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An investigation into the interaction of Aeromonas salmonicida and the gastrointestinal tract of rainbow trout and the implications for oral delivery of a live auxotrophic mutant

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An investigation into the interaction of *Aeromonas salmonicida* and the gastrointestinal tract of rainbow trout and the implications for oral delivery of a live auxotrophic mutant.

By

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ABSTRACT

Furunculosis is a disease of great economic importance to the salmon farming industry. The aetiological agent of the disease is *Aeromonas salmonicida,* probably the most studied pathogen of non mammalian hosts. Little information exists however on the most fundamental aspects of the infection process or the basis of acquired immunity. Although oral vaccination is an ideal solution to the problems of immunising farmed salmonids, poor immunogenicity has always limited the use of this route of administration. In mammals oral vaccination with live auxotrophic mutants has been shown to elicit both humoral and cellular immunity and provide protection against challenge. The recent development of an *aroA* mutant of *A. salmonicida* has enabled investigation into the response of rainbow trout to a live oral vaccine. Initially, the role of gastric acidity in the destruction of the live bacteria was investigated *in vitro. Aeromonas salmonicida, Yersinia ruckeri* and *Escherichia coli* were compared for acid resistance. Using a system of low pH buffers appropriate to the acidity found in the gut of rainbow trout it was found that *A. salmonicida* was readily killed by a pH of 4.0 or less. Resistance to low pH increased during stationary phase and 0.002% of bacteria grown for 72 hours were able to survive at least six hours exposure to pH 3.0, compared with 90% of *Yersinia ruckeri* exposed to the same conditions. The effect of culture medium, oxygen availability, and the pH of the culture medium were also investigated. Whilst growth in BHIB or in a shaking incubator did not alter the resistance of *Aeromonas salmonicida* to pH 3.0 growth in TSB adjusted to pH 5.5 increased survival of the bacteria by ten fold. It was apparent that the bacteria would probably require protection from the gastric acidity. The uptake and localisation of the bacteria was investigated by recovery and enumeration of viable bacteria from the stomach and intestinal mucus as well as the kidney, spleen and liver of fish vaccinated orally , anally and by IP injection. The presence of the bacteria was monitored between 5 minutes and 72 hours. Bacteria were found in the organs within 5 minutes of delivery regardless of route. The largest numbers were found following IP injection and the lowest following oral intubation. Oral administration of the vaccine protected by prior administration of sodium bicarbonate increased the number of bacteria found in the fish and increased the persistence within the tissues. The effect of growth conditions on the uptake and localisation was also investigated and no increase in uptake was found. Oral delivery of the bacteria in TSB increased the number of bacteria found within the organs to levels above that found following IP injection in TSB. Poor uptake has been reported for killed oral vaccines suggesting that the bacterium was able to invade the host via the gut mucosae. The interaction between trout intestine and *A . salmonicida* was investigated using TEM and SEM and the ability of the vaccine strain to invade cell monolayers was compared with that of an A-layer deficient strain and *Y. rukeri.* Infection of the trout intestine *in vitro* and *in vivo* led to pathological damage to the epithelium. There was also evidence of phagocyte infiltration and it was apparent that these cells were destroyed by contact with the bacteria. Invasion of tissue culture cells was assessed by acridine orange staining and enumeration of viable internalised bacteria. *Aeromonas salmonicida* appears to be invasive but its cytotoxic ECPs appear to kill most of the cells it enters. The specific and non-specific humoral immune response to vaccination was investigated. There was evidence of a limited effect on various nonspecific immune response parameters but there was no detectable antibody response to the vaccine. There is evidence that cellular immunity is preferentially stimulated by live vaccines and any future work should involve an investigation of the systemic and mucosal cellular response.

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All experimental work involving animals was carried out under the personal Home Office Licence number 30/03268.

Authors Declaration.

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

This study was financed with the aid of a studentship from the Science and Engineering Research Council.

A programme of advanced study was undertaken, which included Courses in biological electron microscopy and attendance at selected lectures on the MSC course in Applied Fish Biology.

A number of scientific seminars and conferences were attended at some of which work was presented.

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Signed. .....

Date .....

Abbreviations.

λ	Lambda phage
ADCC	Antibody dependant cellular cytotoxicity
ANOVA	Analysis of variance
APC	Antigen presenting cell
aph	Aminoglycoside phosphotransferase
Ap^r	Ampicillin resistance
ASC	Antibody secreting cell
ATR	Acid tolerance response
BHIB	Brain heart infusion broth
BSA	Bovine serum albumin
cDNA	Complimentary DNA
CFT	Complement fixation test buffer
CFU	Colony forming unit
CMI	Cell mediated immunity
DNA	Deoxyribonucleic acid
EBSS	Eagle's balanced salts solution
ECM	Extracellular matrix
ECP	Extracellular products
EDTA	Ethylene diaminotetraacetic acid
EGC	Eosinophilic granulocyte
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene-bis-(oxyethylenenitrilo)tetraacetic acid
EHEC	Enterohaemorrahgic *Escherischia coli*

EIEC	Enteroinvasive *Escherischia coli*
ELISA	Enzyme linked immunosorbant assay
EPEC	Enteropathogenic *Escherischia coli*
ERM	Enteric redmouth
ETEC	Enterotoxogenic *Escherischia coli*
FCA	Freunds complete adjuvant
FCS	Foetal calf serum
FHA	Filamentous haemagglutinin
FIA	Freunds incomplete adjuvant
g	Grams
GALT	Gut Associated Lymphoid tissue
GCAT	Glycerophospholipid: cholesterol acyltransferase
GTP	Guanosine triphosphate
HBSS	Hank's balanced salts solution
HEWL	Hen egg white lysozyme
HFR	High frequency of recombination site
HI	Hyperosmotic bath immersion
HRP	Horseradish peroxidase
HUS	haemolytic-uraemic-syndrome
Ics	Intra-inter cellular spread
IM	Intramuscular injection
IP	Intraperitoneal injection
Ipa	Invasion plasmid antigens
ipg	Invasion plasmid gene
IPTG	Isopropylβ-D-Thiogalactopyranoside
IROMPS	Iron restricted outer membrane proteins

Kan^r	Kanamycin resistant
kDa	Kilo Daltons
LB	Luria Bertani
LD_{50}	Dose leading to mortality in 50% of recipients
LLO	Listeriolysin
LM	Light microscopy
LPS	Lipopolysaccharide
LT	Heat labile toxin
LTD_4	Leucotriene D_4
LVSEM	Low voltage scanning electron microscopy.
MALT	Mucosal Associated Lymphoid Tissue
MDCK	Madin-Darby Canine Kidney cells
MEM	Minimal essential medium.
mg	milligram
MOI.	Multiplicity of infection
MPS	Mononuclear phagocyte system
mRNA	messenger RNA
MW	Molecular weight
mxi	Membrane excretion of invasion plasmid antigens
Nal^r	Nalidixic acid resistant
NCS	Newborn calf serum
ng	nanogram
NK cell	Natural killer cell
Olm	Organelle like movement
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PLA_2	Phospholipase 2 activity
PLCγ	Phospholipase C-γ
PMN	Polymorphonuclear leucocytes
PMSF	Phenylmethylsulfonyl fluoride
PTK	Protein tyrosine kinase
PTKases	Protein tyrosine kinases
PTPases	Protein tyrosine phosphatases
r.DNA	Recombinant DNA
rRNA	Ribosomal RNA
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
spa	Surface presentation of invasion plasmid antigens
SRBC	Sheep red blood cells
TEM	Transmission electron microscopy
Tn	Transposon
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UHT	Ultra heat treated
UV	Ultraviolet
YOP	Yersinia outer membrane proteins
YpkA	Yersinia protein kinase A
°C	Degrees Celcius
μg	microgram

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Finally, my wife for all her help in the final preparation of this thesis.

Think where a man's glory begins and ends.

And say my glory was I had such friends.

W. *B. Yeats*

Dedication.

I would like to dedicate this thesis to my family.

My mother who started me on this road and who's love and support over the last 28 years has been a central part in my life and never more so than in the last seven years.

My wife, Antonella, who's understanding, affection and help have kept me going when things looked bleak and particularly through the long dark hours before this thesis was finally finished.

My children Alexander and Elisia who have seen much less of me than they should and who have reminded me there are more important things to care about than the next assay.

Thank you all, love Steve.

'It used to be so simple once upon at time....

Because the universe was full of ignorance all around and the scientist panned through it like a prospector crouched over a mountain stream, looking for the gold of knowledge among the gravel of unreason, the sand of uncertainty and the little whiskery eight-legged swimming things of superstition.

Occasionally he would straighten up and say things like 'Hurrah, I've discovered Boyle's Third Law.' And everyone knew where they stood. But the trouble was that ignorance became more interesting, especially big fascinating ignorance about huge and important things like matter and creation, and people stopped patiently building their little houses of rational sticks in the chaos of the universe and started getting interested in the chaos itself - partly because it was a lot easier to be an expert on chaos, but also because it made really good patterns that you could put on a t-shirt.'

T. Pratchett
Witches Abroad, 1991.

Chapter One.

General Introduction.

1.1 Introduction: *Aeromonas salmonicida*.

Aeromonas salmonicida is the etiological agent of furunculosis, one of the most serious and economically important infections of salmonid fish. The disease is named after the furuncles characteristic of sub acute and chronically infected fish. However, these 'boil-like' lesions are rarely seen during acute infections that are characterised by a rapidly fatal septicaemia.

It is generally accepted that the bacterium was first isolated from a trout hatchery in Germany by Emmerich and Weibel (1894). They called the bacteria 'Bacillus der Forellenseuche' or Bacillus of contagious trout disease (Austin and Austin, 1987). Lehmann and Neumann (1896) reclassified the organism into the genus Bacterium as *B. salmonicida,* and later still it was reclassified in the genus *Aeromonas* (Griffin *et al.*, 1953).

It was assumed that *A. salmonicida* had a predilection for salmonids but, recently both typical and 'atypical' strains of *A. salmonicida* have been isolated from an increasingly wide variety of salmonid and non-salmonid fish. At the present three subspecies of the organism are recognized: subsp. *salmonicida*, subsp. *achromogenes* and subsp. *masoucida* (Popoff, 1984).

1.2 Morphology, Ecology, and Culture of *Aeromonas salmonicida.*

The organism is Gram-negative, non-motile, and is most often observed as coccobacilli occurring in pairs, chains or clumps under light microscopy. It is an obligate fish pathogen capable of successfully infecting a wide range of host species.

The survival of the pathogen outside the host has been extensively investigated by numerous workers (Arkwright, 1912; Horne, 1928; Williamson, 1929; Smith, 1962; Cornick *et al.*, 1969; McCarthy, 1980; Allen, 1982). Unfortunately, caution must be employed when considering the results of several of these studies as they employed tap water, distilled water or presterilised water that is not typical of the environment in which the bacteria would normally be found. Nevertheless, it appears that *A. salmonicida* is capable of surviving for

prolonged periods in fresh, brackish and sea water. Much of the data on exactly how long the organism will survive is contradictory. Thus, in unsterilised fresh water recovery of viable organisms was observed from as little as 24 hours to as long as 19 days (Horne, 1928; Williamson, 1929; Smith, 1962; Allen, 1982). In unsterilised sea water survival varied between 24 hours to 8 days (Williamson, 1929; Duncan, 1932; McCarthy, 1980), in brackish water survival was shown to be between 16 and 25 days (Smith, 1962; McCarthy, 1980). Sterilisation of river water was shown to increase the survival of the organism to between 28 (20-25°C) and 63 days (10°C) (Cornick *et al.*, 1969), thus temperature also appears to influence survival; this is supported by the results of McCarthy (1980) who conducted his experiments at temperatures between 11°C and 13°C and found longer survival times than most other studies. Allen (1982) showed that the original inoculum and nutrient availability also affect survival. She found that if the original inoculum was low (10^1 - 10^2 CFU cm^{-3})the numbers decreased so as to be unrecoverable after 72 hours, whereas if the inoculum was greater (10^5 - 10^6 CFU cm^{-3}) the bacterial survived and multiplied up to 10^8 CFU cm^{-3} within the 72 hours. Furthermore, the addition of low concentration nutrient broth (0.005% Brain Heart Infusion Broth (BHIB) w/v) led to an increase in the number of bacteria and this increase was found in both the high and low inoculum groups. McCarthy also investigated the survival of the bacteria in sediment from a fish farm. He found that up to 10^5 CFU survived for at least 29 days and probably longer as the experiment was terminated prematurely because the dialysis bags containing the bacteria began to decompose. Michel and Dubois-Darnaudpeys (1980) showed that *A. salmonicida* survived and grew in sterilised river sediments for up to 10 months although pathogenicity was lost after 8 to 9 months. They conceded that in unsterilised river sediment *A. salmonicida* would have to compete with large numbers of other bacteria and that this would undoubtedly limit the proliferation of the pathogen. It has been suggested that *A. salmonicida* can enter a viable but non-culturable state under conditions of environmental stress (Allen-Austin *et al.*, 1984). It is known that *Vibrio*

cholerae enters such a state in the aquatic environment (Singleton *et al.*, 1982; Xu *et al.*, 1982), and such dormant cells of *V. cholerae* and *E. coli* were shown to be capable of infecting rabbit ileal loops (Austin and Austin, 1987). However, Rose *et al.* (1990) failed to find viable dormant *A. salmonicida* in sea water.

The organism can be readily isolated from skin lesions, blood or kidney of affected fish, both diseased and apparently healthy carrier fish. The micro-organism grows well on most nutrient media but tryptone soya agar (TSA) is favoured by most laboratories (Austin and Austin, 1987); as there is no recognised selective growth medium. Small, circular, raised, translucent colonies develop within 48 hours at 22-25°C. *A. salmonicida* is psychrophilic and, will not grow at 37°C, although Austin (1993) reported the recovery of atypical isolates that were capable of growth at 37°C. All strains are normally non-motile, oxidase-positive and fermentative, McIntosh and Austin (1991) described isolates that were capable of producing functional flagella. Typical isolates (Subsp. *salmonicida*) produce a brown, diffusible pigment, although this may not develop for up to ten days in some instances. A comprehensive list of the biochemical characteristics was presented by Austin and Austin (1987) and will not be reproduced here.

1.3 Epizootiology of Furunculosis.

Furunculosis of salmonid fish has been recorded in all parts of the world including Australasia (Trust *et al.*, 1980). There have been some very damaging epizootics of wild fish populations. The seriousness of the infections in Scotland during the 1920's led to the formation of the Furunculosis Committee in 1929. This committee described the disease as occurring in riverine populations of Atlantic salmon (*Salmo salar*) with outbreaks of limited mortality beginning in June associated with elevated or rising water temperature. The fish were beginning to ascend the river to reach the spawning grounds by November. Epidemic mortality sometimes occurred in late summer if water levels became low, temperatures rose and many fish became trapped in river pools (Mackie *et al.*, 1930, 1933, 1935; McCarthy and

Roberts, 1980; Austin and Austin, 1987; Hastings, 1988). Smith (1962) described the occurrence of easily isolated *Aeromonas salmonicida* from more than 40% of dead and dying spent Atlantic salmon. This indicated that the postspawning mortality was at least partly due to furunculosis.

It is in salmonid cultivation that furunculosis has had a major economic impact in recent years. Most, if not all, species and age groups of salmonid fish may be affected by furunculosis, though some species seem to be more susceptible than others. Rainbow trout appear to be remarkably resistant (Cipriano, 1983, Ellis, 1987) to the disease when compared to Atlantic salmon and brown trout. The risk is especially high if fish are overcrowded, but outbreaks can be caused by a variety of stressors such as handling, grading, transporting or following transfer of smolts to sea water (Hastings, 1988).

Blake and Clarke (1931) suggested that furunculosis was only spread by infected fish or by material that has come into contact with them. This is possible, but, as described in Section 1.2 above the organism can probably survive in water and river sediment and presumably these bacteria could infect fish.

Certainly infected fish contain large numbers of bacteria. McCarthy (1980) showed that material from furuncles contains up to 10^8 CFU cm^{-3} and Rose (1990) found 10^{10} CFU cm^{-3}. He suggested that this latter figure means it would require only 1μl of furuncle material to be ingested to cause mortalities. McCarthy (1980) also showed that bacteria remained viable for 32 days in fish muscle tissue and 40 days in the water from tanks in which dead fish had been kept, thus suggesting a probable source of infection for healthy fish.

Fish may also act as a source of infection by becoming asymptomatic carriers of the organism. The role of carrier fish was recognised very early in the study of the disease. Horne (1928) found that 17% of fish examined contained *A. salmonicida* in the blood and concluded that carriers played an important role in the transmission of the disease. The introduction of carrier fish into fish farms may be the main cause of furunculosis spread (Rose, 1992).

McCarthy (1977) emphasised the problems of undetected carriers in experimental transmission studies and reported the experimental transmission of furunculosis between carrier and *A. salmonicida* free fish in fresh water. Early workers suggested that the kidney was the main site of carriage in carrier fish (Blake and Clarke, 1931), and that the intestine was of secondary importance (Mackie *et al.*, 1930, 1933, 1935). Klontz and Amderson (1968) detected *A. salmonicida* antigen in the gut wall of otherwise apparently healthy fish and suggested that the intestine was the primary site of carriage. Recently, it was found that *A. salmonicida* is predominantly found on the outer surfaces of carrier fish. Upon injection of fish with the surface material of carrier fish resulted in 78% mortality compared with only 21% in fish injected with blood. This suggests that either the bacteria can be carried at several sites on carrier fish or the fish investigated in these studies have been at different points in the development of the clinical disease; the carrier state is unstable and carrier fish regularly develop the clinical disease when under stress (Rose, 1990).

Ideally, prevention of contamination of the water supply by wild fish and maintenance of strict hygiene would be the control of choice, but these precautions, especially the former are rarely practicable. Removal of all fish showing furuncles, treatment with antibiotics and potentiated sulphonamides and improvement of environmental conditions remains the usual method of management of outbreaks (Hastings, 1988).

1.4 The Pathology and Histopathology of Furunculosis.

Apart from the furuncles, the clinical signs of the disease are somewhat variable depending on the form of the disease. In very young fish and especially Atlantic salmon smolts, losses may be heralded by little more than loss of appetite, darkening of the skin, lethargy, gathering at tank outlets and death (McCarthy and Roberts, 1980). Haemorrhaging may occur at the bases of fins and in the abdominal wall. Histopathological examination of such fish reveals toxic cardiac necrosis, especially of the atrial lining, with small foci of bacteria present in cardiac, haemopoietic and gill tissue, but little in the way of host response.

Splenomegaly and inflammation of the lower intestine are common features of chronic infections but in acute outbreaks fish may die rapidly with few signs. The histopathology of furunculosis in older fish is often indistinguishable from that of any other Gram-negative haemorrhagic septicaemia (McCarthy and Roberts, 1980).

The furuncles are, in fact, misnamed as the lesions bear no resemblance to the 'boils' found in humans. They are raised, dark tumefactions, often found on the back and sides of fish, which eventually ulcerate to release clear blood-stained fluid into the water. The exudate is very rich in micro-organisms and is probably a major source of transfer of infection (Rose, 1990). The furuncle results from focal localization of bacteria in the dermis or occasionally the epidermis, where they excite an early response of hyperaemia in hypodermis and dermis, with a fibrinous oedema, followed by a massive infiltration of macrophages and some polymorphonuclear leucocytes. There is liquefactive necrosis at the centre of the lesion with deposition of strands of fibrin, along which bacteria may be distributed, as well as many inflammatory cells. The latter often have melanin granules within their cytoplasm and since such pigmented cells are also seen in circulating blood and renal sinuses, they may be derived from the haemopoietic tissue, where fragmentation of melano macrophage cells is one of the first signs of endotoxic septicaemia (McCarthy and Roberts, 1980). Another feature of the disease is the degranulation of intestinal and to a lesser extent gill eosinophilic granulocytes (Ellis, 1985b; Vallejo and Ellis, 1989).

1.5 Virulence Factors.

Mackie *et al.* (1930, 1933, 1935) noted that the virulence of *Aeromonas salmonicida* strains decreased when kept in laboratory culture. They also showed that the histopathology of the disease indicated a marked leucopenia and proteolysis of certain tissues suggesting the possession of virulence determinants. The nature of these virulence determinants has been the subject of very intensive investigation for many years. Comparison of avirulent and virulent cultures by serology (reviewed by McCarthy and Roberts, 1980) indicated a

remarkable homogeneity amongst all. According to Munro (1984) the error of these results was due to not distinguishing the presence of an extra layer in the cell wall and in not recognizing that virulence was lost in the absence of the property of self-agglutination (Evenburg *et al.*, 1982; Evenburg and Lugtenerg, 1982), This layer is commonly referred to as the Additional or A-layer (Udey, 1977), and is associated with rough (as opposed to smooth) colony morphology.

The presence of the A-layer was unable to account for the lesions found in infected fish and it was Karlsson (1962) who first described the production of extracellular toxins by *A. salmonicida* when he discovered an extracellular haemolysin. The first demonstration of the *in vivo* effects of the extracellular toxins was by Klontz *et al.* (1966) by injecting saline extracts of cells which produced haemopoietic necrosis in fish. The role of the surface components, e.g. the A-layer and the extracellular toxins are discussed below.

1.5.1 The A-layer protein and other cell surface components and their role in virulence.

The surface of *Aeromonas salmonicida* is probably the best characterised of all bacterial fish pathogens (Trust, 1986). Most virulent forms of the pathogen have the capacity to produce a paracrystalline surface-protein array, known as the A-layer, whereas this layer is absent in many avirulent strains (Udey and Fryer, 1978; Ishiguro *et al.*, 1981). Mutations that result in a loss of ability to produce the A-layer are accompanied by a loss of virulence (Ishiguro *et al.*, 1981) This was demonstrated by the behaviour of a single-site Tn5 insertion mutant A449-TM1 that could produce A-protein subunits but was unable to translocate them across the outer membrane (i.e. a mutant isogenic for the A-layer) Belland and Trust, 1985). While the LD_{50} for the parent was $<10^3$, the mutant was avirulent (Trust, 1986).

Electron microscopy showed that the A-layer exhibits two tetragonal patterns with p4 symmetry (Stewart, *et al.*, 1986). A diagrammatic representation of a model of the surface structures of *A. salmonicia* is presented in Fig. 1.1. Image analysis indicates that both patterns

are composed of two different morphological units. Biochemical and genetic analysis shows that the A-layer has a single protein subunit of 50-54 kDa (Udey and Fryer, 1978; Kay *et al.*, 1984; Bjønsdottir *et al.*, 1992), so the morphological subunits appear to represent the same protein arranged in alternating reverse directions (Trust, 1986). The gene *vapA* that encodes the A-layer protein subunits has been cloned and sequenced (Belland and Trust, 1987; Chu *et al.*, 1991). Noonan and Trust (1995a) used immunogold labelling of sections of A449-TM1 and showed large amounts of the A-layer subunits localised in an enlarged periplasm. Molecular characterisation of the Tn*5* mutation resulted in the identification of the *aspE* gene (Trust *et al.*, 1996). AspE was shown to be an ATP binding protein bearing remarkable homology to a number of transmembrane export proteins. Despite the mutation in *aspE* A449-TM1 was capable of secreting a wide range of extracellular proteins and it has been shown that the mutant secretes the same level of proteases and haemolysin as the parent strain (Noonan *et al.*, 1993; Noonan and Trust, 1995a). This suggests that AspE is part of a protein secretion pathway specific for the A-layer. Given the importance of the A-layer in the virulence of the bacteria it is reasonable that it should have a specific pathway devoted to its secretion (Trust *et al.*, 1996).

In addition to the A-layer the surface of *A. salmonicida* is characterised by the presence of LPS with antigenically cross reactive O-polysaccharide side chains (O-chains) of homologous chain length (Chart *et al.*, 1984; Shaw *et al.*, 1992). These chains appear to tether the A-layer to the cell surface and mutants lacking the O-chains release assembled A-layer into the culture supernatant (Belland and Trust, 1985; Garduño *et al.*, 1995). Garduño *et al.* (1994) suggested that the A-layer and the O-polysaccharide are coassembled. The gene *abcA* was recently shown to be required for the expression of *vapA* and the biogenesis of the O-chains (Chu and Trust, 1993; Chu *et al.*, 1995; Noonan and Trust 1995b)

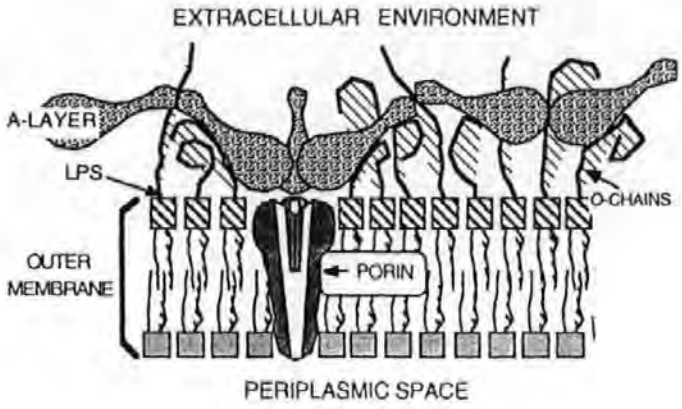


Figure 1.1 The surface structures of *Aeromonas salmonicida*. *(After Gardùno* ***et al.****, 1994).*

It has been shown that both the high and low molecular weight LPS from *Aeromonas salmonicida* and the A-layer protein are strong immunogens in fish (Evenberg *et al.*, 1985); furthermore the dominant immunogenicity of the LPS and A-layer protein was demonstrated in rainbow trout by Hastings and Ellis (1990) and Atlantic salmon by Lund *et al.* (1991). Bjønsdottir *et al.* (1992) compared the immunogenicity of the A-layer with and without LPS and showed that the presence of the LPS appears to enhance the antigenicity of the A-layer. They also suggested that this was probably due to an adjuvant effect from the LPS.

Munn *et al.* (1982) investigated the effect of the A-layer and LPS in the resistance of the bacteria to serum killing. Using normal human, rabbit and trout sera and immune sera from rabbits and trout they showed that virulent strains of *A. salmonicida* displayed high or intermediate resistance to the bactericidal activity of complement in the presence and absence of antibodies. In immune trout serum the A-layer was responsible for the protection, and in normal sera both the A-layer and LPS conferred protection.

Recently, Noonan and Trust (1995c) described the characterisation of a mutant of *A. salmonicida* that possessed altered surface morphology. Strands and blebs of material were observed protruding from the disorganised cell surface. The material was shown to contain

both LPS and A-layer protein. The mutation did not affect the virulence of the organism following bath challenge but upon intra peritoneal administration the virulence of the mutant was enhanced compared with the parental type. They proposed that the enhanced virulence resulted from either, increased secretion of haemolysin and protease, or alternatively, the greater exposure of the host's cells to outer membrane material around the bacterial cells leading to the increased exposure of the host to the GCAT-LPS complex. No biochemical evidence for either proposal was found. They found no detectable increase in the secretion of either extracellular toxin. The second proposal could in theory increase the toxic effect of the GCAT/LPS compound on the host but no evidence for this was presented. They also suggested that the protrusions and blebs may provide false targets for host complement thus sparing the bacterial membrane from lytic activity.

Garrote *et al.* (1992) described the presence of a capsule in *A. salmonicida.* The capsule was produced in bacteria grown in an excess of glucose. Apparently the capsule prevented the binding of monoclonal antibodies to either the A-layer or the O-chains since cells grown in conditions favourable for capsule production were undetected by ELISA. This may confirm the observation by Evenberg *et al.* (1985) of a polysaccharide material in strains of *A. salmonicida* . Garduño *et al.* (1993) showed that the capsule produced *in vivo* prevented binding of anti A-layer protein antibodies to the cell surface. In contrast they did not find the same result when the bacteria were grown on glucose rich agar (Garduño and Kay, 1995).

It is clear then that *Aeromonas salmonicida* has an impressive array of cell associated virulence factors. It is unfortunate then that there is little correlation between the antibody response to the A-layer or the LPS and protection for if there were the search for an effective bacterial vaccine would be easier.

1.5.2 Extra Cellular Virulence Factors

It was in 1980 that the first evidence that *Aeromonas salmonicida* produced a potent toxin was published (Munro *et al.*, 1980), and in 1981 it was shown that the extracellular

products (ECPs) could, on their own, produce the classic lesions associated with furunculosis (Ellis, *et al.*, 1981). During the last 15 years there have been many attempts to purify and analyse the ECPs and to identify their role in the pathogenesis of the disease. These attempts have met with only limited success probably because as, Ellis (1991) stated, the basic assumption that the ECPs produced *in vitro* would be the same as those produced *in vivo* is an unsafe one. The only way to demonstrate that a strain is avirulent because it does not produce a particular component of the ECPs is to show that it lacks the necessary gene (Ellis, 1991).

In common with almost all bacteria *A. salmonicida* produces an array of substances which are secreted into the extra-cellular medium and these are normally termed the Extra Cellular Products (ECPs). The ECPs from *A. salmonicida* contains a large number of proteins, many of which have an enzyme activity and which are generally considered to be virulence factors. The enzymic activities of several of them have been identified, Table 1.1.1 was drawn from Ellis (1991) and summarises the extracellular products of *A. salmonicida.* The role of these ECPs in the pathogenesis of furunculosis is still far from clear despite the obvious importance of this information in the search for a vaccine against the disease. It would appear that two of the toxins identified, the serine protease and the GCAT/LPS are those responsible for the pathogenic effects observed during a typical infection (Ellis, 1991). The MW of the protease produced by different strains has been controversial ranging from 11 to 87 kDa depending on culture conditions. Price *et al.* (1989) showed that the MW of the protease produced by all typical isolates is 70 kDa. It has also been shown that the role of the protease is nutritive being produced to digest the host's tissues releasing amino acids for use by the bacteria (Sakai, 1985a; Sakai, 1985b; Lee and Ellis, 1991). Ellis (1987) showed that the α2-macroglobulin of salmonids has an inhibitory effect on the 70 kDa protease. Ellis *et al.* (1988a) showed that some virulent strains do not produce the protease during normal culture. This led to its role in the virulence of the disease being questioned but it was shown

that these strains did produce the protease *in vivo*. Purified protease is lethal to fish (Fyfe, 1986); the amounts required to kill the fish are quite high (2.4μg/g of fish) (Lee and Ellis, 1989). The other major toxin is the phopsholipase glycerophospholipid cholesterol acyltransferase (GCAT) which is complexed with LPS (Lee and Ellis, 1990). The purified enzyme has a molecular weight of 25 kDa but once complexed with the LPS this increases to 200 kDa and the enzyme becomes heat stable and more resistant to proteolysis. The gene encoding the GCAT has been cloned and sequenced (Nerland, 1996). It was found that the gene encoded a protein of 335 amino acid residues of a deduced MW of 35,380 Da. The homology between this gene and the corresponding one from *A. hydrophila* was 92.9% for the nucleotide sequence and 93.7% for the deduced amino acid sequence. The purified GCAT/LPS has an LD_{50} of 45 ng/g of fish (Lee and Ellis, 1990). Furthermore, it was shown to be haemolytic for salmonid, but not human erythrocytes, and leucocytolytic and cytolytic in salmonids and RGT-2 cells. These activities include many of those described in Table 1.1.1 (Cipriano *et al.*, 1981; Titball and Munn, 1983; Nomura *et al.*, 1988) indicating that the GCAT/LPS is responsible for them all.

Only typical isolates of *A. salmonicida* possess an inducible siderophore-mediated iron uptake system (Hirst *et al.*, 1991). Although, both typical and atypical strains can grow under iron restricted conditions and express Iron Restricted Outer Membrane ProteinS (IROMPS) (Chart and Trust, 1983; Aoki and Holland, 1985; Hirst *et al.*, 1994; Hirst and Ellis, 1996). Both atypical and typical isolates were shown to be able to utilise haem compounds without the expression of siderophores (Hirst *et al.*, 1994) and therefore, typical isolates must contain at least two pathways for obtaining iron; the siderophore dependent and the siderophore independent mechanisms. The extra cellular protease was found to be capable of degrading host transferrin but it was not clear if this process led to the release of iron. The presence of proteolytic activity correlated with the ability of atypical strains to obtain iron, indicating that these isolates can release iron from host transferrin (Hirst and Ellis, 1996).

The IROMPS are surface bound determinants expressed under conditions of iron restriction (Hirst and Ellis, 1994). There are four proteins of MW 82, 77, 72, 70 kDa, these proteins mediate transfer of iron captured by siderophores into the cell.

Table 1.1.1 The extracellular products of *Aeromonas salmonicida*. (*After Ellis (1991)*)

Factor	Reference
Proteases:	
70 kDa protease - (caseinase serine protease)	Price *et al.*, (1989)
Gelatinase (metalloprotease)	Rockey *et al.*, (1988)
Toxins Damaging cell membranes:	
Leucocytolysin	Fuller *et al.* (1977)
Cytotoxic glycoprotein	Cipriano (1982)*
T-lysin (haemolysin)	Titball and Munn (1983)*
H-lysin (haemolysin)	Titball and Munn (1985)
Enterotoxin	Jiwa (1983)
Salmolysin	Nomura *et al.* (1988)*
G CAT	Buckley *et al.* (1982)
G CAT/LPS	Lee and Ellis (1990)
Other factors:	
Lipopolysaccharide	MacIntyre *et al.* (1980)
Siderophore	Chart and Trust (1983)
Brown pigment	Donlon *et al.* (1983)
Esterases	Hastings and Ellis (1988)
Amylase	Campbell *et al.* (1990)
Ribonuclease	Campbell *et al.* (1990)
Aryl-sulphatase	Campbell *et al.* (1990)
α-Glucosidas	Campbell *et al.* (1990)
α-Mannosidase	Campbell *et al.* (1990)
Phospholipase C	Campbell *et al.* (1990)
Lysophospholipase	Campbell *et al.* (1990)

* Activities probably attributable to the GCAT/LPS

1.6 The Ideal Furunculosis Vaccine

An ideal vaccine for furunculosis would provide solid protection for up to two years, this would be required for the protection of Atlantic salmon kept in sea water cages prior to consumption. It would be advantageous if the vaccine was also effective against atypical strains of *A. salmonicida*. Since asymptomatic carriers are important reservoirs of infection, an ideal vaccine would not only prevent clinical disease but also eliminate carrier infections. The method of administration of the vaccine should be convenient and cost-effective for large numbers of fish. Therefore, oral or immersion vaccines would be preferable to injection (Ellis, 1988; Hastings, 1988). This would be an ideal vaccine but there is little to no chance of these objectives being achieved. The abolition of the carrier state is a particularly unlikely outcome of any vaccination regime. Indeed if it were not for the apparent success observed in the first immunisation experiment (Duff, 1942) vaccination against furunculosis may well have been abandoned by now.

1.7 Early Vaccine Design

The earliest attempt to immunise fish was made by Duff (1942). The vaccine culture was grown for 4 days at 22-25°C on fish peptone in Roux flasks. He used 1 or 2 year old cutthroat salmon (*S. clarkii*) and fed them standard amounts of chloroform killed cells for 64 days (40 days was insufficient for protection) and the fish were kept at between 2-14°C. The food was a viscous mixture of beef liver and tinned salmon and very different to modern feeds. This may have contributed to the success of the vaccine by protecting antigens from degradation in the stomach, allowing their passage into the intestine and hence their exposure to the GALT. The principal method of challenge was by bath exposure at 19°C to standardized amounts of homologous bacteria, but intra peritoneal and cohabitation studies were also performed. The challenge was such that 50-90% of the control fish were killed, indicating that an overdose of the pathogen had not been delivered. The results of the bath exposure were 75% and 71% mortality for the controls and 24 and 26% in the orally

immunised fish over two consecutive years. Cohabitation gave 30% for controls and 10% for immunized fish and intra muscular challenge 90% and 68% respectively. Death occurred over approximately 10 days, the pathological signs were those of acute furunculosis with occasional appearance of subcutaneous or open lesions; *A. salmonicida* was isolated from the dead fish.

Agglutination titres were measured, showing more fish with elevated levels in the immunized group. However, this group also contained fish with no agglutinins. The agglutinin titres ranged from 0 to 320 with a mean of 80. Duff also reported that despite coming from a hatchery with no history of furunculosis agglutinin titres of up to 80 were recorded in the control fish although the mean was about 20. This observation has been reported by many subsequent workers.

Snieszko and Friddle (1949), used an oral vaccine of heat-killed whole bacterial cells, and reported that it did not protect brook trout (*Salvelinus fontinalis*) against intraperitoneal challenge. They did show, however, the potency of sulphonamide drugs to control furunculosis. This method of control was so successful that until the emergence of drug resistant strains the development of vaccines was largely forgotten.

1.8 Oral vaccine

Oral vaccination is very attractive since it is suitable for mass administration to fish of all sizes, imposes no stress on the fish because handling is not required and therefore does not interfere with routine husbandry. Furthermore, oral vaccination is the only method suitable for extensive pond rearing of fish since catching the fish prior to immersion or injection is impractical (Hastings, 1988). However, there are some important intrinsic limitations of oral vaccination. Large amounts of killed vaccines are required and this increases the cost and there is the uncertainty of individual dosages since fish consume different amounts of food. Unfortunately, the most important limitation of oral vaccines is

their poor potency and many trials with many different vaccines have resulted in low or inconsistent levels of protection, which also seem to be of limited duration (Ellis, 1995).

A major problem seems to be the destruction of antigens in the stomach and foregut before they reach the immune sensitive areas of the lower gut (Rombout *et al*,. 1985). Future developments will need to overcome this problem, possibly by encapsulating the vaccine so that it survives passage through the stomach. Other relevant research will be the development of suitable adjuvants for oral vaccines.

1.9 Vaccine Trials

Klontz and Anderson (1970) reported on a series of vaccine trials conducted during the late 1960s. They produced an oral vaccine based on an earlier observation that a water soluble extract from disrupted cells of virulent cultures was toxic for salmonids. They prepared a toxoid vaccine (called FSA) by precipitating the toxin with alum. The FSA vaccine was fed to juvenile coho salmon, which received a natural challenge 94 days after feeding commenced. Some 58% of control but none of the immunised fish died. Furthermore, trials at two hatcheries were conducted using the vaccine and there were mortalities of 11.4% and 22-37% for the control fish but 5.6% and 0.7% for the immunized fish. Unfortunately, large scale trials based on a commercially produced FSA vaccine conducted at 3 hatcheries failed to show protection. The major conclusion from this result was that the specification for producing the FSA vaccine did not assay the important antigen(s) necessary for conferring immunity and so the commercial vaccine may have lacked the necessary potency.

Udey and Fryer (1978), using strains Sil 67 A^+ and SS 70 A^-, compared their effectiveness in four vaccine formulations, formalin-killed whole cells either fed or injected intraperitoneally with FCA, fed formalin-killed whole cells + $Al(OH_3)$, and the FSA vaccine preparation of Klontz and Anderson (1970). Oral vaccines were fed for 45 days. All groups were exposed to a natural epizootic occurring at the hatchery by using effluent water from a

pond with active furunculosis. None of the oral vaccines gave protection, but in the intraperitoneally immunised group mortality was 2% instead of 10% in the controls. Fed fish showed no significant increase in agglutinin titre 16-64, when compared with either the control fish 16-32 or intraperitoneally injected fish 32-2000. A final group, fed vaccine and injected the day feeding began had agglutinin titres of only 32-128. The authors concluded that the dual vaccine gave good evidence of suppression of the humeral antibody response.

Michel (1979) performed two vaccine trials with rainbow trout using a formalin killed *A. salmonicida* suspension. In the first experiment the fish were given the vaccine either orally or by intraperitoneal injection, antibodies were found in the injected animals but no protection was found upon challenge with a virulent strain. Antibody titres were similar following immunisation with A-layer positive and A-layer negative bacteria. In the second study fingerlings were immunised by injection but were challenged either intraperitoneally or intra muscularly. Protection was observed in the latter case. In the intraperitoneally injected fish mortalities were the same as for the controls and in both these groups carriers were identified up to 3 months later. This study showed that protection and antibody response are not always correlated and that the route of immunisation, pathogenicity of the strains and the mode of challenge can affect the outcome.

Smith *et al.* (1980) tested an oral vaccine and two others administered by hyper osmotic bath immersion (HI) to brown trout. The oral vaccine (1) was based on a different *A. salmonicida* isolate from that used in the HI vaccines (2 and 3). Vaccine 1 consisted of SDS-disrupted cells fed for 30 days and vaccines 2 and 3 of ultrasonically disrupted cells. Following laboratory immunization fish were transferred to a farm with an annual history of furunculosis. Cumulative mortalities from all causes for vaccines 1, 2 and 3, were 35, 62 and 62% respectively and 86% for control fish. The authors reported the isolation of a strain of *Aeromonas salmonicida* from the dead immunized fish with properties (not recorded) different from either of the two strains used in the vaccines. None of the 3 vaccines elicited

a humoral or gut secretory agglutinin response. All three vaccines did elicit a CMI response as measured by the leucocyte inhibition test. This response was highest (75% of fish responding) when leucocytes of immunized fish were tested against homologous antigen but was less for the heterologous antigen from strain isolated from the dead vaccinated fish. This was the first measurement of CMI response to a furunculosis vaccine, although the report of Udey and Fryer (1978) implicates the CMI suppressing humoral activity when the oral vaccine apparently interfered with the intraperitoneal vaccine. The observation that the naturally occurring challenge strain was antigenically different from the vaccine strains was supported by the results of the CMI assay and this gives one avenue of explanation for the generally poorer protection observed in the field compared with laboratory trials of vaccines.

Cipriano (1982) did several experiments; in one, brook trout were subjected to ECP immunization by bath immersion, oral immunisation and by intraperitoneal injection. Fish were injected with 140μg ECPs, fed 140μg ECPs over 10 days or bathed in ECP protein at 140 μg cm^{-3}. The efficacy of vaccination was assessed by agglutinin titre, which was 2458 for intraperitoneally injected fish, 512 for the bath immersion group and 154 for the orally immunised group. The authors concluded that bathing offered the best compromise, and further studies were initiated to determine which of the four main fractions of ECP (I-IV) from virulent and avirulent culture eluted from DEAE Sephadex were important in conferring protection. For immunization each brook trout received 5μg of protein by intraperitoneal injection and was bath challenged 21 days later. Regardless of whether ECP from virulent or avirulent cultures was used only fraction IV conferred significant protection, 17% and 10% mortalities compared with 77% and 71% for the controls in duplicate experiments. Rabbit antiserum raised against ECP fraction IV formed a single band against a homologous antigen in agar gel diffusion plates but did not react against A-layer protein or LPS, so showing that fraction IV was a separate entity.

Johnson and Amend (1984) showed that a single dose of vaccine via bath immersion was significantly less effective than injection but that both immunised groups were protected when compared with the controls. They also found a 19% difference in the mortalities induced in two control groups treated identically. Multiple exposure by bath immersion appeared to increase the level of protection afforded significantly.

Ellis *et al.* (1988b) investigated the extracellular protease as a protective antigen by passive immunisation. Rabbit antisera was raised to a protease producing strain and a protease deficient strain. The antisera was shown to be essentially similar except for the presence of antibody to the protease. The antisera was injected into rainbow trout that were then challenged. The antiserum to the protease deficient strain showed minimal protection when compared with control fish injected with normal rabbit serum. Significant protection was obtained in the fish injected with the antiserum to the protease positive strain particularly when the fish were challenged with the heterologous and more virulent protease deficient strain. This indicated that the extracellular protease was a good candidate for the production of a vaccine.

Hastings and Ellis (1988) investigated the humoral immune response of rainbow trout and rabbits following immunisation with native and formalin inactivated ECP of *A. salmonicida*. Rabbit antibodies to seven ECP components were found in the sera from animals immunised with the native ECP and to 14 components in those animals given formalin inactivated ECP. In trout antibodies to just four antigens were found with either the native or inactivated ECP. This result showed why passive immunisation with rabbit antisera gave good protection while immunisation of fish with ECPs leads to poor protection. The authors suggested that the antigens may require modification prior to use in a vaccine but it seems quite likely that the inability of the trout to recognise as many antigens may be a feature of the trout immune system rather than the nature of the antigen. If this were the case the

alteration of the antigen or presentation with adjuvants may well have little effect on the outcome.

Ellis *et al.*, also in 1988c presented of the ECP in a variety of ways, i.e. native and formalinised ECP given with or without Freunds complete adjuvant and administered either intraperitoneally or intra muscularly. Specific antibodies to the protease or haemolysin were not found in any of the experimental groups. In rabbits given similar treatments at least 15 antigens were recognised including the protease and haemolysin. This finding indicates that rainbow trout are unable to recognise the full range of antigens recognised by the mammalian immune system. The results of this experiment also contradicted the findings of both Shieh (1985) and Sakai (1985a) who both suggested that protection found after immunisation with ECP was due to a specific antibody response.

In 1989 Marquis and Lallier investigated the efficacy of passive immunisation against *A. salmonicida* infection in brook trout, *Salvelinus fontinalis*. They produced hyper immune rabbit serum and challenged the trout by immersion. The fish were then passively immunised at the time of challenge or at various times post challenge. The passive immunisation was shown to be protective when administered up to 48 hours post challenge but after this time it was not effective. The authors showed that the protection lasted for 35 to 41 days. This investigation again indicates that there are components of the salmonid immune system that can kill virulent *A. salmonicida* in the presence of neutralising antibodies, unfortunately the fish appeared unable to produce such antibodies.

Lund *et al.* (1991) compared the immunogenicity of cell associated and extra cellular antigens in Atlantic salmon and rabbits. The animals were immunised with formalin killed whole cells and formalin inactivated ECPs either separately or in combination. The production of antibodies was studied by ELISA and Western blotting. These results confirmed that more proteins were immunogenic in rabbits compared to salmon but did show

that in both species some antigens are dominant including the protease, the A-layer and the high and low molecular weight LPS.

Tatner (1991) attempted to increase the protection afforded by immunisation of Atlantic salmon with *A. salmonicida* ECPs by modifying them. The antigens were either given as an unmodified solution, particularised onto polystyrene beads, coated on to sheep red blood cells, mixed with FCA or conjugated with Ficoll. The unmodified ECP gave a relative percent survival of 35% and the particularised antigen improved this to 67%, however, none of the other treatments showed any improvement in protection. This indicates that the ECP can induce a higher level of protection when particularised but apparently not when otherwise modified the reasons for this finding are unclear.

McIntosh and Austin (1993) presented the results of immunisation of Atlantic salmon with cell wall deficient or L-forms of *A. salmonicida.* They showed that L-forms produced by lysozyme-EDTA treatment provided good protection from challenge against two virulent strains. Furthermore the protection afforded by these L-forms was greater than that provided by inactivated whole cells or benzyl penicillin induced L-forms. These results agreed with the results of Cipriano (1982) and question the role of the A-layer protein in furunculosis vaccines.

Hirst and Ellis (1994) investigated the potential of the IROMPS as protective antigens. Compared with outer membrane proteins prepared from *A. salmonicida* grown under iron replete conditions the IROMP preparations gave significant protection against natural and heterologous challenge. Passive immunisation of Atlantic salmon with Atlantic salmon antisera containing anti IROMPS antibodies and rabbit anti IROMPS antisera were shown to confer significant protection against a heterologous bath challenge. Both salmon antisera to IROMPS preparations and the rabbit monospecific anti-IROMPS antibodies were also shown to be bactericidal *in vitro.* This indicated that the IROMPS proteins may be very important protective antigens against furunculosis.

Mutants of *A. salmonicida* that were deficient for the production of the A-layer, O-antigens or both were tested for their suitability as live vaccines in rainbow trout. All the mutants were shown to be attenuated and immersion vaccination of fish with $5x10^7$ CFU cm^{-3} followed by an identical immunisation 14 days later resulted in significant protection by all strains from challenge with heterologous virulent strain of *A. salmonicida* 5 weeks later. The levels of protection observed were all greater than or equal to that produced by injection of bacterin. However only this last group was shown to have measurable agglutinin titre and Western blots using the immune fish sera failed to reveal any major differences in antigen recognition by fish from any of the experimental groups (Thornton *et al.*, 1994).

Midtlying *et al.* (1996) conducted a series of experiments to investigate the efficacy and the side effect of intraperitoneal immunisation of Atlantic salmon. They found that following the administration of a variety of vaccine formulations with several different adjuvants four out of ten groups were significantly protected against challenge after 6 weeks. At three months and six months only two of the groups showed significant protection both had received the vaccine in mineral oil. The fish in these groups were increasingly protected with time and this correlated with the appearance of high antibody titres. They concluded that only the use of mineral oil induced durable protective immunity to virulent waterborne infection.

Currently several commercial bacterial vaccines are available. These are manufactured by several companies, and while most are designed for use with salmonids there is a vaccine against vibriosis for use in cod. Four vaccines are available to protect fish from furunculosis, Furovac Injection, Furovac Immersion, Furogen and Furogen b. The first two are made by Aquaculture vaccines Ltd. and the others by Aqua Health Ltd. It has to be assumed that these vaccines are protective but there seems to be no information available in the normal literature discussing them.

1.10 The aims of this study.

The information presented above shows that there has been extensive and varied research into the biology of *Aeromonas salmonicida* and attempt at its control. Unfortunately despite many attempts at producing a vaccine, efficacy has often been poor and even when successful in the laboratory, field trials of vaccine preparations have often resulted in failure. The exact nature of the protective antigens has yet to be established and there is also a lack of correlation between antibody response and protection. There were indications that the cellular response may be important (Smith *et al.*, 1980). This was confirmed recently by Marsden *et al.* (1996) using the *aroA* mutant (see below). The passive protection of fish given immune sera indicates that some cellular immune mechanism may be operating and enhanced but not dependant on the presence of high antibody titre.

It is well known that killed whole bacterial cell preparations are only poor stimulators of cellular immunity whereas live vaccines are very good at stimulating a cellular response. In mammalian vaccine research live vaccines have been extensively employed and currently one of the most popular live vaccine strategies involves the specific attenuation of otherwise virulent bacteria by genetically disabling part of a biosynthetic pathway. In 1993 Vaughan, Smith and Foster reported the construction of an *aroA* mutant of *A. salmonicida*. These bacteria were attenuated in Atlantic salmon and brown trout and persisted in the host for up to 14 days and elicited a good degree of protection (a 253 fold increase in the LD_{50}) when administered IP.

Our laboratory has a history of investigating the fate of soluble antigens following oral delivery. Jenkins (1992) investigated the delivery of model antigens to tilapia and Lavelle (1994) investigated the delivery of similar antigens to rainbow trout. For the present investigation it was decided to investigate the fate and subsequent immune response following oral delivery of bacterial antigen. Dr Vaughan had kindly supplied the laboratory with the *aroA* mutant of *A. salmonicida* and this was chosen as the model bacterial antigen. The work

described here investigated the use of the *aroA* mutant strain of *A. salmonicida* as an orally delivered vaccine.

Work by Lavelle (1994) indicated that *A. salmonicida* is one of the growing number of bacteria recognised as being able to invade host epithelial cells. Furthermore, it has been recently suggested that *A. salmonicida* is a facultative intracellular pathogen within macrophages (Garduño *et al.*, 1993). There is a tradition within the laboratory of under taking a literature review of a topic. Jenkins (1992) reviewed oral vaccination and the use of adjuvants and Lavelle (1994) reviewed the comparative immunology of the gastrointestinal tract of mammals and teleosts. Since there were indications that *A. salmonicida* was an invasive pathogen and as *A. salmonicida* biology has been reviewed comprehensively many times it was decided that the literature review in this thesis should be on bacterial invasion and the methods used to investigate the phenomenon and this is presented in Chapter Two.

Lavelle (1994) had investigated the fate of protein molecules in the gut of rainbow trout using an *in vitro* model system. Since *A. salmonicida* is often cultured from the intestine of infected fish the effect of the gastrointestinal enzymes on the bacteria was not investigated. The acidic environment of the stomach has often been quoted as one reason that oral vaccines are less effective than other methods. Certainly, bacteria are often killed by the low pH of the gastric secretion so it was decided to investigate the effect of low pH on *A. salmonicida* using an *in vitro* model system this is presented in Chapter Three.

There was relatively little information on the uptake and tissue localisation of live *A. salmonicida* cells following oral vaccination . Still less information on the effect of growth conditions on the uptake and localisation of the pathogen was available so it was also decided to investigate this process. Jenkins (1992) and Lavelle (1994) had investigated the uptake of soluble antigens over a time course using ELISA. In this instance ELISA was unsuitable as it was the localisation of viable cells that was important. Therefore, viable count techniques were employed and this investigation is presented in Chapter Four.

As stated above Lavelle (1994) had produced evidence that *A. salmonicida* was invasive. The possibility that the bacteria can probably enter the tissues of the host by invading the intestinal epithelium was of considerable interest and so this investigation is presented in Chapter Five.

The nonspecific immune response has been implicated in the defence of the host fish from infection by *A. salmonicida* this part of the immune response is rarely investigated during vaccine trials and because of the live nature of the vaccine in this case it was decided to investigate the non-specific immune response following administration of the vaccine, this investigation is presented in Chapter Six.

The investigation of the persistence of the bacteria in the host, the specific immune response of the rainbow trout following vaccination, and protection against challenge provided by vaccination is presented in Chapter Seven.

Chapter Two.

A review of the current literature on the molecular basis of bacterial pathogenicity and bacterial invasion of host cells at the intestinal mucosae.

2.1 Introduction.

The past twenty years have seen great advances in experimental biology, advances resulting from our rapidly growing knowledge of the molecular basis of life. This great leap in our understanding, has for the main part, been brought about by the development of genetic manipulation techniques, the so-called recombinant DNA (r.DNA) technology (Weinberg, 1991). As it was the patient research of microbial geneticists that led to development of recombinant DNA technology it is appropriate, therefore, that for microbiologists in general and for those studying microbial pathogenesis in particular it has become a powerful and enabling tool (Dougan, 1989; Finlay and Falkow, 1989). It is not the intention to review all the work involving recombinant DNA technology and microbial pathogenesis or live bacterial vaccine construction. These areas are far too large and complex to be dealt within a piece of work of this size. This review is intended as a survey of the literature that deals with the interaction of the host and bacteria at mucosal surfaces and in particular invasion of the host at via the intestinal mucosae.

2.2 The impact of recombinant DNA technology on the study of pathogenicity.

The ability to colonise the host and cause disease involves a complex series of functions requiring several bacterial factors (Mims, 1987; Finlay and Falkow, 1989). The task of the microbiologist investigating bacterial pathogenesis is to identify and characterise these factors and determine what contribution each makes to virulence. Even in diseases like cholera which are apparently caused by the production of one virulence factor such as a toxin, many additional genes are necessary to cause the disease. These genes encode products involved in adherence, invasion, regulation, bacterial growth, and many other functions. Prior to the advent of modern molecular biology, pathogenicity studies involved biochemical purification of virulence factors and assay of the effects of these factors in an animal or *in*

vitro model systems (Finlay, 1992). The results of these studies were often difficult to interpret as the purified component rarely behaved in the same way when removed from the pathogenic organism. The use of molecular genetics has made it possible to dissect virulence factors *in situ* and has given scientists the opportunity to create isogenic strains with which to study virulence factors (Finlay, 1992).

2.3 Koch's Postulates the old and the new.

In the 1800's Robert Koch provided a set of criteria that can be used to demonstrate whether a given bacteria is involved in the pathogenesis of a disease or not. These criteria are known as Koch's postulates. They are: the bacterium should always be isolated from individuals with the disease or its products should be found in the parts of the body affected by the disease; the bacterium should be isolated from the lesions of an infected person and maintained in pure culture; the pure culture when inoculated into a susceptible human volunteer or experimental animal, should produce the symptoms of the disease; the same bacterium should be re-isolated from the lesions of the experimentally infected individual. Koch's postulates were developed to aid in the identification of the bacteria responsible for the major epidemic disease such as plague, cholera and tuberculosis. In the light of new information on the nature of host-pathogen interactions some assumptions made by Koch appear unsound. First, the postulates imply that the production of disease is entirely dependent on the bacterium and this is known not to be the case, the factors affecting the susceptibility of the host may be at least as important in determining the disease process. Second, Koch's postulates place heavy emphasis on the culturing of the etiological agent. Two examples of bacteria that have not yet been cultured in laboratory media are *Mycobacterium leprae* and *Treponema pallidum,* bacteria implicated in leprosy and syphilis respectively. However, despite the lack of pure cultures few microbiologists will dispute that these bacteria are the causative agents of these diseases. Third, the 2nd and 4th postulates

assume that all individuals of the species are equally virulent and there is quite clear evidence that strains or serotypes of species can differ greatly in their virulence (Salyers and Whitt, 1994). Lastly, there is often a problem finding suitable human volunteers and there are several diseases for which there is no acceptable animal model.

As stated above with the development of r.DNA technology it is possible to study virulence factors away from the bacteria they were isolated from. This molecular 'dissection' of pathogens is the crucial advance gene cloning and r.DNA technology has provided.

In the light of this biochemical dissection of pathogens Falkow (1988) provided a set of 'molecular Koch's postulates' which can be used to establish which genes are responsible for virulence. Falkow's 'molecular Koch's postulates' are; the gene should be present in all virulent strains of the organism and not present, not expressed or mutated to a less virulent form in avirulent strains; upon disruption of the gene in a virulent strain there should be a reduction in its virulence and introducing the cloned wild type gene into an avirulent mutant should increase its virulence. In the case of the gene being one of a group of important virulence factors or where two or more copies of a similar gene exist as a fall back mechanism this criterion would have to be modified; the product of the gene must be produced at some point in the infection of the host; antibodies to the gene product should be protective or in the case of cell-mediated immunity being more important than the antibody response, the gene product should induce protective immunity.

These molecular postulates have gained wide acceptance among molecular microbiologists. Isberg and Falkow (1985) succeeded in cloning the invasin gene from *Yersinia pseudotuberculosis* into *E. coli* that resulted in the normally noninvasive recipient becoming invasive, demonstrating the role of the gene and its product in pathogenesis. Even so, there are problems with these postulates; first, it is often difficult to show which genes are expressed during the infection process rather than *in vitro*; secondly, it is often difficult to express virulence factors in non-virulent hosts especially heterologous hosts; thirdly,

antibodies or cell mediated immunity against virulence factors are not always protective. One example of this is the A-layer of *A. salmonicida*, this protein layer is always seen in virulent strains and absence of the layer results in reduced virulence (Munro, 1984). The layer is also immunogenic. However, antibodies to this protein are not protective (Lund *et al.*, 1991).

2.4 The use of molecular techniques to investigate bacterial pathogenesis.

Recombinant DNA techniques involve use of restriction enzymes to cleave DNA, to ligate the cleaved DNA into a vector, and to propagate the DNA in a suitable host. Two main approaches to defining virulence factors at the molecular genetic level exist (Fig. 2.1). The first is to construct a gene bank of the pathogen of interest and use this to clone a gene encoding a putative virulence factor. The gene can only be cloned if there is a suitable screening method available.

The cloned locus and the encoded product can then be studied at the molecular level in a vector host. Mutations in the gene encoding the putative virulence factor are then constructed and the mutated gene moved into the virulent parental strain to replace its wild-type allele. The virulence of the mutants is assayed and compared with the virulence of the wild type.

The second approach involves the production of banks of mutants (usually constructed using a transposon) which are screened for the loss of a virulence property and appropriate mutants identified. The mutated gene can be cloned (using the transposon as a tag) and the parental gene isolated and studied. The original transposon mutants can also be studied for virulence (Finlay, 1992; Dillon, *et al.*, 1985). Most of the experimental evidence reviewed below has been produced using the power of r.DNA technology. This is a very technical field of experimental biology so it is appropriate to describe and discuss the techniques commonly used in molecular microbial pathogenicity investigations at this point.

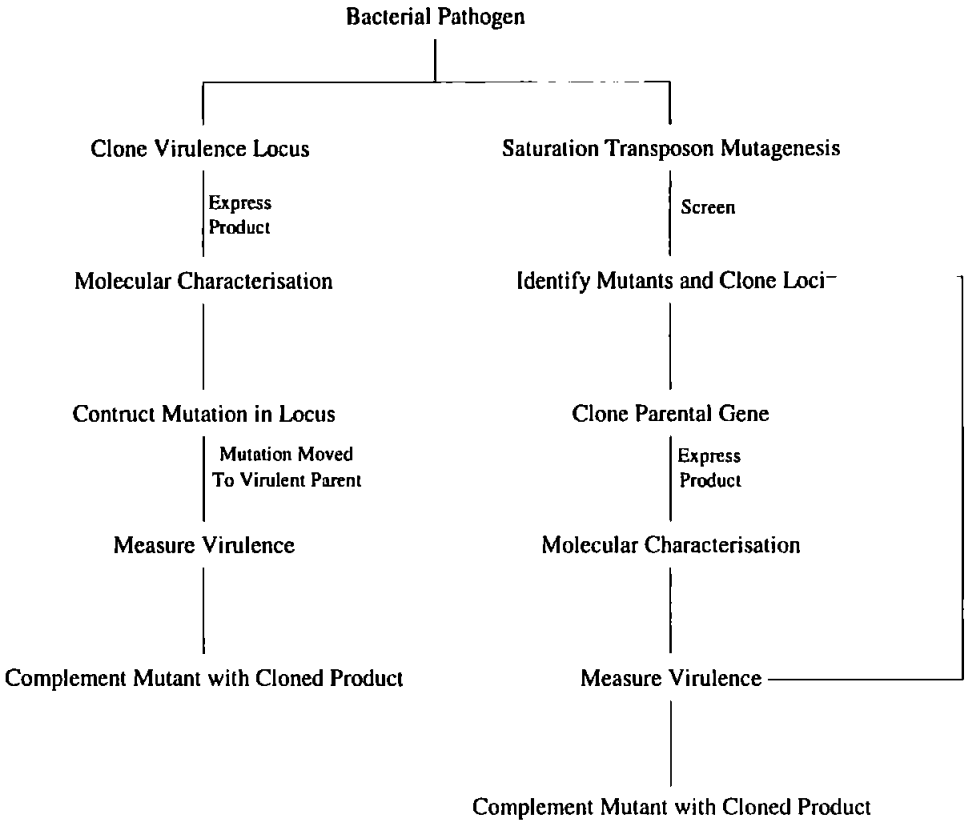


Figure 2.1 Common approaches to study bacterial virulence factors (after Finlay, 1992)

2.4.1 Gene Cloning: Taking genes and making copies.

Molecular cloning represents a mechanism for breaking down complex genetic process into smaller units that can be studied more effectively. Using cloning techniques it is possible to isolate a single DNA segment from a highly heterogeneous mixture and propagate that segment to a high degree of homogeneity (amplification) within a bacterial cell. The DNA to be cloned must be combined with a vector (i.e. plasmid, phage or cosmid, described below) which is capable of autonomous replication within a bacterium. As bacterial hosts can be easily detected and grown, large quantities of specific DNA can be recovered, purified and used (Dillon *et al.*, 1985).

The process of cloning a gene generally begins with the segmentation of the DNA to be cloned, the size of segments depends on the cloning vector and the subsequent use of the DNA. The segmentation can be achieved by physical forces but restriction endonucleases are more commonly used. The DNA segments are then mixed *in vitro* with an appropriate ratio of the vector and the two are joined by a ligase. The resultant chimeric molecule is then introduced to a suitable host (transformation). A suitable host permits expression of the cloned DNA and the vector to multiply (amplification of copy number). Progeny cells that contain and are expressing the cloned DNA are then selected often by using an antibiotic resistance gene in the vector.

To act as a vector, a genetic element must posses properties in addition to the ability to undergo autonomous replication. There have to be nonessential regions within its nucleotide sequence that contain a single cleavage site for at least one endonuclease. It is at this point that the DNA will be cleaved and foreign DNA inserted without affecting the replication of the vector or the ability to select recombinant cells. It must carry genes that confer a recognisable phenotype on the host cells thus allowing the selection of only recombinant hosts. It must contain other endonuclease restriction site than the one used to insert the foreign DNA so the foreign sequence maybe recovered (failing this, the original site must be regenerate following recombination). Furthermore, to reduce the biohazard associated with recombinant molecules and organisms the vector must be unable to survive outside the host, have a limited host range and be incapable of transmission via conjugation (Dillon *et al.*, 1985).

At present there are three types of vector commonly used for cloning research in the field of microbial pathogenicity the plasmid, the phage, and the cosmid. Plasmids are covalently closed circular double stranded DNA molecules identified in a number of prokaryotic genera (Broda, 1979). Large plasmids are limited to 1-3 copies per cell, the smaller ones can have more than ten copies and some small plasmids are able to continue

replicating even in the presence of the protein synthesis-inhibiting antibiotics chloramphenicol (Clewell and Helinski, 1972; Betlach *et al.*, 1976; Chang and Cohen, 1978). Smaller plasmids have the further advantage of increased transformation frequency with decreasing size (Collins and Hohn, 1978). Two main types of *E. coli* DNA bacteriophages have been extensively used for gene cloning. They are the temperate phage Lambda (λ), and the filamentous phage M13. The former contains a linear duplex DNA molecule 50kb in size. There have been numerous alterations of the phage and these λ derivatives have proven useful in genetic manipulations (Williams and Blattner, 1980). These insertional vectors have only one type of endonuclease site that allows cloning by the addition of foreign DNA to form phages up to 105% of the phage's original size. Substitutional vectors with two or more endonuclease sites allow the replacement of up to 25% of the phage DNA with foreign DNA. Lambda vectors are generally used for building gene libraries or DNA banks (Blatner *et al.*, 1978; Maniatis *et al.*, 1978). There is a minimum length of DNA in λ required for viable virus particle formation. Consequently the cloning of small DNA's Fragments (1kb) which in λ would account for only 1-2% of the DNA recovered, is much less efficient than cloning the same fragment in a small plasmid such as pBR322, where cloned fragments of that size would represent 20% of the DNA recovered (Maniatis *et al.*, 1982).

The filamentous phage M13 carries a single strand of DNA 6407 nucleotides in length. Its small size compared with phage λ and the ease with which high titres are reached (50-100 copies per cell), make M13 an attractive vector for cloning. Furthermore its single-strandedness allows for the direct nucleotide sequencing of the cloned DNA by the dideoxynucleotide method of Sanger *et al.* (1977). A comprehensive review of the use of the filamentous phages as cloning vectors is provided by Barnes (1980) and Zinder and Boeke (1982).

Cosmids were originally designed to clone and propagate large segments of genomic DNA, they are a sophisticated type of lambda-based vector being hybrids between a phage

DNA molecule and a bacterial plasmid. Cosmids are designed to take advantage of the fact the enzymes that package the λ DNA molecule into the protein coat need only the *cos* sites to function. The *in vitro* packaging reaction works not only with λ genomes but with any molecule that carries *cos* sites separated by 37-52 kb of DNA. Thus a cosmid is basically a plasmid that carries a *cos* site. It also requires a selectable marker and a plasmid origin of replication, as cosmids carry no λ genes and so do not produce plaques and need to be selected for just like a plasmid vector (Brown, 1995). There is the potential to propagate about 45 kb of foreign DNA in a cosmid which makes them very attractive vectors. Technical problems in their use exist. The most important problem being, the logistical problem of handling a large number of recombinant bacterial colonies and screening them by hybridisation. This means that they are used mostly in circumstances where their twofold greater capacity is of significant advantage (Sambrook *et al.*, 1989).

2.4.2 Expression vectors: Making your protein.

Following the demonstration by Chang and Cohen (1974) that a gene for ampicillin resistance (Ap^R) originating from *S. aureus* could function in an unrelated organism *E. coli*. It was widely assumed that genes from any bacterium could be expressed in any other. This belief was based on the expectation that, in parallel with the universal nature of the genetic code, other parts of the gene-to-phenotype biochemical pathway would also be universal. This idea was strengthened when genes from two lower eukaryotes *Saccharomyces cerevisiae* (Struhl *et al.*, 1976) and *Neurospora crassa* (Vapnek *et al.*, 1977) were also expressed in *E. coli*. However these favourable reports were followed by a whole series that indicated many cloned genes could not be expressed in their new host. An explanation of these failures can be found by careful consideration of the steps involved in the gene-to-phenotype process (Old and Primrose, 1989).

Synthesis of a functional protein depends upon transcription of the appropriate gene, efficient translation of the mRNA and often post-translational processing and

compartmentalisation of the nascent polypeptide (Alberts *et al.*, 1989). A failure to perform any one of these functions properly can result in the failure of the gene to be expressed (Old and Primrose, 1989). Transcription requires the presence of a promoter recognised by the host cells RNA polymerase. Efficient translation, however, requires the presence of a ribosome binding site 'Shine-Delgarno' sequence, and a start codon (AUG) in the mRNA sequence (Shine and Delgarno, 1975; Steitz, 1979). The post transcriptional modifications often include the cleavage of the 'signal sequence' that directs the passage of the polypeptide through the cell membrane (Blobel and Doberstein, 1975; Inouye and Beckwith, 1977). Furthermore, it is known that the short polypeptides encoded by genes that have undergone nonsense mutation are rapidly degraded in *E. coli* (Old and Primrose, 1989) while the wild type proteins are stable. Thus it may be that foreign proteins would be rapidly degraded in the new host if their configuration or amino acid sequence did not protect them from intracellular proteases.

The first requirement of expression of a cloned gene is clearly the presence of a recognised promoter sequence. Cloning vectors do not always possess such a sequence, therefore, vectors used for the isolation and amplification of a particular fragment of DNA may not be the best for expression of that fragment. There are a number of vectors designed specifically for this purpose. These expression vector molecules carry in addition to all the requirements of a good cloning vector, the DNA sequences essential for the transcription and translation of cloned genes. Ideally it is important that the promoter sequences be powerful yet controllable as continuous high level expression may be harmful to the host cell. The promoter, *pL*, of bacteriophage λ (eg plasmid pKC30, Shimatake and Rosenberg, 1981; Rosenberg *et al.*, 1983), the *lac* promoter of *E. coli* (eg plasmid p*β-gal*13C of Goeddel *et al.*, 1979), the *trp* promoter of *E. coli* and *Serratia marcesens* (eg plasmids p*trp*L1 of Edman *et al.*, 1981, and the pBN series of Nichols and Yanofsky, 1983), the promoters of penicillin resistance genes (eg, plasmid pKT287 of Talmadge *et al.*, 1980 and plasmid pSYC193 of

Chang *et al.*, 1982) and the *rec*A promoter have also been used (Shirakawa *et al.*, 1984) the *rec*A gene is normally repressed by the product of the *lex*A gene but is induced by the presence of substances like nalidixic acid that damage DNA.

To ensure efficient translation the cloned genes must be inserted into the vector downstream of a start codon and the Shine-Delgarno sequence. Many vectors have been produced that fulfil these requirements in each case this is done by fusion of the cloned gene with genes on the vector. Thus, the cloned genes have the *N*-terminus portions of the plasmid genes. A number of such vectors were described in detail by Bernard and Helinski (1980).

2.4.3 Altering Genes: Transposon mutagenesis.

A transposon is a genetic element that can change its position within or between cellular genomes. The insertion of a transposon into a gene eliminates expression of that gene ('knock out mutant'). This method of mutagenesis has the advantage over traditional methods in that the transposons contain an identifiable marker, often an antibiotic resistance gene, this allows identification and cloning of the interrupted gene. For this technique to be of value in pathogenicity experiments there must be a method for accurately assaying the virulence of the mutants. This may involve measuring loss of virulence in an *in vivo* infection model but more often involves assays of loss of enzymic activity, adherence, cytotoxicity or invasion.

Transposon Tn5 was used by Weiss *et al.* (1983) to investigate *Bordatella pertussis.* By saturating the bacterial chromosome with Tn5 and screening by several methods mutants were identified that lacked the pertussis toxin, the haemolysin and the filamentous haemagglutinin. They also identified a locus involved with the regulation of these and several other virulence factors. This research was the foundation for a great deal of work that has resulted in the virulence factors of *B. pertussis* being extensively characterised.

Mini-transposons lack the transposase activity that is necessary for the mobility of transposons. These were created to prevent mutagenesis due to the transposons' ability to insert additional copies and in some cases excise and reinsert following the initial insertion

event. The mini-transposon contains an antibiotic marker flanked by the end sequences of a transposon that are essential for successful transposition. For the initial event the transposase activity is supplied *in trans* by a gene carried on a plasmid. When the mini-transposon inserts it is 'locked' in to position and cannot transpose (Elliott and Roth, 1988; Hughes and Roth, 1988).

Transposon Tn*phoA* is another specialised transposon used extensively in the study of virulence factors. The transposon encodes an alkaline phosphatase gene that lacks its signal sequence (Manoil and Beckwith, 1985). Insertion of Tn*phoA* downstream and in-frame with a signal sequence results in export of alkaline phosphatase outside the bacterial cytoplasm, resulting in a colour change in colonies when grown on a suitable substrate. This allows the identification of genes encoding membrane, periplasmic and secreted products. Since most virulence factors are expressed on the surface of bacterial cells, where they interact with the host, Tn*phoA* has been invaluable in identifying products involved in bacterial pathogenesis.

This procedure has been used to identify loci in *Salmonella cholerae-suis* that are necessary for the organism to penetrate through polarised host epithelial cells (Finlay *et al.*, 1988a). Most of the insertion mutants were *Pho*$^-$, approximately 2% (626) of the transposon mutants were *Pho*$^+$. When screened, 42 were unable to penetrate polarised epithelial monolayers cultured on permeable supports. Several of these mutants were subsequently shown to have reduced virulence in mice or were avirulent on the basis of oral LD_{50} values. Most transposons (including Tn*phoA*) do not insert randomly. Of the 42 Tn*phoA* mutants of *Salmonella cholerae-suis*, 20 were within the same gene, while the remainder inserted at unique sites. A further complication in the use of Tn*phoA* mutants is that foreign protein is expressed at the surface of the pathogen and could affect virulence and other pathogenic traits through alterations in the bacterial surface composition and surface charge.

2.4.4 Delivery of transposons: Suicide plasmids.

Delivery of transposons can be achieved in several ways. One of the most often used techniques involves vectors called suicide plasmids. These are plasmids that have special requirements for replication that are only present in the host bacterium (Weiss *et al.*, 1983; Miller and Mekalanos, 1988). Once a suicide plasmid containing a transposon is moved (usually by conjugation) into a recipient organism, the transposon inserts a copy into the recipient DNA. The suicide plasmid cannot replicate in the recipient organism and is quickly diluted out. Appropriate antibiotic selection allows identification of transposon mutants, without the presence of the delivery vector (Finlay, 1992).

Suicide plasmids can be used to deliver genes that undergo homologous recombination within the recipient, replacing the resident gene. To demonstrate that a particular locus codes a virulence factor, the gene must be deleted or mutated in an otherwise virulent strain and loss of virulence must be proved. This is most often accomplished by constructing a mutant allele and moving it onto a suicide plasmid, followed by delivery into a virulent parental strain. Recombination via a *recA*-mediated event then takes place, and the parental (wild type) allele is replaced with an altered one that can be selected for. Recombinants of this kind can then be assayed for virulence. As mentioned above to fulfil the molecular Koch's postulates virulence should be restored and this can be achieved by complementation *in trans* by the cloned parental allele (Falkow, 1988; Finlay, 1992).

2.4.5 Conjugation.

A classical technique used to identify virulence factors utilises conjugation to move virulence loci located on the chromosome into avirulent hosts. This technique has been used most widely in *E. coli* and *Salmonella spp.* Acquiring a set of strains possessing a well-distributed high frequency of recombination (HFR.) sites to use as donors is usually difficult. A set of these strains exists for *E. coli*, however only a small collection exists for *S. typhimurium* (Sanderson and Roth, 1988). Simon (1984) described an approach that overcomes this problem in *Salmonella spp.* Random insertion of a Tn*5-Mob* transposon into

the chromosome creates several sites which can be mobilised provided the transfer functions are supplied *in trans.*

2.4.6 Gene Mapping.

Virulence factors are often clustered on the chromosome and so once a virulence gene has been identified it is often useful to map its position. This can provide information on the gene product's function and regulation of expression (Finlay, 1992). New techniques are being developed to accomplish this task; one example was described in 1988 by Youderian *et al.* A series of transposon insertions evenly distributed around the chromosome of *S. typhimurium* each containing a locked in Mu*d*-P22 prophage, can be used to amplify about 3 minutes of the chromosome. DNA isolated from these transposon insertion mutants can then be immobilised on membrane supports and probed with the DNA encoding the virulence gene. This allows the gene to be mapped to within 3 minutes on the chromosomal map. This technique though useful is limited to a small number of pathogens and most often a combination of methods will be used (Yoneda *et al.*, 1979). Generally these will include pulse field electrophoresis (Schwartz and Cantor, 1984), partial digestion (Smith and Bernsteil, 1976), exonuclease digestion (Legerski *et al.*, 1978), double digests (Jorgensen *et al.*, 1979; Tenover *et al.*, 1980) of DNA and Southern blotting (Southern, 1975) using the cloned gene of a known virulence factor.

2.4.7 DNA Sequencing.

DNA sequencing (Sanger *et al.*, 1977; Maxam and Gilbert, 1977; Hindley, 1983) can generate a lot of information besides the primary structure of the gene product. Using computer programs such as DNASIS™ (Hitachi Software LTD, UK) predictions on secondary and tertiary structure can be made; hydrophobic and hydrophilic regions can be identified, and the portions of the product that are likely to be antigenic can be highlighted. The sequence can also be compared to the sequences of many other genes logged in gene databases, this allows identification of regions of homology between gene sequences. Allowing identification

of functional domains and structures like signal sequences although, DNA sequence homology searches do not always provide any information on the function of the predicted gene product. A good example of the usefulness of this technique in pathogenicity studies is provided by the work on the thiol-activated cytolysins (Geoffroy and Alouf, 1984). These toxins are produced by bacteria from four genera of Gram positive bacteria namely; *Streptococcus, Bacillus, Clostridium,* and *Listeria.* The initial characterisation of these toxins suggested a similar mode of action and possible common evolutionary origin (Kehoe and Timmis, 1984). However these workers failed to demonstrate any homology when they hybridised cloned toxin genes with other species known to produce thiol-activated cytolysins. Kehoe's group then suggested that the genes may have undergone substantial divergence and that only the functional regions might be conserved. This could only be shown by identifying conserved regions in the primary structure of the proteins. This was confirmed by Kehoe *et al.* (1987) by comparing the amino acid sequences of streptolysin O and pneumolysin predicted from the DNA sequence of the coding genes. It was found that when the amino acid sequences were aligned, 42% of the residues matched exactly and the alignment of the sequences using similar residues showed even higher homology.

2.5 Bacterial Infection.

Pathogenic microorganisms have a survival strategy that requires that they multiply on or within a living host. The usual outcome of the interaction between microorganism and host is a subclinical infection that results in both organisms being 'better off' for their association. This is because the host will usually become resistant to subsequent serious infection and the microorganism can grow to an extent that will enable it to be successfully transmitted to another host.

Successful pathogens must be able to perform the following functions: first, gain entry into a host (ideally the preferred host species); second, find a unique niche within the host;

third, evade, circumvent, or exploit the host's innate defence mechanisms; fourth, multiply; and finally exit the host in a manner designed to maximise the likelihood of transmission to another susceptible host (Finlay and Falkow, 1989). This last requirement means that in some cases the microorganism must be able to survive for some time outside the host. Furthermore, if the microorganism's survival strategy requires that it remains within the host for long periods then it will need to cope with the host's adaptive immune system.

2.6 Interactions Between Bacteria and the Mucosal Surfaces of the Host.

The mammalian exterior surface is covered by skin, with a relatively impermeable dry, horny outer layer that acts as a physical barrier which bacteria find difficult to penetrate. The structure of skin demonstrates the compromise between the need to protect the body, yet at the same time maintain sensory communication with the outside world and give mechanical mobility.

The dry, protective skin cannot cover all body surfaces. The conjunctiva, a transparent layer of living cells, replaces the skin over the surface of the eye. Living layers of cells are also found lining the alimentary canal, to facilitate the absorption of food, in the lungs to allow gaseous exchange and finally, in the urinogenital tract, where urine and sexual products are secreted into the exterior. Collectively, these surfaces are called the mucous membranes, since the cells on all these surfaces are covered by a fluid film containing mucin, a complex hydrated gel that waterproofs and lubricates. The mucosal surfaces are damp and warm and provide a very good environment for microorganisms; furthermore, penetration of the mucosal surfaces is far more easily accomplished than in the case of the intact outer skin. One striking feature of acute infectious illnesses all over the world is that most of them are either respiratory or dysentery-like in nature. They are not necessarily the most severe infections,

but for sheer numbers they are the type that matter. Thus for much of the time infectious agents are restricted to the respiratory and especially the intestinal tract (Mims, 1987).

2.6.1 Colonization of Mucosal Surfaces.

Bacterial colonization of mucosal surfaces requires that the organism achieve the following objectives: first, establish close proximity to the mucosa; second, avoid being swept away; third, acquire essential nutrients for growth; fourth, replicate at a rate sufficient to maintain or increase their population; and fifth, resist local defences (Arp, 1987). Mechanisms by which bacteria maintain proximity to the mucosal surface can be categorised by increasing intimacy from association to adhesion and finally invasion.

2.6.2 Association.

Association is a nonspecific term for the localization of bacteria on a surface; it does not specify the mechanisms involved, (Marshall, 1984). The term is used here to describe a loose and reversible attachment to the mucosal surface, specifically as a prerequisite to adhesion and then invasion of the mucosae. Studies with *Vibrio cholerae* and *Salmonella typhimurium* have shown that bacterial association with the intestinal mucosa is influenced by chemotactic stimuli (Allweiss *et al.*, 1977; Freter *et al.*, 1981; Freter *et al.*, 1981). *In vivo* the bacteria must express flagella and the required range of taxin receptors. They are then able to exploit regions of optimal substrate availability and to penetrate the mucous blanket to enhance contact with receptors on the epithelial surface (Freter, 1981).

Lee *et al.* (1987) demonstrated significant antigenic cross-reactivity between flagellar proteins of gastrointestinal *Campylobacter* spp. pathogenic for humans and of gram negative spiral bacteria that colonise the intestinal mucosa of rodents. They believed that there may be a conserved portion of the flagellin molecule that is functionally important for colonization of the mucous layer and that bacteria sharing this niche also share this conserved portion of the flagellin molecule. Furthermore, Attridge and Rowley (1983) postulated that the flagella of *V. cholerae* may function as adhesins.

2.6.3 Adhesion.

The term adhesion is used to describe the relatively stable, irreversible attachment of bacteria to a surface (Jones and Isaacson, 1983). Specific adhesion requires the interaction of specialised complementary molecules in a ligand-receptor fashion between surfaces of bacteria and substratum. This interaction typically occurs between bacterial surface proteins and carbohydrate-containing molecules of the eucaryotic membrane (Lark, 1986). An adhesin is any bacterial structure or molecule that mediates adhesion, and adhesins that agglutinate erythrocytes are also called haemagglutinins (Isaacson, 1985).

During the course of infection, bacteria colonise body sites by sequentially engaging their surface-bound adhesins with cognate receptors available on epithelial cells, endothelial cells, leucocytes, or the extracellular matrix. It is generally accepted that this recognition process is required to establish bacteria at a given site (Hoepelman and Tuomanen, 1992). Although less rigorously demonstrated it is also believed that address recognition by adhesins can determine which host is targeted by which pathogen. For example the adhesins of *Bordatella pertussis* appear to carry sufficient specificity to discriminate between cilia and macrophages and distinguish human from other mammalian cells (Saukkonen, *et al.*, 1992).

Adhesins appear to play a substantial role in determining the outcome of a prokaryotic-eukaryotic interaction. They may initiate invasion, either by themselves or by engaging a cascade of secondary molecules. They have inherent abilities to incite, subvert, or co-opt host defence systems (Hoepelman and Tuomanen, 1992). The more complex the adhesin, the wider the array of possible outcomes. Adhesins can even be toxins, one example is the pertussis toxin that has the ability to interact with a repertoire of glycoproteins and glycolipids on many types of eukaryotic cell (Tuomanen and Weiss, 1985). This capability confers upon this and other toxins the ability to act as an adherence bridge for the whole bacterium and also to mediate cellular intoxication by presentation of the enzymically active toxic subunit.

There is a large amount of information available on the structures and sequences of bacterial adhesins. Those described so far in the literature are composed of protein (Jones and Isaacson, 1983), polysaccharides (Gibbons and Van Houte, 1975; Marcus and Baker, 1985), lipoteichoic acid (Beachey, 1981), or conjugates of these. The majority of adhesins which have been characterised at the molecular and genetic level are surface proteins of gram-negative bacteria (Jones and Isaacson, 1983; Lark, 1986). These proteinaceous adhesins have the capacity for specific receptor interactions comparable to those of antibody molecules. Proteinaceous adhesins can be divided into those with fimbrial morphology and those lacking definite size and shape.

The fimbrial adhesins of *E. coli* and many other species have been reviewed elsewhere, (Sellwood, *et al.*, 1975; Gaastra and de Graaf, 1982; Jones and Isaacson, 1983; Isaacson, 1985; Klemm, 1985) and will not be dealt with in any great detail here.

Bordatella pertussis displays at least seven potential adhesins; four types of fimbriae and three nonfimbrial adhesins, filamentous haemagglutinin (FHA), which despite its name does not appear to have fimbrial morphology (Ashworth, Irons and Robinson, 1985; Ashworth, *et al.*, 1985), pertussis toxin, and pertactin. Both the FHA and the pertussis toxin are unusual in that they bind to both cilia and the bacterial surface to form adherence bridges, (Tuomanen and Weiss, 1985; Tuomanen, 1986). Importantly, Tuomanen (1986) has shown that other pathogenic bacteria, pretreated with these adhesins, acquire the specific ability to bind to cilia *in vitro* and *in vivo*. The carbohydrate recognition domains of pertussis toxin have been localised to the otherwise homologous subunits S2 and S3 (Saukkonen, *et al.*, 1992). Further examination of prokaryotic carbohydrate-binding proteins reveals a kinship between toxin subunit S2 and S3 and the adhesion fimbriae PapG, a similar kinship between sialic acid-binding fimbriae and toxins has also been reported recently (Morschhauser *et al.*, 1990). Interestingly, the pertussis toxin carbohydrate binding domains also have a strong structural homology with eukaryotic C-type lectin domains (but not with plant lectins), (Saukkonen *et*

al., 1992). This indicates that both toxin and fimbrial recognition units are related to the broader family of C type lectin of eukaryotes (Hoepelman and Tuomanen, 1992). Such a structural relationship can be extended to a functional homology between the adhesin molecules and a subset of the C-type lectins the selectins, (Hoepelman and Tuomanen, 1992). Endothelia express selectin (e.g., ELAM and GMP-140) to promote leucocyte adhesion, and recent work by Hoepelman and Tuomanen suggests that both S2 and S3 are active in assays of selectin activity.

Much work has been done on the carbohydrate receptors for prokaryote adhesins and indeed much is known about the oligosaccharide domains to which they attach, however, the molecules bearing these domains are virtually uncharacterised. In marked contrast to this, one group of receptors has recently received much attention: the large family of eukaryotic adhesion molecules, the integrins. This family of glycoproteins mediates cell-cell and cell-extracellular matrix recognition between eukaryotic counterparts. Characteristically integrins bind to proteins containing the Arg-Gly-Asp triplet (Ruoslahti, 1991; Hynes, 1992). A surprisingly large number of pathogens have cooped the existing integrin-based system of addresses and ligands. Three strategies appear to be used to gain entry into the system. The first can be termed masking, whereby adsorption of the natural ligand for the integrin onto the bacterial surface (eg C3bi for *Legionella pneumophila*) smuggles the pathogen along natural reaction pathways. The second can be called ancillary ligand recognition whereby the pathogen binds to carbohydrates on the glycosylated integrins rather than to the Arg-Gly-Asp recognition site. The third process, true mimicry, is apparently the rarest and the pathogen can adopt one of two routes; either expression of an integrin analogue to promote interactions with endothelial cells e.g., *Candida albicans* (Gustafson, *et al.*, 1991) or expression of proteins containing the Arg-Gly-Asp binding motif e.g., *Bordetella pertussis* FHA (Relman, *et al.*, 1990).

Given that integrins can communicate with the internal cytoskeleton, it has recently become possible to explain and appreciate the observations by of intracellular rearrangements. Particularly the coalescing of actin filaments, under the point of contact of a pathogen with the external surface of the cell. Such cytoskeletal rearrangement is a response that may become characteristic of intracellular pathogens as they engineer their own uptake, often beginning with recognition of integrins (Sansonetti 1991a,b; Tilney and Portnoy, 1989; Falkow, 1991; Isberg, 1991)

Although the receptor chosen necessarily restricts the possible fates of the bound pathogen, virtually every pathogen has more than one adhesin, thereby conferring the capability to interact with more than one receptor and their attendant signal transduction systems. Thus, the outcome of an adherence interaction of a particle as complex as a pathogen is more realistically dependent not only on the identity of the receptor involved but also on the sequence in which receptors are bound, the communication between receptors and the state of the receptors on a target cell.

Apparently, pathogens recognise that receptors exist in active and inactive states and can exploit these properties. In the case of *B. pertussis*, activation of an integrin is achieved by a cascade coordinated by two adhesins. In this system, the binding of the B oligomer of pertussis toxin to macrophage carbohydrates first up regulates the integrin CR3. The activated CR3 then in turn binds to the adhesin FHA, leading to bacterial uptake into the macrophage. Binding of the toxin or FHA alone leads to significantly less ingestion than in cells "primed" by preligation to the toxin. This cooperative process is analogous to the interactions between selectins and integrins in the process of leucocyte transmigration across endothelia. The binding of selectins to leucocyte glycoconjugates upregulates integrins which in turn mediate actual transmigration of the leucocyte across the endothelium (Lo *et al.,* 1991). Since pertussis toxin subunits have properties of selectins and FHA binds to the integrin CR3, it appears that *B. pertussis* has co-opted existing communication pathways between eukaryotic

surface molecules which govern leucocyte mobility to promote its own entry into macrophages. It is possible that other pathogens have evolved to take advantage of eukaryotic receptors that have a vital function to the host. Since these are likely to be highly conserved and thus the pathogen optimises its chance of successfully infecting the host.

2.6.4 Invasion.

Many bacterial pathogens are content to adhere to the mucosal surface and presumably they are able to obtain all the nutrients they require and defend themselves from the host's local defences e.g. *Vibrio cholerae*, enterotoxigenic *E. coli*. Other pathogens opt for a more intimate association with the host and these organisms actually invade the host cells, e.g. *Renibacteriaum salmonarum*. It is still unclear, at a molecular level, how bacterial adherence leads to either extracellular colonisation or invasion of the epithelial cells (Falow, 1987, Isberg, 1991). For viral pathogens, invasion of host cells is an essential part of the life cycle since they cannot replicate extracellularly, this is not so for most bacteria and why many of them do invade cells is not clear. Certainly, they do evade some of the host's defences by adopting an intracellular mode of life, and they also gain access to new sources of nutrients, however, they are also presented with a new set of problems. A successful intracellular pathogen must have mechanisms to enter host cells, avoid being killed, multiply, and provide for the escape of their progeny to new host cells (Moulder, 1985).

Furthermore, assuming that the intestinal epithelium is poorly protected from invasion is incorrect. The invasive organism must overcome the combined effects of peristaltic movements of the gut, mucus secretion, the presence of antibacterial substances such as lactoferrin and lysozyme, secretory antibodies, the tight junctions between epithelial cells and the highly differentiated nature of the brush border at the apical surface of enterocytes. Assuming these barriers can be surmounted the bacteria will still face numerous sub-epithelial macrophages and lymphocytes.

The Peyer's patches of the ileum and the lymphoid solitary follicles of the colon and rectum, are intra-mucosal lymphoid structures covered by a differentiated epithelium of microfold or M-cells (Bye *et al.*, 1984; Neutra *et al.*, 1987). These cells specialise in transporting luminal antigens to antigen presenting cells (APCs) that are present in the pockets formed by the M-cells. These APCs present the transported antigen to the lymphocytes present in large numbers in the adjacent lymphoid follicle. These cells provide protection at the mucosal surfaces and have been shown to migrate to the mucosal associated lymphoid tissue preferentially. The antibody secreting cells (ASC) produce IgA that can be transcytosed across the epithelium into the lumen as secretory IgA.

Several of the bacterial pathogens discussed below clearly, but not exclusively, use the M-cells to cross the epithelium (Sansonetti, 1991a,b).

2.7 Enteric diseases of humans and other mammals.

A bacterial enteric disease can be defined as either a localised disease caused by the presence of bacteria within the intestine or a systemic disease that is caused by a bacteria which entered the viscera via the gastrointestinal tract. From this point on this review will concentrate on the bacteria that enter intestinal epthielial cells either as means of gaining access to the viscera or because the epithelial cells are the preferred sites of infection. Within the Enterobacteriaceae family four genera contain species capable of entering mammalian epithelial cells: *Yersinia, Escherichia, Shigella* and Salmonella. The Gram positive bacterium *Listeria monocytogenes* also enters mammalian epithelial cells.

2.7.1 Disease caused by *Yersinia enterolytica* and *Yersinia pseudotuberculosis.*

Among the many species of the *Yersinia* genus, only *Y. pestis, Y. pseudotuberculosis* and *Y. enterolytica* are adapted to multiply at the expense of a living host (Cornelis, 1994). *Y. pestis*, the agent of the most feared human disease, plague, and *Y. pseudotuberculosis* are both primarily pathogens of rodents. *Y. enterolytica* is a common human pathogen that causes

gastrointestinal syndromes of varying severities, ranging from mild self-limiting diarrhoea to mesenteric adenitis evoking appendicitis. Systemic infection by *Y enterolytica* is unusual but reactive arthritis and *erythema nodosum* are common complications (Cover and Aber, 1989). Though human infection by *Y. pseudotuberculosis* is much less common than *Y. enterolytica* there is a greater likelihood of the infection becoming systemic and there is usually no diarrhoea. All three species of *Yersinia* show an affinity for lymphoid tissue and demonstrate a remarkable ability to resist the nonspecific immune response of the host (Lian *et al.*, 1987b; Hanski *et al.*, 1989, 1991; Simonet *et al.*, 1990).

2.7.2 Disease caused by *Escherichia coli.*

Many strains of *E. coli* are known to cause disease; these strains are grouped into virotypes based on the type of disease caused. Of the five virotypes of *E. coli* there are three that cause invasive disease, the enteroinvasive *E. coli* (EIEC), enterohaemorrahgic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) which are the virotypes of primary interest here. Of the other two virotypes enterotoxigenic *E. coli* (ETEC) causes a disease similar but usually less severe than *Vibrio cholerae* and enteroaggregative *E. coli* (EAggEC) which causes a persistent diarrhoea in children due to the secretion of a toxin very like the heat stable toxin of *V. cholerae.*

EIEC strains cause a disease that is indistinguishable from Shigellosis except that they do not produce Shiga toxin that may explain why HUS is not seen as a complication of infection by strains of this virotype. Since the mechanism of cell entry and cell to cell spread of EIEC is virtually identical to that of *Shigella spp.* EIEC will not be discussed below. Enterohaemorrhagic *E.coli* (EHEC) has only recently been recognised as a cause of serious disease. Unusually for enteric pathogens this virotype seems to cause outbreaks of paediatric diarrhoea in the developed rather than the third world. Usually, such outbreaks are rarely fatal, outbreaks caused by EHEC are very serious, because these strains can cause haemolytic-uraemic-syndrome (HUS) which can lead to fatal kidney failure. EHEC binds to cultured

mammalian cells and show the same attachment and effacement phenomenon as seen with EPEC. However, EHEC probably does not invade mucosal cells as readily as *Shigella*, EIEC or EPEC and the role of invasion in the pathogenesis of this disease is unclear. It is likely that the similarity of this condition to Shigellosis is due to the production of a toxin virtually identical to Shiga toxin.

EPEC like ETEC can cause a severe and often fatal infection in children and infants, and EPEC strains are recognised as a major cause of paediatric diarrhoea and death. Interestingly, countries where ETEC is a problem have relatively little EPEC and vice versa. Non-immune adults are also susceptible to infection with EPEC and this type of diarrhoea can be a cause of "traveller's tummy" which can also be caused by ETEC. This virotype will be discussed below starting at Section 2.7.10 (Jerse and Kopecko, 1995).

2.7.3 Disease caused by *Shigella flexneri.*

In 1889 Shiga isolated the etiologic agent of bacillary dysentery. Again DNA sequence homology shows that the genera *Shigella* and *Escherichia* are more similar than the strains in other species (Falkow and Mekalanos, 1990). Despite this similarity the genus *Shigella* is unlikely to be reclassified and is at this time divided into four species, *S. boydii, S. dysenteriae, S. flexneri* and *S. sonnei.* Shigellosis is an invasive disease of the human colon, at present worldwide it is of major concern in the overcrowded areas of the third world, where *Shigella flexneri* causes the endemic form of the disease and *Shigella dysenteriae* 1 causes devastating epidemics (Sansonetti, 1992; Parsot, 1994). The clinical signs of shigellosis range from mild diarrhoea to severe dysentery with blood, mucus and pus in the stools (Parsot, 1994). Epidemiological studies indicate that *Shigella* is transmitted via the faecal-oral route (Wharton *et al.*, 1990). *Shigella* are highly infectious organisms for humans since it only requires between 50 to a few hundred bacteria to cause an infection (DuPont *et al.*, 1989; Gorden and Small, 1993). *Shigella dysenteriae* expresses a potent cytotoxin, Shiga toxin, that cleaves the 28s rRNA of eukaryotic cells. (Parsot, 1994). The two subunits of this

toxin are encoded by the *stxA* and *stxB* genes that are almost identical to the genes specifying the Shiga-like toxin of *E. coli* (Parsot, 1994). It is this toxin implicated in one of the most serious complications of dysentery haemolytic uremic syndrome HUS. This is a form of severe kidney failure that sometimes develops in children infected with *Shigella dysenteriae.*

2.7.4 Disease caused by *Salmonella species*

'Salmonellae are among the most resourceful and successful of human pathogens, and as such have long beguiled microbiologists, epidemiologists, and clinicians' (Rubin, *et al.*, 1977). While the clinical importance of the *Salmonellae* is, well-established classification of this genus of bacteria seems particularly problematical. Modern methods of speciation based on DNA-DNA homology and 16S rRNA sequence show that the *Salmonellae* and *E. coli* have about 90% overall DNA homology. Today this would result in both genera being classified as one species (Salyers and Whitt, 1994). Such reclassification is, however, extremely unlikely, most microbiologists subscribe to another option which splits the genus into three species, *S. typhi* the cause of typhoid fever, *S. cholerasuis* which is primarily a pathogen of swine and only occasionally of humans, and *S. enteritidis* that contains many serotypes (ca.2000) but even this convention is often ignored. For example the serotype *typhimurium* (that is normally a pathogen of mice) is a subgroup of *S. enteritidis* and should be classified as *S. enteritidis* var. *typhimurium* but this classification is almost never seen in the literature with virtually everyone referring to it as *S. typhimurium.*

Four main clinical syndromes caused by *Salmonella spp.* Although each syndrome maybe caused by each species, each species is usually associated with a given syndrome. The most common syndrome is gastroenteritis and this is usually caused by one of the *S. enteritidis* serotypes. Gastroenteritis (food poisoning) is usually a non-life threatening disease characterised by nausea and vomiting 8-48 hrs after ingestion. Diarrhoea, abdominal pain and often fever follows later in the infection. Enteric fever is usually associated with *S. typhi.*

This disease is characterised by prolonged fever, sustained bacteraemia, activation of the reticuloendothelial system, and dysfunction of many organ systems. The incubation period for enteric fever is usually 1-2 weeks and the disease symptoms last longer than gastroenteritis, this syndrome is occasionally fatal. *S. choleraesuis* is the species that is most often associated with *Salmonella* bacteraemia. In this case there may be no apparent enteric manifestations of infection (Taussig, 1990). The fourth syndrome is the chronic carrier state. In individuals with this condition *Salmonella* can be detected in the stools at 10^6-10^9 organisms per gram for over a year (Finlay, 1994). The carrier state seems to occur most often after infection with small numbers of bacteria and may be asymptomatic.

2.7.5 Disease caused by *Listeria monocytogenes.*

Listeria monocytogenes was first characterised by Murray *et al.* (1926) following an outbreak in laboratory animals. However it was not until the 1980's that a link was established between human disease and the consumption of *Listeria*-contaminated foodstuffs (Schlech *et al.*, 1983). Immunosuppressed individuals, pregnant women, foetuses and neonates are most susceptible to infection. Human listeriosis is characterised by an extremely high mortality rate (higher even than for *Clostridium botulinum* poisoning), with clinical features including meningitis or meningo-encephalitis, septicaemia, abortion, and perinatal infections (Gray and Klinger, 1966).

2.7.6 *Yersinia* invasion: the nature of the entry process.

The enteropathogenic *Yersinia* have been shown to interact productively with a small number of cultured mammalian cell types, including nonphagocytic cells; epithelial, fibroblast, and B-cells as well as phagocytic cells; macrophages and polymorphonuclear leucocytes (Miller *et al.*, 1988; Isberg, 1990). Soon after intimate attachment to the surface of host cells, the bacteria are internalised within vacuoles. This process has been described as a parasite directed phagocytosis (Moulder, 1985). Within minutes, the infected host cells undergo rapid and global changes in cell shape, cytoskeletal architecture, and nuclear

membrane integrity. These disruptions are reflected in a failure of the mammalian cells to respond to extracellular stimuli, ultimately resulting in their rounding and detachment from the extracellular matrix (ECM) (Portnoy *et al.*, 1981; Goguen *et al.*, 1986; Rosqvist and Wolf-Watz, 1986).

2.7.7 *Yersinia*invasion: The identification of genes and gene products involved in the invasion process.

The invasion of eukaryotic cells by the enteropathogenic *Yersinia spp.* is the best characterised of the invasion processes discussed in this review. It is mediated by the *Yersinia inv* gene product invasin. The *Y. enterolytica* and *Y. pseudotuberculosis inv* genes are highly homologous and encode invasin, a 92 or 103kD outer membrane protein (Isberg *et al.*, 1987; Young *et al.*, 1990). The *Y. pseudotuberculosis* invasin was identified by its capacity to confer upon noninvasive *E. coli* the ability to invade cultured mammalian cells (Isberg and Falkow, 1985). Only isolates of *Y. enterolytica* and *Y. pseudotuberculosis* that cause enteric disease encode a functional invasin homolog, even though all strains of pathogenic *Yersinia* examined so far contain *inv*-homologous DNA sequences (Miller and Falkow, 1988; Rosqvist *et al.* 1988; Pierson and Falkow, 1991; Simonet and Falkow, 1992).

Invasin is a bacterial surface protein that promotes invasion by interacting with eukaryotic transmembrane proteins belonging to the integrin superfamily, including $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, and $\alpha 6\beta 1$ integrins (Isberg and Leong, 1990). As stated above, integrins bind to various extracellular matrix proteins that contain an Arg-Gly-Asp (RGD) motif such as fibronectin, collagen, and laminin (Hynes, 1992). The integrins are often localised in focal contacts, participate in transducing extracellular signals, and are components of the apparatus that anchors the cell cytoskeleton to the membrane and extracellular surfaces (Hynes, 1992).

Invasin has a much greater affinity for the integrins than the native ligands such as fibronectin (Van and Isberg, 1991), the dissociation constant of the binding of invasin for purified $\alpha 5\beta 1$ is 5×10^{-9} M (Tran Van Nhieu and Isberg, 1991), which is approximately two

orders of magnitude lower than the dissociation constant for fibronectin. This high binding affinity is believed to be important for successful uptake (Isberg, 1991), and it probably serves to cluster integrins at the bacteria-epithelial cell contact areas. The mechanism(s) by which integrins transmit signals to the eukaryotic cell cytoskeleton is as yet unknown. However, a number of events are known to be associated with integrin stimulation. Platelets can be activated through stimulation of several different transmembrane receptors, and two separate activation pathways involve the binding of the ECM proteins collagen and laminin to the integrin $\alpha 2\beta 1$ and $\alpha 6\beta 1$, respectively. After these proteins bind to their receptors, fibrinogen is released and binds to another integrin $\alpha IIb\beta 3$, leading to platelet aggregation and tyrosine phosphorylation of multiple proteins (Shattil and Brugge, 1991). Invasin-mediated binding of *Y. pseudotuberculosis* to the platelet receptor $\alpha 6\beta 1$ has been shown to mediate platelet activation and aggregation (Simonet *et al.*, 1992). This interaction can lead to the entry of *Y. pseudotuberculosis* into platelets, suggesting that a common transmembrane signalling pathway is involved in platelet activation and bacterial entry (Bliska *et al.*, 1993). Furthermore, integrin clustering appears to trigger host protein tyrosine kinase (PTK) activity (Juliano and Haskill, 1993), which in turn causes the rearrangement of cytoskeletal elements such as actin, talin, and filamin. These proteins are associated with, and are probably essential for invasin promoted bacterial uptake (Finlay and Falkow, 1988; Young *et al.*, 1992; Rosenshine and Finlay, 1993). This concept while not proven is supported by the results of several studies. Invasin promoted bacterial uptake can be specifically blocked by PTK inhibitors (Rosenshine, Duronio and Finlay, 1992). Further, PTK activation occurs in response to integrin clustering by fibronectin or antibodies (Kornberg, *et al.*, 1991; Guan *and Dixon*, 1990).

YadA is another major outer membrane protein that is thought to form a fibrillar matrix on the surface of *Y. enterolytica* and *Y. pseudotuberculosis* (Kapperaud *et al.*, 1987) when they are grown at 37°C. YadA is a polymer of 200-240 kDa formed by the association

of approximately 50 kDa subunits secreted via the classical pathway (Skurnik and Wolf-Watz, 1989). The name YadA stands for Yersinia adhesin, because its presence makes the bacteria adherent to epithelial cells (Heeseman and Gruter, 1987). It is also responsible for binding to collagen fibres (Emody *et al.*, 1989; Schulze-Koops *et al.*, 1992) and fibronectin (Tertti *et al.*, 1987; Schulze-Koops *et al.*, 1992). While these characteristics tend to suggest a role in colonisation, some observations in *Y. enterolytica* suggest that YadA plays a major role in the defence against the nonspecific immune system. The presence of YadA reduces the deposition of C3b at the bacterial surface of *Y. enterolytica* by binding factor H which leads to a rapid catabolism of C3b (Tertti *et al.*, 1987; Pilz *et al.*, 1992; China *et al.*, 1993). This has two consequences; first it inhibits the formation of the complement membrane attack complex, which inhibits killing and secondly, it prevents opsonisation, which reduces phagocytosis and killing by polymorphonuclear leucocytes (China *et al.*, 1994). The chromosome of *Y. enterolytica* and *Y. pseudotuberculosis* encodes two independent pathways for attachment to and entry into cultured mammalian cells *inv* and *ail* (Miller and Falkow, 1988; Isberg, 1990) and only the former has been discussed here. *In vivo* internalin appears to play the vital role in promoting entry into intestinal tissue during infection (Pepe and Miller, 1993). Surprisingly concomitant loss of internalin and YadA by *Y. pseudotuberculosis* results in a marked increase and not decrease in virulence (Rosqvist *et al.*, 1988).

2.7.8 *Yersinia* invasion: major virulence determinants.

All pathogenic *Yersinia* contain plasmid pYV a 70-kb plasmid maintained at about seven copies per cell (Vanooteghem and Cornelis, 1990) this plasmid has genes that can be roughly grouped into three types; *ysc* protein secretion, *vir*, regulation of gene expression and *yop* the effector molecules. The genetic maps of this plasmid are similar for all three *Yersinia* species except for a quadrant containing at least *yopE* (yersinia outer membrane protein E) and *yadA* (Biot and Cornelis, 1988; Forsberg *et al.*, 1987; Forsberg and Wolf-Watz, 1988). The plasmid encodes several other genes implicated in *Yersinia* virulence in addition to the two

mentioned already, namely *yopH, yopQ, yopM, yopO (ypkA), yopP, yopB,* and *yopD* (Mulder *et al.,* 1989) as well as the genes encoding the V antigen, flanked by *lcrG* (Skrzypek and Straley, 1993) and *lcrH* (Perry *et al.*, 1986). Insertion mutants in *yopE, H, Q, M, O, P, ylpA* and *yadA* result in the loss of expression of that protein and no change to the overall phenotype. Insertion mutations in the operon *lcrGVHyopBD* lead to the inability to grow at 37°C (Mulder *et al.,* 1989). A contiguous 20 kb region of pYV called the Ca^{2+} dependency region is required for the production of all Yops. Insertion mutation in the region defined a series of pleiotropic loci called *vir* (because they condition virulence) in *Y. enterolytica* W22703 and *lcr* (because they condition the low calcium response) in *Y. pseudotuberculosis* and *Y. pestis.* Some of the loci have been characterised. However, the information is not yet complete these loci are *virA (lcrA), virB (lcrB), virC lcr(C), virF lcr(F)* and *virG* (Goguen *et al.*, 1984; Yother and Goguen, 1985; Yother *et al.*, 1986; Cornelis *et al.*1986, 1987, 1989).

The Yops were initially described as outer membrane proteins (Portnoy *et al.*, 1981; Straley and Brubaker 1981; Bolin *et al.*, 1985) but this status has been revised and they are now considered as secreted proteins (Heesemann *et al.*, 1984, 1986; Michiels *et al.*, 1990). Some of the Yops (LcrV, YopM, Q, R) are soluble in the culture medium but others (Yop H, E, O, B, D, P, N) have a propensity to aggregate as visible filaments in the culture (Michiels *et al.*, 1990). The Yops are highly conserved between the three species but no homology exists between the individual Yops in a single species (Forsberg and Wolf-Watz, 1988; Bolin and Wolf-Watz, 1988; Michiels and Cornelis 1988; Michiels *et al.,* 1990.) Patients suffering an infection (Martinez, 1983) or mice experimentally infected with *Y. enterolytica* grown at low temperature (Sory and Cornelis, 1988) develop antibodies against the Yops indicating that they are produced *in vivo.* The functions of some of the Yops were reviewed by Cornelis (1994). Interestingly they do not have the classical NH_2-terminal secretory signal peptide (Forsberg and Wolf-Watz, 1988; Michiels *et al.* 1991; Reisner and Straley, 1992).

The 8.5 kb *virC* locus constitutes a single large operon composed of 13 genes called *yscA* to *yscK*. Homologues of *yscJ, yscF* and *yscC* have been found in other animal pathogens including *Shigella flexneri* (Allaoui *et al.*, 1992a, 1993; Andrews and Maurelli, 1992; Venkatesan *et al.*, 1992) and *Salmonella typhimurium* (Galan *et al.* 1992a) and also in plant pathogens (Fenselau *et al.*, 1992; Gough *et al.*, 1992; Xiao *et al.*, 1992). The entire *lcrB (virB)* region of *Y. pseudotuberculosis* has been sequenced (Cornelis, 1994). It appears that the *virB (lcrB)* locus is a counterpart of the *spa* locus from *Shigella flexneri* (Venkatesan *et al.*, 1991; Sasakawa *et al.*, 1993) and the *spa* locus of *Salmonella typhimurium* (Groisman and Ochmann, 1993).

2.7.9 *Yersinia*invasion: The molecular basis for subversion of the host cell signalling pathways.

The Carboxy-terminal 262 amino acid domain of YopH is homologous to the catalytic domains of eukaryotic protein tyrosine phospatases (PTPases) (Guan and Dixon, 1990). The family of PTPases act in partnership with the protein tyrosine kinases (PTKases) to regulate several important aspects of cell growth and development (Fischer *et al.*, 1991). PTPases are thought to play specific roles in signal transduction and cellular physiology and not simply to act antagonistically to PTKases. YopH is specific for tyrosine phosphate, and is active against synthetic peptides *in vitro,* and eukaryotic proteins such as the insulin receptor which are phosphorylated on tyrosine (Guan and Dixon, 1990). A study of the kinetic properties of homogeneously purified *Yersinia* PTPase has show that it is the most active PTPase known, with a k_{cat} value 25-fold higher than that of mammalian PTP1 enzyme (Zhang *et al.*, 1992).

Insertional inactivation of *yopH* has no measurable effect on bacterial gene expression, growth or cell viability in culture. However, a mutant strain of *Y. pseudotuberculosis* that encodes a metabolically inactive form of YopH (C403A) was significantly less virulent than the wild type for mice (Bliska *et al.*, 1991). Tyrosine phosphorylated host cell proteins are rapidly dephosphorylated by *Y. pseudotuberculosis* YopH during infection of human epithelial

cells and murine macrophages (Bliska *et al.*, 1991, 1992). The host proteins dephosphorylated by YopH are unknown, but two macrophage proteins that are specifically co-isolated from cell lysates with YopHC403A have been shown to have intrinsic PTKase activity (Bliska *et al.*, 1992). Thus it seems that YopH disrupts host cell signal transduction by dephosphorylating activated PTKases.

In early studies it was found that YopH contributed to the ability of *Yersinia* to inhibit uptake by phagocytic cells (Gougen *et al.*, 1986; Lian *et al.*, 1987a,b; Rosqvist *et al.*, 1988). The molecular basis for this antiphagocytic effect is now understood in terms of the molecular action of YopH. Bacterial entry into cultured cells facilitated by invasin can be inhibited by the PTPase activity of YopH, presumably as a direct result of its action on host cell phosphoproteins (Bliska *et al.*, 1993). However, it may well be that other pathways used by macrophages and neutrophils to take up and destroy *Yersinia* are the primary targets of YopH.

A second protein that acts synergistically with YopH to counter host phagocytosis is YopE (Rosqvist *et al.*, 1990) Together these two proteins are largely responsible for the physiological alteration that lead to rounding and detachment of mammalian cells from the ECM (Rosqvist *et al.*, 1990). Although a molecular function for YopE has not been established, the action of this protein on host cell target leads to the disruption of actin filaments (Rosqvist *et al.*, 1992).

An 82 kDa Yop has recently been shown to have extensive homology with the family of eukaryotic serine/threonine protein kinases (Galyov *et al.*, 1993). This Yop yersinia protein kinase A (YpkA), is autophosphorylated *in vitro*, but other physiological substrates of the enzyme have not yet been identified. Because serine and threonine phosphorylation are common modifications in prokaryotes, this kinase may act as a Yersinia substrate. However, it is equally possible that like YopH, it exerts its primary function by subverting host cell signal transduction.

The interaction of enteropathogenic *Yersinia* with cultured cells is proposed to occur in two sequential steps. Initially, the bacteria communicate with the host cytoskeleton through the β1 integrins and thus exploit existing signal transduction pathways to intimately attach to and enter the target cell. Since invasin and YadA are regulated in an opposite way by temperature, they may act sequentially during infection to promote these intimate attachments (Bolin *et al.*, 1982; Isberg *et al.*, 1988). Accordingly invasin has been shown to have a primary role in the colonisation of the murine Peyer's patches by *Y. enterolytica* (Pepe and Miller, 1993) and *Y. pseudotuberculosis* (Simonet and Falkow, 1992). In the second step the bacteria produces and presents proteins to the host cell that act to interfere with existing signal transduction and cytoskeletal function. The bacterial and/or host molecules that facilitate production and presentation of the Yops have not been identified as yet, but it is unlikely that bacterial entry into host cells is required (Heesemann and Laufs, 1985; Rosqvist *et al.*, 1990). At the molecular level the Yops are proposed to act synergistically to interfere with several key host functions involving tyrosine phosphorylation (YopH), actin microfilament stability (YopE), serine/threonine phosphorylation (YpkA), and G protein-linked receptor stimulation (YopM). The biological outcome of this multifactorial process is the capacity of the bacteria to enter the reticuloendothelial system and delay the development of a cellular immune response (Bliska *et al.*, 1993).

2.7.10 EPEC Invasion: the nature of the entry process.

A number of studies have shown that EPEC are able to adhere to epithelial cells and cause the cytoskeletal rearrangements known to be associated with the attaching and effacing lesion (Moon *et al.*, 1983). These structural changes have been observed in animal models, infected humans and tissue culture assays (Donnenberg and Kaper, 1992). The affected cells show effacement and loss of the microvilli but also a distinct formation of dense 'pedestals' of filamentous actin under the site of bacterial adherence (Knutton *et al.*, 1989). EPEC strains enter cultured mammalian cells at a low efficiency (Donnenberg and Kaper, 1992). Inhibition

of microfilament, microtubule or PTKase function block this entry mechanism (Donnenberg et al., 1989; Francis *et al.*, 1991; Rosenshine *et al.*, 1992) which suggests that the attaching and effacing lesion results from exploitation of host cell signal transduction and cytoskeletal functions by EPEC (Donnenberg and Kaper, 1992; Rosenshine and Finlay, 1993).

2.7.11 EPEC invasion: genes and gene products involved in host cell entry.

Whilst *Yersinia* invasin functions in adhesion, induction of internalisation, and anchors the bacterium to the cytoskeleton via integrin linkages, each of these functions appears to be encoded separately in EPEC. At least three surface exposed products are involved in internalisation of EPEC the products are encoded by the *eae, cfm*, and *bfp* genes.

Adhesion to the eukaryotic cell surface and coadhesion of bacteria is mediated by the plasmid coded bundle forming pilus *bfp* (Giron, *et al.*, 1991). The *bfp* does not appear to participate in invasion but by bringing the bacteria into close contact with the eukaryotic cell it greatly increases the rate of invasion. Mutants lacking this plasmid have reduced kinetics for developing the attaching and effacing lesion. There are other genes required for this initial adherence (Donnenberg *et al.*, 1990), and furthermore, additional or alternative adhesins may function similarly in EPEC and other *E. coli* strains.

Following attachment, probably via the bundle forming pilus, to the epithelial cell surface EPEC elicit an "attaching and effacing" phenotype in the epithelial cell (Moon, *et al.*, 1983), this localised breakdown in the structure of the brush border microvilli, accompanied by the assembly of highly organised cytoskeletal structures within the epithelial cell just below the bacteria (Knutton, *et al.*, 1989). The structure is formed of actin filaments, myosin, α-actinin, ezrin, talin, and tyrosine phosphorylated proteins (Knutton *et al.*, 1989; Finlay, *et al.*, 1992; Manjarrez-Hernandez *et al.*, 1992; Rosenshine, *et al.*, 1992).

The formation of this structure appears to be triggered by activation of specific host PTK activity by EPEC and a rise in intracellular Ca^{2+}. This is apparently released from intracellular stores since the addition of a drug that blocks the release of intracellular Ca^{2+}

blocks the rise in calcium ions (Baldwin *et al.*, 1991). This observation led to the suggestion by Baldwin and coworkers (1991) that the activation of PLC-γ and production of inositol triphosphate and diacylglycerol are responsible for the effacement of the microvilli and other physiological alterations in the epithelial cell. The increase in calcium ions could result from the inositol triphosphate mediated release of Ca^{2+} from intracellular stores (Baldwin *et al.*, 1991). The induction of this PTK activity and Ca^{2+} release appears to be mediated by the product(s) of the *cfm* gene(s), since a class of chromosomal transposon insertion mutants have been shown to be able to induce attachment but are defective for entry (Donnenberg *et al.*, 1990). These products probably interact with epithelial cell receptors to induce tyrosine phosphorylation of several epithelial cell proteins, including a predominant 90kDa protein (Rosenshine *et al.*, 1992). This phosphorylation triggers in turn the formation of the unorganised protein aggregates beneath the adherent bacteria.

The protein aggregates must be rearranged into an organised structure before internalisation can occur, this nucleation process is dependant on the activity of the *eae* gene product the 94kDa outer membrane protein intimin. Intimin has significant homology with the amino terminal domains of the *Yersinia* invasins (Jerse and Kaper, 1991). It may well achieve this by interacting with a host transmembrane receptor which in turn nucleates the cytoskeletal aggregate. Unlike invasin, intimin is not sufficient to induce bacterial entry of the host cell (Donnenberg *et al.*, 1990). However, if *cfm* function is impaired bacterial cells can still invade provided that there are bacteria in the adherent micro colony that have full *cfm* function. In contrast each bacterial cell must have intimin on its surface if it is to be internalised. Thus, *cfm* mutants can be complimented *in trans* by wild type bacteria but *eae* mutants cannot (Rosenshine *et al.*, 1992). Interestingly, both *Yersinia* and EPEC can adhere to the epithelial surface even if not metabolically active or even viable (Finlay, *et al.*, 1989).

2.7.12 *Shigella* invasion: The nature of the entry process.

The invasion process of *Shigella* includes two major steps; the first, involves invasion of the epithelial lining of the colon (Takeuchi *et al.*, 1965), and the invasion of the phagocytes resident within the lamina propria of the intestinal villi. The process leads to confluent foci of a strong inflammatory response resulting in abscesses and ulceration (Takeuchi, *et al.*, 1968). Transmission electron microscopy (TEM) performed following experimental infection of continuous lines of epithelial cells, such as HeLa cells, with *Sh. flexneri*, has shown that the bacteria are internalised via an active phagocytic process (Hale and Bonventre, 1979; Hale, *et al.*, 1979; Clerc and Sansonetti, 1987).

Shigella lyses the membrane of the phagocytic vacuole and thereby gains access to the cytoplasm only a few minutes after internalisation has been completed. Following escape the bacteria multiply with a generation time of about 40 min (Sansonetti *et al.*, 1986). Protein synthesis of the infected cell is quickly blocked (Hale and Formal, 1981), furthermore, *Sh. flexneri* is the first bacterial pathogen reported to induce apoptosis of infected macrophages (Clerc *et al.*, 1987; Zychlinsky, 1992). This was demonstrated by the distinctive ultrastructural morphology of the cells with vacuolated cytoplasm and heavily condensed chromatin and by the appearance of cellular DNA in discrete 200 kb fragments.

Extracellular *Shigella* are nonmotile organisms, however Ogawa and coworkers (1968) showed by phase contrast microcinematography that intracellular bacteria move into the entire cytoplasm of the infected cell. The movement of the bacteria was seen to be random and sometimes led to the formation of structures that protruded from the cell surface containing bacteria at their tip this form of movement is called Intra-Inter Cellular Spread (Ics). This Ics movement can be reversibly inhibited by cytochalasin D which prevents polymerisation of monomeric actin (G-actin) into filaments (F-actin), (Bernardini *et al.*, 1989; Pal *et al.*, 1989). There is apparently another independent movement mechanisms that operates in different cell lines. In chicken embryo fibroblasts, which have a highly organised cytoskeleton, intracellular

Shigella interact with and progress along stress fibres, a movement called organelle like movement (Olm) (Vasselon *et al.*, 1991). Olm has not yet been characterised at a molecular level. From the site of entry bacteria move toward the nucleus and start forming a micro-colony. Unlike the Ics movement described in detail below the bacteria do not become covered with F-actin. In CaCo-2 cells (a permanent cell line derived from human colonic carcinoma cells) the bacteria were observed to move along the actin filament ring of the peri-junctional area (Vasselon *et al.*, 1992).

Electron microscopy studies have allowed a detailed ultra-structural analysis of the protrusions seen in Ics movement. They have a diameter of about 0.5μm and a length of up to 20 μm (Kadurugamuwa *et al.*, 1991; Prévost *et al.*, 1992: Sansonetti *et al.*, 1994). These protrusions have been shown to extend from one cell into adjacent cells indicating that the protrusions can allow cell-cell spread of bacteria without release into the extracellular medium. The studies described below (Section 2.4.13) confirmed the importance of Ics in the dissemination of bacteria from the primary infected cell. *Shigella* become covered with polymerised actin within two hours of entering the cell, as incubation progresses they lose the coat and the tail begins to form at one pole. There is a striking similarity between this phenomenon and that seen in *Listeria monocytogenes* movement (Tilney and Portnoy, 1989; Mounier *et al.*, 1990) (section 2.7.20).

In addition to actin, several other cellular proteins, e.g. vinculin and plastin, but not myosin, are associated with the polymerised structure that trails behind intracellular bacteria (Kadurugamuwa *et al.*, 1991; Prévost *et al.*, 1992). Sansonetti *et al.* (1994) used a cell line that does not produce cell adhesion molecules and transfectants expressing L-CAM or N-cadherin, to show that cell adhesion molecules are required for the cell-cell spread of *Shigella*. Cadherin was shown to be important for the structural organisation of the protrusion and the internalisation of a protrusion by the adjacent cells.

2.7.13 *Shigella*invasion: The identification of genes and gene products involved in the invasion process.

The presence of a large plasmid of 200 kb is necessary for the expression of the invasive phenotype of *Shigella sonnei* (Sansonetti, Kopecko and Formel, 1981) and invasive isolates of *Shigella flexneri* (Sansonetti, Kopecko and Formel, 1982) and EIEC (Harris *et al.*, 1982; Sansonetti *et al.*, 1983a). Hybridisation studies showed a high degree of homology between the large plasmids of the invasive *Shigella spp.* and EIEC (Sansonetti *et al.*, 1983a). Furthermore, mobilisation of the large plasmid from *Shigella flexneri* to *E. coli* K12 gave rise to a recombinant strain that was able to invade HeLa cells (Sansonetti *et al.*, 1983b).

The use of minicell-producing strains of *Sh. flexneri, Sh. sonnei* and EIEC by Hale and co-workers (1983) led to the characterisation of 15-20 proteins encoded by the virulence plasmid. Minicells are small, spherical, nucleate cells which are produced continuously during the growth of certain mutant strains of bacteria (Old and Primrose, 1989).
Seven proteins were found to be common to *Sh. flexneri* and enteroinvasive *E. coli* 0143 by 2-dimensional electrophoresis and designated a-g (Hale *et al.*, 1985). Of these seven proteins, four were recognised by the sera from humans and monkeys convalescing from shigellosis (Oaks *et al.*, 1986). These four proteins and the genes coding for them have been designated as Invasion plasmid antigens (Ipa) (Buysse *et al.*, 1987) the molecular weights of these polypeptides are IpaA, (78 kDa), IpaB (62 kDa), IpaC (43 kDa), and IpaD (39 kDa).

Cloning of the genes required for the induction of phagocytosis by epithelial cells was first achieved in a strain of *Sh. flexneri* serotype 5a. Two strategies, cloning and transposon mutagenesis, were used to identify genes responsible for the invasive phenotype. A library of cosmids containing 45 kb DNA fragments of the pWR100 plasmid from *Sh. flexneri* 5 was introduced into a strain of *Shigella* lacking the virulence plasmid. The recombinant strains were screened for their ability to enter HeLa cells. A common region of about 37 kb was present in the cosmids that conferred the entry phenotype. This region allowed the expression

of the IpaA, IpaB, IpaC, and IpaD antigens (Maurelli *et al.*, 1985). Kato and coworkers (1989) used a similar strategy to identify the invasion region on the plasmid of *Sh. sonnei*. Sasakawa *et al.* (1986) isolated over 300 Tn*5* insertions in pMYSH6000, the virulence plasmid of *Sh. flexneri* 2a, and screened the mutants for their ability to invade LLC-MK2 cells. This led to the identification of a 30 kb fragment, the integrity of which was required for the expression of the invasive phenotype. The restriction map of this fragment was shown to be very similar to that of the entry region of *Sh. flexneri* 5a and *Sh. sonnei*. The combined effort of many workers in different laboratories has resulted in the elucidation of the complete nucleotide sequence of the 30.5 kb fragment that is necessary and apparently sufficient for the entry of *Sh. flexneri* into epithelial cells *in vitro* (Buadry *et al.*, 1988; Venkatesan *et al.*, 1988; Adler *et al.*, 1989; Sasakawa *et al.*, 1989; Buysse *et al.*, 1990; Venkatesan and Buysse, 1990; Allaoui *et al.*, 1992 a,b; Andrews and Muarelli, 1992; Venkatesan *et al.*, 1992; Sasakawa *et al.*, 1993). This fragment contains 33 genes clustered in two regions transcribed in opposite orientation.

The genes encoding the IpaA, IpaB, IpaC and IpaD proteins are clustered in a locus that comprises eight genes in the order *icsB, ipgA, ipgC ipaB, ipaC, ipaD* and *ipaA*. The transcriptional organisation of this locus was analysed by northern blotting and S1 nuclease protection experiments, cloning into a promoter probe vector and by studies of the effect of polar insertions on the expression of downstream genes. In addition to the *icsB* promoter, a promoter necessary for the full expression of the *ipa* genes was found upstream from *ipgB*, and internal weaker promoters were identified upstream from *ipgA* and *ipaD* (Baudry *et al.*, 1987; Venkatesan *et al.*, 1988; Sasakawa *et al.*, 1989; Allaoui *et al.*, 1992a).

The role of these genes in entry was first investigated using transposon insertion mutants constructed either on the large virulence plasmid or on cosmids that conferred entry (Maurelli *et al.*, 1985; Sasakawa *et al.* 1986; Baudry *et al.*, 1987; Watanabe *et al.*, 1990). Transposon insertion in *igaA, ipaB, ipaC,* and *ipaD*, but not in *ipaA*, abolished invasion of

epithelial cells. Complementation analysis using various recombinant plasmids indicated that *ipaB, ipaC,* and *ipaD* were involved in virulence, as assessed by the formation of plaques on epithelial cell monolayers, but that the upstream *ipg* genes were not (Sasakawa *et al.*, 1989). The *ipaB, ipaC* and *ipaD* genes carried on the large virulence plasmid of *Sh. flexneri* 5 have been inactivated by allelic replacement with a gene mutagenised *in vitro* by insertion of a nonpolar cassette (High *et al.*, 1992: Ménard *et al.*, 1993). Each mutant was unable to enter HeLa cells, this phenotype was complemented by recombinant plasmids carrying only a wildtype copy of the mutated gene. The three mutants were not impaired for adhesion to HeLa cells but were unable to induce actin polymerisation at the site of attachment of bacteria and host cell membrane.

Shigella lyses the membrane of the phagocytic vacuole shortly after internalisation and thus gains access to the cytoplasm. This membranolytic activity is reflected by the contact haemolytic activity expressed by invasive *Shigella* (Sansonetti *et al.*, 1986). The three mutants created by High *et al.* (1992) and Ménard *et al.* (1993) were nonhaemolytic suggesting that the three Ipa proteins are also involved in escape from the phagosome. Zychlinsky *et al.* (1994) confirmed the role of the IpaB, C and D proteins in the lysis of the phagocytic vacuole, using a macrophage cell line that allows internalisation of noninvasive bacteria; the *ipa* mutants remained trapped in the phagosome and were not cytotoxic.
The *ipa* mutants are defective in invasion but not in secretion of the remaining Ipa proteins, whereas *mxi* and *spa* mutants cannot secrete these proteins. Ménard and coworkers (1993) suggested that this implies a role for the IpaB, IpaC and IpaD acting together to induce phagocytosis as well as escape from the phagosome. However, the mode of action of the Ipa proteins still has to be elucidated.

The work by Hale and co-workers (1983) on the proteins expressed by the virulence plasmid indicated that several of them including the Ipa A, B, C and D antigens were associated with the outer membrane of the bacterium. This was confirmed by the reactivity

of monoclonal antibodies directed against IpaB and IpaC with whole bacteria in an ELISA (Mills *et al.*, 1988; Hromockyj and Maurelli, 1989). Secretion of IpaB and IpaC into the culture medium was shown by Andrews *et al.* (1991), and Allaoui *et al.* (1992b) demonstrated by SDS-PAGE and Coomassie blue staining of concentrated culture medium that virulent *Shigella flexneri* secretes ten polypeptides. Menard and co-workers (1993) showed that IpaA and Ipa D are also secreted.

A locus that contains many genes whose products are involved in the surface presentation and secretion of the Ipa proteins was identified by constructing non-invasive mutants, by transposon mutagenesis and allelic replacement, followed by characterisation of the phenotype of the mutants (Andrews *et al.*, 1991; Allaoui *et al.*, 1992b, 1993a; Venkatesan *et al.*, 1992; Sasakawa *et al.*, 1993). These genes were called *mxi* (**m**embrane **e**xcretion of **i**nvasion plasmid antigens) and *spa* (**s**urface **p**resentation of invasion plasmid **a**ntigens). Allaoui and coworkers (1992b) suggested that all the *mxi* and *spa* noninvasive mutants were deficient for both surface presentation and secretion of the four Ipa polypeptides as well as at least four other proteins. Since Ipa B, IpaC and IpaD are necessary for invasion, the noninvasive nature of the *mxi* and *spa* mutants is most likely a consequence of this deficiency.

The entire region containing the *mxi* and *spa* genes is designated the *mxi-spa* secretion locus. It contains twenty four genes organised in two operons (Andrews *et al.*, 1991; Tobe *et al.*, 1991; Allaoui *et al.*, 1993a; Sasakawa *et al.*, 1993). Complementation studies and construction of nonpolar mutants have indicated that several genes in this region such as *ipgD* (**i**nvasion **p**lasmid **g**ene), *ipgF, spa15* and ORF10, were neither involved in secretion or presentation of the Ipa proteins nor in the invasion of HeLa cells (Allaoui *et al.*, 1993a; Sasakawa *et al.*, 1993).

The localisation of some of the *mxi-spa* gene products has been proposed based on preliminary studies and sequence analysis. The NH_2-terminal sequences of IpgF, MxiD, MxiJ and MxiM have features that are characteristic of a signal sequence. MxiJ and MxiM were

shown to be lipoproteins by Allaoui *et al.* (1992b) and proposed to be anchored in the outer membrane by their NH_2-terminal lipid moiety. The COOH-terminal domain of MxiD has significant sequence similarities with the COOH-terminal moiety of both *Klebsiella oxytoca* PulD protein (d'Enfert *et al.*, 1989) and protein IV of filamentous bacteriophages (Peters *et al.*, 1985; Luiten *et al.*, 1985) and may be involved in the targeting of MxiD to the outer membrane (Allaoui *et al.*, 1993b). MxiA was detected in the inner membrane by Andrews *et al.* (1991) and subsequent sequence analysis suggested that the protein consists of two domains, an NH_2-terminal domain composed of six membrane spanning regions and a COOH-terminal domain located in the cytoplasm (Andrews and Maurelli, 1992). The presence of internal hydrophobic segments in Spa9, Spa15, Spa24, Spa29 and Spa40 suggests that these proteins might also be located in the inner membrane (Venatesan *et al.*, 1992; Sasakawa *et al.*, 1993).

2.7.14 *Shigella* invasion: The identification of genes involved in intracellular movement.

The gene *virG* was identified in plasmid PMYSH6000 of *Shigella flexneri* 2a 40kb away from the invasion region of the large virulence plasmid. Transposon insertion mutants of this gene were able to invade cultured cells but were unable to spread cell-cell (Makino *et al.*, 1986; Lett *et al.*, 1989). The gene *icsA* was identified by Bernardini *et al.* (1989) on plasmid PWR100 of *Sh. flexneri* 5, this *icsA* mutant was unable to induce protrusion formation and failed to induce the formation of actin filaments at the poles of bacteria seen with the wild type strain. Both *virK* and *icsA* genes are referred to as *icsA* below for reasons of clarity.

The *icsA* gene encodes a 130 kDa protein first detected in the outer membrane (Lett *et al.*, 1989; Bernardini *et al.*, 1989; d'Hauteville and Sansonetti, 1992). It has been shown that about 50% of IcsA is secreted into the culture medium as a 95 kDa cleavage product (Goldberg *et al.*, 1993). Sequencing of the NH_2-terminal region of the 95 kDa protein indicates that cleavage occurs after residue Ala-52, downstream from a region that has the

characteristics of a signal sequence. A polypeptide of 37 kDa, corresponding to the COOH-terminal part of the 130 kDa precursor has been identified by western blotting analysis of *Shigella* whole cell extracts (Nakata *et al.*, 1992, 1993).

Secretion of IcsA is independent of the *mxi* secretion apparatus and may not require determinants carried by the large virulence plasmid, since *mxi* and *spa* mutants (Allaoui *et al.*, 1992b, 1993b; Venkatesan *et al.*, 1992) and an *E. coli* strain carrying a recombinant plasmid expressing the *icsA* gene secreted IcsA.

Bacteria grown *in vitro* have surface bound IcsA at the distal poles of dividing cells (Goldberg *et al.*, 1993). This unipolar distribution is observed in intracellular bacteria after infection of HeLa cells (Goldberg *et al.*, 1993) and correlates with the unipolar reorganisation of F-actin seen at the surface of dividing bacteria (Prévost *et al.*, 1992). Furthermore, labelling with an anti-Ics antiserum indicated that IcsA was also present in the actin tail that trails bacteria in the cytoplasm and in the protrusions implicated in the cell-cell spread of bacteria (Goldberg *et al.*, 1993). This would seem to indicate that the surface-bound and the secreted form of IcsA interacts with either F-actin or actin associated protein(s). This sort of interaction was certainly proposed by Bernardini *et al.* (1989) based on the phenotype of *icsA* mutants unable to elicit accumulation of polymerised actin on the bacterial surface.

The cell-cell spread of *Shigella* appears to be via protrusions. These extend into adjacent cells and are engulfed by them. The bacteria within the protrusion are then surrounded by two membranes, that of the protrusion itself and the membrane of the engulfing cell. Only lysis of both these membranes will release the bacteria into the new cell thus completing the cycle of intercellular dissemination. Inactivation of the *icsB* gene, which is located in the invasion region of the virulence plasmid resulted in a *Shigella flexneri* strain that was invasive, and able to induce actin polymerisation and the formation of protrusions but unable to spread from cell to cell (Allaoui *et al.*, 1992a) as it remained surrounded by the two membranes. Since the bacteria were able to lyse the phagocytic vacuole during initial

entry but unable to spread from cell to cell different membranolytic activities are probably required to escape from the phagosome and the protrusion.

2.7.15 *Salmonella* invasion: the nature of the entry process.

In a seminal descriptive investigation Takeuchi (1967) found that *S. typhimurium* uniformly penetrated the intestinal epithelium, including both columnar epithelial cells and M-cells of the guinea pig. However, in 1986 Kohbata and coworkers reported that *S. typhi* preferentially invaded and destroyed M-cells in mouse ileum and Popischil *et al.* (1990) found that whilst *S. typhimurium* showed no predilection for any intestinal epithelial type in infected swine, *S. choleraesuis* was located preferentially in ileal M-cells within Peyer's patches. This data seems to suggest that *Salmonella spp.* can invade any intestinal epithelial cell type but that larger numbers enter through M-cells.

Takeuchi (1967) using transmission electron microscopy produced a detailed morphological description of *S. typhimurium* interacting with the intestinal epithelium of experimentally infected guinea pigs. This description typified the interaction of all *Salmonella* with non-phagocytic cells and has been documented by various workers on several occasions; in chick ileocecal mucosa (Popiel and Turnbull, 1985), in ligated murine ileal loops (Kohbata *et al.*, 1986) CaCo-2 polarised human epithelial cell line (derived from colonic carcinoma cells) (Finlay and Falkow, 1990), in HEp-2 cells (Francis *et al.*, 1992, 1993) and in MDCK cells (Galán *et al.*, 1993). Prior to initial bacterial contact with the epithelium the brush border remains intact, however, when the bacteria comes close to the epithelial surface (less than 3.5 μm), microvilli in the immediate vicinity begin to degenerate through elongation, swelling and budding. Often long structures are seen joining the bacteria to the apical surface of the cell although a space between bacteria and cell surfaces is always maintained. As the process continues, the apical membrane close to the bacteria begins to bleb and swell, distorting outwards. These distortions were called 'ruffles' by Francis *et al.* (1993) and were described a similar to the disturbances seen following stimulation by

hormones or oncogenic substances. As the cytoplasm is distorted, the bacteria is internalised within a membrane bound vesicle, often surrounded by the cytoplasmic bleb. Concomitant with this extrusion is a marked increase in localised endocytic activity, resulting in internalisation of many vesicular structures. Often many bacteria were seen to invade one cell whilst adjacent cells were uninfected. Takeuchi (1967) also reported seeing bacteria passing through the tight junctions between cells in the epithelium which apparently resealed after the bacteria passed. Initially each invading bacteria appeared to be enclosed in its own vacuole but at later times these sometimes appeared to fuse. The bacteria, unlike *Shigella,* do not escape from the vacuole but appeared to pass through the epithelium within vacuoles until they were released at the basolateral surface of the host cell (Finlay *et al.*, 1988b). This transcytosis was accomplished within two hours post infection (Finlay and Falkow, 1990). Since most invasive strains of *Salmonella* replicate in the reticulo-endothelial system rather than the intestine this transcytosis might have reasonably been expected. Finlay and Falkow (1988b) showed that *Salmonella choleraesuis* and *Salmonella enteritidis* can replicate within MDCK cells, where the generation time was about one hour and in CHO and Hep-2 cells where the generation time was somewhat slower.

2.7.16 *Salmonella* invasion: The identification of genes and gene products involved in the invasion process.

Several invasiveness loci have been identified in *Salmonella spp.* by several groups. Unfortunately, of those characterised, nearly all are involved in the regulation of invasion, secretion of products to the bacterial surface or motility. Elsinghorst *et al.* (1989) cloned a chromosomal region from *S. typhi* Ty2 that conferred the ability to enter human intestinal epithelial cells (Henle 407) on *E. coli* HB101. Three invasion-positive recombinant cosmids were isolated and restriction endonuclease analysis of the inserts showed a 33-kb region of identity. Transmission electron microscopy of Henle 407 monolayers infected with either *S. typhi* Ty2 or *E. coli* HB101 carrying an invasion cosmid showed intracellular bacteria

contained within endocytic vacuoles. One of the invasion cosmids was mutagenised with transposon Tn5 to identify the cloned sequences that were essential for invasive phenotype. Seven of 92 independent Tn5 insertions within the 33-kb region eliminated the invasive phenotype and revealed at least four separate loci that are required for invasion. Penetration of the epithelial cells was found to be inhibited by the use of cytochalasins B and D, indicating that endocytosis of *S. typhi* is a microfilament-dependant event. The invasion cosmids were found to contain both the *recA* and *srlC* genes indicating that the cloned determinants are located at about 58 minutes on the *S. typhi* chromosome. Using a segment of the cloned *S. typhi* invasion region as a probe, homologous sequences were isolated from *S. typhimurium*. Two independent *S. typhimurium* recombinant cosmids containing the entire 33-kb common region identified in *S. typhi* were isolated, but these cosmids did not confer upon *E. coli* HB101 the ability to invade epithelial cells.

Galán and Curtiss III (1989) reported the isolation of a genetic locus designated, *inv*, that conferred to a noninvasive strain of *S. typhimurium* the ability to penetrate tissue culture cells. A cosmid library of *S. typhimurium* DNA was constructed and an invasion deficient mutant strain DB4673, which was capable of adhering to Henle cells but incapable of penetrating them, was used as a recipient for the cosmid library. A cosmid clone was identified that conferred on DB4673 the ability to enter Henle cells as efficiently as its wild type progenitor. Introduction of this cosmid into *E. coli* HB101 did not confer the ability to invade. Subcloning of the invasive phenotype into plasmid PACYC184 yielded PYA2219. This plasmid was subjected to restriction endonuclease analysis and TN*phoA* mutagenesis to establish more precisely the regions of the locus that conferred the invasive properties. Insertions that completely abolished the invasive phenotype mapped to a region of 3.5 kb to the left of the insert, another group of insertions that reproducibly reduced the invasive phenotype by 5-fold mapped to a 1kb region at the right of the insert.

In vitro transcription/translation analysis of PYA2219 insertion and deletion mutants revealed that at least four genes designated *invA*, encoding a 54kDa protein, invB, encoding a 64 kDa protein, invC, encoding a 47 kDa protein and *invD*, encoding a 30 kDa protein, were involved in the invasive phenotype. The genes *invA*, *invB* and *invC* were arranged in that order in the same transcriptional unit and *invD* was located downstream of the *A,B,C* cluster in an independent transcriptional unit. An insertion mutant in the *invA* gene that eliminated expression of *invA*, *invB*, and *invC* was introduced into wild type strains and showed that the ability of these wild type bacteria to invade Henle 407 cells was reduced by 100 fold. An *in vivo* investigation of the effect of the *invA* mutant on pathogenesis in mice was conducted. The results showed that while there was no detectable difference in the intra-peritoneal LD_{50} for the wild type and mutant strains the per oral LD_{50} values were 50 times higher in the wild type compared with the mutant. Furthermore, tissue localisation of wild type and mutants differed as the mutants failed to colonise the Peyer's patches and the numbers of mutant bacteria detected in the spleen following oral infection were very much lower than in the wild type. If the mice were infected intra-peritoneally no differences between the numbers of wild type and mutant bacteria in the spleen could be detected. InvC has subsequently been characterised as a member of the family of proteins with homology to the β subunit of the F_0F_1 ATPase that is implicated in non-classical secretion of proteins through the membranes of bacterial pathogens of plants and mammals (Eichelberg *et al.*, 1994; Vogler *et al.*1991; Fenselau *et al.*, 1992; Venkatesan *et al.*, 1992).

Nucleotide sequence analysis of *invA* showed a single open reading frame (ORF) capable of encoding a protein of 686 amino acids with a predicted molecular weight of 75,974 Da. A protein of this size was observed when *invA* was expressed in a bacteriophage T_7 RNA polymerase expression system. The predicted protein displays the characteristics of an integral membrane protein. The amino-terminus is largely hydrophobic, with eight potential transmembrane domains, while the carboxy-terminal end is hydrophilic and most likely

located in the cytoplasm. This topology is supported by the location of the *invA-61*::Tn*phoA* productive fusion and also by the fact that a signal less PhoA fusion to the carboxy terminus yielded a hybrid protein with no significant enzymatic activity (Galán *et al.*, 1992a). *invA* shows significant similarity to a new family of bacterial proteins (Galán *et al.*, 1991; vanGijsegem *et al.*, 1993). This family includes *C. crescentus* FlbF (Ramakrishnan *et al.*, 1991), *Yersinia spp* LcrD (Plano *et al.*, 1991; Vitanen *et al.*, 1991; section 2.7.8), *Shigella* MxiA (Galán *et al.*, 1993) and *E. coli* FlhA (Galán *et al.*, 1993) proteins. The precise function of these proteins is not known but there are well-defined phenotypes associated with mutations in these genes. Mutations in *flbF* prevent the expression of a large number of *C. crescentus* flagellar genes (Newton, 1989; Ramakrishnan, *et al.*, 1991). Expression of these flagella genes is coordinately regulated in a complex hierarchy in which *flbF* is at or very near the top (Newton *et al.*, 1989). It has been suggested that FlbF may play a role in the signal transduction across the bacterial membrane that leads to transcriptional activation of other *fla* genes (Ramakrishnan *et al.*, 1991). *lcrD* is involved in the Ca^{2+}-response phenotype of *Yersinia spp.* (Plano *et al.*, 1991). This phenotype is characterised by the Ca^{2+}-dependent growth and expression of a number of outer membrane proteins (Yops). Mutations in *lcrD* renders *Y. pestis* Ca^{2+} independent and affects the expression of genes encoding a number of Yops, which are essential for the display of virulence and host invasion by this bacteria (Plano *et al.*, 1991; Straley and Bowmer, 1986). Recently, it was hypothesised by Plano *et al.* (1991) that LcrD may be involved in the sensing and/or transmembrane signalling of the environmental cues of Ca^{2+}. The *mxiA* gene of *Sh. flexneri* has been shown to be involved in the translocation of the Ipa proteins which are essential for the ability of this organism to penetrate intestinal epithelial cells (Galán *et al.*, 1993). The Ipa proteins are surface located although they lack a typical signal sequence. Mutations in *mxiA* prevent the membrane localisation of the Ipa polypeptides although they do not alter the expression levels of the *ipa* genes.This indicates that MxiA plays a role in the translocation apparatus of the *Shigella*

invasion proteins. Similarly, mutations in *flfA* affect the translocation of a number of *E. coli* flagellar proteins which are believed to be exported by a specific mechanism, since these proteins also lack a typical signal peptide (Vogler *et al.*, 1991; Galán *et al.*, 1993). Thus it appears that *invA* is a member of a new family of proteins that functions either to regulate the expression of other genes or the translocation of other proteins by a novel mechanism.

Ginocchio *et al.* (1992) characterised another gene from the *inv* region that was also unable enter Henle 407 cells. During the sequencing of the *invABC* operon of *S. typhimurium*, an ORF was identified immediately upstream of *invA*. To investigate the involvement of this gene in the invasive phenotype of *S. typhimurium*, a cassette containing the aminoglycoside phosphotransferase (*aph*) was introduced to the middle of the ORF. The mutant gene was then introduced into the chromosome of wild-type *S. typhimurium* SR11 by allele exchange. The correct position of the *aph* cassette was verified by Southern blot hybridisation. The resulting mutant, strain SB109, was tested for its ability to invade Henle 407 cells and was found to be severely impeded in its ability to invade tissue culture cells although not in its ability to attach to the same cells. This indicated that the ORF did indeed code for a protein required for invasion and this gene was called *invE*. It was necessary to investigate the possibility that the reduced invasive ability might be due to downstream effects following mutation of *invE* rather than the mutation of the gene itself. A plasmid was constructed that contained the *invE* gene but not the downstream sequences. If the downstream sequences were required for invasivness this plasmid would not restore the ability to invade. However, it was found that the complementation of *invE in trans* by this plasmid restored the invasive phenotype and thus showed that the gene product of *invE* was required for invasion. The *invE* gene was cloned into a T_7 directed expression system and the product was characterised and shown to have homology with YopN, a surface protein involved in the low-Ca^{2+} response of the invasive *Yersinia* and MxiC of *Shigella* (Sections 2.7.7 and 2.7.13). The distribution of *invE* amongst other *Salmonella* was tested by Southern Hybridisation analysis and was found

to be present in all 91 isolates of the 37 serotypes tested. As discussed in section 2.7.17 below Ca^{2+} is an important intracellular messenger in eukaryotic cells. Wild-type *Salmonella typhimurium* appears to invoke increases in the intracellular levels of Ca^{2+} ions very quickly after initial exposure to the cells. The *invE* mutant failed to evoke this response even after 60 minutes. The *invE* mutants also failed to elicit any of the disruption of the microvillus brush border of MDCK cells following attachment. The *invE* mutants were internalised if the tissue culture cells were infected with equal numbers of mutant and wild type bacteria. The wild-type appears to be able to induce the internalisation of the *invE* mutants, in contrast *E. coli* RDEC-1 was not internalised by Henle cells when it was introduced with equal numbers of wild type *S. typhimurium*.

Interestingly the gene *invH* was discovered at the same time by different groups working on different organisms using different methodologies (Altmeyer *et al.*, 1993). A number of *S. choleraesuis* Tn*phoA* insertion mutants defective in invasion were identified by Finlay *et al.* (1988a). Among these mutants, a group was found (class 6a) that exhibited a significant decrease in their ability to invade cultured epithelial cells. Class 6a mutants mapped to a small region on the *S. choleraesuis* chromosome. An 8kb *Sal*1 fragment from the class 6a mutant 284c, containing the kanamycin resistance gene of tn*phoA* and additional flanking sequences, was cloned into the multicopy vector PKS^- yielding p284c. This plasmid was then used to probe a cosmid gene bank from *S. choleraesuis* SL2824. Of the 650 cosmid clones that were screened 3 separate clones exhibited homology to this probe, sharing an 11 kb *Sal*1 fragment. This 11 kb fragment was subcloned and a detailed restriction map of the cloned region was obtained. The nucleotide sequence of the *S. choleraesuis* DNA surrounding the insertion was sequenced, and only one significant open reading frame was found in the 1000bp sequence. The ORF encodes a 147 amino-acid polypeptide with a predicted molecular weight of 16 452 Da. This polypeptide is virtually identical (only three substitutions in the entire sequence) to the predicted product of a *S. typhimurium* open reading

frame located upstream of *invF* and *invG* two recently identified *S. typhimurium* invasion genes (Kaniga and Galan, 1993), this gene was called *invH*. As with *invA* an insertion mutation was introduced into the middle of *invH* by cloning a cassette containing the *aph* gene into a *Bgl*II site created by site directed mutagenesis. The inactivated gene was recombined by allele exchange into the chromosome of a number of *Salmonella* serotypes and the correct position of *aph* was checked by Southern hybridisation. The resulting strains were assayed for their ability to adhere to and invade cultured epithelial cells. *invH* mutants of *S. typhimurium, S. gallinarum, S. enteritidis* and *S. typhi* were defective in their ability to invade cultured Henle 407 cells. *S. typhimurium* and *S. enteritidis* were the least affected by the *invH* mutation (approximately a 5-fold reduction) whilst the host specific *S. typhi, S. choleraesuis* and *S. gallinarum* were the most affected (approximately a 15-fold decrease). *invH* mutations also rendered most of the *Salmonella* strains deficient in their ability to adhere to the Henle-407 cells. Adherence was most affected in *S. gallinarum* but *invH* mutants of *S. typhi* were not adherence defective. The role of *invH* in the invasion process and the reason for the host range effects are unclear. Since adherence was affected it may be that mutation in *invH* affects invasion by virtue of its role in adhesion. However both the linkage of this gene to the *inv* locus and the ability of *S. typhi invH* mutants to bind to the tissue culture cells and yet be unable to invade them suggests a function specific to invasion. Possibly the gene product may help in docking *Salmonella* in the appropriate place on the cell surface to trigger internalisation. Mutation in other genes of the *inv* locus have affected the ability to invade without affecting adherence to host cells (Galán and Curtiss III, 1989, 1990; Galán *et al.*, 1992a). This suggests that *Salmonella* have a number of mechanisms that can bring the organisms in critical proximity to the eukaryotic cell membrane to allow other gene products to trigger the internalisation event. This idea is supported by the evidence that only a restricted group of *Salmonella* serotypes was significantly affected by the *invH* mutation. Possibly the broad host range *Salmonella* serotypes have a larger variety of adhesion

mechanisms capable of compensating for the deficiency in *invH* (Altmeyer *et al.*, 1993). Unlike mutations in the *invABCDE* genes complementation of the *invH* gene *in trans* with plasmids containing either the *invH* gene or even the whole *inv* region failed to fully restore the invasive phenotype. This may be due to deficient expression of *invH* when present on a plasmid where putative regulatory factors may be inactive or superhelical density of the DNA may not be appropriate (Section 2.8.1 below deals with the factors that may control expression of the invasive phenotype). InvG is a member of the PulD family of proteins, some of which have been shown to be involved in phage assembly or the export of the proteins that lack typical signal peptides that are found in several plant and mammalian pathogens (Kaniga and Galán, 1993; Pugsley, 1993; Gough *et al.*, 1993).

Using low voltage high resolution scanning electron microscopy (LVSEM), Ginocchio *et al.* (1994) were able to show that on coming into contact with the brush border of MDCK cells wild type *Salmonella typhimurium* produce transient surface appendages. These structures were seen only if the bacteria were exposed to the host cells and were apparently lost prior to the start of internalisation. Four *Salmonella typhimurium* strains carrying mutations at *invA, invC, invE* or *invG* were exposed to MDCK cells and examined via LVSEM, the *invA* and the *invE* mutants produced appendages on contact with the host cell but they were much longer than those seen in the wild type bacteria and were not lost, neither of these mutants were internalised. The *invG* and *invC* mutants never produced any appendages and were also not internalised. This data would suggest that not only is the production of the correct form of appendage necessary for internalisation but also that the appendages must be lost or retracted prior to the start of endocytosis. It has been shown that *de novo* protein synthesis is not required for *Salmonella* invasion when organisms are grown under conditions that promote the expression of the invasive phenotype (Lee and Falkow, 1990; MacBeth and Lee, 1993). The addition of chloramphenicol under conditions known to immediately inhibit protein synthesis in *Salmonella typhimurium* (MacBeth and Lee, 1993)

did not affect the production of appendages by the wild-type bacteria or the *invA* or *invE* mutants. This suggests that the structural components of the appendages are already available in the bacterial cell prior to contact with the host cell and that the interaction between host and pathogen provides some sort of signal that induces the assembly of the appendages (Ginocchio *et al.*, 1994).

The number of genes required for *Salmonella* invasion is large. However results indicate that many of the bacterial products required for invasion are similar to virulence factors from other bacteria. This has enabled workers to suggest roles for some of the *Salmonella* gene products. In addition to the similarities of the *invA* product to other bacterial proteins Groisman and Ochman (1993) found at least 9 predicted open reading frames downstream of *invB*. These genes share significant homology with the *Sh. flexneri spa* genes which, as described in section 2.7.13 are required for secretion of the Ipa proteins.

2.7.17 *Salmonella* invasion: The molecular basis of changes in host cell signal transduction pathways.

Membrane ruffling, cytoskeletal rearrangements, and intracellular free Ca^{2+} fluxes occur as part of a global cellular response to mitogens, oncogene expression and growth factors (Bar-Sagi and Ferasmico, 1986; Kadowaki *et al.*, 1986; Miyata *et al.*, 1989). The induction of membrane ruffles is critical for entry of *S. typhimurium*, since mutants unable to induce ruffles are severely impaired in their entry of cultured mammalian cells (Galan *et al.*, 1992b; Ginocchio *et al.*, 1992). In addition membrane ruffling induced by wild type *S. typhimurium* can recruit adherent but noninvasive derivatives of *S. typhimurium* into mammalian cells (Galan *et al.*, 1992b; Ginocchio *et al.*, 1992). The appearance of ruffles in the membrane is accompanied by cytoskeletal rearrangements of profound proportions at the point of bacterial host cell adherence. Furthermore, a number of cytoskeletal proteins, including actin, α-actinin, talin, tubulin, tropomyosin and ezrin accumulate at these sites (Finlay *et al.*, 1991). Whilst the function of all of these proteins in the ruffling of the

membrane is unclear, actin is important in the internalisation of *Salmonella* as inhibitors of actin polymerisation block bacterial entry (Kihlstrom and Nilsson, 1977; Buckholm, 1984; Finlay and Falkow, 1988b).

The increase in the concentration of intracellular Ca^{2+} ions is necessary for internalisation, since chelators and antagonists of Ca^{2+} block *Salmonella* entry into cultured cells and bacteria that are defective for entry do not produce a Ca^{2+} ion increase (Pace *et al.*, 1993). A number of actin bundling proteins such as gelsolin, villin or fimbrin can become actin-severing proteins upon changes in Ca^{2+} ion concentration (Mooseeker, 1985). This could result in the depolymerisation of the subcortical actin and the microfilaments that serve as the structural support for the microvilli, thereby providing free actin monomers for the formation of new cytoskeletal structures necessary for membrane ruffling.

Membrane ruffling, cytoskeletal rearrangements and Ca^{2+} fluxes have also been observed as a consequence of the activation of host cell surface receptors, including the epidermal growth factor receptor (EGFR) (Kadowaki *et al.*, 1986; Moolenar *et al.*, 1984; Rijken *et al.*, 1991). Interestingly, infection of Henle-407 cells by *S. typhimurium* is accompanied by the activation of the EGFR (Galan *et al.*, 1992b). The EGFR is not activated in Henle-407 cells infected with a *S. typhimurium* mutant that is defective for entry, and compensation for this entry defect occurs upon the addition of epidermal growth factor to the infected cells (Galan *et al.*, 1992b). Noninvasive strains of *E. coli* and inert particles such as polystyrene beads may passively enter cells via the membrane ruffles induced by *S. typhimurium* or epidermal growth factor. Nevertheless, this process occurs at a lower rate than for the phenotypic rescue observed for noninvasive *S. typhimurium* (Galan *et al.*, 1992b, Ginocchio *et al.*, 1992). It appears that *S. typhimurium* has subverted this host cell signalling pathway to gain entrance into these cells and that stimulation of the EGFR is one of the earliest steps in the process of internalisation (Bliska *et al.*, 1993).

Subsequent studies have unravelled the signal transduction pathway that follows EGFR activation by *S. typhimurium* in Henle-407 cells and that leads to bacterial entry (Pace *et al.*, 1993). *S. typhimurium* infection leads to the phosphorylation and activation of the mitogen activated protein kinase, which in turn activates phospholipase A_2 (PLA_2). The activation of PLA_2 is required for invasion since its inhibition abrogates entry of *S. typhimurium*. As a result of PLA_2 activity, arachidonic acid is produced and ultimately converted into the leucotrine D_4 (LTD_4) by a variety of enzymes. LTD_4 activates a Ca^{2+} channel, causing an influx of Ca^{2+}, which may induce membrane ruffling. This pathway is consistent with the fact that noninvasive *S. typhimurium* mutants, which are unable to stimulate the EGFR, are internalised by the addition of LTD_4 to the cells (Pace *et al.*, 1993). Although this signalling cascade provides the physiological basis for the Ca^{2+} influx, it neither explains the localised nature of the internalisation event nor provides a direct explanation for the mechanisms underlying the formation of the membrane ruffles and cytoskeletal structures required for internalisation. However, several possible mechanisms that are consistent with this signalling pathway may account for these events. Activation of the EGFR is known to stimulate the translocation of phospholipase C-γ (PLCγ) to the membrane. The translocation of this and possibly other phospholipases may contribute to the reorganisation of the cytoskeleton by releasing profilin from its membrane bound form (Goldschmidt-Clemont *et al.*, 1991; Pace *et al.*, 1993). Profilin that has been shown to increase actin polymerisation, could then participate in forming new cytoskeletal structure required for bacterial entry (Pring *et al.*, 1992). Additional possible elements necessary for this event may include a number of cytoskeletal proteins known to be substrates of the EGFR, such as ezrin, as well as the GTP-binding proteins Rac and Rho, which have been shown to be involved in growth factor-induced membrane ruffling and cytoskeletal rearrangements in cultured cells (Ridley and Hall, 1992; Ridley *et al.*, 1992). Similarly, Ras may also play a role in this process, since microinjection of activated forms of this protein into cultured human fibroblasts causes

membrane ruffling and micropinocytosis (Bar-Sagi and Feramisco, 1986). Interestingly this effect is associated with PLA_2 activity, which has been shown to be necessary for *Salmonella* entry into Henle-407 cells (Pace *et al.*, 1993).

S. typhimurium can enter into a number of cultured mammalian cell lines, some of which do not express the EGFR (Galan *et al.*, 1992b) One interpretation of these results is that *S. typhimurium* can stimulate more than one signal transduction pathway to promote entry into mammalian cells. For example, it has been shown in infections of HeLa cells that the intra-cellular concentration of inositol triphosphate rises during bacterial entry. Presumably this is because of activation of PLC-γ (Ruschkowski *et al.*, 1992) and that this activation is essential for bacterial internalisation (Bliska *et al.*, 1993).

It is not known how *S. typhimurium* triggers alternative signalling pathways. It seems unlikely that *Salmonella* encodes multiple determinants capable of activating multiple signalling receptors. More likely, *S. typhimurium* could encode a molecule(s) capable of interacting with moieties such as carbohydrate modifications that are common to many eukaryotic receptors. The distribution or abundance of receptors on the surface of a mammalian cell would then dictate the specific signal transduction pathway used by the bacteria. A number of lectins, such as wheat germ haemagglutinin or concanavalin A, have been shown to induce mitogenic responses in a variety of cell types (Johnson *et al.*, 1992; Sjolander, 1988; Sjolander and Magnusson, 1988; Slomainy *et al.*, 1992). This might occur by lectin-mediated clustering of glycoprotein transmembrane receptors (Bliska *et al.*, 1993).

2.7.18 *Listeria monocytogenes* invasion: the nature of the entry process.

The importance of the oral route of infection in natural infection with *L. monocytogenes* has been emphasised by the recent outbreaks of listeriosis due to the consumption of contaminated foodstuffs. MacDonald and Carter (1980) suggested that *L. monocytogenes* specifically penetrates the M-cells overlying the Peyer's patches. They observed that *L. monocytogenes* could be cultured from the Peyer's patch-containing intestinal

mucosa of mice following intragastric infection but not from intestinal mucosa from which the Peyer's patches had been removed (MacDonald and Carter, 1980; Marco *et al.*, 1992). However, the penetration of *L. monocytogenes* into intestinal epithelial cells *in vivo* was observed by electron microscopy (Racz *et al.*, 1970, 1972, 1973). Thus, entry into the host may occur via different cell types including epithelial cells and the M-cells. Indeed, *in vitro* studies have shown that *L. monocytogenes* can penetrate and multiply within various epithelial and fibroblast-like cells (Cossart and Mengaud, 1989).

Analysis of *Listeria*-infected cell cultures has revealed a complex series of host-pathogen interactions culminating in the direct dissemination of *L. monocytogenes* from one cell to another (Gaillard *et al.*, 1987; Tilney and Portnoy, 1989; Mounier *et al.*, 1990; Tilney and Tilney, 1993). Host cell infection begins with phagocytosis, in macrophages, or by induced phagocytosis, for nonphagocytic cells. The bacteria are rapidly incorporated into a membrane-bound vacuole that they lyse after about 20 minutes. In the cytoplasm the bacteria multiply with a doubling time of about one hour (Gaillard *et al.*, 1987) and become associated with actin filaments. After about two hours, these filaments are rearranged into tails that mediate bacterial movement through the cytoplasm to the cell periphery. This movement is rapid reaching speeds of about 1 μm/s (Dabri *et al.*, 1990); and is independent of known cellular motor molecules like myosin II. Measurements of the rate of actin tail formation suggest that the force for propulsion is provided by the actin tail polymerisation itself (Sanger *et al.*, 1992; Theriot *et al.*, 1992). When the moving bacteria contact the plasma membrane they induce the formation of pseudopod like projections of the membrane. Contact between these protrusions and adjacent cells results in the internalisation of the bacteria containing projection. In the newly infected cell the bacterium is surrounded by two plasma membranes that must be lysed to initiate a new cycle of multiplication and movement. Thus once *Listeria* is within the cytoplasm of one cell it can spread directly from cell to cell circumventing host defences such as antibody and complement (Tiltney and Portnoy 1989; Sheehan *et al.*, 1994).

This ability probably provides an explanation for the importance of cell-mediated rather than humoral immunity in *L. monocytogenes* infection.

2.7.19 *Listeria monocytogenes* invasion: The identification of genes and gene products involved in the invasion process.

To date two surface proteins have been implicated in the invasion of epithelial cells by *L. monocytogenes*, they are internalin and p60. Internalin is a surface protein of approximately 90kDa, and is encoded by the gene *inlA* (Gaillard *et al.*, 1991). Internalin was identified by screening a library of Tn1545 mutants of *L. monocytogenes* for the loss of invasive phenotype in the intestinal cell line CaCo-2. Three such mutants were obtained. These were unable to adhere to CaCo-2 cells and were defective for entry in a variety of cell lines (Gaillard *et al.*, 1991). In all mutants the transposon had inserted into a region upstream from two ORFs *inlA* and *inlB*. Transcription of these genes was abolished in the noninvasive mutants. *inlA* encodes an 800 amino acid polypeptide whose characteristic features include a signal sequence that was only detected by Dramsi *et al.* (1992) following an earlier sequencing error, two regions of repeats one of which is rich in leucine residues and a carboxy terminal region that is hydrophobic and may be a membrane anchor (Gaillard *et al.*, 1991). The first region of repeats (region A) is made up of 15 highly conserved successive repeats of 22 amino acid residues that display a periodicity of hydrophobic residues and have the consensus sequence 1-NQISDITPL..LTNL..L.L.-22 (where dots represent any amino acids) The second region of repeats (region B) is formed by three successive repeats, the first two of 70 amino acids each and the third of 49 amino acids. In the region common to the three repeats, the same amino acid is found in 27 out of 49 positions. Region B contains no periodicity of nonpolar residues and is dissimilar to region A. The putative anchor is the hexapeptide LPTTGD, considered to be a signature of Gram-positive proteins (Fischetti *et al.*, 1990). When *inlA* is expressed in *L. innocua* it confers invasivness on this otherwise noninvasive nonpathogenic *Listeria* species. Thus, in the genetic background of a closely

related *Listeria* species, internalin expression is sufficient to promote the entry into epithelial cells. Dramsi *et al.* (1992) showed that internalin can be released into the supernatant and that maximum invasion is obtained with exponential cultures that corresponds to the maximal expression of internalin in association with the cell wall. These results provide good evidence that it is the cell wall associated form of internalin that is very important in the invasive phenotype but cannot rule out a role for the released form. The gene *inlB* is located downstream from and is cotranscribed with *inlA*. The *inlB* gene product is a 620 amino acid polypeptide which is structurally analogous to internalin except that there is no hydrophobic COOH-terminal region. No role in invasion has been demonstrated for this gene product (Sheenan *et al.*, 1994).

Low stringency Southern blot analysis demonstrated that several other DNA sequences with homology to *inlA* are present on the chromosome of *L. monocytogenes* and all *Listeria* species (Gaillard *et al.*, 1991). Five other genes with homology to *inlA* (in addition to *inlB*) have now been cloned and sequenced (Sheenan *et al.*, 1994). The role of this multi gene family is now under investigation with the hypothesis that the different *inl* genes may encode surface proteins with different cellular trophisims. Internalin and the proteins of the internalin family are structurally analogous to a number of repeat proteins from Gram positive bacteria (including the F and M proteins of *Streptococcus pyogenes* and the fibronectin-binding proteins of *Staphylococcus aureus*) which are involved in host cell contact and recognition. (Dramsi *et al.*, 1992a; Westerlund and Korhonen, 1992).

Spontaneously occurring mutants of *L. monocytogenes* that produce greatly reduced amounts of p60, the other protein implicated in invasion, display a rough colony morphology and reduced adherence and invasivness in some cell types (Kuhn and Goebel, 1989). These bacteria form long chains in which the bacterial cells are separated by a double septa. The gene encoding p60 *iap* has been cloned, it encodes a protein of 484 amino acid residues with a signal sequence but no further hydrophobic sequences that might serve as membrane

spanning domains (Bubert, 1992; Köhler *et al.*, 1990, 1991). About 75% of p60 is found in the culture supernatant with the remaining 25% found associated with the cell wall (Ruhland *et al.*, 1993). The protein p60 has bacteriolytic activity and on the basis of amino acid sequence homologies, is thought to possess a murein hydrolase activity required for a late step in cell division (Wuenscher *et al.*, 1992). It is an essential protein and complete deletions of p60 are lethal. It remains to be shown whether the effect of its reduced expression on adherence can be explained by the altered morphology of the p60 defective mutants.

Among invasive bacteria, the invasin of *Yersinia pseudotuberculosis* (section 2.7.7) is the only protein to be demonstrated to mediate bacterial invasion of non-phagocytic cells by itself (Falkow *et al.*, 1992). As was discussed above (sections 2.7.13 and 2.7.16) the invasion of *Shigella* and *Salmonella* is clearly multifactorial. Internal is the only invasion factor identified in Gram-positive bacteria. It remains to be established if internalin can by itself mediate bacterial entry of eukaryotic cells or whether it forms a complex with other bacterial components such as the *inlB* gene product or the proteins encoded by the genes of the internalin superfamily (Sheenan *et al.*, 1994).

Following internalisation *L. monocytogenes* escapes from the endocytic vacuole and enters the cytoplasm where it replicates rapidly. Listeriolysin (LLO) is a 58 kDa secreted protein which was the first virulence factor identified in *L. monocytogenes*. It is a member of the thiol-activated cytolysins, of which streptolysin O is the prototype (Smyth and Duncan, 1978). These haemolysins share immunological cross-reactivity and are irreversibly inactivated by cholesterol which is their putative membrane receptor the work by Kehoe's group on these protein toxins was discussed above in section 2.4.7. The lytic activity of these toxins *in vitro* is a two step process, the temperature independent bind of the toxin to the membrane followed by an oligomerisation process leading to pore formation and membrane lysis (Sheenan *et al.*, 1994).

The role of LLO in lysis of the phagosomal membrane was established by genetic analysis of non-Haemolytic mutants, transposon insertions in the LLO structural gene *hly* resulted in production of truncated and inactive proteins (Gaillard *et al.*, 1986; Kuhn, Kathariou and Goebel, 1988; Portnoy, Jacks and Hinrichs, 1988; Cossart andMenguard, 1989) *hly* was shown to be able to restore the wild type phenotype when introduced into LLO defective mutants (Cossart and Menguard, 1989; Mengaud *et al.*, 1989). Electron microscopy studies showed that LLO defective strains were still able to enter cell of a human intestinal epithelial cell line (CaCo-2) but remained within the phagosome and could not get access to the cytoplasm and were thus unable to grow intracellularly (Gaillard *et al.*, 1987). In all cases the virulence of the LLO mutants was severely affected with an increase in LD_{50} of five orders of magnitude. Revertants were obtained and recovery of haemolytic phenotype was correlated with a recovery of virulence.

An elegant experiment using recombinant DNA techniques was preformed that provided yet more evidence of the importance of the *hly* gene. The LLO structural gene was cloned into the noninvasive soil bacterium *Bacillus subtilis* and expressed under the control of an inducible promoter (Bielecki *et al.*, 1990). In the presence of IPTG, this recombinant bacteria exhibited haemolytic activity and following internalisation by a macrophage cell lines, lysed the phagosomal membrane and grew rapidly and extensively in the cytoplasm. In the absence of IPTG the bacteria stayed within the vacuoles where they could survive several hours but could not replicate.

Kathariou *et al.* (1988) used isogenic mutants affected in single amino acid residues in LLO to show a direct correlation between virulence and haemolytic activity, but failed to show correlation between the haemolytic activity of various *L. monocytogenes* strains and their virulence.

LLO and a similar molecule made by *L. ivanovii* are the only known examples of thiol activated cytolysins produced by intracellular pathogens, furthermore, unlike all other thiol-

activated haemolysins, LLO has an acidic pH optimum and is relatively inactive at neutral pH (Geoffroy *et al.*, 1987). These properties may indicate an adaptation of the bacteria to provide maximum haemolytic activity in the acidic pH of the phagolysosome. However, studies with a recombinant strain of *B. subtilis* carrying an IPTG regulated perfringolysin O activity (Portnoy *et al.*, 1992) showed that this strain was far more cytotoxic than the similar strain carrying the LLO gene. Since perfringolysin O is not intended to be produce intracellularly this indicates that the acid pH optimum of LLO may be an adaptation to limit damage to the host cell after escape from the phagolysosome.

2.7.20 *Listeria monocytogenes* invasion: The identification of genes and gene products involved in intercellular spread.

The ability of *L. monocytogenes* to spread within host tissues direct from cell to cell, constitutes an essential pathogenicity determinant. It is reflected by the pathogen's ability to form plaques, ie, zones of destroyed cells on fibroblast monolayers that are covered with a bactericidal overlay prevent extracellular spread and multiplication (Havell, 1986). *L. monocytogenes* unable to form plaques also show strongly reduced virulence in mice, even though these mutants still invade cells, multiply in the cytoplasm and even to replicate transiently in the spleen and liver of infected mice (Kuhn *et al.*, 1990; Goossens and Milon, 1992; Kochs *et al.*, 1992; Dormann *et al.*, 1992). To date three genes have been implicated in the cell to cell spread of *L. monocytogenes*, they are *mpl*, *actA* and *plcB*. These genes are part of the lecithinase operon that is located downstream from the *hly* gene on the chromosome and includes downstream from *plcB*, three small ORFs of unknown function (Vazquez-Boland *et al.*, 1992).

Lecithinase production is a known phenotypic characteristic of *L. monocytogenes* (Fuzi and Pillis, 1962). To characterise the role of lecithinase in the pathogenesis of *L. monocytogenes*, transposon-induced, lecithinase-negative mutants were analyzed. One mutant was strongly affected in virulence and defective in plaque formation due to the inability to

polymerise actin (Kocks *et al.*, 1992). The transposon mutant mapped to *actA*, the second gene in the lecithinase operon. Plasmid insertion mutagenesis in the downstream genes showed that the insertion in *actA* was solely responsible for the lack of actin filament assembly (Kocks *et al.*, 1992) and that the lecithinase-negative phenotype of the *actA* mutant was due to a polar effect of the transposon insertion on transcription of *plcB*. Thus actin polymerisation by *L. monocytogenes* is dependant on the expression of *actA* (Domann *et al.*, 1992; Kocks *et al.*, 1992).

The *actA* product is a surface protein with an apparent molecular weight of 90 kDa that migrates aberrantly on SDS-PAGE gels. The protein is thought to be anchored in the bacterial membrane by its COOH-terminal end since insertion mutants in the coding region lead to secretion of nonfunctional truncated molecules that are not associated with the cell wall Domann *et al.*, 1992; Kocks *et al.*, 1992). At least two-thirds of the molecule protrudes from the cell wall with the potential to interact with components of the cytoskeleton (Kocks *et al.*, 1992). In addition to the membrane associated ActA some ActA can be found in the culture medium (Domann *et al.*, 1992; Niebuhr *et al.*, 1992). Searches of sequence data banks failed to reveal striking similarities between ActA any Known proteins. Nevertheless, weak similarities could be found to the actin-binding protein caldesmon and to human microtubule-associated protein 4 (Kocks *et al.*, 1992). The central part of ActA was shown to contain several proline- and glutamic acid rich repeats which share similarity to a region of vinculin that is rich in proline, aspartic and glutamic acids. The ActA repeat motif also harbours several consensus phosphorylation sites for a cellular protein kinase, casein kinase II.

The mechanism by which ActA mediates actin assembly is not known (Cossart and Kocks, 1994). In order to generate forward propulsion, it would be expected that the actin filament formation would have to be initiated on the bacteria in an asymmetric way. This is indeed the case, ActA is asymmetrically expressed during cell division, resulting in absence of the molecule from the newly formed end and abundance at the other older end (Kocks *et*

al., 1992). ActA has not been detected in the actin tails suggesting that it triggers the actin polymerisation process either by directly interacting with the actin or indirectly by inducing actin polymerisation through a cellular nucleator (Theriot and Mitchson, 1992; Theriot *et al.*, 1994). In the cytoplasm ActA gets phosphorylated by an as yet unidentified host cell derived kinase suggesting that this may be the mode of activation (Brundage *et al.*, 1993).

The gene *plcB* is located downstream from *actA* and encodes the *L. monocytogenes* lecithinase PC_PLC (Vazquez-Boland *et al.*, 1992). This enzyme catalyses the hydrolysis of a broad spectrum of phospholipids (Geoffroy *et al.*, 1991; Goldfine *et al.*, 1992). Through analysis of a *plcB* insertion mutant, evidence has been obtained that this enzyme contributes to the breakdown of the double membrane that surrounds *L. monocytogenes* after cell-cell spread (Vazquez-Boland *et al.*, 1992). On fibroblast monolayers *plcB* mutants produced far smaller plaques than wild type *L. monocytogenes* and electron microscopy showed that these mutants tended to accumulate in two membrane vacuoles in the cytoplasm of newly infected cells. These results suggest that PCPLC and LLO are both important in the cell to cell spread phenotype.

Lecithinase is secreted as a 22 kDa inactive precursor polypeptide that can be detected as a double band on western blots (Geoffroy *et al.*, 1991; Niebuhr *et al.*, 1993). Genetic evidence suggests that the predicted *mpl* gen product, a 57 kDa polypeptide with a signal sequence and propeptide sharing homologies with metalloproteases from other bacterial pathogens (Domann *et al.*, 1991; Mengaud *et al.*, 1991), cleaves the PC-PLC proenzyme in *L. monocytogenes* broth cultures resulting in the mature active 29 kDa form of the phospholipase (Raveneau *et al.*, 1992; Niebuhr *et al.*, 1993; Poyart *et al.*, 1993). It is not clear whether cleavage takes place at the bacterial surface or in solution.

Temm-Grove *et al.* (1994) analysed the actin filament 'comet' tail induced by *L. monocytogenes*. They used the PtK_2, LLC-PK1 and PK_{15} cells of these only LLC-PK1 is capable of reconstructing a polarised epithelium-like brush border that requires the synthesis

of villin (Temm-Grove *et al.*, 1992). The infected cells were treated with fluorescently labelled phalloidin for the detection of actin filaments and specific fluorescently labelled antibodies raised against various protein components of the cytoskeleton. In common with the results of other earlier studies (Dabiri *et al.*, 1990; Sanger *et al.*, 1992), alpha-actinin was seen to colocalise with the actin in the tail and in the actin coat around the bacterial cells, this suggest that alpha-actinin is incorporated into the actin filaments at an early stage in polymerisation. Fimbrin was seen to colocalise with actin in a manor very like alpha-actinin this had previously described by Kocks and Cossart (1993). Interestingly, fimbrin did not appear to be present in the distal end of the tails in any great quantity suggesting that it is lost as the tail ages and raises the possibility that the loss of fimbrin proceeds depolymerisation.

The distribution of ezrin/radixin, members of the family of highly related proteins (the ERM proteins; ezrin, radixin, moesin; (Arpin *et al.*, 1994)), was also analysed in *Listeria* infected cells. Under normal conditions these proteins are restricted to the cortical cytoskeleton and localised in cellular protrusions, such as microvilli, lamellopodia and microspikes (Bretscher, 1982, 1991; Arpin *et al.*, 1994). In *Listeria* infected PK_{15} cells ezrin/radixin was found around the bacterial cells and along the entire length of the actin tails. Like fibrin villin is an actin filament bundling protein that is found exclusively in cellular protrusions. however, is normally only found in the microvilli at the brush border and is not present in any other form of protrusion (Robine *et al.*, 1985). Following infection of LLC-PK1 cells with *L. monocytogenes* villin was found incorporated in the actin filament layer surrounding the bacterial cell wall and in the tail structures. Villin is expressed by specialised epithelial cells *in vivo* and is not always expressed by the tissue culture cells used to investigate *Listeria motility* (Robine *et al.*, 1985). Thus, its involvement in intracellular motility is obviously not vital. Nevertheless, its incorporation into the motile machinery following infection of cells capable of producing it means that the tails of these bacteria contain a complement of actin and actin binding proteins normally only seen in microvilli

(Temm-Grove *et al.*, 1994). Although, there are obvious differences since microvilli do not contain alpha actinin and their core filaments are longer and more tightly packed than the rather short and more loosely arranged actin filaments that compose the *Listeria* tails.

L. monocytogenes is a very efficient parasite. A large number of cell types have been shown to be susceptible to infection, and in all these cases the recruitment of the microfilament system seems to be identical. Thus it is reasonable to assume that the bacteria uses the host cells proteins, and copies its mechanisms for microfilament generation.

2.8 Coordination and control of virulence.

A bacterial pathogen colonises its host for its own benefit. It may colonise the interior or superficial surfaces, infect the host directly or be borne by a vector, and the source of infection may be an individual of the same species, a heterologous host, or infection of the host may follow a period of free living in the external environment. Survival at each stage involves adaptation to a series of mini-environments. A successful pathogen must also avoid or subvert the host immune response and ensure that it can infect new suitable hosts. Pathogenic bacteria must make these adaptations to their cellular compositions using a complement of only a few thousand genes and without the benefit of specialised tissues. Since bacteria are obviously capable of adaptation to changes in environment it follows that they have mechanisms to detect external changes, analyse the information and mount an appropriate response.

2.8.1 Expression of the Invasive Phenotype.

Invasive pathogens do appear to regulate invasion gene expression such that their products are only expressed when needed (Rosenshine and Finlay, 1993). Genes can be arranged as groups under the control of a common regulator such units are called regulons. The regulator is usually a DNA binding protein that recognises and binds to a specific sequence in the control regions of its subservient genes, thereby altering their expression. The

ability of the regulator to carry out its task is under environmental control, through mechanisms such as covalent modification and or cofactor binding. Examples of regulons in bacteria are legion and most of them are concerned primarily with the control of functions essential in commensal life (Neidhardt and Van Bogelen, 1987)

In *Yersinia* the secretion of Yops can only occur in the absence of Ca^{2+} ions and at 37°C. By contrast the expression of *yadA, inv* and *ail* are temperature dependant but independent of Ca^{2+} ion concentration (Dramsi *et al.,* 1993; Cornelis, 1994). Gene *virF* encodes a 30 kDa transcriptional activator which controls the expression of the *yop* genes (Cornelis *et al.*, 1987, 1989), of *yplA* (China *et al.*, 1990), of *yadA* (Martinez, 1989; Michiels *et al.*, 1991) and also the *virC* operon. VirF is a DNA binding protein of the AraC family of regulators (Cornelis, *et al.*, 1989). Transcription of the *yop* genes and the *vir* locus including *virF*, is strongly thermodependant (Cornelis *et al.*, 1986, 1987, 1989; Forsberg and Wolf-Watz, 1988; Goguen *et al.*, 1984; Mulder *et al.*, 1989; Hoe *et al.*, 1992; Straley and Bowmer, 1986; Yother *et al.*, 1986). Transcription of a cloned *virF* is thermodependant in a *Y. enterolytica* strain cured of PYV (Cornelis *et al.*, 1989) indicating that *virF* is itself thermoregulated by a chromosomal gene.

Mutagenisation of a *Yersinia enterolytica* strain that carries a *lacZ::yopH* fusion, identified two chromosomal mutants that transcribed *yopH, yopE* and *yadA* strongly at 28°C but nevertheless did not secrete the Yops at low temperature (Cornelis *et al.*, 1991). Transcription of *virF* was increased at 28°C which may account for the increased transcription of the regulon. Even though elements of the *yop* operon were over expressed at low temperature in the mutants there was still an increase in expression at 37°C indicating that the normal control mechanisms had not been abolished.

In both mutants the transposon had inserted into the *ymoA* gene the product is YmoA although there is no sequence homology between YmoA and HU, IHF, or H1 (H-NS) it is very

likely that YmoA is a histone-like protein. The properties of *ymoA* mutants are similar to the *Shigella virR* gene mutants.

Cornelis (1994) suggested that the poor transcription of the *yop* regulon at 25°C in the presence of VirF could be due to an inadequate conformation of the promoter (Lambert *et al.*, 1992). Indeed, in *ymoA* mutants, the *yopH* promoter is active at 37°C in the absence of VirF and it is extremely active at 25°C in the presence of VirF (Lambvert *et al.*, 1992). This indicated that chromatin structure may be involved in transcriptional activation of the *yop* genes. Possibly the *yop* regulon promoters, *in vivo*, are more susceptible to VirF activation at 37°C. Thus, chromatin structure can influence transcription in addition to the effect of VirF.

The mechanism of the Ca^{2+} control is very complex and is poorly understood (Cornelis, 1994). Transcription of *yop* and *vir* genes is reduced in the presence of Ca^{2+} ions (Cornelis *et al.*, 1987; Leung *et al.*, 1990; Forsberg and Wolf-Watz, 1988; Bölin *et al.*, 1988; Price *et al.*, 1989; Mulder *et al.*, 1989). The *lcrGVHyopBD* operon is involved in this regulation but the mechanism is unknown as yet (Price and Straley, 1989; Bergman *et al.*, 1991).

The *S. flexneri ipa* invasion genes are also temperature regulated. Strains that are invasive at 37°C become noninvasive when grown at 30°C (Maurelli *et al.*, 1984). The temperature regulated expression of *ipa, mxi* and *spa* operons is under the control of a regulatory cascade that involves two transcriptional activators, VirB and VirF, which are encoded by the large plasmid and by the product of a chromosomal gene, designated *virR*, respectively. Transposon insertions in a gene located immediately downstream from the *ipa* operon led to a noninvasive phenotype (Maurelli *et al.*, 1985; Sasakawa *et al.*, 1988). This gene was designated *virB* in *Sh. flexneri* 2a (Adler *et al.*, 1989), *ipaR* in *Sh. flexneri* 5 (Buysse *et al.*, 1990), and *invE* in *Sh. sonnei* (Watanabe *et al.*, 1990). It encodes a 36 kDa protein homologous to ParB of plasmid P1 and SopB of plasmid F, two DNA-binding proteins

involved in plasmid partitioning (Parsot, 1994). The *virB* mutants, which did not produce the Ipa proteins, were altered in the transcription of the *ipa, mxi* and *spa* operons. Overproduction of VirB from recombinant plasmids led to an increased transcription of the invasion operon even at 30°C (Watanabe *et al.*, 1990; Tobe *et al.*, 1991). The VirB binding site(s) on the promoters of the *ipa, mxi* and *spa* operons have not yet been identified. Expression of *virB* is positively regulated by VirF.

The *virF* gene is located about 40kb away from the invasion region of the virulence plasmid PMYSH6000 of *Sh. flexneri* 2a (Sakai *et al.*, 1986 a, b). Sequence analysis indicates that the 30 kDa protein belongs to the AraC family of transcriptional activators. Transposon insertions in *virF* abolished transcription of the *virB* gene, which resulted in the lack of expression of the invasion genes (Sakai *et al.*, 1988; Adler *et al.*, 1989; Tobe *et al.*, 1991). Deletion analysis indicated that activation of *virB* promoter by VirF requires a DNA segment extending 110 bp upstream from the *virB* transcription start site (Tobe *et al.*, 1993). The *virF* gene is expressed at 30°C, in contrast to the *virB* that is transcribed only at 37°C. At 30°C, over expression of *virF* from recombinant plasmids did not enhance transcription of *virB* (Tobe *et al.*, 1991); this is likely to be due to the binding of the chromosomally encoded VirR protein on the *virB* promoter at 30°C (Tobe *et al.*, 1993).

The *vir* gene was identified following transposon mutagenesis of a *Sh. flexneri* strain carrying a transcriptional *mxi-lac* fusion (Maurelli and Sansonetti, 1988). Since the invasion genes are expressed at 37°C but not at 30°C (Maurelli *et al.*, 1984), the parental strain could grow on lactose only at 37°C. A mutant carrying a Tn*10* insertion in a gene that was designated *virR* was selected for a Lac^+ phenotype at 30°C. Transduction of the *virR*::Tn*10* mutation onto wild type *Shigella* resulted in a strain that was invasive at both 30°C and 37°C, confirming that the *virR* gene was involved in the temperature-regulated expression of the invasion phenotype. The *virR* mutation was shown to be allelic to the *osmZ, drdX, bglY* and *pilG* mutations identified in *E. coli* (Dorman *et al.*, 1990; Göransson *et al.*, 1990; Hulton *et*

al., 1990; May *et al.*, 1990). The corresponding wild type gene encodes the histone-like protein H1 (H-NS) which may induce local change in DNA supercoiling, thereby modulating gene expression (Hulton *et al.*, 1990). This protein was recently shown to bind the *virB* promoter and repress its activity at 30°C (Tobe *et al.*, 1993).

S. typhimurium invasivness, like *Shigella flexneri,* appears to be regulated by environmental factors that would be affected on entry to the intestine. These include low oxygen levels, growth phase, DNA supercoiling, and other unknown factors (Ernst, Dombroski and Merrick, 1990; Lee, Falkow and Isberg, 1990; Galán and Curtiss, 1990; Kusters *et al.*, 1993). However, the precise molecular mechanisms are not well documented in the current literature.

2.9 Evolutionary relationships of genes coding for invasion.

The species capable of invasion constitute a phytogenetically diverse group. There are striking differences in their host ranges, modes of infection and pathogenic potential and this suggests that the ability to enter host cells arose independently in several bacterial lineages (Ochman and Groisman, 1995). Invasive pathogens differ not only in the antigens presented (section 2.4 above) but in the environmental signals controlling their expression and secretion (section 2.5). In spite of these differences, certain elements of the invasion mechanism are common to *Yersinia*, *Salmonella,* and *Shigella*. These pathogens promote their phagocytosis into epithelial cells by inducing actin polymerisation (Clerc, 1987; Finlay and Falkow, 1988a), and, in fact, it is now apparent that these, species, as well as divergent pathogens, harbour a similar cluster of invasion genes (van Gijsegem *et al.*, 1993; Bergman *et al.*, 1994; Forsberg *et al.*, 1994; Maurelli 1994).

2.9.1 Genetic organisation of invasion loci in *Shigella* and *Salmonella*.

This information was dealt with at length above (Section 2.7) and will only be recapped here. In *Shigella* the genetic components responsible for invasion of host epithelial

cells are contained in a 37 kb fragment of the 220 kb virulence plasmid (Sansonetti, Kopecko and Formel, 1981, 1982; Maurelli *et al.*, 1985; Sasakawa *et al.*, 1988). Several proteins are coded for by this region, principally the Ipa proteins, (Buysse *et al.*, 1987; Mills *et al.*, 1988; Venkatesan *et al.*, 1988; Menard *et al.*, 1993). The export of the Ipa proteins is mediated by the products of the *mxi-spa* region that specify dedicated secretion apparatus (Andrews and Maurelli, 1992; Venkatesan *et al.*, 1992; Sasakawa *et al.*, 1993). Aside from the outer membrane protein MxiD (Allaoui *et al.*, 1993a) none of the Ipa, Mxi or Spa proteins has a cleavable N-terminal signal peptide associated with the classical pathway for the secretion of proteins.

In contrast most of the genes implicated in *Salmonella* invasion and virulence map to the chromosome (Finlay *et al.*, 1988a; Stone *et al.*, 1992). Whilst the majority of virulence loci from *Salmonella* have also been seen in nonpathogenic *E. coli*, a large region, apparently unique to the salmonellae, contains many of the genes necessary for host cell invasion (Galan and Curtis, 1991; Groisman and Ochman 1993). Several loci including *hil, orgA, prgH, inv* and *spa* have been mapped to this region (Galan and Curtis, 1989; Ginocchio *et al.*, 1992; Lee *et al.*, 1992; Groisman and Ochman, 1993; Belau and Miller 1993; Jones and Falkow, 1994).

2.9.2 The phylogenetic distribution of machineries that export surface antigens.

As outlined at several points in section 2.4 above, DNA sequences related to the *mxi, inv*, and *spa* loci have been recovered from other plant and animal pathogens. *Yersinia* species, the secretion of plasmid encoded Yops is controlled by the genes, designated *lcr, lsa, vir* and *ysc*, which are also present on plasmid PYV. Although certain virulence genes from *Yersinia* species were known to have counterparts in other pathogens (Allaoui *et al.*, 1992a, 1994; Andrews and Maurelli, 1992; Galan *et al.*, 1992a), it is now apparent that the whole *lcr* complex of *Yersinia* is homologous to the *spa* gene clusters of *Salmonella* and *Shigella* species (Bergman *et al.*, 1994; Forsberg *et al.*, 1994).

The Gram-negative plant pathogens *Erwinia, Pseudomonas,* and *Xanthomonas* contain a gene cluster *hrp* that is required to induce disease in susceptible hosts and the hypersensitive response in resistant plants (Willis *et al.*, 1991). The *hrp* genes control the secretion of harpins which are the protein that provoke the hypersensitive response, these genes not only show a high degree of homology with the *spa* and *lcr* genes but share a similar arrangements (Gough *et al.*, 1993; van Gijsengem *et al.*, 1993; Bergman *et al.*, 1994).

Thus there appears to be a third export pathway distinct from both the *sec*-dependant pathway and that exemplified by the Hly and Cva of *E. coli*, which are responsible for the secretion of haemolysin and colicin V, respectively (Salmond and Reeves, 1993; Ochman and Groisman, 1995).

2.9.3 Functional equivalence of heterologous invasion determinants.

The conservation and structural integrity of the Spa export pathway in divergent pathogens greatly facilitates the analysis of virulence attributes in these organisms (Ochman and Groisman, 1995). Moreover, it has allowed workers in this field to apply a comparative approach to the study of invasive determinants. Among the most striking of the *inv-spa* gene cluster are the relationships between the homologous genes from different species. For example there are genes that show more than 60% homology in *Salmonella* and *Shigella* species flanked by genes of corresponding lengths and orientation but with only 20% homology (Ochman and Groisman, 1995).

Recently, it was shown that in strains of *Salmonella typhimurium* harbouring mutations in *invA* or *spa* upon introduction of the corresponding genes from the *mxi-spa* locus of *Shigella flexneri*, *mxiA* and *spa24*, respectively (Groisman and Ochman, 1993; Ginocchio and Galan, 1995). Since InvA and Sap are amongst the most conserved of genes (above 60% homology) this result might be expected.. However, there are probably several other proteins or segments of proteins that have changed to an extent that would prevent function in other species. For example, *lcrD* from *Yersinia* species could not complement a *Salmonella invA*

mutant, while a chimeric LcrD protein containing the divergent carboxy-terminal portion of InvA was able to restore function in *S. typhimurium* thereby identifying the portion of *invA* required for *Salmonella* invasion (Ginocchio and Galan, 1995).

2.9.4 Ancestry of the *spa* family.

The finding of such well conserved gene clusters in such a wide range of plant and animal pathogens has indicated that the pathogenic mechanisms in these species are much more similar than previously assumed. Nevertheless, despite similarities in structure and function, the physical features of the gene clusters as well as their genomic locations suggest that these sequences were acquired independently by each species (Groisman and Ochman, 1993; Ochman and Groisman, 1995).

The invasion genes of *Shigella* species are borne on a plasmid and have atypically low G+C content, which can be taken as evidence that the genes have been transferred horizontally from a distantly related organism (Hale, 1991). All species of *Shigella* emerged from *E. coli* relatively recently (Ochman *et al.*, 1983) and could not have been the source of the *inv-spa* complex that appears to be ancestral to the salmonellae (Fitts, 1985). By similar reasoning the relatively A+T rich *inv-spa* genes from *Salmonella* could not be the source of the *Shigella* genes (Ochman and Groisman, 1995). Furthermore genes within the *spa* complexes of *Salmonella* and *Shigella* are more similar to each other than to their *lcr* counterparts, which exclude *Yersinia* as the source of these sequences (Ochman and Groisman, 1995). In summary the invasion genes were not part of the common ancestor of these enteric pathogens but were probably acquired from another source independently.

2.10 *In vivo* and *In vitro* Assays of Invasion.

Animal models are very often used to assess the effect of mutations on the virulence of human pathogens and the experiments described in section 2.4 above provide many examples (ligated rabbit ileal loops (Gots *et al.*, 1974), and the induction of

keratoconjunctivitis in guinea pigs (Sereny, 1957) are just two techniques used to study *Shigella* virulence) of the useful contribution such models have made to the understanding of virulence. Such models have their limitations, however, there is no animal model for *S. typhi* and the are obvious differences in the genetic basis for invasion in the accepted alternative *S. typhimurium* (cf section 2.7.16). However, in the absence of a good model for the human pathogen most experimental work has concentrated on the murine model despite the implicit lack of relevance of the results of these investigations to the human disease. Another problem with animal models is the complicated nature of the potential range of host pathogen interactions. Two approaches have been used to overcome this problem. The first is the use of in bred animals like the BALB/c mice that are excellent infection models for enteric pathogens because they all respond to pathogens in a similar way, however, they are not at all like a real population of animals because of the lack of genetic variability that makes them useful models.

Table 2.1 **The organisation of invasion gene clusters from enteric bacteria. Numbers denote percent identity of protein products the *Salmonella typhimurium* homolog (after Groisman and Ochman, 1995).**

Yersinia	***yscC***	***lcrE***	***lcrD***	***yscN***	***yscO***	***yscP***	***yscQ***	***yscR***	***yscS***	***yscT***	***yscU***
% Identity	31	20	42	50	21	21	24	47	41	26	32
Salmonella	***invG***	***invE***	***invA***	***spaL***	***spaM***	***spaN***	***spaO***	***spaP***	***spaQ***	***spaR***	***spaS***
% Identity	46	32	64	58	23	19	24	63	64	45	49
Shigella	***mxiD***	***mxiC***	***mxiA***	***spa47***	***spa13***	***spa32***	***spa33***	***spa24***	***spa9***	***spa29***	***spa40***

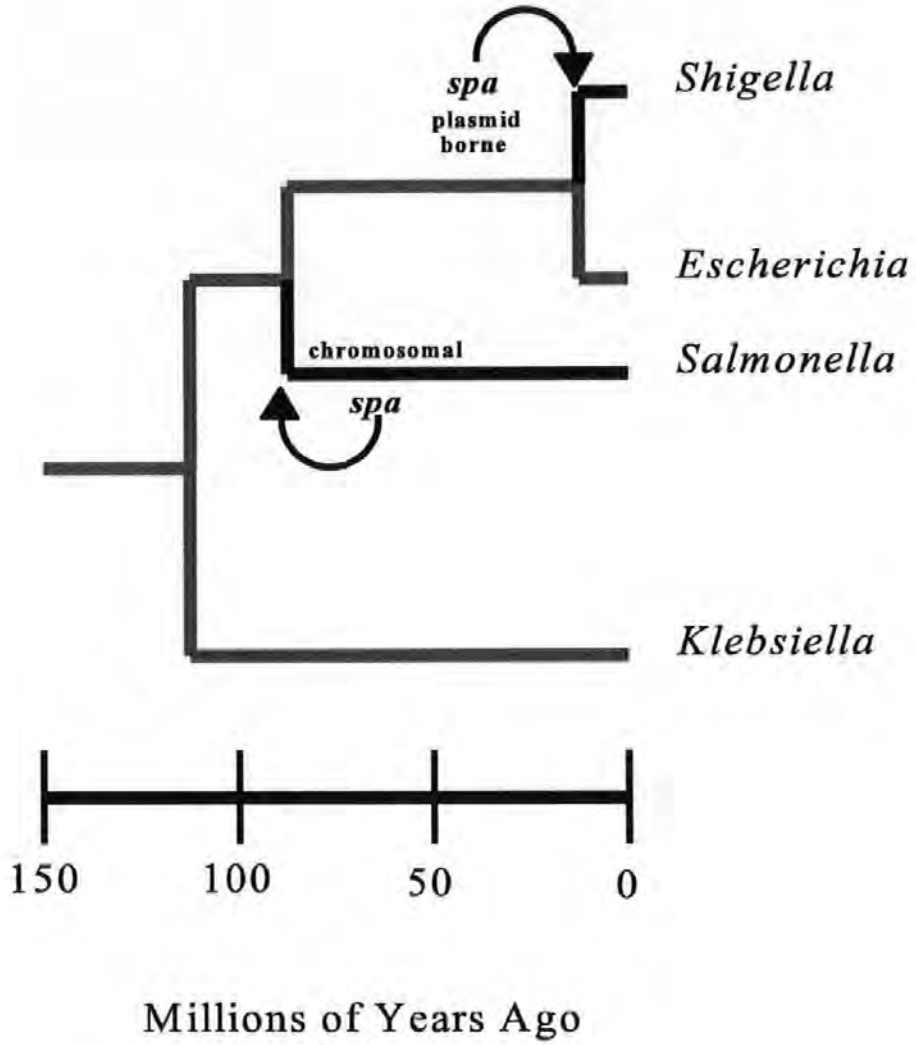


Figure 2.2 **The distribution and ancestry of spa gene clusters in Gram negative enteric bacteria. The time scale was proposed by Ochman and Wilson (1988) (after Groisman and Ochman, 1995).**

The other approach involves the use of cultured cells which provide a simpler and more easily controllable model system are very often used and examples of this use are replete in section 2.7 above.

It cannot be stressed strongly enough that caution must be used when interpreting the results of *in vitro* assays of any kind and especially when the subject under investigation is one as complex as microbial adherence and invasion. It has already been stated that bacteria are capable of controlling the expression of genes according to the prevalent conditions. Since the conditions in a culture flask are unlikely to be like any real environment, the organism is likely to encounter. Being sure that results are not artifactual is hard. Furthermore, many of the investigations quoted above involve transformed cell lines, this is done as these cell lines are well established and relatively easy to work with and they provide a uniform model system. They suffer from one major limitation, i.e. they are transformed and have been grown *in vitro* for a long time their surface markers are unlikely to be identical to cells in the mucosal epithelium of live hosts. Figure 2.3 shows the difference between the exposure of cell surface markers of mucosal epithelium nonconfluent nonpolarised tissue culture cell and a polarised monolayer.

As can be seen in Figure 2.3 in nonconfluent monolayers cell surface marker are exposed on all surfaces of the cell that is very unlike the situation *in vivo*. Furthermore even in polarised monolayers there is no mucus layer to interfere with bacteria/host cell surface interactions.

When *Shigella* enter HeLa cells they do not have to disrupt the microvilli border and can enter directly through the apical surface of the cell. When the human colonic continuous cell line CaCo-2 (Rousset, 1986) is grown to confluency, shigellae do not invade these differentiated cells that express a brush border (Mounier *et al.*, 1992). They rather bind to the outer edge of the islet and subsequently enter peripheral cells. If the confluent monolayer is treated with EGTA that, through Ca^{2+} chelation, impairs the function of cadherins (Volberg

et al., 1986), and opens intercellular junctions and exposes the basolateral pole of the cells bacteria will invade the epithelial lining quite efficiently. *Shigella flexneri* does not recognise a receptor on the apical pole of enterocytes but rather a basolaterally expressed receptor; unlike *Salmonella* that penetrates straight through the apical surface of MDCK and CaCo-2 cells (Finlay *et al.*, 1988b; Finlay and Falkow, 1990).

Therefore, it is likely *in vivo Shigella* often enter via the M-cells of the Peyer' patches, although there is evidence (Sansonetti, 1994) that the *Shigella* induced apoptosis of macrophages induces the migration of PMN into the colonic lumen. *In vivo* and *in vitro Shigella* have been shown to take advantage of this opening of the intestinal barrier to reach the basolateral surface of the enterocytes and invade them (Sansonetti, 1994).

Thus, careful interpretation of results from animal model and tissue culture models is necessary to gain an accurate picture of the events during invasion. The investigations related above appear to have been interpreted carefully and taken together seem to give an incomplete but convincing theory of bacterial invasion.

2.11 Conclusion.

Recombinant DNA techniques have made a huge impact on biology. This review has looked at the techniques involved in manipulating DNA and at the application of these techniques to the investigation of microbial pathogenesis and of invasion by enteric pathogens in particular.

The author has shown that r.DNA techniques have greatly increased our knowledge of the expression of the invasive phenotype, the mechanisms by which bacteria adhere, and invade host cells, how they subvert host cell signal pathways and how the mechanisms for invasion between the bacteria discussed have both striking similarities and obvious differences.

Unfortunately, there was no opportunity to discuss a bacterial pathogen of fish alongside these mammalian pathogens but this field is still a young one and there is unfortunately a paucity of available literature of the molecular basis of invasion of fish epithelial cells by Gram-negative pathogens.

The work by Lavelle (1994) and the work described later in this volume (Chapter 5) represent to the best of this author's knowledge the first attempts to demonstrate an invasive capability for a fish pathogen.

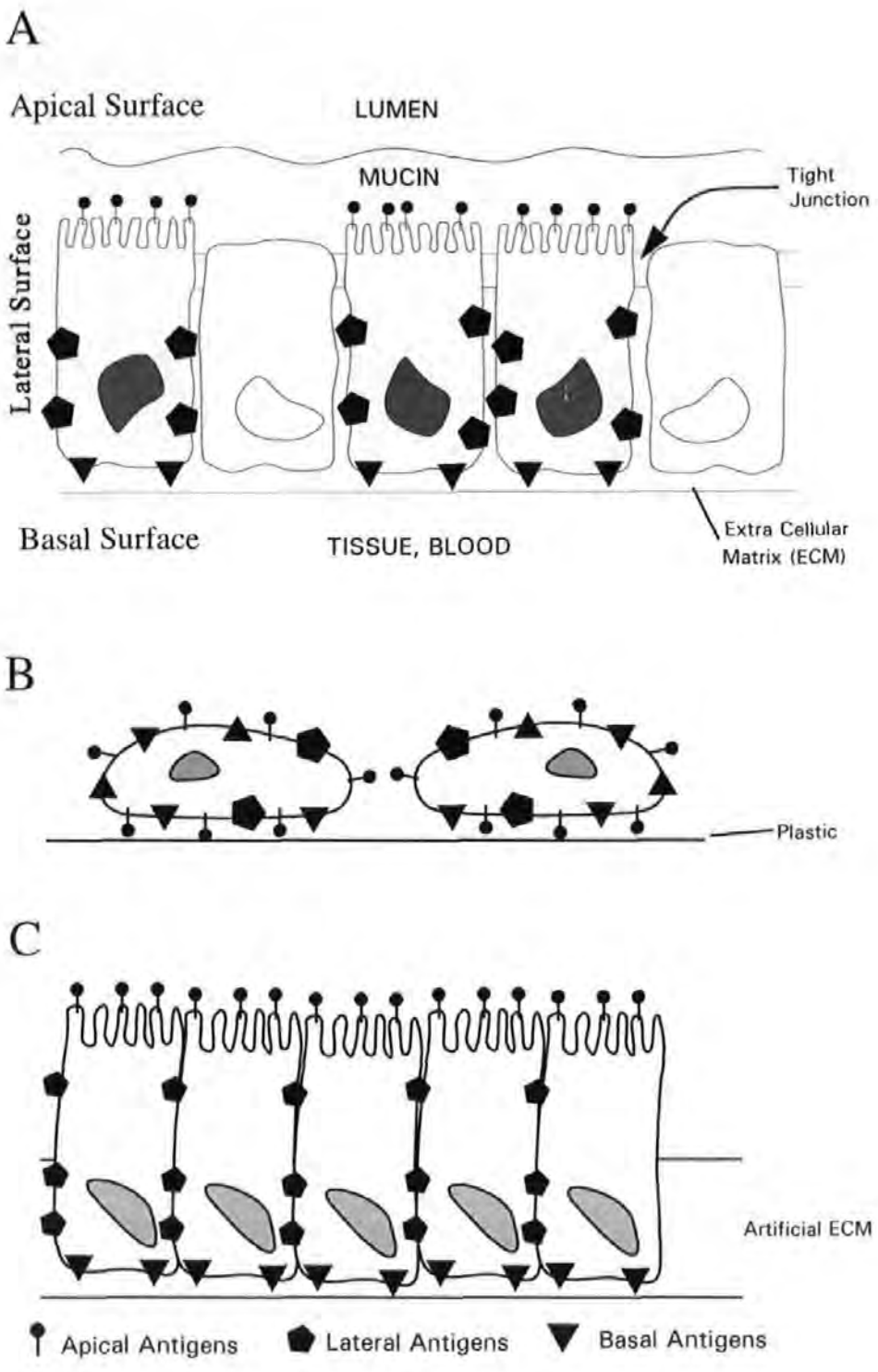


Figure 2.3 A diagrammatic representation of the differences between an actual mucosal surface and tissue culture models. **A**, an actual membrane *in vivo*. **B**, Nonconfluent, nonpolarised tissue culture cells. **C**, polarised monolayer of tissue culture cells. (After Salyers and Whitt, 1994)

Chapter Three.

The effect of low pH on *Aeromonas salmonicida*

3.1 Introduction.

The principal role of the vertebrate gastrointestinal tract has always been regarded as the digestion of nutrient biomolecules, i.e. carbohydrates, lipids and protein into subunits, followed by absorption into the tissues and subsequent egestion of the indigestible components of the food. Consequently, this the absorption of molecules that retain their native activity was not thought possible (Gardner, 1988). A much greater complexity in gut physiology has been revealed by the recognition of the role of the gut in immunological protection and of the capacity for the absorption of macromolecules from the lumen (Udall and Walker, 1982; McGhee and Kiyono, 1993). Also, it has been shown that bacterial pathogens can induce phagocytosis in non phagocytic gastrointestinal epithelial cells and thereby gain entry to the tissues (reviewed in chapter 2).

Previous workers from this laboratory have investigated the use of oral delivery of soluble protein antigen as a means of vaccination. The work by Jenkins (1992) in Tilapia and Lavelle (1994) in Rainbow Trout showed that producing a systemic immune response in teleosts following oral delivery of soluble protein antigens is possible. The present study involves the oral delivery of a dose of live auxotrophic bacteria (*A. salmonicida*) to a host (Rainbow Trout) with the aim of eliciting a protective immune response. In previous work the importance of optimising the delivery of the soluble antigen with respect to adjuvants and gastrointestinal inhibitors has been extensively investigated (Jenkins, 1992; Lavelle, 1994). The delivery of live auxotrophic bacteria raises the further complication of having to optimise conditions for bacteria colonisation of the recipient organism.

In chapter two the colonisation of mammals by bacteria via the mucosal surfaces was discussed in detail. In teleost species the entire interface of the animal with the environment is mucosal in nature and what is known about mammals indicates that the entire fish surface might provide pathogenic bacteria with a way to enter the viscera. However, the gills and the

skin of most fish are subjected to quite vigorous washing as the water passes over the surface and this along with the copious amounts of mucus that are produced might make attachment difficult. Thus the intestinal tract of fish may well provide pathogenic bacteria with a useful natural invasion route, and hence might be suitable for the delivery of live bacterial vaccines.

The digestive tract, however, maintains its internal environment to be as refractive to bacterial colonisation as possible. It does this by the presence of acidic gastric pH., bile salts proteolytic enzymes and very low iron concentrations as a result of lactoferrin activity.

The gastric barrier is commonly cited as an obstacle to the effective oral delivery of intact protein molecules to gastric teleosts (Lillenhaug, 1989; Wong *et al.*, 1992). This idea is supported by the observation of several groups of workers that the anal delivery of soluble antigens and bacterins enhanced both their absorption and the resulting immune response when compared with oral delivery (Johnson and Amend, 1983a, b; Jenkins *et al.*, 1992).

In mammals, the acidity of the gastric compartment has been shown to affect the viability of live orally administered enteric organisms (Gianella *et al.*, 1973) and can alter the immunogenicity of inactivated oral vaccine preparations (Clemens *et al.*, 1986; Sanchez *et al.*, 1993).

The suggestion that the acidic nature of the gastric compartment is the major barrier to enteric infection is about a century old, and is based on the observation that hypochloric and achloric humans are more susceptible to bacterial diarrhoea (Garrod, 1939; Felsen and Osofsky, 1937).

The gastric pH of rainbow trout was found to range between 2.6 and 4.0 following a period of two days starvation. The pH varied between individual animals and feeding with commercial trout pellets resulted in the pH rising to between 5.0 and 7.0 (Lavelle, 1994, personal communication).

Although all enteric pathogens share the oral route of infection *Shigella* species have a uniquely low infective dose. Whilst food borne infection does occur, person to person

transmission is the predominant mode of transmission and this supports the idea that Shigella has a low infective dose (DuPont *et al.*, 1989; Keusch and Bennish, 1989; Hale, 1991). The explanation for this is that in food borne inoculation the dose of bacteria received is usually very high but when human to human contact is responsible the dose is generally much lower. Human volunteer studies have shown that 10 to 500 shigellae cause dysentery in healthy adults (DuPont *et al.*, 1989). In contrast, similar studies have shown the infective dose of *Vibrio cholerae* is 10^8 organisms (Cash *et al.*, 1974) and that of *Salmonella* species is 10^5 to 10^{10} organisms (Blaser and Newman, 1982; Hornick *et al.*, 1970). In contrast, outbreaks of salmonellosis are most often associated with ingestion of common food in which bacteria have undergone extensive replication prior to ingestion. Some epidemiological data estimate a lower infective dose for *Salmonella typhi* than found in volunteer studies (Blaser and Newman, 1982).

Early studies on the sterilising role of the low pH in the stomach showed that many enteric bacteria are killed very rapidly by an acidic environment (Garrod, 1939; Drasar, *et al.*, 1969; DuPont, *et al.*, 1971; Gorden and Small, 1993). Many *in vitro* studies have shown that normal gastric juice, with a pH of less than 4.0, is bactericidal, whereas achloric gastric juice is not (Giannella *et al.*, 1973). Furthermore, bacteria instilled into the normal human stomach are killed within 15 minutes at a pH of less than 3.0, but remain viable in the achloric stomach for at least an hour (Giannella *et al.*, 1973). While other potential gastric antibacterial agents have been suggested (Garrod, 1939), the gastric bactericidal barrier may be primarily pH-hydrochloric acid-dependent (Garrod, 1939; Giannella *et al.*, 1973; Gorden and Small, 1993).

In experiments with human volunteers the infective dose of *Vibrio cholerae* was lowered from 10^8 to 10^4 by administering the inoculum with sodium bicarbonate (Cash *et al.*, 1974). Intestinal colonisation of oral vaccine strains was markedly enhanced by feeding strains with sodium bicarbonate (DuPont, *et al.*, 1972; Herrington *et al.*, 1990; Levine, *et al.*,

1990). This evidence with the early epidemiological investigations into achlorhydria and hypochlorhydria indicates that low pH has a major role in determining the infective dose. Gorden and Small (1993) studied the ability of *E. coli*, *Shigella* species and *Salmonella* species to survive exposure to acid. Strains that survived (≥ 10% of inoculum) exposure to Luria-Bertani (LB) broth acidified with HCl to pH 2.5 for 2 hours were considered acid resistant. In most cases, acid sensitive isolates exhibited less than 0.001% survival after exposure to acid. Of the *Shigella* species tested 75% were acid resistant (between 102% and 13% of the inoculum survived; mean 55%), of the freshly isolated *E. coli* strains 80% were acid resistant (range 113% to 25%; mean 50%) and of the isolates of enteroinvasive *E. coli* 66% were resistant (range, 70% to 17%; mean 32%). In contrast to this none of the isolates of *Salmonella* were acid resistant (<0.001% survival was found for all strains), furthermore, *Salmonella* was shown to be acid sensitive at pH 3.0 as well.

They also showed that the ability of *Shigella flexneri* to survive low pH was dependent on time, pH, and growth phase but not on growth at 37°C or on the presence of the large *Shigella* virulence plasmid. When low pH survivors were retested, they exhibited survival kinetics similar to the parental strain indicating that the ability to survive acid is a stochastic characteristic of the population and not due to the presence of genetically altered variants. They did not show why *Shigella* is more acid resistant than the other species tested, however, it is plain that this degree of resistance to low pH must have a great significance on the oral virulence of *Shigella.*

Shigella was shown to be constitutively resistant to acid exposure and evidence that some bacteria can sense and respond to changes in the environment has been growing and is now a major field of interest. *Salmonella typhimurium* exhibits remarkable versatility in its molecular responses to diverse environmental stresses (Morgan *et al.*, 1986; Aliabadi *et al.*, 1986; Spector *et al.*, 1986, 1988). One possible role for these mechanisms is to overcome the antimicrobial factors and physical barrier's presented by the host (Finlay and Falkow, 1989).

The organism is known to sense various conditions in the environment and respond by coordinately regulating sets of genes that will contribute to survival and, thus, productive infection (Buchmeier and Heffron, 1990; Ernst *et al.*, 1990; Lee and Falkow 1990; Mekalanos, 1992; Valone *et al.*, 1993).

Exposure of *E. coli* and *S. typhimurium* to moderately low pH (pH 6.0-5.0) induces a process that protects the cell from a subsequent challenge at a lower pH (pH 3.4-4.0) (Foster and Hall, 1990; 1991; Raja *et al.*, 1991a; Foster, 1992). The response has been called the acid tolerance response (ATR) in *S. typhimurium* and habituation in *E. coli*, and is characterised by a requirement for protein synthesis during the pre-treatment. The purpose of the moderate acid shock appears to be to induce a system(s) which will prevent a lethal drop in internal pH.

Foster and Hall (1990) found that the synthesis of 18 proteins is effected during the ATR (12 increased and 6 decreased) although it was not clear which were actually require for acid tolerance. Analysis of mutants produced good evidence that at least some were required for the adaptive response. Foster *et al.*, (1994) stated that in *S. typhimurium* the expression of at least 43 proteins is increased following acid shock and that at least some of them are essential for survival at low pH. Several recent publications deal with various aspects of this work (Foster and Aliabadi, 1989; Foster and Hall 1990; 1991; 1992; Foster 1991, 1992; Hickey and Hirschfield, 1990; Goodson and Rowbury, 1991; Raja *et al.*, 1991a,b; Rowbury *et al.*, 1992). All of this work involved looking at genetic lesions that caused increased or, more commonly, decreased acid tolerance and thus at the molecular basis of the ATR. The facilities to conduct this work using *A. salmonicida* were not available in this laboratory and in any case this type of investigation was outside the scope of this study.

The aims of this investigation were to use an *in vitro* model to look at the effect of low pH on both *A. salmonicida* 644rB and the aromatic deficient mutant and compare the acid tolerance of *A. salmonicida* with *Y. ruckeri* and *E. coli* that are both enteric bacteria (of

teleosts and mammals, respectively) and may, therefore, be acid tolerant. Both the effect of growth phase on acid tolerance, and the effect of culture conditions during the growth of the bacteria were studied. This was done to show if the bacterial vaccine needed to be protected from the acidity of the gastric mucus, or if by manipulating the conditions during the growth of the vaccine making the bacteria tolerant of low pH thus enabling survival in the stomach of the trout is possible. This represents the first attempt to investigate factors affecting the acid sensitivity of *A. salmonicida* and the first attempt to altered culture conditions to enhance resistance of a live orally delivered vaccine to the gastric barrier.

3.2 Materials and Methods.

3.2.1 Growth Media, Reagents and Chemicals.

Unless otherwise stated growth media were obtained from Oxoid (Basingstoke), and the other reagents and chemicals were obtained from BDH (Poole, Dorset), Sigma Chemical Company (London) and Labm (Toddington).

3.2.2 Bacterial Strains.

The bacterial species and strains used in this work were:

Escherichia coli strain K12 DH5 obtained from University of Plymouth stock maintained on TSA at 4°C and subcultured to produce working cultures.

Yersinia ruckeri strain (18/83) taken from the University of Plymouth stock. This strain had been kept in liquid nitrogen using skimmed milk as a cryo-protectant since 18/6/85. It was supplied to the University by Dr C J Rogers of the Ministry of Agriculture, Fisheries and Food laboratories at Weymouth, Dorset.

Two strains of *Aeromonas salmonicida* 644rB and its aromatic deficient mutant, 644rB *aroA*::Kan^r both are A layer positive. These were obtained from Dr L Vaughan, Department of Microbiology, Trinity College, Dublin, Ireland. *A. salmonicida* 644Rb was isolated from a clinical outbreak of furunculosis and *in vivo* passaged, it was supplied to Dr Vaughan by the Fish Disease Group, University College, Galway, Ireland. These strains were supplied to this lab as cultures on agar slopes. These were subcultured into brain heart infusion (BHI) broth on arrival. Following incubation at 20°C for 48 hours the bacterial cultures were pelleted (2,000*g*, 10 min), washed once by centrifugation in phosphate buffered saline (PBS) and resuspended in 10 cm^3 of PBS/10% v/v sterile glycerol as a cryo-protectant. These cultures were then stored as 1 cm^3 aliquots in sterile screw cap microcentrifuge tubes (Sarstedt), snap frozen in liquid nitrogen and transferred to a freezer at -70°C.

3.2.3 Routine Culture of Bacteria.

Unless otherwise stated all bacteria were cultured in tryptone soya broth (TSB), unshaken, or on tryptone soya agar (TSA) at 20°C. *A. salmonicida* 644Rb and *aroA*::Kan^r were subcultured weekly, to produce working cultures, by inoculating 100 cm^3 of TSB in 250 cm^3 conical flasks (one loop of stock culture) for 48 hours at 20°C. When 644Rb *aroA*::Kan^r was being grown Kanamycin was included in the growth media at 40µg cm^{-3} and Nalidixic acid at a concentration of 30µg cm^{-3}. For 644Rb only Nalidixic acid was included.

E. coli K12 DH5 was subcultured weekly, by growth in 100 cm^3 of TSB in 250 cm^3 conical flasks for 24 hours at 37°C (unshaken).

Yersinia ruckeri was subcultured weekly, from liquid nitrogen store by streak plating on TSA and growth at 25°C for 24 hours. Single colonies were then used to inoculate TSB.

3.2.4 Enumeration of Bacteria.

Unless otherwise stated viable bacteria were counted using the Miles and Misra technique (Miles *et al.*, 1938). Serial tenfold dilutions were made in PBS and bacteria grown on TSA, *E.coli* at 37°C and *Y. ruckeri* at 25°C, both for 24 hours before counting and *A. salmonicida* at 20°C for 48 hours prior to counting.

3.2.5 Production of standard curve for the rapid spectrophotometric enumeration of A. salmonicida.

Standard curves of absorbance at 590 nm against viable count of *A. salmonicida* colony forming units (CFU) suspended in PBS and in TSB were constructed for the rapid assessment of bacterial numbers. Forty-eight hour cultures of *A. salmonicida* 644Rb and *aroA*::Kan^r were pelleted (2 000*g*, 10 min) and washed once in either PBS or TSB and resuspended in 2 cm^3 of PBS or TSB. From these suspensions the following dilution series were made; 1:1, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200, 1:500 and 1:1000, the absorbance of 1 cm^3 aliquots of these suspensions were measured using a Cecil CE1010 spectrophotometer (Cecil, UK), and serial tenfold dilutions were made from the original suspensions so the viable

numbers could be assessed using the Miles and Misra technique. Linear regression was performed on the standard curves (using the graphics programme, Fig P 6.0a on an Opus 386sx 100% IBM PC compatible computer) to give a regression equation that could be used to calculate the number of CFU cm^{-3} of bacterial suspension for absorbance values in the range of the curve.

3.2.6 Construction of growth curves for the experimental bacteria.

In order that acid sensitivity could be assessed at different points in the growth cycle of the bacteria accurate growth curves were produced. Working cultures were produced as described above (section 3.2.3) and for *E. coli,* and *A. salmonicida* 100μl of the working culture was inoculated into 100 cm^3 TSB. One colony from the *Y. ruckeri* streak plate was inoculated into 100 cm^3 TSB.

The cultures of both strains of *A. salmonicida* were sampled at 0, 2, 4, 6, 8, 10, 12, 19, 21.5, 23, 25, 27, 29, 48 and 72 hours. The culture of *E. coli* was sampled at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 22, 33 and 76. *Y. ruckeri* was sampled at 0, 1, 2, 3, 4.25, 5, 11, 12, 13, 26, 56 and 76 hours.

All the cultures were diluted and CFU cm^{-3} calculated in the same way. Briefly, 500μl aliquots were removed from the cultures and serially diluted tenfold. The numbers of viable bacteria were enumerated using the technique of Miles and Misra (Section 3.2.4).

3.2.7 The resistance to low pH exhibited by the experimental bacterial strains.

Acid resistance of the four bacterial strains was assessed in lag, log, early, mid and late stationary growth phases as judged from the growth kinetic graphs produce as described above (Section 3.2.6). The timing of these phases was different for each species and is shown in Table 3.2.1. The acid sensitivity test was carried out in sterile citrate/phosphate buffer pH 3.0, 4.0 and 5.0 to a final volume of 20 cm^3. The buffer was sterilised by autoclaving for 15 minutes at 121 °C. The volumes of culture added to the buffer and the dilution values are given for each bacterial strain in Table 3.2.2. One cm^3 aliquots were removed from the buffer

immediately following inoculation (0 time) and then every hour for 6 hours. Initially, the removed aliquots were directly used to make the dilutions for plating out. This was changed so that the bacteria were pelleted by centrifugation (13500 rpm, 5 minutes, MSE microcentrifuge) and resuspended in 1 cm^3 of PBS.

One cm^3 of the original culture was removed and centrifuged in the same way. The numbers of viable bacteria were found using the Miles and Misra technique, and the percentage of CFU surviving exposure to each of the pH levels for each time point was calculated from the viable count of this unexposed sample.

Table 3.2.1 The time in hours that each culture was tested.

Bacteria	Time in hours for each Growth Phase				
	Lag	Log	Early-Stationary	Mid-Stationary	Late-Stationary
E. coli	0,75	4	13	48	74
Y. ruckeri	1	3	13	49	75
A. salmonicida 644Rb and *aroA*::Kan^r	2	10	26	48	74

Table 3.2.2 The volumes of bacterial culture used to inoculate the buffer and the resulting dilution factor.

Bacteria	Growth Phase									
	Lag		Log		Early Stat		Mid Stat		Late Stat	
	vol. cm^3	dil.	vol. cm^3	dil.	vol. cm^3	dil.	vol. cm^3	dil.	vol. cm^3	dil.
A. salmonicida 644Rb	2	1:10	2	1:10	1	1:20	1	1:20	1	1:20
A. salmonicida 644Rb *aroA*::Kan^r	2	1:10	2	1:10	1	1:20	1	1:20	1	1:20
Y. ruckeri	2	1:10	1	1:20	1	1:20	1	1:20	1	1:20
E. coli	2	1:10	1	1:20	1	1:20	1	1:20	1	1:20

3.2.8 Effect of Prior Growth Conditions on Survival of *Aeromonas salmonicida.*

A. salmonicida aroA and the wild type were grown under a number of growth conditions, before exposure to low pH buffers. This was done to investigate the effect of environmental factors on the low pH sensitivity of *A. salmonicida.* In brief, bacteria were grown under a range of conditions to see if acid sensitivity could be reduced. These conditions included: i) growth in BHI; ii) increased aerobiosis; preconditioning to low pH, iii) pH 6.0, iv) pH.5.5, v) pH 5.0; vi) preexposure to low temperature and low nutrient levels. The protocol for exposure to low pH citrate/phosphate buffers was the same as described in section 3.2.7.

i) Growth of bacteria in Brain Heart Infusion Broth

Bacteria were grown unshaken at 20°C to late stationary phase in BHI broth in 250 cm^3 conical flasks. The volume transferred to the buffer and the final dilution was the same as shown in Table 3.2.2.

ii) Growth of bacteria in aerobic conditions.

Bacteria were grown in an incubator shaker (New Brunswick Scientific, NJ, USA) rotating at 100 rpm 20°C. Bacteria were grown in 100 cm^3 of TSB in 250 cm^3 conical flasks. The volume transferred to the buffer and the final dilution was the same as shown in Table 3.2.2.

iii) Growth of bacteria in TSB at pH 6.0.

Bacteria were grown unshaken at 20°C in TSB. The broth was acidified before sterilisation (autoclave 15/15) with concentrated hydrochloric acid. A sample of sterilised broth was taken under aseptic conditions and the pH was tested and found to have remained at 6.0. One cm^3 of a 48-hour culture (normal TSB) was taken and used to inoculate the acidified broth. After 48 hours 1 cm^3 was taken and added to another 100 cm^3 of acidified TSB, this culture was grown for 72 hours and 1 cm^3 of this culture was used

to inoculate the low pH buffers. The volume transferred to the low pH buffers and, therefore, the final dilution was the same as shown in Table 3.2.2.

iv) Growth of bacteria in TSB at pH 5.5.

Bacteria were grown unshaken at 20°C in TSB. The broth was acidified prior to sterilisation (autoclave 15 minutes, 121°C) with concentrated hydrochloric acid. One cm^3 of a 48 hour culture was used to inoculate the acidified broth. This failed to produce any growth of bacteria so $1cm^3$ of bacterial culture grown at pH 6.0 / 48 hours was used to inoculate fresh TSB pH 5.5. There was limited growth in this culture and after 48 hours 1 cm^3 was removed and added to fresh TSB pH 5.5 and this culture was grown to late stationary phase. The bacteria were exposed to the low pH buffers as described above.

v) Growth of bacteria in TSB at pH 5.0.

Bacteria were grown unshaken at 20°C in TSB. The broth was acidified prior to sterilisation (autoclave 15/15) with concentrated hydrochloric acid. One cm^3 of a 48-hour culture (normal TSB) was used to inoculate the acidified broth pH 5.0. No growth was detected after 72 hours. As for the growth of bacteria in TSB pH 5.5 a sample of bacteria grown at pH 6.0 was used to inoculate pH 5.5 TSB this also failed to produce any growth as did the addition of bacteria grown in TSB pH 5.5. Following this failure the attempt to grow bacteria in TSB pH 5.0 was abandoned.

vi) Growth of bacteria in TSB followed by exposure to a cold nutrient poor media.

Bacteria were grown for 72 hours in TSB at 20°C unshaken. Ten cm^3 of the culture was removed centrifuged at 4,800*g* for ten minutes and resuspended in sterile PBS chilled to 10°C this suspension was transferred to

an incubator at 10°C and left for 24 hours. Bacteria from this cooled suspension were exposed to low pH citrate/phosphate buffers as described above section 3.2.7. This procedure was done to simulate release of bacteria from a nutrient rich environment such as a furuncle into a cold nutrient poor environment like fresh or sea water.

3.3 Results.

3.3.1 Production of a standard curve for the rapid spectrophotometric enumeration of *A. salmonicida*.

The original intention was to produce the growth kinetic curves and the spectrophotometric calibration curve at the same time by sampling every two hours for 48 hours two 1 cm^3 aliquots were removed from the cultures of the *aroA* and the wild type. One sample from each culture was serially tenfold diluted and bacterial numbers were estimated by the spread plate counting technique and the other sample from each culture was tested for absorbance at 590 nm. This technique failed for two reasons; firstly it was very complex and time consuming, and secondly the increase in absorbance of the culture happened over a short period during the 48 growth period and as a consequence of this there were too few points in the standard curve to give an accurate estimate of bacterial numbers.

The two standard curves shown in Fig 3.3.1 were constructed by growing cultures of *A salmonicida* strains for 48 hours. This was followed by washing, to remove non-cellular molecules that might have introduced error into the calibration curve, and resuspension in either PBS or TSB producing the most concentrated bacterial suspension possible. This concentrated suspension was serially diluted 1/10 to 1/1000. One cm^3 was tested in the spectrophotometer against an appropriate blank and 0.5 cm^3 was used to make the next dilution. Further dilutions were necessary and so 1/2, 1/5, 1/20, 1/50, 1/200, and 1/500 were produced to construct a useable calibration curve. Serial tenfold dilutions were continued past 1/1,000 up to 1/10,000,000,000. The numbers of viable bacteria in the last five of these dilutions were estimated by the spread plate technique.

It was found subsequently that the calibration curve for bacteria washed and resuspended in PBS was more reliable than the calibration curve for bacteria washed and resuspended in TSB. This was believed to be due to differences in the aliquot to aliquot

absorbance of the TSB. Subsequently, and because of this the PBS standard curve was always used. Furthermore, the curve was most accurate at absorbencies between 1.2 and 1.8 and so samples of bacteria were adjusted to an absorbance of approximately 1.5 by eye before spectrophotometric testing. A worked example of the calculation of bacterial numbers using the regression equation for the standard curve is given at Fig 3.3.1.

3.3.2 The growth kinetics of *A salmonicida, Y ruckeri*, and *E. coli.*

The results of this work are presented graphically in Fig. 3.3.2 (A B and C). The growth kinetic curves for both *A. salmonicida* 644rB and *aroA*::kan^r at 20°C are plotted in part A, they are in the same figure to illustrate how similar the two were. Lag phase lasted approximately two hours and is followed by a logarithmic growth phase that lasted for a further 16 hours. Stationary phase started at approximately 22-24 hours post inoculation. The last samples were taken at 72 hours, at this point the number of bacteria in the culture had not started to decline and therefore the start of death or decline phase was not identified for either strain. Doubling time for both strains was about two hours during log phase.

The growth kinetics of *Yersinia ruckeri* 18/83 is presented in part B Fig 3.3.2. These bacteria were grown at the higher temperature of 25°C and the bacteria showed a shorter doubling time of 45 minutes and consequently, shorter lag and log phases at 1 and 8.5 hours respectively. Stationary phase started at 10 hours post inoculation and again when the last sample was taken at 76 hours decline phase had not yet started.

The growth kinetics of *E. coli* K12 DH5 are presented in part C Fig 3.3.2. *E. coli* was grown at 37°C and had the shortest doubling time at 15 minuets and therefore the shortest lag and log phases at 45 minutes and 5 hours, respectively. Stationary phase started at 6 hours post inoculation and decline phase had not started when the last sample was taken. The results of this experiment were used to determine what times post inoculation would be used as the lag, log early, mid and late stationary phases for the investigation into the effect of growth phase on sensitivity to low pH.

3.3.3 The sensitivity of lag phase *A. salmonicida* 644rB, 644rB *aroA*::kan^r, *Y. ruckeri* 18/83, and *E. coli* K12 DH5 to exposure to pH 3.0, 4.0 and 5.0 in citrate/phosphate buffer.

A summary of percentage survival at zero time and 6 hours for all 4 strains at each growth phase and pH is presented in Table 3.3.1. The results are shown in Fig 3.3.3: parts A (pH 3.0); B (pH 4.0); C (pH 5.0). This and all subsequent investigations were conducted over six hours which according to Lavelle (unpublished observations) was the minimum time required for a soluble antigen to be passed from the stomach into the small intestine.

At pH 3.0 neither the aromatic deficient mutant nor the wild type strains of *A. salmonicida* were detected even after as little as five minutes exposure represented as time zero because of the centrifugation time, (samples were centrifuged and the bacteria resuspended in PBS because it was found that bacteria would not grow on the agar plates if they were inoculated with bacteria in the citrate/phosphate buffer). *Y. ruckeri* appeared to survive treatment until around three hours when numbers start to decrease but even after six hours the percentage survival was almost 10% of the original inoculum alive in the buffer. *E. coli* survived less well than *Y. ruckeri,* numbers of bacteria were decreasing after just one an hour and by six hours there were less than 0.1% of the original inoculum still alive.
At pH 4.0 both *A. salmonicida aroA* and 644rB were detected at zero time but not at later time points. Again *Y. ruckeri* survived very well with virtually no change in detectable numbers throughout the six hours. At this pH *E. coli* survived much better with more than 10% surviving six hours exposure.

All the bacteria survived well at pH 5.0 with *E. coli* and *Y. ruckeri* increasing in number at three and four hours although after this time the number of viable *E. coli* did drop, while, there was no drop in the number of *Y. ruckeri.*

3.3.4 The sensitivity of log phase *A. salmonicida* 644rB, 644rB *aroA*::kan^r, *Y. ruckeri* 18/83, and *E. coli* K12 DH5 to exposure to pH 3.0, 4.0 and 5.0 in citrate/phosphate buffer.

Results are presented graphically in Fig 3.3.4: parts A (pH 3.0); B (pH 4.0); C (pH 5.0). Immediately following inoculation of the pH 3.0 buffer *A. salmonicida aroA*::kan^r, *Y. ruckeri*, and *E. coli* were detected but the wild type *A. salmonicida* was not. *E. coli* and *A. salmonicida aroA* :: kan^r, were detected 1 hour following inoculation; no bacteria were detected between two and five hours. At six hours about 0.01% of the original inoculum of *Y. ruckeri* was detected.

At pH 4.0 *E coli and Y. ruckeri* were detected at all time points up to and including six hours. The percentage survival of *Y. ruckeri* remained between 100 and 50% during the six hours exposure but the viability of *E. coli* dropped from between 48 and 100% during the first three hours to less than 1% by six hours. *A. salmonicida* 644rB was only detected immediately following inoculation of the broth and the *aroA* mutant was detected at zero and 1 hour following inoculation during this time the percentage of viable bacteria dropped from 10 to less than 0.1%.

As in lag phase at pH 5.0 all the bacteria were detected at most of the time points. During the six hours the numbers of both *A. salmonicida* strains between 70 and 7% of the original inoculum. The numbers of both *E. coli* and *Y. ruckeri* appeared to increase to greater levels than were originally introduced. Howver *Y. ruckeri* was not detected at 6 hours.

3.3.5 The sensitivity of early stationary phase *A. salmonicida* 644rB, 644rB *aroA*::kan^r, *Y. ruckeri* 18/83, and *E. coli* K12 DH5 to exposure to pH 3.0, 4.0 and 5.0 in citrate/phosphate buffer.

The results of this work are graphically presented at Fig 3.3.5: parts A (pH 3.0); B (pH 4.0); C (pH 5.0). These investigations were again conducted over six hours. At pH 3.0 neither of the *A. salmonicida* strains were detectable at any of the time points between zero and six hours. Both the *Y. ruckeri* and *E. coli* survived for six hours. The percentage survival of *E. coli* fell from slightly less than 100% of the inoculum at time zero to about 3% at six hours. The percentage of *Yersinia* surviving fell from just over 20% at zero time to 10% at one hour. By two hours the percentage of viable bacteria fell to less than 0.001 percent of the inoculum. There appeared to be a recovery in the number of viable bacteria, with the percentage survival rising to approximately 0.05% at six hours.

All of the strains of bacteria survived better at pH 4.0 than at pH 3.0. Both of the strains of *A. salmonicida* were detected up to and including three hours. Between zero time and three hours the percentage survival of the *aroA* mutant fell from approximately 75 to 20%. In the same time period the percentage survival of the wild type strain fell from approximately 9% to 0.1%. Neither of the *A. salmonicida* strains were detected after three hours. The percentage survival for *E.coli* was seen to be between 110% at 1 hour post inoculation and 50%. *Y. ruckeri* percentage survival was constant at around 30-40% during the experiment.

At pH 5.0 the wild type strain of *A. salmonicida* was detected at one to four hours but not immediately following inoculation nor at five or six hours. There was approximately 15% surviving at one hour this fell to 0.08% at four hours. The aromatic mutant was not detected at two or four hours but at the other time points the percentage survival varied from between 100% at zero time and approximately 60% at the rest of the time points. The percentage survival of *Yersinia* and *E. coli* was seen to fall from around 50 - 60% at zero time, to less

than 10% at two hours. The number of CFUs detected for both these species increased at three hours to 110% and 50% respectively and remained at over 100% until the end of the experiment at six hours.

3.3.6 The sensitivity of mid stationary phase *A. salmonicida* 644rB, 644rB *aroA*::kan^r, *Y. ruckeri* 18/83, and *E. coli* K12 DH5 to exposure to pH 3.0, 4.0 and 5.0 in citrate/phosphate buffer.

Results are presented graphically in Fig 3.3.6: parts A (pH 3.0); B (pH 4.0); C (pH 5.0). In the pH 3.0 buffer wild type *A. salmonicida* was undetectable for the whole six hours. The aromatic mutant was detected at approximately 0.01% of the inoculum for three hours but was not detectable at four hours post inoculation or later. *Yersinia* showed survival at around 100% of the inoculum for the six hours of the assay. *E. coli* showed a steady decrease from 100% at zero time, to less than 1.0% at five hours, however, at six hours there was an increase in the number of CFU's detected to approximately 5% of the original inoculum.

At pH 4.0 the *A. salmonicida* wild type was detected at zero time at 1% of the inoculum by two hours this percentage had dropped to 0.02%, from three hours on it was not detected. The aromatic mutant was present at approximately 30% of the inoculum at zero time and the percentage survival fell steadily to approximately 0.03% after six hours. The percentage survival of *E. coli* remained at around 75% and *Y. ruckeri* remained at around 90% over the six hours of the assay.

At pH 5.0 the percentage survival of the *A. salmonicida* 644rB remained between 15% and 5% throughout the six hours. The percentage survival of the mutant at zero time was 50% and by six hours this had fallen to 20%. Both the *Y. ruckeri* and the *E. coli* strains tested showed an increase CFU's so that the percentage survival at six hours was greater than 100% for both species.

3.3.7 The sensitivity of late stationary phase *A. salmonicida* 644rB, 644rB *aroA*::kan^r, *Y. ruckeri* 18/83, and *E. coli* K12 DH5 to exposure to pH 3.0, 4.0 and 5.0 in citrate/phosphate buffer.

Results are presented graphically in Fig 3.3.7: parts A (pH 3.0); B (pH 4.0); C (pH 5.0). Both strains of *A. salmonicida* survived six hours in citrate/phosphate buffer at pH 3.0. The aromatic mutant was detected at levels of between 0.01 and 0.0003%, of the number of CFU in the inoculum. The wild type was not detected at 1 hour post inoculation but at all other times was found at levels between 0.001 and 0.0001% of the original. *Y. ruckeri* was detected at levels of between 90 and 50% of the number introduced and *E. coli* was seen to fall from 115 to 6% at 6 hours.

At pH 4.0 the wild type *A. salmonicida* strain was detected at the start of the experiment and after two hours but was not found at any other time. The aromatic mutant strain was found at all points from the start until six hours. The percentage survival fell from 20 to 0.5% during that time. *Y. ruckeri* was present at levels of between 77 and 80% of the number introduced to the buffer. *E. coli* was found to survive treatment at levels between 200 and 1000% of the inoculum.

Again all of the species of bacteria were found to survive treatment at pH 5.0. The *A. salmonicida* wild type strain was found to survive at levels of between 10 and 30% except at four hours when viable bacteria at 2% of the introduced number were detected. The *aroA* mutant was shown to survive at levels of between 50 and 10%. *Y. ruckeri* was detected at levels between 75% and 130% of the inoculum, and *E. coli* at between 130 and 300%.

3.3.8 The effect of prior growth conditions on the ability of *A. salmonicida* 644rB, 644rB aroA::kan^r to withstand exposure to pH 3.0, 4.0 and 5.0 in citrate/phosphate buffer.

The experiments were again conducted over six hours but this time only the two strains of *A. salmonicida* were used. The results of the experiments are presented graphically in Figs 3.3.8 to 3.3.19. The Figures are presented in pairs on facing pages; data on each pair of facing pages is derived from the same experiments but on the left-hand page shows the percentage survival and of the right-hand page shows the actual numbers of CFUs that these percentages represent.

The results in Fig 3.3.8 and 3.3.9 parts A and B show the effect of growth to late stationary phase in TSB at 20°C, these results were extracted from the same data used for Fig 3.3.7. Both strains clearly survive exposure at pH 5.0 much better than they do pH 3.0 and to a lesser extent pH 4.0 and this trend is seen under most growth conditions used in this investigation. The inability to detect bacteria of the wild type strain following exposure to pH 4.0 may be a genuine result but alternatively may represent an error in the experimental technique.

Even in the pH 3.0 groups where the percentage survival is small (between 0.01 and 0.001%) this is the equivalent of around 10,000 CFUs. Also of interest is the kinetics of acid sensitivity in *A. Salmonicida,* the drop in viable number happens very quickly, usually between the inoculation of the buffer and the subsequent removal of a sample and washing in PBS. After that, the numbers of bacteria remain almost constant for the remainder of the experiment.

At pH 5.0 the number of bacteria of both strains surviving for six hours was approximately 1,000,000,000 though this represents just 10% of the inoculum.

Bacteria grown for 70 hours in BHI (Fig. 3.3.10 and 3.3.11) rather than TSB showed similar survival profiles at pH 5.0 (10%) and pH 3.0 (0.001%), but both strains showed reduced survival at pH 4.0 (0.001%) from two hours on.

When grown in a shaking incubator (Fig. 3.3.12 and 3.3.13) in TSB for 72 hours at 20°C both strains showed reduced survival at pH 3.0, 4.0 and 5.0 when compared with bacteria grown static in TSB or BHI. The effect was most obvious for the aromatic mutant at pH 5.0; the percentage surviving was reduced to 0.01% and the numbers of CFUs viable at six hours was $7x10^5$ compared with the unshaken values of 25% and $5x10^8$ CFU. At pH 3.0 despite the reduction in the percentage survival (0.0001%, from 0.002%) the numbers surviving were similar in the shaken and unshaken groups ($9x10^3$ and $2x10^4$, respectively). Growth in TSB at pH 6.0 (Fig 3.3.14 and 3.3.15) resulted in improved survival at pH 3.0 (between 0.01 and 0.005%) compared with percentage survival of 0.002% when grown in normal TSB. Numbers of viable bacteria were slightly higher at around $5x10^5$ for the wild type, compared with $1x10^4$ CFUs in the bacteria grown in normal TSB. The results for the aromatic mutant were very variable and no clear difference was seen.

In TSB pH 5.5 (Fig 3.3.16 and 3.3.17) the wild type strain showed an increase in survival at pH 3.0 ,from 0.001% to 0.05%. A concomitant decrease in survival at pH 5.0 from approximately 20% to 0.05% was also seen. The aromatic mutant showed a decrease in survival at pH 5.0, from 30% to 1% at zero time to 0.008% at five hours. No bacteria were detected at six hours but this may be the result of an experimental error. At pH 3.0 survival increased from 0.002% to 0.01% this represents an increase in viable bacteria from $1x10^4$ to $1x10^5$ CFUs. There was little difference between the results for pH 4.0 in this or any other experiment.

It did not prove possible to grow either *A. salmonicida* strain at pH 5.0 and this part of study was abandoned.

Growth in TSB for 72 hours followed by 24 hours in PBS at 10°C (Fig 3.3.18 and 3.3.19) resulted in the wild type bacteria surviving at approximately 50% at pH 5.0; about 1% at pH 4.0; and at pH 3.0 the percentage survival dropped from 9% initially to 0.003%, after six hours. This corresponds to $1x10^8$, $1x10^6$ and $5x10^3$ CFUs in the buffer at pH 5.0, 4.0 and 3.0, respectively. The *aroA* strain had percentage survival rates of 50%, 0.3% and 0.001% at pH 5, 4 and three respectively after six hours exposure, corresponding to viable bacteria at $1x10^7$, $5x10^5$ and $5x10^3$ CFUs.

Table 3.3.1 A summary of the percentage survival immediately following and after 6 hours exposure to citrate phosphate buffer.

	Citrate Phosphate Buffer pH						
	3.0		4.0		5.0		
Time Hours	0	6	0	6	0	6	***Species***
Lag Phase	61.90	0.05	95.23	22.86	61.90	10.47	*E. coli*
	69.49	5.423	100.0	93.22	0.00	508.47	*Y. ruckeri*
	0.00	0.00	0.4	0.00	9.33	4.67	*aroA*
	0.00	0.00	7.81	0.00	78.91	7.18	*644rB*
Log Phase	0.00	0.00	48.48	0.97	84.84	121.21	*E. coli*
	0.02	0.01	67.36	55.79	49.47	0.0	*Y. ruckeri*
	0.009	0.00	7.91	0.00	29.17	27.08	*aroA*
	0.00	0.00	0.00	0.00	71.15	0.48	*644rB*
Early Stationary Phase	76.00	2.6	57.00	73.00	67.00	170.0	*E. coli*
	22.07	0.03	40.00	32.75	49.27	141.37	*Y. ruckeri*
	0.00	0.00	52.22	0.00	111.1	88.89	*aroA*
	0.00	0.00	8.89	0.00	0.00	0.00	*644rB*
Mid Stationary Phase	115.8	6.32	73.69	75.79	71.58	326.31	*E. coli*
	76.73	97.55	97.95	89.79	167.34	224.48	*Y. ruckeri*
	0.012	0.00	28.5	0.24	48.00	20.50	*aroA*
	0.00	0.00	1.13	0.00	13.91	3.69	*644rB*
Late Stationary Phase	129.4	6.59	258.82	1047.09	129.41	305.82	*E. coli*
	90.52	56.84	77.89	80.00	77.89	134.73	*Y. ruckeri*
	0.01	0.002	26.10	0.55	31.52	30.17	*aroA*
	0.0007	0.0007	16.67	0.00	26.85	16.67	*644rB*

Figure 3.3.1 Calibration curve for the rapid enumeration of bacteria (*A. salmonicida* 644rB *aroA*::kan^r) using the spectrophotometer.

The absorbance at 590nm was plotted against the number of colony forming units counted using the Miles and Misra techinique, (A) *the calibration curve using bacteria resuspended in PBS.* (B) *the calibration curve using bacteria resuspended in TSB. The regression equation for graph A was used to calculate the numbers of bacteria. The regression equation is shown below with a worked example. The regression analysis for both calibration curves was performed using Fig-P.*

$$\log CFU = 0.822725 \times (A_{590}) + 7.9746$$

Example

Using a 1/10 dilution of a 48hr culture of *A. salmoicida* washed by centrifugation and resuspended in PBS

If the A_{590} of 1 cm^3 of culture was 1.567 then

$$logCFU = (0.822725 \times 1.567) + 7.9746$$

therefore,

$$logCFU = 9.26$$

and so the numbers of bacteria in the 1/10 dilution would be

$$1.84 \times 10^9 \text{ CFU cm}^{-3}$$

and so in the orginal suspension there was

$$1.85 \times 10^{10} \text{ CFU cm}^{-3}$$

Fig 3.3.1 Part A Bacteria suspended in PBS

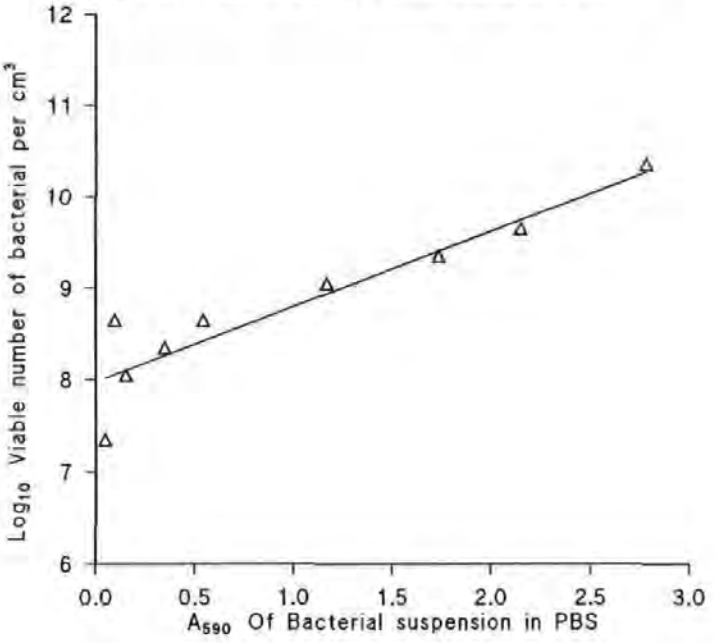


Fig 3.3.1 Part B Bacteria suspended in TSB

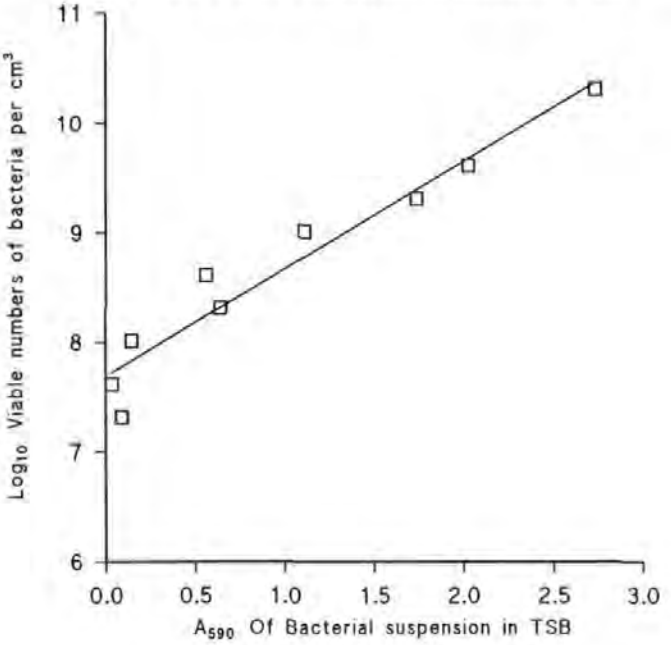
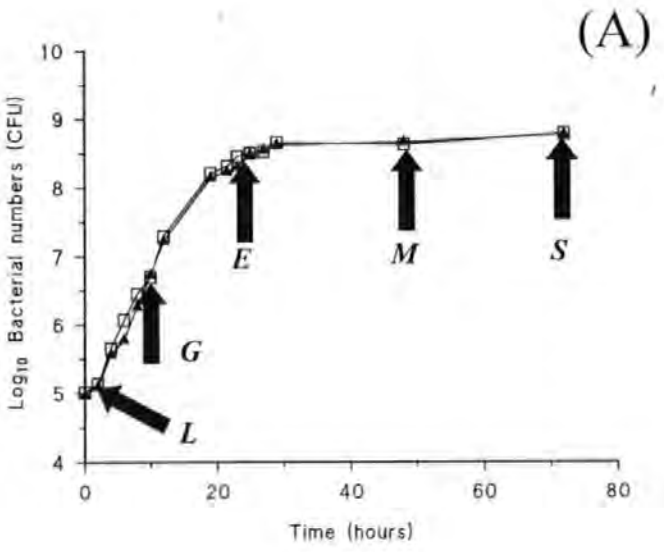
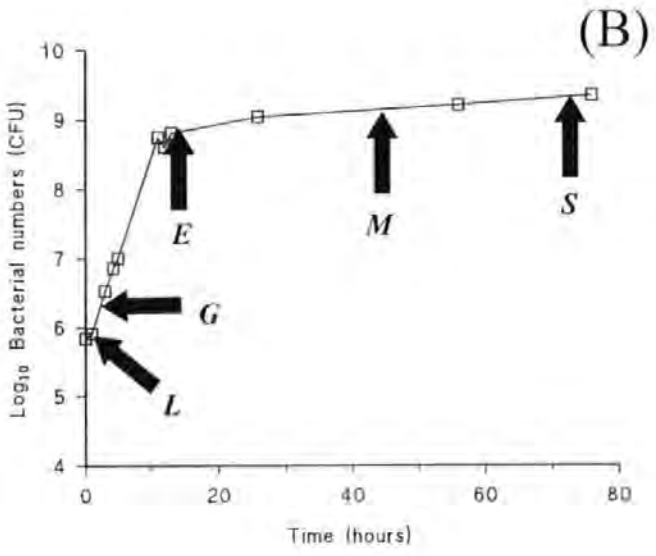


Figure 3.3.2 Growth kinetics of *Aeromonas salmonicida* 644rB and *A. Salmonicida* 644rB *aroA*::kan^r (A), *Yersinia ruckeri* 18/83 (B) and *E. coli* K12 DH5 (C).

The lag (**L**), *log* (**G**), *early stationary* (**E**), *mid stationary* (**M**), *and late stationary* (**S**) *growth phases are indicated.*


(A)
Log10 Bacterial numbers (CFU)
10
9
8
7
6
5
4
0
20
40
60
80
Time (hours)
L
G
E
M
S


(B)
Log10 Bacterial numbers (CFU)
10
9
8
7
6
5
4
0
20
40
60
80
Time (hours)
L
G
E
M
S

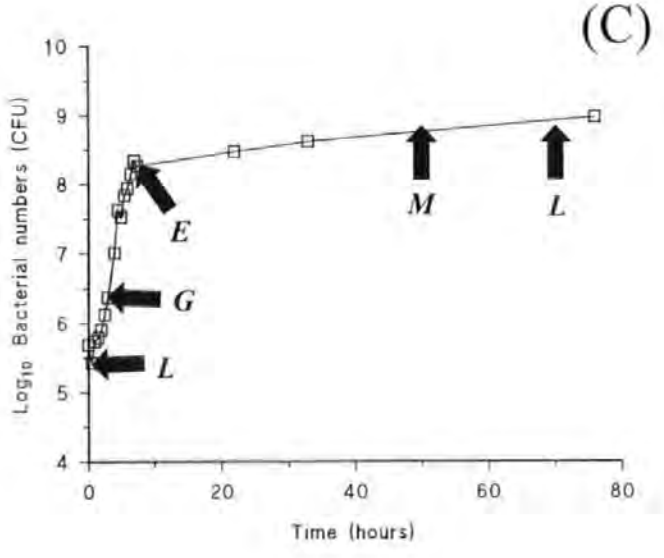

(C)
Log10 Bacterial numbers (CFU)
10
9
8
7
6
5
4
0
20
40
60
80
Time (hours)
L
G
E
M
L

Figure 3.3.3 The survival of bacteria grown to lag phase.

The *effect of exposure to citrate/phosphate buffer pH 3.0* (A) *,pH 4.0* (B), *pH 5.0* (C) *on the survival of* ***Aeromonas salmonicida*** *644rB* (■), ***A. salmonicida*** *644rB* ***aroA****::kan*^{r} (⧄), ***Yersinia ruckeri*** *18/83* (⊠) *and* ***E. coli*** *K12 DH5* (□). *The bacteria were exposed to the buffer for upto six hours. The buffer was inoculated with bacteria grown to lag phase as judged from Fig 3.3.2 above.*

Fig 3.3.3 Part A pH 3.0

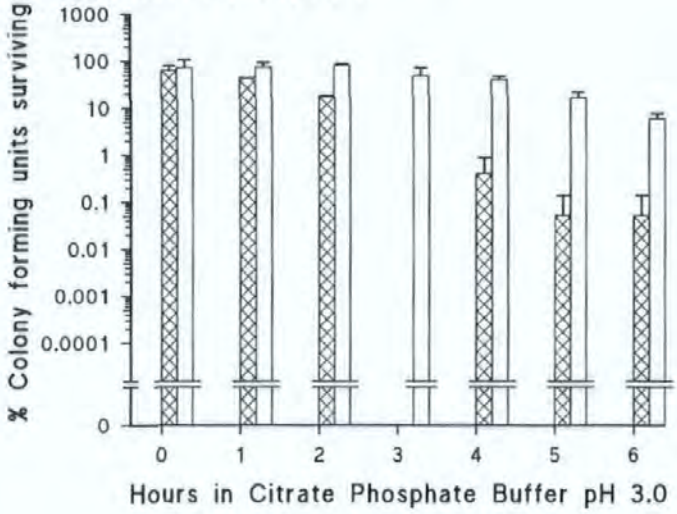


Fig 3.3.3 Part B pH 4.0

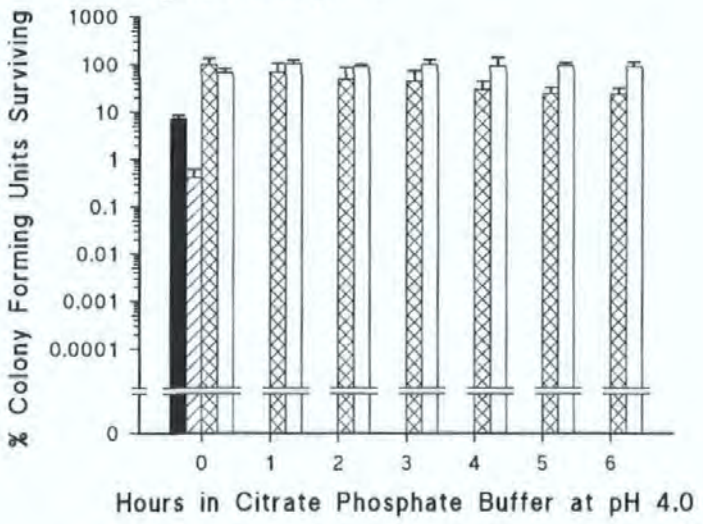


Fig 3.3.3 Part C pH 5.0

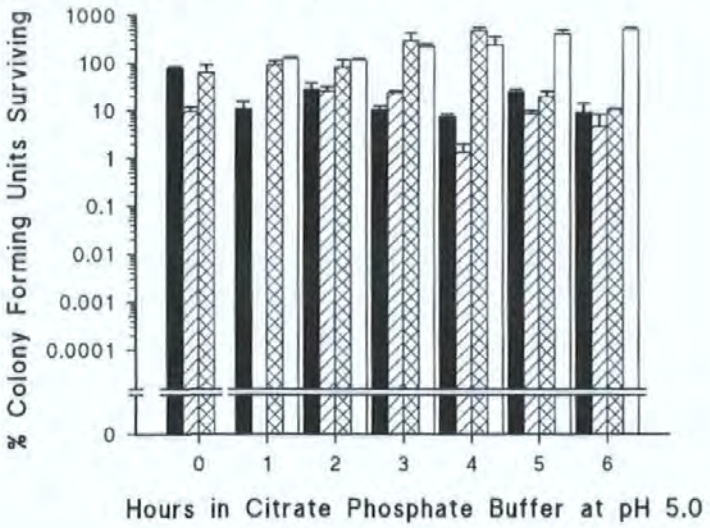


Figure 3.3.4 The survival of bacteria grown to log phase.

The effect of exposure to citrate/phosphate buffer pH 3.0 (A) *,pH 4.0* (B), *pH 5.0* (C) *on the survival of* ***Aeromonas salmonicida*** *644rB* (■), ***A. salmonicida*** *644rB* ***aroA***::*kan*^{r} (⧄), ***Yersinia ruckeri*** *18/83* (⊠) *and* ***E. coli*** *K12* DH5 (□). *The bacteria were exposed to the buffer for upto six hours. The buffer was inoculated with bacteria grown to log phase as judged from Fig 3.3.2 above.*

Fig 3.3.4 Part A PH 3.0

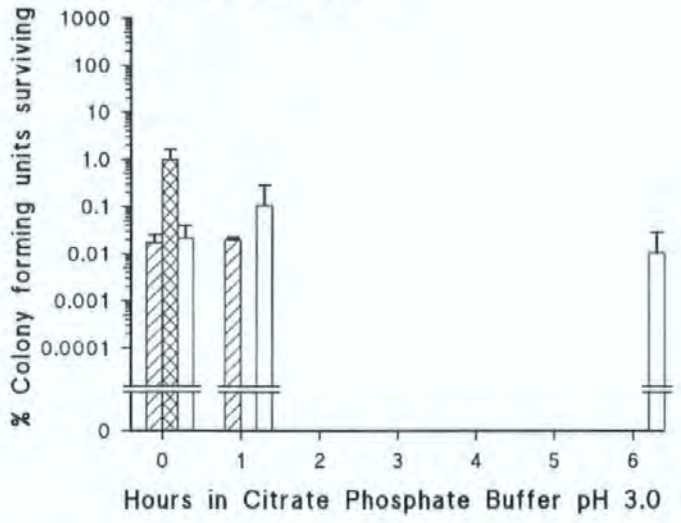


Fig 3.3.4 Part B pH 4.0

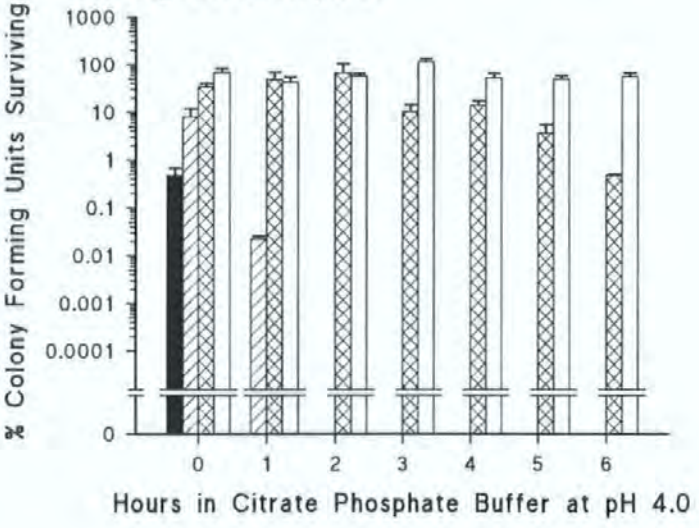


Fig 3.3.4 Part C pH 5.0

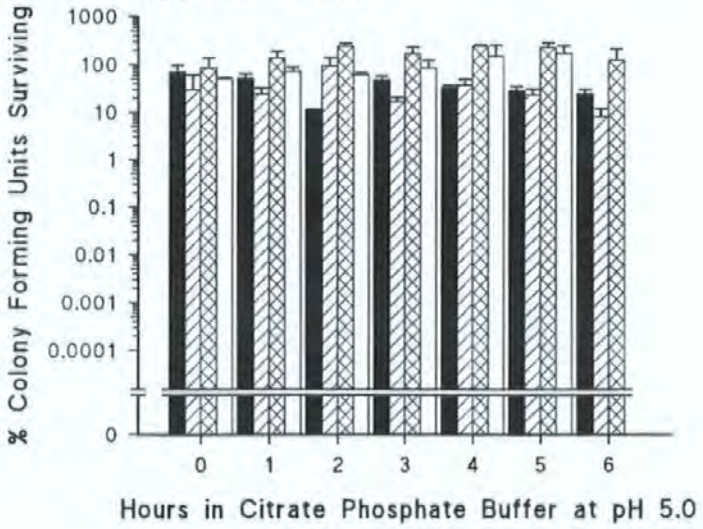


Figure 3.3.5 The survival of bacteria grown to early stationary phase.

The effect of exposure to citrate/phosphate buffer pH 3.0 (A) *,pH 4.0* (B), *pH 5.0* (C) *on the survival of* ***Aeromonas salmonicida*** *644rB* (■), ***A. salmonicida*** *644rB aroA::kan*^{r} (⊠), ***Yersinia ruckeri*** *18/83* (⊠) *and* ***E. coli*** *K12 DH5* (□). *The bacteria were exposed to the buffer for upto six hours. The buffer was inoculated with bacteria grown to early stationary phase as judged from Fig 3.3.2 above.*

Fig 3.3.5 Part A pH 3.0

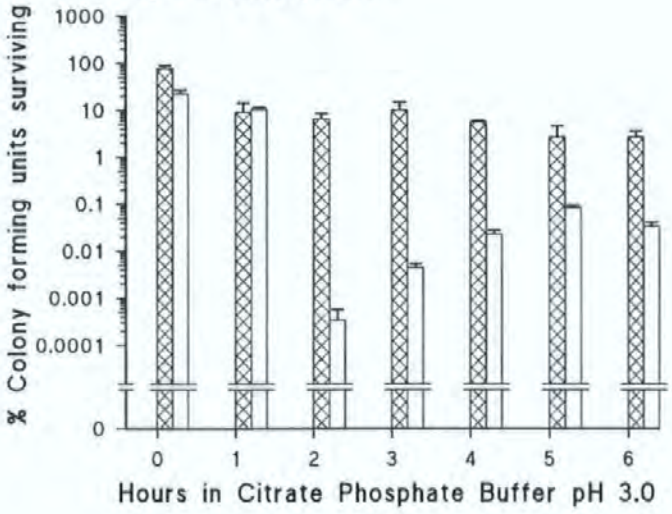


Fig 3.3.5 Part B pH 4.0

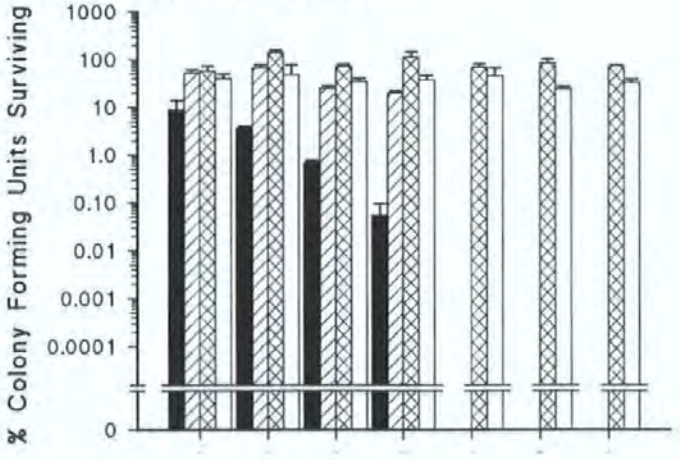


Fig 3.3.5 Part C pH 5.0

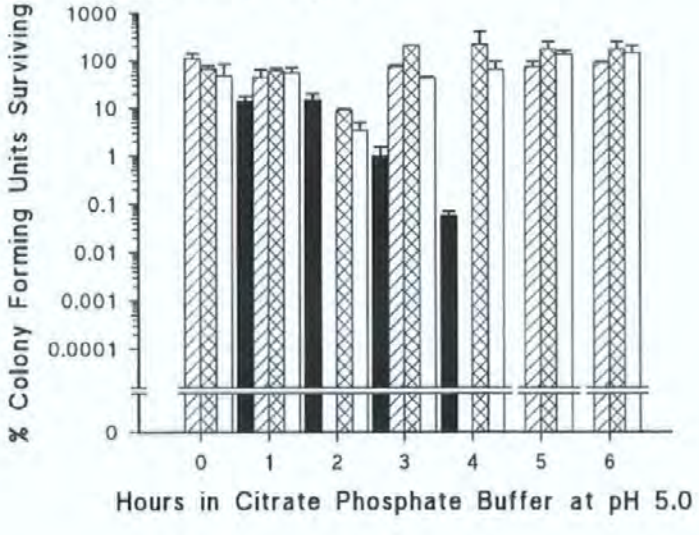


Figure 3.3.6 The survival of bacteria grown to mid stationary phase.

The effect of exposure to citrate/phosphate buffer pH 3.0 (A) *,pH 4.0* (B), *pH 5.0* (C) *on the survival of* ***Aeromonas salmonicida*** *644rB* (■), ***A. salmonicida*** *644rB* ***aroA***::*kan*^{r} (▨), ***Yersinia ruckeri*** *18/83* (⊠) *and* ***E. coli*** *K12 DH5* (□). *The bacteria were exposed to the buffer for upto six hours. The buffer was inoculated with bacteria grown to mid stationary phase as judged from Fig 3.3.2 above.*

Fig 3.3.6 Part A pH 3.0

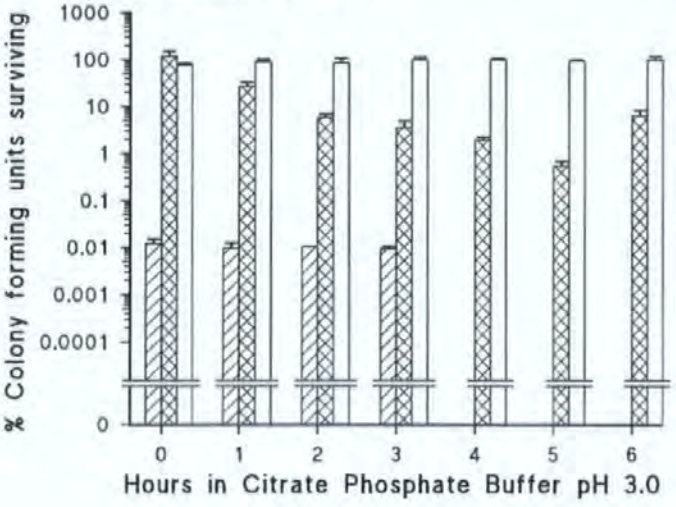


Fig 3.3.6 PartB pH 4.0

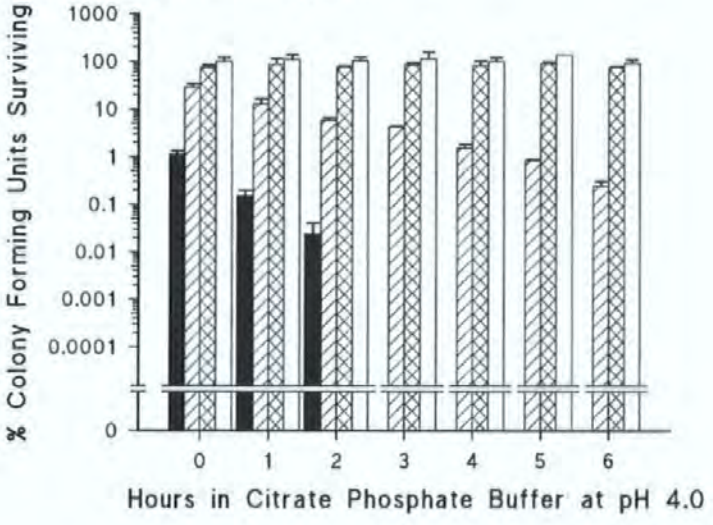


Fig 3.3.6 Part C pH 5.0

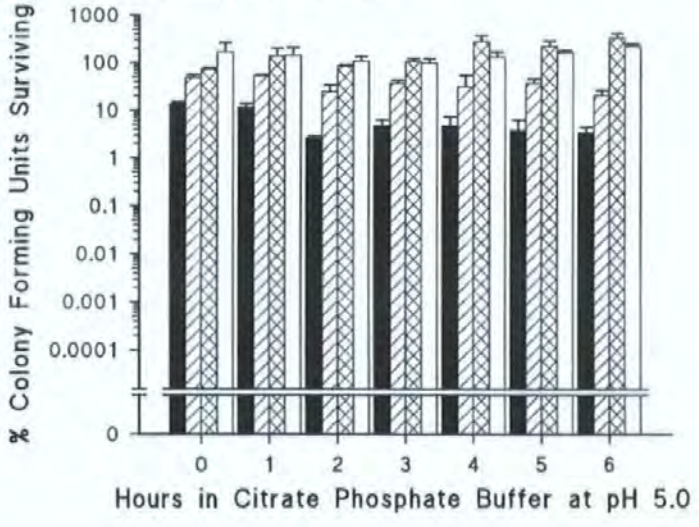


Figure 3.3.7 The survivial of bacteria grown to late stationary phase.

The effect of exposure to citrate/phosphate buffer pH 3.0 (A) *,pH 4.0* (B), *pH 5.0* (C) *on the survival of* ***Aeromonas salmonicida*** *644rB* (■), ***A. salmonicida*** *644rB* ***aroA****::kan*^{r} (⧄), ***Yersinia ruckeri*** *18/83* (⊠) *and* ***E. coli*** *K12 DH5* (□). *The bacteria were exposed to the buffer for upto six hours. The buffer was inoculated with bacteria grown to late stationary phase as judged from Fig 3.3.2 above.*

Fig 3.3.7 Part A pH 3.0

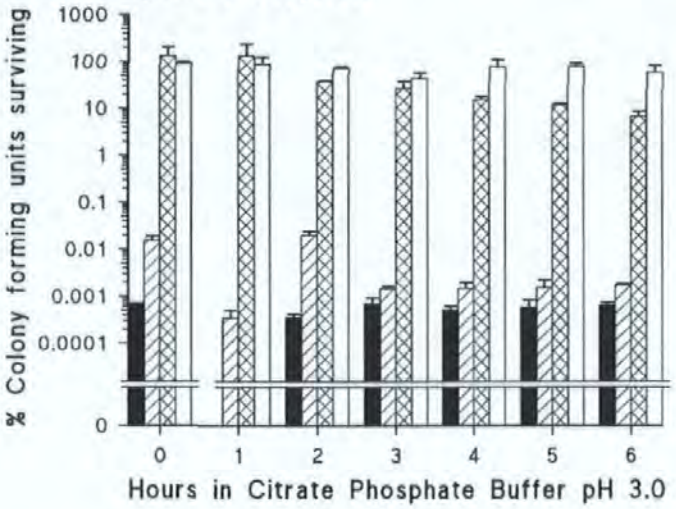


Fig 3.3.7 Part B pH 4.0

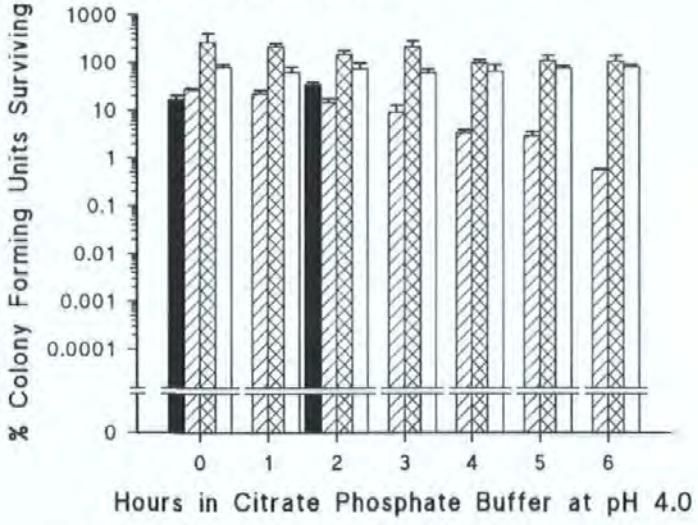


Fig 3.3.7 Part C pH 5.0

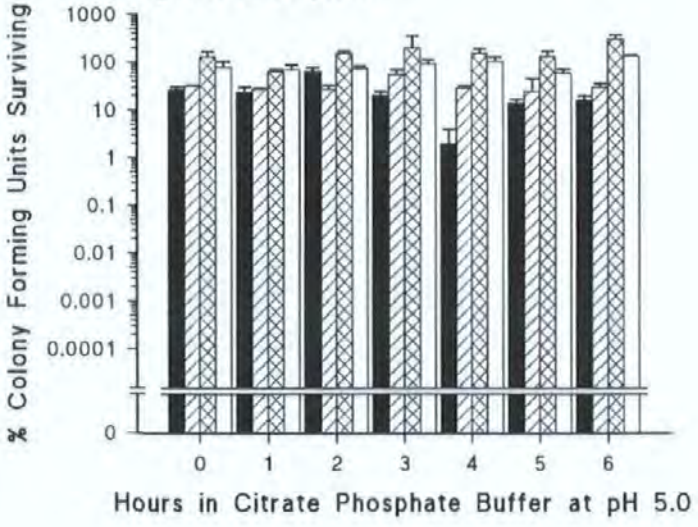


Fig 3.3.8 Part A *A.salmonicida* 644rB

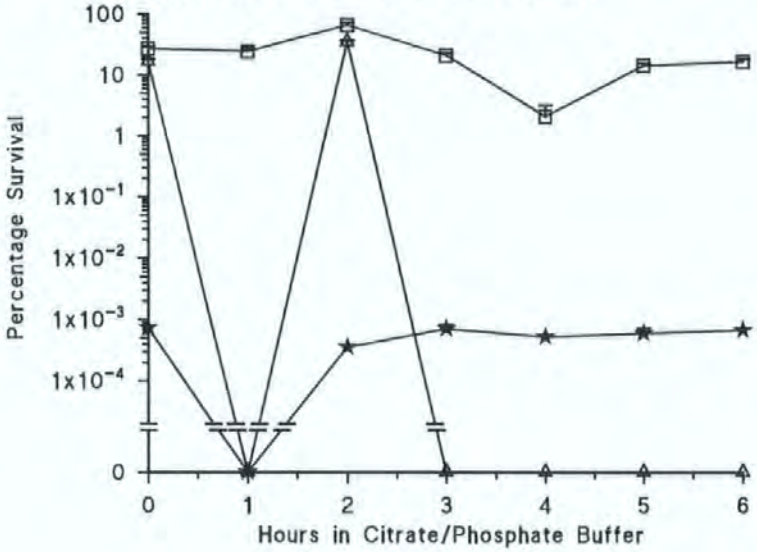


Fig 3.3.8 Part B *A.salmonicida aroA*

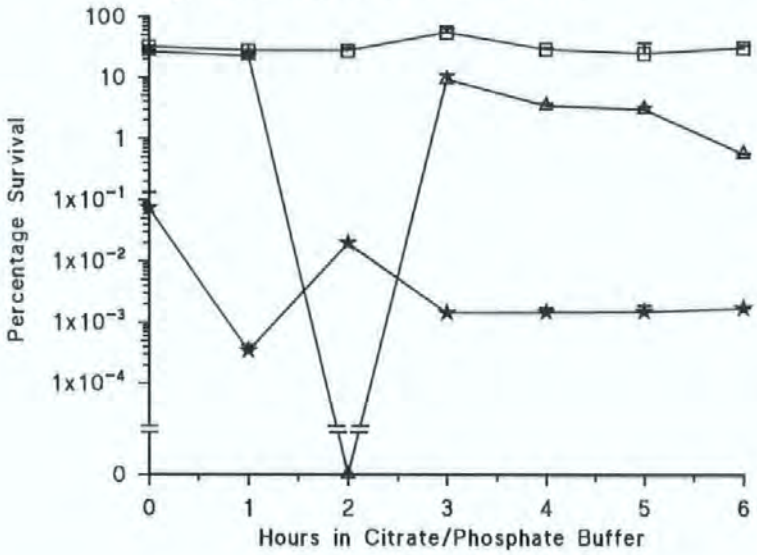


Figure 3.3.8 The percentage survival of *A. salmonicda* grown to late log phase.

The percentage survival of ***A. salmonicida*** *644rB* (A) *and* ***A. salmonicida*** *644rB* ***aroA****::kan*^{r} (B) *following upto six hours exposure to citrate/phosphate buffer pH 3.0* (★), *pH 4.0* (▲), *pH 5.0* (■). *In both cases the bacteria were grown in TSB for 74 hours prior to inoculation of the buffer. The error bars represent* SDn-1 *(n=3).*

Fig 3.3.9 Part A *A.salmonicida* 644rB

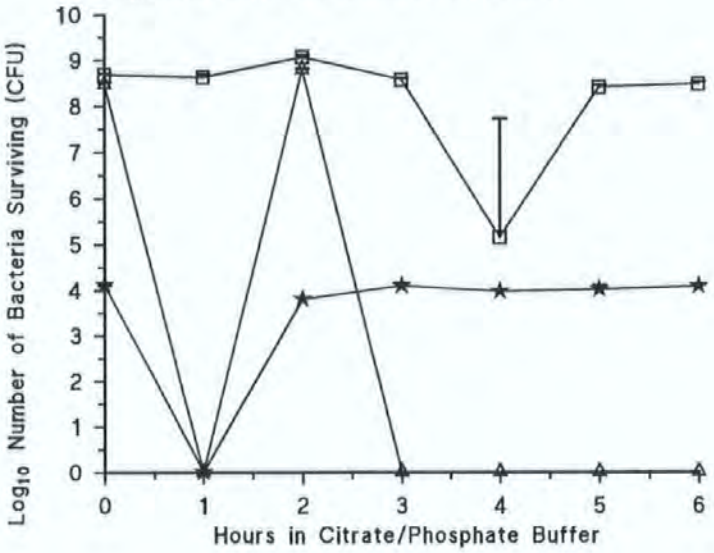


Fig 3.3.9 Part B *A.salmonicida aroA*

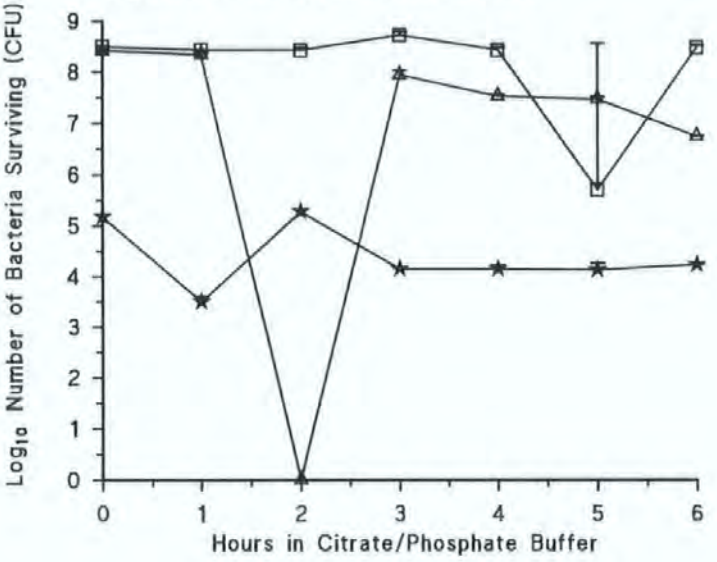


Figure 3.3.9 The number of surviving *A. salmonicida* after growth to late stationary phase.

The number of ***A. salmonicida*** *644rB* (A) *and* ***A. salmonicida*** *644rB* ***aroA****::kan*^{r} (B) *surviving upto six hours exposure to citrate/phosphate buffer pH 3.0* (★), *pH 4.0* (▲), *pH 5.0* (■). *In both cases the bacteria were grown in TSB for 74 hours prior to inoculation of the buffer. The error bars represent* SDn-1 *(n=3).*

Fig 3.3.10 Part A *A.salmonicida* 644rB

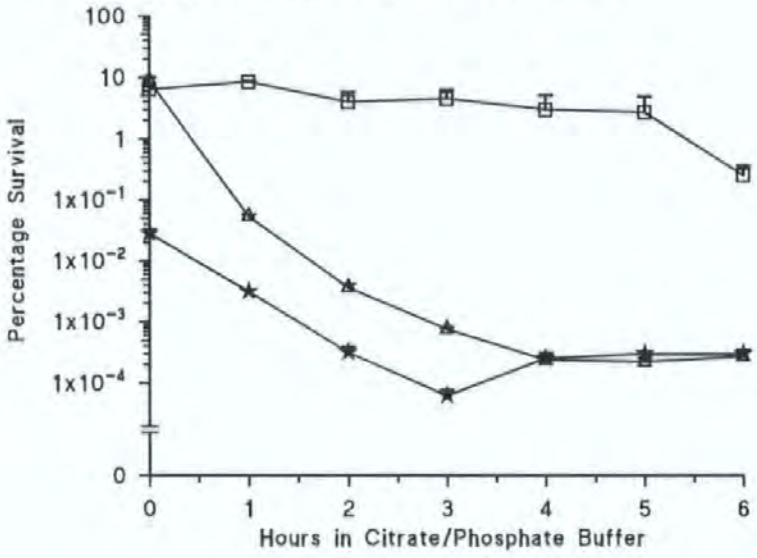


Fig 3.3.10 Part B *A.salmonicida aroA*

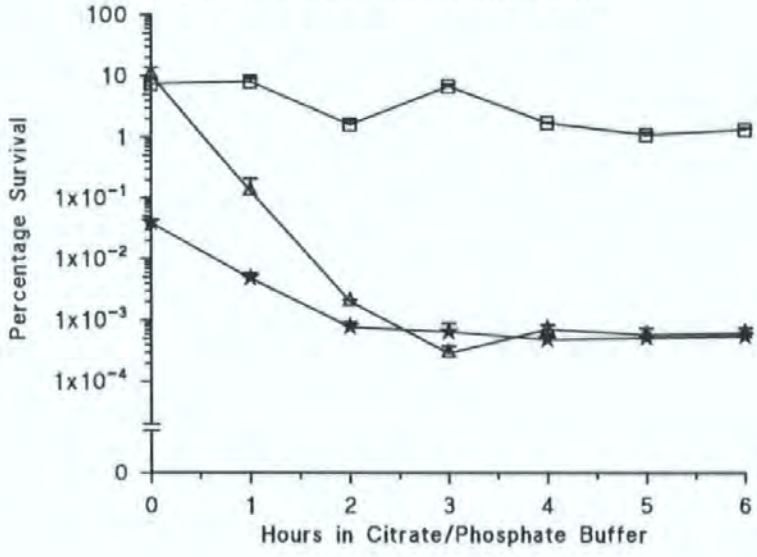


Figure 3.3.10 The effect of growth in BHIB of the percentage survival of *A. salmonicida*. *The percentage survival of* ***A. salmonicida*** *644rB* (A) *and* ***A. salmonicida*** *644rB* ***aroA****::kan*^{r} (B) *following upto six hours exposure to citrate/phosphate buffer pH 3.0* (★), *pH 4.0* (▲), *pH 5.0* (■). *In both cases the bacteria were grown in Brain heart infusion broth for 70 hours prior to inoculation of the buffer. The error bars represent* SDn-1 *(n=3).*

Fig 3.3.11 Part A *A.salmonicida* 644rB

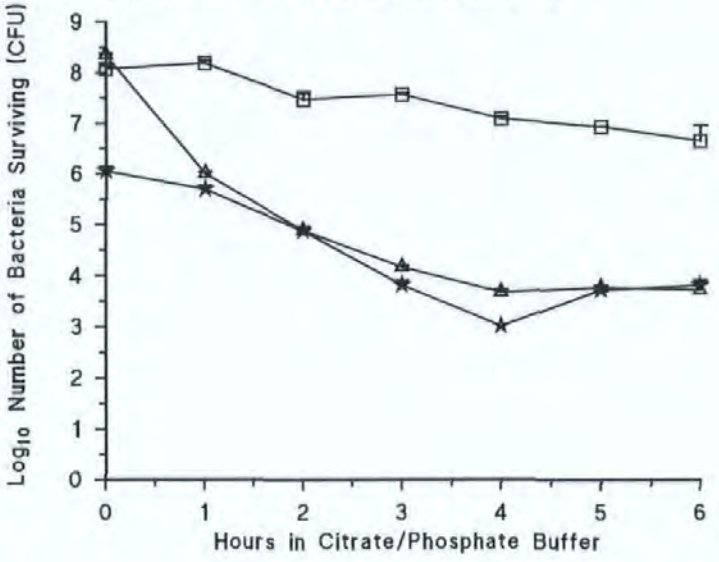


Fig 3.3.11 Part B *A.salmonicida aroA*

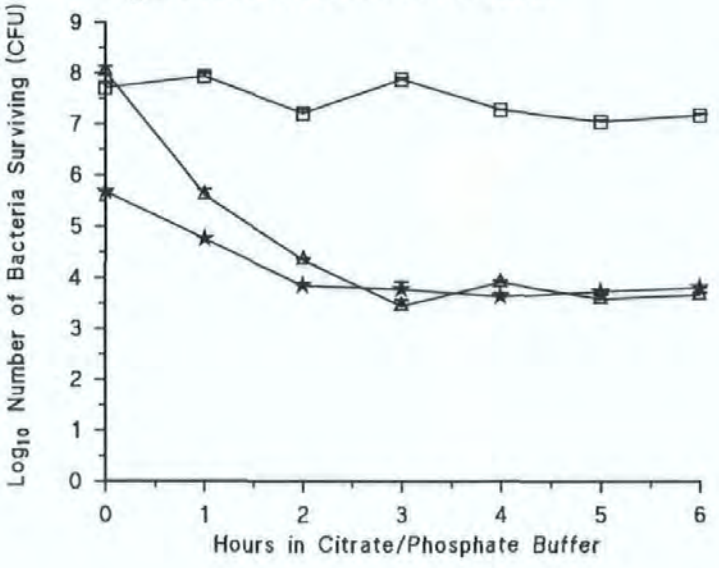


Figure 3.3.11 The effect of growth in BHIB on the number of bacteria surviving.

The number (CFU) of ***A. salmonicida*** *644rB* (A) *and* ***A. salmonicida*** *644rB* ***aroA****::kan*^{r} (B) *surviving upto six hours exposure to citrate/phosphate buffer pH 3.0* (★), *pH 4.0* (▲), *pH 5.0* (■). *In both cases the bacteria were grown in Brain heart infusion broth for 70 hours prior to inoculation of the buffer. Error bars represent* SDn-1 *(n=3).*

Fig 3.3.12 Part A *A.salmonicida* 644rB

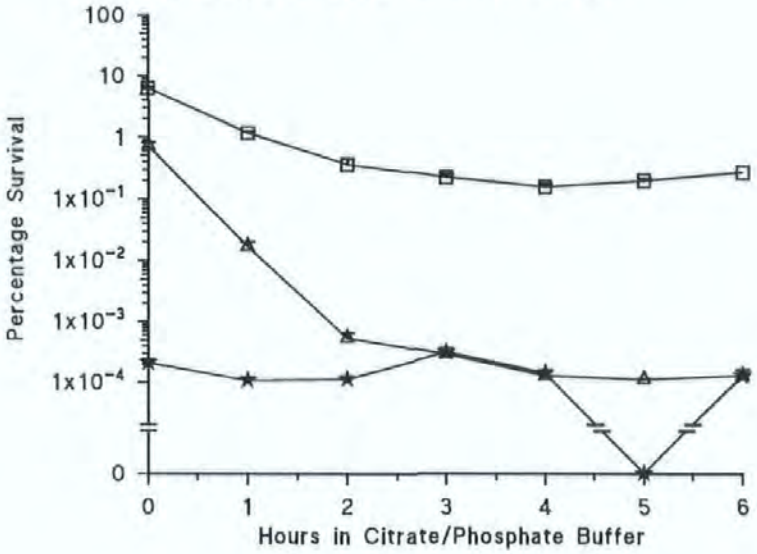


Fig 3.3.12 Part B*A.salmonicida aroA*

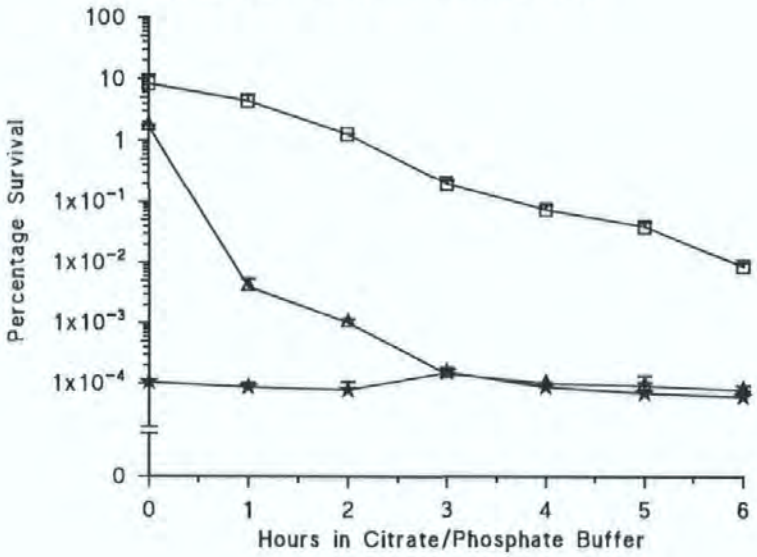


Figure 3.3.12 The effect of growth in a shaking incubator of the percentage survival.

The percentage survival of ***A. salmonicida*** *644rB* (A) *and* ***A. salmonicida*** *644rB* ***aroA****::kan*^{r} (B) *following upto six hours exposure to citrate/phosphate buffer pH 3.0* (★), *pH 4.0* (▲), *pH 5.0* (■). *In both cases the bacteria were grown in TSB for 72 hours shaken at 100rpm in a shaking incubator prior to inoculation of the buffer. Error bars represent* SDn-1 *(n=3).*

Fig 3.3.13 Part A *A.salmonicida* 644rB

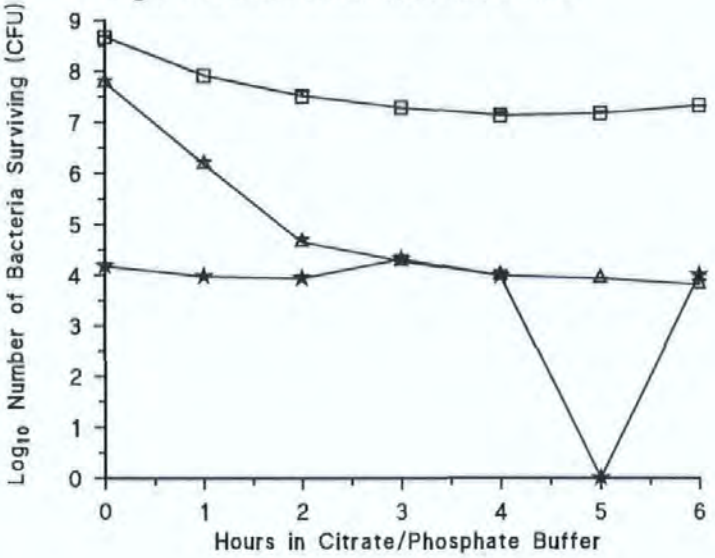


Fig 3.3.13 Part B *A.salmonicida aroA*

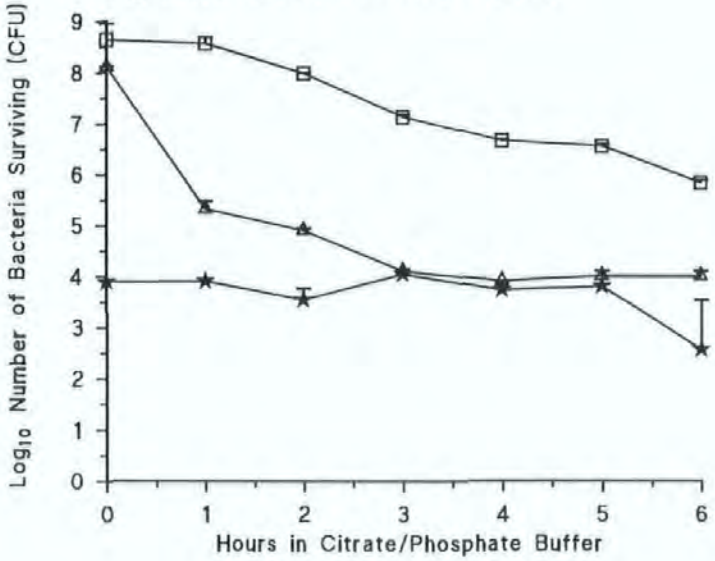


Figure 3.3.13 The effect of growth in a shaking incubator on the number of bacteria surviving.

The number (CFU) of ***A. salmonicida*** *644rB* (A) *and* ***A. salmonicida*** *644rB* ***aroA****::kan*^{r} (B) *surviving upto six hours exposure to citrate/phosphate buffer pH 3.0* (★), *pH 4.0* (▲), *pH 5.0* (■). *In both cases the bacteria were grown in TSB for 72 hours shaken at 100rpm in a shaking incubator prior to inoculation of the buffer. Error bars represent* SDn-1 *(n=3).*

Fig 3.3.14 Part A *A.salmonicida* 644rB

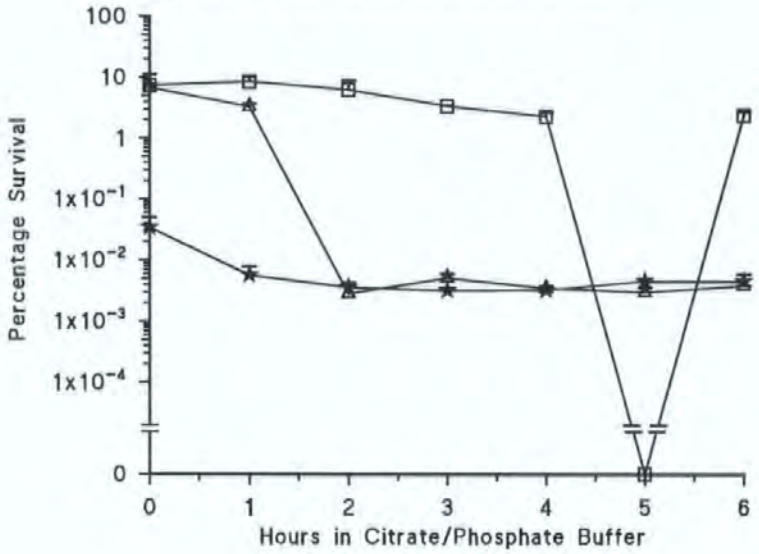


Fig 3.3.14 Part B *A.salmonicida aroA*

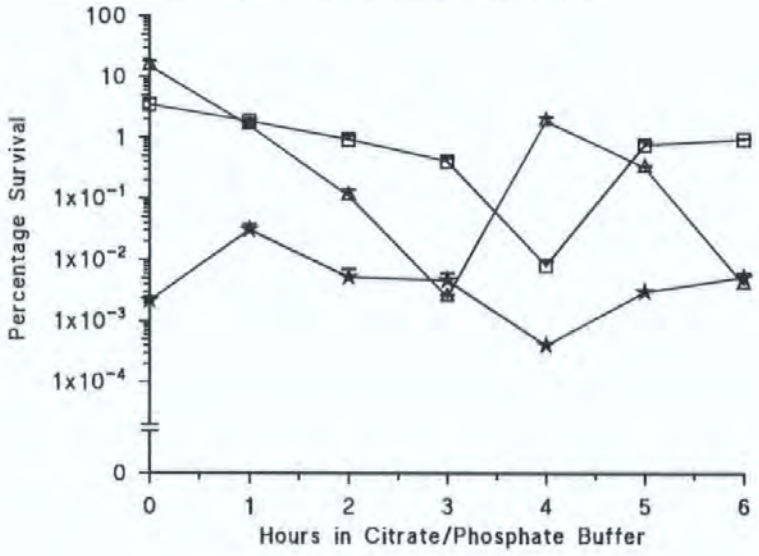


Figure 3.3.14 The effect of growth at pH6.0 on the percenatge survival.

The percentage survival of ***A. salmonicida*** *644rB* (A) *and* ***A. salmonicida*** *644rB* ***aroA****::kan*^{r} (B) *following upto six hours exposure to citrate/phosphate buffer pH 3.0* (★), *pH 4.0* (▲), *pH 5.0* (■). *In both cases the bacteria were grown inTSB acidified to pH6.0 for 48 hours then transfered to fresh TSB pH 6.0 for for 72 hours prior to inoculation of the buffer. Error bars represent* SDn-1 *(n=3).*

Fig 3.3.15 PartA *A.salmonicida* 644rB

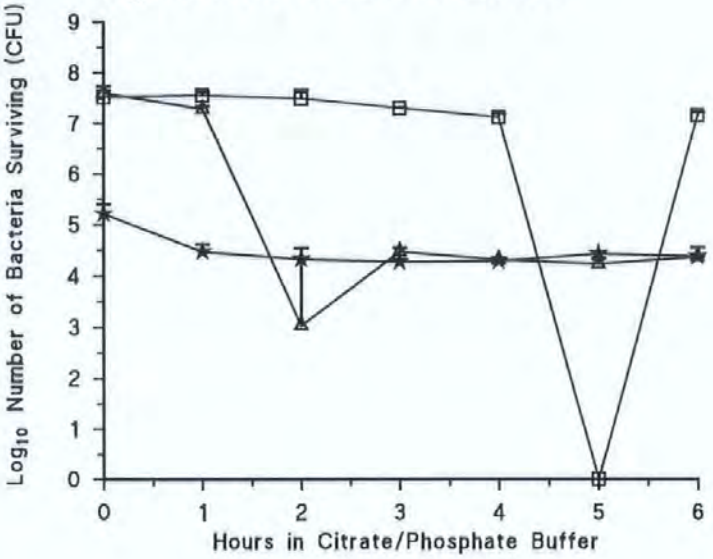


Fig 3.3.15 Part B *A.salmonicida aroA*

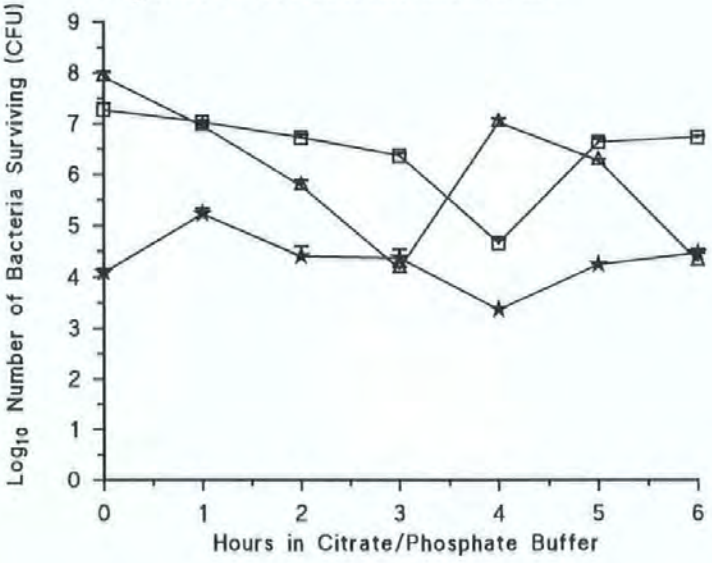


Figure 3.3.15 The effect of growth at pH 6.0 on the number of bacteria surviving.

The number (CFU) of ***A. salmonicida*** *644rB* (A) *and* ***A. salmonicida*** *644rB* ***aroA****::kan*^{r} (B) *surviving upto six hours exposure to citrate/phosphate buffer pH 3.0* (★), *pH 4.0* (▲), *pH 5.0* (■). *In both cases the bacteria were grown inTSB acidified to pH6.0 for 48 hours then transfered to fresh TSB pH 6.0 for for 72 hours prior to inoculation of the buffer. The error bars represent* SDn-1 *(n=3).*

Fig 3.3.16 Part A *A.salmonicida* 644rB

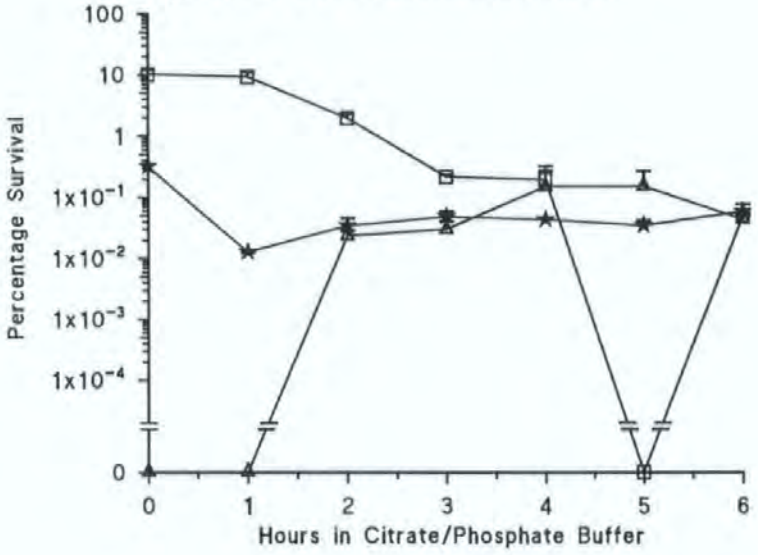


Fig 3.3.16 Part B *A.salmonicida aroA*

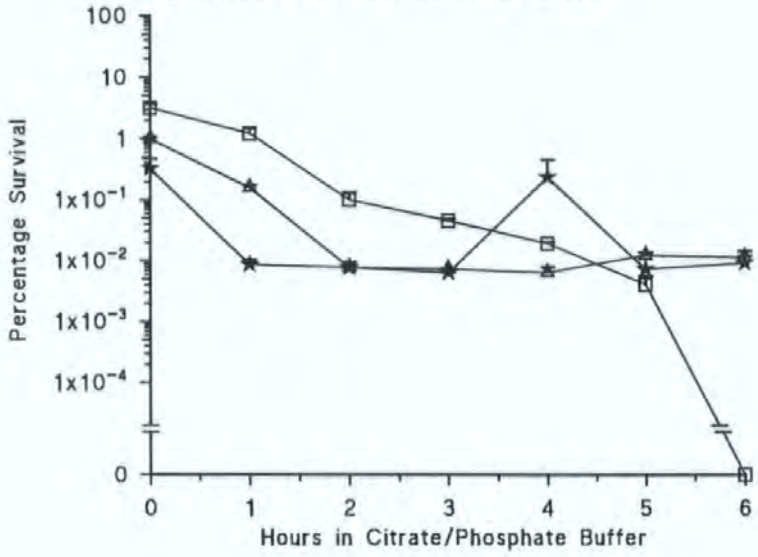


Figure 3.3.16 The effect of growth at pH 5.5 on the percentage of bacteria surviving.

The percentage survival of ***A. salmonicida*** *644rB* (A) *and* ***A. salmonicida*** *644rB* ***aroA***::*kan*^{r} (B) *following upto six hours exposure to citrate/phosphate buffer pH 3.0* (★), *pH 4.0* (▲), *pH 5.0* (■). *In both cases the bacteria were grown inTSB acidified to pH6.0 for 48 hours then transfered to TSB acidified to pH 5.5 for for 48 hours followed by a further growth cycle to late stationary phase in TSB pH 5.5 prior to innoculation of the buffer. Error bars represent* SDn-1 *(n=3).*

Fig 3.3.17 Part A *A.salmonicida* 644rB

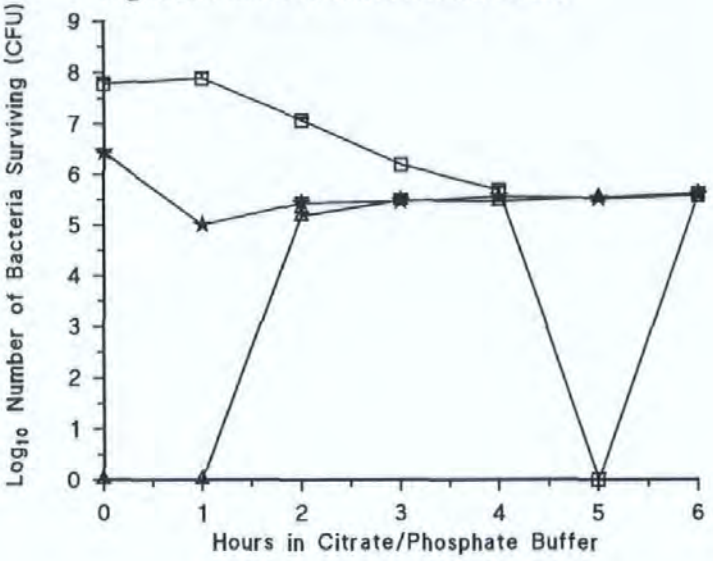


Fig 3.3.17 Part B *A.salmonicida aroA*

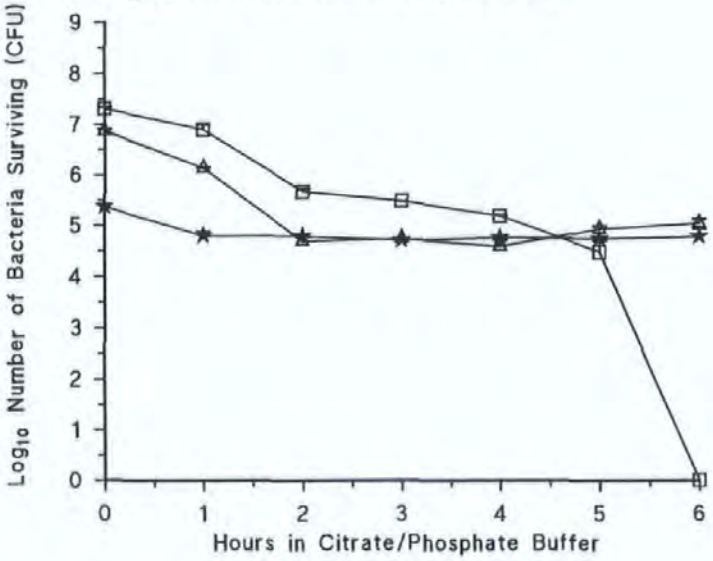


Figure 3.3.17 The effect of growth at pH 5.5 on the number of bacteria surviving.

The number (CFU) of ***A. salmonicida*** *644rB* (A) *and* ***A. salmonicida*** *644rB* ***aroA****::kan*^{r} (B) *surviving upto six hours exposure to citrate/phosphate buffer pH 3.0* (★), *pH 4.0* (▲), *pH 5.0* (■). *In both cases the bacteria were grown inTSB acidified to pH6.0 for 48 hours then transfered to TSB acidified to pH 5.5 for for 48 hours followed by a further growth cycle to late stationary phase in TSB pH 5.5 prior to inoculation of the buffer. The error bars represent* SDn-1 *(n=3).*

Fig. 3.3.18 Part A *A.salmonicida* 644rB

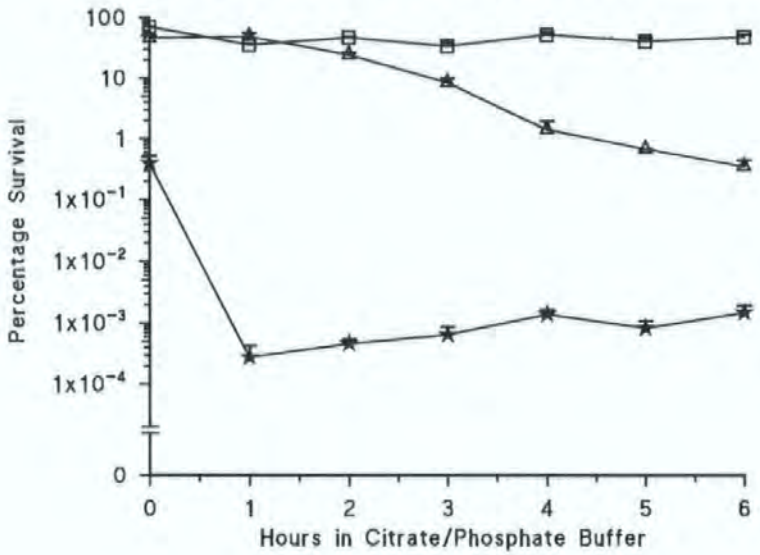


Fig. 3.3.18 Part B *A .salmonicida aroA*

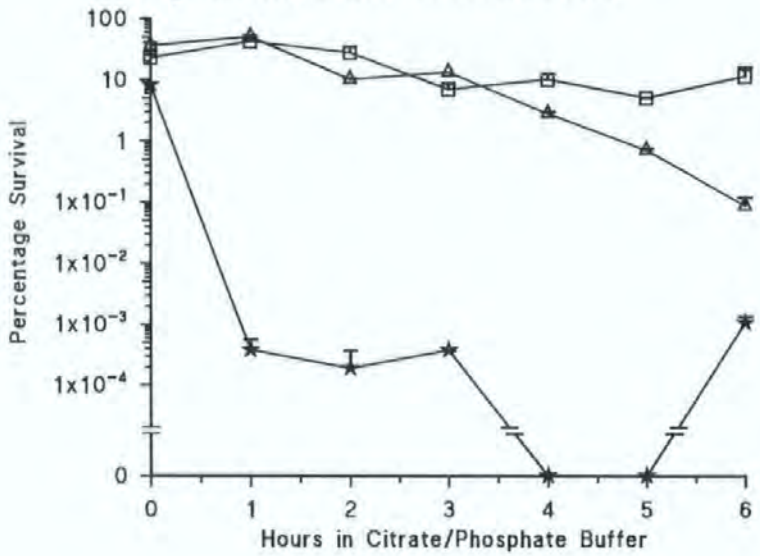


Figure 3.3.18 The effect exposure to low temperature and low nutrient avaliability on low pH resistance.

The percentage survival of ***A. salmonicida*** *644rB* (A) *and* ***A. salmonicida*** *644rB* ***aroA****::kan*^{r} (B) *following upto six hours exposure to citrate/phosphate buffer pH 3.0* (★), *pH 4.0* (▲), *pH 5.0* (■). *In both cases the bacteria were grown inTSB for 72 hours then transfered to PBS at 10 °C for 24 hours prior to inoculation of the buffer. Error bars represent* SDn-1 *(n=3).*

Fig 3.3.19 Part A *A.salmonicida* 644rB

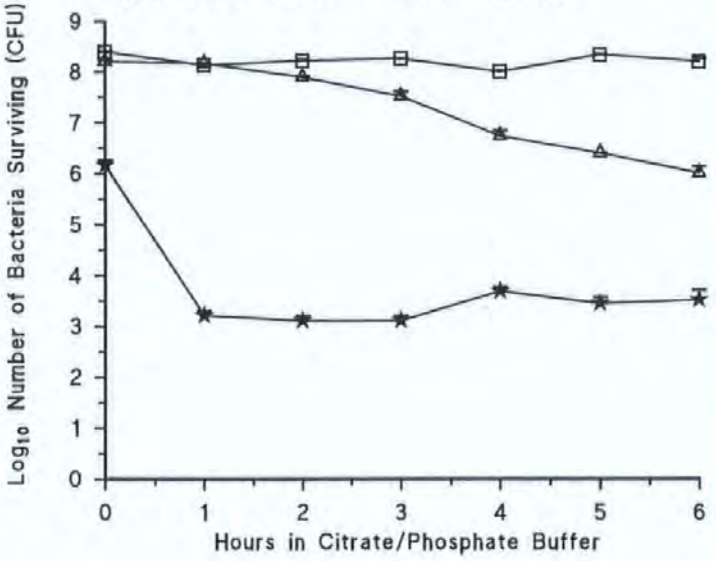


Fig 3.3.19 Part B*A.salmonicida aroA*

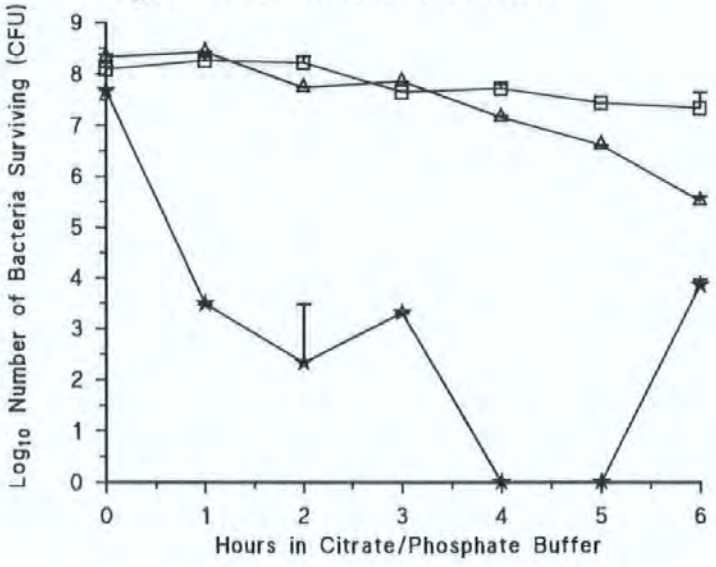


Figure 3.3.19 The effect of growth at low temperature and low nutrient availability on the number of bacteria surviving.

The number (CFU) of ***A. salmonicida*** *644rB* (A) *and* ***A. salmonicida*** *644rB* ***aroA****::kan*^{r} (B) *surviving upto six hours exposure to citrate/phosphate buffer pH 3.0* (★), *pH 4.0* (▲), *pH 5.0* (■). *In both cases the bacteria were grown inTSB for 72 hours then transfered to PBS at 10 °C for 24 hours prior to inoculation of the buffer.Error bars represent* SDn-1 *(n=3).*

3.4 Discussion.

The use of live auxotrophic bacteria as oral vaccines in mammals is a relatively new technique (Hioseth and Stocker, 1981), and to the author's knowledge the work presented in this thesis represents the first attempt to use the *aroA* mutant of *A. salmonicida* as an oral vaccine in Rainbow Trout.

Previous work on the oral delivery of soluble antigens and bacterins has shown that the gastric acid and gastrointestinal enzymes, represent an important barrier to successful immunisation (Johnson and Amend, 1983 a; b, Jenkins, 1992; Lavelle, 1994). The effect that the gastric barrier would have on immunisation with live bacteria was uncertain and this work has attempted to show, using an *in vitro* model system how damaging the stomach acid would be to live *Aeromonas* cells.

The usefulness of *in vitro* models of gastrointestinal function was demonstrated by Lavelle (1994). He looked at the effect of gastric and intestinal enzymes on soluble protein antigens, and showed that the gastric enzymes represented a barrier to successful immunisation with soluble antigens. Furthermore, he also demonstrated *in vivo* that the administration of gastric inhibitors protected antigens from proteolysis thus confirming the prediction of the model system.

It was unclear if live *Aeromonas salmonicida* cells would require similar protection or if they would be able to resist the acidic environment in the stomach. Parker (1985) had shown that *A. salmonicida* would not grow in broth at pH less than 5.5 but he did not investigate the survival of the bacteria in the inoculum over time and so no data was available on the effect of low pH on the survival of this species. Whipple and Rohovec (1994) investigated the procedure for processing fish viscera for incorporation into fish food. As part of this work they looked at the ability of *A. salmonicida* to withstand exposure to

citrate/phosphate buffer at pH 4.0. They found that the bacteria was undetectable after just 90 minutes at 22°C.

It was decided to investigate the effect of stomach acid and not to investigate the effect of stomach enzymes for two reasons: firstly, the literature suggested that the acid was the most important anti-bacterial component of the gastric environment (Garrod, 1939; Felsen and Osofsky, 1937; Giannella *et al.*, 1973; Gorden and Smal, 1993); and secondly, the investigation of the effect of trout gastric enzymes would require the sacrificing of a great many experimental animals which invalidates one of the most important reasons for using an *in vitro* model, the reduction in the number of animals and their replacement with other techniques.

It was apparent that there would be a requirement for an accurate and reliable method for rapid enumeration of viable bacteria and the use of spectrophotometry provided the most convenient means of achieving this. Parker (1985) had used A_{590} of bacterial culture to estimate the number of viable bacteria, this calibration curve may have been good enough for the purposes of this work, however, it was decided to produce a new standard curve using the using the aromatic mutant of *A. salmonicida* 644rB and the wild type. Initially, the attempt involved monitoring the growth of culture of the two strains every two hours for 48 hours. At each time bacteria were removed from the culture and one aliquot was diluted and counted using the spread plate technique and another was measured for absorbance at 590 nm. This standard curve that did not give reproducible results. This was because the Absorbance took many hours to increase above that of the blank followed by a very short period of increase before the A_{max} was reached, resulting in too few points on the calibration curve.

To produce the spectrophotometric standard curve cultures of both the wild type and the aromatic mutant were grown for 48 hours and washed twice by centrifugation and resuspension in PBS following the third centrifugation the samples were resuspended in either a very small volume of TSB or PBS. In this way concentrated cultures were produced which

were then diluted and the standard curves shown in Fig 3.3.1 were produced. Testing of these two calibration curves showed that the PBS curve gave the most reproducible results even though the regression equation for this line was not as good a fit as the one for the TSB curve. This was thought to be due to the difference in the nature of PBS and TSB.

The growth kinetic curves produced in this way were also not satisfactory due probably to experimental error brought on by fatigue (as the method required sampling every two hours for 48 hours) and also having too many things to do at one time. However, they provided a good estimate of the kinetics of *A. salmonicida* growth which proved very useful when estimating the timing and minimum number of sampling points required to give a growth kinetic curve. The kinetic curve shown in Fig 3.3.2 was produced over 72 hours but readings were concentrated at those times.

The growth curves for both strains of *Aeromonas* and the strains of *E. coli* and *Y. ruckeri* were used to establish time points during the growth cycle that would have bacteria in different metabolic states. Both *E. coli* and *Y. ruckeri* are known enteric organisms of mammals and fish respectively. They were, therefore, expected to be resistant to low pH to some extent. Furones (1990) showed that a strain of *Y. ruckeri* would grow at pH as low as 4.5 in BHIB modified with HCl. Since the route of infection for *A. salmonicida* has not been established with any certainty, no assumptions were made as to its ability to withstand acid stress.

Following the work of Gorden and Small (1993) on *Shigella* it was decided to use 1% survival after 6 hours exposure as the minimum for a bacteria to be described as resistant to treatment at pH 3.0, 4.0 or 5.0.

Other than the wild type strain of *A. salmonicida* at log and early stationary phase the data indicates that all of the species of bacteria used could withstand 6 hours exposure to a pH of 5.0 at all of the growth phases tested. This result was expected and this was why pH 5.0 was chosen as the upper pH limit.

Both *A. salmonicida* strains were undetectable at pH 3.0 in lag, log and early stationary phases. The aromatic mutant survived pH 3.0 during mid-stationary phase for 3 hours, interestingly, the wild type was not detected at this growth phase. At late stationary phase both the wild type and the aromatic mutant were able to survive pH 3.0 for 6 hours even though this was a very small percentage of the inoculum (0.0007 and 0.002, respectively). Thus neither strain could be described as resistant to exposure to pH 3.0.

Survival of the *A. salmonicida* strain at pH 4.0 was also dependant on growth phase. In lag and log phase survival was poor, in early stationary phase both *Aeromonas* strains survived for 3 hours at pH 4.0, at mid stationary phase the *aroA* strain survived 6 hours but the wild type was detected for only 2 hours. This survival pattern was repeated during late stationary phase. It is unclear why there should be a difference between the resistance of the *aroA* strain and the wild type but as the levels of surviving bacteria were very low the wild type may have been present but have remained undetected.

The results for *E. coli* and *Yersinia ruckeri* were very different to the results of *A. salmonicida* resistance. *E. coli* was sensitive to pH 3.0 during lag and log phase only and *Y. ruckeri* was sensitive during log phase and early stationary phase. At pH 4.0 and 5.0 both species survived very well and in several instance appeared to be dividing.

Since *A. salmonicida* grown in TSB was clearly sensitive to the levels of acid that are commonly found in the stomach of rainbow trout (Lavelle, per. com.), and since in late stationary phase the bacteria become more resistant it was decided to attempt to alter the environmental conditions during the growth of the bacteria to seen if acid resistance could be induced.

Parker (1985) found that by growing *A. salmonicida* in BHI rather than TSB its ability to bind to macrophages was increased and similarly McCarthy (1983) showed that there was an apparent increase in virulence if *A. salmonicida* was grown in BHIB rather than TSB. This was possibly due to the fact that BHI is derived from animal matter and TSB is derived from

plant matter. This may mean that there substances in the BHIB of unknown nature that affected the bacteria. BHIB was not used routinely for culturing *A.* salmonicida because of the potential health risk of handling large amounts of media derived from nervous tissue and offal derived from cattle.

If BHIB does increase virulence and or attachment by *A. salmonicida* it may because growth in this media more accurately models growth of the bacteria *in vivo* for instance in a furuncle. If natural infection of fish occurs via the oral route the bacteria might prepare itself by inducing acid tolerance or adopting an enhanced state of readiness to cope with low pH. Bacteria were grown to late stationary phase in BHIB to investigate this possibility, however, the results of this investigation showed that there was no increase in acid tolerance and in fact at pH 4.0 both *Aeromonas* strains were more sensitive than when grown in BHI compared with growth in TSB.

It is known that bacteria can detect and respond to environmental conditions (Karem and Foster, 1993). Oxygen levels have been shown to have an impact on the acid resistance of different bacteria (Karem and Foster, 1993; Gorden and Small, 1993). Often acid resistance correlates with anaerobiosis, cultures were grown with shaking to see whether acid tolerance decreased. It was found that no appreciable difference could be seen in *A. salmonicida 644* rB but in the *aroA* mutant there was a decrease in the survival at pH 3.0, 4.0 and 5.0. This may represent a change due to increased oxygen concentration or because it was only seen in the auxotrophic mutant it may represent a decrease in the ability to survive due to increased growth rate because the bacteria were grown with shaking.

The growth of *A. salmonicida* in TSB at pH 6.0 and 5.5 increased survival at pH 3.0 by 10 and 100 fold, respectively. Interestingly, there was a decrease in the ability of both strains to survive at pH 5.0 when grown at pH 5.5. Since it was necessary to grow both strains at pH 6.0 prior to induce growth at pH 5.5 it would appear that some form of adaption is taking place. This may involve changes in DNA topology or in the expression of outer

membrane proteins for instance changes in the proton pump complement or it may reflect a change the internal complement of proteins. Alternatively, and perhaps most likely it involves a combination of these factors.

In *Salmonella* environmental influences affect DNA topology, it is well known that DNA gyrase plays a major role in the creation and maintenance of negative supercoiling in the cell. Additionally, DNA gyrase activity is dependent on the ratios of ATP and ADP (Westerhoff *et al.*, 1988). Recently, several environmental stresses that affect DNA topology, have been shown to affect ATP/ADP ratios (Hsieh *et al.*, 1991, a; b). It was found that in *E. coli* undergoing salt shock, both supercoiling and the ATP/ADP ratio increase initially before dropping to a steady state level higher than that seen in low salt cultures. Similarly, anaerobic growth yielded a transient drop in both supercioling and ATP/ADP ratios before increasing and eventually reaching levels higher than those seen under aerobic conditions (Hsieh *et al.*, 1991 a). It is possible that changes in external pH, and thus ΔpH, alter the proton motive force which consequently alters the production of ATP through the Mg^{2+}- dependant H^+-translocating ATPase (Karem and Foster, 1993).

Foster and Hall (1990) found that the synthesis of 18 proteins is affected during the ATR (12 increased and 6 decreased) although it was not clear which were actually required for acid tolerance. Analysis of mutants produced good evidence that at least some were required for the adaptive response. Foster *et al.*, (1994) stated that in *S. typhimurium* the expression of at least 43 proteins is increased following acid shock and that at least some of them are essential for survival at low pH. These data suggest that the regulation of pH resistance is a complex multifactorial process which needs to be investigated at the molecular level.

Since *in vivo* bacteria may be released from furuncles into salt or fresh water which would be low in nutrients it decided to model this by growing bacteria to late stationary phase and transferring them to PBS. It was further decided to hold the bacteria in the PBS at 10°C

to model low temperature water. This treatment did not seem to affect the ability off the bacteria to withstand low pH exposure.

The investigation has shown that *Aeromonas salmonicida* is apparently much more sensitive to low pH than either *E. coli* and *Y. ruckeri*. It is possible that this reflects the nature of the bacteria both the latter species are specialist enteric commensals/pathogens and the ability to withstand low pH may be essential in achieving colonisation of the host. This does not mean that *A. salmonicida* does not infect fish via the oral route merely that it is not specialised to do so. This would make it similar to both *Salmonella spp* and *Vibrio cholerae* which are both sensitive to low pH.

Furthermore, it may be that it is not important for *A. salmonicida* to be acid resistant because it is protected during its time in the stomach by other factors ingested at the same time. This is supported by the finding by Rose (1990) that salmon could be infected orally by intra gastric intubation of diluted furuncle material which is high in protein which may protect the bacteria from the acid. Furthermore, whilst the percentage survival to 6 hours was poor this represents 10,000 CFU surviving the gastric barrier.

The increase in resistance seen in both strains of *A. salmonicida* during the growth cycle may reflect an adaptation to virulence ie bacteria in the water column are likely to be in stationary phase and thus it would be advantageous to the bacteria to be prepared for ingestion if in stationary phase. Alternatively, it may represent a change in the expression or activity of membrane proteins or an accumulation of secondary metabolites within the cell that protect it from the low pH.

Finally, the approach adopted provided an *in vitro* means of assessing the ability of bacteria to survive the gastric barrier. The next chapter deals with the *in vivo* fate of bacteria following intra gastric intubation and the results of the two chapters will be discussed together in the general discussion below. In conclusion the results of this work indicate that *A. salmonicida* will probably need protection from the gastric environment.

Chapter Four.

The uptake and tissue localisation of *Aeromonas salmonicida* aromatic deficient mutant.

4.1 Introduction.

The development of new live vaccines has been constrained by, among other factors, fears that an attenuated pathogen might revert to virulence (Germanier, 1984; Winther and Dougan 1984). Modern genetic techniques can now be used to construct stable, genetically defined, attenuated bacterial strains suitable for widespread use (Dougan *et al.*, 1988). The use of such organisms as vaccines and as carrier vehicles for delivery of heterologous antigens is an approach being increasingly applied in mammals (reviewed by Chatfield *et al.*, 1992, 1993; Dougan, 1994) and more recently in piscine species (Leong, 1993).

Research into the use of attenuated bacterial species as live vaccines and as carriers for mammalian hosts is ongoing. The species under investigation include *Yersinia enterolytica* (Van Damme *et al.*, 1992), *E. coli* (Newland *et al.*, 1992), *Bordetella pertussis* (Roberts *et al.*, 1990), *Bacillus anthracis* (Ivins *et al.*, 1990), *Shigella flexneri* (Verma and Lindberg, 1991) and *Pasteurella haemolytica* (Homchampa *et al.*, 1992) but the organism at which most effort has been directed is *Salmonella*. This is because apart from *E. coli*, *Salmonella* is the most genetically defined of bacterial species and genetic transfer between the two species is relatively easy to accomplish. DNA manipulation and characterisation work can therefore be carried out in *E coli* and the DNA introduced into *Salmonella* (Chatfield *et al.*, 1993)

Salmonella infections remain a serious world health problem. These pathogens form a closely related group of species that can cause a variety of diseases, ranging from localised gastro-enteritis to disseminated infections involving organs of the mononuclear phagocyte system (MPS), i.e. the spleen, liver, lymph nodes and bone marrow. In man, *Salmonella typhi*, the cause of typhoid is the most dramatic example of invasive salmonellosis although other salmonella species can cause systemic infections. Work on the development of new typhoid vaccines has shown that live attenuated salmonella strains can elicit protective immunity and induce secretory, humoral and cellular anti-*Salmonella* responses in the host (Chatfield *et al.*, 1992, 1993). The

lack of a realistic small mammalian model for typhoid infection has meant that much work has been done with other *Salmonella* species such as *S. typhimurium, S. dublin* and *S. enteriditis*. These strains cause invasive infections in mice that resemble typhoid in humans (Collins, 1971; Collins, 1974; Hormaeche, 1979). The use of the murine model has allowed the development of several rationally attenuated salmonella strains that have the potential to protect against typhoid and other salmonella infections that can also be used for the oral delivery of heterologous antigens.

Early attempts to attenuate salmonella involved the use of mutants of *S. typhi* that were dependant on streptomycin for growth *in vitro* (Reitman, 1967; Mei, *et al.*, 1974). The human oral vaccine strain Ty21a (Gilman *et al.*, 1977) was produced by chemical mutagenesis and while much useful information was gained from this strain it has subsequently been superceeded by strains produced using more defined genetic techniques. The most exhaustive studies have focused on the production of genetically defined auxotrophic mutants. Following observations made in the 1950's (Bacon *et al.* 1950; Bacon *et al.*, 1951); Hoiseth and Stocker (1981) described the production of *S. typhimurium* strains harbouring transposon insertions in the *aro*A gene that codes for 5-enolpyruvylshikimate-3-phosphate synthase. Salmonella *aro*A strains were unable to synthesis aromatic amino acids and proved to be highly attenuated in mice and were shown to be an excellent single dose oral vaccines against salmonellosis (Chatfield *et al.*, 1992,1993). Subsequently *aro*A mutants of *Salmonella* have been tested extensively in mice (Killar and Eisenstien, 1985; O'Callaghan *et al.*, 1988; Stocker *et al.*, 1982), calves (Stocker *et al.*, 1982; Smith *et al.*, 1984) and sheep (Mukkur *et al.*, 1987) and found to be attenuated and able to induce protective immunity.

Much of the work has been done, using the murine model organism BALB/c, these mice have a well established susceptibility to salmonella infection. The LD_{50} for salmonella in an intravenous challenge is $<10^2$ and for oral challenge *c.* 10^6. Dougan *et al.* (1988) showed that mice orally vaccinated with a dose of 10^{10} double *aro* mutant (*aro*A and *aro*C) were well

protected against the highest challenge dose of $1.2x10^{10}$ organisms which is extremely high, (the LD_{50} for the challenge strain was between $5.01 x10^{6}$ and $3.16 x10^{7}$ organisms). Jones *et al.* (1991) showed that oral vaccination with 10^{10} organisms (*aro*A, *aro*D mutants) of *S. typhimurium* to calves provided a significant degree of protection against an oral challenge with 10^{8} organisms. The development and effectiveness as a vaccine of an aromatic dependant mutant *A. salmonicida* was dealt with in Chapter One. In brief, Vaughan *et al.* (1993) described the development of an aromatic *aroA* mutant of *A. salmonicida* and they showed that when injected IP into brown trout there was a significant increase in serum bacterial agglutination titre and in the LD_{50} when vaccinated fish were challenged by IM injection with a virulent bacteria.

Marsden *et al.* (1996) also using an *aroA* mutant of *A. salmonicida* found that IP immunisation caused a dose dependent increase in specific antibody titre and demonstrated that the vaccine induced significantly greater proliferation of T rather than B cells.

Lavelle (1994) described that both this *aro*A strain and the parental strain 644rB seemed capable of inducing cytoskeletal rearrangements within enterocytes isolated from the intestine of rainbow trout. These rearrangements appeared to cause the bacteria to be internalised by the enterocytes. This phenomenon, termed invasion, was discussed in detail in Chapter Two and the experimental investigation of *A. salmonicida* invasion is the subject of Chapter Five, therefore, the subject of will not be dealt with at length here. However, it should be noted that the ability of an orally delivered live bacterial vaccine to invade the epithelial cells of its host and gain access to the viscera is very desirable. This is because it raises the possibility of producing a relevant immune response at the mucosal surfaces and systemically. Many of the vaccination experiments carried out with aromatic deficient mutants of *S. typhimurium* have relied on this dual nature. This strain of *A. salmonicida* appeared to have an invasive capability and has been shown to be an effective immunogen when given IP, suggesting that it might be suitable as an oral vaccine and a means to deliver foreign antigens to salmonids.

Since the first successful oral vaccination of salmonids against furunculosis was reported by Duff (1942) a very large amount of research effort has been directed at achieving a cheap and effective oral vaccine. These attempts have at best had mixed results, Sniesko and Friddle (1949), Udey and Fryer(1978) and Michel (1980) all failed to induce protective immunity by oral vaccination. Using IP injection of vaccines Spence *et al.* (1965) and Paterson and Fryer (1974) demonstrated protection, while Michel (1980) and Palmer and Smith (1980) could not. Direct immersion and hyperosmotic procedures have been shown to provide effective immunisations by Cipriano and Starliper (1982).

It has been proposed that *A. salmonicida* can enter the host at each of the body surfaces; i.e. gill, gut and skin (Ellis, 1981; Munro, 1984; Hodgkinson *et al.*, 1987). Studies have indicated the importance of the skin (Kanno *et al.*, 1989), and the gills (Alexander *et al.*, 1985; Effendi and Ausitn, 1995) as sites of bacterial uptake. The theory that *A. salmonicida* can infect fish via the oral route had been the subject of much debate. Blake and Clarke (1931), Markwardt and Klontz (1989) and Rose (1990) have described infection in salmonids via the oral route, while others (Krantz *et al.*, 1964; McCarthy, 1977) have not been able to demonstrate infection via this route. Treasurer and Laidler (1994) showed that cleaner wrasse kept in sea cages with Atlantic salmon became infected with *A. salmonicida*. Infection apparently followed the consumption of dead infected salmon by the wrasse showing that this species, at least, can be orally infected.

Hodgkinson *et al.* (1987) showed that the physiological state of the bacterial cells was an important factor in their uptake into fish. Growth of bacteria in nutrient deficient broth resulted in the much higher numbers of bacterial cells being found within the tissues of the fish when compared to the number found when bacteria were grown in the normal broth. The bacteria were presented by immersion in a suspension containing 10^4 CFU cm^{-3} and were detected in the fish tissues within 2 minutes. The effect of growth condition and the rapidity of internalisation indicates that the entry of bacterial cells into the fish is a process in which the bacteria plays an

active part and may suggest that stress can increase the expression of proteins necessary for invasion.

Work with *Salmonella* has shown that the ability to invade mammalian cell lines varies with oxygen availability, growth phase, and osmolarity (Ernst *et al.*, 1990; Galan and Curtiss, 1990; Lee and Falkow, 1990; Schiemann and Shope, 1991) these conditions are known to affect the superhelicity of bacterial DNA and this has been implicated in the global control of virulence determinant expression (Dorman *et al* 1990). Prolonged inhibition of bacterial protein synthesis has also been shown to effect invasivness (MacBeth and Lee, 1993) while protein synthesis *per se* has been shown to be unnecessary at the time of invasion (MacBeth and Lee, 1993; Galan and Ginocchio, 1994). This indicates that the invasive process is regulated in response to external conditions. The response of bacteria including *A. salmonicida* to low pH was dealt with, in Chapter Three above.

For an oral vaccine to be effective sufficient antigens must be delivered to the GALT to initiate an immune response at the mucosal surface. Alternatively, immunologically relevant quantities must be taken up into the viscera and exposed to the areas of the fish that are responsible for systemic immunity.

The aim of the work presented in this Chapter was to investigate the fate of *A. salmonicida aro*A delivered to rainbow trout. The first part of the investigation aimed to ascertain whether bacteria could be recovered from the gastrointestinal mucus, the kidney, spleen and liver, of fish immunised by oral and anal intubation and IP injection and to investigate the kinetics of bacterial uptake following immunisation.

The effect on uptake of prior administration of sodium bicarbonate to neutralise the stomach acid was investigated. Lavelle (1994) showed that sodium bicarbonate increased the uptake of intact HGG into blood. The effect on uptake of some environmental factors during the growth of the vaccine was investigated; as was the effect of administering the vaccine in TSB to provide nutrients; in β-glucans that have an immunopotentiating effect in rainbow trout and in

skimmed milk to see if the presence of protein could protect the bacteria from the stomach acid as suggested by Rose (1990). The effect of the dose of bacteria in the oral vaccine was also investigated.

4.2 Materials and Methods

4.2.1 Growth Media, Reagents and Chemicals.

Unless otherwise stated growth media were obtained from Oxoid (Basingstoke), all other reagents and chemicals were obtained from BDH (Poole, Dorset), Sigma Chemical Company (London) and Lab m (Toddington).

4.2.2 Bacterial Strains.

Two strains of *Aeromonas salmonicida*, Strain 644rB and its aromatic deficient mutant, 644rB *aroA*::Kan^r both are A layer+. The details of these strains were given above (Section 3.2.2).

4.2.3 Routine Culture of Bacteria.

Unless otherwise stated all bacteria were cultured as described above (Section 3.2.3).

4.2.4 Enumeration of Bacteria.

Unless otherwise stated viable bacteria were counted using the Miles and Misra technique (Miles *et al.*, 1938). Serial tenfold dilutions were made in PBS 0.5 cm^3 in 4.5 cm^3 and bacteria grown on TSA containing appropriate antibiotic (section 3.2.1 above) and incubated at 22°C for 48 hours prior to counting.

4.2.5 Rapid spectrophotometric enumeration of A. salmonicida.

The standard curves of absorbance 590nm against viable count of *A. salmonicida* colony forming units (CFU) suspended in PBS, as described above (Section 3.2.5) were used throughout this work.

4.2.6 Experimental animals.

Adult rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) 100 - 250 g of both sexes were maintained in a freshwater recirculating system at 14°C ± 1°C and fed to satiation on a commercial trout pellet preparation. Prior to infection with bacteria the fish were removed from

the stock aquaria and moved to the experimental aquaria which were freshwater recirculating systems at 15°± 1°C. Prior to infection they were starved for 48 - 72 hrs.

4.2.7 Growth and administration of bacteria as a vaccine for investigation into the fate of bacteria over a 72 hours time course.

Bacteria were routinely cultured as described above. Prior to intubation or injection the bacteria were grown for 48 hrs to mid stationary phase, washed by centrifugation at 2000*g* for 10 minutes, resuspended in PBS followed by centrifugation and resuspension in between 6 and 10cm^3 of PBS and enumerated using the rapid spectrophotometric method described above. The concentration of bacteria was adjusted to approximately 2.0×10^{10} CFUcm^{-3}.

The bacteria were administered orally via a soft silicon tube external diameter of about 2 mm attached to a 19*g* needle on a 5 cm^3 syringe. The tube was pushed between 4 and 5 cm into the oesophagus so that the end was situated in the stomach and 0.5 cm^3 of bacterial suspension was then expelled. The procedure for anal intubation was similar, a silicon tube, external diameter 1mm was attached to a 19*g* needle on a 5cm^3 syringe. The tube was inserted into the anus and pushed 3 to 4 cm into the rectum of the fish. 0.5cm^3 of bacterial suspension was then expelled. For the IP injection a 25*g* needle was pushed into the abdominal wall of the fish so that the tip of the needle was in the peritoneal space the needle was then tilted and pushed between 1.5 to 2.0 cm just under the internal surface of the abdominal wall. 0.5 cm^3 of bacterial suspension was expelled.

4.2.8 Sampling of fish over the 72 hour time course.

At 5, 15, 30, 60mins, 6, 12, 24, 48 and 72 hours, three fish per treatment were sacrificed by a sharp blow to the back of the head. Each fish was carefully dissected aseptically. A lateral incision was made with a scalpel approximately 3 cm in front of the pelvic fins. Using scissors a longitudinal incision was made running from the anus to the membrane separating the abdominal organs from the heart.

The spleen was removed from the surrounding adipose tissue and between 0.1 and 0.4g of tissue was placed into sterile snap-top microcentrifuge tubes. A portion of the liver was dissected and about the same mass of tissue was placed into another tube. A clamp was fixed on the stomach as close as possible to the head end and using scissors the stomach was cut away from the oesophagus. The gastrointestinal tract was removed from the body cavity. Adipose tissue was dissected away as was the remaining liver and bile duct. The stomach was cut away from the intestine immediately distal to the pyloric sphincter. The gastric mucus was squeezed carefully from the stomach by compressing the stomach between two fingers and passing the fingers along the stomach from the oesophageal end to the pyloric end. Mucus was squeezed out through the pyloric sphincter into a sterile bijou bottle. The intestinal mucus was removed by gently but firmly squeezing the intestine between two fingers and running them along the intestine from the pyloric end so that the mucus was forced out through the anal end into another sterile bijou bottle. The kidney sample was removed by carefully dissecting away the membranes covering the tissue and then scooping out a portion of head kidney using a spatula. This tissue was placed into another snap-top microcentrifuge tube.

All the samples were weighed. The organ samples were then broken down using a micropestle (Eppendorf). When some tissue disruption had occurred 0.5 cm^3 of sterile saline containing appropriate concentrations of kanamycin and nalidixic acid was added to the tissue and when no further disruption occurred another 0.5cm^3 of saline was added.

Disruption of the extracted tissue by incubation in collagenase over night at 20°C was also used. However it proved impossible to isolate bacteria following incubation and so this method was not pursued.

To the mucus samples 5.0 cm^3 of saline/antibiotics was added and mixed using a whirly mixer (Fisons).

The number of bacteria in each sample was calculated by colony count carried out as described, with the modification that 140 mm plastic Petri dishes were used to plate the samples

on. This allowed 45 drops to be placed on each plate, this was the equivalent to the number required for three dilutions each plated in triplicate for all of the sites sampled per fish.

4.2.9 The effect of prior administration of sodium bicarbonate.

To examine the effect of prior administration of sodium bicarbonate on the fate of orally delivered bacteria, 1 cm^3 of a 35mg cm^{-3} solution of sodium bicarbonate was orally administered to the fish 1 hour prior to oral intubation with bacteria. The sodium bicarbonate solution was given in exactly the same way as described for the oral delivery of bacterial suspension above. Bacteria were grown and delivered as described previously. Fish were killed and samples taken between 6 hours and 72 hours post oral intubation.

4.2.10 The recovery of bacteria following administration of 10^8 CFU following the oral administration of sodium bicarbonate.

Bacteria were grown, washed, enumerated and administered as described previously except that they were diluted to a concentration of $2x10^8$ CFU cm^{-3} in saline. Fish were killed and samples taken 12 hours after intubation.

4.2.11 The effect of growth medium on the fate of orally delivered bacteria.

Following the routine weekly subculturing of bacteria 1 cm^3 of bacterial suspension was transferred to furunculosis broth, brain heart infusion broth, 10% normal strength TSB and TSB pH adjusted to 6.0. Bacteria were grown unshaken for 48 hours at 22°C, and then washed by centrifugation as described above. The numbers of bacteria were again adjusted to $2x10^{10}$ CFUcm^3 and administered to the fish as described above 1 hour after administration of sodium bicarbonate. Fish were sacrificed and tissue and mucus sampled 12 hours after oral intubation.

4.2.12 The effect of oxygen availability during culturing on the fate of orally delivered bacteria.

Bacteria were grown in TSB in an anaerobic jar (GasPak, BBL) for 72 hours or in TSB shaken at 100 rpm for 48 hours. In both cases bacteria were grown at 22°C. The cultures were washed, resuspended and delivered as described above 1 hour after oral administration of sodium bicarbonate solution.

4.2.13 The effect of bacterial growth phase on the fate of orally delivered bacteria.

Bacteria were grown in TSB at 22°C unshaken for either 12 hours to mid log phase or 72 hours to late stationary phase. Bacteria were washed, resuspended, enumerated and orally delivered to the fish as described above 1 hour after oral administration of sodium bicarbonate. Fish were sacrificed and tissue and mucus samples taken 12 hours after oral intubation.

4.2.14 The effect of the delivery medium on the fate of orally delivered bacteria.

Bacteria were grown, washed and enumerated as described above. The bacteria were administered in one of three ways either in a 50% v/v UHT milk/saline solution with no prior administration of sodium bicarbonate, in a 10% w/v glucan/saline suspension with no prior administration of sodium bicarbonate or in TSB following oral administration of sodium bicarbonate.

4.2.15 The identification of *Aeromonas salmonicida aroA* colonies on the tryptone soya agar plates.

The number of colonies that had grown to countable size within 48 hours were counted and assumed to be *A. salmonicida;* the plates were then left for several days to see if the colony morphology (circular, buff convex colonies about 1 to 2mm in diameter) and to see if the characteristic brown diffusible pigment was produced. Occasionally, colonies were removed and grown on Coomassie Blue agar to confirm the identification of *A. salmonicida.*

4.3 Results.

4.3.1 The uptake and localisation of *Aeromonas salmonicida aroA* following intubation and injection of rainbow trout with a suspension of live bacteria.

The orally intubated fish were sampled at 5, 15, 30, and 60 minutes and 6, 12, 24, 48 and 72 hours. Anally intubated fish were not tested at 15 minutes and the IP fish were not tested at 15 minutes, or 12 and 72 hours. The kidney, spleen, liver, gastric and intestinal mucus of the orally intubated fish was tested at each time point. The gastric mucus of the anally intubated fish was only tested after 24 hours and the gastrointestinal mucus of IP injected fish was not tested. Tables 4.3.1 to 4.3.3 show the numbers of viable bacteria recovered from each site in CFU g^{-1} of sample for individual fish. Fig 4.3.1 (A, B, C and D) shows the mean numbers of bacteria recovered per group at each time point.

Between five minutes and six hours a steady increase in the recovery of bacteria from the organs of the orally intubated fish was found. From twelve to seventy-two hours very few fish were found to have viable bacteria in their organs. Bacteria were found in the intestinal mucus of orally intubated fish in large numbers from 15 minutes but recovery was sporadic throughout the 72-hour time course. The highest recovery of bacteria from the gastric mucus occurred at 15 minutes and again bacteria were recovered until 48 hours but this was very variable.

The anally intubated fish showed similar pattern of uptake and tissue localisation as the orally intubated fish. Higher numbers of bacteria were recovered from the anally intubated fish compared with the orally intubated group between 5 minutes and 6 hours. However, after six hours virtually no bacteria recovered from the organs of anally intubated fish, whereas, some orally intubated fish had quite high numbers of bacteria in the spleen and liver even after 48 hours. No bacteria were recovered from the gastric mucus of the anally intubated fish. Bacteria were found in the intestinal mucus of orally and anally intubated fish at 72

hours but at most time points higher numbers were recovered from the mucus of anally intubated fish.

Bacteria were recovered from the organs of most of the IP injected fish up to and including 48 hours. The numbers of bacteria recovered were always higher in this group than either of the other two groups. The IP injected fish were not tested at 72 hours because in preliminary investigations mortalities occurred after 48 hours. Occasionally mortalities also occurred in the anally and orally intubated fish. The data of bacterial recovery from these fish were not included in the results as they were invariably very high and obviously anomalous. In these cases the test was repeated with new fish. While the gastrointestinal mucus of the IP injected fish was not tested for bacteria as part of the experiment, mortalities were found to have very high numbers of bacteria in their intestinal mucus. The mortalities in the intubated group usually showed extensive intestinal inflammation and evidence of the destruction of the intestinal epithelium.

4.3.2 The effect of prior oral administration of sodium bicarbonate on the uptake and localisation of *Aeromonas salmonicida aroA* following oral intubation.

Bacteria administered orally without bicarbonate were taken up by rainbow trout and found to localise in most tissue samples between five minutes and six hours. Therefore, the effect of prior administration of sodium bicarbonate was only investigated from six to seventy-two hours. Table 4.3.4 shows the numbers of bacteria recovered per gram of sample from each fish tested and shows the data from the orally intubated group that did not receive sodium bicarbonate for comparison. Fig. 4.3.2 (A, B, C and D) compares the uptake and localisation of bacteria in the kidney, spleen liver and intestinal mucus; from the oral and anally intubated groups described above with that of the group given sodium bicarbonate.

At six hours the sodium bicarbonate did not appear to enhance the uptake of the bacteria into the tissues. However, far higher numbers were recovered from the gastrointestinal mucus of all of the fish in this group compared with the other oral group.

From twelve hours on the recovery of bacteria from the group given bicarbonate was usually greater than that from either the anal or oral (without bicarbonate) groups. At 72-hours viable bacteria were recovered from all of the sites tested except the intestinal mucus.

4.3.3 The effect of dose on the uptake and localisation of bacteria following oral delivery.

The effect of dose was investigated at twelve hours post immunisation for two reasons it was a convenient time point and very few bacteria were recovered at this time point following oral immunisation without bicarbonate. No bacteria were recovered from fish orally intubated with 10^8 CFUs without bicarbonate. If the same dose was given after the administration of sodium bicarbonate bacteria were recovered from all of the samples taken. The numbers recovered were far greater than those of fish given 10^{10} CFUs without bicarbonate. The numbers recovered from the kidney and spleen were very similar to those of fish given 10^{10} CFU and bicarbonate but the numbers of bacteria recovered from the liver, and gastrointestinal mucus was greater in the latter group. The data for individual fish is presented in Table 4.3.5 and the comparison of the 10^8 and 10^{10} CFU with bicarbonate are compared in Fig. 4.3.3.

4.3.4 The effect of growth media on the uptake and localisation of orally delivered bacteria.

The effect of growth in furunculosis broth, TSB pH 6.0, BHIB, 10% TSB and normal TSB on the uptake and localisation of bacteria at twelve hours post immunisation was investigated. Growth conditions did not appear to affect the uptake or localisation of bacteria. No bacteria from the TSB pH 6.0 group were found in the gastric mucus. Bacteria from all groups were found in the kidney, spleen and liver of at least some fish. The bacteria grown in 10% TSB were found in the tissues in the lowest numbers.

4.3.5 The effect of oxygen availability during growth on the uptake and localisation of bacteria.

Bacteria were grown unshaken, in an anaerobic chamber and shaken 125 rpm. All were grown at the same temperature. The uptake and localisation of the three groups were compared at twelve hours after intubation. The bacteria grown unshaken were present in the organs of the fish in the greatest numbers. Bacteria grown anaerobically were only found in the liver and the gastrointestinal mucus. Bacteria grown with shaking were not isolated from any of the organs but bacteria were present in large numbers in the gastrointestinal mucus. The data for individual fish is presented in Table 4.3.7 and the means for each group are presented in Fig.4.3.5.

4.3.6 The effect of growth phase on the uptake and localisation of bacteria.

Bacteria were grown either for 12 hours to mid log phase, for 48 hours to mid stationary phase or for 72 hours to late stationary phase. The effect of growth phase on uptake and localisation was investigated at twelve hours post immunisation. Bacteria were only found in the kidney of only one fish from the mid log phase group but were found in the spleen and liver of the other fish. One individual had very high numbers of bacteria in the spleen and liver. The numbers of bacteria in the organs fish immunised with late stationary phase bacteria were very similar to the mid stationary phase group. The data for individual fish is presented in Table 4.3.8 and the means for each group are presented in Fig.4.3.6.

4.3.7 The effect of delivery system on the uptake and localisation of bacteria.

The effect of administration of bacteria suspended in either 50% (v/v) skimmed milk or ß-glucan without bicarbonate and TSB or saline with bicarbonate on the uptake and localisation of bacteria at 12 hours post immunisation was investigated. The suspension of bacteria in skimmed milk appeared to protect them from the gastric acid as well as sodium bicarbonate as tissue localisation was very similar to that of the saline + bicarbonate group, although no bacteria were recovered from the gastric mucus. Suspension in ß-glucan also

appeared to protect the bacteria as tissue localisation was again similar to the saline + bicarbonate group. The delivery of bacteria in TSB had a very marked effect on the uptake and localisation of the bacteria as each of the tissues contained in excess of 10^4 CFU per gram. There was no increase in the number of bacteria in the gastrointestinal mucus of this group. The data for individual fish is presented in Table 4.3.9 and the means for each group are presented in Fig.4.3.7.

4.3.8 Survival of bacteria in the gastric mucus.

Bacteria were found to survive in the gastric mucus of rainbow trout following delivery in saline with or without prior administration of sodium bicarbonate. This data is presented in Figs. 4.3.8 and 4.3.9. Bacteria were found to survive in the gastric mucus until at least 48 hours but not 72 hours after oral intubation in saline. The number of bacteria recovered was increased by prior administration of sodium bicarbonate, almost all of the fish tested had bacteria in the gastric mucus and bacteria were still found in the gastric mucus 72 hours after administration.

Table 4.3.1 **Number of viable *Aeromonas salmonicida aroA* (CFU) recovered per gram of tissue or mucus sample following oral intubation.** *Fish were administered with 10^{10} CFU in 0.5 cm^3 saline containing 40µg cm^{-3} kanamycin and 30 µg cm^{-3} nalidixic acid.*

Time	5 Minutes			15 Minutes			30 Minutes			60 Minutes			6 Hours		
Fish No.	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Kidney	$2.71x10^6$	$1.3x10^4$	0.00	$1.0x10^5$	$2.03x10^3$	$1.0x10^4$	0.00	$3.89x10^2$	$2.93x10^3$	$2.65x10^2$	$1.32x10^3$	$4.07x10^2$	$2.69x10^3$	$4.91x10^3$	$4.22x10^3$
Spleen	0.00	$1.83x10^4$	0.00	$4.44x10^3$	$8.42x10^3$	$1.13x10^3$	0.00	0.00	$2.09x10^2$	$1.22x10^4$	$1.13x10^4$	$1.93x10^4$	$7.39x10^3$	$1.11x10^4$	$6.8x10^3$
Liver	$2.14x10^6$	0.00	0.00	$4.23x10^3$	$8.64x10^2$	68.6	0.00	83.3	$9.31x10^2$	$7.8x10^3$	$8.79x10^3$	$9.56x10^3$	$7.06x10^3$	$1.2x10^4$	$8.81x10^3$
Stomach Mucus	$5.5x10^8$	$6.67x10^7$	0.00	0.00	$1.27x10^{10}$	$3.0x10^9$	0.00	0.00	$6.17x10^8$	0.00	0.00	$2.62x10^9$	0.00	$9.26x10^4$	0.00
Intestinal Mucus	0.00	$2.22x10^2$	0.00	$2.29x10^8$	$1.28x10^7$	0.00	0.00	0.00	$3.27x10^7$	0.00	0.00	0.00	0.00	$3.86x10^8$	0.00

Time	12 Hours			24 Hours			48 Hours			72 Hours		
Fish No.	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Kidney	0.00	0.00	0.00	40.7	52.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Spleen	0.00	0.00	0.00	0.00	0.00	0.00	$8.33x10^2$	0.00	$2.29x10^3$	0.00	0.00	0.00
Liver	0.00	0.00	0.00	0.00	0.00	0.00	0.00	$2.58x10^2$	0.00	0.00	0.00	0.00
Stomach Mucus	0.00	0.00	$2.66x10^5$	$5.97x10^6$	$1.0x10^6$	0.00	0.00	0.00	$1.46x10^7$	0.00	0.00	0.00
Intestinal Mucus	0.00	0.00	0.00	0.00	$5.7x10^9$	$5.560x10^6$	0.00	$4.17x10^4$	0.00	0.00	$5.05x10^4$	0.00

Table 4.3.2 **Number of viable *Aeromonas salmonicida aroA* (CFU) recovered per gram of tissue or mucus sample following anal intubation.** *Fish were administered with 10^{10} CFU in 0.5 cm^3 saline containing 40 μgcm^{-3} kanamycin and 30 μgcm^{-3} nalidixic acid.*

Time	5 Minutes			15 Minutes			30 Minutes			60 Minutes			6 Hours		
Fish No.	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Kidney	$1.11x10^4$	$8.56x10^2$	0.00	ND	ND	ND	98.00	$3.66x10^4$	$4.0x10^4$	$1.41x10^5$	$3.11x10^5$	$1.46x10^6$	$3.65x10^4$	$4.39x10^4$	$3.03x10^4$
Spleen	$1.75x10^4$	0.00	0.00	ND	ND	ND	0.00	$5.56x10^4$	$1.0x10^3$	$1.11x10^5$	$3.15x10^5$	$1.70x10^5$	$2.38x10^4$	$5.21x10^4$	$3.07x10^4$
Liver	$6.49x10^4$	$9.2x10^4$	0.0	ND	ND	ND	0.0	$5.77x10^5$	$1.79x10^3$	$7.94x10^4$	$7.29x10^4$	$1.0x10^4$	$4.29x10^4$	$5.21x10^4$	$2.93x10^4$
Stomach Mucus	ND	ND	0.00	0.00	ND	ND	0.00	0.00	ND	0.00	0.00	ND	0.00	ND	0.00
Intestinal Mucus	$7.04x10^9$	$9.26x10^9$	$5.26x10^9$	ND	ND	0.00	$9.77x10^8$	$1.67x10^8$	$6.77x10^8$	$3.65x10^{10}$	$4.12x10^{10}$	$2.80x10^{10}$	$2.82x10^{10}$	$4.62x10^{10}$	$1.17x10^{10}$

Time	12 Hours			24 Hours			48 Hours			72 Hours		
Fish No.	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Kidney	0.00	64.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Spleen	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Liver	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0	0.00	0.00	0.00	0.00
Stomach Mucus	0.00	0.00	ND	0.00	ND	0.00	0.00	0.00	ND	0.00	0.00	0.00
Intestinal Mucus	$3.85x10^7$	$5.0x10^6$	$8.39x10^3$	0.00	$6.2x10^4$	$2.5x10^8$	$1.27x10^8$	0.00	$9.62x10^6$	$8.54x10^3$	0.00	$3.1x10^6$

Table 4.3.3 **Number of live *Aeromonas salmonicida aroA* (CFU) recovered per gram of tissue or mucus sample following IP injection.**
Fish were administered 10^{10} CFU in 0.5 cm^3 saline containing 40 $\mu g cm^{-3}$ kanamycin and 30 $\mu g cm^{-3}$ nalidixic acid. with approximately ^{1010} CFU in 0.5 cm^3 saline.

Time	5 Minutes			15 Minutes			30 Minutes			60 Minutes			6 Hours		
Fish No.	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Kidney	$2.19x10^5$	$4.17x10^6$	$2.96x10^4$	ND	ND	ND	$1.72x10^7$	$9.24x10^6$	$6.67x10^3$	0.00	$1.28x10^7$	$1.25x10^5$	0.00	$3.50x10^5$	0.00
Spleen	0.00	$4.25x10^7$	0.00	ND	ND	ND	$1.3x10^8$	$6.83x10^5$	$9.88x10^3$	0.00	$3.13x10^7$	$1.57x10^7$	0.00	$1.41x10^8$	0.00
Liver	$7.02x10^6$	$9.55x10^6$	$3.75x10^6$	ND	ND	ND	$1.07x10^5$	$1.58x10^6$	$8.77x10^3$	0.00	$2.63x10^6$	$2.78x10^6$	0.00	$2.78x10^4$	0.00
Stomach Mucus	ND	ND	0.00	0.00	ND	ND	0.00	0.00	ND	0.00	0.00	ND	0.00	ND	0.00
Intestinal Mucus	0.00	ND	0.00	ND	ND	0.00	0.00	0.00	ND	0.00	0.00	0.00	0.00	ND	0.00

Time	12 Hours			24 Hours			48 Hours			72 Hours		
Fish No.	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Kidney	0.00	0.00	0.00	$5.83x10^5$	$3.37x10^7$	0.00	$8.33x10^3$	0.00	$1.12x10^5$	0.00	0.00	0.00
Spleen	0.00	0.00	0.00	$4.05x10^5$	$1.22x10^7$	0.00	$1.32x10^5$	0.00	$1.17x10^4$	0.00	0.00	0.00
Liver	0.00	0.00	0.00	$3.13x10^5$	$7.15x10^4$	$4.84x10^5$	$3.45x10^5$	0.00	$2.27x10^4$	0.00	0.00	0.00
Stomach Mucus	0.00	0.00	ND	ND	ND	0.00	0.00	0.00	ND	0.00	0.00	0.00
Intestinal Mucus	0.00	0.00	0.00	0.00	ND	ND	0.00	ND	0.00	0.00	ND	0.00

Table 4.3.4 **The effect on the number of live bacteria (CFU) recovered per gram of tissue or mucus sample following oral administration of sodium bicarbonate.**

*35 mg of sodium bicarbonate was given to each fish 1 hour prior to oral intubation with approximately 10^{10} CFU **A. salmonicida aroA**. Shaded values are taken from table 4.3.1 above for comparison.*

Time	6 Hours			12 Hours			24 Hours			48 Hours			72 Hours		
Fish No	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Kidney	0.00	$2.56x10^3$	$8.77x10^2$	$530x10^2$	72.5	$7.33x10^2$	0.00	$1.28x10^2$	75.8	0.00	$3.21x10^3$	$6.75x10^4$	$1.11x10^4$	50	$3.13x10^3$
Kidney	$2.69x10^3$	$4.91x10^3$	$4.22x10^3$	0.00	0.00	0.00	40.7	52.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Spleen	0.00	$1.3x10^2$	$2.38x10^2$	0.00	$1.75x10^2$	$3.7x10^3$	0.00	0.00	72.5	$1.14x10^5$	$7.18x10^4$	$8.25x10^4$	$6.39x10^3$	$4.42x10^4$	$6.83x10^3$
Spleen	$7.39x10^3$	$1.11x10^4$	$6.8x10^3$	0.00	0.00	0.00	0.00	0.00	0.00	$8.33x10^2$	0.00	$2.29x10^3$	0.00	0.00	0.00
Liver	0.00	0.00	$2.84x10^5$	66.7	$2.0x10^2$	$1.07x10^5$	$4.17x10^2$	$4.76x10^2$	$6.71x10^2$	$8.52x10^3$	$6.33x10^5$	$2.13x10^4$	$4.64x10^3$	$8.89x10^3$	$8.89x10^3$
Liver	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Stomach Mucus	$6.25x10^8$	$1.56x10^{10}$	$2.08x10^{10}$	$1.39x10^8$	$5.68x10^8$	$5.42x10^7$	0.00	0.00	0.00	0.00	$3.13x10^5$	$8.33x10^4$	$1.75x10^4$	$3.33x10^4$	$3.67x10^4$
Stomach Mucus	0.00	$9.26x10^4$	0.00	0.00	0.00	$2.66x10^5$	$5.97x10^6$	$1.0x10^6$	0.00	0.00	0.00	$1.46x10^7$	0.00	0.00	0.00
Intestinal Mucus	$8.72x10^8$	$2.79x10^8$	$7.25x10^8$	$7.18x10^{10}$	$1.59x10^{10}$	$9.26x10^{10}$	$4.23x10^8$	$5.95x10^9$	$3.35x10^9$	$6.17x10^8$	$8.33x10^7$	$8.33x10^8$	0.00	0.00	0.00
Intestinal Mucus	0.00	$3.86x10^8$	0.00	0.00	0.00	0.00	0.00	$5.69x10^9$	$5.56x10^6$	0.00	$4.17x10^5$	0.00	0.00	$5.05x10^4$	0.00

Table 4.3.5 **The number of viable bacteria (CFU) recovered per gram of tissue or mucus sample recovered 12 hours after oral intubation of fish with either 10^8 or 10^{10}.**

	Dose intubated and other treatment											
	10^8 CFU No Bicarbonate			10^8 CFU With Bicarbonate			10^{10} CFU No Bicarbonate			10^{10} CFU With Bicarbonate		
Fish No	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Kidney	0.00	0.00	ND	$3.89x10^2$	$8.05x10^2$	$4.67x10^2$	0.00	0.00	0.00	$5.3x10^2$	72.5	$7.33x10^2$
Spleen	0.00	0.00	ND	72.5	$6.25x10^2$	$4.84x10^2$	0.00	0.00	0.00	0.00	$1.75x10^2$	$3.7x10^3$
Liver	0.00	0.00	ND	$6.67x10^2$	$1.03x10^3$	$5.8x10^2$	0.00	0.00	0.00	66.7	$2.0x10^2$	$1.07x10^5$
Stomach Mucus	0.00	0.00	ND	$1.63x10^7$	$5.36x10^6$	$1.67x10^6$	0.00	0.00	$2.66x10^5$	$1.39x10^8$	$5.68x10^8$	$5.42x10^7$
Intestinal Mucus	0.00	0.00	ND	$6.05x10^8$	$7.09x10^7$	$2.71x10^8$	0.00	0.00	0.00	$7.18x10^{10}$	$1.59x10^{10}$	$9.26x10^{10}$

Table 4.3.6 **The effect of culture medium on the number of live bacteria *A. salmonicida aro A* (CFU) recovered from tissue of mucus samples 12 hours after oral intubation.**
The shaded values for immunisation with 10^{10} CFU in saline + bicarbonate were taken from Table 4.3.4.

	Furunculosis Broth			Brain Heart Infusion Broth			10% Normal TSB			TSB pH 6.0			10^{10}		
Fish No.	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Kidney	$4.0x10^2$	$1.33x10^2$	$5.56x10^2$	0.00	69.4	0.00	$1.26x10^2$	0.00	95.2	$2.38x10^3$	0.00	$1.67x10^3$	$5.3x10^2$	72.5	$7.33x10^2$
Spleen	0.00	$5.13x10^2$	$5.56x10^2$	0.0617	$1.15x10^2$	0.00	52.1	$1.45x10^2$	$3.7x10^2$	$7.58x10^2$	0.00	$1.39x10^2$	0.00	$1.75x10^2$	$3.7x10^3$
Liver	$3.79x10^2$	$2.08x10^2$	$1.15x10^3$	0.0595	0.00	$2.38x10^2$	95.2	$3.09x10^2$	0.00	$7.89x10^2$	$7.94x10^2$	$1.54x10^3$	66.7	$2.0x10^2$	$1.07x10^5$
Stomach Mucus	ND	$1.45x10^7$	0.00	$1.23x10^7$	$8.19x10^7$	$1.71x10^6$	0.00	$7.92x10^8$	$8.09x10^6$	0.00	0.00	0.00	$1.39x10^8$	$5.68x10^8$	$5.42x10^7$
Intestinal Mucus	ND	$1.11x10^7$	0.00	$2.92x10^7$	$2.98x10^8$	$1.74x10^9$	$4.35x10^9$	$4.87x10^{10}$	$2.23x10^{11}$	$1.9x10^9$	0.00	$2.08x10^6$	$7.18x10^{10}$	$1.59x10^{10}$	$9.26x10^{10}$

Table 4.3.7 **The effect of oxygen availability on the number of live bacteria (CFU) recovered from tissue of mucus samples 12 hours after oral intubation.** *Bacteria were either grown in an anaerobic jar, for 72 hours; in a vigorously shaking incubator, for 48 hours or unshaken for 48 hours. A dose of 10^{10} CFU* ***A. salmonicida aroA*** *following the administration of 35 mg of sterile sodium bicarbonate. The shaded values for immunisation with 10^{10} CFU in saline + bicarbonate were taken from Table 4.3.4.*

	Dose intubated and other treatment								
	Anaerobic			**Shaken 100 rpm + Bicarbonate**			**10 CFU + Bicarbonate**		
Fish No	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Kidney	0.00	0.00	0.00	0.00	0.00	0.00	$5.3x10^{2}$	72.5	$7.33x10^{2}$
Spleen	0.00	0.00	0.00	0.00	0.00	0.00	0.00	$1.75x10^{2}$	$3.7x10^{3}$
Liver	$1.03x10^{3}$	0.00	0.00	0.00	0.00	0.00	66.7	$2.0x10^{2}$	$1.07x10^{5}$
Stomach Mucus	$6.33x10^{7}$	$4.86x10^{4}$	0.00	0.00	$5.30x10^{9}$	$2.51x10^{10}$	$1.39x10^{8}$	$5.68x10^{8}$	$5.42x10^{7}$
Intestinal Mucus	$3.43x10^{9}$	$6.48x10^{7}$	$1.03x10^{8}$	$4.46x10^{10}$	$8.91x10^{9}$	$8.33x10^{9}$	$7.18x10^{10}$	$1.59x10^{10}$	$9.26x10^{10}$

Table 4.3.8 **The effect of growth phase on the number of live bacteria (CFU) recovered from tissue of mucus samples 12 hours after oral intubation.** *Fish were immunised with a dose of 10^{10} CFU **A. salmonicida aroA** following the administration of 35 mg of sterile sodium bicarbonate. The bacteria were grown in TSB for 12, 48 or 72 hours. The shaded values for immunisation with 10^{10} in saline + bicarbonate were taken from Table 4.3.4.*

	Growth Phase								
	Log Phase (12 hours) + Bicarbonate			Late Stationary Phase (72 hours) + Bicarbonate			Mid Stationary Phase + Bicarbonate		
Fish No	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Kidney	0.00	$1.15x10^2$	0.00	0.00	$1.22x10^3$	$2.5x10^2$	$5.3x10^2$	72.5	$7.33x10^2$
Spleen	$1.34x10^6$	0.00	$3.3x10^2$	0.00	$1.61x10^2$	$4.61x10^3$	0.00	$1.75x10^2$	$3.7x10^3$
Liver	$3.82x10^5$	$1.59x10^2$	$1.15x10^2$	$1.39x10^2$	98.0	$3.03x10^3$	66.7	$2.0x10^2$	$1.07x10^5$
Stomach Mucus	$7.38x10^8$	0.00	$6.25x10^8$	$4.84x10^8$	$1.07x10^9$	$7.85x10^9$	$1.39x10^8$	$5.68x10^8$	$5.42x10^7$
Intestinal Mucus	$7.33x10^9$	$1.98x10^{10}$	$9.43x10^9$	$1.27x10^{10}$	$4.81x10^9$	$7.85x10^9$	$7.18x10^{10}$	$1.59x10^{10}$	$9.26x10^{10}$

Table 4.3.9 The effect of delivery medium on the number of live bacteria (CFU) recovered from tissue of mucus samples 12 hours after oral intubation. *Fish were immunised with a dose of 10^{10} CFUs of **A. salmonicida aroA** following the administration of 35 mg of sterile sodium bicarbonate. The shaded values for immunisation with 10^{10} in saline + bicarbonate were taken from Table 4.3.4.*

Bacteria delivered in	50% v/v UHT Milk solution in saline No Bicarbonate			10%Glucan Suspension in sterile saline No Bicarbonate			Tryptone Soya Broth Following Bicarbonate			0.85% Saline Following Bicarbonate		
Fish No	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Kidney	$5.56x10^{2}$	0.00	$1.19x10^{2}$	0.00	69.0	$3.47x10^{2}$	$5.0x10^{4}$	$4.56x10^{4}$	$2.99x10^{4}$	$5.3x10^{2}$	72.5	$7.33x10^{2}$
Spleen	$5.56x10^{2}$	$4.76x10^{2}$	$2.22x10^{3}$	$4.55x10^{2}$	0.00	0.00	$3.45x10^{4}$	$4.0x10^{4}$	$1.98x10^{4}$	0.00	$1.75x10^{2}$	$3.7x10^{3}$
Liver	$7.58x10^{2}$	$1.11x10^{3}$	$8.73x10^{2}$	$1.28x10^{3}$	$1.07x10^{3}$	$7.02x10^{5}$	$3.88x10^{4}$	$4.27x10^{4}$	$1.59x10^{4}$	66.7	$2.0x10^{2}$	$1.07x10^{5}$
Stomach Mucus	0.00	0.00	0.00	0.00	0.00	$1.96x10^{3}$	0.00	$3.95x10^{9}$	0.00	$1.39x10^{8}$	$5.68x10^{8}$	$5.42x10^{7}$
Intestinal Mucus	$2.21x10^{8}$	$8.33x10^{7}$	$1.27x10^{9}$	$7.62x10^{7}$	$2.9x10^{8}$	$3.85x10^{7}$	0.00	$2.46x10^{7}$	$3.54x10^{9}$	$7.18x10^{10}$	$1.59x10^{10}$	$9.26x10^{10}$

Figure 4.3.1 The uptake and localistation of live *A. salmonicida aro A* mutants following immunisation.

Live bacteria were detected in the tissue and gastro-intestinal mucus of rainbow trout from 5 minutes to 72 hours following oral(□) and anal (⊠) intubation and from 5 minutes to 48 hours for intraperitoneal injection (■). No fish were injected IP at 12 hours and no fish were anally intubated or injected IP at 15 minutes. The dose given to the fish was approximately $1x10^{10}$ cfu in 0.5 cm^3 of saline containing 40 $\mu g\ cm^{-3}$ of kanamycin and 30 $\mu g\ cm^{-3}$ of nalidixic acid. (A) the number of CFU detected per gram of kidney; (B) the number of CFU detected in the spleen; (C) the number of CFU detected in the liver; (D) the number of CFU detected in the intestinal mucus. The error bars show the SDn-1 (n=3)

Fig. 4.3.1 Part A Kidney

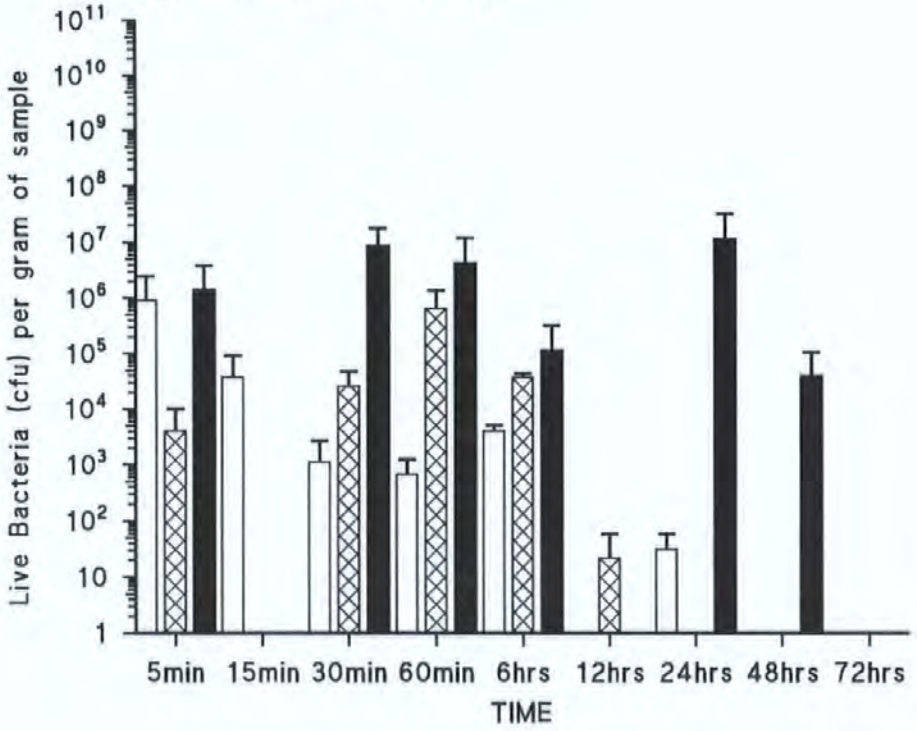


Fig. 4.3.1 Part B Spleen

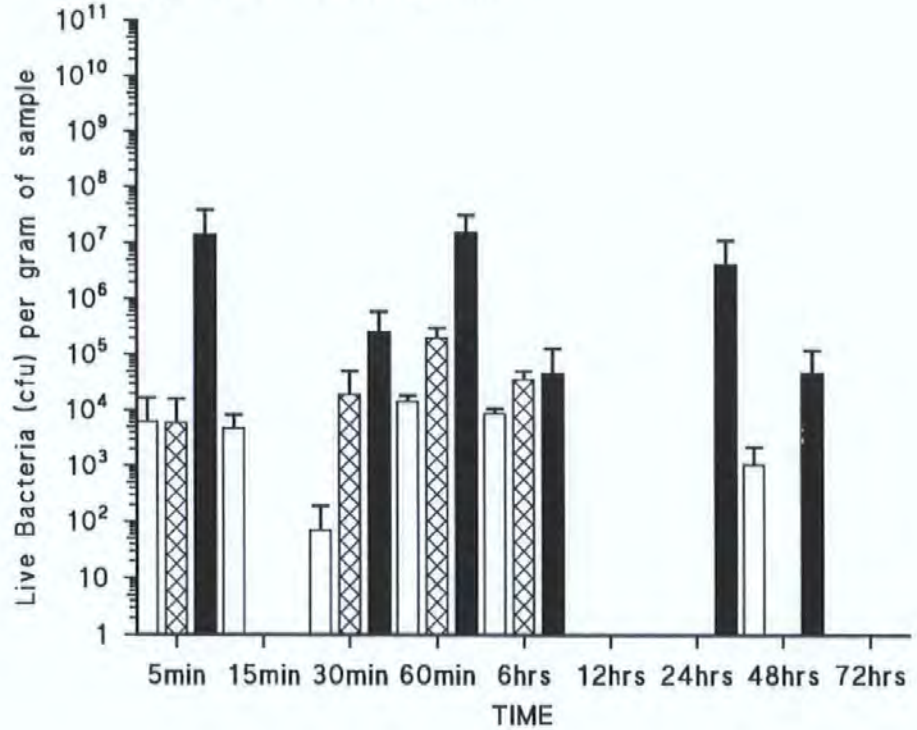


Fig. 4.3.1 Part C Liver

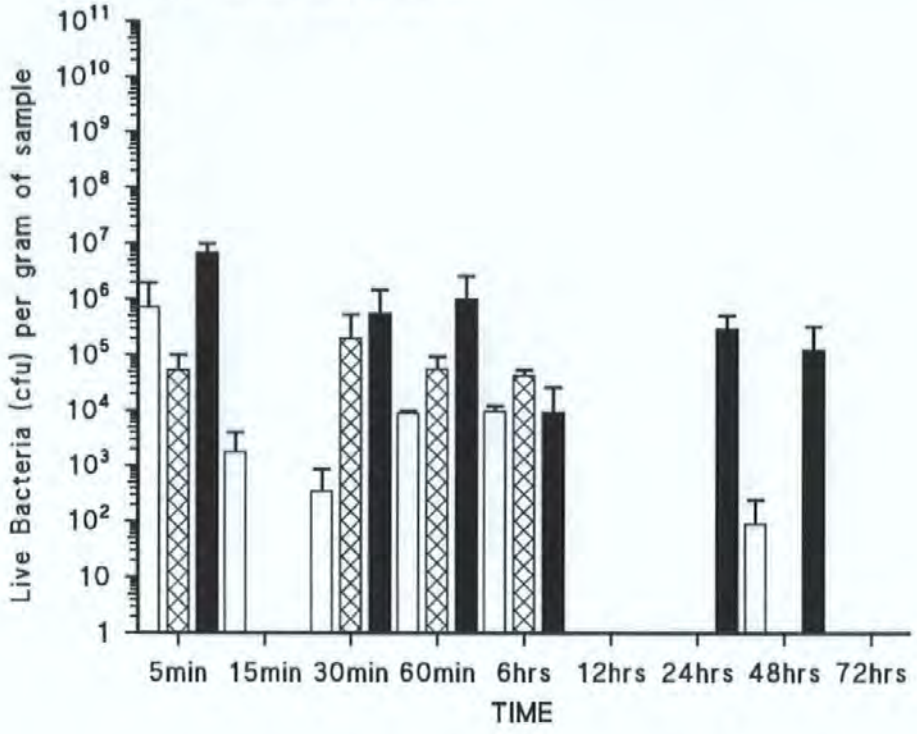


Fig. 4.3.1 Part D Intestinal Mucus

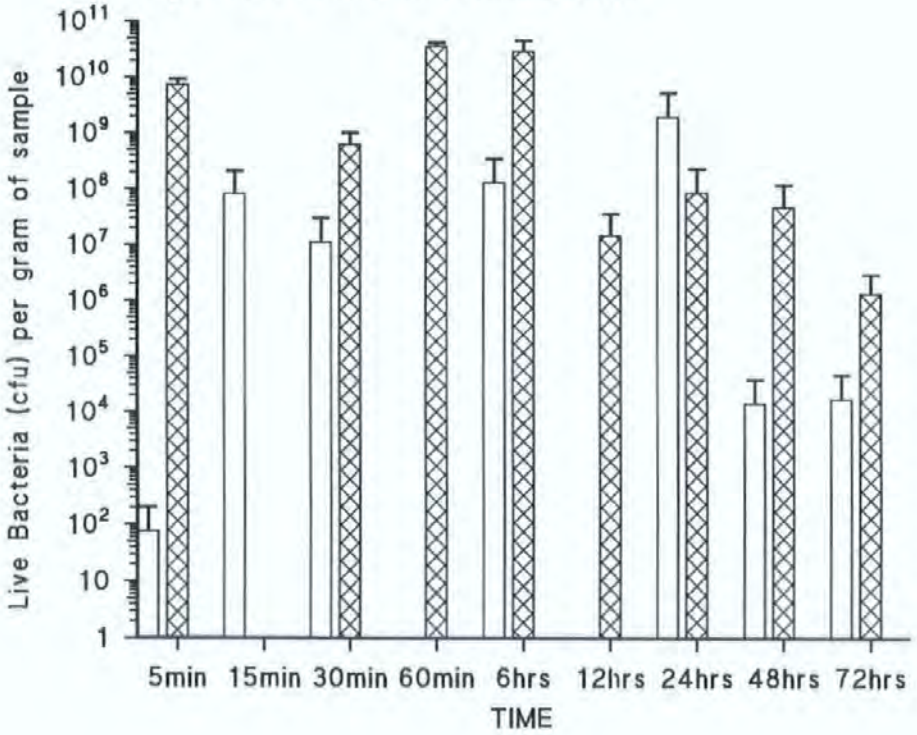


Figure 4.3.2 The uptake and localisation of live *A. salmonicida aro A* mutants after neutralisation of gastric acidity.

Bacteria were detected in the tissues and gastro-intestinal mucus of rainbow trout following anal (⊠), oral(□) intubation and oral intubation 1 hour after fish were administered 1 cm^3 of a 35mg cm^{-3} solution of sodium bicarbonate (■) of approximately $1x10^{10}$ cfu in 0.5 cm^3 of saline containing 40 $\mu g\ cm^{-3}$ of kanamycin and 30 $\mu g\ cm^{-3}$ of nalidixic acid. (A) the number of CFU detected per gram of kidney; (B) the number of CFU detected in the spleen; (C) the number of CFU detected in the liver; (D) the number of CFU detected in the intestinal mucus. The errors bars show the SDn-1 (n=3).

Fig. 4.3.2 Part A Kidney

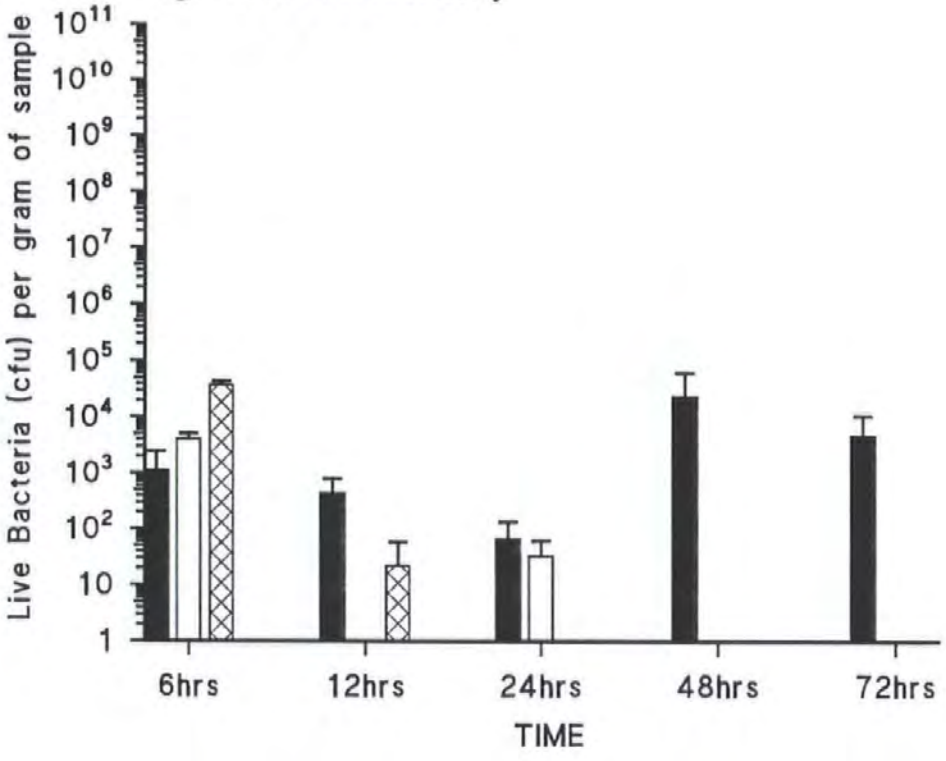


Fig. 4.3.2 Part B Spleen

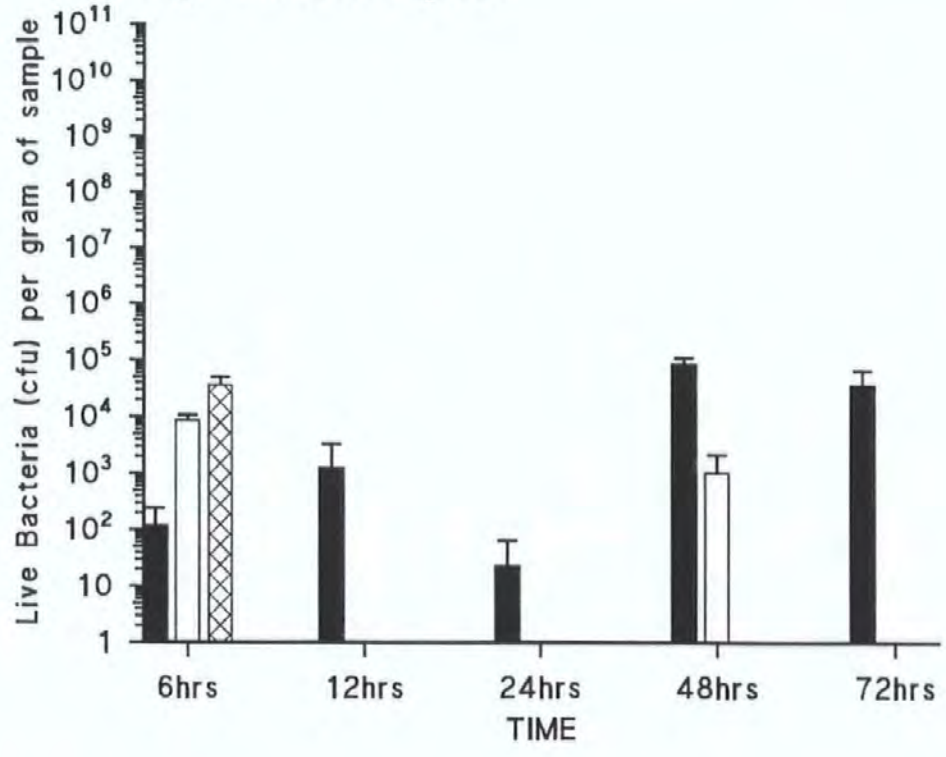


Fig. 4.3.2 Part C Liver

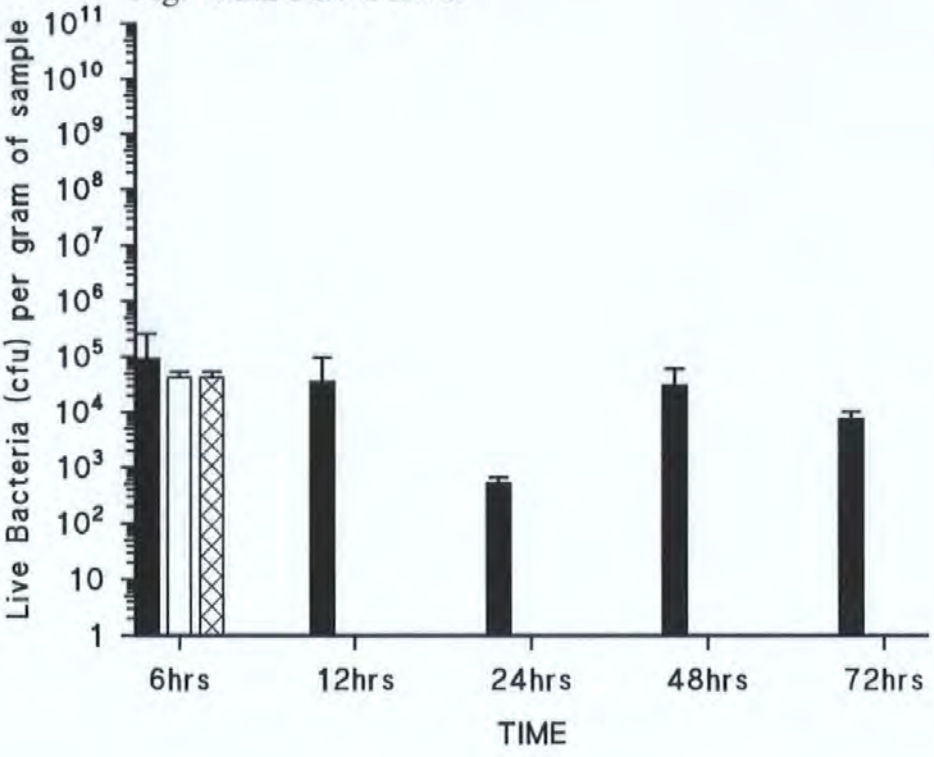
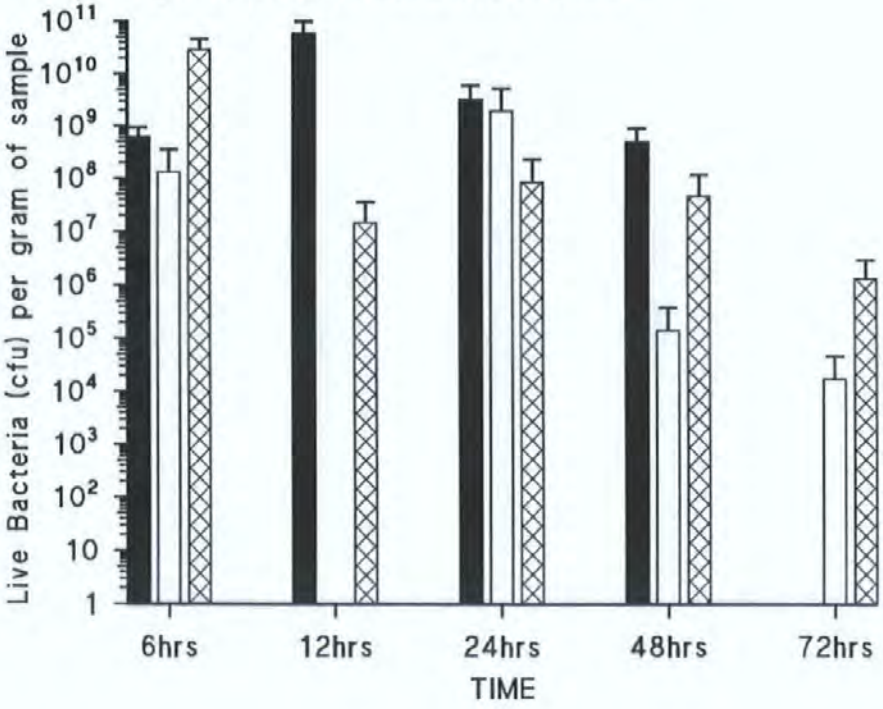


Fig. 4.3.2 Part D Intestinal Mucus



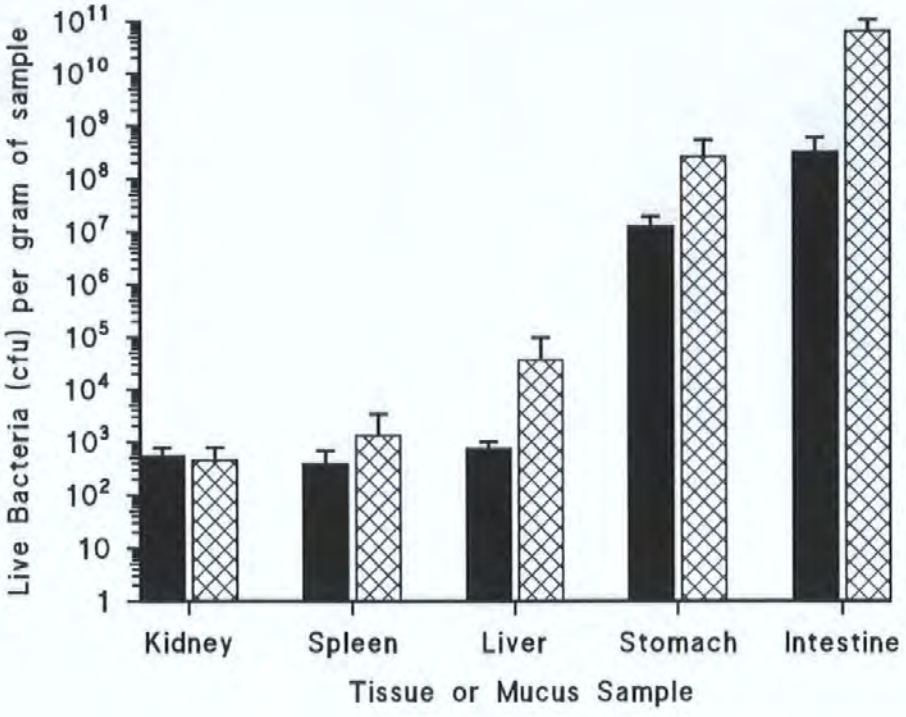


Figure 4.3.3 The effect of oral vaccine dose on tissue localisation of bacteria.

The number of live bacteria (cfu) detected in the kidney, liver, spleen, stomach mucus and intestinal mucus of fish that were orally intubated with either $1x10^{8}$ (■) or $1x10^{10}$ (⊠) cfu of ***A.salmonicida aro A*** *1 hour after oral administration of $1cm^{3}$ of a sterile 35mg cm^{-3} solution of sodium bicarbonate.*

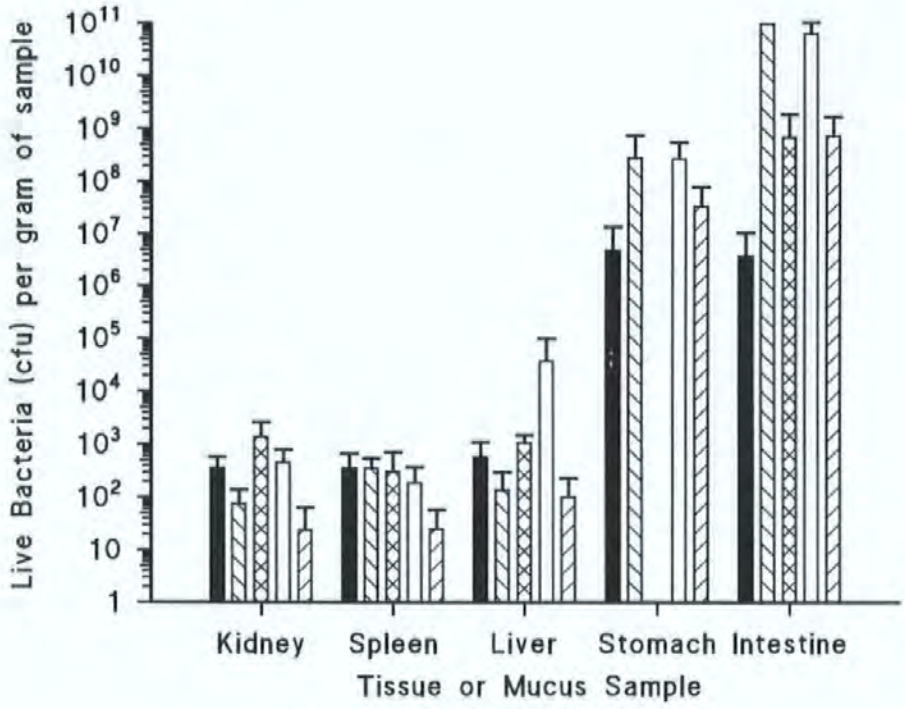


Figure 4.3.4 The effect of culture medium on tissue localisation of bacteria.

The number of live bacteria (cfu) detected in the kidney, liver, spleen, stomach mucus and intestinal mucus of fish that were orally intubated with approximately $1x10^{10}$ cfu of ***A. salmonicida*** *1 hour after oral administration of $1cm^3$ of a sterile 35mg cm^{-3} solution of sodium bicarbonate. The bacteria were grown for 48 hours at 22 °C in Furunculosis Broth (■), Brain, Heart Infusion Broth (▨), 10% normal strength Tryptone Soya Broth (▧), Tryptone Soya Broth at pH 6.0 (⊠) and TSB (□). Error bar show the SDn-1 (n=3).*

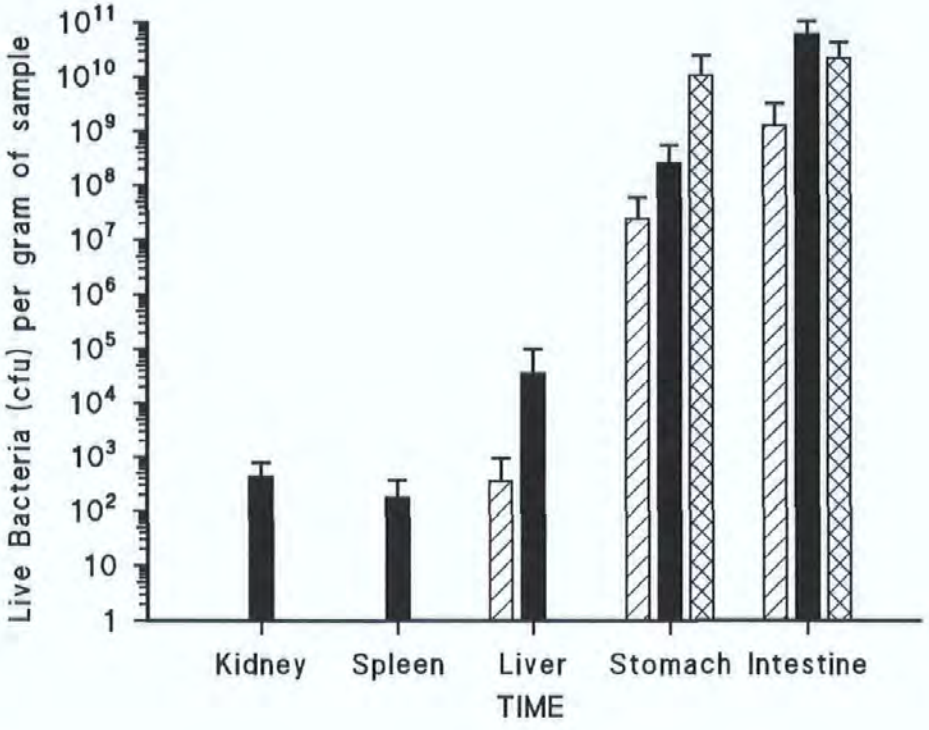


Figure 4.3.5 The effect of oxygen availability during culture on the tissue localisation of bacteria.

The number of live bacteria (CFU) detected in the kidney, spleen, liver, stomach mucus and intestinal mucus 12 hours after fish were orally intubated with approximately 10^{10} CFU of ***A. salmonicida*** *given 1 hour after oral administration of 1 cm^3 of a sterile 35 mg cm^{-3} solution of sodium bicarbonate. The bacteria were grown either for 72 hours in an anaerobic jar (▨), 48 hours shaken at 125 rpm (▩) or 48 hours unshaken (■). All cultures were grown at 22 °C in TSB. The error bars show SDn-1 (n=3).*

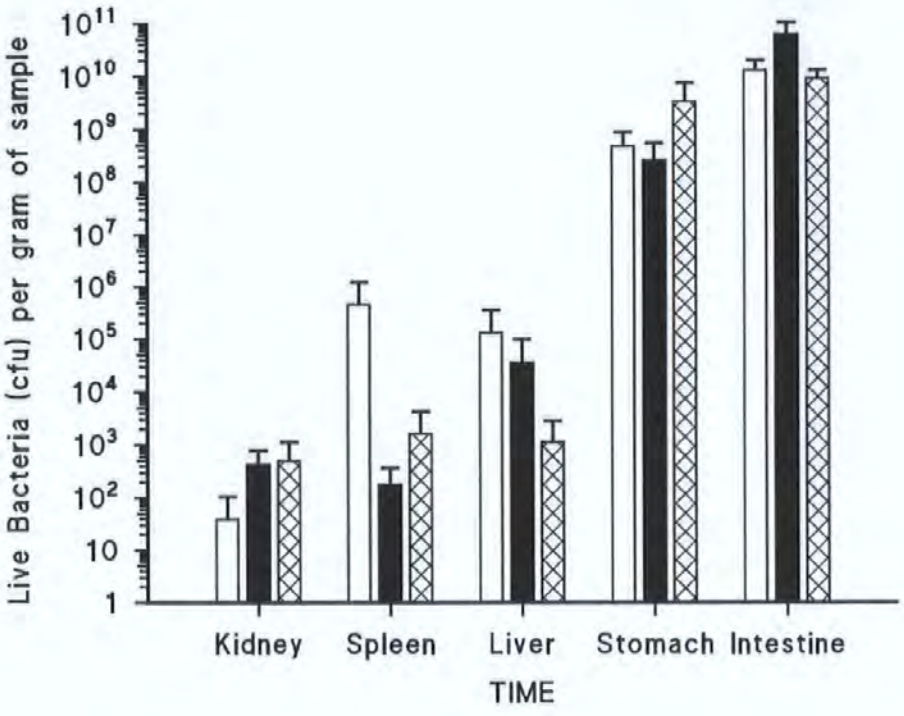


Figure 4.3.6 The effect of growth phase on the tissue localisation of bacteria.

The number of live bacteria (CFU) detected in the kidney, spleen, liver, stomach mucus and intestinal mucus 12 hours after fish were orally intubated with approximately 10^{10} CFU of ***A. salmonicida*** *given 1 hour after oral administration of 1 cm^3 of a sterile 35 mg cm^{-3} solution of sodium bicarbonate. The bacteria were either cultured for 12 hours, mid log phase, (□), 48 hours, mid stationary phase, (■) or for 72 hour, late log phase, (⊠). All cultures were grown statically at 22 °C. The error bars show the SDn-1 (n=3).*

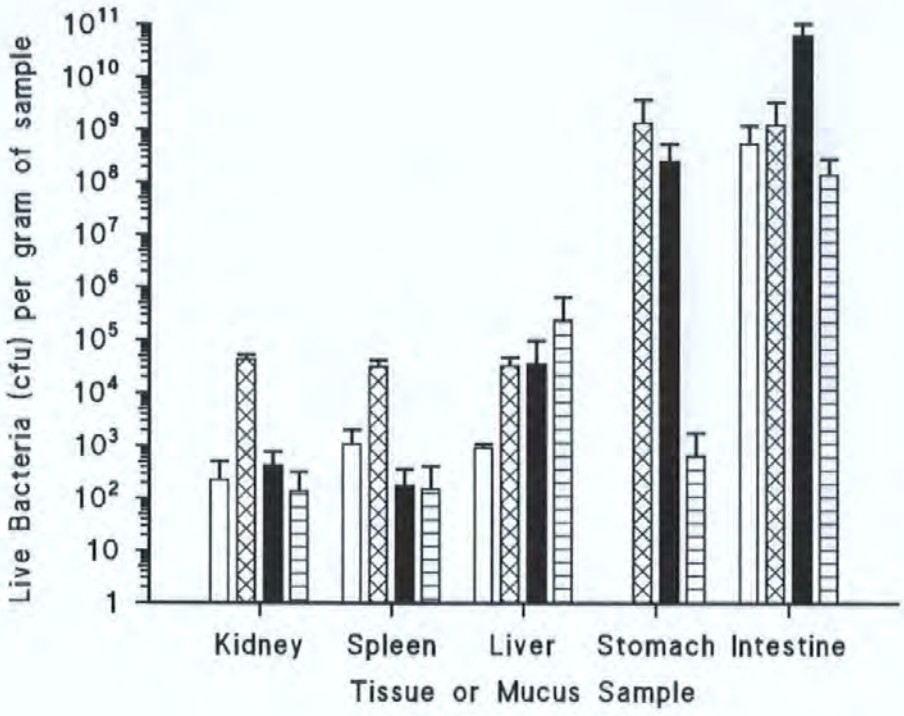


Figure 4.3.7 The effect of oral delivery medium on the tissue localisation of bacteria. *The number of live bacteria (CFU) detected in the kidney, spleen, liver, stomach mucus and intestinal mucus 12 hours after fish were orally intubated with approximately 10^{10} CFU of* ***A. salmonicida*** *given. The bacteria were grown statically for 48 hrs at 22 °C. They were administered to the fish in 50% v/v UHT milk/saline (□) with no prior administration of sodium bicarbonate; in a 10% glucan/saline (⊟) suspension also with no prior administration of sodium bicarbonate; in TSB (⊠) or in saline (■) both given 1 hour following oral administration of 1 cm^3 of a sterile 35 mg cm^{-3} solution of sodium bicarbonate. The error bars show SDn-1 (n=3).*

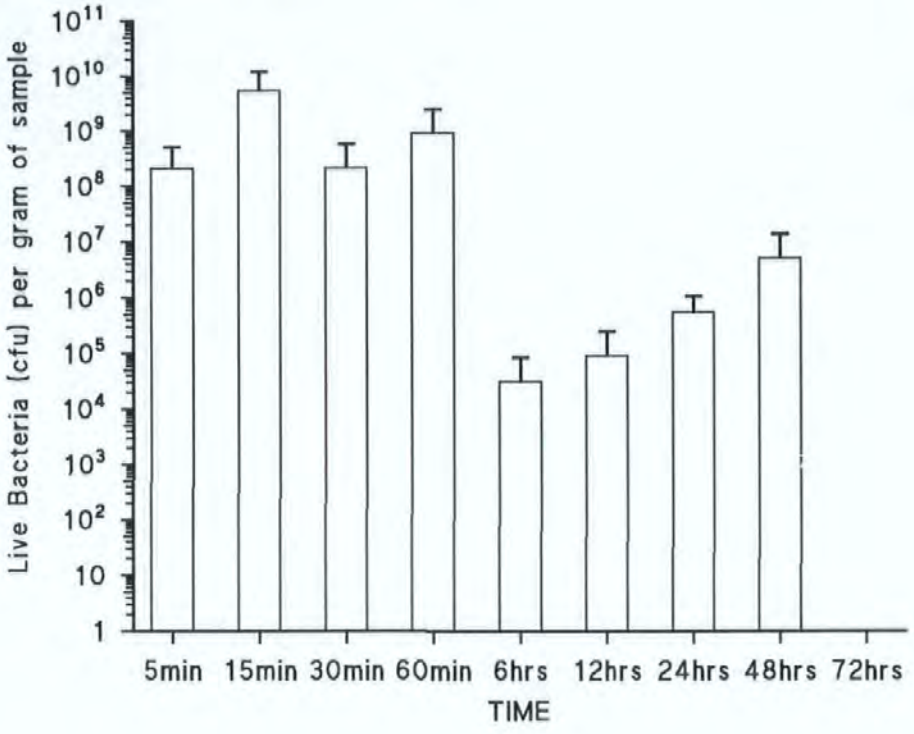


Figure 4.3.8 The survival of bacteria in the stomach of rainbow trout following oral delivery bacteria in saline.

The number of live bacteria (CFU) detected in the stomach mucus after fish were orally intubated with approximately 10^{10} CFU of ***A. salmonicida*** *given suspended in 0.5cm^3 of saline containing 40μgcm^{-3} kanamycin and 30μgcm^{-3} nalidixic acid. The error bars showSDn-1 (n=3).*

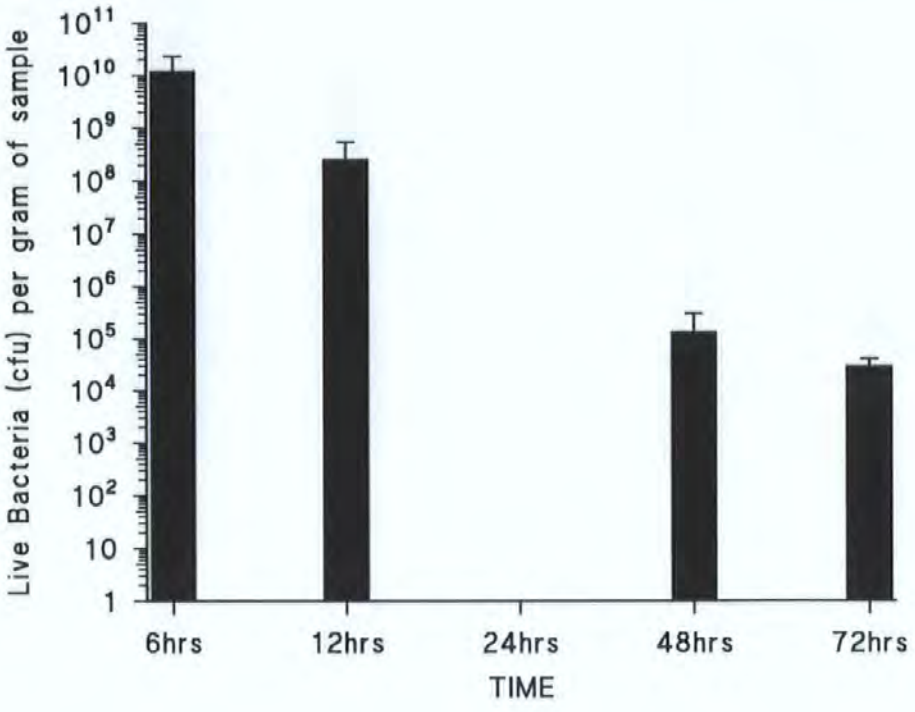


Figure 4.3.9 The effect of prior administration of sodium bicarbonate on the survival of bacteria in the stomach of rainbow trout following oral delivery bacteria in saline.

The number of live bacteria (CFU) detected in the stomach mucus after fish were orally intubated with approximately 10^{10} CFU of ***A. salmonicida*** *given suspended in 0.5cm^3 of saline containing 40$\mu g cm^{-3}$ kanamycin and 30$\mu g cm^{-3}$ nalidixic acid. Given 1 hour following oral administration of 1 cm^3 of a sterile 35 mg cm^{-3} solution of sodium bicarbonate. The error bars showSDn-1 (n=3).*

4.4 Disscussion.

The approach adopted in this study represents the first attempt to identify the fate of *A. salmonicida aro*A when delivered orally to rainbow trout. It was the fate of live cells that was of primary interest and consequently it was essential that a viable counting technique was applied. This brought with it its own problems, viable counts are notoriously inaccurate and therefore, all results must be viewed with caution. Furthermore, it is imperative that all techniques are carried out aseptically and that reagents and equipment are sterile. The most striking feature of the results was the variation between individual fish within groups. Therefore, the data was presented in the Tables to demonstrate this variability and in the Figures show the data averaged, illustrating the general trends between the groups and treatments. The variability of the results in this work complicates interpretation but careful examination of the results does suggest some trends and some observations are quite marked.

During the 5 minutes to 72 hour time course for oral, anal and IP routes, the highest numbers of bacteria detected in the tissues were found in those fish injected IP with the vaccine. Since IP injection introduces the vaccine directly into the space in which the organs are contained this is perhaps not surprising. It is also not surprising that the highest levels of bacteria in the intestinal mucus were found in those fish that were anally intubated, and that bacteria were only isolated from the gastric mucus of fish intubated orally.

The observation that bacteria delivered orally and anally could be readily detected in the organs of the experimental animals is extremely interesting. Tatner *et al.* (1984) found very little tissue localisation following the oral delivery of live *A. salmonicida* cells. It is important to note that in that study 10^6 bacteria were delivered orally as opposed to 10^{10} here. This is a very substantial difference (10000x) and this may explain the differences in results.

The pattern of accumulation of bacteria within the spleen and kidney is similar for both oral and anal groups the peak numbers appear around 60 minutes followed by a decline

by 6 hours. In the liver the peak number of bacteria in the anal group seems to be at around 30 minutes and this might reflect the route of colonisation following intubation since the blood from the vessels around the intestine flows first to the liver. Anal delivery appears to result in larger numbers of bacteria in the organs than oral delivery. There are two probable explanations for this; first by avoiding the low pH in the gastric compartment more bacteria are able to invade the host, the second involves the nature of the technique involved, when intubating anally not damaging the mucosal surface when inserting the tube is almost impossible. This is because the lumen in the intestine is much smaller than the oesophagus and stomach and the mucus in the stomach is thicker and therefore protects more efficiently against mechanical damage. If the mucosal surface is damaged then *A. salmonicida* would be able to exploit it. Effendi and Austin (1995) investigated the uptake of *A. salmonicida* by brushing the bacteria onto the suface of the fish with a squirrelk hair brush. It is very unlikely that this could be done without causing mechanical damage to the mucus membranes and any such damage would cast doubt upon the results. The need to isolate site of infection and then study the upatke of the bacteria is manifest but brushing the bacteria on to the fish cannot be the best way forward.

The localisation pattern is similar to that found by Tatner *et al.* (1984) and as she described this pattern ties in well with the reticulo-endothelial system of fish and the known pathology of infection. However, Tatner *et al.* (1984) found this pattern following IP injection rather than oral intubation.

McCarthy (1977) found that when bacteria were introduced into the stomach they were eliminated from the gastrointestinal tract within two days. The results in this investigation clearly show that there are bacteria within the tract at 48 and 72 hours after oral and anal intubation.

Few bacteria from the oral and anal groups were isolated from the organs after six hours but following IP injection bacteria were isolated from the tissues at 48 hours which was

the last time point tested for the IP group. The IP group were not tested at 72 hours as during preliminary experiments significant mortalities occurred between 48 and 72 hours after injection with 10^{10} CFUs. The absence of bacteria from the organs of the intubated fish contrasts with the presence of numerous bacteria in the intestine at 24, 48 and 72 hours.

Fish orally intubated with bacteria after administration of sodium bicarbonate showed a different uptake and localisation profile. This group was not tested for the presence of bacteria before six hours because the presence of bacteria to six hours had already been demonstrated. The aim of giving bicarbonate was to enhance both the uptake and persistence of the bacteria in the host. There appeared to be a peak in the number of bacteria in the organs at six hours; followed by a decline in the numbers of bacteria in the organs until 12 hours; with a new peak at 48 hours sustained until 72 hours.

The extremely high number of viable bacteria detected per gram of gastric mucus six hours after intubation following the administration of bicarbonate shows that the bicarbonate has overcome the antibacterial action of the low pH of the gastric secretions. The response of this strain of *A. salmonicida* to low pH was discussed fully in Chapter Three of this thesis. The sensitivity of unprotected bacteria to the gastric acid, and the subsequent protection afforded by the sodium bicarbonate, was in keeping with the results of the work described earlier and that work of Lavelle (1994) on the effect of pH on the fate of soluble protein when orally administered.

In mammalian systems bicarbonate is often used to protect live vaccines. Li *et al.* (1993) gave different groups of human volunteers either a single dose of $2x10^9$ CFU of *Shigella flexneri aro D* mutant on day 0 or three doses of $2x10^9$ CFU on days 0, 2 and 4. In each case the volenteers were starved for two hours prior to administration of the vaccine, they consummed 2 g of sodium bicarbonate followed 10 minutes later by the vaccine suspended in saline. The triple dose illicited a stronger immune response than the single dose. Jones *et al.* (1991). Immunised calves with a *Salmonella typhimurium aroA/aroD* mutant strain.

Calves recieved 10^{10} CFU after administration of an antiacid solution. Seven out of eight experimental animals survived a challenge with a virulent strain 28 days after immunisation.

When Vaughan *et al.* (1993) looked at the persistence of this strain of bacteria in brown trout on day two between $1x10^5$ and $1x10^4$ were present in the whole kidney and the bacteria persisted until day 14 when all of the kidneys tested proved negative. Nnalue and Stocker (1987) showed that the persistence of an *aro*A mutant of *S. choleraesuis* was an essential factor in determining the level of protection conferred by the vaccine. Presumably this is because a bacteria that fails to persist will also have failed to establish the limited infection thought to be the foundation of the good protection live oral vaccines produce when compared with dead nonreplicating antigens (Lycke and Svennerholm, 1990)

Lavelle (1994) working with rainbow trout and Jenkins (1992) working with tilapia noted that antigen uptake into the blood occurred in a biphasic pattern. Both workers noted that when soluble antigens are given orally to trout there is a peak in the amount detectable in the blood at around six hours and another at between 12 and 24 hours. It seems likely that this biphasic pattern is caused by some of the inoculum being forced into the intestine during intubation this is then assimilated rapidly accounting for the first peak. As the gastric contents are later voided naturally into the intestine there is a second peak in antigen accumulation. This may account for the presence of bacteria in the organs at early time points after oral intubation but peaks in bacterial numbers at six and again at 12 to 24 hours were not found. This demonstrates the difference between the delivery of soluble and particulate antigens. The peak in the uptake of bacteria that occurred at 48 hours following bicarbonate administration, may have been due to colonisation of the host by bacteria from the intestine. This seems to indicate that bacteria are taken up by the distal end of the intestine as it takes about 48 to 72 hours for food to pass through the intestine.

The delivery of $1x10^8$ bacteria showed that with bicarbonate much smaller doses of bacteria can cause similar accumulation within the tissues. This is important since most

mammalian vaccines described so far use $1x10^{10}$ CFU as a standard oral dose. This many bacteria is expensive and difficult to produce on a commercial scale especially with a relatively slow growing bacteria like *A. salmonicida* and would be very difficult to administer in the field. If the same or similar level of protection could be achieved using this smaller dose, the vaccine might become a practical commercial proposition. The importance of the sodium bicarbonate was demonstrated as no bacteria were detected following intubation with $1x10^{8}$ without bicarbonate and the levels of bacteria seen in the $1x10^{8}$ + bicarbonate group at 12 hours were far greater than the number seen in the $1x10^{10}$ - bicarbonate group.

The growth medium used to culture the bacteria prior to intubation appeared to have little effect on the numbers of bacteria found in the organs or the gastrointestinal mucus. Parker (1985) investigated the effect of growth medium on the attachment of *A. salmonicida* to RTG-2 cells. He found that bacteria grown in BHI and furunculosis broth adhered to the cell line more frequently than those grown in TSB. The results of this study do not bear out his observations. This might be due to the very different nature of the assay systems or may indicate that attachment and uptake at mucosal surfaces *in vivo* is dependant on different factors than adhesion *in vitro*.

Work on *Salmonella typhimurium* by Ernst *et al.* (1990), Galan and Curtiss (1990), Lee and Falkow (1990); Schiemann and Shope (1991) and MacBeth and Lee (1993) has shown that anaerobiosis and other environmental factors are important in enhancing bacterial invasiveness. The result of the investigation of the effect of oxygen availability shows that shaking the culture during growth appears to have a strong negative effect on the numbers of bacteria found in the tissue. Parker (1985) found that A-layer positive bacteria adhered to cell monolayers more efficiently that shaken ones the results here apparently confirm his finding and show that they have a relevance to uptake *in vivo*.

In this case anaerobic growth did not increase the number of bacteria found in the host's organs. Growth phase has also been shown to be an important factor in determining

bacterial invasivness (Ernst *et al.*, 1990) and late log phase has been shown to be the point in the growth cycle that *Salmonella typhimurium* are most invasive. Bacteria from the late log phase grown under anaerobic conditions were not used in this study because of the established acid sensitivity of this strain during the log phase (Chapter 3). Furthermore, producing 10^{10} bacteria from a stationary phase culture is much easier than from log phase cultures.

It would useful to measure invasion in a cellular assay system using bacteria grown to late log phase to investigate if the increase in invasiveness is likely to be worth the extra effort involved in producing such a vaccine. The results of the growth phase experiments in this study indicated that growth phase had little effect on the uptake and localisation of bacteria.

The medium in which the bacteria are delivered did seem to affect the uptake and localisation of bacteria following oral immunisation. Delivery of bacteria in 50% v/v UHT milk/saline provided protection from the gastric acid comparable to that provided by the sodium bicarbonate. Rose (1990) suggested the reason that he could infect fish by intragastric challenge while many others (McCarthy, 1977; Tatner, *et al.*, 1984; Markwardt and Klontz, 1989) had not succeeded was because the diluted furuncle material, digested muscle tissue, in which he delivered the bacteria may have protected the bacteria in the stomach. Since the UHT skimmed milk is largely a protein solution and did protect the cells in the stomach this lends some weight to this theory. It also appears that the thick suspension of glucans also had a protective effect on the bacteria since localisation was similar to that seen in the bicarbonate group.

The administration of bacteria in TSB following the oral dose of sodium bicarbonate appeared to give the highest levels of bacteria in the organs. This is consistent with practice in the delivery of oral vaccines to mammalian hosts.

The evident localisation of bacteria in the tissues and the enhanced uptake and persistence following administration of sodium bicarbonate suggests that this strain of *A. salmonicida* may be suitable for use as an oral vaccine if delivered in the correct way.

Chapter Five.

An investigation into the invasive properties of *Aeromonas salmonicida.*

5.1 Introduction.

Invasion, the process by which bacterial pathogens enter host epithelial cells by inducing endocytosis by normally non-phagocytic cells, is an important virulence mechanism for several bacterial species (Tang *et al.*, 1993). Invasion by mammalian bacterial pathogens has been extensively investigated and has been thoroughly reviewed on many occasions, most recently by Miller (1996) and as part of the literature review of this thesis. It is not the intention, therefore, to review this work here, but to indicate why invasion may be an important, although under-investigated virulence mechanism of fish pathogens and to examine the possibility that *A. salmonicida* is an invasive pathogen.

The incomplete state of understanding of the nature of the biology and pathogenicity of *A. salmonicida* has been described as one of the greatest obstacles to the development of an effective vaccine (Austin, 1984). It is not clearly understood how the bacterium is disseminated and investigations into its route of infection have produced contradictory results and the skin, lateral line, gills, and gut, have all been implicated (McCarthy, 1977; Ellis, 1981; Tatner *et al.*, 1984; Hodgkinson *et al.*, 1987).

It is known that *A. salmonicida* is an obligate pathogen and therefore, must ensure regular infection of its host species to replicate. Furthermore, furunculosis is a septicaemic disease and so *A. salmonicida* apparently must usually enter the viscera of the host to replicate most efficiently. Unlike mammals that have keratinised skin, the interface between a fish and its environment consists entirely of mucosal epithelia. The ability to invade the epithelial cells of the skin, gills or gastrointestinal tract or preferably all three would enable the bacteria access to the host and this would presumably be a great advantage.

Unfortunately, investigations into the interaction between *A. salmonicida* and the cells of its hosts have been limited to those cells, which have an immune function like macrophages and neutrophils (Garduno and Kay, 1992, 1995; Olivier *et al.*, 1992; Sharp and Secombes,

1993; Lamas and Ellis, 1994a, b; Weeks-Perkins and Ellis, 1995). It has been suggested that the A-layer enhances bacterial adhesion to macrophages and also that *A. salmonicida* can probably live as a facultative intracellular pathogen within macrophages, thereby avoiding the humoral defence mechanisms (Garduño *et al.*, 1993).

Despite this work on bacteria/macrophage interactions, no reports of investigation of the invasive capacity of *A. salmonicida* or any other bacterial pathogens of fish were found in the current literature. To the best of this author's knowledge the only work on the invasive capacity of *A. salmonicida* was completed by Lavelle (1994), although, Grey and Kirov (1993) showed that adhesion to Hep-2 cells by *A. veronii* and *A. caviae* was greater in bacteria isolated from clinical samples rather than the environment. Nishikawa *et al.* (1994) showed that *Aeromonas spp.* (not *A. salmonicida*) could invade monolayers of CACO-2 cells. They also showed that the DNA of the *Aeromonas* strains positive for invasion did not hybridise with the *E. coli eae* and *ipa*B probes associated with the attaching and effacing ability and invasion respectively. This suggested that while mesophilic *Aeromonas spp.* can invade epithelial cells; they use a different mechanism to that of enteropathogenic *E. coli* (EPEC).

Lavelle (1994) looked at the ability of *A. salmonicida* to invade isolated rainbow trout enterocytes using transmission and scanning electron microscopy, and used the acridine orange staining technique of Miliotis (1991) to visualise internalised bacterial cells. He also used the acridine orange technique to investigate invasion of tissue culture cells, and showed, using fluorescently labelled anti-actin antibodies, that internalised bacteria are surrounded foci of actin filaments. This last result indicates that *A. salmonicida* may invade by a different mechanism than *A. hydrophila* as Nishikawa *et al.* (1994) failed to show actin polymerisation during invasion of a human cell line by *A. hydrophila*.

Lavelle (1994) also showed that cytochalasin D which prevents actin filament formation, also prevented invasion of cells by *A. salmonicida*. Unfortunately, Lavelle's results were qualitative in nature. Furthermore, the use of isolated enterocytes infected in

suspension provides a very poor experimental model for the invasion of an epithelial membrane. This is because the cells are under great stress when removed from the basement membrane and isolated from other cells and because very different antigens and receptors are exposed when the cells are in suspension. The result's presented in Chapter four above also showed that *A. salmonicida* could infect rainbow trout via the gastrointestinal tract. Together this evidence suggested that *A. salmonicida* may be invasive and showed that further investigation was worthwhile.

The aims of the present study were to investigate by electron microscopy the ability of *A. salmonicida* to invade the epithelial cells of the gastrointestinal tract of rainbow trout. To improve upon the work of Lavelle this part of the investigation was performed on gut epithelial cells *in situ*, using either excised tissue or by exposing the gut to bacteria *in vivo*. It was also intended quantitatively to assay the ability of *A. salmonicida* to invade tissue culture cells. The invasive ability of a virulent A-layer positive strain of *A. salmonicida* was compared with that of an A-layer negative strain and a strain of *Yersinia ruckeri*.

5.2 Materials and Methods.

5.2.1 Bacterial strains.

The bacterial strains used in this investigation were *Aeromonas salmonicida* 644rB, *A. salmonicida* MT004 and *Yersinia ruckeri* 18/83. The details for 644rB and 18/83 are given above (Section 3.2.1). MT004 is an avirulent strain of *A. salmonicida* that lost the ability to produce the A-layer during laboratory subculture. This strain was taken from the University of Plymouth culture collection where it had been stored in liquid nitrogen. It was originally obtained from Dr A. E. Ellis (SOAEFD) in 1988.

5.2.2 Routine culture of bacteria.

Bacteria were cultured as described above (Section 3.2.2) except that antibiotics were not added to either the agar or broth in which they were grown.

5.2.3 Enumeration of bacteria.

Bacteria were counted using the method of Miles and Misra (Miles *et al.* 1938). This has also been described above (Section 3.2.3).

5.2.4 Rapid spectrophotometric enumeration of bacteria.

The number of *Aeromonas salmonicida* in working suspensions was estimated using the standard curve and regression equation described above (Section 3.2.5). The number of *Yersinia ruckeri* was estimated by measuring absorbance at 625nm against a PBS blank and using the following regression equation of Furones (1990).

$$CFU = 1.62 \times A_{625} + 7.9$$

5.2.5 Experimental animals.

Prior to the investigation of the uptake and localisation of acridine orange stained cells *in vivo* fish were kept in an experimental aquarium. These were freshwater recirculating systems at 15 °C ± 1 °C. Before infection they were starved for 48 - 72 hours.

Before the investigation of the invasion of *A. salmonicida* 644rB of isolated fish intestine rainbow trout were kept in the challenge aquarium (Section 6.2.5), at 13°C ± 1°C and starved for 48 hours before they were sacrificed and the intestine removed.

For the electron microscopy investigation of the cellular interaction of *A. salmonicida* 644rB and rainbow trout intestinal cells, the fish were kept in the challenge aquarium (Section 6.2.5), at 13°C ± 1°C and starved for 48 hours before anal intubation with either a bacterial suspension or TSB.

5.2.6 Cell Culture.

Two cell lines were used during this investigation they were, Madin-Darby Canine Kidney Cells (MDCK) which is a polarised cell line with a brush border and EPC cells that are an epithelial-like cell line derived from carp. Both cell lines were grown in Minimal Essential Medium (MEM.) with Earle's salts, supplemented with 10% (v/v) new born calf serum, 0.1% (v/v) glutamine, and 1% (v/v) non essential amino acids. MDCK cells were grown at 37°C and ECP cells at 15°C. Prior to incubation the 5% CO_2 (v/v) was added to the tissue culture flasks. The split ratio for both cell lines was 1:4. During routine culture of the MDCK cells Penicillin and Streptomycin were also added to the culture medium to final concentrations of 100 IU and 100 μg cm^{-3}.

5.2.7 Uptake of acridine orange stained *A. salmonicida* by rainbow trout.

Bacteria grown as described above (Section 4.2.7), were washed once by centrifugation and resuspended in a known volume of PBS. The number of viable bacteria was estimated using the spectrophotometric method described above (Section 5.2.4) and the concentration of bacteria adjusted to $2x10^{10}$ cm^{3}. This suspension was sedimented by centrifugation and resuspended in an identical volume of PBS containing 0.001% (w/v) acridine orange. The bacteria were administered by oral and anal intubation and IP injection as described above (Section 4.2.7).

One hour after vaccination the fish were sacrificed by a blow to the head. The fish were then dissected and the tissue processed as described above (Section 4.2.8). Smears of mucus and tissue samples were made on glass microscope slides and these were examined using a UV microscope.

5.2.8 Infection of trout intestinal epithelia *in vitro.*

Rainbow trout 250-350g were sacrificed by a blow to the head. The gut, from the pyloric caecae to the anus was excised immediately. The lumen was washed three times with Hank's balanced salts solution (HBSS) by pipetting into the proximal end and gently squeezing the solution along the intestine.

The proximal end of the intestine was tied off using sterile thread. Some samples were treated with Mucolex (s-carboxymethyl-L-cysteine) to remove the mucus from the surface of the epithelium. The Mucolex was pipetted into the gut which was gently massaged and the mucolex and mucus was gently squeezed out. The gut was then infected by the addition of 10^{10} bacteria in 1 cm^3 of MEM. The distal end was then closed with sterile thread, and the gut was immersed in MEM. and incubated for 2 hours. Some sections were treated with Mucolex after incubation.

Following incubation the gut was divided into small and large intestine. The tissue samples were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for three hours. These samples were then processed for either Scanning Electron Microscopy (SEM) or Transmission Electron Microscopy (TEM) as described below.

5.2.9 *In vivo* infection of trout intestinal epithelia.

Aeromonas salmonicida 644rB was grown as described above (Section 4.2.7). Adult rainbow trout 250-300g were anally intubated with 10^{10} CFU in 0.5 cm^3 of TSB. The negative control fish was intubated with 0.5 cm^3 of TSB. The fish were left for 5, 15, 30 and 60 minutes or 6 and 18 hours then sacrificed and the gut excised. Tissue was taken from three areas, the large intestine, small intestine and the boundary between these two. The tissue samples were laid on to sterile filter paper with the lumenal surface uppermost. The paper stopped the tissue from rolling up. The tissue samples were placed in primary fixative, 2.5% glutaraldhehyde in 0.1M sodium cacodylate buffer pH 7.2 immediately and left in the fixative for at least 1 hour at 4°C. These samples were then processed for TEM.

5.2.10 Infection of cell monolayers MDCK cells.

Cells were passaged at least four times before they were used in an experiment. MDCK cells were subcultured into 30mm diameter Petri dishes at a density that caused them to become 80% confluent overnight before infection. *Aeromonas salmonicida* 644rB was grown in TSB overnight to late log phase and for 48 hours to mid stationary phase. The bacteria were washed twice by centrifugation and resuspension in PBS. The numbers of bacteria were estimated by the spectrophotometric method and confirmed by viable counts. The duplicate aliquots of bacterial suspension were centrifuged again and resuspended in HBSS to give multiplicities of infection (MOI.) of 10 and 100 in each 500μl. This volume of bacterial suspension was added to each Petri dish and incubated at 25°C for either one or two hours. The negative controls were incubated with HBSS for the same periods.

After incubation, the plates were washed three times with 1cm^3 of HBSS and triplicate plates of control and infected cells were either stained with acridine orange as described below or lysed. The number of internalised bacteria were measured as described below.

Twelve Petri dishes were infected with bacteria prestained with acridine orange as described above. These plates were infected in triplicate with both MOI. and incubated for 1 or 2 hours, washed five times with 1 cm^3 of HBSS then counter stained with crystal violet as described below.

5.2.11 Infection of cell monolayers EPC cells.

EPC cells were passaged four times before they were used in an experiment. Petri dishes 30mm in diameter containing washed and sterilised cover slips were set with cells as described for the MDCK cells. Duplicate dishes were infected with *A. salmonicida* 644rB, *A. salmonicida* MT004 and *Yersinia ruckeri* at a MOI. of 25 in 1.0 cm^3 of Earle's Balanced Salts Solution (EBSS). The cells were incubated for 1 hour with the bacteria, negative controls with EBSS only. They were then stained with Acridine orange and counterstained with crystal violet as described below.

Bacterial invasion of ECP cells was also investigated by viable counting of invading bacteria. EPC cells were cultured in ninety six-well tissue culture plates. These were infected in quintuplicate with *A. salmonicida* 644rB, *A. salmonicida* MT004 and *Yersinia ruckeri* at a MOI. of 25 in 0.1 cm^3 of EBSS. The cells and bacteria were incubated for 15 minutes, 1 hour or 3 hours. The invading bacteria were counted as described below. Five control wells per time point were incubated with EBSS only. To investigate the effect of the bacteria on the cell line a duplicate set of wells were treated identically except that the monolayers were not lysed as described below but fixed in 100% methanol and stained with Giemsa.

5.2.12 Transmission electron microscopy.

Samples were fixed in 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for at least 1 hour at 4°C. The tissue was washed twice for 5 minutes in 0.1 M sodium cacodylate buffer. The tissue was then dissected further to give samples of between 1 and 2mm^3. These were postfixed in 1% osmium tetroxide in sodium cacodylate buffer for 1 hour at 4°C. The samples were washed again twice for 5 minutes in 0.1 M sodium cacodylate

buffer before dehydration. The tissues were dehydrated through a series of ethanols (30%, 50%, 70%, 90%) for 5 minutes per change and twice for 10 minutes in absolute alcohol. Samples were then infiltrated with Spurrs resin by adding to mixture in ratios of absolute alcohol: resin of 3:1, 1:1, 1:3 for two hours each then incubating in pure resin for two hours and then overnight. The samples were placed in pure resin in coffin moulds and allowed to polymerise for 12 hours at 60°C. Ultrathin ('gold') sections were cut using a Reichert OmU3 Ultracut microtome and mounted on copper mesh grids. Sections were routinely stained with aqueous uranyl acetate for 15 minutes and washed in distilled water. Counterstained with Reynolds lead citrate for 15 minutes washed in distilled water, dried and observed under a Jeol JEM 1200 - EXII transmission electron microscope operated at 80 kV.

5.2.13 Scanning electron microscopy.

Samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 3 hours at 4°C, transferred to fresh fixative and stored overnight at 4°C. After fixation the tissues were washed twice for 5 minutes in 0.1 M sodium cacodylate buffer before dehydration. The tissues were dehydrated through a series of ethanols (30%, 50%, 70%, 90%) for 5 minutes per change and twice for 10 minutes in absolute alcohol. Amyl acetate was used as the intermediate fluid in the following ratios absolute ethanol to amyl acetate; 3:1, 1:1, 1:3 and two changes of pure amyl acetate each change took five minutes. The tissues were then critical point dried using a Polaron E500 critical point dryer, sputter coated with gold-palladium in an Emitech K550 sputter coating unit for 2 minutes at 15 mA for the control tissues and three minutes at 10 mA for the infected tissues; nominal thickness 10nm.

5.2.14 Acridine orange staining of infected cell monolayers.

This procedure was adapted from that of Miliotis (1991). The Petri dishes containing MDCK cells were washed three times with 1 cm^3 of HBSS to remove as many extracellular bacteria as possible. They were then stained with 1 cm^3 of acridine orange in PBS 0.001% (w/v) for one minute and counter stained for 5 minutes with crystal violet 0.05%. The Petri

dishes containing MDCK cells infected with prestained bacteria were washed five times with HBSS to remove extracellular bacteria and then counterstained as described above.

The EPC cells were washed three times in 1 cm^3 of EBSS, stained for 45 seconds with 0.001% acridine orange in PBS, then counterstained for 30 minutes in 0.05% crystal violet. An antifade mounting solution (90% glycerol, 10% PBS, 2.5% DABCO) was applied to the cells and the cover slips were sealed to the microscope slide with clear nail varnish. The cells were viewed under a Canon Vannox research microscope. Photographs were taken onto Fujicolour super G 400 ASA film.

5.2.15 Enumeration of viable intracellular bacteria.

Monolayers were infected and incubated for various times with bacteria. At the end of the incubation period unbound bacteria were removed by washing the monolayer three times with the appropriate balanced salt solution. To the MDCK cells 100μg of kanamycin in 0.5 cm^3 was added, to the ECP cells 150μg of kanamycin was added in 100μl.

The kanamycin was allowed to incubate with the cells for 2 hours. Kanamycin can only kill extracellular bacteria. The bacteria used in this investigation had been shown to be killed by the concentrations of kanamycin used in a preliminary experiment. After two hours the monolayers were washed five times. The MDCK cells were washed with 1.0 cm^3 of HBSS and the ECP cells were washed with 200μl of EBSS. The monolayers were then lysed by the addition of 1% (v/v) Triton X-100 in PBS; this concentration did not affect the viable count of the bacteria used here even after 2 hours. 1.0 cm^3 of this solution was added to the MDCK cells and 200μl was added to the EPC cells.

The cells were observed under an inverted microscope to ensure the monolayer had lysed. The suspension of lysed cells and bacteria was serially diluted ten fold twice. The viable bacteria were then counted by the method of Miles and Misra (1938) as described above.

5.3 Results.

5.3.1 Uptake of acridine orange stained bacteria by rainbow trout.

The staining of *A. salmonicida* with acridine orange was not shown to affect the viability of the cells. However, the stain was shown to be very stable as even after 48 hours of growth on TSA the colonies fluoresced green under UV light and unstained colonies did not. Bacteria were not detected in the liver or spleen of any of the fish examined. They were found in the kidney and could be seen as small spots of green fluorescence when visualised at under a x10 objective but were clearly seen to be bacteria under a x40 objective.

There were very few bacteria in any of the kidney samples but the kidney from the IP injected fish contained most approximately 10 bacteria per smear. The kidney smears from orally and anally intubated fish never contained more than three bacteria and most contained none. The bacteria were seen to be extra cellular.

5.3.2 *In vitro* infection of rainbow trout intestinal cells.

The results of the SEM and TEM examination of the *in vitro* infected gut tissue are presented in Table 5.3.1. Bacteria were seen in all of the SEM samples but were only observed adhering to the epithelial cells in one of the small intestine samples. By TEM bacteria were seen adhering to intestinal epithelial cells in one tissue sample from both large and small intestines and in these samples bacteria were also seen associated with epithelial cell debris. The Mucolex had no apparent effect on either the adherence or internalisation of bacteria. Large amounts of mucus were seen in all of the tissue samples so the Mucolex seemed ineffective in removing the mucus layer or was replaced during incubation with the bacteria.

5.3.3 Infection of trout intestine *in vivo*.

The results of TEM observation of the interaction of bacteria and intestinal epithelial cells are presented in Table 5.3.2. Electron micrographs are presented in Plates 5.3.1 to 5.3.4 The Plates are each described in detail in the legend, full descriptions will not be given again here but the finding of the electron microscopic study will be summarised.

The control tissues were undamaged by processing and sectioning before examination. These tissues were well preserved. Observation of the gross structure of the gut samples revealed villi, with clearly defined layers. Transmission electron microscopy showed the epithelium predominantly consisted of two cell types, absorptive epithelial cells and goblet cells. The epithelial cells were seen to have the usual complement of organelles and a well-defined brush border. The goblet cells were seen to be full of lightly staining vesicles that contain the mucus, the cell body of the goblet cell was darkly staining and few organelles were visible. No bacteria were seen in any of the control samples.

Bacteria were only visualised in two of the experimental samples, the large intestine at 2 and 6 hours. Even in these samples the bacteria themselves were few but signs of tissue damage were frequently observed. Many examples of epithelial cells with disrupted brush borders were observed. Many of these cells also contained many large vacuoles and many more small microvesicles. These features were unique to the experimental tissue. Furthermore, the lumen of these samples were often seen to contain debris that was from damaged epithelial cells. Membrane bound vesicles and mitochondria both apparently in a good state of preservation were seen free in the lumen. Some cells were seen to form large processes as the cytoplasm was forced into the lumen; these structures were apparently membrane bound. The contents of these large cytoplasmic processes were predominantly granular with only a few small vesicles; the organelles were seen to remain within the cell.

The debris of cells that did not seem of epithelial origin were also frequently seen in the lumen. Cells were not found in the lumen of the control tissue. The debris consisted of

apparently intact nuclei and cell organelles that were apparently without a plasma membrane. The preservation of the cellular fragments was usually good. Bacteria were usually seen close to or within this sort of debris. Many bacteria appeared to have a fuzzy outline suggesting that they might have produced a capsule. Often the bacteria were seen in small groups of between 3 and 5 cells but were sometimes seen singly.

5.3.4 Invasion of MDCK monolayers.

A. salmonicida 644rB was found to adhere strongly to the surface of MDCK cells when monolayers that had not been counterstained were observed. After counter staining most of the previously fluorescent bacteria were no longer visible confirming that the fluorescence had been quenched by the crystal violet. However, some bacteria clearly not associated with host cells were still seen, suggesting that quenching was incomplete. By focusing up and down through the monolayer some bacteria were seen to be intracellular, however, the evidence for invasion of MDCK cells was unconvincing. The use of bacteria prestained with acridine orange reduced the background fluorescence slightly, but did not improve the efficiency of the counter staining.

When the number of invading bacteria was estimated by viable count, it was shown that there was virtually no detectable invasion of these monolayers by *A. salmonicida.*

5.3.5 Invasion of EPC monolayers.

When investigated by acridine orange staining a clear difference was seen between *A. salmonicida* 644rB and both *A. salmonicida* MT004 and *Y. ruckeri* 18/83 the UV light micrographs are presented in Plates 5.3.5-5.3.7. The cells infected with MT004 were virtually free of bacterial cells. The EPC cells had taken up the acridine orange stain themselves and the nuclei of dividing cells were particularly clear but there was very little evidence for the invasion of the EPC cells by MT004. The evidence for invasion by *Y. ruckeri* was more convincing as more bacteria were seen associated with the monolayer, but these were still very few and often only single bacterial cells. *A. salmonicida* 644rB was associated very strongly

with the EPC cells, most of which were seen to have more than ten bacteria per cell. As before the counterstaining evidently failed to quench the fluorescence of extracellular bacteria completely but by focusing through the monolayer many EPC cells appeared to have internalised the bacteria associated with them.

When investigated by viable count a different picture emerged Fig 5.3.1. *Aeromonas salmonicida* 644rB was the most invasive bacteria initially, with the A-layer negative mutant MT004 next and *Y. ruckeri* was shown not to enter EPC cells within the first 15 minutes. The levels of intracellular *A. salmonicida* of both strains then appeared to remain constant for the next three hours. The number of intracellular *Y. ruckeri* increased steadily for the three hours and eventually reaching more than 1% of the inoculum invading. This was the percentage of internalisation decided upon before the start of the experiment as the critical value for calling a bacterial stain invasive. The growth phase appeared not to affect the internalisation of any of the three bacteria used.

The cytopathic effect of the bacteria on the monolayers was investigated at the same time as the assay determining the number of viable bacteria internalised. These cells were treated the same as the other cells except that after killing of the extracellular bacteria with kanamycin the monolayers were not lysed but fixed and stained with Giemsa Fig. 5.3.2. Both strains of *A. salmonicida* caused significant damage to the monolayers after both 1 hour and 3 hours incubation. The cultures grown overnight cased more damage than the 48 hour cultures but no difference between the A-layer positive and negative strains was observed. *Yersinia ruckeri* did not appear to cause any damage to the monolayers.

Table 5.3.1 Summary of SEM and TEM observations of the interaction of bacteria and trout intestinal epithelial cells following in vitro infection.

Internalised bacteria could not be seen using SEM. Ma = Mucolex added after infection, Mb= Mucolex added before incubation.

Specimen	Tissue	Bacteria Observed	Adherent Bacteria	Internalised Bacteria
SEM1(Ma)	Small Intestine	Yes	Not Seen	Not visible
SEM2	Small Intestine	Yes	Not Seen	Not visible
SEM3(Ma)	Large Intestine	Yes	Not Seen	Not visible
SEM4	Large Intestine	Yes	Not Seen	Not visible
SEM5(Mb)	Small Intestine	Yes	Yes	Not visible
SEM6	Large Intestine	Yes	Not Seen	Not visible
TEM1(Ma)	Small Intestine	Not Seen	Not Seen	Not Seen
TEM2	Small Intestine	Yes	Yes	Yes
TEM3(Ma)	Large Intestine	Not Seen	Not Seen	Not Seen
TEM4	Large Intestine	Yes	Not Seen	Not Seen
TEM5(Mb)	Small Intestine	Not Seen	Not Seen	Not Seen
TEM6	Large Intestine	Yes	Yes	Yes

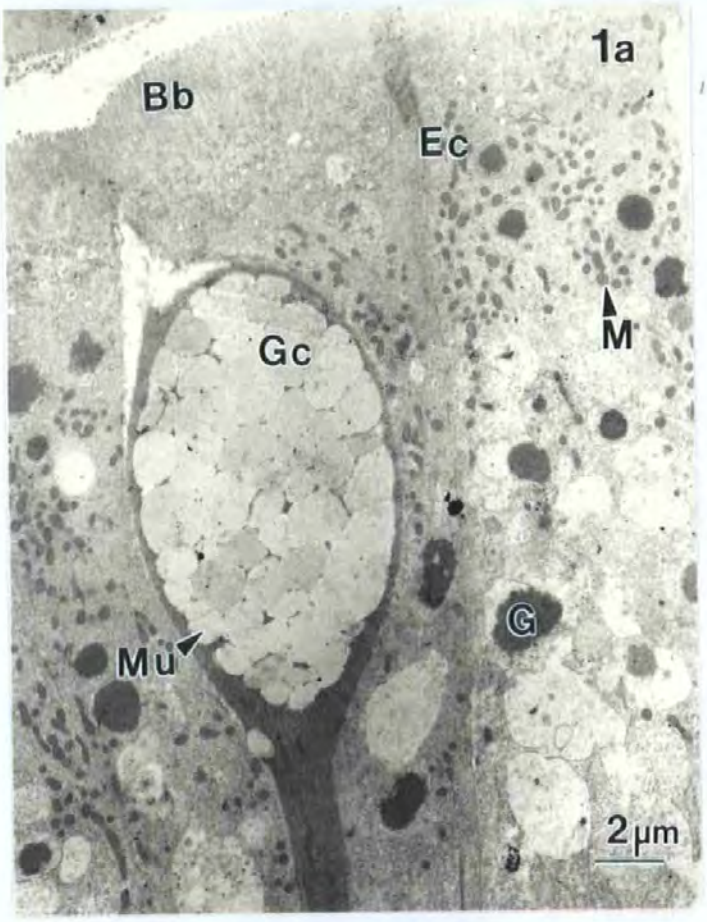
Table 5.3.2 Summary of TEM observations of the interaction of bacteria and trout intestinal epithelial cells following in vivo infection.

Incubation Time	Tissue	Bacteria Observed	Adherent Bacteria	Internalised Bacteria
5 Minutes	Small Intestine	No	Not Seen	Not Seen
	Large Intestine	No	Not Seen	Not Seen
	Boundary	No	Not Seen	Not Seen
15 Minutes	Small Intestine	No	Not Seen	Not Seen
	Large Intestine	No	Not Seen	Not Seen
	Boundary	No	Not Seen	Not Seen
30 Minutes	Small Intestine	No	Not Seen	Not Seen
	Large Intestine	No	Not Seen	Not Seen
	Boundary	No	Not Seen	Not Seen
1 Hour	Small Intestine	No	Not Seen	Not Seen
	Large Intestine	No	Not Seen	Not Seen
	Boundary	No	Not Seen	Not Seen
2 Hours	Small Intestine	No	Not Seen	Not Seen
	Large Intestine	Yes	Not Seen	Not Seen
	Boundary	No	Not Seen	Not Seen
6 Hours	Small Intestine	No	Not Seen	Not Seen
	Large Intestine	Yes	Not Seen	Not Seen
	Boundary	No	Not Seen	Not Seen
18 Hours	Small Intestine	No	Not Seen	Not Seen
	Large Intestine	No	Not Seen	Not Seen
	Boundary	No	Not Seen	Not Seen

Plate 5.3.1 **Electron micrographs of normal intestinal epithelia from rainbow trout.**

Plate 1a *Epithelial cells from the side of a villus in the large intestine of rainbow trout. The goblet cell* (**Gc**) *can be seen between two epithelial cells* (**Ec**). *Inside the goblet cell are numerous mucous containing vesicles* (**Mu**). *The cell body of the goblet cell is darkly staining but the mucus vesicles are not. The brush border* (**Bb**) *of one epithelial cell can be seen as can the lumen of the gut and the brush border of a cell on the other side of the lumen. The epithelial cell contain numerous mitochondria* (**M**) *and darkly staining granules* (**G**) *of approximately 1 to 2.5μm in diameter.*

Plate 1b *An epithelial cell from another location in the large intestine. The microvilli* (**Mv**) *that form the brush border are more clearly evident than in Plate 1a. Again there are numerous mitochondria* (**M**) *and granules* (**G**). *The presence of vesicles* (**Ve**) *between 2 and 2.5μm in diameter is shown. The position of the plasma membrane* (**Pm**) *between two adjacent epithelia cells is also shown.*


1a
Bb
Ec
M
Gc
G
Mu
2μm

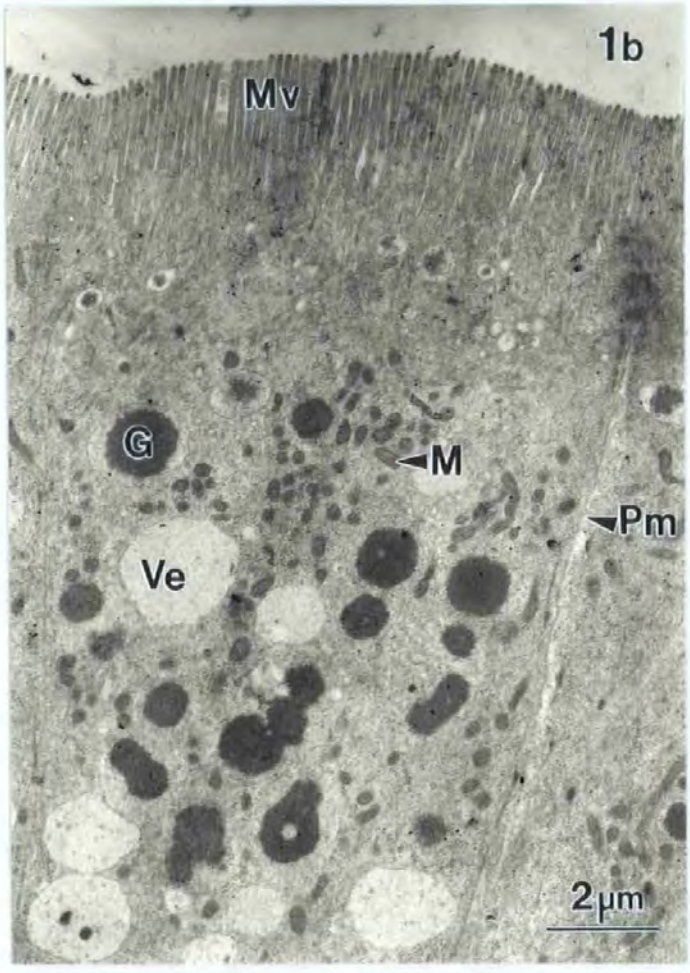
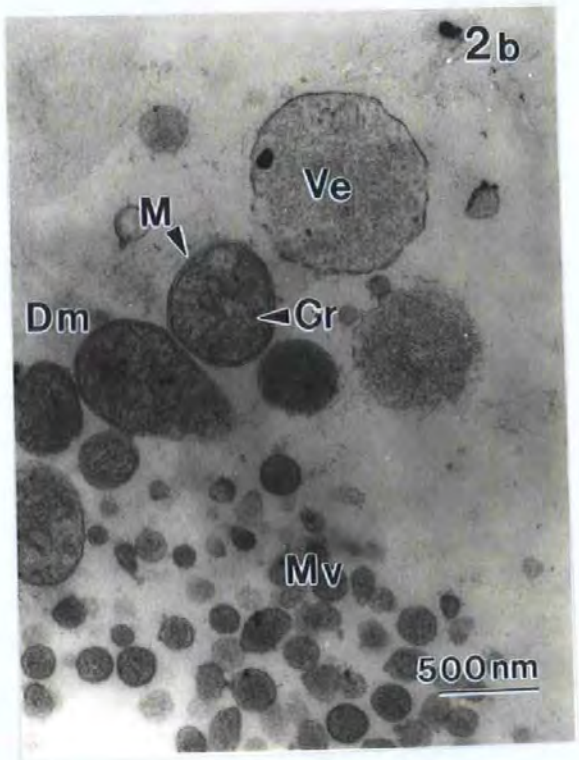

1b
Mv
G
M
Pm
Ve
2μm

Plate 5.3.2 **Electron micrographs of intestinal epithelia from rainbow trout infected with *Aeromonas salmonicida* 644 rB.**

Plate 2a *An epithelial cell and the lumen* (**Lu**) *of the gut can be seen. The epithelial cell has a clearly defined brush border* (**Bb**) *and contains a large vacuole* (**Va**) *of between 2 and 2.5μm in diameter. The lumen appears to contain cellular debris. Microvilli* (**Mv**) *are seen in cross section and there are numerous vesicles* (**Ve**).

Plate 2b *A section from the top left corner of Plate 2a taken at a higher magnification. Mitochondria* (**M**) *are clearly present in the lumen. The ultrastructure of the mitochondria is good and the double membrane* (**Dm**) *and cristae* (**Cr**) *can be seen. A vesicle* (**Ve**) *is also seen this structure has one membrane.*


2b
Ve
M
Dm
Cr
Mv
500nm

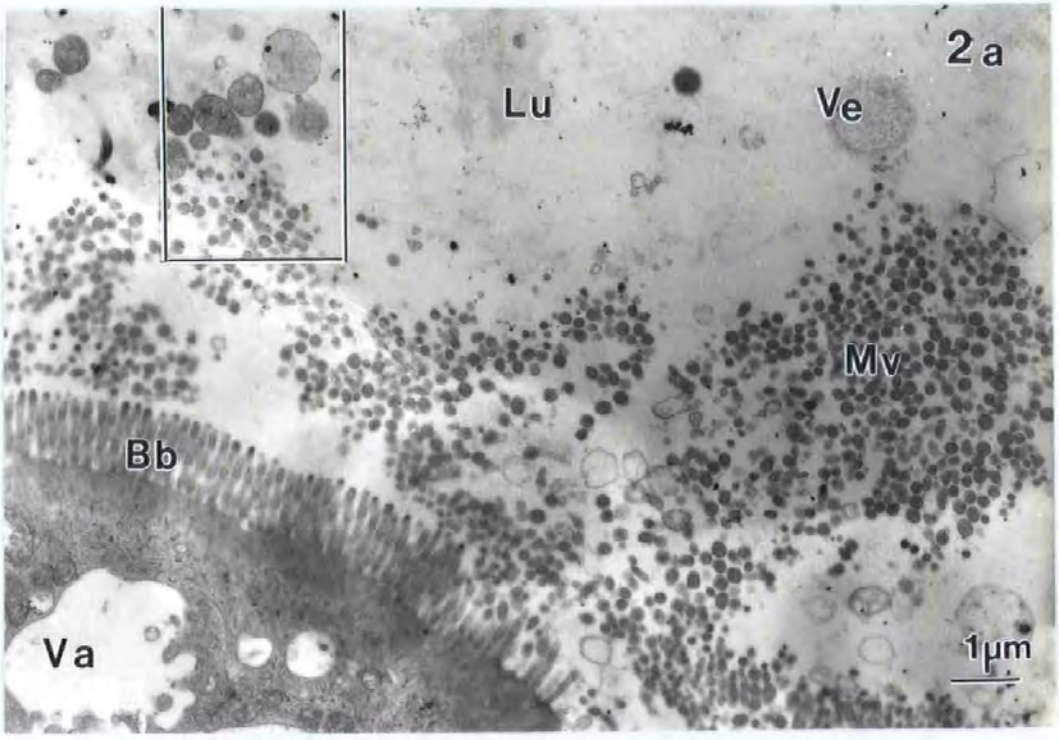
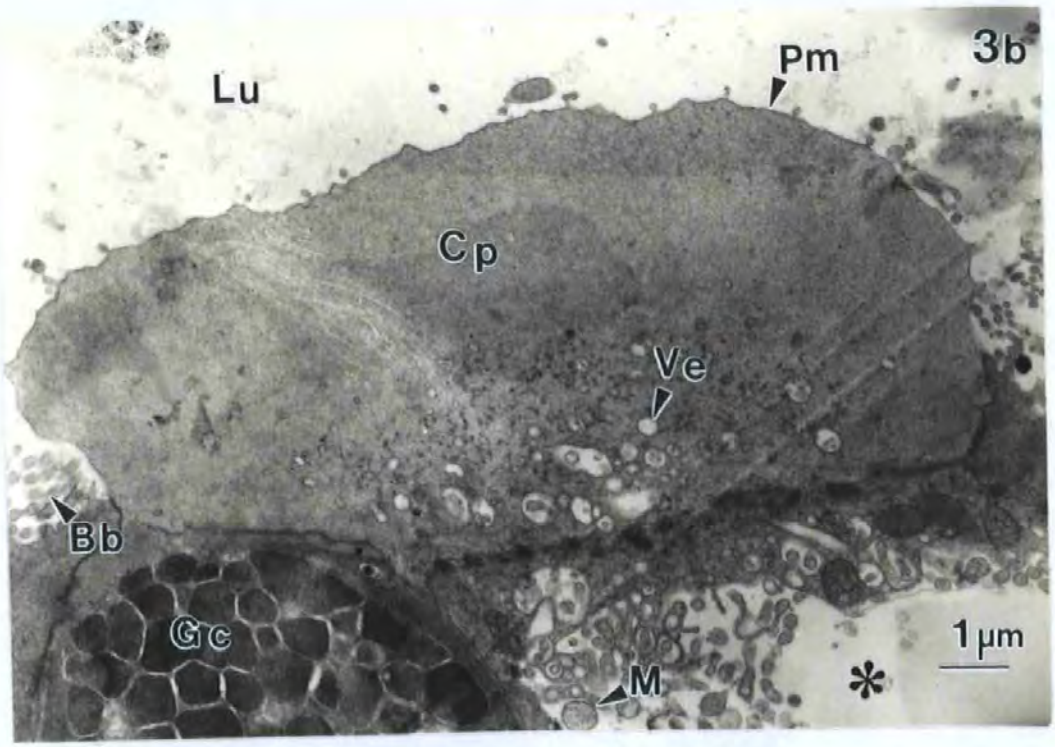

2a
Lu
Ve
Mv
Bb
Va
1μm

Plate 5.3.3 **Evidence of the cytopathic effect of *Aeromonas salmonicida*.**

Plate 3a *Epithelial cells* (**Ec**) *and a goblet cell* (**Gc**) *from the large intestine of rainbow trout 6 hours after being infected with* ***Aeromonas salmonicida.*** *The epithelial cells contain large vacuoles* (**Va**) *of between 3 and 7μm in diameter. They also contain numerous microvesicles* (**Ve**). *The brush border* (**Bb**) *appears to be disorganised. One epithelial cell* (*) *appears to have lost most of its cytoplasm into a membrane bound cytoplasmic process* (**Cp**) *(Plate 5.3.3b). However, the cell still contains mitochondria* (**M**) *and other membrane bound organelles. The brush border of this cell is completely disorganised. The mucus vesicles in the goblet cell appear to be electron dense.*

Plate 3b *The cytoplasmic contents of an epithelial cell* (*) *appear to have been lost into a cytoplasmic process* (**Cp**) *but appear to be contained within the plasma membrane* (**Pm**) *and not free in the lumen* (**Lu**). *The cytoplasmic process contains small vesicles* (**Ve**) *but no mitochondria* (**M**), *which appear to have remained within the cell proper. The formation of the cytoplasmic process has destroyed the structure of the brush border* (**Bb**) *that can still be seen on the neighbouring cell but also appears to be disorganised.*


Lu
Pm
3b
Cp
Ve
Bb
Gc
M
1 µm

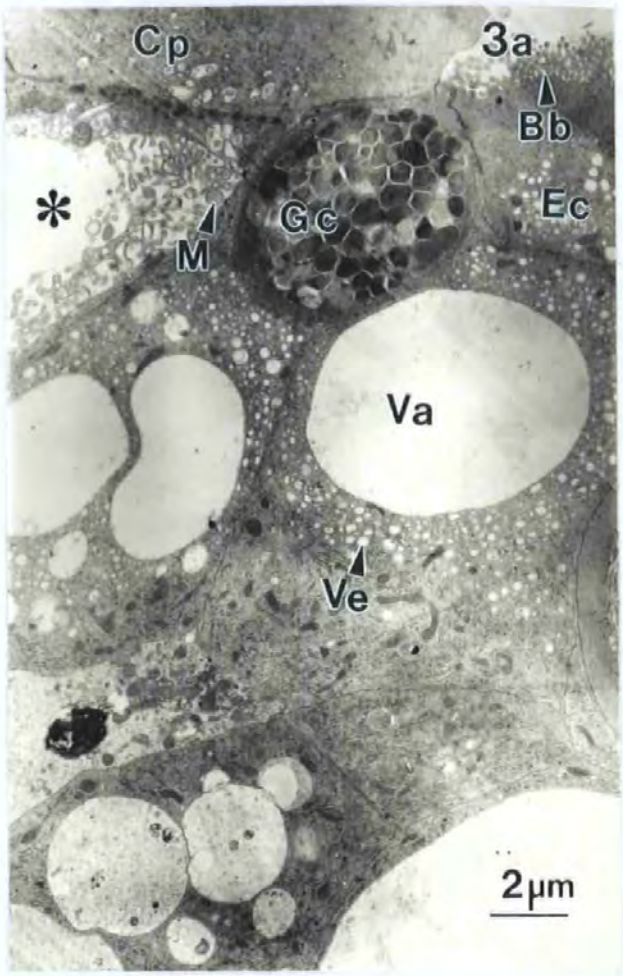
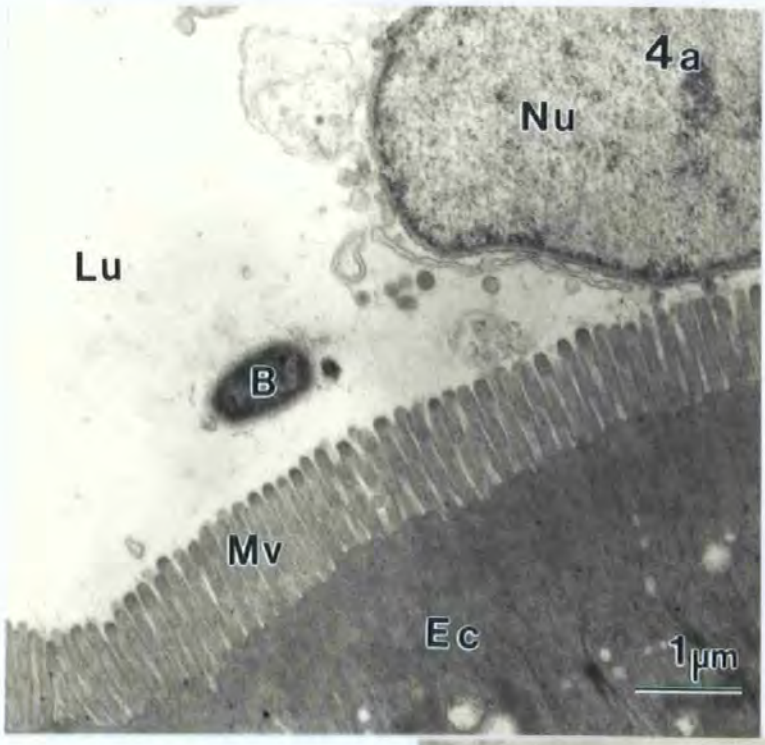

Cp
3a
Bb
Ec
Gc
M
Va
Ve
2µm

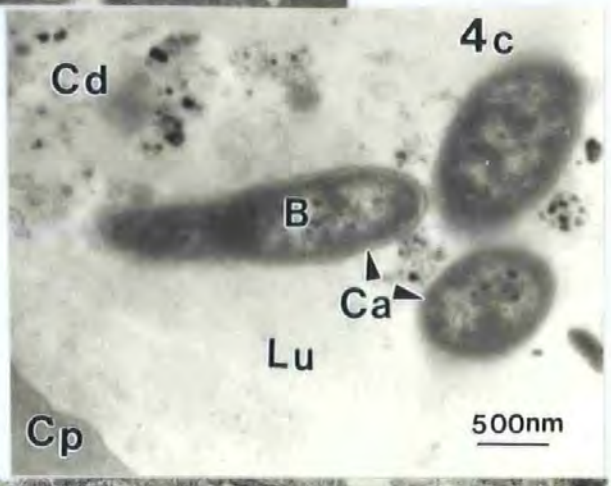
Plate 5.3.4 Debris from nonepithelial cells in the lumen of the large intestine.

Plate 4a *Bacterial* (**B**) *cell can be seen close to the microvilli* (**Mv**) *of an epithelial cell* (**Ec**) *and the debris of another cell. The nucleus* (**Nu**) *of the dead cell is apparently free in the lumen* (**Lu**).

Plate 4b *Another dead cell in the lumen, closely associated with the microvilli* (**Mv**) *of the brush border of an epithelial cell. The nucleus* (**Nu**) *and nuclear envelope* (**Ne**) *are present and apparently well preserved. The other cellular organelles are also well preserved; mitochondria* (**M**), *smooth endoplasmic reticulum* (**SEr**) *and the rough endoplasmic reticulum* (**REr**) *can be clearly seen as can the ribosomes* (**r**) *on the REr. A bacterial cell* (**B**) *is present close to the dead cell and epithelium.*

Plate 4c *Three bacterial cells found within the debris of a cell* (**Cd**) *in the lumen* (**Lu**). *The ultrastructure of the bacteria is clearly visible and the three cells may be surrounded by capsular material* (**Ca**). *Some membrane bound cell debris can be seen close to the bacteria cells* (**Cp**).


4a
Nu
Lu
B
Mv
Ec
1µm


4c
Cd
B
Ca
Lu
Cp
500nm

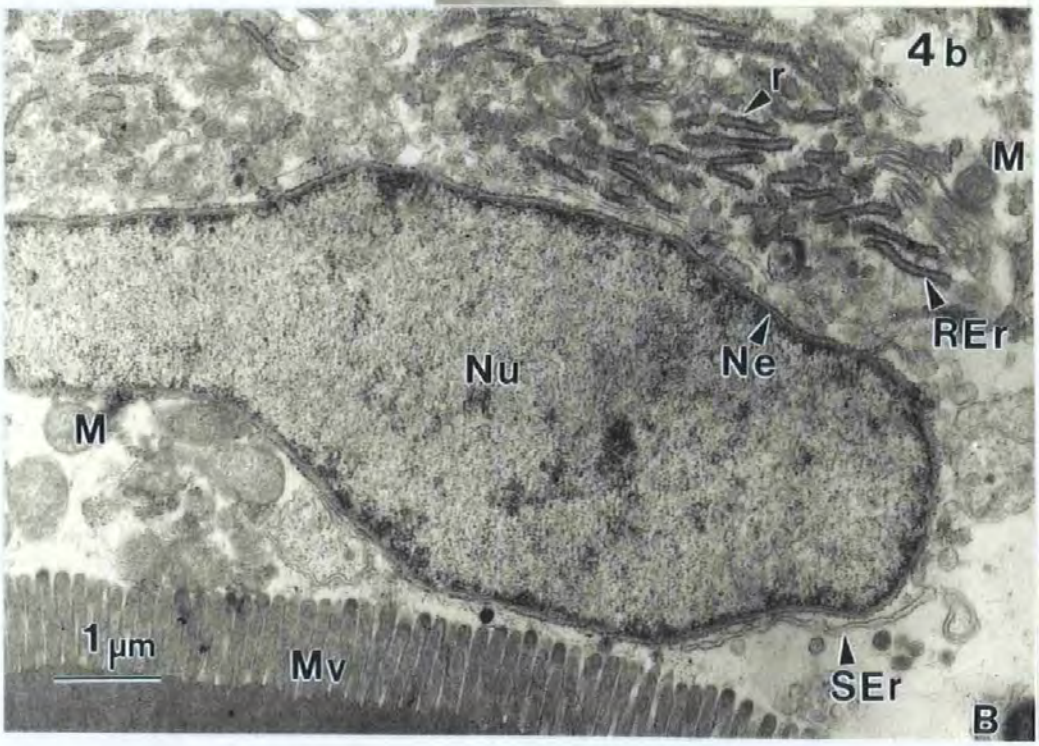

4b
r
M
REr
Ne
Nu
M
1µm
Mv
SEr
B

Plate 5.3.5 **EPC monolayers infected with *A. salmonicida* MT004 and stained with acridine orange.**

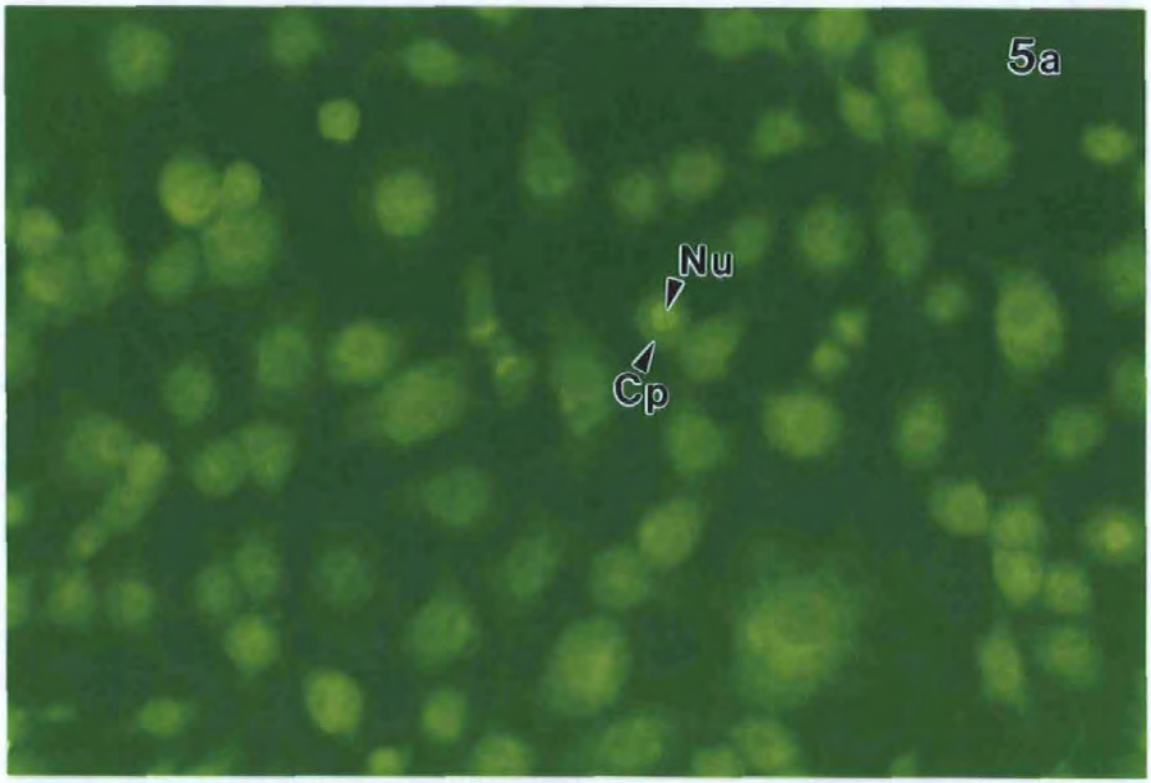
Plate 5a *The monolayer is subconfluent. The acridine orange has stained the cytoplasm* (**Cp**) *and the nucleus* (**Nu**) *of the EPC cells. No bacteria can be seen. Magnification x1714.*

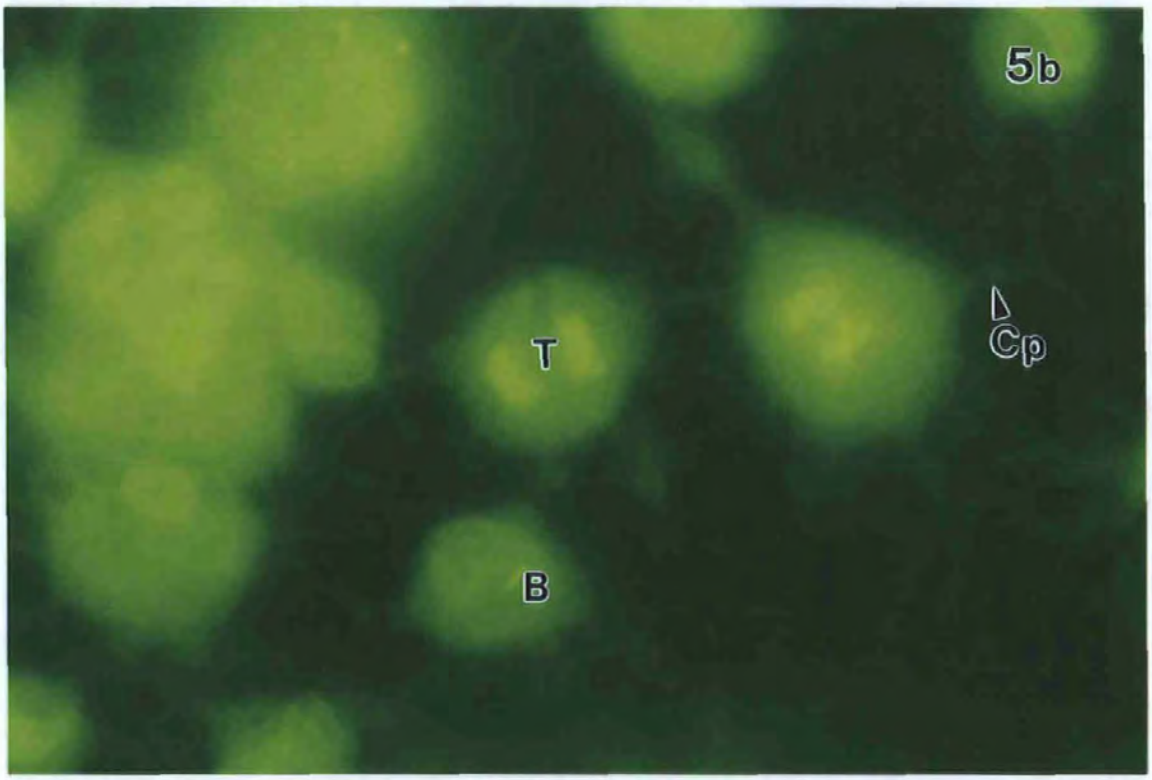
Plate 5b *A high magnification micrograph of a monolayer. An EPC cell is undergoing mitosis and is in telophase* (**T**). *The EPC cells have cytoplasmic processes* (**Cp**) *extending into the surrounding area. The bacterial cell* (**B**) *was the only one seen within an epithelial cell infected with MT004. Magnification x4286.*

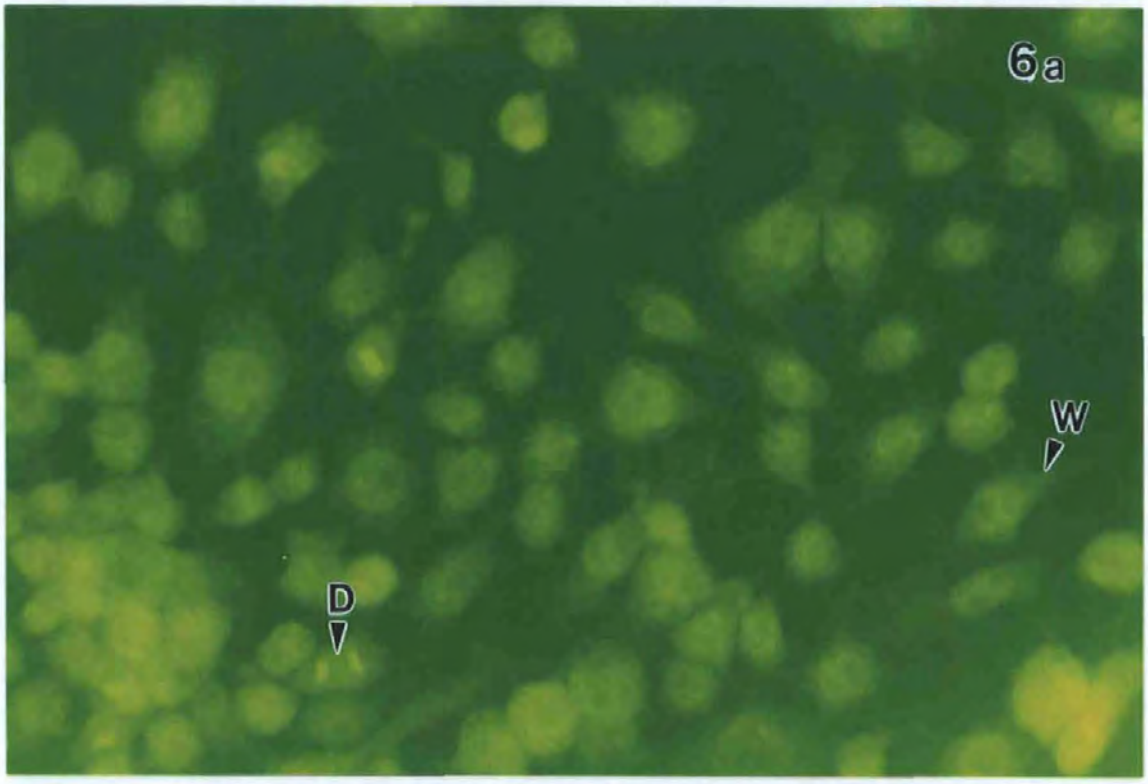
Plate 5.3.6 **EPC monolayers infected with *Yersinia ruckeri* 18/83 and stained with acridine orange.**

Plate 6a *The monolayer is subconfluent. A dividing* (**D**) *cell can be seen and the 'web-like'* (**W**) cytoplasmic processes are visible. *No bacteria can be seen. Magnification x1714.*

Plate 5b *A high magnification micrograph of a monolayer. Again the nucleus* (**Nu**) *and the cytoplasm* (**Cp**) *have taken up the stain. The bacterial cell* (**B**) *was one of very few seen within an epithelial cell. Magnification x4286.*


5a
Nu
Cp


5b
Cp
T
B


6a
W
D

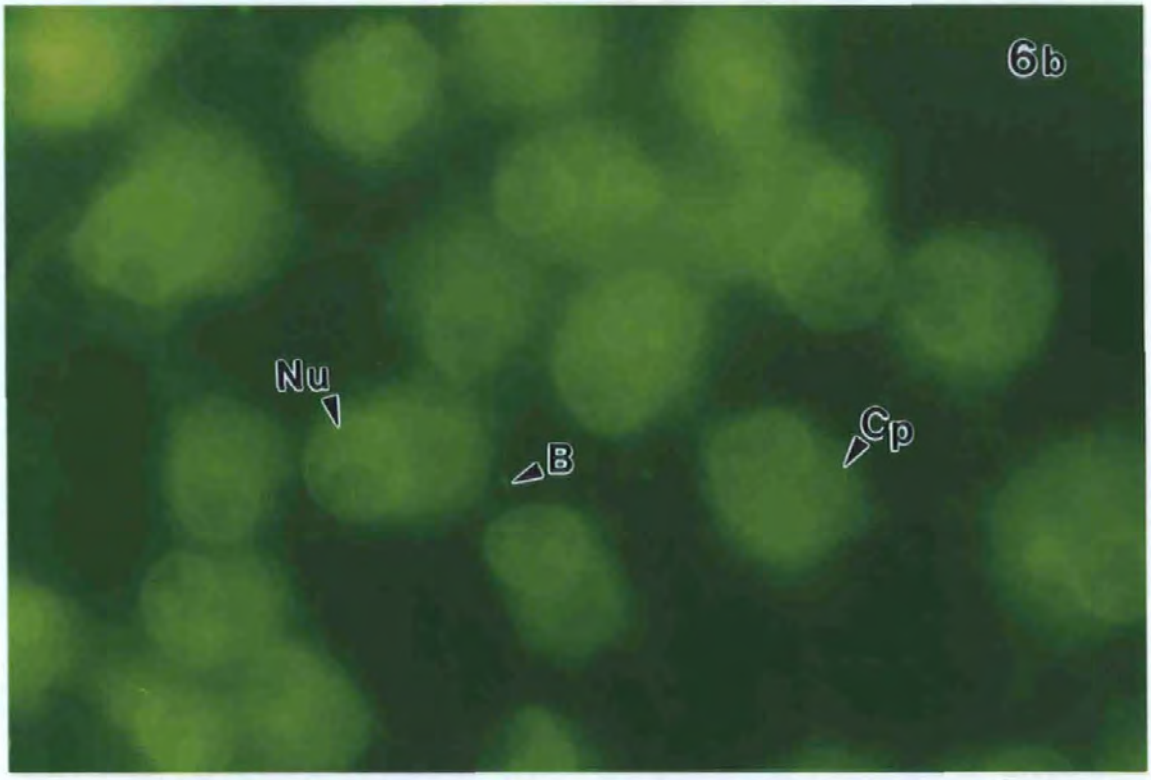
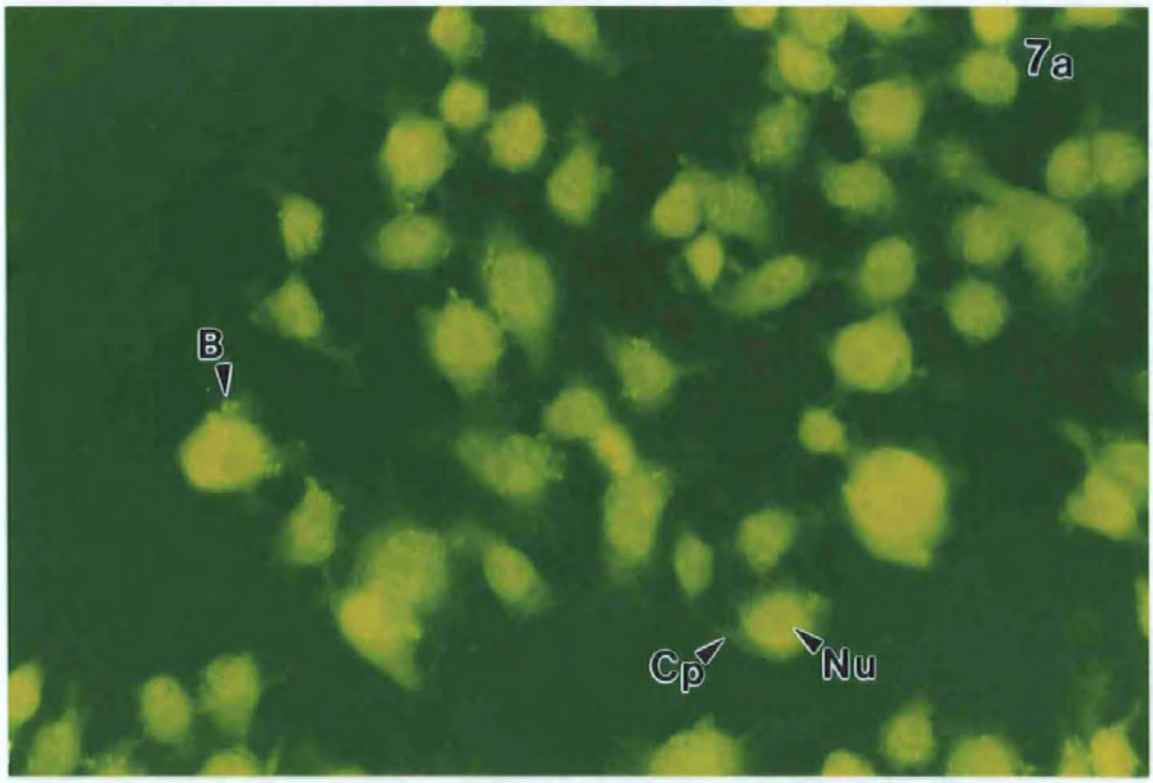

6b
Nu
B
Cp

Plate 5.3.7 **EPC monolayers infected with *Aeromonas salmonicida* 644rB and stained with acridine orange.**

Plate 7a Thin strands of cytoplasm (**Cp**) can be seen spreading out from the more intensely stained nuclei (**Nu**). Many bacteria (**B**) can be seen associated with EPC cells.

Plate 7b At higher magnification it is possible to see that each EPC cell is associated with many bacterial cells (**B**). The nuclei (**Nu**) and cytoplasm (**Cp**) of these cells appears to fluoresce more brightly than uninfected cells.


7a
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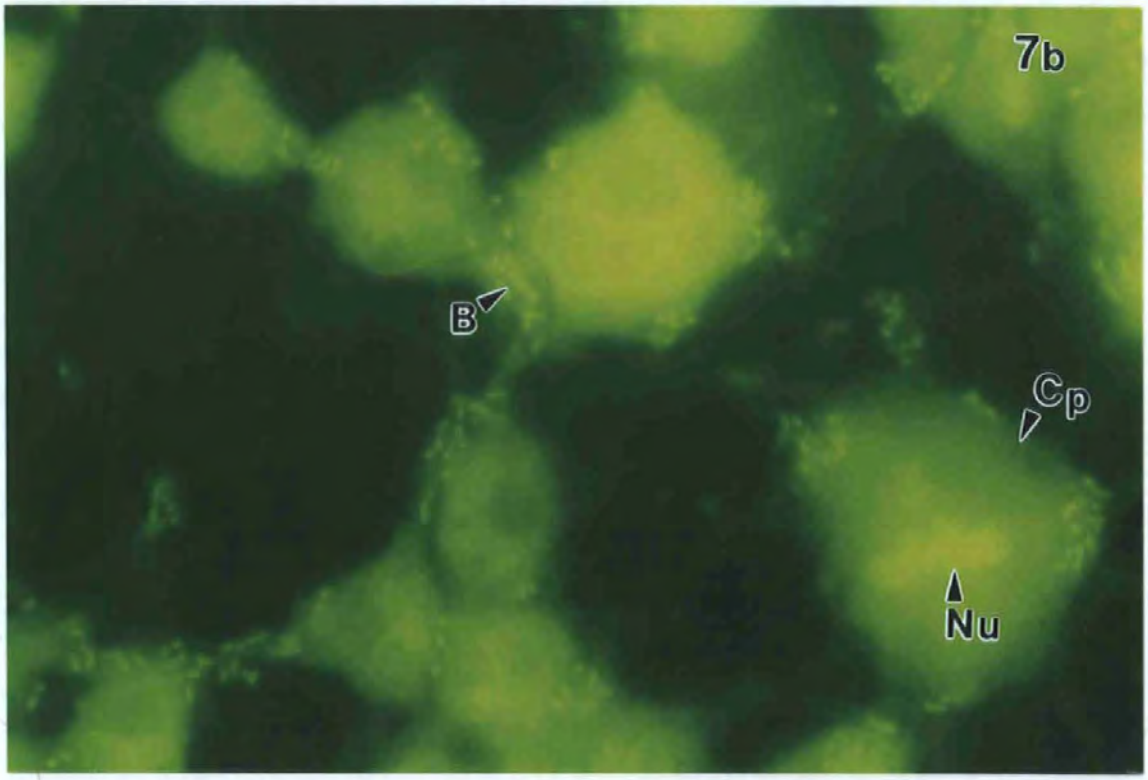

7b
B
Cp
Nu

Figure 5.3.1 Viable bacteria recovered from EPC monolayers.

The number of viable bacteria (CFU) recovered from monolayers of EPC cells. Bacteria were incubated with the EPC cells for 15 minutes, 1 hour and 3 hours at 15 °C. At the end of the incubation period extracellular bacteria were killed by incubation for two hours with kanamycin at 150μgcm^{-3}. After careful washing the monolayers were lysed with Triton X-100 to release intracellular bacteria. The bacteria used, were ***Y. ruckeri*** *18/83 (■),* ***Aeromonas salmonicida*** *MT004 (●), and* ***Aeromonas salmonicida*** *644rB (▲). The bacteria were grown either overnight (Part A) or for 48 hours (Part B) prior to infection of the monolayers. The error bars represent the SDn-1 (n=5).*

Fig. 5.3.1 Part A Bacteria Grown Overnight

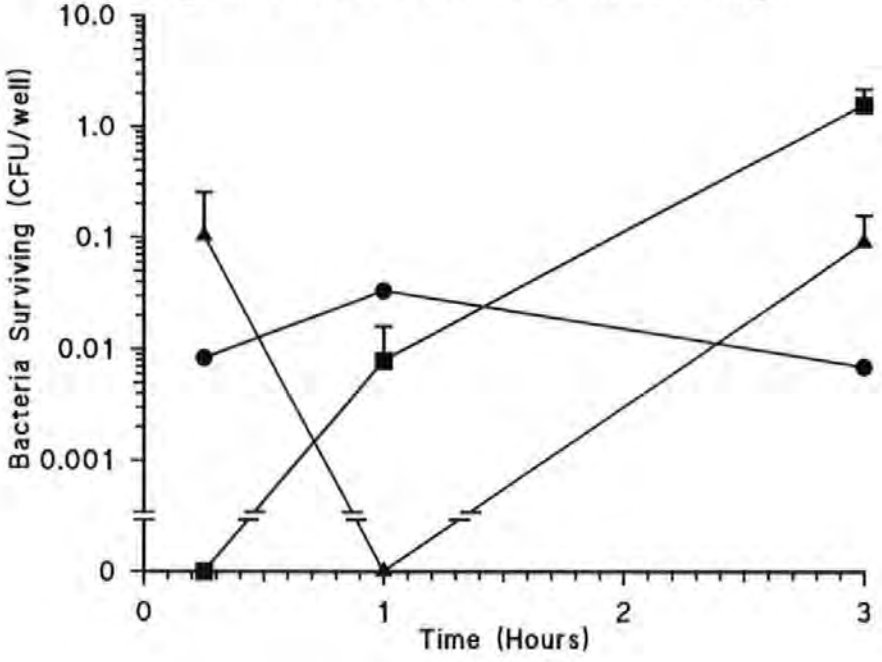
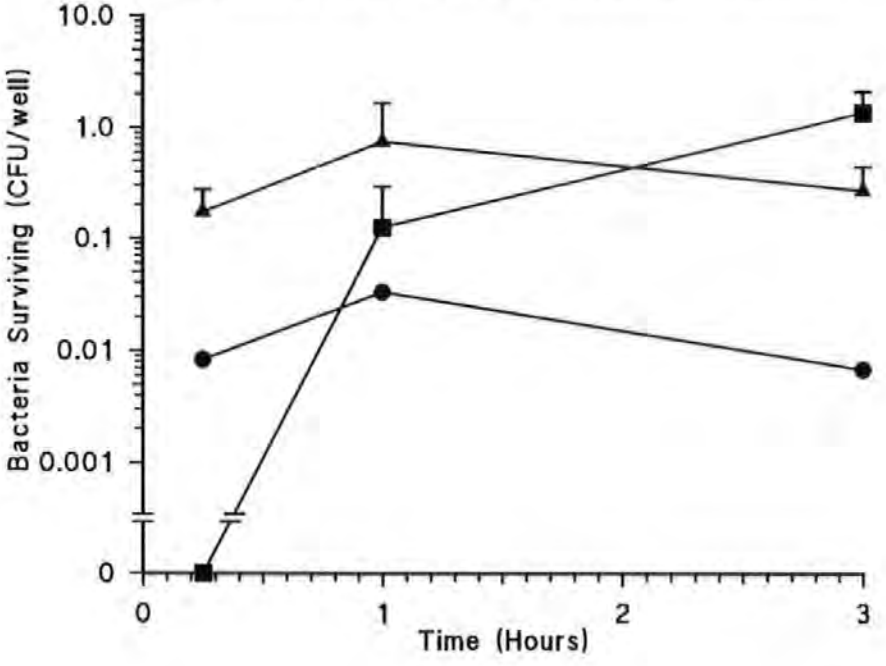


Fig. 5.3.1 Part B Bacteria Grown For 48 Hours



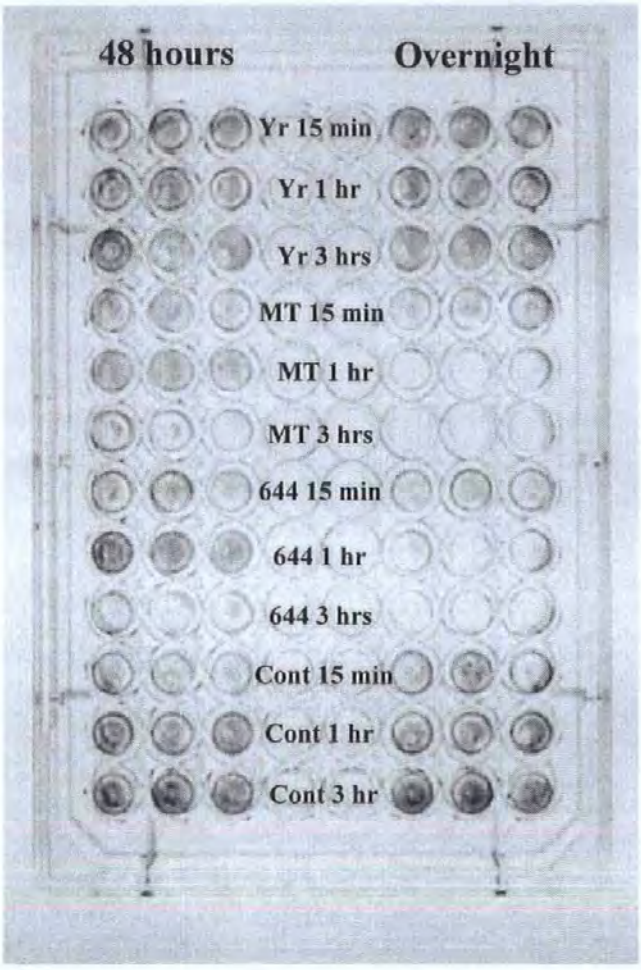


Figure 5.3.2 Viability of EPC cells following infection of monolayers with bacteria. *Bacteria were incubated with the EPC cells for 15 minutes, 1 hour and 3 hours at 15 °C. At the end of the incubation period extracellular bacteria* were *killed by incubation for two hours with kanamycin at 150μgcm^{-3}. The monolayers were carefully washed and the cells fixed by the addition of 100% methanol. The monolayers were stained with Giemsa to show to what extent the monolayers were damaged by the bacteria. The bacteria used were* ***Y. ruckeri*** *18/83,* ***Aeromonas salmonicida*** *MT004* and **A. salmonicida** 644rB. *The bacteria were grown either overnight or for 48 hours prior to infection of the monolayers. The control wells were incubated for the same time intervals as the experimental wells but only HBSS was added to them.*

5.4 Discussion.

A range of techniques were used to investigate the interaction of *A. salmonicida* and the intestinal epithelial cells of rainbow trout and tissue culture fish and canine cells. The investigation was preliminary in nature but has yielded valuable information.

Bacteria stained with acridine orange prior to being intubated or injected into rainbow trout were seen to localise in the kidney, but not the spleen or liver. The use of this technique to study uptake and tissue localisation was limited however, as it was less sensitive than the detection of viable bacteria described earlier (Chapter Four).

The *in vitro* investigation of invasion of rainbow trout intestinal epithelia failed to show any evidence of the mechanism of uptake of *A. salmonicida* by epithelial cells. However, bacteria were found adhering to the surface of epithelial cells and were seen amongst the debris of dead cells. This suggested that the bacteria were damaging the epithelial cells.

The *in vivo* investigation also failed to show any bacteria being taken up by host cells but did show that infection of the intestine by bacteria causes dramatic changes in the morphology of the epithelial cells. The appearance of large vacuoles and the presence of many microvesicles were also observed as was disorganisation of the brush border. Yamamoto (1966) examined the intestine of rainbow trout and goldfish using TEM. He described the presence of large vacuoles in the goldfish intestine but found no evidence for them in rainbow trout, indicating that the vacuoles observed here were most likely associated with infection by *A. salmonicida*.

Many cells that did not appear to be of epithelial origin were also seen in the lumen. They appeared to be well preserved except that they did not possess a plasma membrane. Bacteria were usually seen close to the debris of these cells. The GALT of rainbow trout has been described (Davidson, 1991) and for other species of teleost (Doggett, 1989; Rombout

et al., 1993). Large and small intestinal macrophages were described in rainbow trout by Davidson (1991) and in carp by Rombout *et al.* (1985, 1989b). In the latter study small cells were found in the lamina propria and the large cells were found between epithelial cells. Neutrophils have also been described in the digestive tract of carp (Rombout *et al.*, 1989) and rainbow trout (Sharp, 1990). The presence of macrophages and neutrophils in teleost cutaneous mucosa has also been demonstrated (Mittal *et al.*, 1980; Peleteiro and Richards, 1985; Davidson, 1991) and Vallejo and Ellis (1989) described the presence of eosinophilic granular cells in the gut and likened them to mast cells.

The attachment of an array of bacterial pathogens to epithelial surfaces is accompanied by recruitment of host defence cells as manifested by transepithelial migration of neutrophils (PMN). For example migration of PMN across renal tubular, urinary transitional, or bronchial epithelia represents the histological definition of acute bacterial pyelonephritis (Ivanyi *et al.*, 1983), acute cystitis (Koyama *et al.*, 1991), and acute bronchitis (Tsujimura *et al.*, 1980), respectively. Similarly, transmigration of PMN across intestinal epithelia is a hallmark of bacterial enterocolitis, exemplified by salmonellosis (Takeuchi, 1967; Yardley and Donowitz, 1977; Day *et al.*, 1978; McGovern and Slavuten, 1979; Kumar *et al.*, 1982). McCormick *et al.* (1993) showed that the attachment of *Salmonella typhimurium* to human intestinal epithelial cells leads to transepithelial migration of PMN whereas similar densities of *E. coli* failed to stimulate a directed migration. Furthermore, it appeared that the mechanism of transepithelial migration was distinct from the classical pathway by which bacteria normally stimulate migration, it was shown by McCormick *et al.* (1995) that the attachment of *Salmonella* initiate the formation of a gradient in the subepithelial matrix that is chemotactic for PMN. It is possible that the dead cells seen in these sections were neutrophils or macrophages that had migrated into the lumen in response to the presence of *A. salmonicida*. It is known that *A. salmonicida* has a cytotoxic effect on macrophages and neutrophils (Olivier *et al*, 1992; Lamas and Ellis, 1994a). However, both cell types are capable of killing *A.*

salmonicida (Sharp and Secombes, 1993; Lamas and Ellis, 1994a). It has also been suggested that the bacteria can live as a facultative intracellular parasite in peritoneal macrophages (Garduño *et al.*, 1993b). The cell debris may represent those cells that have taken up bacteria and been killed by them. It is possible that many other phagocytic cells have taken up bacteria and killed but there was no evidence for this.

Lavelle (1994) showed that *A. salmonicida* invaded isolated enterocytes very rapidly, within 5 minutes but that after 30 minutes incubation no bacteria could be found inside the enterocytes but they were seen amongst cellular debris after this time. This confirms the observations *in vivo* and *in vitro* (discussed below) that *A. salmonicida* lyses the cells it invades or becomes associated with.

The presence of large vacuoles in the cytoplasm of the epithelial cells may be associated with this cytopathic effect; however, bacteria were not seen to be associated with these cells suggesting that internalisation of viable bacteria was not required for the production of vacuoles. The development of the large cytoplasmic processes may well be linked to Lavelle's observation of actin polymerisation at the site of bacterial adhesion, but again, bacteria were not seen at the sites of these lesions. Therefore, the role of *A. salmonicida* in the formation of these processes cannot be confirmed.

A. salmonicida appeared to be only poorly invasive for MDCK cells. This cell line is of mammalian origin and was therefore, a poor model for the uptake of a psychrophilic bacteria like *A. salmonicida.* The cell line was chosen because of its well-defined brush border that provides a more realistic *in vitro* invasion model than non polarised cell lines like EPC and AS2 cells. Invasion is a process that is dependent on both the eucaryotic and bacterial cell. While MDCK cells were grown at 37°C the invasion assay took place at 25°C which was at the tolerance limit for both the bacteria and MDCK cells. It is not particularly surprising that the bacteria failed to be invasive, but it was unfortunate, as at present there is no fish cell line with a defined brush border and MDCK cells would have provided a good *in*

vitro model. The failure of the crystal violet to quench the fluorescence of extracellular bacteria was not described by Lavelle (1994), but has been reported by Nishikawa *et al.* (1994) when investigating the invasion of CACO-2 cells by strains of *A. hydrophila* and *A. sobria*, both intestinal pathogens of humans. This resistance to counterstaining was attributed to the surface proteins of these mesophilic Aeromonads. These workers increased the counter staining time to 30 minutes and reported that this was effective in quenching extracellular bacteria.

The EPC cells appeared to be capable of taking up all of the bacteria tested to some degree. These monolayers were incubated for an hour with *Y. ruckeri* and, *A. salmonicida* 644rB and MT004 stained with acridine orange and counterstained with crystal violet for 30 minutes. Large numbers of the A-layer positive *A. salmonicida* 644rB were associated with the cells in the monolayer, but very few *Y. ruckeri* and virtually no A-layer negative *A. salmonicida* MT004 were seen. The visualised bacteria all fluoresced bright green indicating that the cells were viable.

The detection of viable bacteria after killing of extracellular bacteria and lysis of the EPC cells showed that, initially *A. salmonicida* 644rB invaded the EPC cells more effectively than the other two bacterial strains. However, the level of an invasion did not appear to increase over the three hours of the assay whereas the numbers of invading *Y. ruckeri,* were seen to rise throughout this period.

It had been shown in a preliminary assay that after incubation for two hours with $100\mu gcm^{-3}$ of kanamycin the number of viable bacteria dropped to approximately $2x10^{-5}$ % of the original inoculum. The percentage surviving in the cell invasion assay were considerably greater than this and the concentration of kanamycin had been increased to $150\mu gcm^{-3}$ making it very unlikely that these results were due to the presence of antibiotic resistant bacteria.

Since at least 1% of the inoculum of *Y. ruckeri* had invaded the monolayer within the three hours it was termed an invasive strain. The levels of either of the *A. salmonicida* strains failed to reach this level and so they appeared to be non invasive. The growth phase of the bacteria did not appear to affect the ability to invade EPC cells.

These conclusions were complicated by the observation of very profound cytopathic effect following incubation of *A. salmonicida* with the monolayers. Both strains of *A. salmonicida* were found to cause the destruction of monolayers after three hours and had induced very serious damage after just 1 hour. The amount of damage caused was dependent on growth state with the overnight cultures being more cytotoxic than the 48 hour cultures. MT004 appeared to be more damaging than 644rB despite being apparently unable to adhere to the monolayer. This suggests that the cytopathic effect was caused not by invasion but by the ECPs that are produced in great quantities by MT004. The GCAT and serine protease of *A. salmonicida* are both capable of producing this sort of damage and the GCAT is known to have a membrane lysing activity (Lee and Ellis, 1990).

It is possible, therefore, that *A. salmonicida* 644rB if not MT004, is invasive and this is being masked because cytotoxic products are destroying the cells it invades. *A. salmonicida* 644rB apparently invades EPC cells more rapidly than *Y. ruckeri.* This might be due to autoaggregation increasing the rate it settles on to the monolayer and hence speeds the rate of invasion. This does fit with the observation that large numbers of *A. salmonicida* were associated with each invaded EPC cell but only one *Y. ruckeri* cell was observed to invade each EPC cell.

The invasion of the host via mucosal epithelial cells is an important step in the pathogenesis of many microorganisms and to date this phenomenon has not been studied with reference to bacterial pathogens of fish. Lavelle (1994) showed that isolated enterocytes and an Atlantic salmon cell line could be shown to internalise the bacteria. He also showed that *Y. ruckeri* was not invasive, a finding contradicted by this study.

No evidence was found either to support or contradict Lavelle's speculation that *A. salmonicida* invades using a similar process to the 'attachment and effacing' invasion mechanism of EPEC strains.

Most of his work was performed on isolated enterocytes which are a poor model for the investigation of invasion, since the basolateral surfaces are exposed to the bacteria when this would not normally happen *in vivo*. This may explain why Lavelle (1994) was able to show that *A. salmonicida* was internalised with such frequency by these cells. This effect was discussed with reference to *Shigella* in chapter two of this thesis (Mounier *et al*, 1992).

The presence of nonepithelial cells in the lumen of the gut suggests that infection by *A. salmonicida* induces the migration of professional phagocytic cells through the epithelium and into the lumen where they phagocytose the bacterial cells. It is known that *A. salmonicida* can kill macrophages but it has also been suggested that it can live within macrophages as an intracellular parasite. This would provide a potentially important mechanism of dissemination within the host. This hypothesis would explain the presence of bacteria close to and within the debris of these cells and may account for the lack of bacteria within the epithelial cells.

The infection of cells *in vitro* allows the delivery of a known multiplicity of infection. Lavelle (1994) routinely used a MOI. of 100 as he found this gave the optimal level of observable invasion. The delivery of a MOI. of 100 to an entire trout intestine is most impractical. This may well explain why very few bacteria were seen.

The A-layer positive strain was found associated with the monolayer to a much greater extent than the A-layer negative strain. The importance of the A-layer in the virulence of *A. salmonicida* has long been established (Udey and Fryer, 1978; Ishiguro *et al.*, 1981; Munro, 1984; Belland and Trust, 1985; Trust, 1986; Noonan and Trust, 1996). Udey and Fryer (1978) showed that A-layer positive strains of *A. salmonicida* could adhere to fish intestinal cells as well as human, rabbit and fish leucocytes. The mechanism of binding is poorly understood especially when compared to adhesins such as the K88 fimbrial adhesin of *E. coli* and the

adhesins of *Bordatella pertussis* discussed above (Chapter Two). It is thought that nonspecific hydrophobic interactions between the A-layer and the host cell are the most important means of adhesion (Titball and Munn, 1985). Hydrophobic interactions are recognised as important in the adhesion of bacteria to mammalian cells (Macura, 1987; Doyle and Rosenburg, 1990; Wibawan *et al.*, 1992). They appear to mediate adhesion by overcoming the weak electrostatic forces that repel bacteria and host cell over short distances due to mutual negative charges (Jones, 1977). Parker (1985) found that *A. salmonicida* adhered most effectively to fish cells suggesting the presence of specific recognition factors, this was confirmed by this study, as there was a much lower level of adherence to MDCK cells compared to ECP cells. Comparision of the amino acid sequence of the A-layer protein revealed similarities with the K88 fimbriae of *E. coli* (Evenberg and Lugtenburg, 1982) providing confirmation of the likely role of the A-layer in colonising the host. The role of the A-layer in the invasion of EPC cells was confirmed by this investigation.

Aeromonas salmonicida causes a septicaemic disease suggesting that it must be able to penetrate the mucosal membranes of the host. The work by Lavelle (1994) suggested that this might be accomplished by invading directly through the epithelial cells of the intestine since he showed that the bacteria were capable of invading isolated enterocytes and an Atlantic salmon cell line. The work presented here confirms the ability of *A. salmonicida* to invade fish tissue culture cells. However, it questions the relevance of this ability as an invasion strategy. *In vivo* there is evidence, although limited, which suggests that the bacteria may induce an inflammatory response that causes infiltration of phagocytic cells into the lumen of the gut. The bacteria may then live as facultative intracellular parasites and be transported around the fish within the phagocyte (Garduno *et al.*, 1993b). The data described in chapter four suggested that the bacteria accumulate within the tissues in a pattern that suggests they are spread by the reticuloendothelial system. The bacteria may be transported around that system within phagocytic cells.

There is evidence that the bacteria induce significant pathological lesions in the epithelial cells of the intestine and the phagocytic cells. There is no obvious advantage to the bacteria in damaging the phagocytes but this may only happen when the phagocyte takes up several bacteria. The ability of *A. salmonicida* to avoid phagocytic killing and its ability to kill phagocytes is well documented.

The investigation of invasion by fish pathogens is of great importance. However, it is inhibited because of a lack of a good *in vitro* model. The difficulty is in establishing the normal route of infection, and in this case the cytotoxic activity of the bacteria. The use of molecular biology to create invasion/infection deficient mutants would certainly aid the investigation and should be pursued. The ineffectiveness of crystal violet in quenching extracellular *A. salmonicida* may mean that further TEM work should be done to demonstrate that bacteria are internalised by the fish cells.

In conclusion, this study has added information on the invasion of enterocytes *in vivo* and has quantified the invasiveness of *A. salmonicida* but many questions are left unanswered.

Chapter Six.

The effect of the live attenuated vaccine strain of *Aeromonas salmonicida* on rainbow trout nonspecific immune parameters.

6.1 Introduction.

Vaccination against furunculosis has been attempted for more than 50 years but the protection can be described as inconsistent at best (Munro 1984; Ellis 1985a; Hastings 1988; Midtlyng, *et al.*, 1996; Chapter 1). One of the major problems with effective vaccine design has been the lack of correlation between antibody response and protection (Cipriano and Heartwell, 1986; Austin and McIntosh, 1988). Furthermore, Olivier *et al.* (1985) showed that adjuvant alone administered to coho salmon gave protection to coho salmon from infection with *A. salmonicida,* and the nonspecific activation of macrophages was believed to be responsible for this result.

The nonspecific immune response has often been purported to be of greater importance in the protection of fish than of mammals because of the apparently less effective specific immune response. It is known that the primary immune response of fish is slower than that of mammals and that fish are apparently able to recognise and respond to fewer antigens when compared to rabbits (Hastings and Ellis, 1988). Nonspecific immunity in fish has been the subject of several reviews (Ellis, 1981; Alexander, 1985; Alexander and Ingram, 1992). It is not the intention to review the full range of humoral nonspecific immune factors in this work, but to concentrate on those factors that were investigated namely serum and skin mucus lysozyme, complement activity, serum protein level, α-2 macroglobulin, and the antibacterial activity of serum and mucus.

Lysozyme is one of the best characterised of all enzymes, its molecular weights, amino acid sequences, nucleotide sequences, conformations and functions are all well known (Alexander and Ingram, 1992). Lysozyme primarily attacks structures containing β 1-4 linked N acetylmuramine and N-acetylglucosamine which are components of bacterial cells walls. The enzyme has also been shown to have a role in the defence against viruses, neoplasmas and possibly fungi and eukaryotic parasites (Alexander and Ingram, 1992). Lysozyme has

previously been found to increase in the serum of fish following exposure to forgein material (Fletcher and White, 1973).

Two lysozymes (types I and II) were isolated from rainbow trout by Grindle and coworkers (1988) and the antibacterial activity of these enzymes was compared with that of hen egg white lysozyme (HEWL) (Grindle, 1989) in order to evaluate their role in protection. The activities of the three enzymes was tested against a range of Gram negative pathogens commonly found in fish on Norwegian fish farms. Grindle found that, the two forms of the rainbow trout lysozyme were equally potent against *Y. ruckeri* but that the type II enzyme was more effective against *V. salmonicida*, *A. salmonicida,* and *V. anguillarium* than either the type I or HEWL. No evidence of a synergistic effect between the two forms of lysozyme was found. The HEWL was much less effective against the fish pathogens than the rainbow trout lysozyme and this may be evidence of a species specific adaption of the enzyme.

Rainger and Rowley (1993) investigated the antibacterial activity of the serum and mucus of rainbow trout following immunisation with *A. salmonicida.* They found that the level of lysozyme in the serum of fish injected with bacteria increased peaking at ten days post immunisation, whereas the primary antibody response peaked at day 30. They also found that the levels of lysozyme in the serum of fish vaccinated with saline alone also increased significantly peaking at day ten. The increase in the fish receiving bacteria was significantly greater than the increase in the control fish however. Rainger and Rowley postulated that the increase in lysozyme concentration in the saline injected fish could be due the presence of small quantities of endotoxin in the apparently sterile saline or that the increase was stress related and due to handling and injection. This may be so however, Möck and Peters (1990) showed that the usual response to stress in rainbow trout was a decrease in the concentration of serum lysozyme. They were unable to demonstrate the presence of lysozyme in the mucus of the vaccinated trout and concluded that the concentration of mucus lysozyme was lower than found in the serum. This result contradicts the findings of Lindsay (1986) who found a

higher concentration of lysozyme in the mucus than in the serum of rainbow trout. Jørgensen *et al.* (1993) found that the levels of serum lysozyme were increased following the administration of yeast-cell-wall glucan, and that the bacteriocidal activity of the rainbow trout macrophages also increased. This was apparently due to an increase in the ability of the macrophages to produce reactive oxygen species.

In an investigation into the effects of infection of Atlantic salmon with *A. salmonicida* on various nonspecific immune parameters Møyner *et al.* (1993) found that serum lysozyme concentrations increased from day 4 post infection reaching a maximum on day 9, also, symptomatic fish and survivors had significantly more lysozyme than control fish and furthermore, acutely infected fish had higher levels than chronically infected individuals.

The role of complement in the immune response of fish was reviewed by Sakai (1992) and only information of direct relevance will be discussed here. Complement is an important component of the immune response to bacteria. It has a bacteriocidal activity without mediation by antibody, and can act as an opsonin to enhance the phagocytic activity of macrophages and neutrophils (Sakai, 1992). The complement cascade can be activated in two ways the immunoglobulin dependant pathway (classical) and the immunoglobulin independent pathway (alternative). High haemolytic complement activity in rainbow trout serum which was comparable to that of human sera was described by Nonaka and coworkers (1981a). The presence of C5 and C3 equivalent components was also demonstrated (Nonaka *et al.*, 1981b; 1984). Complement levels are usually determined using the complete haemolysis assay CH_{50} and the spontaneous haemolysis assay SH_{50} The CH_{50} assay measures the level of complement mediated haemolysis of RBC sensitised with antibody, the SH_{50} measures the level of haemolysis of an unsensitised heterologous RBC. Sakai (1981) found that the SH_{50} was approximately half the CH_{50}. Since spontaneous haemolysis disappears when serum is heat treated the activation of the alternative pathway by the RBC was believed to be responsible for the activity.

The bactericidal activity of rainbow trout complement against *A. salmonicida* was demonstrated by Sakai (1983). Normal trout sera having high CH_{50} and SH_{50} activities were shown to exhibit high killing activities for live *A. salmonicida*, whereas heat inactivated serum and LPS pretreated serum did not. Sakai (1984) showed that the ECPs of *A. salmonicida* cause nonspecific activation of rainbow trout complement and suggested that the complement was responsible for the inactivation of the ECPs. However, Ellis (1987) showed that serum inactivation of the *A. salmonicida* serine protease was due to the action of α-2 macroglobulin, a serum protease inhibitor. This suggestion is supported by the observation that normal serum levels of α-2 macroglobulin correlate well with susceptibility to *A. salmonicida* (Ellis, 1987; Freedman, 1991)

The bacteria are capable of protecting themselves from complement, however, Munn *et al.* (1982) showed that the possession of the A layer mediates resistance to serum killing by complement.

Garduño *et al* (1993) showed that complement in serum and peritoneal fluid were capable of killing virulent *A. salmonicida* and that bacteria kept within special intraperitoneal chambers were more susceptible to killing than bacteria injected freely into the peritoneal cavity. They suggested that free bacteria were able to infect macrophages and live as facultative intracellular bacteria and so avoid lysis.

Møyner *et al.* (1993) also investigated the effect of infection on the haemolytic activity of complement using the CH_{50} and SH_{50} assays. They found a decrease in the CH_{50} following infection but this was also found in the non-infected controls. The SH_{50} showed no distinct changes during infection nor in the control fish but fish that survived infection both the CH_{50} and SH_{50} were significantly increased compared with the control fish (Møyner *et al.*, 1993).

The same group also investigated total serum protein levels following infection and found that serum protein levels decreased significantly in symptomatic fish and were lowest

in fish with acute furunculosis. Serum protein levels returned to normal levels in fish that recovered from infection. They also investigated serum protease activity and found that activity was increased in fish with acute disease and that there was an inverse relationship between total serum protein levels and protease activity. The activity of protease inhibitor in the serum was lowest in fish with acute disease and significantly lower than in those with chronic infection. In the chronically infected individuals and in surviours the levels were closer to normal but still significantly lower than in the controls (Møyner *et al.*, 1993).

Two groups have noted that fish skin mucus has a natural antibacterial activity (Austin and McIntosh, 1988; Fouz *et al.*,1990). Austin and McIntosh (1988) showed that the antibacterial activity of rainbow trout mucus greatest against *A. hydrophila* a common bacteria in freshwater. Fouz and co-workers in a very similar experiment on turbot skin mucus showed that the antibacterial activity was greatest against *A. salmonicida* but also very high against various *Vibrio spp.* Unfortunately Austin and McIntosh (1988) did not test the antibacterial activity of rainbow trout mucus against *A. salmonicida* so direct comparisons cannot be made, furthermore, whilst Fouz *et al.* (1990) gave the mucus activity as millimetres of bacterial growth inhibition Austin and McIntosh (1988) only say that activity against *A. hydrophila*was greater than 3mm. Both groups attributed the activity to a novel glycoprotein as it was not inactivated by heating to 50°C for 30 minutes nor by exposure to proteinase K. Neither group suggested the mode of action of this antibacterial molecule. Interestingly, many bacteria are known to be able to live in the mucus of fish including *A. hydrophila* (Austin and McIntosh, 1988) and *A. salmonicida* (Cipriano *et al.*, 1992).

Many workers have investigated the role of the nonspecific immune system in protection of salmonids against bacteria and particularly *A. salmonicida.* Some of this work is outlined above and a lot of effort has been associated with the investigation of the role of lysozyme and complement in the immune response. Many workers have found that both lysozyme and complement can play a role in protection. Indeed some workers (Fevolden *et*

al., 1994; Lund *et al.*, 1995; Hollebecq *et al.*, 1995) have investigated the use of nonspecific immune parameters as a marker for breeding inherently resistant fish, with varying, but inconclusive results.

As part of the on going investigation into the use of the live attenuated *aroA* mutant of *A. salmonicida* 644 rB, it was decided to investigate serum and mucus lysozyme activity, serum protein, antibacterial activity in serum and mucus and spontaneous haemolytic activity of complement.

This work was conducted over three independent experiments and only the first was specifically devoted to the nonspecific immune response the second also investigated the persistence of the live bacterial cells in the host and the third investigated the specific immune response to the vaccine and challenge with the wild type bacteria *A. salmonicida* 644rB. The results of the persistence as well as the specific immune response and challenge are given in Chapter 7 below.

6.2 Materials and Methods.

6.2.1 Bacterial strains.

The bacterial strain used for the immunisation of the experimental animals was *Aeromonas salmonicida* 644Rb *aroA*::kan^r. The details of this strain were given above (Section3.2.1).

6.2.2 Routine culture of bacteria.

Unless otherwise stated all bacteria were cultured as described above (Section 3.2.2).

6.2.3 Enumeration of bacteria.

Unless otherwise stated viable bacteria were counted using the Miles and Misra technique (Miles *et al.*, 1938). Serial tenfold dilutions were made in PBS (Oxoid, UK) 0.5 cm^3 in 4.5 cm^3 and bacteria grown on TSA containing appropriate antibiotic (Section 3.2.2 above) and incubated at 22°C for 48 hours before counting.

6.2.4 Rapid spectrophotometric enumeration of *A. salmonicida*.

The standard curves of absorbance 590nm against viable count of *A. salmonicida* colony forming units (CFU) suspended in PBS, as described in section 3.2.5 above were used to determine the numbers of CFUs in the vaccine preparations prior to immunisation. The estimations were confirmed by viable count.

6.2.5 Experimental animals.

There were three immunisation experiments. Prior to the start of each experiment the rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) were kept in the stock aquarium under the care of the aquarium technicians. During this time they were kept in freshwater recirculating system at 14°C ± 1°C and fed to satiation on a commercial trout pellet preparation.

For the first experiment adult female rainbow trout 350-500g were removed from the stock aquarium and transferred to the challenge aquarium in two stages with six days between

each stage. This was done to allow the biological component of the filter systems to mature. The challenge aquarium contained four 220 gallon tanks with a recirculating freshwater system maintained at 13°± 1°C by a room cooling system. The fish were randomly assigned to one of four groups (n=12); each group was kept in a separate tank. The fish were then allowed to acclimatise to the experimental conditions for a further six days before immunisation. The fish were fed lightly throughout the experiment but were starved for 48 hrs before and 24 hours following immunisation.

In the second experiment adult female rainbow trout 150-200g were again transferred to the challenge aquarium in two stages. They were again randomly allocated one of four groups (n=21). The fish were allowed to acclimatise and were fed as described above.

In the third experiment 240 rainbow trout 40-50g were transferred to the challenge aquarium in four stages over a period of 14 days. The aquarium was maintained at 14°± 1°C. The fish were randomly allocated to four groups (n=70). They were acclimatised and fed as before.

6.2.6 Production of the bacterial vaccine.

The vaccine was produced in different ways for each of the three experiments, however, some aspects were constant for each experiment. Bacteria were routinely cultured as described above (Section 3.2.2.). To produce the vaccine a 48-hour working culture was used to inoculate fresh TSB. These cultures were grown for a further 48 hours washed once by centrifugation and resuspension in PBS followed by a further centrifugation step, and resuspension in a known volume of PBS. The number of CFUs in the suspension was estimated using the rapid spectrophotometric method described above (Section 3.2.5.). The bacteria were sedimented by centrifugation and the concentration of bacteria was adjusted to the level required for each of the experimental groups. The details for each of the three experiments are given below.

i) The working culture was used to inoculate 12 x 100 cm^3 of TSB in 250 cm^3 conical flasks. Centrifugation was performed in 50 cm^3 centrifugation tubes at 2,000 *g* for 10 minutes. The bacteria were finally resuspended at the following concentrations; $2x10^{10}$ CFUs cm^{-3} in 10 cm^3 of TSB, $2x10^8$ CFUs cm^{-3} in 10 cm^3 of TSB and $2x10^8$ CFUs cm^{-3} in 10 cm^3 of saline.

ii) The working culture was used to inoculate 20 x 100 cm^3 of TSB in 250 cm^3 conical flasks. The initial sedimentation and washing of the bacterial cells was done by centrifugation in 250 cm^3 centrifugation pots at 6,000 *g* for 30 minutes. The sedimentation step following the estimation of bacterial concentration was performed in 50 cm^3 centrifuge tubes 6,000 *g* for 30 minutes. The bacteria were finally resuspend at concentrations of; $2x10^{10}$ CFUs cm^{-3} in 20 cm^3 of TSB, $2x10^8$ CFUs cm^{-3} in 20 cm^3 of TSB and $2x10^7$ CFUs cm^{-3} in 20 cm^3 of saline.

iii) The working culture was used to inoculate 9 litres of TSB in conical flasks of various sizes between 250 cm^3 and 5 litres. Centrifugation was performed in 500cm^3 centrifugation pots at 6,000 *g* for 30 mins, the sedimentation step following the estimation of bacterial concentration was performed in 50 cm^3 centrifuge tubes at 6,000 *g* for 30 minutes. The bacteria were finally resuspend at concentrations of; $2x10^{10}$ CFUs cm^{-3} in 60 cm^3 of TSB, $2x10^8$ CFUs cm^{-3} in 60 cm^3 of TSB and $2x10^7$ CFUs cm^{-3} in 60 cm^3 of TSB.

6.2.7 Administration of the bacterial vaccine.

For each of the three experiments the bacteria were administered either orally or intra-peritoneally. For oral intubation the fish was held so that the ventral surface was uppermost and the mouth of the fish was held open by inserting a thumb between the jaws. Oral administration was performed via a soft silicon tube external diameter of 2 mm pushed onto a 19 *g* needle attached to a 5 cm^3 syringe. The tube was pushed 4 to 5 cm into the oesophagus so that the end was located in the stomach and 0.5 cm^3 of bacterial suspension was expelled.

Unless otherwise stated orally intubated fish were given 1cm^3 of a 35mg cm^{-3} solution of sodium bicarbonate. This was administered oral in the same manner described for the vaccine. For intraperitoneal injection the fish were held so that their ventral surface was uppermost the 25 *g* needle was pushed through the abdominal wall of the fish so that the tip of the needle was just in the peritoneal space the needle was then tilted and pushed between 1.5 to 2 cm just under the internal surface of the abdominal wall, and 0.5 cm^3 of bacterial suspension expelled.

In each experiment the four groups of fish were given different treatments these were:

i) In experiment one the groups of fish were given treatments as follows, the negative control group was given 0.5 cm^3 of TSB orally, the low dose oral group was given 0.5 cm^3 of 2.0x10^8 CFU cm^{-3} in TSB, the high dose oral group was given 0.5 cm^3 of 2.0x10^{10} CFU cm^{-3} in TSB, and the IPgroup was given 0.5 cm^3 2.0x10^8 CFU cm^{-3} saline.

ii) In experiment two the groups of fish were given treatments as follows, the high dose oral group was given 0.5 cm^3 of 0.5 cm^3 of 2.0x10^9 CFU cm^{-3} in TSB, the second high dose oral was given 0.5 cm^3 of 2.0x10^{10} CFU cm^{-3} in TSB but was not given sodium bicarbonate, the IP group was given 0.5 cm^3 of 2.0x10^8 CFU cm^{-3} in TSB intra-peritoneally, and the low dose oral group was given 0.5 cm^3 2.0x10^8 CFU cm^{-3} TSB orally. NB due to high level mortalities a second IP was set up and was given 0.5 cm^3 2.0x10^7 CFU cm^{-3} TSB intra-peritoneally.

iii) In experiment three the groups of fish were given treatments as follows, the high dose oral group was given 0.5 cm^3 of 2.0x10^{10} CFU cm^{-3}, the negative control group B was given 0.5 cm^3 of TSB orally, the IP group was given 0.5 cm^3 2.0x10^9 CFU cm^{-3} and the low dose oral group D was given 0.5 cm^3 of 2.0x10^8 CFU cm^{-3}.

6.2.8 Sampling of the fish.

Each of the three experiments were aimed at achieving different ends and so the methods used to obtain samples were not the same throughout the three investigations. This was primarily because in experiment one the fish were kept alive and returned to the tank after sampling whereas in experiments two and three the fish were sacrificed after sampling and used to investigate the persistence of the bacteria in the host or the specific immune response respectively (methods and results presented in Chapter 7 below).

i) In experiment one the skin mucus was collected by placing the fish into a plastic bag and squeezing the sides of the fish through the bag from the head to the tail the mucus was collected from the bag using a Pasteur pipette and transferred to a glass bijoux bottle. The mucus was used immediately. This was only done when sampling three days before vaccination.

Blood samples were collected from the caudal sinus after the mucus was collected to prevent contamination of the mucus with blood. From each fish 0.25 - 0.5cm^3 of blood was removed using a 21g hypodermic needle attached to a 2 cm^3 syringe. The blood samples were transferred to glass bijoux bottles and allowed to clot at room temperature for 3 - 4 hours. Following centrifugation, the serum was transferred to microcentrifuge tubes (Starstadt) and used immediately for all of the assays except the total serum protein assay, the remainder frozen at -70°C and the total serum protein assay was performed on this. All fish from each of the four groups were sampled three days prior to immunisation (D-3). At immunisation 6 of the fish from each group were marked with Alcian blue using a Panjet marker so that each group was divided into two pools A and B. Starting the day after immunisation (D+1) the six fish in pool A (unmarked) were sampled and pool B was sampled the next day. These pools were then sampled every other day until day 7 after immunisation when the experiment was terminated.

ii) In experiment 2 the blood was removed as before save that between 1.0 and 2.0 cm^3 of blood was taken. The blood samples were transferred to glass bijoux bottles and allowed to clot at room temperature for 3 - 4 hours. Following centrifugation, the serum was transferred to microcentrifuge tubes (Starstadt) and some was used immediately in the alternative complement assay and the remainder frozen at -70°C. The fish were then sacrificed by a sharp blow to the back of the head. The head to the posterior edge of the operculum and the area around the anal fin (the location of the needle wound) were covered in tissue to prevent contamination of the mucus with blood. The mucus was removed using two double ended cotton buds for each fish. The buds were wiped along the side of the fish from head to tail and the ends of the buds were cut off and placed in a glass Bijoux bottle. Two cm^3 of saline was added to each Bijoux and the water and cotton buds vortexed using a 'Whirlymixer' for 1 minute. The mucus suspension (1.5 cm^3) was transferred to microcentrifuge tubes and frozen at -70°C.

iii) In experiment three the blood was collected and treated as described for experiment two and again the fish were sacrificed by a blow to the back of the head. The head and needle wound were again covered with tissue and the mucus scraped toward the tail with the handle of a No 4 scalpel. Once at the tail end the mucus was collected and transferred to microcentrifuge tubes using a 200 μl tip on a Finpette (Jencons) digital pipettor set to 100 μl. The mucus was then diluted 1/10 in saline and stored at -70°C.

6.2.9 Lysozyme assay.

The levels of serum and skin mucus lysozyme were measured using a lysoplate assay. 100 cm^3 of 1% purified agar in PBS, containing 0.25 mg cm^{-3} *Micrococcus lysodeikticus* was prepared. This was done by adding 1 gram of agar to 90 cm^3 of distilled water. The agar was autoclaved and stored at room temperature until required. It was then melted in a steamer and

allowed to cool to 55°C when 10 cm^3 of PBS containing 25 mg of *Micrococcus lysodeikticus* was added. The agar was poured into 20 cm diameter plates and allowed to cool to room temperature. Serial twofold dilutions (2.0 - 0.1 mg cm^{-3}) of Hen Egg White Lysozyme (HEWL) were prepared in PBS stabilised with 1% BSA. Wells were cut into the agar 4 mm in diameter and 25 μl of standards, serum or skin mucus samples were pipetted into each well. Each plate had a set of standards and the diameter of the cleared zones was deemed to be proportional to the natural log (ln) of lysozyme concentration. A regression equation for the concentration against clearance diameter was produced for each plate and the concentration of lysozyme in each of the standards was calculated using that equation. The calculations were performed using Quattro Pro 6.0™ for Windows™.

6.2.10 Antimicrobial activity of serum and mucus.

Two methods were used for this assay. The first was an adaption of the method of Fouz *et al.* (1990). Briefly, 6 mm diameter discs of blotting paper were used to absorb 20μl of mucus or serum samples. These discs were then placed onto TSA in 15 cm^2 glass culture plates seeded with 10^4 - 10^5 CFU.

In the second assay, 500 cm^3 of molten TSA were allowed to cool to 45°C and seeded with bacterial cells to a final concentration of 10^5 CFU cm^{-3}. The agar was poured (250 cm^3) into 15 cm^2 culture plates and allowed to set. Four mm wells were cut into the agar and 25 μl of serum or mucus was added to each well.

In each experiment the diameter of the zone of bacterial growth inhibition surrounding the paper or well was measured. The antibacterial activity was expressed as mm of inhibition.

6.2.11 Alternative complement assay.

Sheep red blood cells (SRBC) were obtained suspended in Alsever's solution (Tissue Culture Services Ltd.). They were stored at 4°C and prior to use were washed four times, by centrifugation at 750 *g* for 5 minutes followed by resuspension in complement fixation test buffer (CFT) pH7.2. Following the final centrifugation the SRBC were resuspended to an

approximate 5% concentration again in CFT. One part SRBC suspension was removed and added to 14 parts distilled water. The absorbance of this solution was measured against a distilled water blank at 540 nm. A 5 % suspension (ie 10^9 SRBC cm^{-3}) has an absorbance of 0.700 at 540 nm (Ingram, 1990). The approximate 5% suspension was adjusted to an accurate 5% suspension using the following formula (Kabat,1971):

$$Vf = Vi \times \frac{Absorbance\ of\ Haemolysate}{0.700}$$

Where V*i* is the initial volume of the approximate 5% suspension and V*f* is the final volume to which V*i* has to be adjusted to give an accurate 5% suspension.

The 5% suspension was then further diluted in CFT to give a 2% suspension, and this suspension that was used in the alternative complement activity assay.

The level of spontaneous haemolytic activity in the serum was measured by the haemolysis of unsensitised sheep red blood cells (SRBC). Two fold serial dilutions, (starting with 100 μl of serum in 100 μl of CFT), were prepared in a 96 well, round bottomed, microtitre plate. 50µl of 2% SRBC in CFT were added to each well, the plate was incubated for 1 hour at 20 °C followed by overnight incubation at 4°C. The natural haemolytic activity was expressed as the Log_2 of the highest dilution yielding complete haemolysis. The end point was the highest serum dilution where there was complete lysis of the SRBC.

6.2.12 Total serum protein.

The total serum protein level was determined using the Sigma total serum protein assay Kit (Cat. No. 541-2). The assay was performed by following the manufacturers instructions. Briefly, for each serum sample to be processed 1 cm^3 of reagent was transferred to a UV quality semi-micro cuvette. The standards were prepared (in the range 20-80 g l^{-1}) from the Sigma serum protein assay stand solution which contained 80 g of protein per litre

and 20μl of each added to a cuvette containing the reagent the blank was prepared by adding 20 μl of distilled water to the reagent. 20 μl of each of the experimental samples was added to 1 cm^3 of reagent. The blank, standards and experimental samples were thoroughly mixed with the reagent and left for 10 minutes. The absorbance 540nm was measured using a Phillips PU8700 spectrophotometer. The concentrations of the standards were entered into the spectrophotometer and the accessory software produced a standard curve and calculated the concentration of protein in each of the serum samples.

6.2.13 Serum protease activity.

The serum protease activity was determined using 1 % purified agar gels containing 1% bovine casein. Gels were poured onto 10 x 10 cm glass plates, and 4μl samples were added to 2 mm diameter wells. The gels were left in a moist box at 20°C overnight, the protein was precipitated using 5 % acetic acid and the gel dried using a hair drier. The gel was then stained with Coomassie Brilliant Blue G (Sigma). The activity of the crude ECP preparation (Section 7.2.14) was also measured in this way.

6.3 Results.

6.3.1 General points.

The effects of vaccination with a live attenuated bacterial strain on the nonspecific immune response was investigated in three experimental stages. Only the first stage was conducted solely to investigate the nonspecific response, stage two was also designed to investigate the persistence of live bacterial cells within the tissues of the recipient fish and stage three was primarily intended to investigate the specific immune response followed by a small scale challenge investigation. The large variation between individual fish given the same treatment represented by the error bars (SD n-1) for each of the data points in Figs. 6.3.1 - 6.3.5 was an outstanding feature of these experiments.

6.3.2 Experimental infection and mortalities.

On the ninth day after immunisation during experiment one there were mortalities in both the orally immunised groups. These were followed by yet more on day 10 and 11 when mortalities also occurred in the control group and in the IP immunised group. All mortalities were tested for the presence of *A. salmonicida* in the kidney or intestinal mucus but none were found.

In experiment two the IP immunised fish were given a dose of $1x10^8$ CFUs in TSB rather than in saline as in experiment one. There were 5 mortalities on day one after immunisation, 10 on day two and 3 on day 3. All the mortalities were found to have *A. salmonicida* in the kidney and intestinal mucus, furthermore, on dissection the peritoneal membranes and the intestine were found to be inflamed, and there was evidence of localised tissue destruction, this will be discussed in Chapter 7 below. There were no mortalities in the replacement group which was give $1x10^7$ CFU in TSB.

There were no mortalities in any of the groups in experiment three following immunisation. However, on day 15 a major leak developed overnight in the negative control

fish tank leading to the loss of most of those fish. The remainder were removed to the stock aquarium whilst the cause of the leak was identified and the chemical filtration material was replaced. Following this the control fish were live bled and no mucus samples were taken.

6.3.3 Serum lysozyme activity.

Serum lysozyme levels for the three experiments presented graphically in Fig. 6.3.1 parts A, B and C and the results for the individual fish in each experiment are given in Tables 6.3.1 - Table 6.3.10.

During experiment one fish in each group were divided into two pools at immunisation and these pools were sampled every other day following immunisation (n=6) and returned to the tank. All of the assays except the serum protein assay were performed on the day of sampling. Three days before immunisation most fish had serum lysozyme levels of between 1 and 3 mg cm^{-3} regardless of experimental group, there was no significant difference between any of the experimental groups (p=0.99). From day 3 there was a general trend for the serum lysozyme levels in all groups to increase. At day 4 post immunisation there was a significant difference between the IP immunised group and the other three groups (p=0.01). The increase in the levels of serum lysozyme in the other three groups on days 5 and 6 meant that there was no significant difference between the four groups on those days. On day seven there was a highly significant difference between all four groups (p<0.0006). This was due to the lysozyme activity of the IP group being significantly greater than that of any of the other groups (negative control p=0.01; $1x10^{8}$ p=0.01; $1x10^{10}$ oral p=0.02) there was no significant difference between the other groups. There was a significant increase in the serum lysozyme levels for three of the groups over the course of the experiment (negative control p=0.02; $1x10^{8}$ oral p=0.12 NS; $1x10^{10}$ oral p=0.04; IP p=0.02).

In experiment two the sample sizes were smaller (n=3) than in experiment one and the fish were dead sampled so each days results are from different fish. The main aim of this experiment was the determination of the persistence of the vaccine by viable count, therefore

only the complement assay was performed on the day of sampling all the other assays were performed on serum and mucus that was frozen on the day of sampling. These samples were originally stored at -70°C but due to a freezer fault that went uncorrected they were partially defrosted and had to be transferred to -20°C.

There was no significant difference between the four groups on day 2 (p=0.44), The IP group showed an increase in lysozyme level at day 4 and day 6 with a further but slower increase until day 10. At day 10 the level of serum lysozyme in the IP group was significantly higher than at day 2 (p=0.04) and higher than the other groups (p=0.01).

In experiment three the main aim was to investigate the specific immune response so again only the complement assay was performed the same day the rest of the assays were performed on sera that had been stored at -70°C but as described for experiment two had to be moved to -20°C. The number of samples available for determining the nonspecific immune response was variable because much of the serum from the IP and negative control groups was used during the specific immune response investigation.

The serum lysozyme levels for both the orally immunised groups were highest at seven days post immunisation and fell over the course of the experiment. There were no apparently important differences between the groups and no obvious trend other than that already mentioned. The wide differences in number of samples for the groups meant that the significance of the results was not tested.

6.3.4 Alternative complement activity.

Serum complement levels for the three experiments are presented graphically in Fig. 6.3.2 parts A, B and C and the results for the individual fish in each experiment are given in Tables 6.3.1 - Table 6.3.10.

The alternative complement activity was determined using the SH_{50} assay. This was performed on fresh sera for all of the experiments. In experiments one and two there was no apparent change in the level of alternative complement activity. In experiment three however,

there was an obvious change in titre. Comparison of the change in complement activity for each group over the six weeks showed that there was a significant change in the activity of the IP group (p=0.01) and a highly significant change in both of the orally immunised groups ($1x10^{10}$ p=0.0005; $1x10^{8}$ p=0.0001) whereas there was no significant change in the negative control group (p=0.08). For each group the change involved an increase in titre peaking in week four. The complement titres of the immunised fish at week 4 were not significantly different but all were significantly different to the negative control ($1x10^{10}$ oral p<0.004; $1x10^{8}$ oral 0.01; IP p=0.03).

6.3.5 Total serum protein levels.

Total serum protein levels for the three experiments are presented graphically in Fig. 6.3.3 parts A, B and C and the results for the individual fish in each experiment are given in Tables 6.3.1 - Table 6.3.10.

The most striking observation is that the levels of serum protein in the groups from the first experiment are very similar and that in both the second and third experiments the variation is quite pronounced but there is no apparent pattern to the variation.

6.3.6 Serum antibacterial activity.

Total serum protein levels for the three experiments are presented graphically in Fig 6.3.4 parts A, B and C and the results for the individual fish in each experiment are given in Tables 6.3.1 - Table 6.3.10.

As with the serum protein levels the antibacterial activity of the samples from the first experiment appear to be very similar between groups and to have changed very little during the course of the experiment. In experiments two and three however, the results were very variable but there seemed to be no pattern to this variation in may be relevant that the serum protein levels and the serum antibacterial activity levels were both variable.

6.3.7 Skin mucus antibacterial activity and skin mucus lysozyme activity.

Skin antibacterial activity levels for experiments two and three are presented graphically in Fig 6.3.5 parts A, B and C and the results for the individual fish in each experiment are given in Tables 6.3.1 - Table 6.3.10.

During the first experiment skin mucus samples were taken three days before immunisation, only. Neither antibacterial activity nor lysozyme activity could be detected in these samples and subsequently skin mucus was not collected.

Mucus samples were collected throughout experiments two and three and tested for antibacterial activity and lysozyme activity in the same way as serum samples. It was not possible to demonstrate lysozyme activity in the mucus of any of the fish tested. There was measurable antibacterial activity. This was very variable in some fish there was no measurable activity and in others from the same group and time point there was activity. In experiment three there was no negative control mucus available from week 3 onward.

6.3.8 Serum protease.

The serum protease assay failed to demonstrate a serum protease activity. Digestion of casein was shown in the crude ECP controls but the zones around the serum samples were darker than the surrounding agar suggesting that the serum far from digesting the agar increased the protein concentration around the wells.

6.3.9 Individual data Tables.

The individual results are presented in Tables 6.3.1 to 6.3.10 so that the reader can see how variable the results for some of the assays were, furthermore it is possible to see that there was no relationship between results in one assay and results in another.

Fig 6.3.1 Part A Experiment One

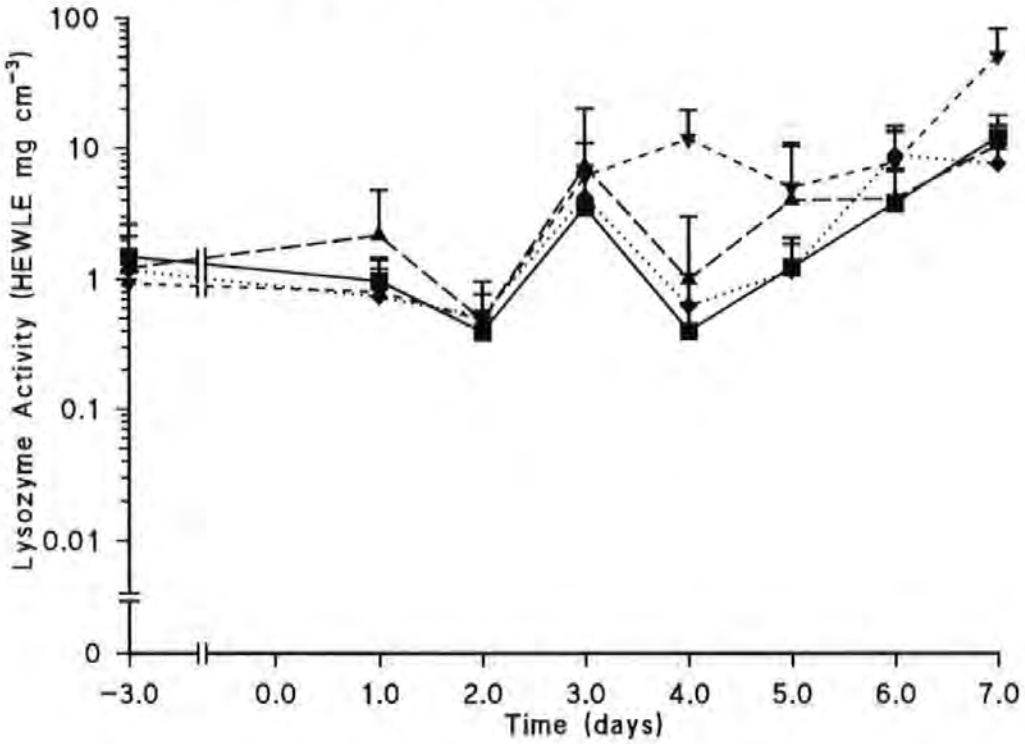


Fig 6.3.1 Part B Experiment Two

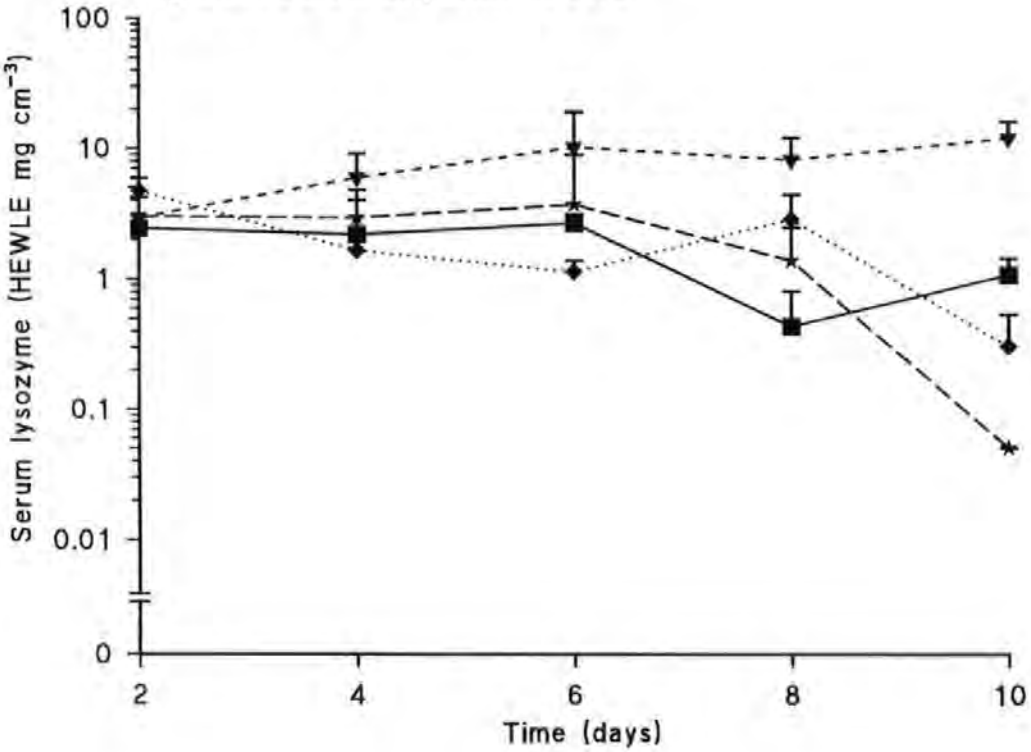


Fig 6.3.1 Part C Experiment Three

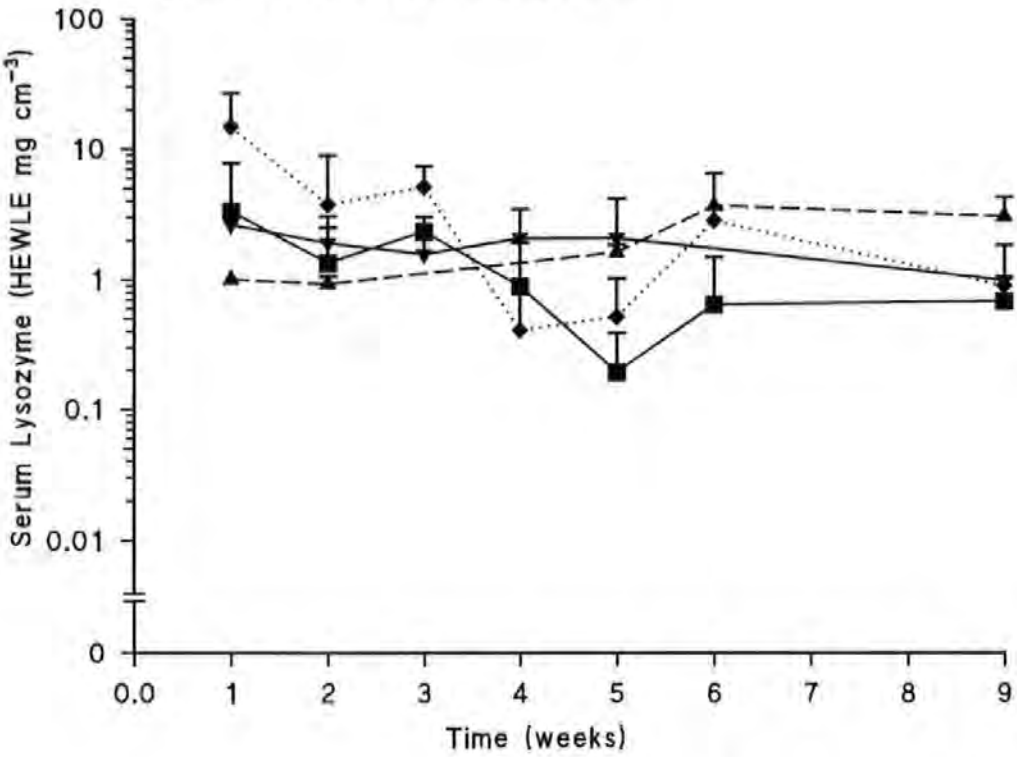


Figure 6.3.1 Serum lysozyme activity.

Lysozyme activity was measured be a lysoplate method and activity was calculated from a set of five standards on each plate. Activity is expressed as hen egg white lysozyme equivalents mg cm^{-3} serum (HEWLE).

Part A Experiment one; Part B Experiment Two; Part C Experiment Three. Error bars represent SDn-1. For all parts data points for each group are 1x10^{10} orally in TSB (■); 1x10^{10} orally in saline (★); IP (▼); 1x10^{8} orally in TSB (◆); negative control(▲).

Fig 6.3.2 Part A Experiment One

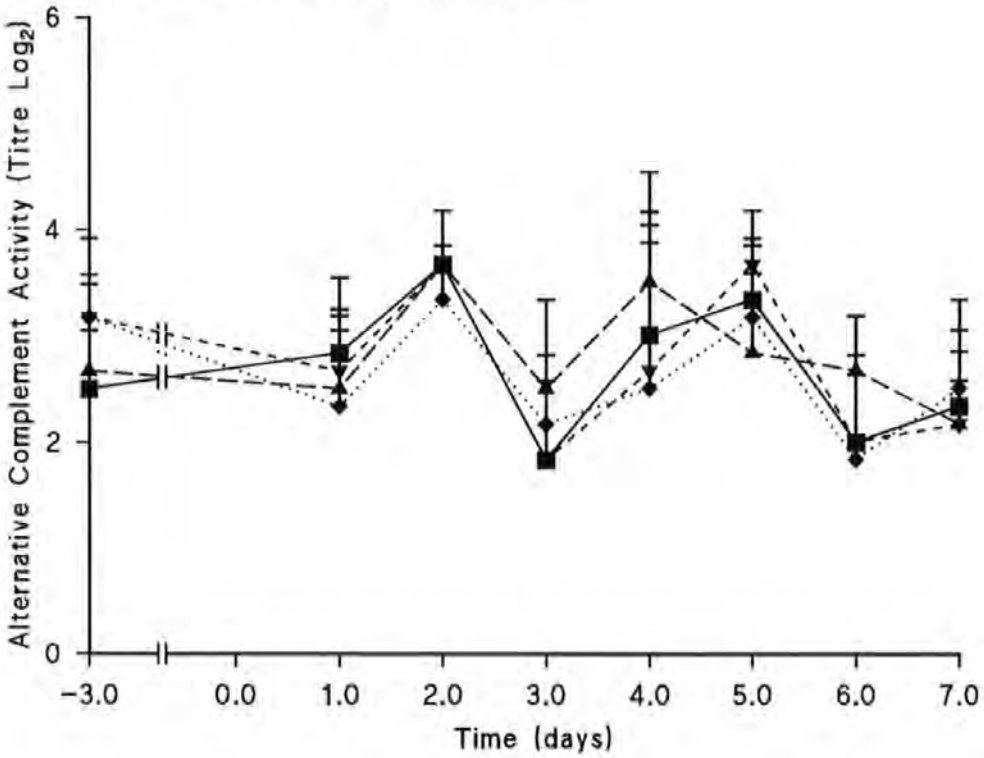


Fig 6.3.2 Part B Experiment Two

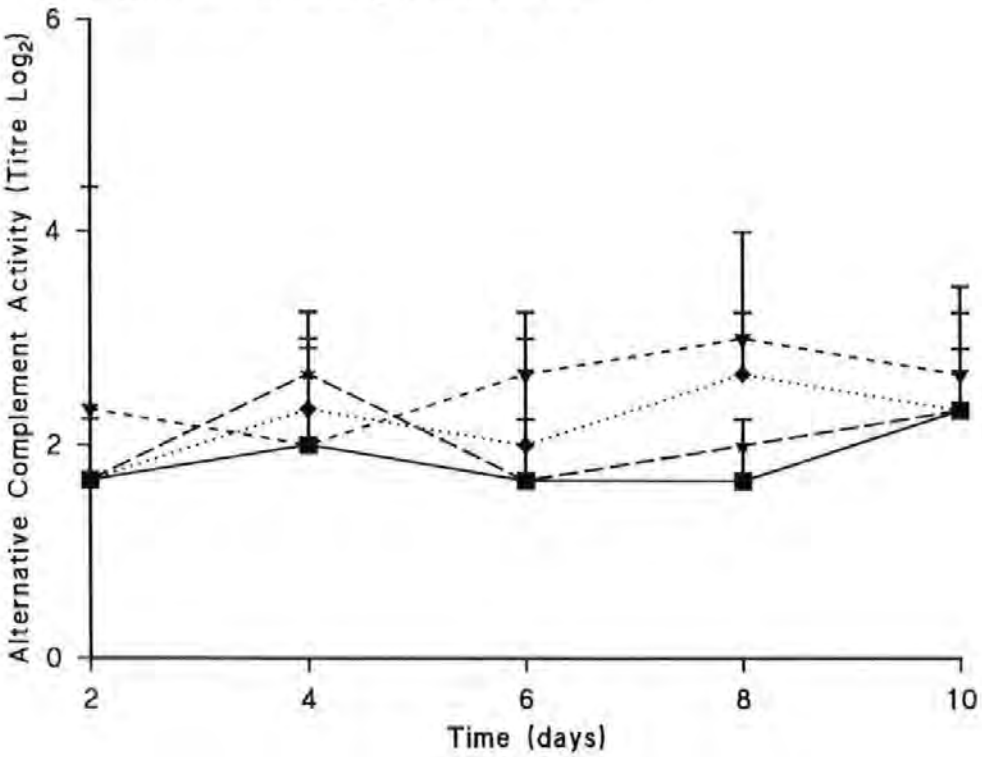


Fig 6.3.2 Part C Experiment Three

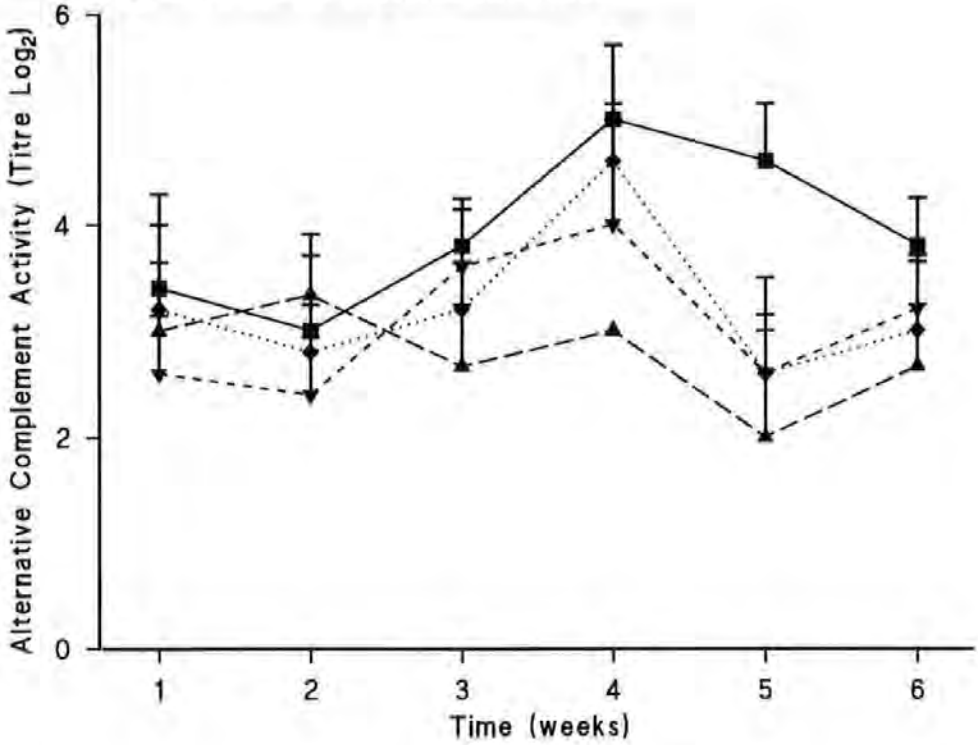


Figure 6.3.2 Alternative complement Activity.

Alternative complement activity was measured by SH_{50}. Activity is expressed as the log_2 of the highest dilution of serum to give 100% lysis of SRBC.

Part A Experiment one; Part B Experiment Two; Part C Experiment Three. Error bars represent SDn-1. For all parts data points for each group are $1x10^{10}$ orally in TSB (■); $1x10^{10}$ orally in saline (★); IP (▼); $1x10^{8}$ orally in TSB (◆); negative control(▲).

Fig 6.3.3 Part A Experiment One

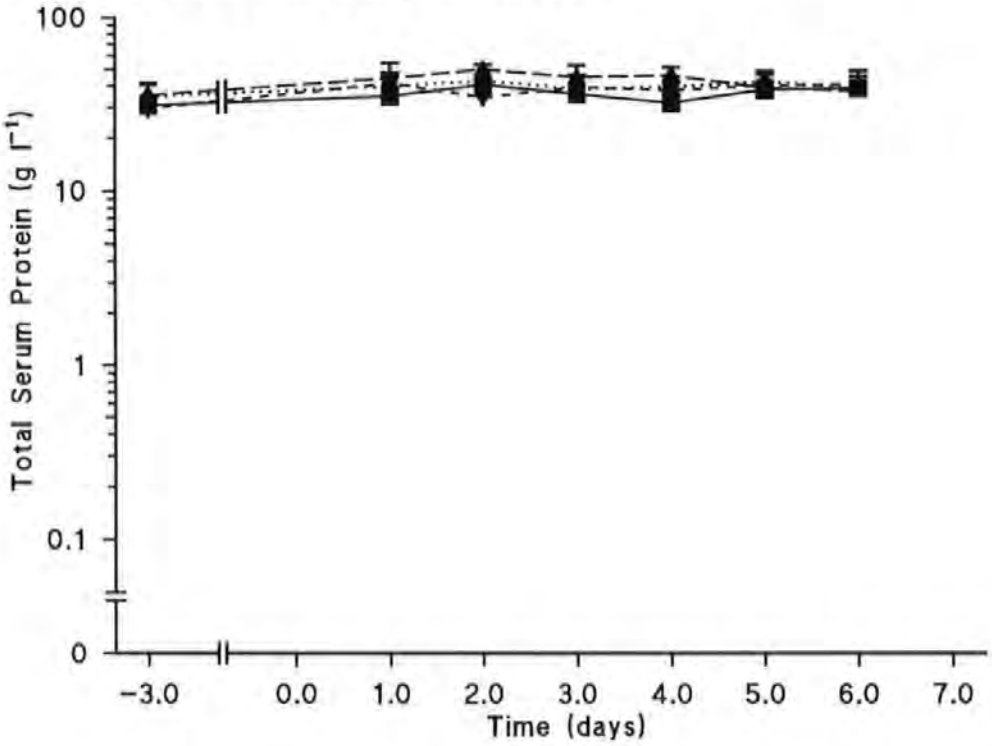


Fig 6.3.3 Part B Experiment Two

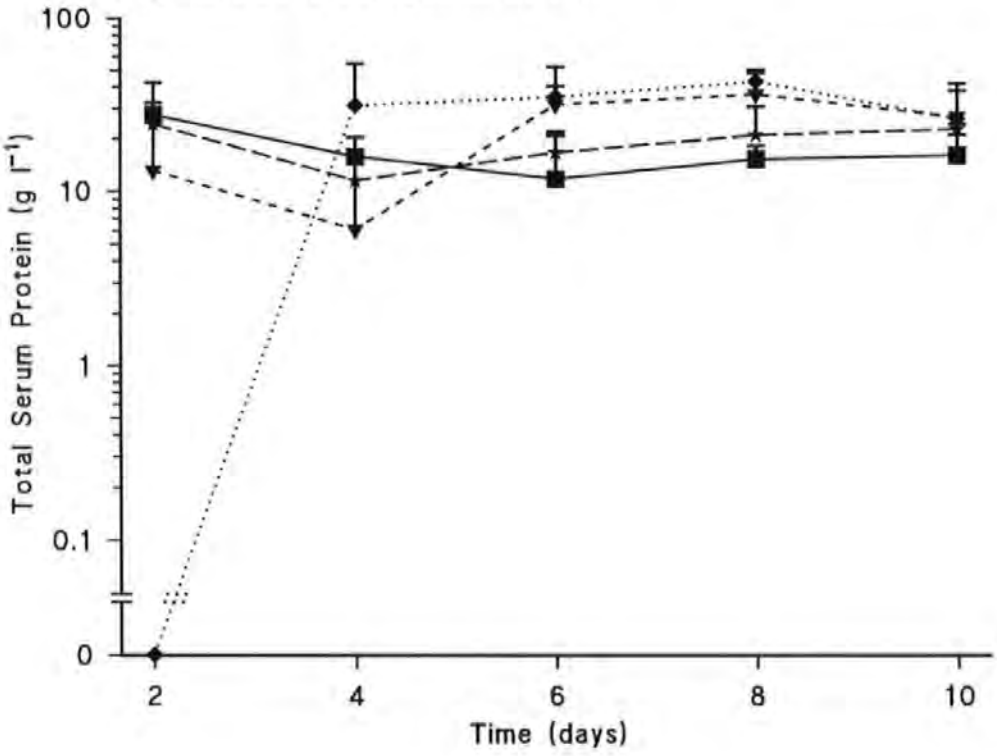


Fig 6.3.3 Part C Experiment Three

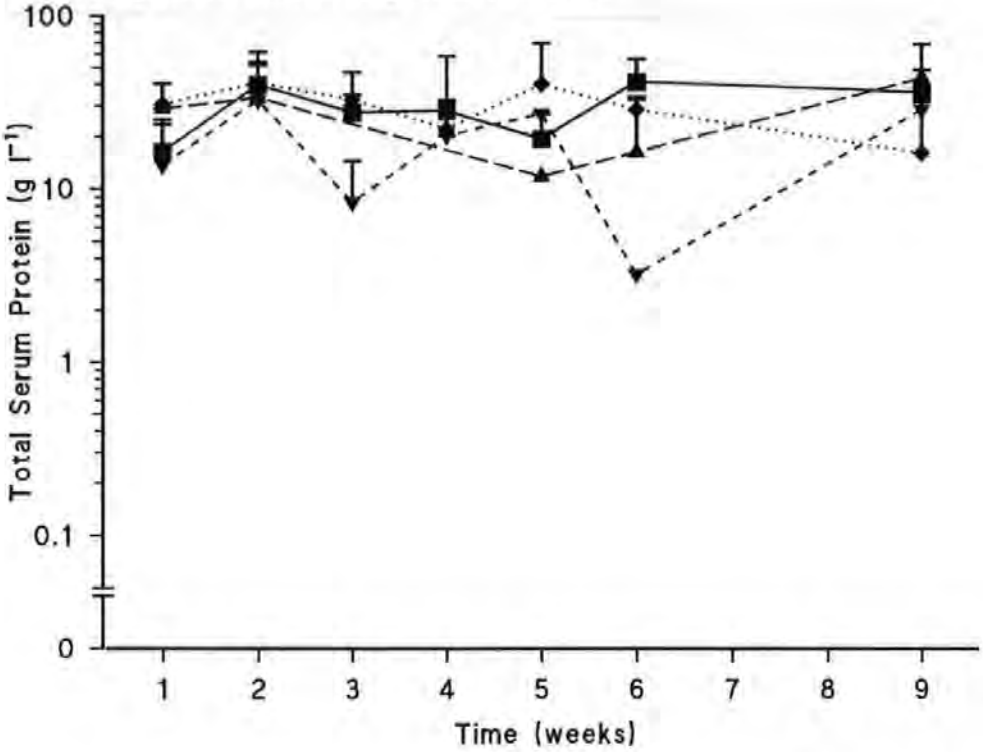


Figure 6.3.3 Total serum protein

Total serum protein was determined using the Sigma total serum protein assay kit. A calibration curve was constructed using the Sigma serum protein standards and this was used to calculate the serum protein levels of the trout. Total serum protein is expressed as grams of protein per litre of serum.

Part A Experiment one; Part B Experiment Two; Part C Experiment Three. Error bars represent SDn-1. For all parts data points for each group are $1x10^{10}$ orally in TSB (■); $1x10^{10}$ orally in saline (★); IP (▼); $1x10^{8}$ orally in TSB (◆); negative control(▲).

Fig 6.3.4 Part A Experiment One

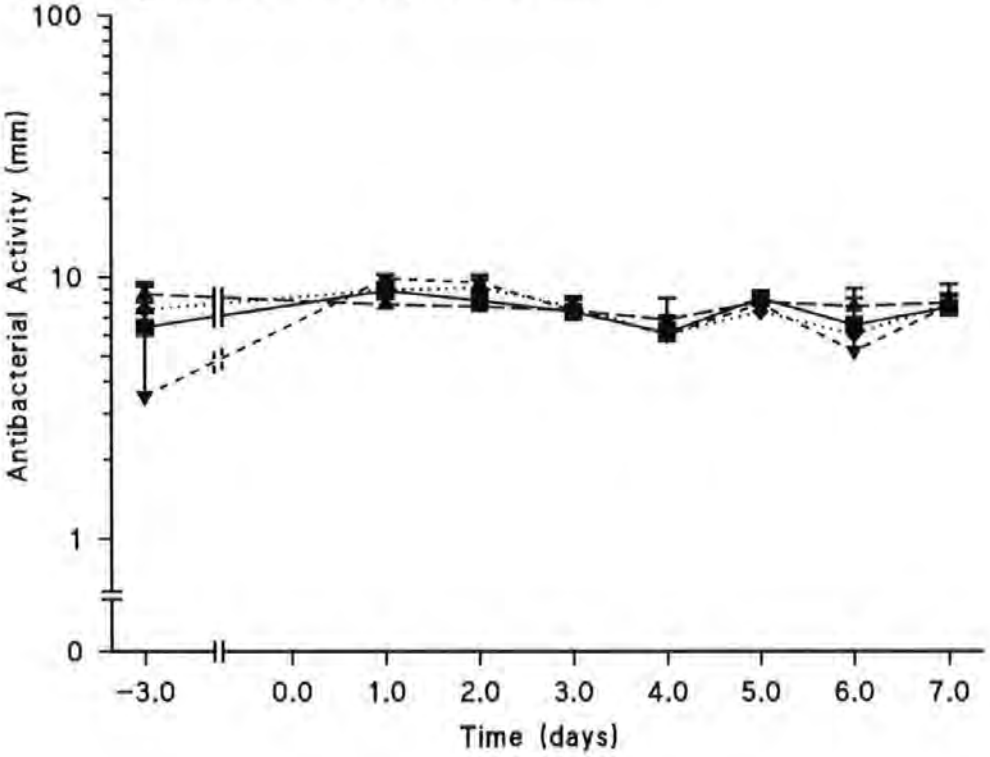


Fig 6.3.4 Part B Experiment Two

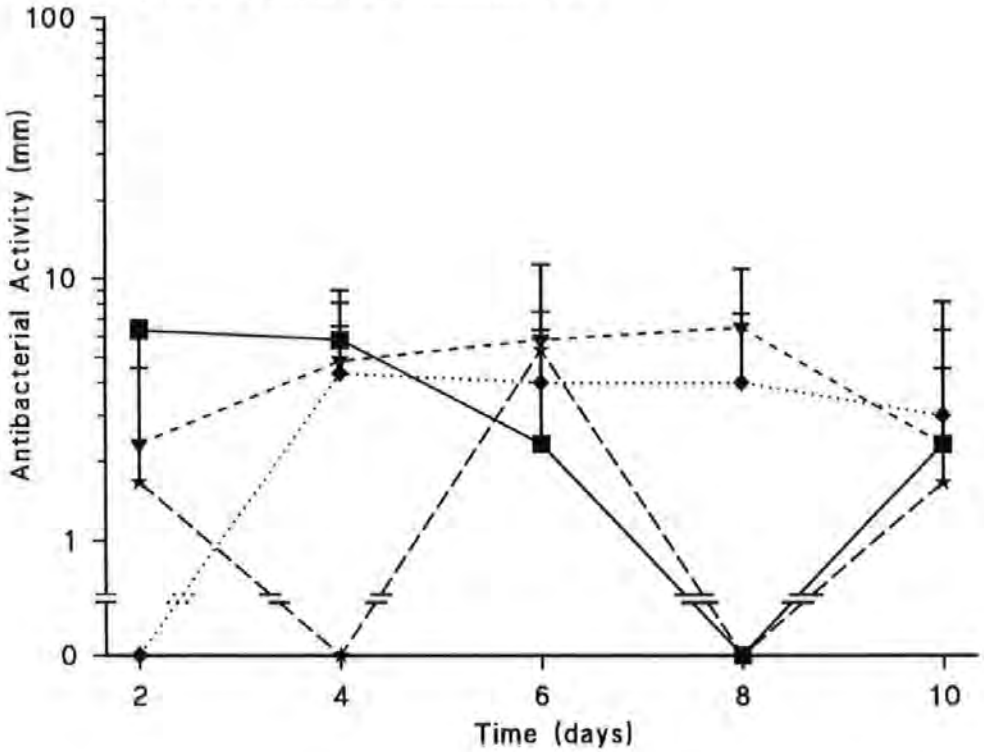


Fig 6.3.4 Part C Experiment Three

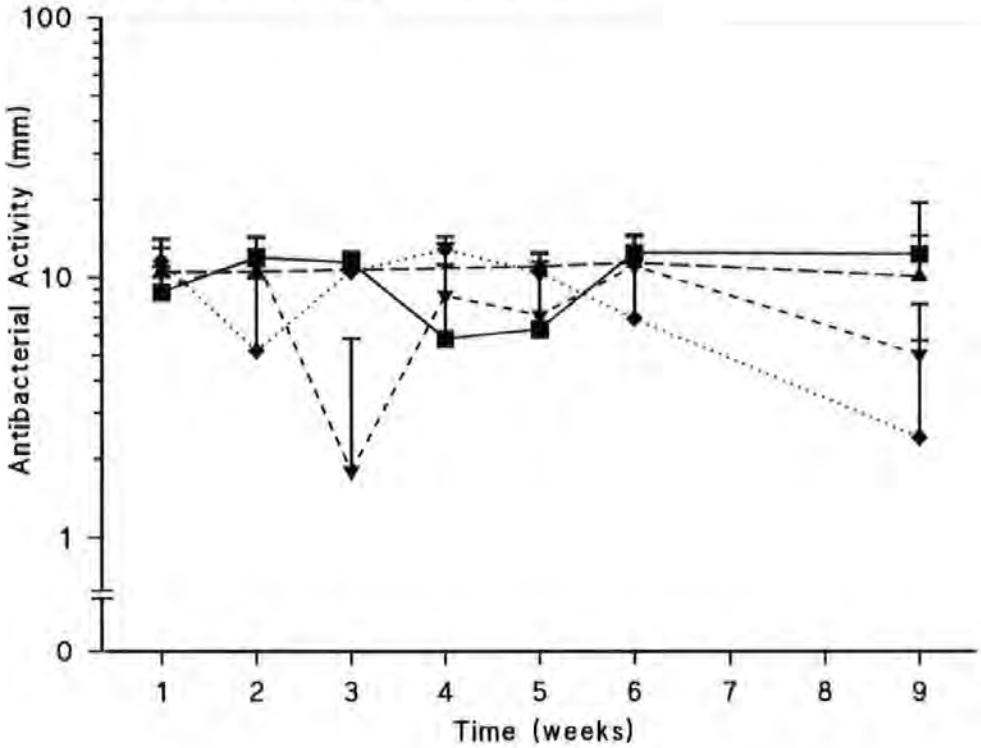


Figure 6.3.4 Serum antibacterial activity.

In experiment one the antibacterial activity of serum was measured by means of a impregnated filter paper diffusion assay, in experiments two and three it was measured by a well diffusion assay. Antibacterial activity is expressed as the diameter of the zone of growth inhibition in millimetres.

Part A Experiment one; Part B Experiment Two; Part C Experiment Three. Error bars represent SDn-1. For all parts data points for each group are $1x10^{10}$ orally in TSB (■); $1x10^{10}$ orally in saline (★); IP (▼); $1x10^{8}$ orally in TSB (◆); negative control(▲).

Fig 6.3.5 Part A Experiment Two

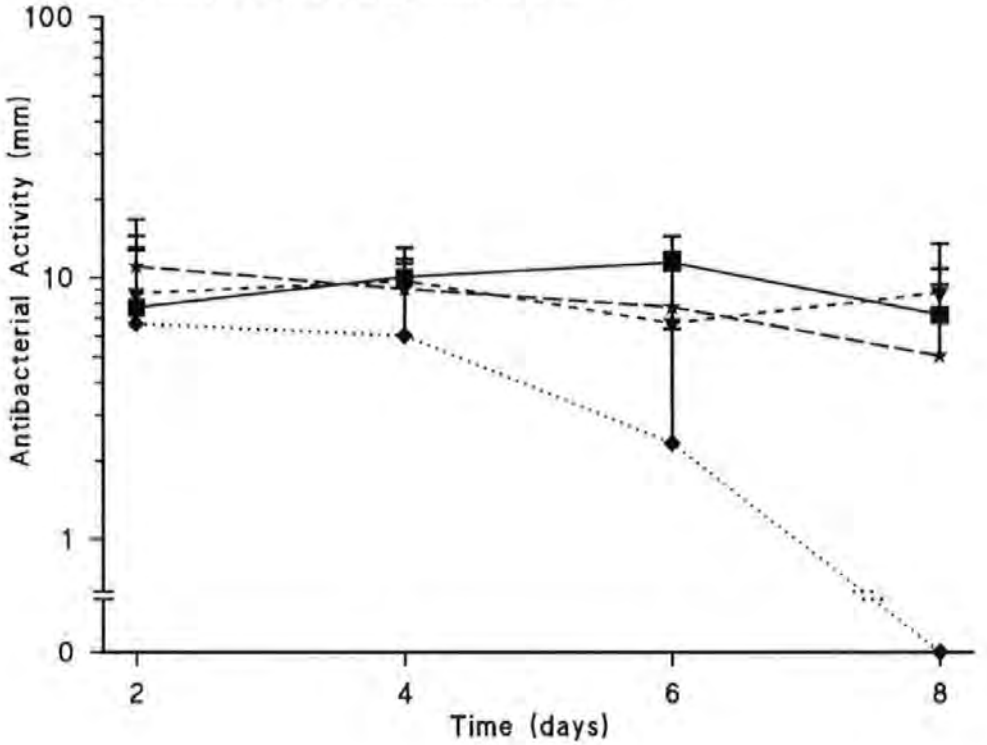


Fig 6.3.5 Part B Experiment Three

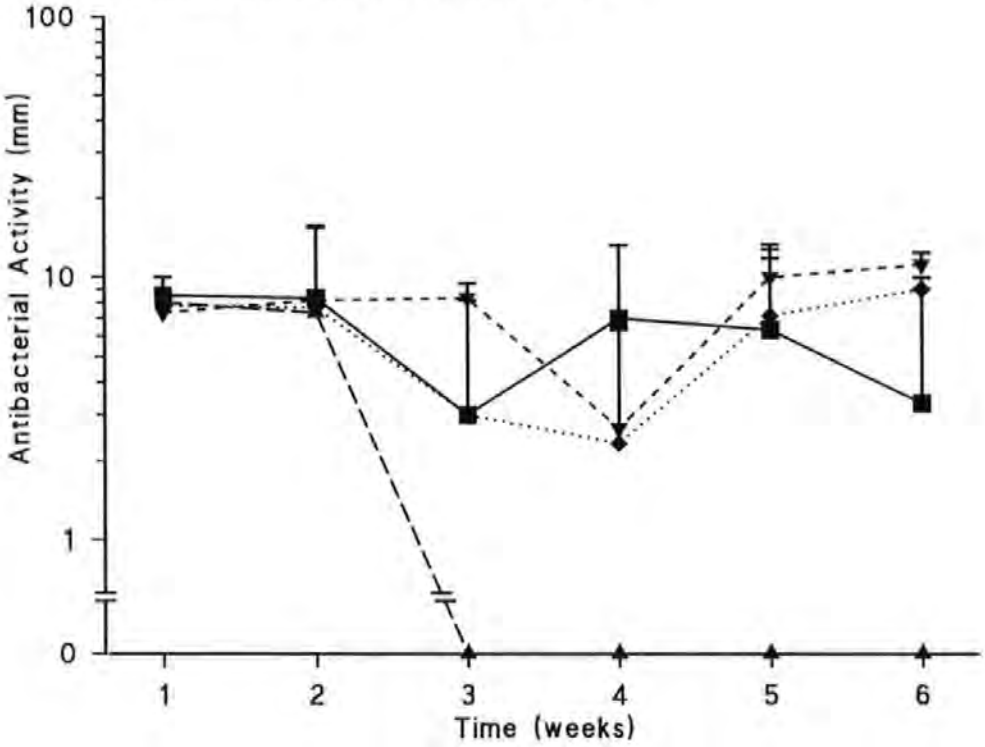


Figure 6.3.5 Antibacterial activity in skin mucus.

The antibacterial activity of skin mucus was measured by a well diffusion assay. Antibacterial activity is expressed as the diameter of the zone of growth inhibition in millimetres. Skin mucus samples were not taken from the negative control fish in experiment three after week two.

Part A Experiment one; Part B Experiment Two; Part C Experiment Three. Error bars represent SDn-1. For all parts data points for each group are $1x10^{10}$ *orally in TSB* (■); $1x10^{10}$ *orally in saline* (★); *IP* (▼); $1x10^{8}$ *orally in TSB* (◆); *negative control*(▲).

Day No	Pool	Part A Negative Control				Part B High Oral Group			
		Serum lysozyme levels HEWLE (mg cm^{-3})	Total Serum Protein (g l^{-1})	Serum Antibacterial Activity (mm)	Alternative Complement Pathway Activity (log_2)	Serum lysozyme levels HEWLE (mg cm^{-3})	Total Serum Protein (g l^{-1})	Serum Antibacterial Activity (mm)	Alternative Complement Pathway Activity (log_2)
		1.20	39.80	8.00	3	1.85	38.70	7.00	4
		4.39	36.60	8.00	3	1.20	29.90	6.50	4
		1.20	33.40	7.50	4	1.20	35.10	8.00	3
		1.20	26.10	7.50	2	1.85	34.40	8.00	3
		1.20	44.50	9.00	2	1.20	32.70	7.00	4
-3	A & B	1.20	32.40	9.00	3	1.20	23.70	8.00	3
		2.85	34.70	9.00	2	1.20	41.20	8.00	3
		0.02	nd	8.00	3	0.78	36.30	7.00	2
		0.78	nd	9.00	4	0.78	39.40	7.00	4
		nd	nd	0.00	nd	0.78	28.20	0.00	4
		nd	nd	7.00	nd	1.10	40.30	0.00	4
		nd	nd	8.00	nd	0.96	39.50	9.00	3
		3.75	40.00	7.00	2	0.27	43.30	9.00	3
		6.05	32.80	8.00	2	0.89	29.60	9.00	3
		0.89	53.30	6.50	3	0.89	46.30	9.00	2
+1	A	0.70	44.90	8.00	3	1.14	41.20	9.00	3
		0.70	58.10	8.50	3	1.14	38.00	8.50	3
		0.00	35.80	9.00	2	0.00	38.10	9.00	0
		0.34	47.30	7.00	4	0.55	38.80	8.50	3
		0.34	52.50	8.50	4	0.89	44.70	8.00	3
+2	B	1.44	52.20	9.00	4	0.27	41.60	9.50	3
		0.27	48.90	6.00	4	0.34	45.00	9.50	4
		0.21	42.70	7.50	3	0.70	41.40	10.00	3
		0.27	51.30	8.00	3	0.34	42.70	9.00	4
		4.25	36.00	7.00	3	4.25	33.30	7.00	2
		32.62	33.50	8.00	3	6.39	26.90	7.00	2
		0.37	52.60	8.00	3	0.83	42.60	8.50	2
+3	A	0.55	45.60	8.50	3	6.39	34.20	8.00	2
		0.55	51.20	7.00	1	4.25	47.50	7.00	2
		4.25	47.50	6.00	2	2.83	44.50	8.50	3
		0.11	49.10	7.00	3	0.40	38.10	6.50	3
		0.00	41.60	8.00	3	0.76	35.50	7.00	3
		5.00	39.40	5.00	4	0.55	43.40	5.50	0
+4	B	0.55	53.90	8.00	4	1.42	42.90	5.50	2
		0.16	45.20	8.00	4	0.55	35.80	5.50	3
		0.04	43.40	5.00	3	0.00	39.40	6.00	4
		17.63	32.80	8.00	3	1.94	32.60	7.00	3
		1.03	35.70	8.00	4	1.03	40.00	7.00	4
		1.42	39.70	8.00	3	0.29	42.00	7.00	3
+5	A	0.55	44.20	8.00	3	1.94	39.50	8.00	2
		0.03	38.80	8.00	2	1.03	52.00	7.00	4
		2.66	39.50	8.00	2	0.55	44.20	8.00	3
		1.33	54.10	7.00	3	3.43	36.00	7.00	0
		2.50	29.10	7.00	3	2.50	36.40	8.50	3
+6	B	4.69	36.60	8.00	3	12.08	35.10	7.00	2
		1.82	41.10	8.00	2	12.08	41.30	6.50	2
		4.69	38.90	7.50	2	4.69	43.40	7.00	2
		8.82	20.30	8.50	3	16.56	37.40	0.00	2
		12.08	nd	7.50	3	2.50	nd	7.00	3
		4.69	nd	6.00	2	2.50	nd	7.00	2
+7	A	16.56	nd	10.00	2	4.69	nd	8.00	3
		12.08	nd	9.00	2	8.82	nd	8.00	3
		8.82	nd	8.00	2	16.56	nd	8.00	2
		6.43	nd	7.00	2	8.82	nd	9.00	2

Table 6.3.1 Individual Nonspecific immune parameters for fish from experiment one.

Part A the negative control group (0.5 cm^3 of TSB per fish orally) and Part B the high oral dose group ($1x10^{10}$ CFUs per fish in TSB). All fish were sampled three days before immunisation (day 0) and each pool (A or B) was sampled alternately every day until day 7. Oral immunisations were with ***A. salmonicida aroA*** *with a prior dose of sodium bicarbonate.* ***nd*** *indicates that there was insufficient serum to conduct the assay.*

Table 6.3.2 Individual Nonspecific immune parameters for fish from experiment one.

Part A the low dose oral group ($1x10^8$ CFUs per fish in TSB) and Part B the IP group ($1x10^8$ CFUs per fish in saline). All fish were sampled three days before immunisation (day 0) and each pool (A or B) was sampled alternately every day until day 7. Oral immunisations were with ***A. salmonicida aroA*** *with a prior dose of sodium bicarbonate.* ***nd*** *indicates that there was insufficient serum to conduct the assay.*

Day No	Pool	Part A Low Oral Dose				Part B IP Group			
		Serum lysozyme levels HEWLE (mg cm^{-3})	Total Serum Protein (g l^{-1})	Serum Antibacterial Activity (mm)	Alternative Complement Pathway Activity (log_2)	Serum lysozyme levels HEWLE (mg cm^{-3})	Total Serum Protein (g l^{-1})	Serum Antibacterial Activity (mm)	Alternative Complement Pathway Activity (log_2)
		0.78	40.80	9.00	3	1.20	42.60	0.00	1
		1.20	34.10	9.00	3	1.85	25.10	6.00	2
		2.85	32.20	9.00	3	1.85	35.70	0.00	2
		1.20	35.90	8.00	3	1.20	32.40	0.00	4
		2.85	32.00	7.00	2	0.78	31.90	7.00	3
-3	A & B	2.85	32.10	0.00	2	0.78	33.00	7.00	3
		1.20	26.50	8.00	3	0.78	21.40	7.00	3
		0.78	26.80	7.50	3	0.78	32.20	0.00	3
		0.00	30.70	8.00	2	1.20	21.10	7.00	3
		0.00	26.00	0.00	3	1.20	36.90	0.00	3
		1.85	41.90	8.00	3	2.85	25.90	7.00	4
		0.00	nd	6.50	3	0.00	nd	0.00	3
		0.89	35.30	8.50	2	0.70	37.30	10.00	3
		0.89	35.40	9.00	3	0.17	46.40	9.00	3
		1.83	41.60	7.50	3	1.14	36.70	10.00	3
+1	A	0.70	35.50	9.50	3	1.83	38.00	10.00	2
		0.55	28.70	9.00	3	0.89	36.10	10.00	3
		0.89	31.10	9.50	3	0.00	51.70	10.00	2
		0.44	35.20	8.50	4	0.55	35.50	9.50	4
		0.34	41.10	8.00	3	0.34	38.80	10.00	3
+2	B	0.34	34.40	7.50	4	0.55	27.40	9.50	3
		0.44	44.00	7.00	4	0.44	29.20	10.50	4
		0.55	41.20	9.50	3	0.44	38.80	9.00	4
		0.21	44.70	8.00	4	0.55	33.60	8.50	4
		1.25	42.10	7.00	2	6.39	44.90	8.50	2
		2.83	33.30	7.50	3	6.39	34.40	7.00	3
		9.60	35.30	6.00	2	4.25	40.70	7.50	2
+3	A	6.39	28.10	7.00	2	14.43	45.00	6.00	2
		0.00	39.70	8.00	0	4.25	29.10	7.50	0
		0.83	34.20	8.50	2	0.00	39.50	8.00	2
		0.16	40.20	6.00	0	12.87	35.80	6.00	4
		0.55	31.20	6.50	3	12.87	44.10	8.00	2
		0.55	28.20	6.00	4	9.39	38.80	6.00	3
+4	B	0.55	29.10	6.50	4	24.16	38.10	5.00	0
		0.55	28.60	5.50	4	9.39	31.20	6.00	4
		0.00	32.10	6.00	3	0.00	35.50	5.00	3
		1.94	39.90	8.50	4	0.00	44.00	7.00	4
		1.03	34.40	8.00	3	0.00	45.10	9.00	3
		0.55	34.50	8.50	4	12.87	34.90	9.00	3
+5	A	0.76	36.50	8.50	3	1.94	29.10	7.00	4
		1.94	39.20	7.50	3	5.00	44.70	8.00	4
		1.94	39.70	8.50	3	9.39	39.90	7.00	4
		8.82	34.70	6.50	1	0.00	35.90	6.50	3
		4.69	30.60	7.00	2	4.69	36.70	0.00	1
+6	B	1.33	31.90	6.50	3	8.82	48.70	6.00	2
		1.33	45.40	6.00	2	8.82	29.00	6.00	2
		2.50	44.90	7.00	2	16.56	50.00	6.00	2
		3.43	41.30	6.00	2	6.43	41.10	6.50	2
		8.82	nd	9.00	2	31.09	nd	7.00	3
		8.82	nd	7.00	3	80.00	nd	7.00	2
+7	A	8.82	nd	7.50	2	80.00	nd	7.00	3
		8.82	nd	6.50	2	42.60	nd	8.00	3
		22.69	nd	7.50	2	58.38	nd	8.00	2
		12.08	nd	8.00	3	0.00	nd	9.00	0

Table 6.3.3 **Experiment two, Group A, individual results for the assays of nonspecific immune status.** *Samples for days 1 to 6 were obtained following oral immunisation with $1x10^{10}$ CFU of* ***A. salmonicida aroA*** *in TSB given with a prior dose of sodium bicarbonate.*

		Assays of nonspecific immune status				
Day No	Fish No	Serum lysozyme levels HEWLE (mg cm^{-3})	Total Serum Protein (g l^{-1})	Serum Antibacterial Activity (mm)	Alternative Complement Pathway Activity (log_2)	Skin mucus Antibacterial Activity (mm)
	1	2.68	44.70	7.00	2	0.00
2	2	2.28	17.90	6.00	1	10.00
	3	2.28	19.70	6.00	2	13.00
	1	1.95	18.50	6.00	2	7.00
4	2	2.28	18.50	6.50	2	10.00
	3	2.28	10.40	5.00	2	13.00
	1	2.28	16.50	0.00	1	12.00
6	2	3.15	0.00	0.00	2	12.00
	3	2.51	18.80	7.00	2	10.00
	1	0.65	18.70	0.00	2	10.00
8	2	0.00	14.20	0.00	2	11.50
	3	0.65	12.90	0.00	1	0.00
	1	0.65	21.90	7.00	3	nd
10	2	1.28	11.90	0.00	3	nd
	3	1.28	14.50	0.00	1	nd

Table 6.3.4 **Experiment two Group B individual results for the assays of nonspecific immune status.** *Samples for days 1 to 6 were obtained following oral immunisation with $1x10^{10}$ CFU of* ***A. salmonicida aroA*** *in saline given with a prior dose of sodium bicarbonate.*

		Assays of nonspecific immune status				
Day No	Fish No	Serum lysozyme levels HEWLE (mg cm^{-3})	Total Serum Protein (g l^{-1})	Serum Antibacterial Activity (mm)	Alternative Complement Pathway Activity (log_2)	Skin mucus Antibacterial Activity (mm)
	1	2.28	14.90	5.00	2	11.00
2	2	2.28	30.50	0.00	2	9.00
	3	4.35	27.30	0.00	1	13.00
	1	2.51	4.80	0.00	3	9.00
4	2	4.94	14.50	0.00	2	10.00
	3	1.28	15.20	0.00	3	8.00
	1	0.65	21.60	6.00	1	10.50
6	2	0.65	13.90	5.00	2	12.50
	3	9.72	14.40	5.00	2	0.00
	1	1.28	10.50	0.00	2	8.00
8	2	2.51	29.90	0.00	2	0.00
	3	0.33	22.80	0.00	2	7.00
	1	0.05	21.00	0.00	3	nd
10	2	0.05	22.70	0.00	2	nd
	3	0.05	24.60	5.00	2	nd

Table 6.3.5 **Experiment two Group C individual results for the assays of nonspecific immune status.** *Samples for days 1 to 6 were obtained following intra-peritoneal immunisation with 1x10^7 CFU of* ***A. salmonicida aroA*** *in TSB.*

		Assays of nonspecific immune status				
Day No	Fish No	Serum lysozyme levels HEWLE (mg cm^{-3})	Total Serum Protein (g l^{-1})	Serum Antibacterial Activity (mm)	Alternative Complement Pathway Activity (log_2)	Skin mucus Antibacterial Activity (mm)
	1	2.68	24.90	7.00	4	16.00
2	2	6.00	14.40	0.00	3	10.00
	3	nd	nd	nd	nd	nd
	1	4.56	0.30	0.00	1	8.00
4	2	9.57	17.90	7.00	2	12.00
	3	3.56	0.00	7.50	3	9.00
	1	7.47	21.00	0.00	3	0.00
6	2	20.10	36.00	11.00	2	10.00
	3	2.78	37.50	6.50	3	10.00
	1	4.56	49.00	6.00	2	7.00
8	2	12.25	35.40	7.50	4	11.00
	3	7.47	24.50	6.00	3	8.00
	1	7.47	19.90	7.00	2	nd
10	2	15.69	16.00	0.00	3	nd
	3	12.25	44.40	0.00	3	nd

Table 6.3.6 **Experiment two Group D individual results for the assays of nonspecific immune status.** *Samples for days 1 to 6 were obtained following oral immunisation with 1 x 10^8 CFU of* ***A. salmonicida aroA*** *in TSB given with a prior dose of sodium bicarbonate*

		Assays of nonspecific immune status				
Day No	Fish No	Serum lysozyme levels HEWLE (mg cm^{-3})	Total Serum Protein (g l^{-1})	Serum Antibacterial Activity (mm)	Alternative Complement Pathway Activity (log_2)	Skin mucus Antibacterial Activity (mm)
	1	3.70	0.00	0.00	2	0.00
2	2	4.35	0.00	0.00	1	8.00
	3	6.00	0.00	0.00	2	12.00
	1	0.49	11.00	0.00	2	10.00
4	2	4.35	25.50	7.00	2	8.00
	3	0.07	57.10	6.00	3	0.00
	1	1.27	15.90	6.00	3	7.00
6	2	1.27	50.60	6.00	2	0.00
	3	0.84	38.60	0.00	1	0.00
	1	1.27	50.90	12.00	2	0.00
8	2	2.88	36.00	0.00	3	0.00
	3	4.35	42.30	0.00	3	0.00
	1	0.56	33.80	9.00	3	nd
10	2	0.24	12.40	0.00	2	nd
	3	0.11	32.40	0.00	2	nd

Table 6.3.7 Experiment three, Group A, individual results for the assays of nonspecific immune status. *Samples for weeks 1 to 6 were obtained following oral immunisation (week 0) with 1 x 10^{10} CFU of* ***A. salmonicida aroA*** *in TSB given with a prior dose of sodium bicarbonate. Week nine was three weeks post-challenge (IP) with* ***A. salmonicida*** *644rB.* ***nd*** *indicates that the test was not done due to lack of serum,* ***tnd*** *indicates that the test was not done for another reason,* ***snt*** *indicates that the sample was not taken.*

Week No	Fish No	Assays of nonspecific immune status				
		Serum lysozyme levels HEWLE (mg cm^{-3})	Total Serum Protein (g l^{-1})	Serum Antibacterial Activity (mm)	Alternative Complement Pathway Activity (log_2)	Skin mucus Antibacterial Activity (mm)
One	1	2.54	10.20	8.00	4	10.00
	2	0.35	15.00	8.00	4	8.50
	3	1.55	8.80	8.00	3	8.00
	4	11.12	30.70	11.00	2	7.50
	5	nd	nd	nd	4	7.00
Two	1	0.95	30.20	12.00	4	8.00
	2	3.25	54.70	13.50	3	8.00
	3	0.58	44.90	11.50	2	9.00
	4	0.35	23.30	11.00	3	8.00
	5	1.55	43.10	11.50	3	9.00
Three	1	2.54	19.60	13.00	4	9.00
	2	2.54	22.80	11.00	4	0.00
	3	1.98	39.00	12.00	4	0.00
	4	1.98	28.10	11.00	4	0.00
	5	2.54	26.50	10.00	3	0.00
Four	1	2.54	24.90	10.00	5	0.00
	2	1.21	nd	8.00	5	9.00
	3	0.00	12.50	0.00	6	12.00
	4	0.45	22.60	11.00	5	0.00
	5	0.21	78.80	0.00	4	0.00
Five	1	0.45	13.60	0.00	5	9.00
	2	0.30	32.20	0.00	4	10.00
	3	0.21	20.50	8.00	4	0.00
	4	0.00	14.10	10.50	5	0.00
	5	0.00	14.80	13.00	5	0.00
Six	1	0.21	37.60	15.00	4	0.00
	2	0.65	49.90	13.00	4	0.00
	3	3.03	16.20	12.00	4	10.00
	4	2.07	45.90	13.00	4	0.00
	5	0.21	53.40	9.00	3	0.00
Nine	1	0.56	30.40	0.00	tnd	snt
	2	0.56	33.90	16.50	tnd	snt
	3	1.27	27.10	13.00	tnd	snt
	4	0.56	27.70	17.00	tnd	snt
	5	0.39	56.10	14.50	tnd	snt

Table 6.3.8 Experiment three, Group B, individual results for the assays of nonspecific immune status. *Samples for weeks 1 to 6 were following sham oral immunisation (week 0) with 0.5 cm^3 of TSB given with a prior dose of sodium bicarbonate. Week nine was three weeks post-challenge (IP) with* ***A. salmonicida*** *644rB.* ***nd*** *indicates that the test was not done due to lack of serum,* ***tnd*** *indicates that the test was not done for another reason,* ***snt*** *indicates that the sample was not taken.*

		Assays of nonspecific immune status				
Week No	Fish No	Serum lysozyme levels HEWLE (mg cm^{-3})	Total Serum Protein (g l^{-1})	Serum Antibacterial Activity (mm)	Alternative Complement Pathway Activity (log_2)	Skin mucus Antibacterial Activity (mm)
	1	nd	nd	11.00	3	8.00
	2	1.00	28.70	10.00	4	8.00
One	3	nd	nd	nd	3	0.00
	4	nd	nd	nd	2	0.00
	5	nd	nd	nd	4	8.00
	1	1.00	30.40	12.50	3	6.50
	2	0.76	28.30	6.00	3	8.50
Two	3	1.00	40.50	13.00	3	7.00
	4	0.76	28.70	12.00	4	0.00
	5	nd	nd	nd	3	0.00
	1	nd	nd	nd	3	snt
Three	2	nd	nd	nd	3	snt
	3	nd	nd	nd	2	snt
	1	nd	nd	nd	3	snt
Four	2	nd	nd	nd	3	snt
	3	nd	nd	nd	3	snt
	1	1.32	11.60	11.00	1	snt
Five	2	1.73	nd	11.00	2	snt
	3	1.73	nd	9.00	3	snt
	1	6.78	8.20	8.50	3	snt
Six	2	2.27	4.90	14.50	3	snt
	3	1.73	34.60	11.00	2	snt
	1	4.11	38.10	15.00	tnd	snt
	2	3.06	68.70	7.00	tnd	snt
Nine	3	13.42	20.10	8.00	tnd	snt
	4	1.70	74.80	7.00	tnd	snt
	5	0.70	29.70	7.00	tnd	snt

Table 6.3.9 Experiment three, Group C, individual results for the assays of nonspecific immune status. *Samples for weeks 1 to 6 were obtained following intra-peritoneal immunisation (week 0) with 1 x 10^7 CFU of* ***A. salmonicida*** *in TSB. Week nine was three weeks post-challenge (IP) with* ***A. salmonicida*** *644rB.* ***nd*** *indicates that the test was not done due to lack of serum,* ***tnd*** *indicates that the test was not done for another reason,* ***snt*** *indicates that the sample was not taken.*

Week No	Fish No	Assays of nonspecific immune status				
		Serum lysozyme levels HEWLE (mg cm^{-3})	Total Serum Protein (g l^{-1})	Serum Antibacterial Activity (mm)	Alternative Complement Pathway Activity (log_2)	Skin mucus Antibacterial Activity (mm)
One	1	nd	23.40	10.50	3	9.00
	2	1.02	nd	nd	3	7.00
	3	2.97	0.00	0.00	2	6.00
	4	nd	nd	nd	2	8.00
	5	3.87	3.80	12.00	3	0.00
Two	1	3.87	23.20	12.00	3	0.00
	2	nd	nd	nd	3	8.00
	3	1.34	33.30	15.00	3	10.50
	4	1.02	45.70	12.00	2	8.00
	5	1.34	57.40	8.00	2	14.00
Three	1	1.74	8.30	0.00	4	9.00
	2	3.87	16.70	0.00	3	9.00
	3	1.34	6.20	0.00	4	7.00
	4	0.78	0.00	9.00	4	nd
	5	0.00	10.20	0.00	3	nd
Four	1	3.87	19.90	10.00	5	8.00
	2	nd	nd	0.00	4	0.00
	3	2.27	nd	13.00	5	0.00
	4	0.00	nd	11.00	3	nd
	5	nd	nd	nd	3	nd
Five	1	4.77	26.70	8.00	4	7.00
	2	3.73	26.50	0.00	2	12.50
	3	0.66	nd	12.00	2	10.50
	4	1.08	nd	nd	2	nd
	5	0.00	nd	8.50	3	nd
Six	1	0.00	3.20	11.00	3	10.00
	2	0.00	nd	nd	4	11.00
	3	nd	nd	nd	3	12.50
	4	nd	nd	nd	3	nd
	5	nd	nd	nd	3	nd
Nine	1	0.39	28.20	6.00	tnd	snt
	2	0.70	25.50	0.00	tnd	snt
	3	1.26	23.30	6.00	tnd	snt
	4	0.21	29.50	6.00	tnd	snt
	5	2.28	31.90	7.00	tnd	snt

Table 6.3.10 Experiment three, Group D, individual results for the assays of nonspecific immune status. *Samples for weeks 1 to 6 were obtained following oral immunisation (week 0) with 1 x 10^8 CFU of* ***A. salmonicida aroA*** *in TSB, given with a prior dose of sodium bicarbonate. Week nine was three weeks post-challenge (IP) with* ***A. salmonicida*** *644rB.* ***nd*** *indicates that the test was not done due to lack of serum,* ***tnd*** *indicates that the test was not done for another reason,* ***snt*** *indicates that the sample was not taken*

Week No	Fish No	Assays of nonspecific immune status				
		Serum lysozyme levels HEWLE (mg cm^{-3})	Total Serum Protein (g l^{-1})	Serum Antibacterial Activity (mm)	Alternative Complement Pathway Activity (log_2)	Skin mucus Antibacterial Activity (mm)
One	1	6.12	44.00	9.00	3	8.00
	2	34.67	21.00	13.00	3	0.00
	3	12.87	37.70	12.00	4	6.50
	4	4.77	23.90	12.00	3	9.00
	5	nd	25.20	nd	3	7.50
Two	1	12.87	42.00	9.00	3	0.00
	2	1.77	75.50	9.00	3	7.00
	3	1.38	28.50	8.00	3	0.00
	4	1.38	22.10	0.00	3	9.00
	5	1.08	34.30	0.00	2	16.00
Three	1	nd	nd	10.00	3	9.00
	2	1.39	55.10	10.50	4	0.00
	3	nd	33.30	10.50	3	0.00
	4	6.88	17.90	12.00	3	nd
	5	6.88	22.60	9.50	3	nd
Four	1	0.00	17.80	11.50	5	0.00
	2	1.01	31.60	11.00	5	7.00
	3	1.01	27.80	13.00	4	0.00
	4	0.00	27.00	15.00	5	nd
	5	0.00	5.40	13.50	4	nd
Five	1	1.01	31.20	12.00	2	10.50
	2	0.00	81.70	8.00	3	11.00
	3	0.00	55.30	9.00	2	0.00
	4	0.53	8.10	13.00	3	nd
	5	1.01	21.30	10.00	3	nd
Six	1	nd	nd	nd	4	10.00
	2	0.00	20.00	0.00	3	8.00
	3	nd	nd	nd	2	9.00
	4	1.92	29.50	0.00	3	nd
	5	3.63	34.50	11.50	3	nd
Nine	1	0.00	nd	0.00	tnd	snt
	2	0.12	41.60	6.00	tnd	snt
	3	0.70	35.10	6.00	tnd	snt
	4	2.28	nd	0.00	tnd	snt
	5	1.26	nd	5.00	tnd	snt

6.4 Discussion.

The work presented in this chapter is to the author's knowledge the first investigation into the nonspecific immune response in rainbow trout following immunisation with a live *aro* mutant vaccine. The specific response following intraperitoneal immunisation has been investigated and the results presented (Vaughan *et al.*, 1993; Marsden *et al.*, 1996). It has been shown that for this vaccine at least there is a good correlation between protection and agglutinating antibody titre, however, this is not proof that the antibody response is the protective element of the immune response. Marsden *et al.* (1996) has shown that there is a significant stimulation of T-cells following immunisation with *A. salmonicida aroA*. It is also possible that the nonspecific response is enhanced in someway by this vaccine.

The results of experiment one show that there is a significant increase in the concentration of serum lysozyme following IP injection of $1x10^8$ CFUs in saline, there was also an increase in the level of lysozyme in the serum of the fish receiving the highest oral dose and unfortunately in the negative control. These findings were similar to those of Rainger and Rowley (1993) who found an increase in the in the level of serum lysozyme following peaking at day 10. Unfortunately, the results of the present study can not be directly compared to those of Rainger and Rowley since they expressed lysozyme activity in units and not mg cm^{-3} and because experiment one did not continue for ten days because of the mortalities in the orally immunised group. They found an increase in lysozyme concentration following injection with saline and suggested that this might be due to the presence of endotoxin in the saline. In the present experiment the fish were not injected with the saline but were intubated. This makes it unlikely that the increase in lysozyme concentration was caused by endotoxin since Gram negative bacteria are present in the environment all the time fish must be constantly swallowing endotoxin and without a subsequent rise in lysozyme concentration. Rainger and Rowley rejected the possibility that the increase in activity was

due to the stress induced in the fish by handling but in this case there seems to be no other like explanation. It is important that the increase in lysozyme concentration in the IP immunised group was still significantly greater than that in the negative control.

The results of the second experiment are more difficult to interpret as it was not possible to include a negative control as there were not enough tanks in the challenge aquarium. The negative control was discarded as the main aim of the experiment was to investigate factors that might effect the persistence of the vaccine in the host and it had already been demonstrated that without prior injection or intubation the fish did not harbour any *A. salmonicida* . It is clear from these results that there is not the same sort of peak seen in experiment one however, the samples start at day +2 and the level may have already risen following immunisation. Certainly the level of lysozyme in the serum of the IP immunised fish is significantly greater at day +10 compared with day +2 and this is not the case for any of the other groups. Again the results of experiment three are difficult to interpret because the sample sizes are not the same for each group and the results were very variable. It is plain that the orally immunised groups did have higher serum lysozyme concentrations at 7 days post immunisation than found subsequently and this may indicate an increase due to immunisation but the differences were not significant. It is interesting that these results are also similar to the findings of Møyner *et al.* (1993) who found that following experimental infection with virulent *A. salmonicida* serum lysozyme levels increased from day four peaking at day nine.

The mortalities that occurred at the end of experiment one were worrying at it was thought that they might have been due to the vaccine. Microbiological testing of the mortalities failed to show the presence of any bacteria in the peritoneal cavity or kidney and there were no *A. salmonicida* colonies identified in the intestinal mucus. Furthermore it was subsequently shown that this vaccine did not persist for nine days following oral intubation or IP injection (Chapter 7). This indicated that the mortalities were probably due to the stress caused by handling and bleeding every other day. Whilst none of the assays here demonstrated

that there was a definite effect on the health status of the fish, save the lysozyme results described above, this can not be proven however the appearance of the blood samples did deteriorate over the time course and this was indicative of anaemia.

There were no obvious effects on the levels of alternative complement activity during the either of the short time course experiments but there was a significant increase in SH_{50} titre around week four in all the immunised groups during the long term experiment. This is in keeping with the finding of Møyner *et al.* (1993) who also showed that alternative complement activity was not changed during infection with bacteria but was significantly greater after recovery. This may indicate that the vaccine stimulated similar changes in the complement system as are found during and following a real infection. It would have been useful to have accumulated data on the classical pathway complement activity of the serum of vaccinated fish. Hollebecq *et al.* (1995) failed to correlate antibacterial activity in salmonid serum with alternative complement activity and suggested that the classical pathway was more important certainly Møyner *et al.* (1993) showed that CH_{50} titres did decrease in infected salmon and although this was not a significant decrease it may indicate a depletion in the complement pool. The serum protein assay indicated that immunisation caused no demonstrable effect on the level of serum protein this is also in keeping with the results of Møyner *et al.* (1993) who showed that only symptomatically infected fish had reduced serum protein levels.

The antibacterial activity of the serum was consistent throughout experiment one and all groups had very similar activities. In experiments two and three the activities were much more variable as were the serum protein results. This indicates that these results may be related even though it was not possible to demonstrate correlation between serum protein level and serum antibacterial activity. It was also not possible to correlate antibacterial activity with alternative complement activity indicating that the classical pathway may be more important in serum killing than the alternative pathway, as discussed above. It is certainly possible that

the complement activity may have been damaged when the samples from experiments two and three were partially thawed and moved to a lower temperature freezer. It is even more likely that the thawing of samples from experiment three to make working dilutions for the specific immune response assays damaged complement activity. This exposed the major flaw in the way these experiments were conducted. It would have been much better to conduct all the nonspecific immunity assays on the day of sampling as was done during experiment one. However, as mentioned above the primary aims of experiments two and three were not the investigation of the nonspecific immune response and in both cases there just was not enough time available on the day of sampling to do all the necessary work.

It was not possible to demonstrate the serum protease activity in samples from immunised or control rainbow trout despite demonstrating zones of clearance around wells containing crude ECP extract. This was apparently because the protein in the serum enhanced the staining in the zone of diffusion around the well making it darker than the rest of the caseinase agar. This result contradicts that findings of Møyner *et al.* (1993) who demonstrated serum protease activity in infected fish and noninfected controls.

The presence of antibacterial activity in the skin mucus was interesting because in confirms the findings of Austin and McIntosh (1988) and Fouz *et al.*(1990) and implicates the mysterious glycoprotein, that both groups said was responsible for this activity but did not identify in the defence of rainbow trout at the skin surface. Since it did not prove possible to demonstrate the presence of lysozyme in the skin mucus confirming the findings of Rainger and Rowley (1993) a role for lysozyme in this antibacterial activity must be discounted. Unfortunately complement levels in skin mucus were not tested but to the best of the authors knowledge this has not been done. There was no correlation between serum antibacterial activity, serum protein levels, alternative complement activity or skin mucus antibacterial activity so discussion of the interrelationship of these factors is not possible.

In conclusion, there was evidence that vaccination with live attenuated bacteria had a stimulatory effect on the levels of serum lysozyme and alternative complement activity but had no effect on total serum protein, serum antibacterial activity or skin mucus antibacterial activity. The large amount of individual variation between experimental animals means that sample sizes should be larger than was possible here, furthermore live bleeding and mucus collection would allow trends in individual fish to be followed over time. The mortalities that occurred in experiment one indicate that live bleeding at least of the type done here could be problematic. It would have been very useful to have data on the classical complement activity of the sera as this may well be more relevant than the alternative complement activity. Also substances other then antibodies can activate complement by the classical pathway in mammals for instance C-reactive protein (Kaplan and Volanakis, 1974), mannose-binding protein (Ikeda *et al.*, 1987) and lactoferrin (Rainard, 1993) and it may well be that investigation of these substances as well as classical complement activity could be useful. Certainly, Michel *et al.* (1991) showed that natural antibodies and furunculosis resistance increase in parallel although he was not able to demonstrate the functional properties of these molecules they might also be worth further investigation. Lastly, it would also be very helpful to have some measure of the activation of the cellular nonspecific immune response to consider in parallel with these humoral assays since one side of the immune response rarely functions without the other.

Chapter Seven.

The specific immune response in rainbow trout following immunisation with *Aeromonas salmonicida aroA*

7.1 Introduction.

Of the many known bacterial pathogens affecting non-human hosts, *Aeromonas salmonicida* is one of the most intensively investigated. This is probably because of its economic importance, complex pathogenic mechanisms and the great difficulty in developing an effective vaccine. Most of the published work on *A. salmonicida* has been extensively reviewed elsewhere (Austin and Austin, 1987; Munro and Hastings, 1993; Noonan and Trust, 1996; Trust *et al.*, 1996), furthermore the current state of knowledge of bacterial vaccines for fish has been recently reviewed (Hastings, 1988; Newman, 1992; Ellis, 1995; Vaughan *et al.*, 1996) as was the current state of oral vaccination of fish (Ellis, 1995). These topics were also dealt with as part of the general introduction of this thesis and no purpose would be served by covering this ground again here. Therefore, it is intended to look at the factors affecting successful vaccination of fish such as the lack of correlation between antibody response and protection the inherent variability within experimental groups and pond effect.

Much of the recent work has concentrated on the immunisation of fish with putative virulence factors from *A. salmonicida* grown *in vitro*. Ellis (1991) has already observed that this strategy may be fundamentally flawed since virulence factors expressed *in vivo* may be quite different from those produced *in vitro*. By growing bacteria in rainbow trout within intraperitoneal implants Gardùno *et al.* (1993) showed that *A. salmonicida* becomes resistant to host-mediated bacteriolysis, phagocytosis, and oxidative killing and that these properties were lost upon subculture *in vivo*. The resistance to bacteriolysis and phagocytosis was associated with the production of a capsule composed of acidic polysaccharide. The capsule prevented the underlying A-layer from being labelled with gold labelled antibodies. Thornton *et al.* (1993) showed that bacteria grown *in vivo* express several unique antigens. Antiserum raised in rabbits against *in vivo* grown cells was approximately 10 times more sensitive in detecting *A. salmonicida* in fish kidney than antiserum raised against *in vitro* grown bacteria.

These data confirm Ellis's speculation furthermore, the capsule produced *in vivo* has been shown to have different properties to the capsule produced by *A. salmonicida* grown on glucose rich agar (Garduño and Kay, 1995). It has also shown that virulent but apparently protease deficient strains of *A. salmonicida* produced the protease *in vivo* (Ellis *et al.*, 1988a).

The growth conditions for the bacteria can obviously affect the production of virulence factors and one reason for using live attenuated mutants of virulent bacterial strains is that they can in theory produce the full range of *in vivo* virulence determinants. Furthermore, they can act as carriers for heterologous proteins and thus become multivalent vaccines (Chatfield *et al.*, 1989, 1992, 1993; Dougan, 1989,1994).

One of the main obstacles to the development of an effective *A. salmonicida* vaccine has been the apparent lack of correlation between the production of an antibody response and the degree of protection (Michel, 1979; Michel and Faivre, 1982). Midtlying *et al.* (1996) showed that protection did correlate with antibody response, but even so correlation between two factors is not proof of a causal relationship. This has also been observed during experiments with *Vibrio* vaccination, where the protective role for antibodies was demonstrated by passive immunisation experiments (Harrell *et al.*, 1975,1976) but then was contradicted when immunisation by routes that do not normally produce a good antibody response, i.e. oral (Kawai *et al.*, 1981) and by immersion (Croy and Amend, 1977) gave good protection. This may indicate that an alternative but immunologically relevant response is stimulated when the vaccine is delivered via a mucosal surface which does not necessarily manifest itself in a measurable humoral response.

The vaccination of rainbow trout against enteric redmouth (ERM) caused by *Y. ruckeri* has also been very successful, in the field and in the laboratory. Tebbit *et al.* (1981) reported the field trial of the vaccine in 22 million rainbow trout at three sites over two years and the combined results indicated an 84% reduction in mortality due to ERM. Unlike the *Vibrio* vaccine for which the protective antigens are known (Chart and Trust, 1984; Evelyn, 1984)

the protective antigens for ERM are unidentified, but the presence of agglutinating antibodies does not appear to be correlated with protective immunity.

Vaccine efficacy is investigated by comparison of control and experimental group responses to immunisation and/or challenge. These comparisons can only demonstrate significant differences if the intra-group variation is less than the inter-group variation (Michel, 1980). Unfortunately, the intra group variation is often very large in experiments involving fish (Michel *et al.*, 1984). This variation may be caused by the inability of fish to regulate their internal environment, certainly in mammals there is usually very little intra group variation and this is often enhanced by the use of inbred strains of experimental animals. Michel *et al.* (1984) suggested that for a change in vaccine dose in the order of one dilution step requires an experimental group of 75 to be significantly different and this is more than twice the number of mammals generally required.

Furthermore, the most carefully prepared experiment may be adversely effected by environmental factors beyond the control of the investigator. Often significantly different results can be achieved from apparently identical groups, kept under apparently identical conditions and subjected to similar treatments (Michel *et al.*, 1984). This is known as group effect or 'pond effect' and Michel *et al.* (1984) presented data that demonstrated that the influence that this effect can have on the experimental infection of rainbow trout with *A. salmonicida*. In our laboratory a similar effect was seen in fish undergoing a nutrition trial. Fish were assigned to three experimental treatments and then separated into two groups per treatment. For one of these treatments the growth of the fish in each of the groups were very different (Roper, unpublished observations).

Recently, Vaughan *et al.* (1993) reported the development of an aromatic deficient mutant of *A. salmonicida* and showed that it persisted in detectable numbers for 12 days and provided a significant protection for Atlantic salmon and brown trout against challenge with a virulent strain and precipitated in a significant increase in agglutinin titre. Marsden *et al.*

(1996) have presented results showing that a similar vaccine can induce a specific cellular and antibody response in rainbow trout following intraperitoneal injection.

This work aimed to investigate the persistence of the *aroA* mutant following oral and intraperitoneal immunisation of rainbow trout since persistence and *in vivo* multiplication of live vaccines is believed to be an important factor in the immune response afforded by them. It was also intended to investigate the kinetics of the serum and mucosal antibody response following administration of the vaccine orally and by intraperitoneal injection using a dot blot assay. The proteins from fractionated bacterial cells and the ECPs were used to identify which bacterial antigens were immunogenic and determine if there was a difference in the antigen recognition between orally and IP immunised fish using both dot blot and Western blotting. Finally, the protection afforded by the immunisation against challenge with the homologous parental strain was investigated.

7.2 Materials and Methods

7.2.1 Bacterial strains.

The bacterial strain used in the immunisation of the experimental animals was *Aeromonas salmonicida* 644rB *aroA*::kan^r. The strain used in the LD_{50} test and used to challenge the experimental animals was *Aeromonas salmonicida* 644rB. The details of these strains are given Section 3.2.1.

7.2.2 Routine culture of bacteria.

Unless otherwise stated all bacteria were cultured as described in section 3.2.2.

7.2.3 Viable count.

Unless otherwise stated viable bacteria were counted using the Miles and Misra technique (Miles *et al.*, 1938). Serial tenfold dilutions were made in PBS (Oxoid, UK) 0.5 cm^3 in 4.5 cm^3 and bacteria grown on TSA containing the appropriate antibiotics (Section 3.2.2) and incubated at 22°C for 48 hours before counting.

7.2.4 Rapid spectrophotometric enumeration of *A. salmonicida*.

The standard curves of absorbance at 590nm against viable count of *A. salmonicida* colony forming units (CFU) suspended in PBS (as described in Section 3.2.5) were used to determine the numbers of CFUs in the vaccine preparations prior to immunisation. The estimations were confirmed by viable count (Section 7.2.3).

7.2.5 Experimental animals.

Prior to the start of each experiment the rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) were kept in the stock aquarium; during this time they were kept in a freshwater recirculating system at 14°C ± 1°C and fed to satiation on a commercial trout pellet preparation.

In the persistence experiment adult female rainbow trout 150-200 g were transferred to the challenge aquarium in two stages. The challenge aquarium contained four 220 gallon

tanks each with a recirculating freshwater system maintained at 13°± 1°C. The fish were randomly allocated to one of four groups (n=21), each group being kept in a separate tank. The fish were allowed to acclimatise to the experimental conditions for a further six days before immunisation. The fish were fed lightly throughout the experiment but were starved for 48 hrs before and 24 hours following immunisation.

In the specific immune response experiment 240 rainbow trout 40-50g were transferred to the challenge aquarium in four stages over a period of 14 days. The aquarium was maintained at 14°± 1°C. The fish were randomly allocated to one of four groups (n=70). The fish were allowed to acclimatise to the experimental conditions for a further six days before immunisation. The fish were fed lightly throughout the experiment but were starved for 48 hrs before and 24 hours following immunisation

In the LD_{50} test 48 fish were transferred to four 117 litre tanks in the laboratory which were maintained at about 15°C. The fish were randomly allocated to one of 4 groups (n=12). These fish were challenged the day they were moved.

7.2.6 Production of the bacterial vaccine.

The vaccine was produced in different ways for the persistence and specific immune response experiments, however, some aspects were constant for each experiment. Bacteria were routinely cultured as described above (Section 3.2.2.). To produce the vaccine a 48-hour working culture was used to inoculate fresh TSB. These cultures were grown for a further 48 hours washed once by centrifugation and resuspension in PBS followed by a further centrifugation step and resuspension in a known volume of PBS. The number of CFUs in the suspension was estimated using the rapid spectrophotometric method described above (Section 3.2.5.). The bacteria were sedimented by centrifugation and the concentration of bacteria was adjusted to the level required for each of the experimental groups. The details for each of the experiments are given below.

i) *The investigation of the persistence of the vaccine.*

The working culture was used to inoculate 20 x 100 cm^3 of TSB in 250 cm3 conical flasks. The initial sedimentation and washing of the bacterial cells was done by centrifugation in 250 cm^3 centrifugation pots at 6,000 *g* for 30 minutes. The sedimentation step following the estimation of bacterial concentration was performed in 50 cm^3 centrifuge tubes 6,000 *g* for 30 minutes. The bacteria were finally resuspended at concentrations of; $2x10^{10}$ in 20 cm^3 of TSB, $2x10^8$ in 20 cm^3 of TSB and $2x10^7$ in 20 cm^3 of saline.

ii) *The investigation into the specific immune response.*

The working culture was used to inoculate 9 litres of TSB in conical flasks of various sizes between 250 cm^3 and 5 litres. Centrifugation was performed in 500cm^3 centrifugation pots at 6,000 *g* for 30 minutes, the sedimentation step following the estimation of bacterial concentration was performed in 50 cm^3 centrifuge tubes at 6,000 *g* for 30 minutes. The bacteria were finally resuspended at concentrations of; $2x10^{10}$ in 60 cm^3 of TSB, $2x10^8$ in 60 cm^3 of TSB and $2x10^7$ in 60 cm^3 of TSB.

7.2.7 Production of the bacteria for the LD_{50} test.

A working culture of *A. salmonicida* 644rB was used to inoculate 100 cm^3 of TSB in a 250 cm^3 conical flask. This was incubated at 20°C for 48 hours. Bacterial suspensions at $2x10^4$ CFUs cm^{-3}, $2x10^3$ CFUs cm^{-3}, $2x10^2$ CFUs cm^3 and $2x10^1$ CFUs cm^{-3} each in TSB were made as described for the production of the vaccine and the viable count confirmed also as described above. Bacteria were delivered by IP immunisation as described above (Section 4.2.7). Each group of twelve fish received one of the doses of bacteria.

7.2.8 Administration of the bacterial vaccine.

The bacteria were administered either orally or intraperitoneally, as described above (Section 4.2.7). Unless otherwise stated orally intubated fish were given 1cm^3 of a 35mg cm^{-3} solution of sodium bicarbonate one hour before immunisation. The sodium bicarbonate was administered orally in the same manner described for the vaccine.

In each experiment the four groups of fish were given different treatments these were:

i) *The investigation of the persistence of the vaccine.*

The groups of fish were given treatments as follows, the high dose oral group A given 0.5cm^3 of 2.0x10^{10} CFUcm^{-3} in TSB, the second high dose oral group was given 0.5cm^3 of 2.0x10^{10} CFUcm^{-3} in TSB orally but was not given sodium bicarbonate, the IP group was given 0.5 cm^3 of 2.0x10^{10} CFU cm^{-3} in TSB intraperitoneally, and the low dose oral group was given 0.5 cm^3 2.0x10^8 CFU cm^{-3} TSB. The initial IP group suffered high mortality and was replaced with a group given 0.5 cm^3 2.0x10^7 CFU cm^{-3} TSB intraperitoneally.

ii) *The investigation of the specific immune response.*

The groups of fish were given treatments as follows; the high dose oral was given 0.5 cm^3 of 2.0x10^{10} CFU cm^{-3} in TSB, the negative control group was given 0.5 cm^3 of TSB orally, the IP group was given 0.5 cm^3 2.0x10^7 CFU cm^{-3} in TSB and the low oral dose group was given 0.5 cm^3 of 2.0x10^8 CFU cm^{-3} in TSB.

7.2.9 Collection of samples for the investigation of the persistence of the vaccine.

Samples of kidney, spleen and intestinal mucus were collected, processed and viable bacteria enumerated as described above (Section 4.2.8).

7.2.10 Collection of blood and secretions from the fish for the investigation of the specific immune response.

Each week for 2 weeks following immunisation 5 fish were sacrificed per group. From week 3 to week 6, three fish from the control group were bled, mucus and bile samples

were not taken and the fish were returned to the tank alive. Samples of skin mucus, serum, bile and intestinal mucus were taken from the sacrificed fish and antibodies to *A, salmonicida aroA* were assessed by dot blot and Western blotting (Sections 7.2.19 and 7.2.20). The antibody titres for the control fish were treated as baseline levels against which the titres for the immunised groups were determined. Fish were starved for 48 hours prior to sampling to ensure the presence of bile in the gall bladder and so that there would be very little food in the intestine.

i) Serum

Blood samples were drawn from the caudal sinus of fish via a 23*g* x 25mm needle into a 2.5 cm^3 syringe. Immediately after removal of blood the fish were sacrificed by a sharp blow to the head before the collection of the other samples. Blood was transferred to sterile 7 cm^3 bijoux bottles and left at room temperature for 4 to 5 hours to clot. Once the blood had clotted the serum was removed with a sterile glass Pasteur pipette and transferred to 1.5cm^3 micro-centrifuge tubes and centrifuged at 13,000 rpm for 5 minutes in a microcentrifuge (MSE, UK) to remove any blood cells. Serum was then placed in a new sterile microcentrifuge tube and stored at - 70°C until required.

ii) Cutaneous mucus

Cutaneous mucus was collected immediately after sacrifice. If the fish was bleeding from the head or from the needle wound that part of the fish was covered in tissue to limit the risk of contamination of mucus with blood. Mucus was removed from the dorsal surface of the fish by gently scraping the skin with the handle of a scalpel from the operculum to the tail to minimise scale detachment. Mucus was collected from the tail of the fish using a 200 μl pipette tip and a Finnpipette Digital pipette set to 100 μl. The mucus was transferred to a microcentrifuge tube and 1 cm^3 saline was added. The mixture was vortexed for 1 minute and the supernatant removed and stored at -70°C until required.

iii) Bile

After collection of cutaneous mucus and serum, fish were dissected ventrally to expose the digestive tract. Bile was collected from the gall bladder via a 23*g* needles into a 1cm^3 syringe and stored undiluted at -70°C until required.

iv) Intestinal mucus

The digestive tract from immediately posterior to the pyloric caecae to the anus was excised. Mucus was collected by holding one end of the intestine with forceps and gently squeezing the intestine between the first finger and thumb moving along the intestine slowly. The mucus was collected in 15 cm^3 centrifuge tube (Falcon, UK) and the volume made up to 2 cm^3 with saline and 50 μl of 100mM Phenylmethylsulfonyl fluoride (PMSF) was added. The mixture was vortexed for 1 minute and then centrifuged at 4,800*g* and 1cm^3 of the supernatant was removed and stored at -70°C until required.

7.2.11 Confirmation of the cause of death of fish in the LD_{50} test.

Fish were observed for symptoms of acute furunculosis ie; darkening of the skin, loss of appetite, lethargy, haemorrhage at the base of the fins and reddening of the vent. Mortalities were removed and dissected. Sterile swabs were taken from the peritoneal cavity and of the intestinal mucus and small samples of kidney tissue were taken. These samples were smeared onto Coomassie Blue agar. The cause of death was confirmed as furunculosis if at least two of the samples from each fish gave rise to blue colonies that produced a brown diffusible pigment. The LD_{50} was calculated by the method of Reed and Muench (1938).

7.2.12 Production of rabbit antiserum to rainbow trout IgM.

Rabbit anti-rainbow trout IgM antibodies had previously been prepared by Dr. A. B. Wrathmell. Rainbow trout IgM was purified from the serum of fish immunised i.p. with 5 mg HGG emulsified in Freunds incomplete adjuvant. Serum was separated by gel filtration chromatography on a ACA-22 column (LKB, Bromma, Sweden). Fractions were tested by ELISA for anti HGG activity, protein concentration was measured and the reactive fractions

were pooled and dialysed against 200mM Tris saline, pH 7.5 to remove azide. The second separation step was performed by anion exchange chromatography using a 0.5 M sodium acetate stepped gradient at room temperature (Burgess, 1988; Grayson *et al.*, 1991; Lavelle, 1994). A mono-Q HR5/5 column was connected to a Fast Protein Liquid Chromatography system (FPLC) (Pharmacia, Milton Keynes, UK). Fractions were monitored at 280nm (UV-1, Pharmacia) and peaks collected (Frac-100, Pharmacia). Fractions were again assessed for anti-HGG activity by indirect ELISA, reactive fractions were pooled and 200 μg of the resultant protein administered sub-cutaneously (SC) to Dutch rabbits. After 24 days the rabbits were given a booster injection of 150 μg of the protein and three weeks later blood samples were taken and tested by immunoelectrophoresis (Hudson and Hay, 1988) against rainbow trout IgM (anti-HGG reactive fractions from the column as described above). The precipitin band after being excised and homogenised in 1.5 cm^3, pH 7.2 was used to immunise the rabbits five weeks after the first booster immunisation. The animal was re-immunised 6 months later as described above and bled 8 months after the final injection.

7.2.13 Production of the *Aeromonas salmonicida aroA* whole cell antigens.

A 48 hour culture of *A. salmonicida aroA* was used to inoculate 9 x 100 cm^3 of TSB in 250 cm^3 conical flasks. Bacteria were allowed to grow for forty eight hours at 20°C unshaken. The bacteria were then centrifuged at 2000 g for 10 minutes and washed three times in cold PBS, the resulting pellets of bacterial cells were each resuspended in 5 ml of cold and transferred to 50 cm^3 centrifuge tubes (Falcon, UK) that had been cooled to 4°C and then kept on ice, these tubes were used as sonication vessels.

Sonication was performed within a class two safety cabinet. The micro sonication probe attached to a W-385 sonicator (Heat Systems, Ultra Sonics Inc., N.Y., USA) was placed into each bacterial cell suspension, in turn, with the tip approximately 5 mm from the bottom of the vessel which was surrounded by ice. The vessels were secured to the same stand as the sonicator using a clamp.

The sonicator was tuned according to the manufacturers instructions and then set to run for 5 minutes on a 50% duty cycle each cycle was 2 seconds long. If sonication was incomplete after 5 minutes the vessel was returned to the ice bucket and allowed to cool for 5 minutes then sonicated for a further 5 minutes.

7.2.14 Production of the *Aeromonas salmonicida aroA* extra-cellular protein antigens.

Extra cellular proteins were prepared by the cellophane membrane overlay method of Munro *et al.* (1980). TSA (250 cm^3) containing kanamycin 40 $\mu g\ cm^{-3}$ and nalidixic acid 30 $\mu g\ cm^{-3}$ was poured into two 25 cm x 25 cm 100 cm^3 Pyrex culture plates. Whilst the agar was still damp a 30 cm^2 piece of sterile cellophane membrane was placed on to the surface of the agar so that both the agar and the side of the plate were covered by the membrane. Any spare membrane was cut away using scissors and 20 cm^3 from 100 cm^3 of a 48 hour culture of *A. salmonicida aroA* was pipetted onto the membrane. The plates were incubated for a further 48 hours and the bacterial culture and broth was removed from the overlay by pipette. The bacterial suspension was transferred to two 50 cm^3 centrifuge tubes (Falcon) and centrifuged for 30 minutes at 6000 *g* in an MSE 18 centrifuge. The supernatants were removed and the protein content was measured using the method of Bradford (1976) using a Biorad Bradford Protein Assay Kit. The ECP solution was then aliquoted into 100 and 200 µl volumes and stored at -20°C until required.

7.2.15 Cellular fractionation of *A. salmonicida* 644rB *aroA* :: kan^r.

Bacteria were grown for 48 hours at 20°C as a confluent monolayer on 15 cm diameter Petri dishes. The growth was scraped from the agar using a glass microscope slide and transferred to universal bottles and placed on ice. The bacterial cells were suspended in 5 cm^3 *25 mM* Tris-HCl, pH 7.4 containing 1 m*M* EDTA, transferred to sonication vessels and sonicated as described above.

Following sonication the bacterial suspensions were poured into prechilled 50 cm^3 centrifuge tubes (Oakridge, US) containing 20 cm^3 25 m*M* Tris HCl, pH 7.4. Whole

bacteria were sedimented by centrifugation in a MSE 18 centrifuge *5000 g*, at 4°C, for 30 minutes, with no brake.

The supernatants containing the bacterial envelopes were carefully poured into a second set of Oakridge centrifuge tubes ensuring that whole bacteria did not enter the fresh tubes. Bacterial envelopes were sedimented by centrifugation *45,000 g*, at 4°C, for 1 hour, with no brake. The supernatants were collected and stored on ice and the centrifuge tubes were allowed to drain on to a double layer of tissue paper. When drained 1 cm^3 of 25 m*M* Tris HCl, pH 7.4 was added to each tube and the envelope pellet resuspended by repeatedly drawing into and out of a 1 cm^3 pipette tip using a fresh tip for each tube. Exactly 19 cm^3 of 25 m*M* Tris HCl, pH 7.4 was added to each tube and the contents were mix by gentle rocking. Disposable semi micro UV quality cuvettes (Hughes and Hughes LTD, UK) were filled with the envelope suspension using one cuvette for each centrifuge tube and the absorbance (A_{280}) of each suspension was measured with a Phillips PU 8700 spectrophotometer using 25 m*M* Tris HCl, pH 7.4 as a blank. Each absorbance unit was approximately equivalent to 1 mg cm^3 protein; by multiplying the absorbance units by 20 the total protein in each suspension could be calculated. The contents of the cuvettes were returned to their respective centrifuge tubes and 20 µl of Sarkosyl® was added for every milligram of protein in the suspension. The screw caps were fastened as tightly as possible and the contents mixed by placing the tubes on a Spiramix 5 (Denley, UK) for 30 minutes at room temperature. The tubes were then transferred to a prechilled centrifuge head and the outer membranes were sedimented *45,000 g*, at 4°C, for 1 hour, no brake. The inner membrane fraction was collected by pouring the supernatant carefully into a cooled universal bottle and stored on ice. The outer membrane fractions were washed by resuspending the pellets in 1 cm^3 of 25 m*M* Tris HCl, pH 7.4 by repeatedly pipetting into and out of a 1 cm^3 pipette tip. To this 29 cm^3 of 25 m*M* Tris HCl, pH 7.4 was added prior to mixing and centrifugation *45,000 g*, at 4°C, for 1 hour, with no brake. Following this centrifugation step the supernatants were discarded and the tubes

inverted and allowed to drain onto a double layer of tissue paper. Once dry the pellets were resuspended in a total volume of 200μl of 25 m*M* Tris HCl, pH 7.4 and stored in a screw capped microcentrifuge tube at -20 °C.

7.2.16 Isolation of lipopolysaccahride from *A. salmonicida.*

To isolate the LPS from *A. salmonicida* outer membranes were prepared as described above except that the starting culture was grown in 8 x 100 cm^3 TSB rather than on TSA plates. This culture was sedimented by centrifugation in 500 cm^3 pots (Nalgene) at 10,000*g*, at 4°C, for 10 minutes, with no brake. The supernatant was carefully discarded and the pellets transferred to the sonication vessels. Outer membrane preparation then continued as described above and products stored overnight at -20°C. There were eight outer membrane suspensions and these were pooled into two suspensions. For each of these 5 cm^3 of 80% aqueous phenol was dispensed into a 15 cm^3 glass centrifuge tube and the tubes were plugged with cotton wool. The centrifuge tubes were then placed into a heated water bath set at 68°C and allowed to equilibrate for 10 minutes. The outer membrane preparations were thawed quickly and suspended in 5 cm^3 of distilled water. Within a fume cabinet each aqueous suspension was added to the preheated phenol solutions and mixed thoroughly, but carefully with a glass Pasteur pipette to form a 'milky' white emulsion. The cotton wool was replaced and the emulsions incubated at 68°C for 15 minutes. Following incubation the cotton wool plugs were removed from the tubes and the suspensions centrifuged at 3,000 *g*, at 15°C, for 45 minutes. The tubes were careful removed from the centrifuge to avoid disturbing the water and phenol phases. As much of the aqueous (upper) phase as possible was harvested whilst ensuring that the white deposit at the phenol-water interface was not disturbed. The water phase from each tube was placed into one clean universal bottle and stored on ice. To the residual phenol phase a second 5 cm^3 volume of distilled water was added and the incubation and centrifugation steps were repeated, and again the aqueous layer was removed, pooled with the previous layer and stored on ice. Approximately 30 cm^3 of dialysis tubing was boiled in

distilled water for 5 minutes. A double knot was tied in one end of the tubing and the aqueous layer was transferred into the dialysis tubing using a Pasteur pipette. The open end of the tubing was sealed with another double knot and placed into 5 l of distilled water which was agitated by a magnetic stirrer. The water was changed five times and finally dialysed overnight in a cold room.

The following morning the dialysis tubing was removed from the water and the dialysed liquid was transferred to a Petri dish and placed in a freezer at -70°C for one hour. The freeze drier was prepared by cooling the chamber to -60°C and the Petri dish containing the LPS suspension was transferred to the freeze drier immediately so that thawing did not occur. The top of the Petri dish was covered with aluminium foil and a hole approximately 3 mm in diameter was made in the foil. The vacuum was applied and lyophilisation was allowed to occur. Lyophilisation took 30 hours until the LPS could be seen as a dry white fluffy deposit in the Petri dish. The LPS was transferred to a pre-weighed microcentrifuge tube and the tube was weighed again to give the yield of LPS. The LPS was suspended in distilled water to a concentration of 5 mg cm^{-3} and stored at -20°C.

7.2.17 Rapid method for the isolation of LPS.

Bacteria were grown for 72 hours in broth culture and sedimented by centrifugation for 10 minutes at 6000 *g*, washed once in PBS, and resuspended to a transmittance of 30% at 525nm. The bacteria suspension was aliquoted into 1.5 cm^3 volumes in screw cap microcentrifuge tubes and the bacterial cells were harvested by centrifugation at 12,000 *g* for 2 minutes. Cells were disrupted by the addition of 50 μl of Laemmli electrophoresis sample buffer and boiling for 10 minutes, followed by the addition of 10 μl of proteinase K solution 2.5 $\mu g\ \mu l^{-1}$ in Laemmli sample buffer and incubated for 1 hour at 60 °C.

7.2.18 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed using the Biometra minigel system and the Pharmacia Phast Gel system. The gels used for the Phast system were purchased precast and were either 4% to 25% gradient gels or 12.5% homogeneous gels. The gels used in the minigel system were pour according to the discontinuous buffer system of Laemmeli (1970) and are outlined in Appendix A. Proteins were separated under reducing condition using a 12.5% separation gel and a 4% stacking gel.

For the separation of whole cell sonicate for Western blot analysis of the antibody response of the trout to the vaccine the 20 well combs supplied with the mingel system were altered using cellotape to form three wells.

For the comparison of protein composition of the outer membrane, inner membrane, cytosolic protein, ECPs and whole cell sonicate the ten well combs supplied were used unmodified.

Total protein was stained for using Coomassie Brilliant Blue R.250. Coomassie Blue stain was added to the gels which were incubated for two hours or overnight at room temperature. Gels were destained in fixative until bands were clearly visible. Molecular weight markers were included in each gel to enable calculation of the molecular weights of the experimental protein bands. The minigels were run at a constant voltage of 195 mV for 45 minutes.

The Phast gels were run and stained according to the conditions in the relevant program supplied with the system.

LPS was also analysed by SDS-PAGE using the minigel system. In this case 12.5% separating gels and 4% stacking gels were cast but the 10% SDS solution was replaced with an equivalent amount of distilled water. The LPS fractions were run at a constant voltage of 195 mV for 45 minutes.

7.2.19 Determination of anti-*A. salmonicida* antibody titre by dot blot.

A dot blot apparatus (Schleicher and Schuell, Germany) was used through out this investigation. The Immobilon-P blotting membrane and its filter paper support were cut to size. The Immobilon-P was prepared by soaking in 100% methanol for 15 seconds, transferred to distilled water for 2 minutes and then allowed to equilibrate in PBS for at least 5 minutes. The filter paper support was dampened with PBS. The dot bot apparatus was assembled including the filter paper and support according to the manufacturers instructions.

Each well was coated with 1μg of whole cell sonicate in 300 μl of coating buffer (Appendix A). This amount was chosen following optimisation of the assay by performing a checker board titration using rabbit antisera to a heterologous strain of *A. salmonicida;* this antisera was subsequently used as a positive control and blocking buffer or normal trout serum were used as a negative controls.

For the analysis of the anti-ECP response of the post challenge fish the wells were coated with 0.08μg of protein.

After coating, unbound sites on the membrane were blocked using 3% skimmed milk in 0.0.5% PBS Tween 20 and then washed three times with 0.05% PBS Tween 20. Serial two fold dilutions (100μl of sample and 100μl of buffer) of serum, mucus and bile samples were prepared in blocking buffer in a round bottomed microtitre plate and 100 μl of each dilution was transferred to the appropriate well in the dot blot apparatus. These samples were incubated at room temperature for 2 hours, and the membrane was then washed three times with 0.05% PBS Tween 20. The dot blots were removed from the apparatus and blocked with blocking buffer for either an hour at room temperature or overnight at 4°C.

A 1/1000 dilution of the secondary antibody (the rabbit anti-trout IgM described above) in blocking buffer was incubated with the dot blot for 1 hour at 37°C in a rotating chamber of a hybridising oven. The blot was then washed twice for 5 minutes with 0.05% PBS Tween 20 and incubated with a 1/2000 dilution of HRP conjugated swine anti-rabbit IgG

(Dako) in blocking buffer for 1 hour as for the secondary antibody. The blot was washed three more times for 5 minutes in 0.05% PBS Tween 20 and developed using FastDAB (Sigma) prepared according to the manufacturers instructions with the addition of 0.003% nickel chloride.

7.2.20 Western blot analysis of rainbow trout serum and mucus antibody response against *A. Salmonicida* whole cell sonicate.

For Western blot analysis of Phast gels the proteins were transferred to nitrocellulose by a semi-dry blotting method. Nitrocellulose and two pieces of blotting paper were cut to the size of the Phast gel and soaked in transfer buffer (Appendix A) for 5 minutes. The electrophoresis bed was heated to 55°C and the nitrocellulose was placed on top of the gel and covered with the two pieces of blotting paper. A heavy weight was placed on top of the blotting paper and the proteins allowed to transfer for one hour. Following transfer Phast gel blots were blocked using 2% PBS Tween 20 for 20 minutes. They were then cut into sections and incubated with various serum samples in order to optimise the blotting process and identify trout anti-*A. salmonicida* antibodies. Primary incubation was with serum samples diluted 1/10 in 0.01% PBS Tween for 45 minutes. Secondary antibody was diluted 1/1000 in 0.1% PBS Tween 20 and incubated for 45 minutes and the tertiary antibody was diluted 1/2000 in 0.1% PBS Tween 20 and again incubated for 45 minutes, all of the incubations were performed at room temperature on a rotating table.

Protein was transferred from the Minigels to Immobilon-P by wet transfer in a Biorad Transfer tank used according to the manufacturers instructions. The Immobilon-P membrane was soaked in 100% methanol, transferred to distilled water for 2 minutes and allowed to equilibrate in transfer buffer (Appendix A) for at least 5 minutes. Transfer took place over 18 hours with a constant current of 30mA.

Following transfer the blots were removed from the transfer buffer and placed in blocking buffer, 3% skimmed milk in 0.01% PBS Tween 20, for 20 minutes and then washed

in 0.05% PBS Tween 20 for a further 20 minutes. The blots were then either stored at 4°C for 3 hours or used immediately.

The blots were put into the Biorad Multiscreen II apparatus which was then assembled according to the manufacturers instructions. Serum, bile and intestinal mucus samples were diluted 1/10 in blocking buffer. Skin mucus samples were applied to the membrane undiluted. Rabbit anti-serum to a heterologous strain of *A. salmonicida* was used as a positive control and blocking buffer was used as a negative control. Primary incubation took place at room temperature for 2 hours following which the blots were washed and incubated with the secondary and tertiary antisera and developed as described for the dot blots.

As for the dot blot all the samples taken were tested against the whole cell sonicate but only the post challenge sera were tested against the ECPs

7.3 Results.

7.3.1 *In vivo* persistence of the live vaccine.

The persistence of the vaccine in the spleen, kidney and intestinal mucus was investigated over a 10-day period following immunisation and the results of this investigation are presented graphically in Fig. 7.3.1 (A, B and C). The first IP immunised group were given 10^8 CFUs in 0.5cm^3 of TSB. It was apparent there were going to be substantial mortalities in this group when the fish were inspected the following day and by day three post-immunisation 85.7% (18 of 21) of the fish had died. *Aeromonas salmonicida* was reisolated from the spleen, kidney and intestinal mucus of the dead fish and the live fish tested on days two and four post immunisation.

The preparation of the vaccine for a second IP group was started on the day after the first immunisation. This second group received 10^7 CFUs cm^{-3} in 0.5 cm^3 of TSB. No mortalities occurred in this group and the results for these fish are presented in Fig. 7.3.1.

A. salmonicida was not isolated from the kidney or spleen of the second IP immunised group. Bacteria were detected in the intestinal mucus of each fish tested on day two but were not found in any of the other samples tested.

Viable bacteria were only detected in the spleen or kidney of very few of the orally immunised fish and only on days 2 and 4. Bacteria were found to persist in the intestinal mucus of most orally immunised fish for up to six days after immunisation. Bacteria were not detected in any of the samples taken from the orally immunised fish on day eight or day 10 post-immunisation

7.3.2 Determination of the challenge dose lethal for 50% of recipients (LD_{50}).

The calculation of the LD_{50} for the challenge strain is presented in Table 7.3.1. The increase in mortality with increasing doses is presented in Fig.7.3.2. No mortalities occurred in the groups receiving 10^1 or 10^2 CFUs, one mortality occurred in the 10^3 group and seven

in the 10^4 group. The first mortalities occurred on day eight and the last on day 14. The LD_{50} was calculated according to the method of Reed and Meunch (1938) and was found to be $1.21x10^3$ CFU. The vaccinated fish were later challenged with three times this dose.

The numbers of bacteria given to the fish in the vaccine experiments and challenge are presented in Tables 7.3.2 and 7.3.3. The values for both the spectrophotometrically estimated numbers of bacteria and the number detected by viable count were always very similar.

7.3.3 SDS-PAGE of the protein fractions and LPS prepared from *A. salmonicida.*

Fractions of the outer membrane proteins, inner membrane proteins, cytosolic proteins, extra cellular products and sonicated whole cells were separated by SDS-PAGE. Digital images of Coomassie Blue stained gels were captured using a digital camera (Kodak) and analysed using the gel electrophoresis analysis software 1-D™ (Kodak). The molecular weight/relative mobility standard curve was calculated by the software from the lanes containing the protein standards. The software then calculated the relative mobility and the relative molecular weight in kDa. of the protein bands in the experimental lanes.

To compare the components in the whole cell sonicate, cytosolic proteins, outer membrane proteins, inner membrane proteins and extra cellular products were run on a 12.5% gel, and this is presented in Fig. 7.3.3. Lanes one and seven contain the high and low molecular weight markers. Lane two contains the cytosolic proteins, lane five the inner membrane proteins and lane six the outer membrane proteins, all of which were obtained during sub-cellular fractionation. Lane three contains the extracellular proteins produced using the cellophane overlay technique and lane four contains the whole cell sonicate.

Visual inspection of the gel enabled the identification of 19 bands in lane two, 5 bands in lane three, more than 40 in lane four, 13 bands in lane five, and 16 in lane six. The digital camera and software were apparently less sensitive than the human eye as fewer bands were identified by software analysis these results are presented in Table 7.3.4. Both the high and

low molecular weight markers appear to contain more bands than should have been present, this may have been due to degradation of the proteins during storage.

A band corresponding to the A-layer protein (A) is clearly visible in all but the cytosolic fraction and in this sample a very faint band could be seen.

Silver staining of LPS showed that the LPS produced by the rapid extraction method contained two groups of LPS molecules, three high molecular weight bands and a large amount of low molecular weight LPS migrating just behind the dye front. This low molecular weight LPS was much more darkly staining than the faint high molecular weight bands. The LPS was not detected in the sample prepared by the method described in Section 7.2.16.

7.3.4 Investigation of the specific immune response of vaccinated fish to *A. salmonicida.*

The specific immune response was measured quantitatively by dot blot and qualitatively by Western blotting. The results of the dot blot assay were expressed as the $\log_{10}$ of the lowest dilution giving a positive reaction compared with the negative controls.

The anti- WCS dot blot was optimised by checker board titration using rabbit anti- *A. salmonicida* antibodies Fig. 7.3.4. The intensity of the colour was shown to be dependent on both the amount of the antigen used to coat the membrane and the dilution of primary antibodies. Furthermore, the titration showed that the production of a coloured precipitate was dependant on the specific binding of the primary antiserum to the antigen. A coating concentration of 1μg per dot was chosen based on this titration. The ECP dot blot was coated with 0.08μg of the antigen as the concentration of protein in the ECPs was much less than in the whole cell sonicate. The rabbit anti-ECP antibodies could detect this concentration of the antigen.

Dot blotting was unable to detect specific antibodies in the bile or skin mucus of any of the fish. Some samples of intestinal mucus appeared to give positive responses in the dot blot but Western blotting showed that this apparent response was due to nonspecific binding. This problem was made worse because the skimmed milk in the blocking buffer was digested by some, though not all, of the intestinal mucus samples making the prevention of nonspecific binding difficult.

Positive serum responses were detected in all of the samples of the IP immunised fish at week six and the titres are presented in Table 7.3.5 the dot blot is presented in Fig 7.3.5. Anti-WCS antibodies were not identified in any of the samples taken before week six. Positive serum responses in orally immunised fish were only detected in week four. Two fish from the low dose group had antibody titres of 1.30 and 1.90 (log_{10}). The dot blot is presented in Fig. 7.3.6, the positive control and the positive serum samples can be seen in columns one, two and six respectively and the negative control can be seen in column 12. A typical blot with no detectable antibody response is presented in Fig. 7.3.7. A positive serum antibody response to ECP was only detected in one IP immunised fish post challenge.

Comparison of dot blots developed using the FastDAB™ without nickel enhancement (Figs. 7.3.4 and 7.3.5) and with enhancement (Figs. 7.3.6 and 7.3.7) show that the dark grey/black colour developed in the presence of nickel made the reaction appear more intense.

Western blotting was optimised using rabbit anti-*A. salmonicida* antibodies. The blots were prepared by running whole cell sonicate on Phast gels and transferring the proteins to nitrocellulose by semi-dry blotting. Using this technique 24 bands recognised by the antiserum were identified Fig. 7.3.8.

All of the serum, mucus and bile samples were tested by Western blot analysis for antibodies against whole cell sonicate proteins separated by SDS-PAGE using the Biometra Minigel system. A Coomassie blue stained gel is presented in Fig. 7.3.9 the bands were analysed by computer and the molecular weights of the proteins are given in Table 7.3.6. The

post challenge serum samples were tested against WCS and also against ECPs and outer membrane proteins.

The positive controls were seen on all of the Western Blots and the negative controls were clearly negative. Before the challenge, no antibodies to specific protein bands were detected by Western blotting in any of the samples from the orally immunised or negative control groups. The serum samples from the IP immunised fish did detect some protein bands at week six but the intensity of the colour reaction was very faint and much less intense that in the positive control and the A-layer was not recognised.

In the post challenge fish there was a band with a molecular weight of approximately 120 kDa in the WCS recognised by both groups of orally immunised fish and the IP immunised fish Fig. 7.3.10. All of the fish from the immunised groups also had a detectable response to one protein band in the ECP sample probably the 70 kDa protease. The positive control antiserum detected at least nine proteins bands and the presence of low molecular weight LPS Fig 7.3.11. When the experimental sera were testing against the ECPs or outer membrane fraction the A layer protein was apparently not detected, though a band corresponding to the A-layer was recognised by the rabbit antiserum in both of these protein samples. The low molecular weight LPS was also detected in the whole cell sonicate, ECP, and outer membrane fraction by the rabbit antisera but not by the trout antisera.

7.3.5 Challenge of vaccinated fish with the homologous strain of *A. salmonicida*.

Very few mortalities occurred in any of the groups; two fish died in the high dose orally immunised group, five fish died in the negative control group, two fish in the IP immunised group and one fish in the low dose orally immunised group. All of these fish were tested for the presence of *A. salmonicida* in the kidney, intestinal mucus and in the peritoneal cavity. The swabs taken from these sites were plated out onto Coomassie Blue Agar blue and colonies that took up the blue stain and produced brown pigment were detected in all of the mortalities confirming the presence of *A. salmonicida*.

Fig 7.3.1 Part A Persistance of Bacteria in the Spleen.

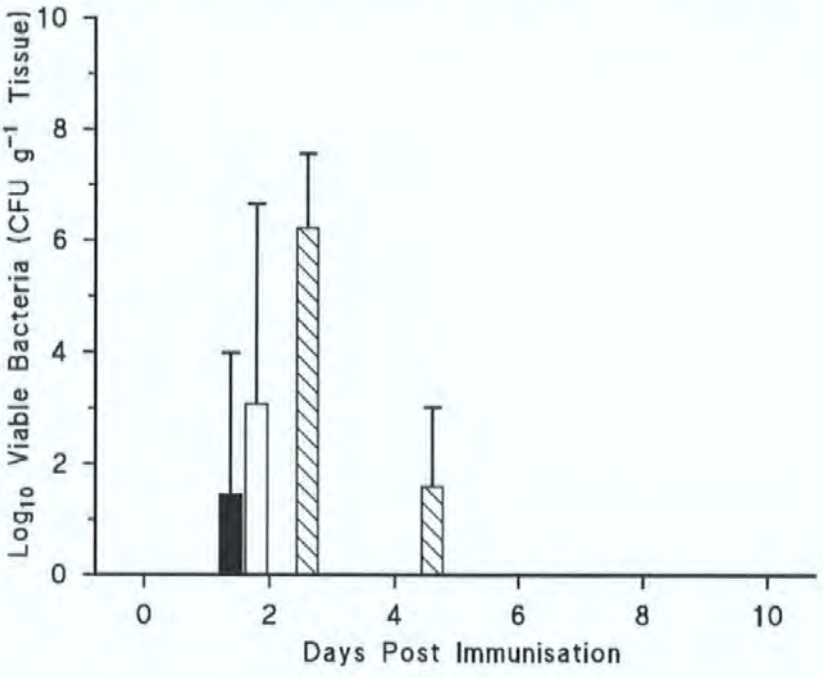


Fig 7.3.1. Part B Persistance of Bacteria in the Kidney.

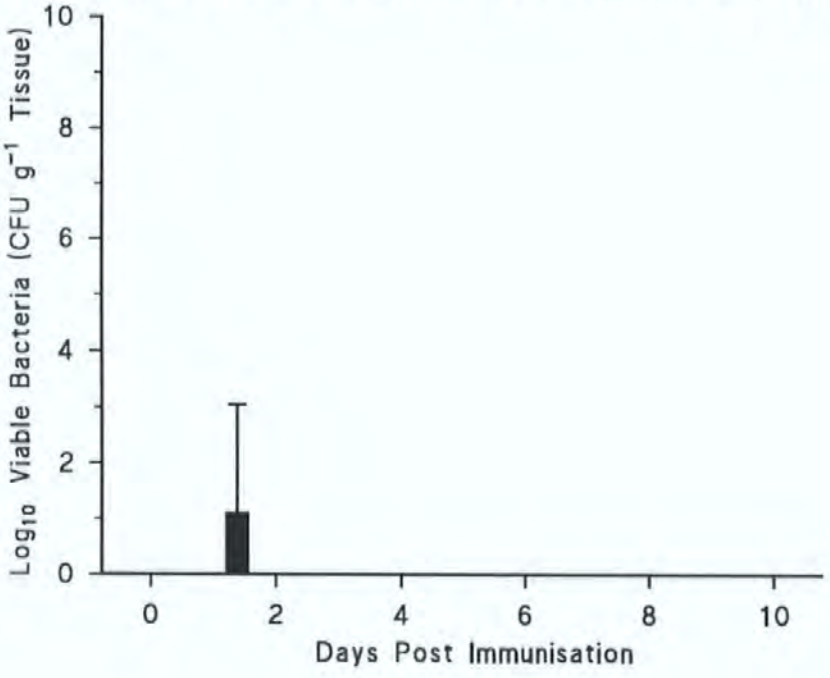


Fig 7.3.1 Part C Persistance of Bacteria in the Intestinal Mucus

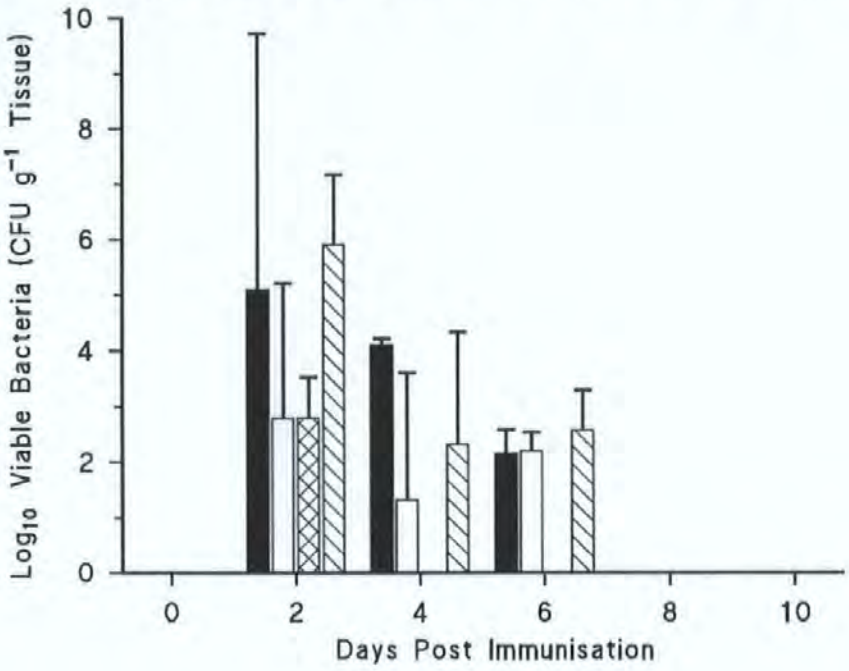


Figure 7.3.1 Persistence of viable bacteria at three sites within vaccinated fish.

The numbers of viable bacteria are expressed as CFU per gram of sample. Data points represent the mean CFU detected (n=3) and the error bars are the SDn-1 for the four experimental groups; (i) 10^{10} CFU orally following administration of sodium bicarbonate (■), (ii) 10^{10} CFU orally without administration of sodium bicarbonate(□), (iii) 10^{7} IP(⊠), (iv) 10^{8} CFU orally following administration of sodium bicarbonate (□).

Table 7.3.1. Estimation of the 50% end point in the LD_{50} challenge experiment.

			Cumulative Values			
Dose of bacteria (CFU/cm^{-3})	No. of Fish Dead	No. of Fish Alive	No. of Fish Dead	No. Fish Alive	Ratio Dead/ Alive	% Dead
10^4	7	5	8	5	7/13	53.85
10^3	1	11	1	16	1/17	5.88
10^2	0	12	0	28	0/28	0
10^1	0	12	0	40	0/40	0

The 50% end point can be calculated using the formula:

$$\frac{A-50}{A-B}\times\log D$$

where A= lowest concentration giving greater than 50% mortality,
B= highest concentration giving less than 50% mortality.
D= dilution factor

In this case:

$$\frac{54-50}{54-6}\times\log 10$$

which equals:

$$\frac{4}{48}\times 1 = 0.083$$

therefore the 50% end point is:

$10^{3.083}$ CFU

or

$1.21\text{x}10^3$ CFU

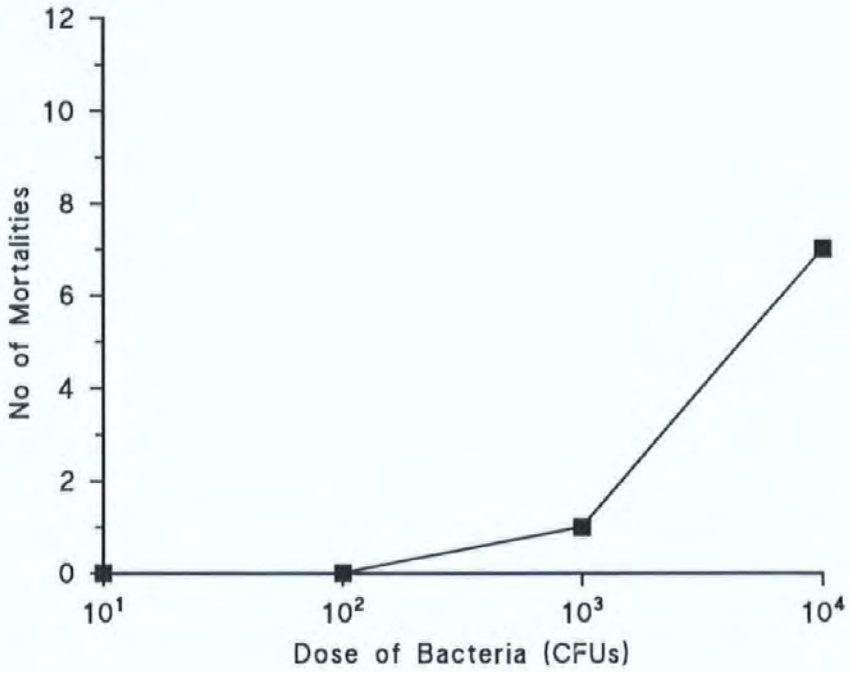


Figure 7.3.2 Increasing mortality with increasing challenge doses.

Table 7.3.2 The dose of bacteria in the vaccine administered to the fish in the persistence investigation. The actual dose given would be half that shown as the numbers of bacteria are for 1 cm^3 of vaccine and only 0.5 cm^3 were given to the fish.

Vaccine Group	Number of Viable Bacteria Administered CFU cm^{-3} (Rapid Estimation A_{590})	Number of Viable Bacteria Administered CFU cm^{-3} (Viable Count)
High Oral Dose prior Sodium Bicarbonate	$2x10^{10}$	$1.85x10^{10}$
High Oral Dose no Sodium Bicarbonate	$2x10^{10}$	$2.01x10^{10}$
IP Group 1	$2x10^{8}$	$2.13x10^{8}$
IP Group 2	$2x10^{7}$	$1.86x10^{7}$
Low Oral Dose prior Sodium Bicarbonate	$2x10^{8}$	$1.98x10^{8}$

Table 7.3.3 The doses of bacteria in the vaccines or in the challenge inoculum administered to the fish. For the vaccinated fish the actual dose given would be half that shown as the numbers of bacteria are for 1 cm^3 of vaccine and only 0.5 cm^3 were given to the fish. The challenge inoculum would also have been half that shown here for the same reason

Vaccine Group	Number of Viable Bacteria Administered CFU cm^{-3} (Rapid Estimation A_{590})	Number of Viable Bacteria Administered CFU cm^{-3} (Viable Count)
High Oral Dose prior Sodium Bicarbonate	$2x10^{10}$	$1.85x10^{10}$
Negative Control	0	0
IP Group	$2x10^{7}$	$1.86x10^{7}$
Low Oral Dose prior Sodium Bicarbonate	$2x10^{8}$	$1.98x10^{8}$
Challenge Dose	$3.63x10^{3}$	$3.89x10^{3}$

Table 7.3.4 The molecular weights of the protein bands identified in Fig 7.3.3.

** denote the position of the A-layer protein.*

Band Number	High Molecular Weight Markers	Cytosolic Protein Fraction	Extra Cellular Proteins	Whole Cell Sonicate	Inner Membrane Fraction	Outer Membrane Fraction	Low Molecular Weight Markers
	MW (KDa)	MW (KDa)	MW (KDa)	MW (KDa)	MW (KDa)	MW (KDa)	MW (KDa)
	Lane Number						
	1	2	3	4	5	6	7
1	116.000	124.731	126.312	123.941	114.838	106.119	66.000
2	97.400	97.981	109.025	122.359	110.769	100.888	45.000
3	66.000	87.358	83.047	120.778	93.062	79.955	36.000
4	45.000	44.690	56.980	116.034	80.973	74.111	29.000
5	29.000	36.740	49.469*	113.662	73.179	56.440	24.000
6		31.051	36.740	110.769	56.440	47.665*	20.100
7			26.243	104.375	48.557*	42.699	14.200
8				98.563	42.699	38.795	
9				90.737	38.795	36.922	
10				83.047	34.451	33.944	
11				76.977		29.994	
12				69.569		28.830	
13				54.338		27.034	
14				48.109*		21.660	
15				45.938		18.330	
16				42.313			
17				38.413			
18				36.378			
19				34.794			
20				31.829			
21				30.143			
22				28.405			
23				26.504			
24				21.140			
25				19.313			
26				16.953			
27				14.397			
28				15.440			

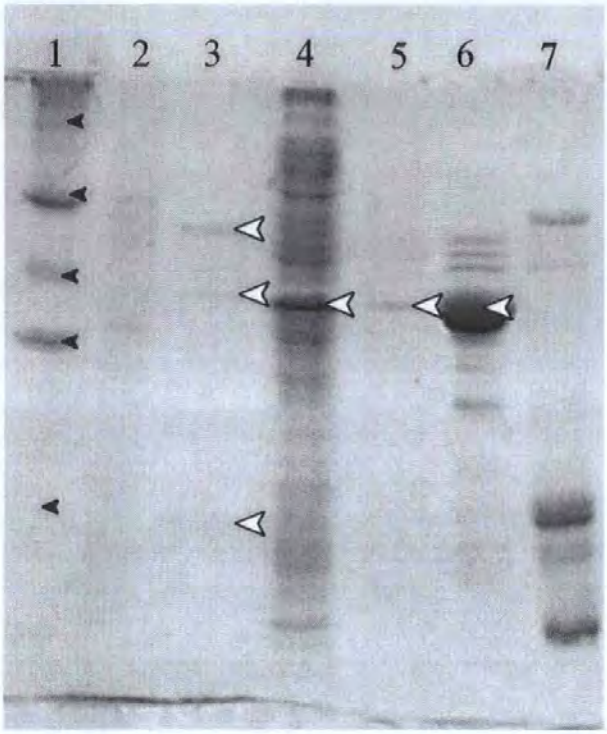


Figure 7.3.3 SDS-PAGE of protein samples from *A. salmonicida aroA*.

Lane one contains the high molecular weight markers, lane two contains the cytosolic proteins, lane three contains the ECPs, lane four the whole cell sonicate, lane five the inner membrane proteins, lane six the outer membrane proteins and lane seven the low molecular weight markers. The large white arrows indicate the position of the A-layer in lanes 3 to 6. In lane 3 they also indicate the position of the 70 kDa protease and the 29 kDa GCAT. The black arrows indicate the position of the molecular weight markers, they were from top to bottom, 116, 97.4, 66, 45, 29 kDa

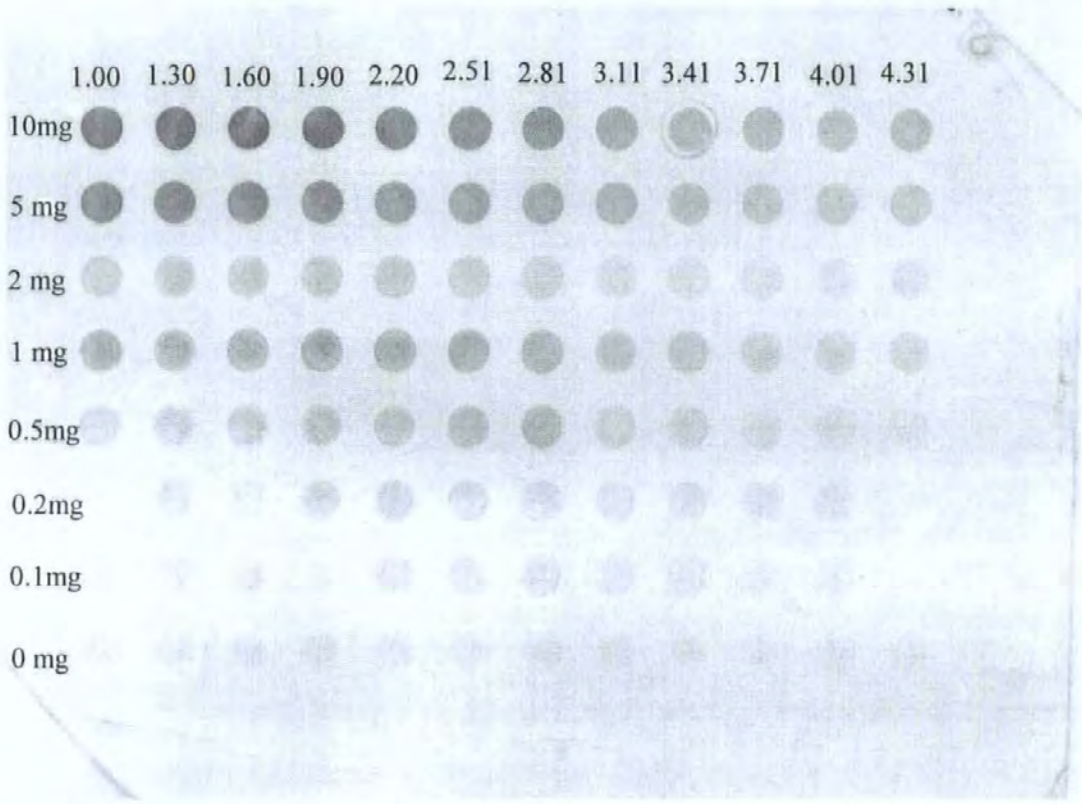


Figure 7.3.4 Optimisation of the dot blot by chequer board titration of antibody and antigen.

Antigen concentrations decreased from the top row to the bottom. The concentrations (mg/well) used to coat the wells in each row are shown to the left of the dot blot. The antibody was serially diluted twofold along the rows, the log_{10} of the dilutions in each column are shown at the top of the dot blot.

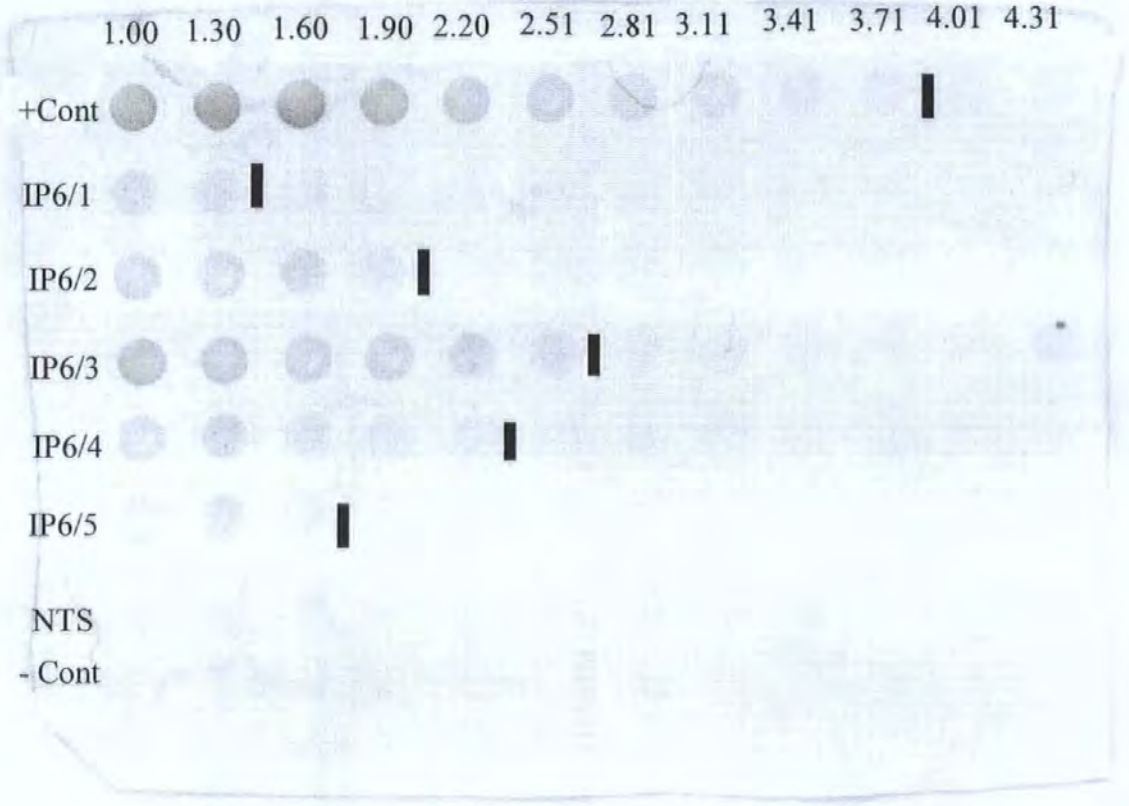


Figure 7.3.5 Dot blot of serum from IP immunised fish six weeks post immunisation. *Antisera was diluted twofold from a starting dilution of 1/10 in column 1. Row one contained rabbit anti-**A. salmonicida** serum as a positive control. Rows two to six contain sera taken from fish six weeks after IP immunisation. Row seven contain normal trout serum and row eight contains blocking buffer with no serum both serving as negative controls.*

Table 7.3.5 Antibody titres (log_{10}) in serum of IP immunised fish six weeks after immunisation.

Sample	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	NTS	neg control	Rabbit
Titre	1.30	1.90	2.51	2.20	1.60	0	0	3.71
Mean			1.92				-	-
SDn-1			0.47			-	-	-

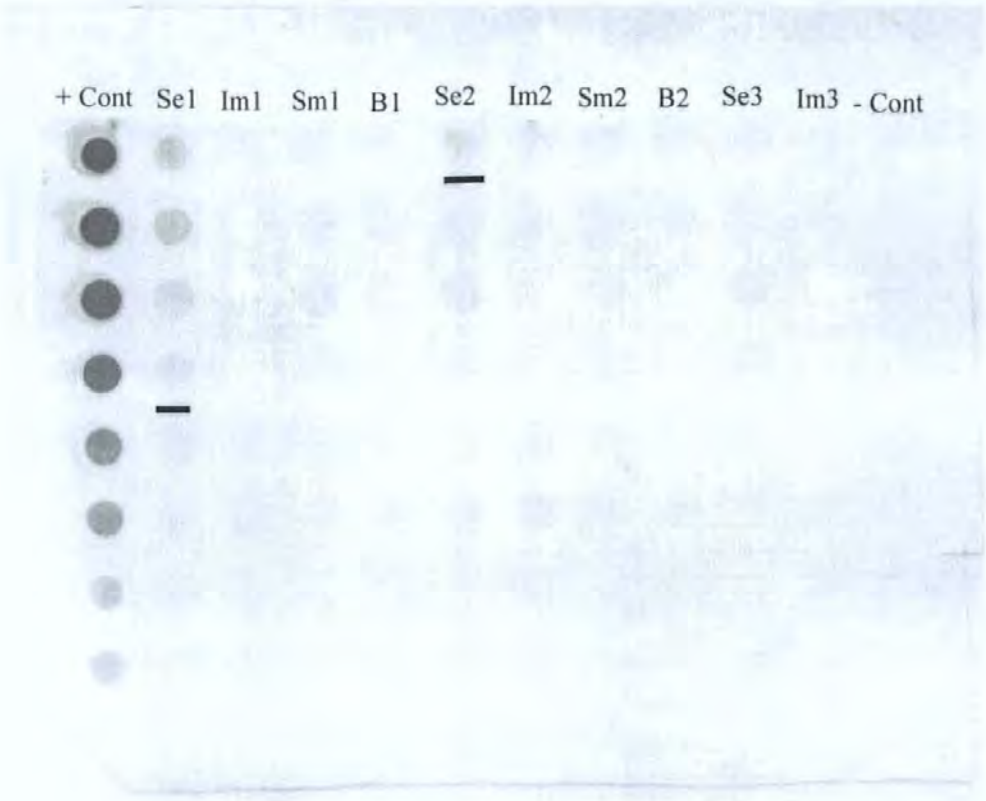


Figure 7.3.6. Positive antibody response in orally immunised fish determined by dot blot.

*Samples of serum, intestinal mucus, skin mucus and bile from fish given the low dose oral vaccine samples were taken four weeks post immunisation. All of the samples were serially diluted twofold from a starting dilution in row one. For serum samples this dilution was 1/10, for intestinal mucus 1/2, skin mucus samples were applied neat and bile samples 1/10. Column one contained rabbit anti-**A. salmonicida** serum as a positive control. Columns two, six and ten contain serum, columns three, seven and eleven contain intestinal mucus, columns four and eight contain skin mucus and columns five and nine contain bile samples. Column twelve contains normal trout serum. The remaining samples for the fish in this group were run on a second dot blot presented in Fig. 7.3.7.*

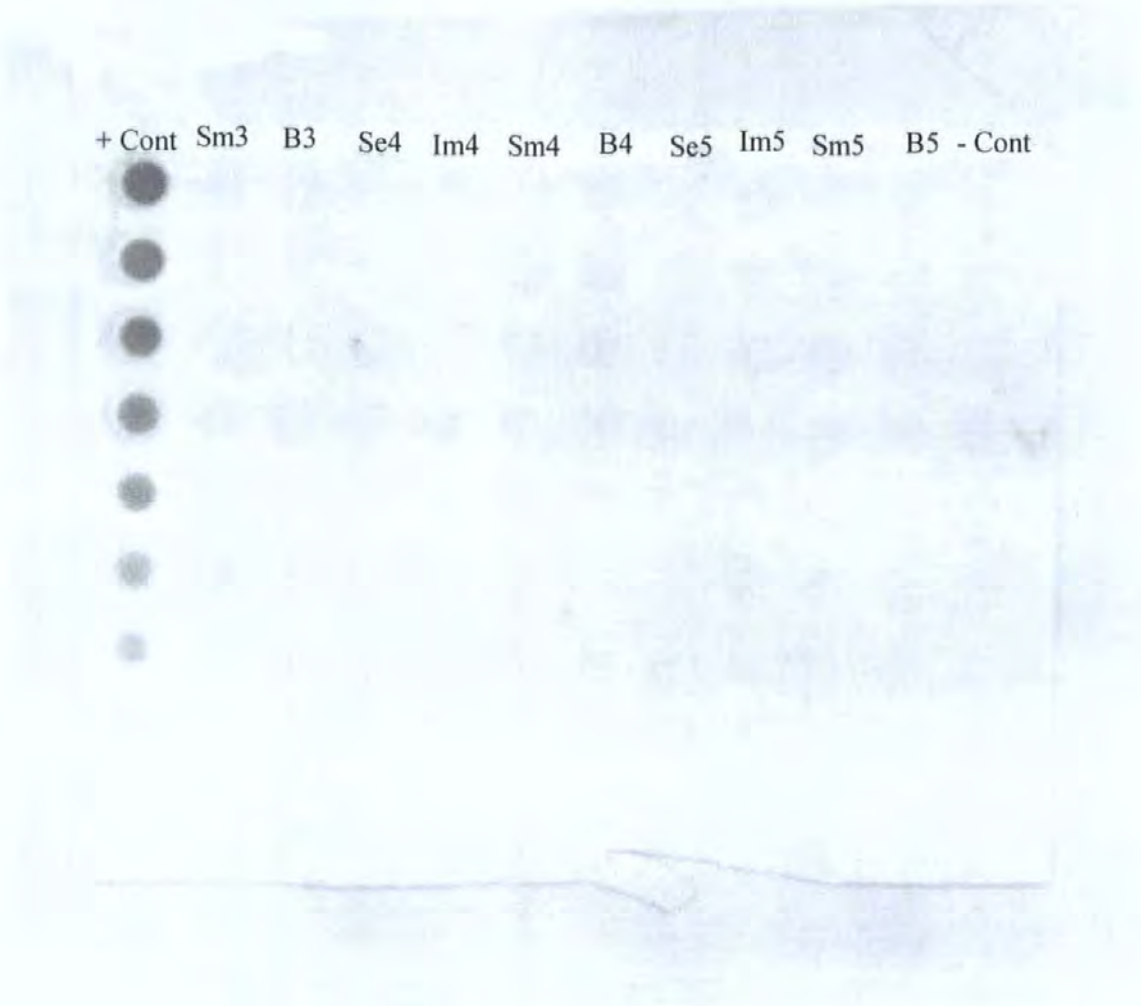


Figure 7.3.7. Negative antibody response in orally immunised fish determined by dot blot

*Samples of serum, intestinal mucus, skin mucus and bile from fish given the low dose oral vaccine samples were taken four weeks post immunisation. All of the samples were serially diluted twofold from a starting dilution in row one. For serum samples this dilution was 1/10, for intestinal mucus 1/2, skin mucus samples were applied neat and bile samples 1/10. Column one contained rabbit anti-**A. salmonicida** serum as a positive control. Columns two, six and 10 contain skin mucus, columns three, seven and 11 contain bile, columns four and eight contain serum and columns five and nine contain intestinal mucus samples. Column twelve contains blocking buffer.*

Figure 7.3.8 Western blot of whole cell sonicate.

The whole cell sonicate was separated using the Pharmacia Phast system on 12.5% gel. A semi dry method was used to transfer the proteins to nitrocellulose. The protein bands were detected using rabbit anti-A. salmonicida primary antiserum and a peroxidase conjugated swine anti-rabbit IgG was used as the secondary.

Table 7.3.6 The molecular weight of the protein bands identified on the gel presented in Fig 7.3.9. ** denotes the A-layer protein.*

Band Number	Whole Cell Sonicate Lane 1	Molecular Weight Markers Lane 2
	MW (kDa)	MW (kDa)
1	298.154	180.000
2	253.846	116.000
3	244.000	84.000
4	160.308	58.000
5	116.000	48.500
6	106.857	36.500
7	105.333	26.600
8	102.286	
9	88.571	
10	85.524	
11	73.167	
12	56.109	
13	53.803	
14	49.769*	
15	47.724	
16	46.038	
17	44.411	
18	41.577	
19	40.592	
20	39.630	
21	38.001	
22	36.221	
23	35.152	
24	33.305	
25	31.746	
26	30.078	
27	28.498	
28	27.001	
29	25.737	
30	24.385	
31	22.966	
32	22.288	

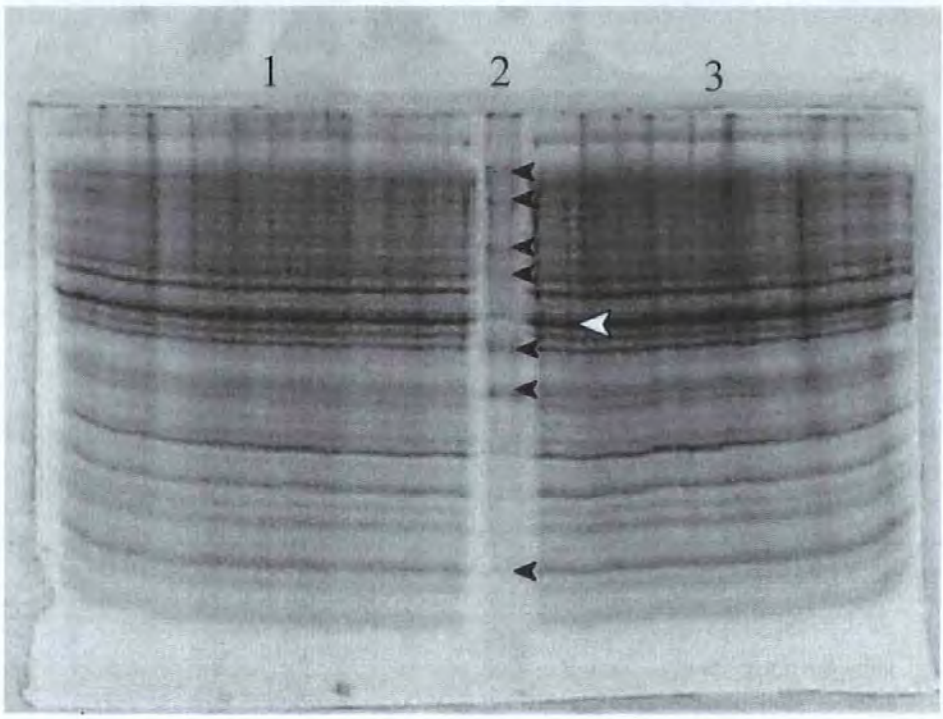


Figure 7.3.9 SDS-PAGE of whole cell sonicate protein samples from *A. salmonicida aroA*.

Lanes one and three contain the whole cell sonicate, lane two contains the Sigma prestained molecular weight markers. The large wells one and three were constructed by wrapping cellotape around a twenty-well comb leaving three wells. This gel shows the protein profile that the samples from immunised and control fish were tested against by Western blotting. The large white arrow indicates the position of the A-layer bands. The small black arrows indicate the position of the molecular weight markers. The weights were, from top to bottom 180, 116, 84, 58, 48.5, 36.5 and 26.6 kDa.

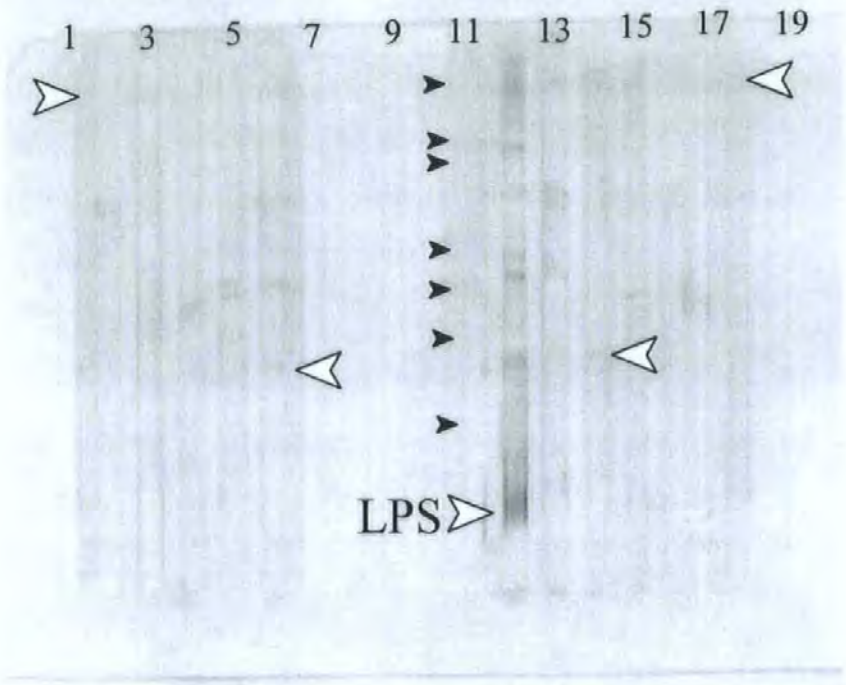


Figure 7.3.10 **A Biorad Multiscreen II Western blot of whole cell sonicate.**

Proteins were separated using the Biometra Minigel System, and transferred to Immobilon-P membrane by a wet transfer method. The Biorad Multiscreen II allows twenty samples to be tested against SDS-PAGE separated proteins. The serum samples of post challenge fish were tested. Wells two to six contain serum from the high oral dose group. Well ten contained the prestained molecular weight markers. Lanes eleven contained the positive control antiserum. Lanes thirteen contained the serum from the IP immunised. Lane 18 contained normal trout serum. Lanes one, nineteen and twenty were blank. The large white arrows indicate the position of bands recognised by the fish serum. The small black arrows indicate the position of the molecular weight markers. The weights were, from top to bottom 180, 116, 84, 58, 48.5, 36.5 and 26.6 kDa.

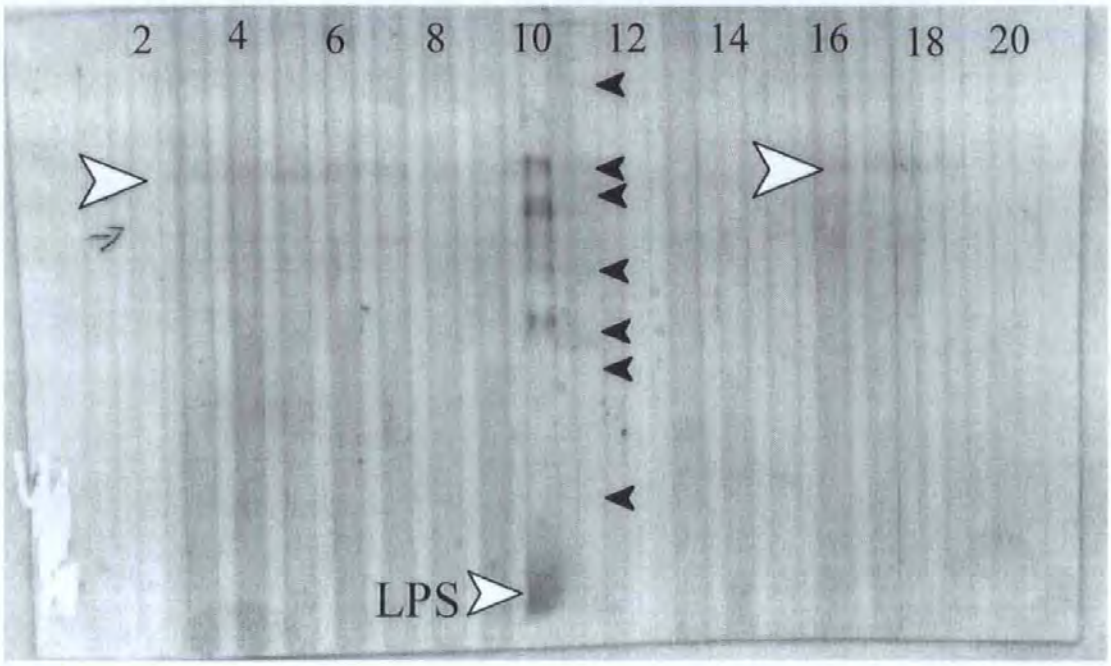


Figure 7.3.11 A Biorad Multiscreen II Western blot of *A. salmonicida aroA* ECP.

Proteins were separated using the Biometra Minigel System, and transferred to Immobilon-P membrane by a wet transfer method. The serum taken from fish from all four experimental groups three weeks after IP challenge was tested for anti-ECP antibodies. Well eleven contained the prestained molecular weight markers. Lane ten contained the positive control antiserum. Lane nineteen contained normal trout serum. Lanes two to five contained serum from the high dose oral immunisation group; six to nine contained serum from the IP immunised group; lanes twelve to fifteen contain serum from the negative control group and lanes sixteen to 19 contained serum from the low dose orally immunised group. Lane one was blank and lane twenty contained blocking buffer. The large white arrows indicate the position of bands recognised by the fish serum. The black arrows indicate the position of the molecular weight markers. The weights were, from top to bottom 180, 116, 84, 58, 48.5, 36.5 and 26.6 kDa.

7.4 Discussion.

This chapter describes the first attempt to use a live mutant strain of *A. salmonicida* as an oral vaccine. The advantages of oral vaccination are obvious and have been reviewed recently (Ellis, 1995). However, most studies conducted using oral immunisation have found that it is less effective at inducing protective immunity than either IP or bath immersion vaccination (Ellis, 1995). The major reasons given for this are the uncertainty of the dose received by individual fish because of different rates of consumption and the destruction of antigens in the stomach and foregut.

Live vaccines have several advantages over killed ones; they are thought to mimic the infection process, may produce protective antigens only expressed *in vivo* and are better at stimulating a cellular immune response than killed whole cell vaccines (Vaughan *et al.*, 1993). In mammals live replicating antigens that multiply in the lumen or ideally in the GALT effectively stimulate local and generalised systemic and secretory immunity (Mestecky, 1987; Mestecky and McGhee, 1989).

The use of live mutants of *A. salmonicida* as vaccines has been described by Cipriano and Starliper (1982), Thornton *et al.* (1991, 1994) Vaughan *et al.* (1993) and Marsden *et al.* (1996). The first of these reports used an avirulent mutant produced by subculture. The second used surface disorganised mutants, one a slow growing aminoglycoside-resistant mutant and the second a rapidly growing pseudo-revertant. Both mutants were avirulent and the pseudo-revertant conferred protection against challenge following vaccination by either IP injection or bath immersion. The study by Thornton *et al.* (1994) was described in Chapter One. Briefly, A-layer and O antigen mutants were shown to be attenuated and able to induce protection against heterologous challenge following immersion. However, only the IP injected fish was shown to have a measurable agglutinin titre. Vaughan *et al.* (1993) and Marsden *et al.* (1996) worked with *aroA* mutants and their findings were described in Chapter

One. Briefly, Vaughan *et al.* (1993) showed that IP immunisation of brown trout protected them against an IM challenge. Marsden *et al.* (1996) reported that the antibody response to the vaccine was dose dependent, and that both B and T cells proliferated *in vitro* when stimulated with bacterial antigens but that T-cells proliferated significantly more than B-cells.

During the uptake and localisation experiments described in Chapter Four all fish immunised IP were given bacteria suspended in saline. For the persistence and specific immune response investigations this was changed to suspension in TSB. This was for two reasons; firstly Vaughan *et al.* (1993) had administered the bacteria as an unwashed suspension in BHIB and secondly the oral uptake and localisation experiments described above showed that delivery of the bacteria in TSB increased tissue localisation.

The very high mortality that occurred in the fish given 10^8 CFU in TSB was disturbing. The first IP immunised group received a dose only half a log dilution higher than that given by Vaughan *et al.* (1993). He reported no mortalities. In this study, there were also no mortalities in the group given 10^7 CFU in TSB, only a log dilution less than that given to the first group. Marsden *et al.* (1996) found that there were occasional mortalities in fish given more than $2x10^9$ CFU suspended in PBS, and in Chapter Four mortalities in fish receiving 10^{10} CFU in saline were reported at 72 hours post immunisation.

It would appear that the attenuation of the organism is very sensitive to both the initial inoculum and the presence of nutrients in the vaccine. This is probably because there is a point at which enough bacteria are present to produce a lethal dose of ECPs. The number of bacteria required to cause this will be lower if there are nutrients in the vaccine as the bacteria can probably grow more effectively. Attenuation will probably also be sensitive to temperature, since at some temperatures the ability of the live vaccine to grow and produce ECPs will be greater than the ability of the salmonid immune system to fight the infection.

Vaughan *et al.* (1993) reported that following IP injection of unwashed cultures viable bacteria were found to persist for at least 12 days but not for 14 days. He also found that the

persistence of live bacteria in the host kidney was dependant on the water temperature with survival of bacteria being greater at 10°C than at higher temperatures (personal communication, 1995). Marsden *et al.* (1996) did not report how long his vaccine persisted. However, the fish were kept in water at 16°C suggesting that persistence would be poor.

In the present study the fish were kept at 13°C. No bacteria were detected in the spleen or kidney following the IP administration of 10^7 CFUs in TSB. Bacteria were isolated from the kidney and spleen of some fish immunised orally; they were only present for 4 days post immunisation. In the orally immunised fish, bacteria persisted for six days but were not found on day eight. That few bacteria were recovered from the IP immunised fish (10^7 CFU) was initially surprising. However, 48 hours after IP immunisation with 10^{10} CFU (Chapter Four) the organs that were found to contain bacteria had between 8.33×10^3 and 3.45×10^5 CFU g^{-1} and no bacteria were recovered from one of these fish. Since the initial dose was 1000 times greater than given in the present study, the absence of bacteria from the organs is less troubling. It is also possible that the bacteria persisted in sites that were not tested. The intestinal tissue is one possibility. It could also be that they colonised macrophages as suggested by Garduno *et al.* (1993). In either case the number of persisting bacteria would have been underestimated.

The persistence of the vaccine is widely believed to be important in the production of a protective immune response (Nnalue and Stocker, 1987; Hormaeche *et al.*, 1991; Vaughan *et al.*, 1993;). The experimental conditions both in the experiment reported here and in that of Marsden *et al.* (1996) suggest that persistence and therefore, the immune response, should be poor. This was not found to be the case indicating that persistence may not be as important as believed. Indeed, Cardenas and Clements (1993) and Cardenas *et al.* (1994) showed protection against typhoid seems to be dependent on the persistence of the vaccine in the host, persistence is not required for the development of antibodies to heterologous antigens carried by the bacteria. Indeed they showed that even if the bacteria were killed prior to

administration an immune response was still induced. They suggested that in this case the bacterial cells acted as an adjuvant.

The fractionation of the bacterial cells was successful and Fig.7.3.3 shows that the fractions all had quite different profiles. The reason for fractionating the bacterial cells was to show which bacterial protein or LPS bands the fish recognised. The A-layer protein could be seen as a dominant band in all but the cytosolic fraction and even there it could be seen but only as a very faint band. The concentration of outer membrane protein solution separated on the gel in Fig 7.3.3. and WCS in Fig 7.3.3. and Fig 7.3.8 was ten times greater than for Western blotting. Samples were diluted for Western blotting to prevent the blot being overloaded with antigens.

An antibody response in the mucus or bile was not demonstrated in any of the fish. This was not wholly unexpected, but it had been hoped that a live oral vaccine may have induced the production of antibodies in the mucosal secretions. This has been found to be the case in mammals (Mestecky, 1987; Dougan *et al.*, 1988; Mestecky and McGhee, 1989; Jones, 1991). It is possible that antibodies were present but that they remained undetected because of a low titre. Though the protective role of an antibody response with a titre of less than 1.0 (log_{10}) must be in question though it is possible that the mucosal antibody response develops more slowly than the systemic response.

The gut mucosa of a number of fish species has been shown to be capable of immunologically responding to orally administered antigens. Gut Associated Lymphoid Tissue (GALT) in fish comprises a repertoire of lymphoid cells capable of such functions as antigen binding, antigen presentation, antibody secretion and cell-mediated immunological activity (Lavelle, 1992). In mammals the existence of a common mucosal immune system possessing a number of features that are distinct from the systemic immune system is well documented (McGhee and Kiyono, 1993). In several cases a lack of correlation between serum antibody responses and protection has been demonstrated, the induction of a mucosal

immune response being the most effective means of protection (Bienenstock and Befus, 1980). A vital component of this mucosal defence is the production of secretory IgA (sIgA), an antibody isotype uniquely adapted to its role in the defence of the gut mucosa and lumen.

The existence of a common immune system in fish has been proposed based primarily on the finding of specific antibodies at mucosal sites remote from that of enteric antigen delivery (Kawai *et al.*, 1981; Kawai and Kusuda, 1983; Rombout *et al.*, 1985; 1989a; 1993). Antibodies have been detected in the bile (Lobb and Clem, 1981a; Lamers, 1985; Rombout *et al.*, 1986; Davidson, 1991; Jenkins, 1992), cutaneous mucus (Harrell *et al.*, 1976; Lobb and Clem, 1981b; Rombout *et al.*, 1986, 1989a; Davidson, 1991; Jenkins, 1992) and in the intestinal mucus (Fletcher and Grant, 1969; Bradshaw *et al.*, 1971; Di Conza and Halliday, 1971; Harris, 1972; Fletcher and White, 1973; Rombout *et al.*, 1986).

However, a protective role for such antibody has rarely been demonstrated although Horne and Blaxendale (1983) demonstrated a reduced adherence of *Vibrio anguillarium* to excised sections of rainbow trout gut in vaccinated fish and reduction in bacterial establishment in the skin mucus of ayu has been demonstrated after oral immunisation (Kawai *et al.*, 1981). IgA is protected from degradation by gut enzymes by the secretory piece and 'J' chain but the evidence for such a component in teleosts is at best contradictory. A 'J' like component of sheepshead IgM was described by Lobb and Clem (1981c) and for rainbow trout by Sanchez *et al.*(1989) but could not be detected in chum salmon or flounder (Kobayshi *et al.*, 1982; Glynn and Pulsford, 1990). The difficulty in detecting IgM in the intestinal mucus may in part be due to the lack of protection from digestive enzymes. In the current experiment intestinal mucus samples were treated with PMSF to inhibit the activity of the gut enzymes but in some of the samples taken this procedure did not work. A second problem is the recovery of antibodies from the mucus. In this author's experience the intestinal mucus of rainbow trout is very viscous and despite thorough mixing in diluent thick strands of mucus were usually evident in the sediment following centrifugation. Since the only supernatant

from these samples was tested for antibody, it is possible that antibodies closely associated with the mucin was not detected.

Smith *et al.* (1980) found that oral vaccination against furunculosis did not result in an enhanced antibody response but did enhance cell-mediated immunity. Marsden *et al.* (1996) also demonstrated that when delivered IP aromatic deficient mutants of *A. salmonicida* preferentially stimulate T-cells.

Live bacterial vaccines have been shown to stimulate cell mediated immunity in mammals. Tagliabue *et al.* (1985) showed that adult human volunteers immunised orally with *S.typhi* Ty21a live vaccine acquired the capacity to express specific cellular immunity against *S. typhi.* Tagliabue *et al.* (1986) showed that the cellular immunity conferred by Ty21a immunisation was of the antibody dependent cellular cytotoxicity (ADCC) type. It has been suggested that the development of a cytotoxic T cell response directed against *Salmonella typhi* infected cells would likely to be important in protection against infection (Levine, 1994). Such a cytotoxic T cell response was recently demonstrated for the first time (Sztein *et al.,* 1995). Following oral immunisation of human volunteers with the attenuated *S. typhi* strain CVD 908, the development of cytotoxic T cells capable of killing a human cell line infected with *S. typhi* was demonstrated. This raises the possibility that CTL might play an important role in immunity to *S. typhi* and similar organisms and also that live attenuated bacteria may be good vectors for antigens against which a cytotoxic T cell response is desirable.

It is quite possible that the lack of mucosal antibody response found in the present investigation is a very poor indicator of the effectiveness of the vaccine at inducing mucosal immunity. It is quite possible that the mucosal surfaces are not protected by antibodies and that the occasional detection of mucosal antibodies is largely irrelevant to the protection of the fish. If this is the case then the investigation of intestinal and skin epithelial lymphocytes

responses should be a matter of priority in future investigations. In particular the development of specific cytotoxic T cells should be investigated.

The antibody titre determined by dot blot showed that IP immunised fish could produce a high titre of antibodies by week six. The lack of antibodies in the serum of most orally immunised fish shows that the vaccine doses used do not normally induce a serum antibody response, despite being taken up into the tissues in quite large numbers. The presence of antibodies in the serum of two orally immunised fish complicates the picture even further. All of the orally immunised fish may have responded with a systemic response to the pathogen and produced antibodies albeit at a lower level than the 1/10 dilution used as the starting point in the dot blot and Western blot. Unfortunately, the samples had to be diluted 1/10 but this was the only way to produce enough sample to run the dot blot and the Western blot. The two responders detected may have produced a greater level of antibodies because they had been previously exposed to *A. salmonicida* antigens or alternatively the result may be due to those fish being naturally high responders to *A. salmonicida*. While the reason for the response of these fish will remain unclear that they were in the minority is very clear.

Western blotting was optimised using the Phast gel, the rabbit anti-*A. salmonicida* antiserum could be seen to detect 24 bands Fig 7.3.8. This blot looks very different to the positive control in Fig. 7.3.11. This may be an inherent limitation of the Multiscreen II apparatus. The Multiscreen II was used in this investigation because it allowed up to 40 samples to be tested at once. Unfortunately, the membrane is tightly held between the two plates of the manifold. This appears to stretch and distort the membrane slightly, more troubling is that the antibody samples are added to one end of the slot in the manifold and allowed to run along the slot. Agitating the sample over the surface of the blot is not possible and not all the antibodies present are necessarily brought into contact with the antigen they recognise. Furthermore, each slot is quite narrow and if the protein mixture is a complex distinguishing between bands could be difficult. In this case this was only a problem with the

rabbit positive control. It is possible that Western blot analysis of the samples using a different system, where agitation was possible and the tracks were wider, clearer results would have been possible. However, the use of the Multiscreen was required by the number of samples to be processed.

The Western blotting analysis of anti-WCS antibodies in serum, mucus and bile failed to detect an antibody response in any fish other than the IP group six weeks post immunisation and this confirmed the dot blot data. The IP immunised fish were seen to have antibody responses to several bands all of which were very faint.

This was interesting because the antibody titre in the week six IP fish was shown to be quite high by dot blot. The problems with the Multiscreen apparatus discussed above may have made detection of antibodies difficult. This would be especially true if the antibodies are of low avidity or affinity. It may also be the case that the antiserum was degraded in the time between the dot blot and the Western blot.

The high oral dose and IP immunised fish post challenge recognised a high molecular weight band, the low oral dose group also appeared to recognise this band but less distinctly. This suggests that lower antibody levels were present in the fish from this group compared with the other two groups. The dot blot did not show an antibody response in the post challenge serum samples to the ECPs or WCS. The post challenge fish recognised a band of 70 kDa in the ECPs that probably corresponds to the serine protease. Interestingly none of the samples tested showed a response to the A-layer protein though it was detected by the rabbit antiserum. The A-layer has been suggested as an important antigen and is immunogenic in fish (Udey and Fryer, 1978; Kay *et al.*, 1984; Trust, 1986; Lund *et al.*, 1991; Bjørnsdottir *et al.*, 1992); it is unclear why these fish did not respond to it. The bacteria may produce a capsule *in vivo,* and this may have masked the A-layer and the high molecular weight LPS. Positive control rabbit anti-*A. salmonicida* antiserum recognised the A-layer and the low molecular weight LPS.

Much of the previous work on oral vaccination against furunculosis was described in Chapter One. Little evidence of a strong antibody response following oral immunisation has been produced (Snieszko and Friddle, 1949; Klontz and Anderson, 1970; Udey and Fryer, 1978; Michel, 1979; Smith *et al.*, 1980; Cipriano, 1982). This does not mean that the fish are unprotected by the immunisation. Kawai *et al.* (1981) and Croy and Amend (1977) showed that fish could be protected from infection by *Vibrio* by oral and bath immersion respectively. However, the antibody response induced by these routes was poor. This again suggests the role of cellular immunity in the protection of fish after mucosal delivery of antigens.

The only way to investigate protection is to challenge vaccinated fish with a virulent strain. In this investigation the LD_{50} was established and the vaccinated fish were given three times that dose. However, very few of the challenged fish died. The negative control group comprised fish that had not been sham vaccinated and had been kept in the stock aquarium. These were brought into the experiment when the original control group died when a filter canister developed a leak overnight draining two thirds of the water from the tank. This group had been kept under a different regime and were larger than the fish in the vaccinated groups and this may have contributed to their ability to withstand challenge.

The main reason for the failure of this challenge, was probably the different conditions under which the vaccinated and LD_{50} fish were kept following challenge. The LD_{50} fish were challenged as they were moved from the stock aquarium. They were kept in much smaller tanks with much smaller filter volumes and this probably meant they were more stressed than the vaccinated and negative control fish.

This means that commenting on the protection offered by the vaccine is impossible. Marsden *et al.* (1996) did not challenge the fish and so no information is available from that source furthermore, the challenge method of Vaughan *et al.* (1993) was unsatisfactory. They used an intra muscular challenge that is even less like a natural infection than the IP challenge

used. Delivery IM means that the bacteria bypass not only the defences at the mucosal surfaces but also the peritoneal macrophages.

The work completed here also suffered from the all-to-frequent problem of, too few fish in too few groups. If this work is continued, increasing the number of fish in each group would be necessary as would separating them into two or more tanks per treatment to compensate for 'tank effect'. Studying the serum antibody response only, would be preferable and to live-bleed the same fish at each time point to show the kinetics of the antibody response would also enhance the results. Better still would be the extraction of circulating and epithelial T cells with subsequent testing of their responsiveness to antigens from the bacteria. Comparison of the oral and IP routes as priming and boosting immunisations should also be investigated and a challenge on a much larger scale would be required.

To conclude, this first attempt at oral immunisation of rainbow trout with a live aromatic deficient mutant of *A. salmonicida* showed that serum antibodies do not appear to be induced by oral immunisation. Furthermore, IP immunisation induced a serum antibody response but not against the A-layer protein or LPS. Neither immunisation route induced a detectable mucosal antibody response. The failure of the challenge made it impossible to comment on the protection conferred by either route.

Chapter Eight.

General Discussion.

8.1 General Discussion.

The work presented in this thesis represents the first attempt to orally immunise rainbow trout with an aromatic deficient recombinant mutant of *Aeromonas salmonicida.* Indeed to this authors knowledge this is the first attempt at oral immunisation with any live attenuated strain of *A. salmonicida.* The complexity of interaction between a live vaccine and its host has been illustrated. The experimental chapters of this thesis are presented in chronological order and so provide a guide to how oral delivery of a live vaccine was approached. The combination of *in vivo* and *in vitro* methods used during this investigation may prove useful in the future as preliminary steps in the rational design of live oral vaccines.

This laboratory has been investigating immune response of teleosts following oral delivery of soluble protein antigens for several years. Jenkins (1992) showed that the uptake of HGG by tilapia was increased when it was delivered with the saponin Quil-A in soluble form and when combined to form Immune-Stimulatory COMplexeS (ISCOMS). He also showed that Quil-A was effective in increasing the serum, bile and skin mucus antibody response to HGG. Lavelle (1994) investigated proteolysis of HGG and BSA by the gastrointestinal enzymes of rainbow trout both *in vitro* and *in vivo.* He also investigated the implication of proteolysis of orally delivered antigens on the stimulation of the humoral response. He showed that modification of the enzymic activity of the gut enzymes could increase the uptake of protein antigens and the subsequent immune response.

Initial work in this study examined the effect of low pH on *A. salmonicida, Yersinia ruckeri* and *E. coli.* The gastric barrier, the destruction of antigens in the stomach, has long been quoted as one of the major reasons for the apparent failure of oral vaccination, and the higher uptake and greater antibody titres achieved by anal intubation have been used to support this. It was unclear how the gastric barrier would affect a live vaccine. Some bacteria, such as *Shigella* are resistant to low pH and are very effective at infecting the host

via the oral route. Other bacteria like *Salmonella spp.* and *Vibrio cholerae* are very sensitive to low pH but still cause gastrointestinal infections.

There is evidence from work on *Salmonella* and *E. coli* that both intrinsic factors such as growth phase and external factors such as oxygen availability, temperature and pH can affect gene expression allowing the bacteria to respond in complex ways to their environment. The first part of the investigation looked at the effect of growth phase on the ability of the three species to survive exposure to low pH. The pH of the rainbow trout stomach was reported as rarely falling below pH 3.0 (Lavelle, personal communication). Therefore, the susceptibility of the bacteria to pH 3.0 - 5.0 was investigated.

A. salmonicida was found to be killed by exposure to pH 3.0 and 4.0 although when grown to mid or late stationary phase bacteria were shown to survive. *Y. ruckeri* and *E. coli* were found to survive exposure to low pH rather better than *A. salmonicida* although they were killed by exposure to pH 3.0 during periods at early points in the growth cycle. All of the bacteria could survive exposure to pH 5.0.

The effect of growth conditions on the survival of *A. salmonicida* was then investigated McCarthy. (1983) showed that virulence increased and Parker (1985) had shown that the adhesion of *A. salmonicida* increased when it was cultured in BHIB instead of TSB. The results showed that growth in BHIB increased the sensitivity of the bacteria to low pH. The effect of oxygen availability during the growth of the bacteria was investigated by comparing the survival of bacteria grown in a shaking incubator with that of unshaken cultures. The increase in oxygen availability appeared to increase the sensitivity of the bacteria. The effect of growth in TSB at a lower pH was investigated; *A. salmonicida* grew well at pH 6.0, grew at pH 5.5 after initial exposure to pH 6.0 but could not be grown at pH 5.0. Bacteria from TSB at pH 6.0 and pH 5.5 were between 10 and 100 times more resistant to low pH exposure, but when grown at pH 5.5 were more sensitive to treatment at pH 5.0. This was evidence that *A. salmonicida* can adapt to growth in media of a reduced pH and this

effects the sensitivity of the organism to further pH stress. The environmental change when bacteria are released from one host into the environment might have provided a cue for increased acid resistance and so bacteria were grown to stationary phase then transferred to PBS at 10°C to simulate conditions upon entry to the environment. There was no increase in the ability of the bacteria to withstand low pH.

The implications for oral delivery of the vaccine is, that some form of protection from the low pH in the stomach would probably be required, as modification of culture conditions did not appear to be very effective at protecting the bacteria.

The data also provides insight into the normal pathogenic mechanisms of the bacteria. Since *A. salmonicida* is obviously sensitive to the pH commonly found in the stomach of rainbow trout the oral dose required would be high like that of *Salmonella* and *V. cholerae* rather than the low inoculum required for infection with *Shigella.* Rose (1990) showed that intragastric intubation with 10^{7} bacteria could cause mortalities in Atlantic salmon and also that furuncle material contains 10^{10} bacteria per gram. The evidence presented in chapter three would suggest that from an inoculum of 10^{7} some bacteria would survive and enter the intestine. Thus it is quite possible that bacteria can infect the host via the enteric route. Furthermore, the assay system used here, involved introducing a small volume of bacterial suspension to a large volume of low pH buffer but *in vivo* the amount of stomach mucus is small and the pH of the gastric compartment can rise sharply if the fish have recently eaten (Lavelle, personal communication) suggesting that *in vivo* survival of bacteria would be better than in this assay system. This was investigated and described as part of the work presented in chapter four.

Chapter four dealt with the uptake and tissue localisation of the bacteria following oral and anal intubation and IP injection. The bacteria were found to localise in the tissues after as little as five minutes regardless of the route of administration. When delivered orally or anally in saline bacteria were found to reach peak numbers in the organs after about 1 hour

which was sustained at least 6 hours but after this the numbers of bacteria decreased and in most cases the numbers of bacteria in the tissues were found to be higher in those fish immunised anally. Bacteria were present in the intestinal mucus for 72 hours and following oral delivery in the gastric mucus for 48 hours. Following IP administration the bacteria were found in the organs in high numbers for at least 72 hours.

The observation that bacteria could persist in the gastric mucus for up to 48 hours apparently contradicts the findings of chapter three. However, the difference in survival was probably caused by the differences in the two systems outlined above. It is quite possible that the delivery of bacteria in 0.5 cm^3 of saline helped to neutralise the gastric acidity. It is also possible that the survival of these bacteria was a result of the fish having higher pH levels than expected Lavelle (1994) found that the acidity of the trout stomach could vary considerably.

The protection of orally delivered bacteria by the administration of sodium bicarbonate before immunisation resulted in increased tissue localisation and prolonged persistence with bacteria being detected for up to 72 hours. The number of bacteria detected in the tissues and mucus of fish given bicarbonate was usually higher than those anally intubated, confirming the observations made *in vitro* and described above.

Oral immunisation with 10^{10} bacteria on a commercial scale would be problematic, and the effect of delivery of a lower dose was investigated. Bacterial suspensions at 10^{8} and 10^{10} were given to fish and the uptake and localisation investigated. No bacteria were found in the fish given 10^{8} without bicarbonate but the organs of fish given 10^{8} after bicarbonate contained more bacteria than the 10^{10} without bicarbonate group despite the 100-times difference in the initial inoculum. This indicated that the lower dose may be capable of inducing an effective immune response.

The effect of growth conditions on the uptake of the bacteria was investigated but there was no evidence that altering the culture medium, the pH or oxygen availability improved uptake.

The delivery of the bacteria in suspended glucans or skimmed milk without prior administration of sodium bicarbonate resulted in very similar levels of uptake to those seen for bacteria administered in saline following bicarbonate administration. This shows that the bacteria could be capable of infecting the host via the gastrointestinal route if associated with a viscous or proteinaceous material. This confirms the findings of Rose (1990). Delivery of the vaccine in TSB substantially increased the numbers of bacteria found in the tissues.

It was concluded that oral immunisation using a combination of prior administration of sodium bicarbonate and delivery of the bacteria in TSB would give the best possible chance of stimulating an immune response

Chapter five investigated the interaction of *A. salmonicida* and the intestinal epithelial cells of rainbow trout. Lavelle (1994) had shown that *A. salmonicida* could invade enterocytes *in vitro* but had not demonstrated the phenomenon *in vivo*. The work presented in chapter four showed that live *A. salmonicida* can be found in the kidney, spleen and liver of rainbow trout following anal and oral intubation. This suggested that *A. salmonicida* could invade the rainbow trout via the intestinal epithelium. This confirmed observations of Rose (1990) and Treasurer and Laidler (1994) who showed that cleaner wrasse kept in salmon sea cages could become infected with *A. salmonicida,* suggesting that the fish had become infected after eating the flesh of dead salmon.

Several methods were employed to demonstrate invasion. Bacteria were stained with acridine orange and smears of kidney, spleen and liver observed using UV light microscopy. Bacteria could be found in the kidney of fish one hour after infection orally, anally, and IP. The number of bacteria seen per smear was too few to make the technique useful in quantifying the uptake of the organism so the attempt was eventually abandoned. The invasion of gut tissue *in vitro* and *in vivo* was investigated using the parental strain *A. salmonicida* 644rB. Tissue samples were observed using SEM and TEM. By SEM bacteria could be seen associated with the epithelium but because of the large deposits of mucus the

interaction between the bacteria and enterocytes could not be determined. By TEM *in vitro* infected tissue was seen to contain bacteria. Close association between these bacteria and the enterocytes was found but there was no evidence to support Lavelle's description of a 'pedestal-like' structure at the site of bacterial attachment.

After *in vivo* infection very few bacteria were seen in contact with the epithelium or in the lumen. This contrasts with the findings of Lavelle (1994) following *in vitro* infection of enterocytes as he readily observed the attachment, adhesion and internalisation of bacteria by the enterocytes. This can be explained by the very different experimental systems used. The isolated enterocytes were at a known concentration and Lavelle (1994) was able to add a known MOI which he optimised for visualisation of any interactions between the cells. The number of enterocytes on the surface of the intestine is much higher than used by Lavelle (1994) but was unknown in this study. Therefore, adding a known multiplicity of infection to the intestine was not possible. Furthermore, isolated enterocytes are poor models for *in vivo* infection work. This is because they are stressed following removal from the intestine and also when in suspension the whole surface of the epithelium is available for the bacteria to attach to. *In vivo,* however, the basolateral surfaces are hidden and the bacteria must either invade via the brush border at the apical surface like *Salmonella* or disrupt the integrity of the membrane like *Shigella.*

Disruption of the epithelium of infected tissue was observed. There were many large vacuoles in the epithelial cells. Some cells were seen to have produced large cytoplasmic processes that were granular in appearance and did not seem to contain organelles which could still be seen within the cell proper. Other cells were seen within the lumen of the gut. They did not appear to be enterocytes but were always seen closely associated with the brush border. These cells were usually associated with bacteria. The most striking feature, however, was that they were always dead. Interestingly, the state of preservation of the cell contents was very good but there was no evidence of a plasma membrane. This suggested the

possibility that the bacteria had caused the translocation of phagocytic cells into the lumen of the gut. It would then appear that the cells internalised the bacteria and at least some of the phagocytes were killed. Garduño *et al.* (1993b) suggested that bacteria are internalised by peritoneal macrophages upon injection and that they are able to live within the phagocytes as intracellular pathogens. If *A. salmonicida* can live within macrophages, it is possible that this ability evolved to aid it in infection of the host. By initiating infiltration of macrophages into the lumen of the gut and then infecting them, the bacteria would not have to penetrate the apical surface of the enterocytes. It is possible that internalised bacteria could be disseminated throughout the tissues by the macrophages. The presence of macrophages and neutrophils in the gut mucosa of teleosts has been described by Davidson (1991), Rombout *et al.* (1985), Rombout *et al.* (1989b) and Sharp (1990). This mechanism of invasion would be similar to that of *Shigella spp. In vivo* they are able to induce the migration of neutrophils across the epithelial membrane and once within the neutrophil they proceed to infect the epithelium whereas, *in vitro Shigella* has been frequently observed invading cells. This difference may again be attributable to the difference between tissue culture monolayers and the intestinal epithelium *per se.*

Invasion of cell monolayers was also investigated. MDCK cells were infected with *A. salmonicida* and invasion observed using acridine orange staining and quantified by viable count following killing of extracellular bacteria and lysis of the monolayer to release intra cellular bacteria. It was found that *A. salmonicida* failed to invade MDCK cells. EPC cells were infected with *Y. ruckeri* and an A-layer positive strain, and an A-layer negative strain of *A. salmonicida.* These cells were also observed by acridine orange staining and the *A*-layer positive strain was seen to interact with the monolayer to a much higher degree than the other strains used. By viable count it was found that *Y. ruckeri* was far more invasive than *A. salmonicida* but that *A. salmonicida* was internalised by some cells within 15 minutes. At the same time it was found that both strains of *A. salmonicida* caused substantial damage to the

monolayers but that *Y. rukeri* did not. This raised the possibility that *A. salmonicida* can invade tissue culture cells *in vitro* but cell lysis makes this very difficult to quantify. Quantification by counting acridine orange stained cells was unreliable as counterstaining was not completely effective at quenching the florescence of extracellular bacteria.

Chapter six presented the investigation into the nonspecific immune response to *A. salmonicida.* The levels of serum and mucus lysozyme, serum protein levels, alternative complement activity and the antibacterial activity of serum and skin mucus were investigated. The investigation was carried out over the course of three separate investigations and only the first was designed specifically to investigate the nonspecific immune response. The investigation showed that within the first 10 days after immunisation the levels of serum lysozyme rise and in the IP immunised group this was a significant increase. The levels of alternative complement activity were seen to increase in the immunised fish after 4 weeks. The largest increase was seen in those fish given 10^{10} CFU orally. This indicates that oral immunisation leads to different stimulation of the immune response when compared with IP immunisation. It was not possible to demonstrate lysozyme activity in the skin mucus but there was an antibacterial activity similar to that found by Austin and McIntosh (1988) and Fouz *et al.* (1990). This activity and the antibacterial activity in the serum were unaffected by immunisation and were therefore, unlikely to be due to lysozyme activity or alternative complement activity.

The persistence of the bacteria, the specific immune response and the protection afforded by the vaccine were investigated and the results presented in chapter seven. The vaccine was found to be lethal to fish given 10^8 in TSB by IP injection whereas 10^7 in TSB IP was not. Marsden *et al.* (1996) described mortalities occurring at doses greater than 10^9 CFU in PBS IP. This was confirmed by the results of preliminary investigations during the work presented in chapter four as a dose of 10^{10} CFU in saline IP led to mortalities within 72 hours. This presents a serious problem for commercial production of a vaccine based on the

aroA mutant. If the fish farmer is not to kill large numbers of fish he must ensure that the dose of vaccine is correct and there is apparently little margin for error. This may well be complicated by such factors as temperature and the health status of the fish. The persistence of the vaccine was found to be poor following IP injection and the bacteria were apparently cleared by 8 days post immunisation following orally administered. This was much quicker than the time period described by Vaughan *et al.* (1993) but was almost certainly caused by a higher water temperature that appears to allow the fish to clear the bacteria more quickly.

It did not prove possible to demonstrate a specific immune response in the majority of fish following oral immunisation. However, the IP immunised group produced an antibody response by 6 weeks that was of a similar level to that found by Marsden *et al.*(1996). The Western blot analysis of the specific immune response showed that the fish failed to respond to the high and low molecular weight LPS or the A-layer. However, very few of the samples gave a positive response following Western blotting and this may have been due to the assay system employed, the low avidity or affinity of the antibodies or destruction of antibodies during storage. As bacteria given orally persisted for a longer period, they may express antigens not expressed *in vitro* and therefore, the fish did not recognise the antigens in the dot blot or Western blot. The IP injected bacteria failed to persist and may not have switched to expression of *in vivo* antigens before being killed. The production of a capsule *in vivo* could explain why the antisera failed to recognise the A-layer and high molecular weight LPS.

The LD_{50} for *A. salmonicida* 644rB was $1.12x10^3$ CFU when delivered in TSB. Three times this dose was given to the vaccinated fish, however, very few fish from any group including the unvaccinated controls, died. This makes it impossible to assess any protection afforded by the vaccine following either oral or IP administration.

Oral vaccination resulted in limited changes in certain nonspecific immune parameters, but resulted in no detectable antibody response. It is quite possible that this has no reflection on the efficacy of the vaccine itself. Cellular immunity may well have been

successfully stimulated despite the poor antibody response. It is known that live vaccines are much better stimulators of cellular immunity. The teleost GALT is much less well defined than its mammalian counterpart and the role of antibody in rainbow trout intestinal mucus is uncertain. Stimulation of the cellular response may be very important. If *A. salmonicida* infects macrophages as part of the infection process then a cellular response would be required to kill infected macrophages. If this were the case then IP challenge would not have measured protection.

The role of the specific humoral immune response in the protection of fish must also be questioned, since furunculosis is rapidly fatal and that following primary immunisation salmonids require at least 3 weeks before a specific humoral immune response is detectale. It would seem likely that the bacteria would have overwhelmed the fish and killed it before the production of an immune response. It is recognised that the secondary antibody response of salmonids is different to that of mammals in that it is due exclusively to clonal expansion of antibody secreting cells and there is no evidence of isotype switching or affinity maturation. If clonal expansion of T-cells also occurs as seem likely (Marsden *et al.*, 1996), the presence of large numbers of these cells may be enough to prevent *A. salmonicida* from establishing an infection especially if phagocytosis and transport by phagocytic cells are important steps in infection. It is also possible that an increase in NK cells in response to immunisation may result in greater protection and this would mean that the specific immune response would be much less important.

In summary, the work presented here has demonstrated that *A. salmonicida* is sensitive to pH at the levels normally found in the gut of rainbow trout. However, it was shown that a small number of bacteria seem to survive exposure to pH 3.0 if they are not actively dividing and that the bacteria can be protected from destruction by the presence of protein or sodium bicarbonate. Furthermore, it was shown that *A. salmonicida* can be found in the tissues following oral intubation and if protected from the stomach acid the numbers of internalised

bacteria can be quite high. This has two implications, first, *A. salmonicida* may well infect its host via the gastrointestinal route and second, that this vaccine if protected from the gastric barrier can be found in the tissues at levels similar to that found following IP injection. The delivery of the bacteria in a nutrient source further boosted uptake. There is evidence that the bacteria can invade tissue culture cells *in vitro* but the cytotoxic properties raise questions about the relevance of this *in vivo.* Examination of intestine infected *in vivo* showed evidence of a pathological effect on the epithelium and some evidence that infection leads to migration of phagocytes from the lamina propria into the lumen. It appears that these cells internalise the bacteria but that some phagocytes are then killed. This may be a mechanism used by *A. salmonicida* for invasion of the host. The oral vaccine did not appear to affect the nonspecific or specific immune response. However, this result may have been different if other factors such as the presence of C-reactive protein, α-2 macroglobulin, classical complement activity, the levels of NK cells, cytotoxic T cells and their activation state, had been monitored.

The evidence that *Yersinia ruckeri* is both resistant to low pH and capable of invading EPC cells indicates that it might well prove a suitable candidate for a live oral vaccine. It may not be worth the effort to develop an oral vaccine against enteric redmouth but the live vaccine might provide an effective carrier of heterologous antigens.

Future work should investigate these aspects. It should also investigate the effect of giving multiple initial doses of oral vaccine, and also the effect of administration of the vaccine orally and IP in combination. This would establish the usefulness of the oral vaccine to boost a primary immune response initially induced by IP injection. A challenge should investigate the effect of the vaccine on the mucosal immunity of the fish and therefore, bath immersion or oral challenge would be more appropriate than by IP or IM injection.

Finally, although much has been written about the difficulties in inducing effective immunity in fish to bacterial pathogens and *Aeromonas salmonicida* in particular, it should be remembered that despite 200 years of experience since Jenner first used the smallpox

vaccine and the many hundreds of recognised human pathogens, immunoprotection in humans has only been achieved to 18 different pathogens. Furthermore most of the effective vaccines are against viral and not bacterial pathogens. In this context it is remarkable that any effective vaccines have been developed for fish.

Appendix A.

Reagents.

Appendix A.

Part A Reagents for Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis.

1) Separating gel buffer.

3.0 *M* Tris-HCl, pH 8.9	36.3 g
Made up to 100cm^3 with distilled water	

2) Stacking gel buffer.

0,5 *M* Tris-HCl, pH 6.7	6.05 g
Made up to 100cm^3 with distilled water	

3) SDS solution.

10% SDS	10 g
Distilled water	90 cm^3

4) Ammonium per sulphate solution.

10% Ammonium persulphate	0.5 g
freshly prepared in Distilled water	5 cm^3

5) Sample buffer.

1% SDS	1 g
10% Glycerol	10 cm^3
0.1% Mercaptoethanol	0.1 cm^3
0.002% Bromophenol Blue	0.002 g
Dissolved in 1/10 strength spacer gel buffer	90 cm^3

6) Electrode buffer.

Tris	6 g
Glycine	28.8 g
SDS	2 g
Made up to with Distilled water 2000cm^3, pH 8.3	

7) Fixative solution.

10% Methanol	200 cm^3
10% Acetic Acid	200 cm^3
80% Distilled Water	1600 cm^3

8) Acrylamide/Bisacrylamide solution.

29% Acrylamide	145 g
1% Bisacrylamide	5 g
Distilled Water	350 cm^3

9) Coomassie blue staining solution.

0.2% Coomassie Blue R250	2 g
50% Methanol	500 cm^3
10% Acetic Acid	100 cm^3
40% Distilled Water	400 cm^3

Gels were made according to the proportions stated in Table A.A.1

Part B Reagents for Western Blotting.

1) Transfer buffer.

Tris	6.1 g
Glycine	28.8 g
Methanol	400 cm^3
Distilled Water	1600 cm^3

2) Ponseau S staining of nitrocellulose.

A) PONSEAU S CONCENTRATE

Ponseau S	2.0 g
Trichloroacetic acid	30 cm^3
Sulfosalicyclic acid	30 cm^3
Distilled water	40 cm^3

B) PONSEAU S WORKING SOLUTION

Ponseau S concentrate	2 cm^3
Distilled water	180 cm^3

C) DETAINING SOLUTION

5% Acetic Acid	5 cm^3
Distilled water	95 cm^3

TABLE A.A.1 The formulation of polyacrylamide gels for SDS-PAGE.

STOCK SOLUTION	SEPARATION GEL										STACKING GEL	
	5%	7.5%	10%	12%	12.5%	13%	14%	15%	17%	20%	3%	4.5%
acryl/bis	3.3	5.0	6.7	8.0	8.35	8.7	9.35	10.0	11.3	13.3	1.0	1.5
sep buffer	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	-	-
stack buffer	-	-	-	-	-	-	-	-	-	-	2.5	2.5
water	13.9	12.2	10.5	9.2	8.85	8.5	7.85	7.2	5.9	3.9	6.3	5.8
SDS*	0.2	0.2	0.20	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02
NH_4perS0_4	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10

3) Washing solution: Phosphate buffered saline Tween 20.

Disodium hydrogen orthophosphate	1.15 g
Potassium dihydrogen orthophosphate	0.2 g
Potassium chloride	0.2 g
Sodium chloride	0.2 g
0.05% Tween 20	0.5 cm^3
Distilled Water to	1000 cm^3

4) Blocking buffer.

3% Skimmed milk	3 g
Washing solution to	100 cm^3

5) Developing solution.

FastDAB™	
Distilled water	15 cm^3
0.003% Nickel chloride	0.45 mg

Part C Reagents for Dot Blotting.

1) Sample buffer.

1% SDS	1 g
0.1% Mercaptoethanol	0.1 cm^3
0.002% Bromophenol Blue	0.002 g
Dissolved in 1/10 strength	
SDS-PAGE spacer gel buffer	90 cm^3

2) Washing solution: Phosphate buffered saline Tween 20.

Disodium hydrogen orthophosphate	1.15 g
Potassium dihydrogen orthophosphate	0.2 g
Potassium chloride	0.2 g
Sodium chloride	0.2 g
0.05% Tween 20	0.5 cm^3
Distilled Water to	1000 cm^3

For wetting membrane and filter paper the Tween 20 was omitted.

3) Blocking buffer.

3% Skimmed milk	3 g
Washing solution to	100 cm^3

4) Developing solution.

FastDAB™	
Distilled water	15 cm^3
0.003% Nickel chloride	0.45 mg

Part D Reagents for Silver Staining of LPS.

1) Fixing solution.

Distilled water	550 cm^3
Ethanol	400 cm^3
Acetic Acid	5 cm^3

2) Oxidising solution.

Periodic Acid	0.7%
Made fresh in Fixing solution.	

3) Staining reagents (mixed in order).

0.1N Sodium Hydroxide	28 cm^3
Ammonium Concentrated	2 cm^3
20% (w/v) Silver nitrate in distilled water	5 cm^3
Distilled water	115 cm^3

4) Developing solution.

Citric acid	50 mg
37%-38% formaldehyde	0.5 cm^3
Distilled water to	200 cm^3

Part E **Molecular weight markers.**

A) LOW MOLECULAR WEIGHT MARKERS

Bovine Serum Albumin	66,000 Da
Egg Albumin	45,000 Da
Glyceraldehyde-3-phosphate dehydrogenase	36,000 Da
Carbonic anhydrase	29,000 Da
Trypsinogen	24,000 Da
Trypsin inhibitor	20,100 Da
Lactalbumin	14,200 Da

B) HIGH MOLECULAR WEIGHT MARKERS

Myosin	205,000 Da
β-Galactosidase	116,000 Da
Phosphorylase	97,000 Da
Bovine Serum Albumin	66,000 Da
Egg Albumin	45,000 Da
Carbonic anhydrase	29,000 Da

C) PRESTAINED MOLECULAR WEIGHT MARKERS

α-2 Macroglobulin	180,000 Da
β-Galactosidase	116,000 Da
Fructose-6-phosphate kinase	84,000 Da
Pyruvate kinase	58,000 Da
Fumarase	48,500 Da
Lactic dehydrogenase	36,500 Da
Triosephosphate isomerase	26,600 Da

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