlaThrLysGlyAlaPheProAlaAspLeuProGlnAlaLysGlnGlnProTrpL
.....^.....
MnlI NciIC Fnu4HI BbvI CviJIGT
DdeI ScrFI HaeIAA CviJIAC
HpaII CviJIGC SecI
CviJIGT Tth111IIG BstNIT
DpnI Fnu4HI
EcoP15
BbvI
Fnu4HI
ScrFI

610 620 630 640 650 660
TTCGGCAACCAAGATCGCCTCAGCTGGCAAAGACGCCAGCCGCGATCTCGCTTGTGACC
euArgGlnProArgSerProGlnLeuAlaLysThrProAlaAlaIleSerLeuValAspG
.....^.....
DpnI AhaIIAC DpnI HincIIITG
MnlI HgaI
DdeI CviJIAC
PvuII Fnu4HI
AluI BstUI

670 680 690 700 710 720
AGAAAGCAATCTGGTTCGACCGCTTCTTATCGGCAAGGGAGCTACAGGCTCCGTTGCAG
lnLysAlaIleTrpPheAspAlaValLeuIleGlyLysGlyAlaThrGlySerValAlaV
.....^.....
TaqI AluI FinI
AhaIIAC CviJIGT
HgaI NlaIV
BcefiXR

730 740 750 760 770 780
TCCCGGCGTACCAGTTCAGCTTACCAGTGGATTTCGCGAATCGCGGGTGCTCACGGTAG
alProAlaTyrGlnPheSerPheThrThrGlyPheAlaGluSerArgValLeuThrValA
.....^.....
NciIC AluI HinfI BstUI AluI
ScrFI HphIR HinfI BbvIR
HpaII BsrI Bsp1286ITT Fnu4HI
CviQI
BsrIR

1150 1160 1170 1180 1190 1200
 CGTTCGCTAATGCTTTCTGGTACAACGGCCAGATGACCTACGGCCAAGGCGTGACCACCG
 roPheAlaAsnAlaPheTrpTyrAsnGlyGlnMetThrTyrGlyGlnGlyValThrThra
^.....^.....^.....^.....^.....
CviQI.....BceFIx.....MaeIII
BceFIx.....CfrICA
CfrICA.....CviJIGC
CviJIGC.....SecI
StyIAA.....Tsp45IG

1210 1220 1230 1240 1250 1260
 ACGACATTACCGGCCACGAAGTACGCGACGGCGTGACCGAAAAGACCAATGGTTTGGTCT
 spAspIleThrGlyHisGluLeuThrHisGlyValThrGluLysThrAsnGlyLeuValT
^.....^.....^.....^.....^.....
Cfr10IAC.....HgaI.....TaqIIa.....BstXI
HpaII.....DraIII.....EcoPI.....EcoPIR
CfrICA.....BceFIx
CviJIGC.....MaeIII
Tsp45IG

1270 1280 1290 1300 1310 1320
 ATGCAAATGAATCTGGCGCAATCAACGAATCGATGTCCGATGTATTCCGGCGAGTTCATCG
 yrAlaAsnGluSerGlyAlaIleAsnGluSerMetSerAspValPheGlyGluPheIleA
^.....^.....^.....^.....^.....
HinfI.....HinfI.....TaqI
HhaI.....ClaI
TaqI

1330 1340 1350 1360 1370 1380
 ACTTGAGCAACGGCAGCTCGGATGACACTGCAGCTAACCGCTGGGCAATCGGCGAAGGCA
 spLeuSerAsnGlySerSerAspAspThrAlaAlaAsnArgTrpAlaIleGlyGluGlys
^.....^.....^.....^.....^.....
BceFIx.....PstI.....BbvI
BbvI.....BbvI.....Fnu4HI
Fnu4HI.....Fnu4HI.....EcoP15
AluI.....AluI
FokI.....NspBIICT

1390 1400 1410 1420 1430 1440
 GCAGCCTTGGTGTGATCCGGAGCATGAAAGATCCCGGAAAGTACGGTGAACCGGCGATCT
 erSerLeuGlyValIleArgSerMetLysAspProGlyLysTyrGlyGluProAlaIleT
^.....^.....^.....^.....^.....
BbvI.....AlwIR.....BstYIAC.....CviQI.....DpnI
Fnu4HI.....DpnI.....AlwIR.....HphI
CviJIAC.....BspMII.....DpnI.....Cfr10IAC
SecI.....HpaII.....HpaII
StyITT.....NlaIII.....HpaII
ScrFI

1450 1460 1470 1480 1490 1500
 ATAAGGGCTCCAAGTGAAGCCAACCGCTACCAACCCGAACGACAACAATGACCAGGGTG
 yrLysGlySerAsnTrpLysProThrAlaThrAsnProAsnAspAsnAspGlnGlyC
^.....^.....^.....^.....^.....
BaniIGT.....CviJIAC.....BstNIA
CviJIGT.....ScrFI
NlaIV.....SecI
MmeIA
BsrI

Alphabetic:			
AflII	CTTAAG	!	:369
AhaIIAC	GACGCC	!	:632 678
AhaIIGC	GGCGCC	!BanICG;BbeI;Eco78I;HaeIIGC;NarI	:201
AluI	AGCT	!CviJIIAT	:2 105 161 193 62
2 701 738 779 875 947 1335	1352 1531 1609 1659 1787		
AlwI	GGATC	!+4N	:1102
AlwIR	GATCC	!-5N;BinIR	:1394 1410
AvaITA	CTCGAG	!SciI;XhoI	:1655
AvaIIT	GGTCC	!	:494 1097
BanIIGT	GGGCTC	!Bsp1286IGT	:476 1445
BbvI	GCAGC	!+8N	:191 232 587 590
1333 1350 1378 1381 1725			
BbvIR	GCTGC	!-12N	:780 948
BceFIx	ACGGC	!+12N	:790 1126 1165 11
80 1228 1330 1552 1567			
BceFIxR	GCCGT	!-13N	:147 681 1092
BclI	TGATCA	!	:1543
BglI	GCCNNNNNGGC	!	:1120 1752
BinI	GGATC	!+4N	:1102
BsmIR	GCATTC	!-1N	:1536
Bsp1286ITCGTGCCC		!	:1136
Bsp1286ITTTGTGCTC		!HgiAIIIT	:768
BspMII	TCCGGA	!	:1099 1396
BsrI	ACTGG	!+1N	:153 747 1453 161
5			
BsrIR	CCAGT	!-1N	:731
BssHII	GCGCGC	!	:388
BstNIA	CCAGG	!EcoRIIA	:393 1493 1739
BstNIT	CCTGG	!EcoRIIT	:595
BstUI	CGCG	!ThaI	:197 269 389 641
763 834 931 1809			
BstXI	CCANNNNNNTGG	!	:1246
BstYIAC	AGATCC	!XhoIIAC	:1409
BstYIGT	GGATCT	!	:1102
Cfr10IAC	ACCGGC	!	:1209 1430 1820
Cfr10IGT	GCCGGT	!	:1798
CfrICA	CGGCCA	!GdiIIRA	:1166 1181 1211 1
553 1568			
CfrITG	TGGCCG	!GdiIIT	:961
ClaI	ATCGAT	!	:864 1289
CviJIIAC	AGCC	!	:212 234 408 592
638 925 1119 1383 1459 1745			
CviJIGC	GGCC	!HaeIII	:366 581 917 942
962 1028 1167 1182 1212 1554 1569 1601 1619 1862			
CviJIGT	GGCT	!	:167 341 477 566
598 708 1446 1670			
CviQI	GTAC	!RsaI	:140 184 728 1160
1421			
DdeI	CTNAG	!	:117 343 490 550
619 913 1024 1597 1606 1780 1843			
DpnI	GATC	!MboI;Sau3AI	:290 337 570 613
644 816 867 1035 1103 1394 1410 1436 1544 1838			
DraIIAT	AGGNCCCT	!PssIAT	:493
DraIII	CACNNNGTG	!	:1227
DsaIGT	CCGTGG	!	:1093
EcoBA	AGCANNNNNNNTCA	!	:286
EcoP15	CAGCAG	!+25N	:588 1379
EcoPI	AGACC	!	:1243 1573
EcoPIR	GGTCT	!	:839 1256
EcoRIPA	AAATCC	!	:71
EcoRIS	AATT	!TspEI	:61 485 527 1586
EspI	GCTNAGC	!	:1605 1842
FinI	GTCCC	!	:720
Fnu4HI	GCNGC	!	:191 213 232 575
587 590 639 780 948 1333	1350 1378 1381 1602 1620 1725		
FokI	GGATG	!+9N	:1340
FokIR	CATCC	!-13N	:464
HaeIAA	AGGCCA	!	:580 916 941 1027
HaeITT	TGGCCT	!	:1861
HaeIIAC	AGCGCC	!	:351 1795
HaeIIGT	GGCGCT	!	:510 792 1823
HgaI	GACGC	!+5N	:632 678 929 1223
HgaIR	GCGTC	!-10N	:1002
HhaI	GCGC	!HinPII	:202 270 318 352
388 390 511 793 932 1276	1796 1824		
HincIITG	GTTGAC	!	:654 1634

HindIII	AAGCTT	!	:1
HinfI	GANTC	!	:240 417 751 759
911 1269 1287			
HpaII	CCGG	!	:364 468 564 723
1100 1210 1397 1414 1431 1582 1799 1821			
HphI	GGTGA	!+8N	:1425
HphIR	TCACC	!-7N	:538 742
MaeI	CTAG	!	:349 877 1789
MaeII	ACGT	!	:250 1696 1708
MaeIII	GTNAC	!	:150 255 516 537
1110 1191 1233 1578			
MfeI	CAATTG	!	:526
MmeIA	TCCAAC	!+20N	:1449 1509
MnlI	CCTC	!+7N	:223 520 541 618
MnlIR	GAGG	!-7N	:492 1814
MseI	TTAA	!	:59 370
NciIC	CCCGG	!	:363 467 563 722
1413			
NlaIII	CATG	!	:176 1403
NlaIV	GGNNCC	!	:201 708 1446 171
6			
NruI	TCGCGA	!	:1808
NspBIIAG	CAGCGG	!	:1514
NspBICT	CCGCTG	!	:214 1358
NspHIGC	GCATGC	!SphI	:175
PleIR	GACTC	!-5N	:240 911
PpuMIATT	AGGTCCT	!	:493
PstI	CTGCAG	!	:1348 1865
PvuI	CGATCG	!	:336 815
PvuII	CAGCTG	!NspBIIAT	:621
Sau96I	GGNCC	!	:494 1097 1618
ScaI	AGTACT	!	:139
ScrFI	CCNGG	!SsoII	:363 393 467 563
595 722 1413 1493 1739			
SecI	CCNNGG	!	:223 362 520 594
1093 1184 1385 1493 1738			
SfaNI	GCATC	!+5N	:463 951 1768
SfaNIR	GATGC	!-9N	:846 888
SspI	AATATT	!	:47
StyIAA	CCAAGG	!	:1184
StyITT	CCTTGG	!	:1385
TaqI	TCGA	!	:423 676 855 865
1290 1318 1656			
TaqIIa	GACCGA	!+11N	:1235
TaqIIb	CACCCA	!+11N	:1736
Tsp45IC	GTAC	!	:537
Tsp45IG	GTGAC	!	:1110 1191 1233 1
578			
Tth111IIA	CAAACA	!+11N	:7 1018
Tth111IIG	CAAGCA	!+11N	:584
Tth111IIRCTGCTTG		!-9N	:1682
Tth111IIRTTGTTTG		!-9N	:379
XhoIIGT	GGATCT	!	:1102
NOT Found (for those classes of cutters selected):			
AatII	GACGTC	!AhaIIAT	:
AccIAG	GTAGAC	!	:
AccIAT	GTATAC	!SnaI;XcaI	:
AccICG	GTCGAC	!HincIICG;SalI	:
AccICT	GTCTAC	!	:
AflIIIIAC	ACACGT	!	:
AflIIIIAT	ACATGT	!NspHIIAT	:
AflIIIIIC	ACGCGT	!MluI	:
AflIIIIIGT	ACGTGT	!	:
AhaIIGT	GGCGTC	!	:
AlwNI	CAGNNNCTG	!	:
AocI	CCTNAGG	!Bsu36I	:
ApaI	GGGCCC	!BaniIGC;Bsp1286IGC	:
ApaLI	GTGCAC	!Bsp1286ITA;HgiAITA	:
AseI	ATTAAT	!VspI	:
Asp700I	GAANNNTTC	!XmnI	:
Asp718I	GGTACC	!BaniTA;KpnI	:
AsuII	TTCGAA	!BstBI	:
AvaICA	CCCGAG	!	:
AvaICG	CCCGGG	!SmaI;XmaI	:
AvaITG	CTCGGG	!	:
AvaIIA	GGACC	!	:
AvrII	CCTAGG	!StyITA	:

BalI	TGGCCA	!CfrITA;HaeITA	:
BamHI	GGATCC	!XhoIIGC	:
BanICA	GGCACC	!	:
BanITG	GGTGCC	!	:
BanIIAC	GAGCCC	!Bsp1286IA	:
BanIIAT	GAGCTC	!Bsp1286IAT;Ecl136II;HgiAIIAT;SacI	:
BbvII	GAAGAC	!+2N	:
BbvIIR	GTCTTC	!-6N	:
BglII	AGATCT	!XhoIIAT	:
BsmI	GAATGC	!+1N	:
Bsp1286IAAGAGCAC		!HgiAIAA	:
Bsp1286IGAGGGCAC		!	:
BspHI	TCATGA	!	:
BspMI	ACCTGC	!+4N	:
BspMIR	GCAGGT	!-8N	:
BstEII	GGTNACC	!	:
Cfr10IAT	ACCGGT	!	:
Cfr10IGC	GCCGGC	!Eco56I;NaeI	:
CfrICG	CGGCGG	!EagI;GdiIIC;GdiIIRC;XmaIII	:
DraI	TTTAAA	!	:
DraIIAC	AGGNCCC	!PssIAC	:
DraIIGC	GGGNCCC	!PssIGC	:
DraIIGT	GGGNCCCT	!PssIGT	:
DsaIAC	CCACGG	!	:
DsaIAT	CCATGG	!NcoI;StyIAT	:
DsaIGC	CCGCGG	!SacII;NspBIIGC	:
Eco31I	GGTCTC	!+1N	:
Eco31IR	GAGACC	!-5N	:
Eco47III	AGCGCT	!HaeIIAT	:
Eco57I	CTGAAG	!+16N	:
Eco57IR	CTTCAG	!-14N	:
EcoBT	TGANNNNNNNTGCT	!	:
EcoKA	AACNNNNNNGTGC	!	:
EcoKG	GCACNNNNNNGTT	!	:
EcoNI	CCTNNNNNAGG	!	:
EcoP15R	CTGCTG	!-27N	:
EcoRI	GAATTC	!	:
EcoRIPG	GGATTT	!	:
EcoRV	GATATC	!	:
FinIR	GGGAC	!	:
FspI	TGCGCA	!	:
GsuI	CTGGAG	!+16N	:
GsuIR	CTCCAG	!-14N	:
HgiEII	ACCNNNNNNGGT	!	:
HincIIICA	GTCAAC	!	:
HpaI	GTTAAC	!HincIITA	:
Ksp632I	CTCTTC	!+1N	:
Ksp632IR	GAAGAG	!-4N	:
MboII	GAAGA	!+8N	:
MboIIR	TCTTC	!-7N	:
MmeIG	TCCGAC	!+20N	:
MmeIRC	GTCGGA	!-18N	:
MmeIRT	GTTGGA	!-18N	:
NciIG	CCGGG	!	:
NdeI	CATATG	!	:
NheI	GCTAGC	!	:
NotI	GCGGCCGC	!	:
NsiI	ATGCAT	!	:
NspHIGT	GCATGT	!	:
PflMI	CCANNNNNTGG	!	:
PleI	GAGTC	!+4N	:
PmaCI	CACGTG	!	:
PpuMIAAC	AGGACCC	!	:
PpuMIAAT	AGGACCT	!	:
PpuMIATC	AGGTCCC	!	:
PpuMIGAC	GGGACCC	!	:
PpuMIGAT	GGGACCT	!	:
PpuMIGTC	GGGTCCC	!	:
PpuMIGTT	GGGTCCCT	!	:
RsrIIA	CGGACCG	!	:
RsrIIT	CGGTCCG	!	:
SfiI	GGCCNNNNNNGGCC	!	:
SnaBI	TACGTA	!	:
SpeI	ACTAGT	!	:
SplI	CGTACG	!	:
StuI	AGGCCT	!HaeIAT	:
TaqIIaR	TCGGTC	!-9N	:
TaqIIbR	TGGGTG	!-9N	:
Tth111I	GACNNNGTC	!	:
Uba26I	GTCTC	!+1N	:
Uba26IR	GAGAC	!-5N	:
XbaI	TCTAGA	!	:

APPENDIX II

Comparison of the amino acid sequences of a variety of bacterial metalloproteases. *: identical residues; .: conserved residues.

B.stearothermophilus (M) : -MNKRAM---LGAIGLAFGLLAA----PIGASAKGESIVWNEQWKTPSFVSGSLLNGGEQ
B.caldolyticus : -MNKRAM---LGAIGLAFGLMAW----PFGASAKGKSMVWNEQWKTPSFVSGSLLGRCSQ
B.stearothermophilus (N) : --MKRKMKMKLVRFGLAAGVAAQVFFLPYNALASTEHVVTWNQQFQTPQFISGDLLKVNGT
B.megaterium : MKKKKQALKVLLSVGILSSSFafahtSSAAPNNVLSTeKYNKEIKSPEFISGKLSGPSSQ
B.thermoproteolyticus : -----
B.cereus : -----
B.polymyxa : -----MKKVWFSLGgAMLLGSVASGASAEssVSGPAQLTPTFHTEQWKAPSSV
B.subtilis (N) : -----MR
B.subtilis (M) : -----
B.amyloliquifaciens : -----
L.monocytogenes : -----
R.salmoninarum : -----
P.aeruginosa : -----
V.cholerae : -----
V.proteolyticus : -----
L.pneumophila : -----
E.carotova : -----

B.stearothermophilus (M) : ALEELVYQYVDRENGTFRlggr-ARdRLALIGKQTDELGH-TVMRFEQRHHGIPVYGTML
B.caldolyticus : ---ELVYRYLDQEKNTFQLGGQ-ARERLSLIGNKLDELGH-TVMRFEQAIAASLCMGAVL
B.stearothermophilus (N) : SPEELVYQYVEKNENKFKFHEN-AKDTLQLKEKKNDNLGF-TFMRFQQTYKGIPVFGAVV
B.megaterium : KAQDVVFHYMNTNKDKYKLGNEsaQNSfKVTEVVKDPVEQATVVRLQQVYNNIPVWGSTQ
B.thermoproteolyticus : -----
B.cereus : -----
B.polymyxa : SGDDIVWSYLNrQKKSLLGVDSSSVREQFRIVDRtSDKSGVSHYRLKQYVNGIPVYGAEQ
B.subtilis (N) : NLTKTSLLLAgLCTAAQMVfVTHASAEESIEYDHTYQTPSYIIEKSPQKPVQNTTQKESL
B.subtilis (M) : -----MGLGKKLSVRVAASfMSLSISLPGVQAAEGHQLKENQT
B.amyloliquifaciens : -----MGLGKKLSVAVAASfMSLTISLPGVQAAENPQLKENLT
L.monocytogenes : -----MKSklICIIMVIAfQAHFTMTVKADSV
R.salmoninarum : -----MKKYyAVTGIALAVGMLCTTQLAGATQAADPSV
P.aeruginosa : ----MKKVSTLDLLFVAImGVSPAaFAADLIDVSKLPSKAAQGAPGVTLQAAVGAGGA
V.cholerae : -MKMIQRPLNWLVLAgAATGFPLYAAQMVtIDDASmVEQA-----LAQQQYSMPAA
V.proteolyticus : -MNKTQRHINWLLAVSAATALPVTAAEMINVDGSLNQA-----LKAAQSQSVAPVE
L.pneumophila : -----MHPNYyLSPLAVAIAlGIAspVKAADPIPL-----QKSSfSEVT
E.carotova : -----

B. stearothermophilus (M) : AAHV-KDGELIALSGSLIPNLDGQPR-----KKAKTVTVQQAEEAIAEQDVTETVTKERP
B. caldolyticus : VAHV-NDGELSSLSGTLIPNLDKRT-L-----KTEAAISIQQAEIAKQDVADRVTKERP
B. stearothermophilus (N) : TAHV-KDGTLTALSGTLIPNLDTKGSL-----KSGKKLSEKQARDIAEKDLVANVTKEVP
B. megaterium : LAHVAKDGTCLKVVSCTVAPDLDKKEKL-----KGQKQVDSKKAIQAAEKDLGFK-----P
B. thermoproteolyticus : -----
B. cereus : -----
B. polymyxa : TIHVKGSGEVTSYLGAVINEDQEEATQGTPPKISASEAVYTAYKEAAARIEALPTSDDT
B. subtilis (N) : FSYLDKHQTQFKLKGNANSHFRVSKTIKDPKTKQTFF-----
B. subtilis (M) : NFLSKKPIAQSELSAPNDKAVKQFL--KKNSNIFKGDPSKSVKLVESTTDALGYKHFRYA
B. amyloliquifaciens : NFVFKHSLVQSELPVSDKAIKQYL--KQNGKVFKGNPSERLKLIDQTTDDLGYKHFRYV
L. monocytogenes : GEEKLQNNQAKKTPADLKALPDSCEAKDFYKNF-----KILDMTKDKLGVTHYTLA
R. salmoninarum : GSLDSSNVVTEFSAQGNVEQITFKSAIKSAPMSSARSAQTSATIPGLKNLFVSAPGSDFS
P. aeruginosa : DELKAIRSTTLP-----NGKQVTRYEQFHNGVRVVGAI-----
V. cholerae : SGFKAVNTVQLP-----NGKVKVRYQQMYNGVPVYG-----
V. proteolyticus : TGFKQMKRVLPL-----NGKVKVRYQQTHHGLPVFN-----
L. pneumophila : QKFQ----LTLPGVMKGAVVSTNSLQFIRQHTDGNKVTHVRMQQYAGFPVFGGYAILHS
E. carotova : -----

B. stearothermophilus (M) : TT---ENGERTRLVIYPTDGTARLAYEVNVRFLTPV-----
B. caldolyticus : AA---EEGKPTRLVIYPDEETPRLAYEVNVRFLTPV-----
B. stearothermophilus (N) : EY---EQGKDTEFVVYVNGDEASLAYVNLNFLTPE-----
B. megaterium : TY---EKSPSSELYVYQNASDTTYAYVNLNFLSPE-----
B. thermoproteolyticus : -----
B. cereus : -----
B. polymyxa : ISKDAEEPSSVSKDTYAEANNDKTLSDKDELSDKASVL-----
B. subtilis (N) : --KLTEVYKGIPIYGFEQAVAMKENKQVKSFFGKVHPQIKDVSV-----TPSISE
B. subtilis (M) : PVVNGVPIKDSQVIVHVDKSDNVYAVNGELHNQSAAKTDNSQKVSSEKALALAFKAIGKS
B. amyloliquifaciens : PVVNGVPVKDSQVIIHVDKSNNVYAINGELNNDVSAKTANSKKLSANQALDHAYKAIGKS
L. monocytogenes : LSSGGYLTNDDEIKVHVTPDNKITFINGDLQQGQLRIT-NQIKITEKNAIEKAFAEIGQS
R. salmoninarum : LNDSSNNYIKRFTQNIAGIPVLGSSITEVLDGQGAVTSAIGAVTSATKGAFFPADLPQAKQ
P. aeruginosa : ---TEVKGPGKSVAQAQRSGHFVANIAADLPGSTTAAVSAEQV-----LAQAKSLKA
V. cholerae : ---TVVVATESSKGISQVYGQMAQQLEADLPTVTPDIESQQA-----IALAVSHFG
V. proteolyticus : ---TSVVATESKSGSSEVFGVMAQGIADDVSTLTPSVEMKQA-----ISIAKSRFQ
L. pneumophila : KNATPSLATAKS-----DEKMNGVIYDGLQAEKGQPKPSFVKNASMALQ
E. carotova : -----

B.stearothermophilus (M) : -----PGNWVYII---DATDGAILNKFNQIDS-----
B.caldolyticus : -----PGNWIYMI---DAADGKVLNKWNQMDE-----
B.stearothermophilus (N) : -----PGNWLYII---DAVDGKILNKFNQLDA-----
B.megaterium : -----PGNYYYFV---DAISGKVLDKYNTIDSVAGPKAD-----
B.thermoproteolyticus : -----
B.cereus : -----
B.polymyxa : KDSKIEAVEAEKSSIAKIANLQPEVDPKAELYYPKGGDLLLVYVTEVNVLEPAPLRTRY
B.subtilis (N) : KKAIHTARRELEASIGKIEYLDGEPKGELYIYPHDGEYDLAYLVRLSTSEPEPGYWHYFI
B.subtilis (M) : PDAV-SNGAAKNSNKAELKAIETKDGSYRLAYDVTIRYVEPEPANWEVLVDAETGSILKQ
B.amyloliquifaciens : PEAV-SNGTVANKNKAELKAAATKDGKYRLAYDVTIRYIEPEPANWEVTVDAETGKILKK
L.monocytogenes : EAHVKSIVGNPVKEKEIILNSRTK----RLVYNIKLIFAEPEVASWIVQVDVETGAILKK
R.salmoninarum : QPWLRRQPRSPQLA-KTPAAISLVDQKAIWFDVAVLIGKATGSAVPAYQFSFTTGFAESR
P.aeruginosa : QGRKT-----ENDKVELVIRLGENNIAQLVYNVSYLIPGEG--LSRPHFVIDAKTGEVL
V.cholerae : EQHAGESLPV-ENESVQLMVRLLDDNQQAQLVYLVDFVASET--PSRPFYFISAETGEVL
V.proteolyticus : QQEKMVAEPATENEKAEMLMVRLLDDNQQAQLVYLVDFVVAEDH--PARPFFFIDAQTGEVL
L.pneumophila : QFKDKYANKQVSEDQVTPMIYIDEKHQAHWAYKVSVLVIHDDRIPERPTAIDAETNPKPF
E.carotova : -----

B.stearothermophilus (M) : ---RQPGGGQPVAGASTVGV-GRGVLGDQKYINTTYSSYYGYYYLQDNTRGSGIFTYDGR
B.caldolyticus : ---AKPGGAQPVAGTSTVGV-GRGVLGDQKYINTTYSSYYGYYYLQDNTRGSGIFTYDGR
B.stearothermophilus (N) : ---AKPGDVKSITGTSTVGV-GRGVLGDQKNINTTYST---YYYLQDNTRGNGIFTYDAK
B.megaterium : VKQAAKPAAPVTGTNTIGS-GKGVLDGDTKSLKTTLSST--YYLQDNTRGATIITYDAK
B.thermoproteolyticus : -----ITGTSTVGV-GRGVLGDQKNINTTYST---YYYLQDNTRGDGIFTYDAK
B.cereus : -----VTGTNKVGT-GKGVLDGDTKSLNTTSLSGSS--YYLQDNTRGATIITYDAK
B.polymyxa : IIDANDGSIVFQYDIIKEAT-GKGVLDGDSKSFTTTASGSS--YQLKDTTRGNGIVITYTAS
B.subtilis (N) : DAKNGKVIESFNAIHEAAGT-GIGVSGDEKSFVDTEQNGR--FYLADETRGKGINTFPAK
B.subtilis (M) : QNK---VEHAAATGSGTTL-KGATVPLNISYEGGK-----YVLRDLKPTGTQIITYDLQ
B.amyloliquifaciens : QNK---VEHAATTGTGTTL-KGKTVSLNISSESGK-----YVLRDLKPTGTQIITYDLQ
L.monocytogenes : QNMLSEVERADTHKDFQALGKGANRLLRPLHVMKINDLFYLVDRTHKGL---IRTFDLK
R.salmoninarum : VLTVAANDGAILNDRDTRKDINRVVCDANSKVIDLEASNADALLKCGKTQANKPTRIEGQ
P.aeruginosa : DQWEGE--AHAEAG--GPGGNQKIGKYTYGSDYGPL-IVNDR----CEMDDGNVITVDMN
V.cholerae : DQWDGI--NHAQATGTGPGGNQKTGRYEYGSNGLPG-FTIDKTGTTCTMNSAVKTVNLN
V.proteolyticus : QTWDGL--NHAQADGTGPGGNKTGRYEYGSDF-PP-FVIDKVGTKCSMNSAVRTVDLN
L.pneumophila : VQWDDVKTEKVQAKGMGFGGNRKIGEYQFGKDLPLLEITRDSSVEMCFMENTDVKVVDN-
E.carotova : -----MKSRPICSVIPPYILHRIANGTDEQRHCAQQTLMHVQSLM

B. stearothermophilus (M) : NRTV-----LPGSLWTDGDNQFTASYDAA-----AVDAHYYAGVVY
B. caldolyticus : NRTV-----LPGSLWADGDNQFFASYDAA-----AVDAHYYAGVVY
B. stearothermophilus (N) : YRTT-----LPGSLWADADNQQFFASYDAP-----AVDAHYYAGVTY
B. megaterium : NRTS-----LPGTLWADTDNTYNATRDAA-----AVDAHYYAGVTY
B. thermoproteolyticus : YRTT-----LPGSLWADADNQQFFASYDAP-----AVDAHYYAGVTY
B. cereus : NRST-----LPGTLWADADNVFNAAAYDAA-----AVDAHYYAGKTY
B. polymyxa : NRQS-----IPGTLTLDADNVWN---DPA-----GVDHAYAAKTY
B. subtilis (N) : NLNETLFTLLSQLIGYTGKEIVSGTSVFNEPA-----AVDAHANAQAVY
B. subtilis (M) : NRQSR-----LPGTLVSSTTKTFTSSSQRA-----AVDAHYNLGKVVY
B. amyloliquifaciens : NREYN-----LPGTLVSSTTNQFTTSSSQRA-----AVDAHYNLGKVVY
L. monocytogenes : HNTDT-----SFGKVVSNTKTNMFTDPEFSS-----AVDAHFYASEVY
R. salmoninarum : AASSVADVNSVYNFLNDTASFYGANTKANDLT-----ALIGN-----
P. aeruginosa : SSTDDSKTTPFRFACPT-----NTYKQVNGAYSPLNDAHFFGGVVFKLY
V. cholerae : GGT--SGSTAFSYACNNS-----TNY-----NSVKTVNGAYSPLNDAHFFGKVVFDMY
V. proteolyticus : GST--SGNTTYSYTCNDS-----TNY-----NDYKAINGAYSPLNDAHFFGKVVFDMY
L. pneumophila : GHKYYSNKPMQFTCKETPDQTSTKYTYTGYSADGYDRDNGAASPTNDALYAGYVIKHY
E. carotova : VSHHPRPEPEKLPAGQANRSIHDAEQQQQLPGKLVRAEGQPSNGDIAVDEAYSYLGVY

B. stearothermophilus (M) : DYYKNVHGRLSYDGSNAAIRSTVHYGRGYNNAFWNGSQMVYGDGDGQ--TFLPFSGGIDV
B. caldolyticus : DYYKNVHGRLSYDGSNAAIRSTVHYGRGYNNAFWNGSQMVYGDGDGQ--TFLPFSGGIDV
B. stearothermophilus (N) : DYYKNVHNRLSYDGNNAAIRSSVHYSQGYNNAFWNGSQMVYGDGDGQ--TFIPLSGGIDV
B. megaterium : DYYKNKFNRNSYDNAGRPLKSTVHYSSGYNNAFWNGSQMVYGDGDGT--TFVPLSGGLDV
B. thermoproteolyticus : DYYKNVHNRLSYDGNNAAIRSSVHYSQGYNNAFWNGSEMUYGDGDGQ--TFIPLSGGIDV
B. cereus : DYYKATFNRRNSINDAGAPLKSTVHYGSNYNNAFWNGSQMVYGDGDGV--TFTSLSGGIDV
B. polymyxa : DYYKSKFGRNSIDGRGLQLRSTVHYGSRYNNAFWNGSQMTYGDGDGDGSTFIAFSGDPDV
B. subtilis (N) : DYYSKTFGRDSFDQNGARITSTVHVKGQWNNAAWNGVQMVYGDGDGSK--FKPLSGSLDI
B. subtilis (M) : DYFYSNFKRNSYDNKGSKIIVSSVHYGTQYNNAAWTGDQMIYGDGDGGSF--FSPLSGSLDV
B. amyloliquifaciens : DYFYQKFNRNSYDNKGSKIIVSSVHYGSRYNNAAWIGDQMIYGDGDGGSF--FSPLSGSMDV
L. monocytogenes : EYYKNVHQLSLDGGKGEIDSFVHYGLNCNNAFWWDGQEILYGDGDGKKN--FKPFSCAKTI
R. salmoninarum : DEGDGLGKAMRAVVRICVTDSONGEQCPFANAFWYNGQMTYQGQVTTD-----DI
P. aeruginosa : RDWFGTSPLTH-----KLYMKVHYGRSVENAYWDGTAMLFGDGATM---FYPLVSLDVA
V. cholerae : QQWLNTSPLTF-----QLTMRVHYGNNYENAFWDGRAMTFGDGYTR---FYPLVDINVS
V. proteolyticus : KDWMNTTPLTF-----QLTMRVHYGNNYENAFWNGSSMTFGDGYST---FYPLVDINVS
L. pneumophila : HDWYGVEALTKSDGSPMQLVMRVHYGQGYENAYWDGKQMTFGDGDGM---MYPLVSLGVG
E. carotova : DFFWKIFQRNSLDAEGLPLAGTVHYGQDYQNAFWNGQQMVFGDGDGKIFNRFTIALDVVA

*** .*. *

B. stearothermophilus (M) : VGHELTHAVTDYTAGLVYQNESGAINESAMSDIFGTLVEFYANRNP-----WEIGEDIYT
B. caldolyticus : VGHELTHAVTDYTAGLVYQNESGAINESAMSDIFGTLVEFYANRNP-----WEIGEDIYT
B. stearothermophilus (N) : VAHELTHAVTDYTAGLIYQNESGAINESAMSDIFGTLVEFYANKNP-----WEIGEDVYT
B. megaterium : IGHELTHALTERSSNLIYQYESGALNEAISDIFGTLVEFYDNRNP-----WEIGEDIYT
B. thermoproteolyticus : VAHELTHAVTDYTAGLIYQNESGAINESAMSDIFGTLVEFYANKNP-----WEIGEDVYT
B. cereus : IGHELTHAVTENSNNLIYQNESGALNEAISDIFGTLVEFYDNRNP-----WEIGEDIYT
B. polymyxa : VGHELTHGVTEYTSNLEYYGESGALNEAFSDVIGNDI-----QRKN-----WLVGDDIYT
B. subtilis (N) : VAHEITHAVTQYSAGLLYQGEFGALNESISDIMGAMADRD-----D-----WEIGEDVYT
B. subtilis (M) : TAHEMTHGVQTETANLIYENQPGALNESFSDVFG-----YFNDED-----WDIGEDITV
B. amyloliquifaciens : TAHEMTHGVQTETANLNYENQPGALNESFSDVFG-----YFNDED-----WDIGEDITV
L. monocytogenes : VGHELTHAVIQYSAGLEYEGQSGALNESFADVFG-----YFIAPNH-----WLVGEDVCV
R. salmoninarum : TGHELTHGVTEKTNGLVYANESGAINESMSDVFGFIDLSNGSSDDTAANRWAIGEGSSL
P. aeruginosa : A-HEVSHGFTEQNSGLIYRGQSGGMNEAFSDMAGEAAEFYMRGKND-----FLIGYDIKK
V. cholerae : A-HEVSHGFTEQNSGLVYRDMSSGINEAFSDIAGEAAEFYMRGNVD-----WIVGADIFK
V. proteolyticus : A-HEVSHGFTEQNSGLVYENMSSGMNEAFSDIAGEAAEFYMKGSVD-----WVVGADIFK
L. pneumophila : G-HEVSHGFTEQHSGLYFGQSGGMNESFSDMAAQAAEYYSVGKNS-----WQIGPEIMK
E. carotova : --HELTHGITENEAGLIYFRQSGALNESLSDVFGSMVKQYHLGQTTEQAD-WLIGAEALLA
 ** . * . * * * . ** * . *

B. stearothermophilus (M) : PGVAGDALRMSDPAKYGDPDHYSKRY-----TGTQ---DNNG-VHTNSGIINKAAYLLS
B. caldolyticus : PGVAGDALRMSDPAKYGDPDHYSKRY-----TGTQ---DNNG-VHTNSGIINKAAYLLS
B. stearothermophilus (N) : PGISGDSLRSMSDPAKYGDPDHYSKRY-----TGTQ---DNNG-VHINSIGINKAAYLLS
B. megaterium : PGTSGDALRMSNPAKYGDPDHYSKRY-----TGSS---DNNG-VHTNSGIINKAAYLLA
B. thermoproteolyticus : PGISGDSLRSMSDPAKYGDPDHYSKRY-----TGTQ---DNNG-VHINSIGINKAAYLLS
B. cereus : PGKAGDALRMSDPTKYGDPDHYSKRY-----TGSS---DNNG-VHTNSGIINKQAYLLA
B. polymyxa : PNICGDALRMSNPTLYDQPHHYSNLY-----KGSS---DNNG-VHTNSGIINKAYYLLA
B. subtilis (N) : PGIAGDSLRSLEDPSKQGNPDHYSNRY-----TGTE---DYGG-VHINSIHNKAAYLLA
B. subtilis (M) : S---QPALRSLSNPTKYNQPDNY-ANYRNLNPTDEG---DYGG-VHTNSGIPNKAAYNTI
B. amyloliquifaciens : S---QPALRSLSNPTKYGQPDNF-KNYKNLNPNTDAG---DYGG-VHTNSGIPNKAAYNTI
L. monocytogenes : RGSRDGRIRSIKDPDKYNQAAHM-KDYESLPLTEEG---DWGG-VHNSGIPNKAAYNTI
R. salmoninarum : GVI-----RSMKDPGKYGEPAIYKGSNWKPTATNPNDNDQGG-VHSNSGVGNKLAFLIT
P. aeruginosa : GSGA---LRYMDQPSRDGRSIDNASQYYNGID-----VHHSSGVYNRAFYLLA
V. cholerae : SSGG---LRYFDQPSRDGRSIDHASQYYSGID-----VHHSSGVFNRAFYLLA
V. proteolyticus : SSGG---LRYFDQPSRDGRSIDHASDYNGLN-----VHYSSGVFNRAFYLLA
L. pneumophila : EDSGYDALRYMDKPSRDGMSIDVADDYGGGLD-----VHYSSGVYNHLFYILA
E. carotova : DGIHGMGLRMSHPGTAYDDELLGIDPQPSHMNEYVNTREDNGGVHLNSGIPNRAFYLLA
 * * * * *

B. stearothermophilus (M) : QGGVHYGVSVNGIGRDKMGKIFYRALVYYLTPTS NFSQLRAACVQAAA-----DLYGSTS
B. caldolyticus : QGGVHYGVSVTGIGRDKMGKIFYRALVYYLTPTS NFSQLRAACVQAAA-----DLYGSTS
B. stearothermophilus (N) : QGGTHYGVSVVGIGRDKLGKIFYRALTOYLTPTS NFSQLRAAAVQSAT-----DLYGSTS
B. megaterium : NGGTHYGVTVTGIGGDKLGKIYYRANTLYFTQSTTFSQARAGLVQAAA-----DLYGSGS
B. thermoproteolyticus : QGGTHYGVSVVGIGRDKLGKIFYRALTOYLTPTS NFSQLRAAAVQSAT-----DLYGSTS
B. cereus : NGGTHYGVTVTGIGKDKLGAIYYRANTQYFTQSTTFSQARAGAVQAAA-----DLYGANS
B. polymyxa : QGGTFHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFS NARA AAVIQAAK-----DLYGANS
B. subtilis (N) : EGGVHHGVQVEGIGREASEQIYYRALTYVVTASTDFSMMKQAAIEAAN-----DLYGEGS
B. subtilis (M) : TK-----LGVSKSQIYYRALTTYLTPTSSTFKDAKAALIQSAR-----DLYG--S
B. amyloliquifaciens : TK-----IGVNKAEQIYYRALTVYLTPTSSTFKDAKAALIQSAR-----DLYG--S
L. monocytogenes : TK-----LGKEKTEQLYFRALKYYLT KKSQFTDAKKALQQA AK-----DLYG--E
R. salmoninarum : DGQTFNGQTVTGIGIAKAAQL-YWAAQRQLTANATYSSLGKALNSACSANVSNNVAGTTA
P. aeruginosa : NSPGWDTR-----KAFEVFVDANRYWTATSNYNSGACGVIRSAQNRNYSAAD-----
V. cholerae : NKSGWNVR-----KGFEVFVANQLYWTNPSTFDQGGCGVVKAAQDLNNTAD-----
V. proteolyticus : NKAGWDVR-----KGFEVFTLANQLYWTANSTFDEGGCGVVKAASDMGYSVAD-----
L. pneumophila : NQPNWNLRL-----MAFDVMVKANMDYWTPTYSTFDEGGCGMLSAAKDLGY-----
E. carotova : IALGGHSWEKAGRIW-----YDTL CDKTL PQNADFEIFARHTIQHA AKRFNH

*

B. stearothermophilus (M) : QEVNSVKQAFNAVGVY-----
B. caldolyticus : QEVNSVKQAFNAVGVY-----
B. stearothermophilus (N) : QEVASVKQAFDAVG VK-----
B. megaterium : QEVISVGKSFDAVG VQ-----
B. thermoproteolyticus : QEVASVKQAFDAVG VK-----
B. cereus : AEVA AVKQSFS AVGVN-----
B. polymyxa : AEATAA AKS FDAVG-----
B. subtilis (N) : KQSASVEKAYEAVGIL-----
B. subtilis (M) : TDAAKVEAAWNAVGL-----
B. amyloliquifaciens : QDAASVEAAWNAVGL-----
L. monocytogenes : DASKKVAEAEAVGVN-----
R. salmoninarum : ANCTQVANAIKAVGIK-----
P. aeruginosa : -----VTRAFSTVGV-----TCPSAL-----
V. cholerae : -----VVA AFNTVGVNASCGTTPPPVGKVLEKGPITGLSGSRGGEDFYTFTVTNSG SVV
V. proteolyticus : -----VEDAFNTVGVNASCGATPPPSGDVLEIGKPLANLSGNRNDMTYYTFTPSSSSSVV
L. pneumophila : -----
E. carotova : TVADIVQQSWETVGVEVRQEFL-----

<i>B.stearothermophilus</i> (M) :	-----
<i>B.caldolyticus</i> :	-----
<i>B.stearothermophilus</i> (N) :	-----
<i>B.megaterium</i> :	-----
<i>B.thermoproteolyticus</i> :	-----
<i>B.cereus</i> :	-----
<i>B.polymyxa</i> :	-----
<i>B.subtilis</i> (N) :	-----
<i>B.subtilis</i> (M) :	-----
<i>B.amyloliquifaciens</i> :	-----
<i>L.monocytogenes</i> :	-----
<i>R.salmoninarum</i> :	-----
<i>P.aeruginosa</i> :	-----
<i>V.cholerae</i> :	VSISGGTGADLYVKAGSKPTTSSWDCRPYRSGNAEQCSISAVVGTTYHVMLRGYSNYSG
<i>V.proteolyticus</i> :	IKITGGTGADLYVKAGSKPTTTSYDCRPYKYGNEEQCSISAQAGTTYHVMLRGYSNYAG
<i>L.pneumophila</i> :	-----NLDDIKKSLSEVTINYQSCYVD-----
<i>E.carotova</i> :	-----

<i>B.stearothermophilus</i> (M) :	-----
<i>B.caldolyticus</i> :	-----
<i>B.stearothermophilus</i> (N) :	-----
<i>B.megaterium</i> :	-----
<i>B.thermoproteolyticus</i> :	-----
<i>B.cereus</i> :	-----
<i>B.polymyxa</i> :	-----
<i>B.subtilis</i> (N) :	-----
<i>B.subtilis</i> (M) :	-----
<i>B.amyloliquifaciens</i> :	-----
<i>L.monocytogenes</i> :	-----
<i>R.salmoninarum</i> :	-----
<i>P.aeruginosa</i> :	-----
<i>V.cholerae</i> :	VTLRLD
<i>V.proteolyticus</i> :	VTLRAD
<i>L.pneumophila</i> :	-----
<i>E.carotova</i> :	-----

APPENDIX III

Abbreviations, molecular weights and classification of amino acids.

Amino acid	3 Let Abbrev.	1 Let Abbrev.	Mol. Weight	Codon	Functional	Hydrophobic	Charge	Chemical
Alanine	Ala	A	89	GCA GCC GCG GCU	HNP	Pho	0	Aliphatic
Arginine	Arg	R	174	AGA AGG CGA CGC CGG CGU	+	Phi	+	Basic
Asparagine	Asn	N	132	AAC AAU	POU	Phi	0	Amide
Aspartic acid	Asp	D	133	GAC GAU	-	Phi	-	Acidic
Cysteine	Cys	C	121	UGC UGU	POU	Phi	0	Sulfur
Glutamic acid	Glu	E	147	GAA GAG	-	Phi	-	Acidic
Glutamine	Gln	Q	146	CAA CAG	POU	Phi	0	Amide
Glycine	Gly	G	75	GGA GGC GGG GGU	POU	Phi	0	Aliphatic
Histidine	His	H	155	CAC CAU	+	Phi	+	Basic
Isoleucine	Ile	I	131	AUA AUC AUU	HNP	Pho	0	Aliphatic
Leucine	Leu	L	131	CUA CUC CUG CUU UUA UUG	HNP	Pho	0	Aliphatic
Lysine	Lys	K	146	AAA AAG	+	Phi	+	Basic
Methionine	Met	M	149	AUG	HNP	Pho	0	Sulfur
Phenylalanine	Phe	F	165	UUC UUU	HNP	Pho	0	Aromatic
Proline	Pro	P	115	CCU CCC CCA CCG	HNP	Pho	0	Imino
Serine	Ser	S	105	AGC AGU UCA UCC UCG UCU	POU	Phi	0	Hydroxyl
Termination	End	*	-	UAA UAG UGA				
Threonine	Thr	T	119	ACA ACC ACG ACU	POU	Phi	0	Hydroxyl
Tryptophan	Trp	W	204	UGG	HPH	Pho	0	Aromatic
Tyrosine	Tyr	Y	181	UAC UAU	POU	Phi	0	Aromatic
Valine	Val	V	117	GUA GUC GUG GUU	HNP	Pho	0	Aliphatic

HNP = Hydrophobic nonpolar
POU = Polar uncharged
Pho = Hydrophobic
Phi = Hydrophilic

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