CHARACTERISATION OF THE IRON UPTAKE MECHANISMS OF *AEROMONAS SALMONICIDA*: ROLE IN VIRULENCE AND PROTECTIVE IMMUNITY

A thesis submitted for the degree of Doctor of Philosophy to the University of Stirling

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DECLARATION

I hereby declare that this thesis has been composed by myself, that it has not been accepted, nor submitted, for any other degrees and the work has been done by myself unless otherwise acknowledged. All sources of information have been acknowledged.

Ian Hirst

Experiments described in Chapter 6,7 and 8 concerning immunisation of Atlantic salmon were performed in collaboration with Drs Tony Ellis and Ian Bricknell, who have given permission for the inclusion of the relevant data in this thesis.

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PUBLICATIONS

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ABSTRACT

The ability of the bacterial fish pathogen *Aeromonas salmonicida* to grow under conditions of iron-restriction *in vitro* was examined in an attempt to identify and characterise the iron uptake mechanisms of the pathogen. Both typical and atypical strains of *A.salmonicida* grew and multiplied in the presence of the synthetic high affinity iron chelators ethylene diamine di (*o*-hydroxyphenylacetic acid) (EDDA) and 2,2'-dipyridyl (Dp) and possessed one or more functional iron uptake mechanisms. The first mechanism involved the inducible production of a phenolate siderophore which was detected using chrome azurol S (CAS) agar. This inducible siderophore-mediated mechanism was possessed only by typical strains of *A.salmonicida*. A second mechanism was identified which enabled both typical and atypical strains to utilise a number of mammalian sources of transferrins (Tf). Typical strains were able to utilise Tf via a siderophore-mediated mechanism; atypical strains were able to utilise Tf via a mechanism involving the proteolytic degradation of Tf by an extracellular metalloprotease. A third mechanism was identified which enabled virulent strains of *A.salmonicida* to utilise haem sources of iron via a constitutive, siderophore-independant mechanism. The mechanism involved a common cell-surface associated haem-binding protein, thought to be the 49-kilodalton (kDa) A-layer protein capable of binding the sulphonated diazo dye, congo red.

Growth under conditions of iron-restriction resulted in the increased synthesis of a number of extracellular virulence factors involved in the pathogenesis of furunculosis, including haemolysin and protease. Iron-restricted growth also resulted in the expression of four iron-regulated outer membrane proteins (IROMPs) of molecular weight 82, 77, 72 and 70kDa which were present and immunologically cross-reactive in eighteen strains of *A.salmonicida* grown in the presence of an iron chelator. The IROMPs were shown by immunoblotting techniques to be expressed *in vivo* during infection, since *A.salmonicida* isolated directly without subculture from the furuncle material of an infected Atlantic salmon expressed IROMPs.

Immunoblotting techniques and an enzyme-linked immunosorbent assay (ELISA) developed to detect the presence of anti-IROMP immunoglobulin (Ig) found the IROMPs to be immunogenic in Atlantic salmon, and in addition, convalescent sera of Atlantic salmon surviving an *A.salmonicida* challenge contained antibodies against the IROMPs.

Outer membrane proteins (OMPs) of *A.salmonicida* were then evaluated for their ability to induce protective immunity in Atlantic salmon. Compared with OMP prepared from *A.salmonicida* grown under iron-replete conditions, IROMPs conferred protection against both natural and experimental heterologous *A.salmonicida* bath challenge. In addition, passive immunisation of Atlantic salmon with an antisera containing antibodies to the IROMPs, and a rabbit anti-IROMP antisera or affinity-purified immunoglobulin G (IgG) also conferred protection against heterologous bath challenge. In addition, an iron-restricted bacterin of *A.salmonicida* containing IROMPs was found to be capable of protecting Atlantic salmon against *A.salmonicida* bath challenge. Both the Atlantic salmon antisera raised against the IROMPs, and the rabbit anti-IROMP antisera were bactericidal against virulent strains of *A.salmonicida* in the presence of complement. Results presented in this thesis indicate that the IROMPs of *A.salmonicida* represent important protective antigens in the vaccination of Atlantic salmon against furunculosis.

ABBREVIATIONS

bio-Tf/Lf	biotinylated transferrin/lactoferrin
BSA	bovine serum albumin
°C	degrees Celsius
CAS	chrome azurol S
cfu	colony-forming units
CR	congo red
2,3-DHBA	dihydroxybenzoic acid
DNase	deoxyribonuclease
ddH_2O	deionised-distilled water
Dp	2,2'-dipyridyl
ECP	extracellular products
EDDA	ethylene diamine di (*o*-hydroxyphenylacetic acid)
ELISA	enzyme-linked immunosorbent assay
$^{59}Fe^{3+}$	radio-labelled iron ion
Fe^{2+}	ferrous iron ion
Fe^{3+}	ferric iron ion
Fe-NTA	ferric nitroltriacetate
FIA	freund's incomplete adjuvant
FITC	fluorescein isothiocynate
FCA	freund's complete adjuvant
g	gram
g	acceleration due to gravity
GHCl	guanidine hydrochloride
h	hour
HDTMA	hexadecyltrimethyl ammonium bromide
HRP-Tf/Lf	horseradish cojugated transferrin/lactoferrin
HU	haemolytic units
Ig	immunoglobulin
im	intramuscular
ip	intraperitoneal
IROMPs	iron-regulated outer membrane proteins
%K	percent killing
kDa	kilodaltons
Lf	lactoferrin
LPS	lipopolysaccharide
M	molar
µM	micromolar
mAb	monoclonal antibody
min	minute
ml	millilitre
mg	milligram
MW	molecular weight
N	normal
N_2	nitrogen gas
nm	nanometre
OMP	outer membrane protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pH	negative log $[H^+]$
PMSF	phenylmethylsulphonyl fluoride
Rf	relative front/retardation factor
RNase	ribonuclease
rpm	revolutions per minute
RPS	relative percent survival

SDS	sodium dodecyl sulphate
TBS	tris-buffered saline
Tf	transferrin
Tris	tris(hydroxymethyl)aminomethane
TSA	tryptone soya agar
TSB	tryptone soya broth
TTBS	tween 20/tris-buffered saline
v/v	volume per volume
w/v	weight per volume

CHAPTER 1:- VIRULENCE MECHANISMS OF *AEROMONAS SALMONICIDA*

1.1 Introduction

Aeromonas salmonicida, the causative agent of furunculosis, continues to be a serious economic threat to the successful farming of Atlantic salmon in Scotland. With the recent emergence of multi-antibiotic-resistant strains of the bacterium, the aquaculture industry has become increasingly dependent upon the use of vaccination as a means of control of furunculosis. However, some fifty years of research has failed to obtain a satisfactory vaccine, like those vaccines developed for some other fish diseases, namely, vibriosis and enteric redmouth disease. The lack of success in the development of a furunculosis vaccine is thought to be due to the inability to identify potential protective antigens important in the pathogenesis of furunculosis. Although recent developments have led to a better understanding of the pathogenesis of furunculosis, it appears that many of the cell-associated and extracellular virulence determinants are poorly immunogenic in the fish, or that they do not elicit protective immune responses.

All of the vaccine work described to date has involved the use of *A.salmonicida* grown *in vitro* under conditions which do not necessarily reflect conditions experienced *in vivo* during infection. It is generally assumed that bacteria grown *in vivo* produce additional cell-associated and extracellular antigens which are essential for growth in this environment. Conditions of severe iron-restriction are experienced by bacterial pathogens growing *in vivo* during infection. Iron-restricted conditions within the vertebrate host comprise an

important non-specific defence mechanism, and similar conditions can be reproduced experimentally *in vitro* by the addition of high affinity iron chelators to the growth medium.

Since very little information is available regarding the ability of *A.salmonicida* to grow under conditions of iron-restriction, the aims of the work presented in this thesis were to examine the ability of *A.salmonicida* to grow under conditions of iron-restriction reproduced *in vitro*; to identify and characterise potential mechanisms of iron uptake and to determine the presence of additional cell-associated or extracellular antigens of *A.salmonicida* when cultured under these conditions which may be immunogenic and act as potential protective antigens in the vaccination of Atlantic salmon against furunculosis.

1.2 Classification of *Aeromonas salmonicida*

The genus *Aeromonas*, a member of the *Vibrionaceae* family, consists of two groups. One containing the mesophilic, motile *Aeromonas* species, *Aeromonas hydrophila*, *A.caviae* and *A.sobria*. The second contains the psychrophilic, non-motile aeromonads contained within the species *Aeromonas salmonicida* (Popoff, 1984).

The species *Aeromonas salmonicida* is classifed into three subspecies: *A.salmonicida* subspecies *salmonicida*, *A.salmonicida* subspecies *achromogenes* and *A.salmonicida* subspecies *masoucida* (Popoff, 1984), all of which are Gram-negative, non-motile, fermentative rods which produce cytochrome oxidase and catalase. The subspecies may be differentiated on the basis of biochemical properties and the ability of *A.salmonicida* subspecies *salmonicida* to produce a brown water-soluble pigment on media containing tyrosine or phenylalanine. Many isolates do

not fit into these subspecies and modifications to the current classification scheme have been proposed (McCarthy & Roberts, 1980; Belland & Trust, 1988; Austin & Austin, 1987). These proposals include the addition of a new subspecies, *A.salmonicida nova* and combining isolates from *A.salmonicida mesoucida* and *A.salmonicida achromogenes* into the single subspecies, *achromogenes* (Rockey *et al.*, 1991).

1.3 History of *A.salmonicida*

The history of furunculosis has been extensively reviewed by McCarthy & Roberts (1980) and Austin & Austin (1987). The first report of *A.salmonicida* was by Emmerlich & Weibel (1894) who isolated the pathogen from diseased brown trout (*Salmo trutta*) from a German hatchery. By the early 1900s furunculosis was found to occur in a number of European countries and by 1902 was confirmed in North America. Furunculosis was first reported in England in 1909 and by 1926 had reached Scotland through movement of infected trout from England. In 1929 a Furunculosis committee was founded and the history and significance of furunculosis in Scotland was documented (Mackie *et al.*, 1930;1933;1935).

At present the geographical distribution of *A.salmonicida* is worldwide. Traditionally a pathogen of salmonids in freshwater, over the years the host range has expanded to include both salmonid and non-salmonid fish in both fresh and sea water. The rapid growth and expansion of commercial salmonid farming in Scotland has led to furunculosis being one of the most serious and devastating diseases of salmonids. In recent years, mortalities due to *A.salmonicida* subspecies *salmonicida* epizootics in Atlantic salmon are estimated to cost in the region of £20 million per annum, establishing furunculosis as the most

serious infectious disease of cultivated salmonid fish in Scotland.

1.4 Clinical and pathological features of *A.salmonicida* infection

A.salmonicida subspecies *salmonicida* referred to as the typical strain is the etiological agent of furunculosis, resulting in an acute haemorrhagic septicaemia often associated with extensive muscle liquefaction in salmonid fish. A number of other diseases associated with *A.salmonicida* infection are recognised. *A.salmonicida* subspecies *achromogenes* and *masoucida*, referred to as the atypical strains, are the etiological agents of ulcerative or atypical furunculosis in salmonids (Paterson, 1983), erythrodermatitis in commercially-reared carp (Bootsma, 1977) and ulcer disease in goldfish (Elliot & Shotts, 1980a,b), resulting in ulcerative inflammatory lesions of skin and muscle. Although *A.salmonicida* subspecies *masoucida* has only been isolated from Japanese salmon, subspecies *achromogenes* has been isolated from both salmonid and non-salmonid fish.

1.4.1 Infections caused by typical *A.salmonicida*

The clinical and pathological features of *A.salmonicida* infection has been reviewed by McCarthy & Roberts (1980). Furunculosis is typically an acute haemorrhagic septicaemia characterised by inconsistent appearance of furuncles and absence of an inflammatory response. Based on different clinical symptoms, four categories of infection are described; peracute, acute, subacute and chronic furunculosis. The severity of furunculosis is generally age-related; peracute symptoms occur in fingerlings, acute symptoms in growing fish

with subacute and chronic forms of the disease being recognised in older fish.

Peracute furunculosis normally affects fingerling fish. Fish darken in colour and die within 2-3 days with little external symptoms. Mortalities are exceptionally high with rapid onset. Histopathological examination reveals cardiac necrosis, especially of the epicardium and myocardium, with bacterial colonisation of the heart, spleen, kidney and gills but little in the way of an inflammatory response.

In growing and adult fish, clinical and pathological features of acute furunculosis are typically those of a haemorrhagic septicaemia with lethargy, darkening of colour, lack of appetite, tachybranchia and haemorrhage. Acute forms of the disease may occur with or without the development of "furuncles". These are the characteristic necrotic or liquefactive lesions of the musculature which resemble but are not true furuncles. Internally, there are characteristic signs of haemorrhagic septicaemia with dissemination of bacteria throughout major organs, enlarged spleen, pale liver with subcapsular haemorrhaging and extensive areas of haemorrhage over abdominal wall, viscera and heart. Liquefaction of the kidney and presence of blood-stained peritoneal fluid are also a common feature. Acute furunculosis is a disease of short duration and high mortality; fish often die within 2-3 days with few, if any, external signs.

Subacute and chronic furunculosis is more common in older fish. Generally this form of the disease is characterised by lethargy, slight exophthalmia, bloody discharge from the vent and presence of haemorrhagic areas at the base of the fins, in the abdominal wall and over the pyloric area, heart and liver. Liquefaction of the kidney, enlarged spleen and inflammation of the lower intestine are common

features of subacute/chronic infection. "Furuncles", a misnomer for the swellings resulting from muscle liquefaction and giving the disease its name are not a consistent or common feature (McCarthy & Roberts, 1980).

1.4.2 Infections caused by atypical *A.salmonicida*

Infections due to atypical strains of *A.salmonicida* among salmonid and non-salmonid fish results in diseases of differing clinical and pathological features and reflect diseases of markedly different pathogenesis. There are few reports of the detailed pathology of atypical *A.salmonicida* infection. Generally, infections such as ulcerative furunculosis, carp erythrodermatitis and goldfish ulcer disease result in a more localised superficial disease characterised by shallow haemorrhagic lesions with little systemic involvement (Paterson, 1983). Mortality due to atypical *A.salmonicida* are normally due to secondary bacterial or fungal infection, and in some instances, septicaemia.

1.5 Pathogenesis of Furunculosis

The pathogenesis of bacterial infection involves a complex series of biochemical interactions between the pathogenic microorganism and the vertebrate host. The outcome of an infection depends on a combination of factors including virulence of the bacterial pathogen, the immune status of the host and the innate resistance of the host.

Important stages in bacterial pathogenicity and virulence are the ability of the invading pathogen to attach to and enter the host,

multiply rapidly *in vivo* by overcoming both specific and non-specific host defence mechanisms and produce disease, causing damage to the host. A number of mechanisms of pathogenicity leading to infection and disease are used by bacterial pathogens and will be reviewed briefly in the following section.

1.5.1 Bacterial Entry and Adherence

The first interaction between the bacterial pathogen and the vertebrate host involves attachment to a eukaryotic cell surface. Some microrganisms multiply at, and remain on, the surface of the host. Others use attachment as the first essential step before proceeding to deeper tissue or the blood. Microbial attachment mechanisms are usually designed to interact with receptors characteristic of a given host. Generally, microbial adherence requires the participation of a receptor and an adhesin. The receptors defined so far are usually specific carbohydrate residues on the eukaryotic cell surface. Bacterial adhesins are typically of protein structure such as fimbriae or non-fimbrial adhesins on the bacterial cell surface which interact with the host cell receptor (Finlay & Falkow, 1989).

Target surfaces for primary attachment of bacterial fish pathogens to the fish host are those in contact with the external environment eg skin, gills and digestive tract. Although the fish host has evolved various protective mechanisms, both specific and non-specific, to prevent microbial entry (Ellis, 1981), pathogenic bacteria have evolved mechanisms to capitalise these sites of environmental contact as points of entry. Organisms which infect these regions have developed specific tissue adherence mechanisms which overcome the constant presence of

non-specific and specific defence factors. These pathogens are highly adapted for their unique niche and this is usually reflected by the molecular structure and function of their specialised adherence factors (Trust, 1986). In addition, the microbial cell envelope is adapted for survival at these sites and provides protection against local host defence systems (Brown & Williams, 1985).

1.5.2 Bacterial Invasion

Invasiveness, the ability of bacteria to enter and proliferate in the host tissues, is another feature of the pathogenesis of bacterial infection. Entry into host cells is a specialised strategy for survival and multiplication utilised by a number of pathogens (Williams *et al.*, 1988). Besides avoiding the extracellular host defence system, intracellular localisation places the organism in an environment potentially rich in nutrients, yet devoid of competing microbes. However, invasive pathogens face additional and quite different requirements than pathogenic microbes which live free in the environment or bound to host surfaces. They must first penetrate the eukaryotic cell surface barrier and gain entry into the host cell. The majority of invasive pathogens exploit existing eukaryotic internalisation pathways enabling the pathogen to gain entry into the host cell. Once inside, it must be able to survive, multiply and escape from the cell. A number of bacteria are invasive including *Salmonella*, *Shigella*, *Escherichia* and *Yersinia* species (Williams *et al.*, 1988).

1.5.3 Bacterial establishment

Having gained access to host tissues and body fluids, invasive bacterial pathogens must be capable of surviving attack from host defence mechanisms in order to proliferate at the site of infection and disseminate. Success of an invading bacterial pathogen is measured by its ability to grow and multiply sufficiently to establish itself within the host or to reach sufficient numbers to enable transmission to another susceptible host. As this occurs, the bacteria may secrete toxins which cause tissue damage. Although some potent bacterial toxins are probably the best-characterised virulence determinants of bacterial pathogens, their actual roles in microbial pathogenicity are often not fully determined (Finley & Falkow, 1989). Toxins are often just one of several virulence factors produced by microbial pathogens and although toxins may represent a principal determinant of bacterial virulence and cause of disease, they may not be the principal determinant of infectivity.

The highly efficient host immune system is made up of many components, each of which is capable of destroying bacteria. Microbial pathogens have evolved a number of ways to escape this system.

Possibly the most effective host defence mechanism against invading bacteria is engulfment and killing by phagocytes; although some pathogens are capable of proliferation within phagocytic cells (Buchanan & Pearce, 1979). Phagocytes are attracted to invading bacteria by various chemotaxins including the early components of the complement cascade. A fundamental requirement of many pathogens is to escape phagocytosis by macrophages and polymorphonuclear phagocytes. The most common means of this is by possession of an antiphagocytic capsule eg

Haemophilus influenzae, *Neisseria meningitidis*, *Escherichia coli*, *Streptococcus pneumoniae* and *Klebsiella pneumoniae* which have a polysaccharide capsule which inhibits their phagocytosis. These capsules are generally weak immunogens and possibly mask more immunogenic underlying bacterial surface structures which would directly activate complement. Another mechanism to avoid specific and non-specific defence mechanism involves the production of enzymes capable of cleaving humoral defence factors like secretory immunoglobulin A (IgA) eg *N.gonorrhoeae*, *N.meningitidis*, *H.influenzae* (Finlay & Falkow, 1989).

In addition, it has long been known that certain bacterial pathogens are killed and often subsequently lysed by exposure to human or animal serum, effects which constitute one of the primary defence mechanisms against bacterial invasion (Taylor, 1983). The ability of a pathogenic bacteria to resist the bactericidal action of complement, known as serum resistance is an important virulence factor seen with bacteria such as *N.gonorrhoeae*, *N.meningitidis*, *E.coli* and *S.typhimurium* (Buchanan & Pearce, 1979). Killing occurs when five terminal proteins of the complement cascade form as a membrane attack complex (MAC) which is deposited on and inserted into the bacterial outer membrane, destroying the structural integrity (Buchanan & Pearce, 1979). The outer membrane then no longer forms an effective protective barrier and the peptidoglycan layer becomes more susceptible to degradative action of serum lysozyme so that killed bacterial pathogens are normally lysed. The classical pathway is usually initiated by the specific interaction of antibodies (generally immunoglobulin M (IgM)) with antigenic determinants such as lipopolysaccharide (LPS) or outer membrane proteins (OMPs). A more

rapid antibody-independent alternative pathway of complement activation, initiated by membrane-associated LPS, is of less significance in the bactericidal effect of serum (Taylor, 1983).

1.5.4 Dissemination within the host

Once the bacterial pathogen has entered the eukaryotic cell, it can often pass through that cell to enter deeper tissue or the blood. Once through host epithelial barriers, bacteria can potentially disseminate throughout the host.

Bacterial pathogens capable of disseminating via the bloodstream usually have developed high affinity iron uptake mechanisms to acquire iron. Although the host contains large amounts of iron, the levels of free ionic iron (Fe^{3+}) available are maintained at very low concentrations due to sequestration by the host iron-binding glycoproteins, transferrin (Tf) in serum and extracellular fluids and lactoferrin (Lf) on mucosal surfaces. These iron-binding glycoproteins have association constants for Fe^{3+} of the magnitude 10^{36}M (Griffiths, 1987a) and are only partially saturated with iron. This results in the amount of free iron *in vivo* (approximately 10^{-18}M) being insufficient for bacterial growth. In addition, much of the vertebrate iron is found intracellularly as haem and haemoglobin and is only accessible through cell lysis. Because of an absolute requirement for iron (Bullen, 1981) a number of bacterial pathogens have developed a variety of high affinity iron uptake mechanisms, such as the production of siderophores and the induction of transferrin-binding or haem-binding proteins to ensure survival *in vivo* (Griffiths, 1987b; Martinez *et al.*, 1990).

1.6 Virulence factors of *A.salmonicida*

In recent years a number of potential virulence factors of *A.salmonicida* have been described which may contribute to the pathogenesis of furunculosis. These virulence factors are classified as being either cell-associated or extracellular. Recent progress has been made toward a better understanding of the nature and significance of these virulence factors.

1.6.1 Cell-associated factors

It has been known for many years that virulent strains of *A.salmonicida* autoagglutinate (McCarthy, 1975) and Udey & Fryer (1978) reported that the possession of an additional protein layer (A-layer) external to the outer membrane and cell wall was reponsible for autoagglutination and was associated with virulence properties. The A-layer was found to be composed of a 49kDa protein subunit (Kay *et al.*, 1981) and hydrophobic in nature (Trust *et al.*, 1983). The role of the A-layer protein array in virulence was confirmed by Ishiguro *et al.*, (1981) since isogenic mutants unable to produce A-layer were found to display a 10^8-fold reduction in virulence (measured by LD_{50}). Attenuated surface-disorganised mutants of *A.salmonicida* which possess an incomplete A-layer have recently been described which are sensitive to the bactericidal action of complement and also membrane antagonists (detergents)(Thornton *et al.*, 1991). These mutants are generally avirulent being unable to survive for greater than 48h *in vivo* although they possess the normal array of both cell-associated and extracellular virulence factors.

Reports of virulent, autoagglutinating strains lacking the A-layer protein (Ward *et al.*, 1985; Johnson *et al.*, 1985; Bernoth, 1990) and the failure to associate presence of A-layer with virulence (Ellis *et al.*, 1988a; Olivier, 1990) however, leave the association of the A-layer protein with virulence of all strains of *A.salmonicida* open to question.

The role of the 49kDa A-layer protein appears to be multifunctional in that it is capable of protecting *A.salmonicida* from the bactericidal activity of both immune and non-immune serum (Munn *et al.*, 1982) since the A-layer protein is capable of depleting complement from serum (Kay & Trust, 1991). In addition, the A-layer protein also protects against the killing activity of the fish's phagocytes (Trust *et al.*, 1983; Graham *et al.*, 1988). Indeed, the A-layer appears to facilitate binding of *A.salmonicida* to macrophages (Trust *et al.*, 1983), and in addition specifically binds immunoglobulin G and M (IgG and IgM) from serum (Phipps & Kay, 1988) presumably either as an Ig-depleting device or as a masking mechanism to avoid phagocytosis. The A-layer has also been demonstrated to have a possible role in iron uptake since it has been demonstrated to bind porphyrins and haem-precursors (Kay *et al.*, 1985), in addition to certain tissue matrix proteins (Kay & Trust, 1991) such as the host basement membrane molecules, fibronectin and laminin (Doig *et al.*, 1992). The A-layer protein of *A.salmonicida* thus represents an important multifunctional virulence determinant (Kay & Trust, 1991).

Avirulent isogenic mutants lacking the A-layer protein (A^- cells) have been used to demonstrate that the A-layer protein of virulent strains is required for efficient entry into both murine and salmonid macrophages where the bacterium is capable of resisting killing by phagocytes. Attachment of A-layer positive (A^+) cells to baby hamster kidney (BHK) and rainbow trout gonad (RTG-2) cells in tissue

culture (*in vitro*) to a greater extent than observed with the isogenic A- mutants suggests a role in adhesion to host cells (Parker & Munn, 1985).

The importance of the A-layer protein as a virulence factor is suggested by the finding that the gene coding for the 49kDa A-layer protein (*vapA*) is conserved among a diverse selection of *A.salmonicida* isolates (Kay & Trust, 1991; Chu *et al.*, 1992) representative of the three subspecies, as well as strains from different geographic sources, from different species of both marine and freshwater fish, and from diseases with markedly different pathogenesis.

Bacterial lipopolysaccharide (LPS) is a major structural component of the outer membrane of Gram-negative bacteria and is composed of three structural regions: lipid A, which is embedded in the outer membrane; core oligosaccharide; and the O-antigen polysaccharide side chains.

The cellular LPS of *A.salmonicida* is found both as a 9 sugar containing branched core oligosaccharide linked to lipid A as well as a complete O antigen containing a linear tetrasaccharide repeat unit joined to core oligosaccharide (Chart *et al.*, 1984a; Shaw *et al.*, 1986). Some of these O antigens project through to the surface of the A-layer (Chart *et al.*, 1984a) and cells stain uniformly with both monoclonal and polyclonal antisera against LPS.

The association of LPS (endotoxin) of the Gram-negative bacterial cell wall released from lysed bacteria, with various metabolic derangements observed in mammals (endotoxic shock) as not been observed with *A.salmonicida* LPS in fish species (Wedemeyer *et al.*, 1968; Paterson & Fryer, 1974).

As with the 49kDa A-layer protein, cellular LPS of both typical and atypical strains of *A.salmonicida* exhibits remarkable structural

(chemical analysis of sugar composition) and immunochemical homogeneity with respect to O-antigen chain length (Chart *et al.*, 1984a) although the O-antigen appears to traverse the A-layer to different degrees in different strains. Recently, however, the homogeneity of *A.salmonicida* LPS has been questioned with the finding of LPS heterogeneity in the species using monoclonal antibody (mAb) techniques (Rockey *et al.*, 1991).

Growth of *A.salmonicida* in the presence of high affinity iron chelators results in the expression of three novel high molecular weight iron-regulated outer membrane proteins (IROMPs)(Chart & Trust, 1983; Aoki & Holland, 1985). Although Chart & Trust (1983) were able to demonstrate the binding of $^{59}Fe^{3+}$ (as $FeCl_3$) by isolated cell membranes containing IROMPs, the role and function of the IROMPs in the iron uptake mechanisms and virulence of *A.salmonicida* has not been determined.

1.6.2 Extracellular factors

The extracellular products (ECP) of *A.salmonicida* are known to be pathogenic and lethal to salmonid fish (Munro *et al.*, 1980; Ellis *et al.*, 1981). The ECP is known to contain a variety of enzymes, including serine proteases and metalloproteases (Sakai, 1985abc; Sheeran *et al.*, 1984; Fyfe *et al.*, 1987; Price *et al.*, 1989), glycerophospholipid:cholesterol acyltransferase (GCAT)(Buckley *et al.*, 1982; Lee & Ellis, 1990) and a variety of partially characterised toxic activities. These include haemolysins (Fyfe *et al.*, 1987; Titball & Munn, 1981;1983;1985; Titball *et al.*, 1985), leukocytolysin (Fuller *et al.*, 1977), cytotoxin (Cipriano, 1982) and a lethal toxin (Nomura *et*

al., 1988).

Evidence that ECP contributes to the pathogenesis of furunculosis has stimulated an intensive research on the different fractions of ECP of typical strains of *A.salmonicida*. However, little is known regarding the extracellular products of the atypical strains of *A.salmonicida* except that atypical ECP was found to be lethal for carp (Pol *et al.*, 1980) and that atypical strains do not appear to produce detectable haemolysin (Hastings & Ellis, 1985) although they produce a toxic 20kDa metalloprotease which is lethal for Atlantic salmon (Gudmundsdottir *et al.*, 1990).

The 70kDa serine protease of *A.salmonicida* possessing caseinase activity is a major component of the ECP and is an important virulence factor responsible for much of the tissue liquefaction produced during disease or by injection of ECP and is present *in vivo* in furuncle lesions (Ellis, 1991). A second metalloprotease capable of digesting gelatin and collagen (Sheeran & Smith, 1981) is produced in low levels by typical strains of *A.salmonicida* (Rockey *et al.*, 1988; Price *et al.*, 1989). Tajima *et al.*, (1983) and Shieh (1985) considered the serine protease to be the major lethal toxin. However, although purified protease was demonstrated to be lethal to fish, the lethal dose was in fact higher than the ECP alone. In addition, if the serine protease in the ECP is inhibited by the serine protease inhibitor phenylmethylsulphonyl fluoride (PMSF), the lethal dose of the ECP was not markedly affected (Ellis *et al.*, 1988a). These findings prompted search for another lethal toxin present in ECP.

The physiological role of the 70kDa protease is probably to degrade fish proteins into sufficiently small peptides for uptake as a source of nutrient for the bacterium, and the protease has only a

limited degree of specificity toward protein substrates (Price *et al.*, 1990). In characterisation studies, the 70kDa serine protease is able to hydrolyse two *p*-nitroanilides which are regarded as specific substrates for thrombin. Like thrombin, the 70kDa serine protease was found to be able to reduce clotting time of rainbow trout blood (Price *et al.*, 1990). This observation may account for the presence of microthrombi in the vasculature and heart in Atlantic salmon with clinical furunculosis and following injection of ECP (Ellis *et al.*, 1981; 1988a).

A further characteristic of the 70kDa protease, indicating an adaptation to activity within the host, is its resistance to antiproteases present in blood and tissue fluids. The protease is resistant to antithrombin III (Price *et al.*, 1990) and α_1-antiprotease but is inhibited by α_2-macroglobulin (Ellis, 1987; Price *et al.*, 1990). The resistance of the serine protease of *A.salmonicida* to the majority of protease inhibitors present in fish suggests a degree of pathogenic adaptation (Ellis, 1991).

Although the 70kDa serine protease alone is not the lethal toxin of *A.salmonicida*, it is lethal for fish (Lee & Ellis, 1989) and it has been demonstrated that in combination with a haemolysin, it is responsible for producing muscle liquefaction, associated haemorrhaging and inflammation associated with furunculosis and furuncle formation (Fyfe *et al.*, 1988; Lee, 1990).

A number of membrane-damaging activities of the ECP have been reported, such as GCAT (Buckley *et al.*, 1982), leukocytolysin (Fuller *et al.*, 1977), cytotoxin (Cipriano *et al.*, 1981), trout-specific haemolysin (T-lysin) and non-specific haemolysin (H-lysin) (Titball & Munn, 1981;1983;1985), haemolysin (Fyfe *et al.*, 1987), salmolysin (Nomura *et*

al., 1988), GCAT and GCAT/LPS (Lee & Ellis, 1990). Most of these activities have not been examined for lethality, although many of them produce lesions in fish. However, only salmolysin and GCAT have been identified as potent lethal toxins of *A.salmonicida* (Ellis, 1991).

The production of haemolytic toxins by *A.salmonicida* has also been demonstrated to contribute to the pathogenesis of furunculosis. The ECP is known to contain components which possess haemolytic activity for trout erythrocytes (Munro *et al.*, 1980). Titball & Munn (1981;1983;1985) reported the existence of two distinct haemolytic activities (T- and H-lysin) and identified important interrelationships between proteolytic and haemolytic activities. Haemolysis of trout erythrocytes was incomplete by the action of haemolysin alone, with complete lysis only demonstrated in the combined presence of caseinase activity (Titball *et al.*, 1985; Rockey *et al.*, 1988; Lee,1990).

Recently, the GCAT enzyme described by Buckley *et al.*, (1982) has been characterised and purified (Lee & Ellis, 1990). It was found to be a protein of 25kDa, which in the native form is complexed with LPS with MW >200kDa. Its phospholipase activity was restricted to glycerophospholipids in that in the presence of cholesterol, the 2-acyl group was transferred to form the cholesterol ester (Ellis, 1991). The GCAT toxin was found to be lethal to Atlantic salmon at 45ngg^{-1}, highly haemolytic for salmonid erythrocytes (but not mammalian), leucocytoxic (salmonid) and cytotoxic (RTG-2 cells). In terms of MW and toxicity, GCAT is similar to the salmolysin toxin described by Nomura *et al.*, (1988).

GCAT is haemolytic for fish erythrocytes but not mammalian erythrocytes due to differences in the phospholipid content of erythrocyte membranes. The optimal substrate for GCAT was found to be

phosphatidylcholine substituted with unsaturated fatty acids (Lee & Ellis, 1990) which comprises 58.6% total phospholipid of Atlantic salmon erythrocyte membranes (Lee *et al.*, 1989).

The role of LPS in the toxin complex is to stabilise the toxin from proteolytic attack and to enhance its haemolytic activity (Lee & Ellis, 1990). This is due to LPS which, having an affinity for the eukaryotic cell membranes, can penetrate cells with a high proportion of phospholipids and unsaturated fatty acids to produce a destabilising effect (Lee & Ellis, 1991b). Resistance of GCAT/LPS to proteolytic attack may have possible significance *in vivo* during the host inflammatory response by protecting the toxin from inactivation by host factors such as leucocyte derived proteases.

Although GCAT/LPS is highly haemolytic *in vitro*, there is no evidence for *in vivo* haemolysis in salmonids with clinical furunculosis, or when ECP or purified cytotoxin are injected (Lee & Ellis, 1991c). The histopathological effects of intramuscular (im) and intraperitoneal (ip) injection of purified lethal cytotoxin are generally not extensive and restricted to degranulation of eosinophilic granular cells (EGCs) in the gills (which may release histamine and lead to perturbation of blood flow through the gills), a limited coagulative necrosis of muscle fibres and restricted haemorrhaging at the site of injection. The purified toxin also results in death in less than 48h, and it is difficult to account for its lethality solely upon histopathological features. It is suggested that metabolic dysfunction may possibly lead to death (Lee & Ellis, 1991c).

Further *in vitro* studies by Lee & Ellis (1991a) indicate that complex interactions between the GCAT/LPS toxin and the fish's serum lipoproteins results in enhanced phospholipase and haemolytic activity

and increased electrophoretic mobility of serum lipoproteins which also results in disturbances in the properties of serum albumin and lipoprotein. Since these serum proteins are involved in the transport and metabolism of lipids and cholesterol, these results provide an insight into the possible perturbation of the fish's metabolism which could play a significant role in the pathogenesis of furunculosis and the mechanism of lethal toxicity exercised by the potent bacterial toxin.

Typical strains of *A.salmonicida* are also known to produce siderophores in the ECP when growing under conditions of iron-restriction (Chart & Trust, 1983). Siderophores are soluble, low MW iron chelators capable of removing Fe^{3+} from host iron-binding glycoproteins present in the vertebrate host and are an important component of the iron uptake mechanisms of bacterial pathogens (Griffiths, 1987b). Chart & Trust (1983) described a siderophore-like activity which was capable of removing $^{59}Fe^{3+}$ from a $^{59}Fe^{3+}$-lactoferrin complex across a dialysis membrane with a 14kDa MW cut off, suggesting it was a siderophore ie soluble and low MW. The chemical identity of the siderophore was not established since it gave negative reaction in assays for phenolate-catechol and hydroxamate siderophores.

1.7 Immunogenicity of *A.salmonicida*

The immune status of the vertebrate host is an important factor in the pathogenesis of bacterial infection. However, natural immunity in Atlantic salmon exposed to *A.salmonicida* infection appears to be missing suggesting that the antigens of *A.salmonicida* are poor immunogens in Atlantic salmon. Of the many cell-associated and extracellular antigens

of *A.salmonicida* only a few are immunogenic in Atlantic salmon and to date none have been identified which are capable of eliciting protective immunity in Atlantic salmon.

1.7.1 Cell-associated factors

To date, the nature of protective cell-associated antigens of *A.salmonicida* remains unknown (Hastings & Ellis, 1988). Much attention has focused on the immunogenicity of LPS and the 49kDa A-layer protein; both of which are thought to be antigenically conserved within the species. Although both cell-associated antigens are immunogenic in salmonids (Hastings & Ellis, 1990; Lund *et al.*, 1991), these antigens fail to elicit protective immunity (Hastings, 1988).

1.7.2 Extracellular factors

Of the 25 extracellular antigens of *A.salmonicida* which are immunogenic in rabbit, only a few are immunogenic in rainbow trout and Atlantic salmon (Hastings & Ellis, 1988; 1990; Lund *et al.*, 1991). Of these, high MW LPS and the 70kDa serine protease are the major immunogenic antigens. However, the protective ability of these antigens has been variable and not reproducible (Shieh, 1985; Ellis *et al.*, 1988b). A detoxified preparation of the lethal toxin, salmolysin, has additionally been found to be immunogenic in white-spotted char (*Salvelinus leucomaenis*) (Kawahara & Nomura, 1989). Recently, Kawahara *et al.*, (1991) immunised white-spotted char with detoxifed salmolysin and found fish had developed protective immunity and a serum anti-salmolysin antibody response, suggesting that salmolysin is a

potential protective antigen in the prevention of furunculosis in white-spotted char.

1.8 Innate resistance of the host

The susceptibility of salmonids to furunculosis appears to differ between species and strains. Generally, rainbow trout are more resistant than other salmonids such as brown trout and Atlantic salmon (Ellis & Stapleton, 1988). The apparent differences in susceptibility of salmonids appears to be due to differential ability to neutralise the protease which may lead to a potential for differential growth rates of the bacterium in the differing salmonid hosts. Ellis & Stapleton (1988) found low ECP:serum ratios which reflect *in vivo* conditions experienced during the initial stages of *A.salmonicida* infection resulted in the enhancement of the proteolytic activity of the ECP in brown trout, with a net inhibition in Atlantic salmon and a 50% inhibition in rainbow trout. Although the mechanism of protease enhancement is unkown it is potentially an important determinant of virulence and may explain the differential susceptibility of salmonids to furunculosis.

Another possible factor in the susceptibility of salmonids to furunculosis is the innate genetic disease resistance (Chevassus & Dorson, 1990) of fish. Recently this was examined by Houghton *et al.*, (1991) who measured the genetic resistance of carp (*Cyprinus carpio* L.) to carp erythrodermatitis caused by an atypical strain of *A.salmonicida* (subspecies *nova*). It was found that certain genetic lines (determined by transferrin phenotype) of carp were more resistant than others and disease resistance was found to be correlated to transferrin phenotype.

1.9 Control of furunculosis

A number of methods may be used to control furunculosis in farmed Atlantic salmon in Scotland, including the exclusion of infected live fish or eggs by legislative policy; ensuring that importation and movement of live fish or eggs is accompanied by the appropriate disease certification and enforcement of a slaughter policy if an outbreak occurs within a "disease-free zone". Exclusion methods of disease prevention are far reaching and include provision of safe water supplies and a water source free of wild fish species which may act as reservoirs of infection. Exclusion methods are difficult to achieve especially when the restocking of farms is considered, due to the possible introduction of asymptomatic carrier fish which can produce clinical disease under conditions of stress.

Since exclusion methods are not always able to prevent furunculosis outbreaks, treatment of infected fish with antimicrobials plays an important role in the control of furunculosis in reducing the severity of outbreaks. At present, four antimicrobials are licenced for use in the aquaculture industry in the United Kingdom; these are oxytetracycline, furazolidone, oxolinic acid and a potentiated sulphonamide. This method, however, suffers from the increasing emergence of chromosomal and plasmid-mediated drug resistant strains of *A.salmonicida* (Aoki *et al.*, 1983; Hedges *et al.*, 1985) and poses the problems of a practical means of administration (normally incorporation into food). Oral administration may be successful if given early in the course of an outbreak, although affected fish are usually inappetant and it is difficult to achieve therapeutic levels of the antimicrobial *in*

vivo. Prophylactic treatment is often administered where fish are at risk from infection eg when transferred to a new environment such as sea transfer of Atlantic salmon smolts.

When a disease is endemic, the vaccination of Atlantic salmon as a potential means of controlling furunculosis is an increasingly popular control measure, since the development of mass-administration methods of fish vaccines made vaccination a practical possibility.

Mass-administration vaccination methods include injection, immersion, bath and oral delivery. Intraperitoneal injection enables the use of adjuvants, however, it requires anaesthetisation of the fish, involves handling stress, is labour intensive and only feasible with fish greater than 15g. Immersion vaccines are generally successful for vibriosis and ERM and the method of administration is simple and rapid, requiring only a few seconds exposure of the fish to the vaccine. Although immersion in these vaccines is effective, the mechanism of antigen uptake is not precisely known. The oral delivery of vaccine to fish enables the mass administration of vaccine to fish of all sizes. However, large doses of oral vaccine are required and even then many trials with different vaccines have resulted in low or inconsistent levels of protection (Ellis, 1988).

The successes achieved with chemically-inactivated adjuvanted whole cell bacterin vaccines against vibriosis, enteric redmouth and Hitra disease has not been achieved with vaccines against furunculosis. Since the early attempts of Duff (1942), 50 years of research and development of an effective furunculosis vaccine has had limited success. Of the few furunculosis vaccines marketed commercially, information regarding the performance of the vaccines suggest efficacy is marginal. This is thought to be due to a lack of knowledge concerning the basis of the

pathogenesis of *A.salmonicida* infection and the immunogenicity of putative virulence factors.

CHAPTER 2:- GROWTH OF *AEROMONAS SALMONICIDA* UNDER CONDITIONS OF IRON-RESTRICTION

2.1 INTRODUCTION

Iron is the fourth most abundant element and the second most abundant metal in the Earth's crust. It exists in two forms: iron (III) (Fe^{3+}, ferric iron) and iron (II) (Fe^{2+}, ferrous iron). In the aerobic environment, soluble ferrous iron (Fe^{2+}) tends to oxidise, hydrolyse and polymerise to form essentially insoluble ferric iron (Fe^{3+}) hydroxide and oxyhydroxide polymers. The solubility product constant for ferric hydroxide has been estimated to be $4x10^{-38}$ and any "free" Fe^{3+} in excess of $2.5x10^{-18}M$ is precipitated as insoluble ferric hydroxides (Bullen *et al.*, 1978; Bullen, 1981).

In the vertebrate host, iron is present in the body fluids at a high concentration (>20μM), however, the quantity of free iron is below $10^{-18}M$, a concentration lower than that required for bacterial growth (0.05-0.5μM). These low iron levels are a reflection of the low solubility product ($4x10^{-38}$) of Fe^{3+} at physiological pH and the presence of intracellular iron-binding proteins such as ferritin, haemosiderin or haem, and extracellular proteins such as transferrin (Tf) and lactoferrin (Lf). These extracellular iron-binding proteins have association constants for iron of approximately 10^{36} and they are normally only partially saturated (20-40%). In addition, during infection the host reduces the total amount of iron bound to serum transferrin. This decrease, termed the "hypoferraemia of infection", can be reproduced experimentally by injection of endotoxin (LPS) (Baker

& Wilson, 1965) which acts by stimulating release of leukocytic pyrogen (interleukin-1, IL-1) from polymorphonuclear leukocytes (PMN s) as a result of an interaction between macrophages with the lipid A component of LPS of Gram-negative bacteria. The subsequent processes which lead to the reduction of serum iron levels are not known. The hypoferraemia of infection has recently been demonstrated in rainbow trout (Congleton & Wagner, 1991) and is considered to be an important non-specific, iron-withholding defence mechanism in salmonid fish.

Iron is an important biological catalyst in electron transport processes and oxygen transport and is essential for a wide variety of metabolic processes in both vertebrates and bacteria, involving iron-proteins such as the cytochromes, catalases, peroxidases and nitrogenases. The importance of iron as a catalyst lies with its possession of unfilled *d* atomic orbitals which are able to undergo changes in oxidation states involving one electron (Griffiths, 1987a). The easy access to two oxidation states, iron (II) and iron (III) allows the iron molecule to interact with electron donors and to participate in redox processes). The ease of electron acceptance can vary over a wide range with redox potentials of the iron-containing enzymes, being able to cover a range of upto 1000 mV. However, it is well recognised that reactions involving oxygen favour a univalent reductive pathway which can lead to the formation of "free radicals" ie unstable intermediates with unpaired electrons (Gutteridge, 1989). Superoxide radical ($O_2^{\cdot}$) produced in many biological processes eg electron transport chain and production by PMN's during phagocytosis can lead to the formation of a more damaging radical species by "Fenton chemistry" which reacts with iron, reducing it to the ferrous state which reacts with hydrogen peroxide to form hydroxyl radicals ($\cdot OH$). These are

highly reactive oxidants capable of causing damage to biological molecules such as DNA and cell membranes (Halliwell & Gutteridge, 1985). Thus, free iron in biological systems is normally sequestered by host iron binding proteins to prevent iron participating in such free radical reactions. This sequestration of iron additionally serves to withhold iron from invading bacterial pathogens (Griffiths, 1987a).

It is well recognised that bacterial virulence can be enhanced in experimental infection by administration of iron and that animals injected with various forms of iron are more susceptible to infection. Effects noted include, for example, the reduced survival time of mice given lethal doses of *S.typhimurium* (Jones *et al.*, 1977), and a decrease in the number of *Yersinia pestis* required to cause infection and/or death of mice injected with haemin (Jackson & Burrows, 1956). The lethal effects of ip administration of *E.coli* and haemoglobin is well documented (Bornside *et al.*, 1968) as is the promotion of *Pasteurella haemolytica* infection in mice by iron (Al-Sultan & Aitken, 1984) by haemoglobin (Chengappa *et al.*, 1982) and the enhancement of *V.anguillarum* infection by iron in the Japanese Eel (*Anguilla japonica*) and Ayu (*Plecoglossus altivelis*) (Nakai *et al.*, 1987). The increase in virulence of *Vibrio parahaemolyticus* by lysed erythrocyte factor (Karunasagar *et al.*, 1984) is also recognised.

It is also known that iron-restriction *in vitro* affects the expression of classical virulence determinants such as bacterial toxins. A number of iron-regulated toxins are recognised; for instance, diphtheria toxin of *Corynebacterium diphtheriae* (Tai *et al.*, (1990), exotoxin A, elastase, alkaline protease of *Pseudomonas aeruginosa* (Bjorn *et al.*, 1978), Shiga toxin of *Shigella dysenteriae* (van Heyningen

& Gladstone, 1953), Shiga-like toxin I of *E.coli* (Weinstein *et al.*, 1988), cholera-like toxin of *Plesiomonas shigelloides* (Gardner *et al.*, 1990) and a cytotoxin of *Pasteurella haemolytica* (Gentry *et al.*, 1986). In addition, production of a number of iron-regulated haemolytic toxins by various bacteria have been identified, for instance, *Listeria monocytogenes* (Cowart & Foster, 1981), *E.coli* (Lebek & Gruenig, 1985), *Serratia marcescens* (Poole & Braun, 1988) and *Vibrio cholerae* (Stoebner & Payne, 1988).

The need of the vertebrate host to maintain iron in the solubilised state for use in metabolic processess, yet unavailable for reacting in systems which produce toxic free-radicals means that the availability of "free" iron must be severely restricted in the healthy host. This serves as an important non-specific defence mechanism against invading bacterial pathogens. The mechanism whereby a pathogen acquires iron *in vivo* thus becomes an important component of the virulence or pathogenic properties of the pathogen.

Little is known regarding the ability of typical and atypical strains of *A.salmonicida* to grown under conditions of iron-restriction *in vitro*. Chart & Trust (1983) examined six typical and three atypical strains of *A.salmonicida* and found them to be able to grow in the presence of the high affinity iron chelators ethylene diamine di (*o*-hydroxyphenylacetic acid)(EDDA), transferrin (Tf) and lactoferrin (Lf), although the atypical strains displayed a reduced ability to sequester iron under these conditions. The aim of this study was to reexamine the ability of *A.salmonicida* to grow under conditions of iron-restriction *in vitro* and to examine how *A.salmonicida* adapts to growth in the iron-restricted environment.

2.2 MATERIALS AND METHODS

2.2.1 Determination of iron levels in Atlantic salmon

Serum iron levels of Atlantic salmon (*Salmo salar*) were determined in order to quantify the amounts of uncomplexed ferric iron available to *A.salmonicida* during infection. Serum iron levels were measured using an iron and total iron-binding capacity kit (Sigma) based on the method described by Persijn *et al.*, (1971). Free serum iron levels were measured using a bleomycin assay described by Gutteridge *et al.*,(1981).

2.2.2 Strains of *Aeromonas salmonicida*

Twenty-nine typical and 14 atypical strains of *A.salmonicida* were examined for their ability to grow under conditions of iron-restriction and are described in Table 2.1. Three typical and one atypical were non-autoagglutinating and presumed to lack the 49kDa A-layer protein. All other *A.salmonicida* strains were autoagglutinating.

2.2.3 MICs of iron chelators

The minimum inhibitory concentrations of five high affinity iron chelators used to induce conditions of iron-restriction were determined; using *A.salmonicida* MT004, MT423 and MT194 in a plate bioassay using various concentrations of the chelator. The iron-chelators (obtained from Sigma unless otherwise stated) included desferrioxamine mesylate (Desferal™; Ciba-Geigy), 2,2'-dipyridyl, ethylene diamine di (*o*-hydroxyphenylacetic acid) (EDDA), bovine

transferrin (Tf) and bovine lactoferrin (Lf) (essentially iron-free).

2.2.4 Preparation of apo-transferrin

Transferrin and lactoferrin were prepared in the iron-free apo-form by the method of Aisen *et al.*, (1970). Tf or Lf (10mgml^{-1}) were dialysed against two-changes of 0.1M citrate and 0.1M acetate buffer (pH 4.5) and two-changes of ddH$_2$O.

2.2.5 Preparation of EDDA

Contaminating iron was removed from EDDA by the method of Rogers (1973). EDDA (5g) was dissolved in 95ml 1N HCl in a water bath, cooled, filtered and diluted to 750ml with cold acetone. The pH of the solution was adjusted to pH 6.0 by the addition of 1N NaOH and left at 4°C overnight. The resulting iron-free EDDA precipitate was filtered and washed with acetone.

2.2.6 Iron-restricted media

Iron-restricted conditions were induced in Tryptone Soya Agar and Broth (TSA/B; Oxoid) by the addition of 2,2'-dipyridyl (100μM), EDDA (100μgml^{-1}), Tf (5mgml^{-1}) or Lf (2mgml^{-1}). For routine use, 2,2'-dipyridyl and EDDA were the chelators of choice. All glassware was washed in 6N HCl and rinsed 10 times with ddH$_2$O obtained from a MilliQ water purification system (Millipore) to remove contaminating iron.

2.2.7 Iron content of iron-restricted media

The free ferric iron content of the iron-restricted media was determined by the method described by Holzberg & Artis (1983). Ten-fold concentrates of the iron-restricted media were prepared and to 125µl the following were added with mixing; 500µl 0.5M sodium acetate (pH 4.5) and 25µl of 1.3M ascorbic acid in order to reduce iron to the ferrous form. Contents were mixed and absorbance at 562nm determined. After 5min, 25µl ferrozine was added and the final absorbance at 562nm determined at 20min. The change in absorbance at 562nm was compared to a calibration curve constructed using iron as standard to determine the amount of iron present.

2.2.8 Growth of *A.salmonicida* in iron-restricted media

Before use bacteria were subcultured three times on iron-restricted media in order to reduce ferric iron stores. *A.salmonicida* grown on TSA or TSA-EDDA were inoculated into 5ml iron-replete or iron-restricted media respectively and incubated overnight at 22°C. One ml of the iron-replete or iron-restricted bacteria were then inoculated into 500ml Erlenmeyer flasks containing 100ml iron-replete or iron-restricted media respectively. The cultures were then incubated at 22°C on a Gallenkamp orbital incubator (150 rpm) for 48-72h. Growth was monitored by measurement of absorbance at 540nm on a Pharmacia-LKB Ultraspec II spectrophotometer.

2.2.9 Effects of iron-restriction upon the extracellular products (ECP) of *A.salmonicida*

The effects of iron-restriction upon the extracellular products (ECP) of four typical and two atypical strains of *A.salmonicida* were examined with respect to extracellular protein, haemolytic and proteolytic activity. The ECPs were obtained from 48h iron-replete and iron-restricted broth cultures by centrifugation (8,000g for 20min at 4°C). Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard with a BioRad protein assay kit. Proteolytic activity was determined by measuring hide powder azure (HPA) digestion as described by Hastings (1986)(see 8.2.9). Haemolytic activity against rainbow trout erythrocytes was determined as described by Hastings (1986)(see 8.2.10).

2.2.10 Growth of *A.salmonicida* in salmonid sera

A.salmonicida was inoculated into heat-inactivated Atlantic salmon or rainbow trout serum (inactivated by incubation at 45°C for 30min) at a concentration of $3x10^3$ colony forming units (cfu) ml^{-1} and incubated at 22°C for 48h. Growth of *A.salmonicida* was monitored by taking 20ul samples, making serial ten-fold dilutions in PBS and inoculating onto TSA plates. The latter were incubated at 22°C for 48h and the number of $cfuml^{-1}$ (mean of three) calculated to give viable counts and the growth rate of *A.salmonicida* in salmonid sera.

2.2.11 Growth of *A.salmonicida in vivo*

A.salmonicida NT1057 (10^8 cfu's) were injected intramuscularly (im) into ten rainbow trout (*Oncorhynchus mykiss*). Fish were killed at 2h, 4h, 12h, 24h and 48h and samples of blood, kidney, liver and spleen were taken for bacteriological analysis on TSA in an attempt to re-isolate *A.salmonicida*. The amount of growth on TSA after incubation at 22°C for 48h was assessed visually and scored as; -,+,++, accordingly.

2.2.12 Utilisation of iron compounds

A plate bioassay was used to determine which iron compounds could supply iron to four typical and two atypical strains of *A.salmonicida*. Approximately 10^8 cfu bacteria ml^{-1} were seeded into TSA containing 200μg EDDA ml^{-1}. Thirty μl of iron compound solution (100μM) were added to 3mm diameter wells punched in the agar. Plates were incubated for 48h at 22°C and then examined for stimulation of growth around the wells.

2.3 RESULTS

2.3.1 Iron levels in Atlantic salmon

The amount of iron present in Atlantic salmon serum was calculated to be 46.2 $\mu g ml^{-1}$ +/- SE 14.0 (n=10) which is comparable to the amounts found in mammalian serum (Persijn *et al.*, 1971). However, using the bleomycin assay of Gutteridge *et al.*, (1981) to detect amounts of

free serum iron, Atlantic salmon serum was found to contain no detectable free iron.

2.3.2 Growth of *A.salmonicida* in iron-restricted media

The minimum inhibitory concentrations of five high affinity iron chelators was determined (Table 2.2). The concentration of iron chelator used to induce conditions of iron-restriction *in vitro* are stated in 2.2.6. At the stated concentrations, the chelators reduced the free iron content of TSB from 385ngml^{-1} to approximately 20ngml^{-1} (Table 2.3). With the exception of one non-autoagglutinating atypical strain, all the *A.salmonicida* strains examined were able to grow and multiply under conditions of iron-restriction. The growth rates of four typical and two atypical strains of *A.salmonicida* monitored in liquid iron-restricted media are represented in Figure 2.1. In all cases of iron-restriction, growth rates were similar to those observed in normal liquid media and liquid media supplemented with 100μM ferric chloride. Minimal growth of *A.salmonicida* was observed on TSA containing 200μg EDDA ml^{-1} (except *A.salmonicida* MT534 which was unable to grow at this concentration) unless an additional source of iron was provided. This feature formed the basis of the subsequent bioassays in which iron-containing compounds were evaluated for their ability to reverse iron-restricted growth.

2.3.3 Effects of iron-restriction upon ECPs of *A.salmonicida*

The extracellular products (ECPs) of typical and atypical strains of *A.salmonicida* grown under iron-replete and iron-restricted conditions

were compared to examine the effects of iron-restriction on extracellular protein and specific haemolytic and proteolytic activity (Table 2.4). In general, *A.salmonicida* produced greater amounts of extracellular protein under iron-replete conditions. With respect to haemolytic and proteolytic activity, specific activities were generally higher in the iron-restricted ECPs of the typical strains of *A.salmonicida*. The atypical strains of *A.salmonicida* used in this study did not possess haemolytic activity. Although atypical strains possessed little proteolytic activity, the specific activity was higher in the iron-restricted ECP of MT194 only.

2.3.4 Growth of *A.salmonicida* in salmonid sera

The ability of two typical and an atypical strain of *A.salmonicida* to grow in heat-inactivated salmonid sera was examined (Figure 2.2). Both typical and atypical strains were capable of rapid growth in Atlantic salmon serum containing no available free iron (detectable by the bleomycin assay) and only very small amounts of complexed iron. The same strains were also capable of rapid growth in rainbow trout serum.

2.3.5 Growth of *A.salmonicida in vivo*

The ability of *A.salmonicida* MT1057, a virulent clinical isolate, to grow *in vivo* was examined at various intervals after im inoculation of 10^8 cfu s of *A.salmonicida*. Specific target organs were cultured and examined for the presence of *A.salmonicida* and the amount of growth was assessed visually. *A.salmonicida* MT1057 was found to be capable of rapid systemic spread throughout the major body organs,

appearing in the kidney within 2h, in the blood within 4h and other body organs within 12h (Table 2.5).

2.3.6 Utilisation of iron compounds by *A.salmonicida*

The ability of *A.salmonicida* to overcome the iron-restriction imposed by 200μg EDDA ml^{-1} by utilising different iron compounds was examined. The results (summarised in Table 2.6) show that the strains examined could use iron only when supplied as the ferric (Fe^{3+}) salt and not as the ferrous (Fe^{2+}) salt. Furthermore, *A.salmonicida* was able to utilise iron when supplied as ferric citrate, ferric ammonium citrate and ferric nitrilotriacetate, compounds which efficiently chelate iron (Williams *et al.*, 1990).

2.4 DISCUSSION

Results of this study confirm earlier reports by Chart & Trust (1983) of the ability of both typical and atypical strains of *A.salmonicida* (with the exception of one non-autoagglutinating atypical strain) to grow in the presence of both natural and synthetic high affinity iron chelators, where the available iron was less than $20ngml^{-1}$. The growth kinetics of representative typical and atypical strains monitored in liquid medium containing the various iron chelators indicated that in all cases of iron-restriction, growth rates were similar to those observed in normal iron-replete media and those supplemented with 100μM $FeCl_3$. The similar growth kinetics of *A.salmonicida* growing under conditions of iron-restriction where there

was a ten-fold reduction of available iron compared with iron-replete medium, indicate that *A.salmonicida* is well adapted for growth under iron-restricted conditions. Similarly, both typical and atypical strains were capable of rapid growth in heat-inactivated salmonid sera confirming observations by Stapleton & Ellis (1988), where there was no detectable free iron, using the bleomycin assay of Gutteridge *et al.*, (1981), due to the sequestration of the free iron by the host iron-binding proteins such as Tf (Congleton & Wagner, 1991). In addition, the ability of a virulent clinical isolate of *A.salmonicida* (MT1057) to grow rapidly in the severely iron-restricted environment encountered *in vivo* and become systemic was examined in rainbow trout. As with most successful systemic bacterial pathogens possessing a high affinity iron uptake mechanism, *A.salmonicida* was found to be capable of rapid systemic spread throughout the major body organs within 12h of im administration.

Using iron bioassays it was determined that typical and atypical strains of *A.salmonicida* were able to utilise iron only as the iron (III) salt and not as the iron (II) salt to reverse growth inhibition imposed by inhibitory concentrations of EDDA. Since iron (III) is highly insoluble in the aerobic environment and normally sequestered by host iron-binding proteins in order to maintain iron in a readily usable form for metabolic processess, *A.salmonicida* must be able to compete with these iron-binding proteins for growth and survival. In addition, *A.salmonicida* was able to utilise the iron salts of weak iron chelating acids such as citrate and nitrilotriacetate which possess weak siderophore activity.

Growth of *A.salmonicida* under condition of iron-restriction results in an increase in the amounts of λ protease and haemolysin activity

present in the extracellular products of the typical strains. Similar increases in the protease activity of non-haemolytic atypical strains was also observed. Since both protease and haemolysin are regarded as important virulence factors in the pathogenesis of *A.salmonicida* infection (Ellis, 1991), it is possible that iron-restricted cells of *A.salmonicida* are more virulent for salmonid fish. Increased virulence of iron-restricted bacteria for the animal host has been reported for a number of bacterial pathogens (Griffiths, 1987b). The iron-regulated synthesis of a number of bacterial toxins including haemolysins is also well established (See 2.1). Since the protease and haemolysin of *A.salmonicida* are known to be produced *in vivo* during infection (Rockey *et al.*, 1988; Ellis, 1991) their possible increased synthesis under these circumstances supports their role in the pathogenesis opf furunculosis. Results with the haemolysins of *E.coli* (Payne, 1988) and *V.cholerae* (Stoebner & Payne, 1988) suggest that the haemolysin of *A.salmonicida* may serve a role in the acquisition of iron by the bacterium.

In view of the virtual absence of freely available iron in normal body fluids of the vertebrate host, and the hypoferraemia of infection, pathogenic bacteria are able to grow and multiply successfully *in vivo* to establish infection. Since all known bacterial pathogens, with the exception of the lactobacilli (Archibald, 1983), require iron for growth and multiplication, they must be able to adapt to the iron-restricted environment found *in vivo* and develop mechanisms for the acquisition of protein-bound iron, or be able to acquire it from liberated haem compounds. A number of mechanisms have been described for bacterial pathogens which are involved in iron acquisition (Griffiths, 1987b). They include: the proteolytic cleavage of Tf, and disruption of the

iron-binding sites in order to releasing iron (Doring *et al.*, 1988); the reduction of a Fe^{3+}-Tf complex, by iron reductase enzymes, to the Fe^{2+} complex with the consequent release of soluble Fe^{2+} (Adams *et al.*, 1990; Cox, 1990; Huyer & Page, 1989; Johnson *et al.*, 1989); direct interactions between bacterial cell surface and Fe^{3+}-Tf complex (Griffithe, 1987b); the production of low molecular weight iron-chelators (siderophores) capable of removing iron from the Fe^{3+}-Tf complex (Griffiths, 1987b) and the utilisation of haem compounds and complexes via haem-binding proteins (Griffiths *et al.*, 1987).

Since *A.salmonicida* has been shown in this study to be able to grow effectively under conditions of iron-restriction, encountered both *in vitro* and *in vivo* during infection, the possible mechanisms for iron uptake utilised by *A.salmonicida* were examined further, as reported in the following chapters.

TABLE 2.1 Strains of *Aeromonas salmonicida*.

Strain	Origin	Source[1]
Typical (Non-autoagglutinating)		
MT004	Atlantic salmon	URL (strain 2862)
MT532	Not known	C.Michel (strain 51/79S)
MT539	Not known	C.Michel (strain 72/78S)
Atypical		
MT534	Carp	D.Evenberg (strain V75/93-W)
Typical (Autoagglutinating)		
MT028	Brown trout	D.H.McCarthy (strain As-3)
MT048	Atlantic salmon	Marine Laboratory
MT423	Atlantic salmon	Å. Adams (strain 184/86)
MT476	Atlantic salmon	Marine Laboratory
MT477	Atlantic salmon	Marine Laboratory
MT478	Atlantic salmon	Marine Laboratory
MT481	Atlantic salmon	Marine Laboratory
MT483	Atlantic salmon	Marine Laboratory
MT486	Atlantic salmon	Marine Laboratory
MT487	Atlantic salmon	Marine Laboratory
MT488	Atlantic salmon	Marine Laboratory
MT489	Atlantic salmon	Marine Laboratory
MT490	Atlantic salmon	Marine Laboratory
MT491	Atlantic salmon	Marine Laboratory
MT492	Atlantic salmon	Marine Laboratory
MT537	Not known	C.Michel (strain 36/75R)
MT538	Not known	C.Michel (strain 72/78R)
MT616	Salmonid	D.Evenberg (strain 152-69)
MT1057	Atlantic salmon	Marine Laboratory
MT1058	Atlantic salmon	AVL
MT1059	Atlantic salmon	Marine Laboratory
MT1060	Atlantic salmon	Marine Laboratory
MT1061	Atlantic salmon	Marine Laboratory
MT1077	Atlantic salmon	Marine Laboratory
MT1078	Atlantic salmon	AVL
MT1080	Atlantic salmon	Marine Laboratory
Atypical		
MT194	Atlantic salmon	Marine Laboratory
MT460	Sea trout	Marine Laboratory
MT482	Atlantic salmon	Marine Laboratory
MT523	Eel	T.Kitao (strain KF8318)
MT525	Atlantic salmon	B.K.Gudmundsdottir (strain 265/87)
MT526	Atlantic salmon	B.K.Gudmundsdottir (strain LON 82)
MT527	Atlantic salmon	B.K.Gudmundsdottir (strain LON 76)
MT528	Atlantic salmon	B.K.Gudmundsdottir (strain 510/SU)
MT529	Atlantic salmon	B.K.Gudmundsdottir (strain 17/87)

TABLE 2.1 Strains of *A.salmonicida* continued.

Strain	Origin	Source[1]
MT530	Sea trout	B.K.Gudmundsdottir (strain Fae II)
MT533	Atlantic salmon	B.K.Gudmundsdottir)(strain Eldi 80)
MT535	Goldfish	D.Evenberg (strain 3.111)
MT536	Carp	D.Evenberg (V76/135)

[1] Unilever Research Limited, Bedford UK; C.Michel, Laboratoire d' Ichtyopathologie, Jouy-en-Josas, France; D.Evenberg, Institute of Molecular Biology and Department of Molecular Cell Biology, University of Utrecht, Netherlands; AVL, Aquaculture Vaccines Limited, Saffron Walden, Essex, UK; D.H.McCarthy, Tavolek Inc., Redmond, Washington, USA; A.Adams, Institute of Aquaculture, University of Stirling, Stirling, Scotland; T.Kitao, Department of Fisheries, Faculty of Agriculture, Miyazaki University, Japan; B.K.Gudmundsdottir, Institute for Experimental Pathology, Fish Disease Laboratory, University of Iceland, Reykjavik, Iceland.

TABLE 2.2 Minimum Inhibitory Concentrations (MIC) of iron chelators.

		MICs against *A.salmonicida*		
		MT004	MT423	MT194
EDDA	(μgml^{-1})	200	200	200
Dp	(μM)	150	150	150
Df	(mM)	0.6	>1	>1
Tf	($mgml^{-1}$)	5	4	5
Lf	($mgml^{-1}$)	2	2	2

EDDA, ethylene diamine di (*o*-hydroxyphenylacetic acid); Dp, 2,2'-dipyridyl;, Df, desferal; Tf, transferrin; Lf, lactoferrin.

TABLE 2.3 Free ferric iron content of iron-restricted media.

Media	Iron content $ngml^{-1}$
TSB	385±11.4
TSB+EDDA	15± 7.0
TSB+Dp	25± 5.9
TSB+Df	20± 4.6
TSB+Tf	10± 2.3
TSB+Lf	15± 2.5

TSB, tryptone soya broth; EDDA, ethylene diamine di (*o*-hydroxyphenylacetic acid); Dp, 2,2'-dipyridyl;, Df, desferal; Tf, transferrin; Lf, lactoferrin. Results are the mean of triplicate values (+/- SE of the mean).

TABLE 2.4 Characterisation of the extracellular products of *A.salmonicida* grown under iron - replete and iron-restricted conditions.

Strain		48h ECP		
		Protein ($\mu g ml^{-1}$)	Specific proteolytic activity (unitsmg^{-1} ECP protein)	Specific haemolytic activity (HUmg^{-1} ECP protein)
MT004	N	176	0.42	0.07
	IR	82	1.13	0.26
MT616	N	223	0.17	0.07
	IR	85	0.54	0.38
MT423	N	300	0.26	0.08
	IR	183	0.51	0.26
MT486	N	73	0.59	0.44
	IR	96	0.86	0.33
MT194	N	31	0.04	0
	IR	19	0.07	0
MT525	N	29	0.04	0
	IR	26	0.04	0

N, iron-replete ECP; IR, iron-restricted ECP

TABLE 2.5 Recovery of *A.salmonicida* MT1057 on Tryptone Soya Agar from Rainbow trout tissues at various times following intramuscular injection of 10^5 bacteria.

Organ	Time (h)				
	2	4	12	24	48
	(presence of *A.salmonicida*)*				
Blood	-	+	++	++	++
Kidney	+	+	++	++	++
Liver	-	+	+	++	++
Spleen	-	-	+	++	++

* presence of *A.salmonicida* assessed visually: -, no growth; +, moderate growth; ++, confluent growth. Two fish were tested at each time.

TABLE 2.6 Utilisation of iron compounds by *A.salmonicida*.

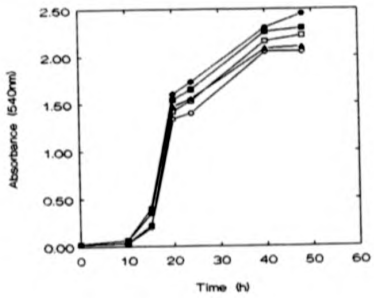
Strain**	Utilisation of*								
	$FeCl_2$	$FeCl_3$	$Fe_2(SO_4)_3$	$Fe(SO_4)$	FeC	FAC	FeN	Fe-NTA	Fe-D
MT004 (T)	0	7±1.0	5±0.5	0	5±0.3	5±1.0	4±0.0	2±0.0	2±0.0
MT616 (T)	0	8±0.9	5±0.4	0	7±0.3	5±0.2	5±0.2	3±0.0	2±0.0
MT423 (T)	0	8±0.7	5±0.4	0	8±0.0	4±0.7	5±0.3	2±0.1	4±1.0
MT486 (T)	0	5±1.0	4±0.0	0	5±1.0	6±1.0	5±0.3	2±0.0	5±0.2
MT194 (A)	0	9±0.0	5±0.0	0	6±1.2	6±0.0	6±0.9	4±0.2	3±0.1
MT525 (A)	0	8±0.0	5±0.3	0	6±0.8	7±0.6	5±1.0	4±0.0	4±0.2

* Utilisation of iron compounds was determined by measuring the zone of growth (in mm) around a 3mm diameter well containing 30µl iron compound solution (100µM) on a plate containing TSA+200 µg EDDA ml^{-1} seeded with 10^5 cfu of one of six *A.salmonicida* strains; $FeCl_2$, ferrous chloride; $FeCl_3$, ferric chloride; $FeSO_4$, ferrous sulphate; $Fe_2(SO_4)_3$, ferric sulphate; FeC, ferric citrate; FAC, ferric ammonium citrate; FeN, ferric nitrate; Fe-NTA, ferric nitrilotriacetate; Fe-D, ferric hydroxide-dextran (iron-dextran). Results are the means of triplicate values (+/- SE of the mean).

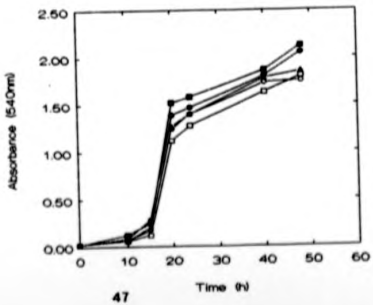
** (T), typical; (A), atypical

FIGURE 2.1 Growth kinetics of *A.salmonicida* in Tryptone Soya Broth (TSB) (●), TSB+ferric chloride ($FeCl_3$) (■), TSB+ethylene diamine di (*o*-hydroxyphenylacetic acid)(EDDA⁻ (▲), TSB+2,2'-dipyridyl (Dp) (○) and TSB+transferrin (Tf) (□). Results are the mean of triplicate values.
A.salmonicida strain:
a) MT004, b) MT616, c) MT423, d) MT486, e) MT194, f) MT525.

a)


Absorbance (540nm)
2.50
2.00
1.50
1.00
0.50
0.00
0
10
20
30
40
50
60
Time (h)

b)


Absorbance (540nm)
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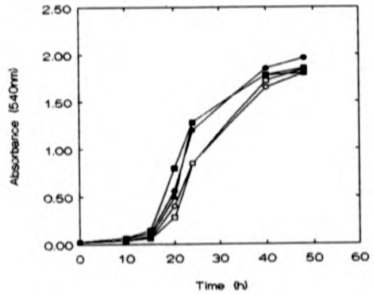
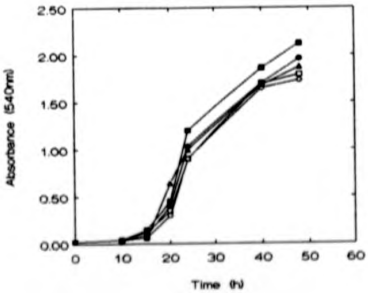
2.00
1.50
1.00
0.50
0.00
0
10
20
30
40
50
60
Time (h)

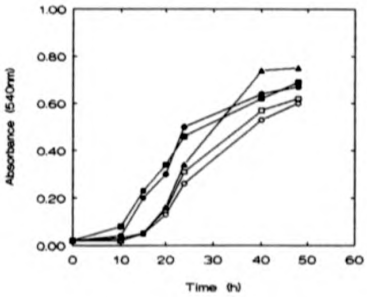
c)


Absorbance (540nm)
2.50
2.00
1.50
1.00
0.50
0.00
0
10
20
30
40
50
60
Time (h)

d)


Absorbance (540nm)
2.50
2.00
1.50
1.00
0.50
0.00
0
10
20
30
40
50
60
Time (h)

e)


1.00
0.80
0.60
0.40
0.20
0.00
Absorbance (540nm)
0
10
20
30
40
50
60
Time (h)

f)

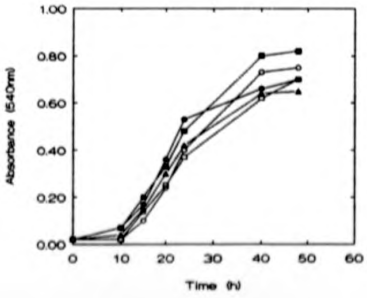

1.00
0.80
0.60
0.40
0.20
0.00
Absorbance (540nm)
0
10
20
30
40
50
60
Time (h)

FIGURE 2.2 Growth kinetics of *A.salmonicida* in heat-inactivated Atlantic salmon serum (a) and rainbow trout serum (b). *A.salmonicida* typical strain MT004 (●), MT423 (■) and atypical strain MT194 (○). Results are the mean of triplicate values.

CHAPTER 3:- SIDEROPHORE PRODUCTION BY *AEROMONAS SALMONICIDA*

3.1 INTRODUCTION

An important factor in bacterial virulence and pathogenicity is the ability of the invading pathogen to grow and multiply successfully in the tissues of the host. Within this environment bacteria must produce the full complement of virulence determinants required for pathogenicity. As was determined in Chapter 2, both typical and atypical strains of *A.salmonicida* were found to:- (1) grow under conditions of iron-restriction *in vitro*, (2) grow and multiply in salmonid sera and (3) be capable of growth *in vivo* during infection with rapid systemic dissemination. In view of the severely restricted iron levels in Atlantic salmon, *A.salmonicida* must possess high affinity iron uptake mechanism(s) to overcome the biological unavailability of iron and compete with the host iron-binding proteins to obtain sufficient iron for growth (Griffiths *et al.*, 1988; Martinez *et al.*, 1990). Low affinity iron uptake mechanisms are thought to operate under iron-replete conditions, however, little is known about the mechanisms involved (Griffiths, 1987b).

The best understood high affinity iron-uptake mechanism possessed by pathogenic bacteria, particularly the Gram-negative bacteria, involves the production of siderophores. These are soluble, low molecular weight, iron-chelating molecules which normally belong to two distinct chemical classes, although siderophores with novel structures are commonly identified (See Table 3.1). An essential component of siderophore-mediated iron-uptake mechanisms is the synthesis of iron-regulated outer membrane proteins (IROMPs) which act as receptors

for siderophores since the molecular weight of siderophores (500-1000 dalton) exceeds the 500 dalton diffusion limit of the small water-filled pores of the outer membrane (OM) of bacteria (Nikaido, 1979). A number of enzymes involved in the uptake and release of iron from the Fe^{3+}-siderophore complex are also required (Neilands, 1982). IROMPs of enteric bacteria are generally in the range 74-84kDa (Griffiths, 1987b) and will be discussed along with IROMPs of *A.salmonicida* in Chapter 6.

One class of siderophore is the phenolate-catechols, for example, enterobactin, produced by enteric bacteria such as *Escherichia, Klebsiella, Salmonella* and *Shigella*. Enterobactin is a cyclic triester of 2,3-dihydroxy-*N*-benzoyl serine with a formation constant for ferric enterobactin the highest recorded (being 10^{52} at neutral pH)(Harris *et al.*, 1979). It is capable of solubilising transferrin-bound iron and transporting it back to the bacterial cell via a 81kDa FepA enterobactin receptor. Enterobactin has been shown to be produced *in vivo* (Griffiths & Humphreys, 1980) and to be a determinant of virulence. Enterobactin-deficient mutants of *Salmonella typhimurium* exhibit reduced virulence for mice and an inability to grow in human serum, though this has been disputed by Benjamin *et al.*, (1985). Enterobactin, and the *V.cholerae* siderophore, vibriobactin (Griffiths *et al.*, 1984), are synthesised from chorismate, which is the final product of the common aromatic (aro) biosynthetic pathway. AroA mutants (evaluated recently as live attenuated vaccines (Dougan *et al.*, 1989)) have a block in the aromatic pathway, making them auxotrophic for two compounds not available in mammalian tissue, enterobactin and *p*-aminobenzoic acid (PABA), and are avirulent possibly due to the lack of a siderophore and an efficient iron uptake mechanism.

The second class of siderophores are the hydroxamates, for example,

aerobactin, a conjugate of 6-*N*-acetyl-*N*-hydroxyamino-2-aminohexanoic acid and citric acid (Griffiths, 1987b). Aerobactin was initially isolated from *Aerobacter aerogenes* (Gibson & Magrath, 1969) and it is synthesised by many bacterial pathogens, in addition to enterobactin, which carry pColV (colicin V) plasmid, which are capable of causing generalised extraintestinal infections in humans and domesticated animals. Aerobactin synthesis plays an important role in virulence of *E.coli* pColV (Williams, 1979) and is capable of solubilising Tf-bound iron (Konopka & Neilands, 1982) and transporting it back into the bacterial cell via a 74kDa IutA aerobactin receptor (Bindereif *et al.*, 1982). Until recently it was not clear how the ability to produce aerobactin conferred an advantage over bacteria already producing enterobactin since the formation constant for aerobactin is considerably less than that for enterobactin. It may be due to aerobactin being recycled by the bacterium (Braun *et al.*, 1984), whereas enterobactin is cleaved by esterases. Also, aerobactin is superior in competing with transferrin for iron, a consequence of its more rapid secretion compared with enterobactin (Der Vartanian, 1988). Since during the invasive process bacteria become intimately associated with host tissue rather than free in the bloodstream, cell-associated iron might form a more readily available source than Tf, if a siderophore can scavenge iron from cells. Indeed aerobactin-producing *E.coli* demonstrate a preference for cell-bound iron whereas enterobactin-producing *E.coli* demonstrate a preference for Tf-bound iron (Brock *et al.*, 1991). In addition, the relative ability of aerobactin and enterobactin to remove Fe and transport it back to the bacterial cell is important as far as infection is concerned. Since the bacteria *in vivo* are exposed to tissue fluids such as serum or mucosal secretions, other factors may be important in

the overall effectiveness of siderophores. For instance Moore *et al.*, (1980) and Moore & Earhart (1981) demonstrated the presence of anti-enterobactin antibodies in serum. In addition, anti-O-polysaccharide antibodies have been demonstrated, which interfere with enterobactin secretion (Fitzgerald & Rogers, 1980) as well as antibodies against the 81kDa FepA ferric-enterobactin receptor (Griffiths *et al.*, 1985a), all of which could reduce the effectiveness of the enterobactin-mediated iron acquisition system in serum and favour aerobactin or other siderophores.

A variety of siderophores, in addition to aerobactin and enterobactin have been identified from many Gram-negative and Gram-positive bacterial pathogens (See Table 3.1). The possession of siderophore-mediated iron uptake mechanisms is generally related to the ability of a pathogen to produce systemic infection. Pathogens which do not produce siderophores, for example, *N.gonorrhoeae* (Norrod & Williams, 1978a) and *Haemophilus influenzae* (Morton & Williams, 1990) which possess an ability to utilise Tf-bound iron by direct interaction, generally produce more superficial and localised infections. Siderophores have also been demonstrated as determinants of virulence in *E.coli* and *V.anguillarum*, and specific plasmid classes, for example, pColV encoding aerobactin (Braun, 1981; Warner *et al.*, 1981), the 74kDa IutA aerobactin receptor protein (Bindereif *et al.*, 1982), the pJM1 encoding anguibactin (Actis *et al.*, 1986) and the 86kDa OM2 anguibactin receptor protein (Actis *et al.*, 1985) have been linked with the ability of these bacteria to cause septicaemic diseases (Crosa, 1989).

In addition to siderophores, a number of siderophore-producing bacterial pathogens have been shown to obtain iron directly from naturally ocurring iron-binding acids such as pyruvate, malate and

citrate (Archibald & DeVoe, 1980). *E.coli* and *N.menigitidis* can use the Fe^{3+}-citrate complex as an iron source and, in addition, *Ps.aeruginosa* which is able utilise citrate as a carbon source, possesses a specific iron-citrate transport system involving specific OMP receptors (Harding & Royt, 1990).

In an earlier study of *A.salmonicida* growing under conditions of iron-restriction, Chart & Trust (1983) found that typical strains were able to sequester iron from a number of high affinity iron chelators by a mechanism involving the production of a siderophore-like actuivity. This siderophore-like activity was characterised by its ability to remove $^{59}Fe^{3+}$ from $^{59}Fe^{3+}$-lactoferrin and pass through a dialysis membrane with a 14kDa MW cut off. Thus, the siderophore-like activity was identified as a siderophore by its solubility, low MW and production only under conditions of iron-restriction. Atypical strains did not appear to produce a siderophore. The siderophore was undetectable by the chemical assays for phenolate-catechol and hydroxamate siderophores but no further information was available. The aims of the present study were (1) to confirm the production of siderophores by *A.salmonicida*, (2) characterise its biological activities and (3) extract the siderophore from culture supernatants and perform a biochemical characterisation.

3.2 MATERIALS AND METHODS

3.2.1 Bacteria and growth conditions

Bacteria were grown in either TSB or on TSA with the exception of *Vibrio anguillarum* which was grown in medium supplemented with 2% (w/v) sodium chloride. Iron-free EDDA (100μgml^{-1})(prepared as described in 2.2.5) was used to induce conditions of iron-restriction. Bacteria were subcultured three times on iron-restricted media to reduce internal ferric iron stores. *A.salmonicida*, *A.hydrophila*, *A.sobria* and *V.anguillarum* strains were grown at 22°C. *E.coli*, *Ent.cloacae* and *S.typhimurium* strains were grown at 37°C. Growth of *A.salmonicida* in liquid media was monitored by measurement of absorbance at 540nm.

3.2.2 Detection of the iron-binding activity of A.salmonicida culture supernatant

Iron-binding activity of *A.salmonicida* iron-restricted culture supernatant was measured by the method of Norrod & Williams (1978b) as follows. Uninoculated culture media, ddH$_2$O and 48h iron-restricted culture supernatant of four typical and two atypical strains was added to iron-free bovine transferrin (Tf) (2.5mgml^{-1} in 0.1M Tris-HCl) and incubated at 22°C for 1h. The binding of iron by Tf in the presence or absence of a siderophore was measured by the absorbance at 470nm after each addition of 20μl of 1mM ferric nitrilotriacetate (Fe-NTA).

3.2.3 Detection of iron reductase activity of A.salmonicida culture supernatant

Iron reductase activity was measured by the method of Dailey & Lascelles (1977) as described by Johnson *et al.*, (1991) using ferrozine, [3-(2-pyridyl)-5,6-bis-4(4-phenylsulfonic acid)-1,2,4-triazine] (Sigma). The reaction mixture contained 400ul of culture supernatant and 1.6ml of 5μM ferrozine and 100μg ferric citrate in 0.01M Tris-HCl (pH7.8). Reactions were performed at 22°C and the change in absorbance was measured at 562nm. Spontaneous reduction of iron by endogenous reductants was measured for 10 min before the addition of reductant. β-nicotinamide adenine dinucleotide (NADH) or β-nicotinamide adenine dinucleotide phosphate (NADPH) were then added to the reaction mixture at a concentration of 2μM. Reductase activity was recorded as the change in absorbance over time. Specific activity was calculated as the amount of product formed per mg of protein per 30 min.

3.2.4 Siderophore assays

Bacteria were screened for the production of siderophores by the method of Schwyn & Neiland (1987) using chrome azurol S (CAS) agar.

Possible catechol siderophore production was assessed in iron-restricted culture supernatant by the colorimetric assays of Arnow (1937) and Rioux *et al.*, (1983) using 2,3-dihydroxybenzoic acid (2,3-DHBA) as standard.

The assay of Arnow (1937) consisted of the following procedure:- to 200ul of culture supernatant or 2,3-DHBA standard, the following were added with mixing; 200μl 0.5N HCl, 200μl nitrite-molybdate solution (10%

(w/v) sodium nitrite + 10% (w/v) sodium molybdate in ddH_2O), 200µl 1N NaOH and 200µl ddH_2O. Absorbance at 509nm was measured and the amount of siderophore present determined by comparison with the calibration curve.

The assay of Rioux *et al.*, (1983) consisted of the following procedure:- to 2.3ml ddH_2O, 200µl 20% (v/v) sulphuric acid and 1ml culture supernatant or 2,3-DHBA standard were added, to which the following addditions were made with mixing; 100µl 1% (w/v) ferric ammonium citrate in 0.09N sulphuric acid; 400ul 2M ammonium flouride, 400ul 1% (w/v) 1,10-phenanthroline (4,7-diphenyl-1,10-bathophenanthroline diaulphonic acid) and 600µl 3M hexamethylenetetraamine. The assay mixture was then heated to 60°C for 1h, cooled to 22°C and the absorbance at 510nm measured. The amount of siderophore present was determined by comparison with the calibration curve.

Possible hydroxamate siderophore production was assessed in lyophilised ten-fold concentrated iron-restricted culture supernatant by the colorimetric assay of Csáky (1948) and in unconcentrated iron-restricted culture supernatant by the assays of Atkin *et al.*,(1970) and Arnold & Viswanatha (1983) using hydroxylamine monohydrochloride and desferrioxamine mesylate (Desferal™, Ciba-Geigy) as standards.

The assay of Csáky (1948) consisted of the following procedure:- 1ml of culture supernatant or hydroxylamine monohydrochloride or Desferal standard was acidified by the addition of 6N sulphuric acid and heated for 30min at 100°C. The sample was then neutralised by the addition of 35% (w/v) sodium acetate to which 0.5ml 1.3% (w/v) iodine in acetic acid followed by 1ml 2% (w/v) sodium arsenite was added. One ml 0.03% α-naphthylamine in 30% (v/v) acetic acid was added and the

absorbance at 526nm measured. The amount of siderophore present was determined by comparison with the calibration curve.

The assay of Atkin *et al.*, (1970) consisted of the following procedure:- 1ml culture supernatant or desferal standard was mixed with 1ml 5mM ferric perchlorate in 0.14M perchloric acid and the absorbance at 480nm measured. The amount of siderophore present was determined by comparison with the calibration curve.

The assay of Arnold & Viswanatha (1983) consisted of the following solutions:- Solution A - Ammoniacal mercaptoacetate (1.25ml 80% (v/v) mercaptoacetic acid and 2.2ml concentrated ammonium hydroxide, made up to 100ml with ddH_2O). Solution B - Ferric nitrate (30mgml^{-1}). Solution C - 22.5 ml solution A + 440μl solution B made up to a volume of 50ml to which 50ml 500mM ammonium carbonate/ammonium hydroxide buffer (pH 8.6) was added. The assay consisted of the following procedure:- 1ml of culture supernatant or desferal standard was added to 0.5ml of solution C with mixing. After 4min in the dark the absorbance at 532nm was measured. The decrease in absorbance at 532nm in the sample relative to the blank is a measure of the amount of siderophore present, compared with the calibration curve.

Crude siderophore preparations were analysed by paper and thin layer chromatography (TLC). Paper chromatography was performed as described by Lemos *et al.* (1988) using butanol:acetic acid:water (120:30:30) or 5% (w/v) ammonium formate in 0.5% (v/v) formic acid as solvent and chromatograms developed with the CAS assay solution of Schwyn & Neilands (1987). TLC was carried out using methanol:water (20:15) to detect anguibactin, the siderophore produced by *V.anguillarum* (Actis *et al.*, 1986) and butanol saturated with 1.7% (w/v) ammonium acetate to detect amonabactin, one of the siderophores produced by

A.hydrophila (Barghouti *et al.*, 1989). Chromatographed material was visualised either by blue fluorescence under ultraviolet light (302nm) or by exposure to 2.7% (w/v) ferric chloride in 2M HCl/3.7% (w/v) potassium ferricyanide spray. Rf values were calculated using 2,3-DHBA as reference.

3.2.5 Production of siderophores in salmonid sera

A.salmonicida NT004 ($3x10^3$ cfu s) were inoculated into heat-inactivated Atlantic salmon and rainbow trout sera and incubated at 22°C for 48h. Aliquots were taken at 12h intervals, centrifuged in an Eppendorf centrifuge at 6500g for 5min to remove bacteria and the supernatant tested for the presence of siderophores using the assay of Arnow (1937). Serum inoculated with PBS served as a control.

3.2.6 Production of siderophores *in vivo*

A.salmonicida NT1057 (10^5 cfus) were injected intramuscularly (im) into five rainbow trout (*Oncorhynchus mykiss*). Fish were killed when showing symptoms of acute furunculosis, dissected and samples of kidney removed for bacteriological examination on TSA. Bacterial colonies were tested for purity and only organs of fish from which *A.salmonicida* was isolated in pure culture were used for further experiments. Entire kidney, liver, spleen and muscle samples from each fish were individually sonicated on ice (4 x 30s) in 2ml PBS. Extracts were centrifuged (3000g 15min at 4°C) in order to remove tissue debris. Organ samples from fish injected with PBS served as controls. For detection of the phenolate-catechol siderophore produced by

A.salmonicida MT1057, extracts were adjusted to pH1.5 by the addition of 1N HCl and extracted with ethyl acetate. The organic layer was collected, evaporated under N_2 to dryness and the residue dissolved in a one-tenth volume of ethyl acetate. Extracts were then subjected to TLC using butanol:acetic acid:water (120:30:30) as solvent and chromatograms visualised as described in 3.2.4.

3.2.7 Citric acid determination

Citric acid production by typical and atypical strains of *A.salmonicida* growing under iron-replete and iron-restricted conditions was determined enzymatically as described by Guerinot *et al.*, (1990) using a citric acid assay kit (Boehringer Mannheim).

3.2.8 Siderophore extraction

Several procedures were used to extract the siderophore from iron-restricted culture supernatant of *A.salmonicida* MT004. The colorimetric assays of Arnow (1937) and Csáky (1948) were used to indicate the success of the extraction procedure. Extracts were evaporated under N_2 and diluted five-fold in ddH_2O for use in the bioassays described in 3.2.10.

For dichloromethane-acetic acid extraction (Ankenbauer *et al.*, 1985), 10ml of culture supernatant was mixed with 5ml of a 90% (v/v) dichloromethane: 10% (10% v/v) acetic acid solution. After mixing, the organic phase was evaporated under N_2 to dryness and the residue dissolved in 1ml ddH_2O.

For ethyl acetate, diethyl ether and chloroform extraction (Hu *et*

al., 1986), 5ml of culture supernatant was adjusted to pH 1.5 by addition of 1N HCl and extracted with 2.5ml of solvent. The organic layer was collected, evaporated under N_2 to dryness and the residue dissolved in 1ml of ddH_2O.

For chloroform-phenol-diethyl ether extraction (Hu *et al.*, 1986), 25ml of lyophilised culture supernatant, concentrated ten-fold was adjusted to pH 2.0 with 1N HCl and extracted with 20ml of chloroform-phenol (1:1v/v). Diethyl ether (15ml) was added to the organic layer, mixed and extracted with 10ml of ddH_2O. The aqueous extract was washed with ether, adjusted to pH 7.0 and evaporated under N_2 to dryness. The residue was dissolved in 1ml ddH_2O.

For methanol extraction (Hu *et al.*, 1986), 40ml methanol was added to the residue obtained from the lyophilisation of 250ml of culture supernatant. The mixture was stirred at 22°C overnight and then centrifuged (1000g, 10min at 22°C) to remove undissolved material. The supernatant was evaporated under N_2 and then suspended in 5ml ddH_2O.

3.2.9 Partial purification of the *A.salmonicida* MT004 siderophore

Attempts to extract a siderophore from the iron-restricted culture supernatant of *A.salmonicida* MT004 were made by the method of Actis *et al.* (1986). Amberlite XAD-7 resin was washed with methanol, acetonitrile and diethyl ether in order to remove residual organic impurities prior to being packed into a 2.5cm x 10cm column in ddH_2O. Five litres of culture supernatant, adjusted to pH 7.0, were applied to the XAD-7 column at a rate of 10 bed volumes per hour. The column was rinsed with 100ml ddH_2O followed by a step gradient of 25ml each of 33%(v/v) and 66%(v/v) methanol-water mixtures. Adsorbed material was

eluted with 75ml pure methanol. Peak column fractions, determined by the colorimetric assay of Arnow (1937), were evaporated under N_2 and diluted five-fold in ddH_2O for use in the bioassays described in 3.2.10. A small amount of the adsorbed material was dissolved in a minimum volume of methanol and tested for purity on Silica G TLC plates. The plates were developed with the following solvent systems: butanol:acetic acid:water (120:30:30); chloroform:methanol:water (35:12:2); methanol:ammonium hydroxide (100:1.5) and ethyl acetate:methanol:acetic acid (80:1:1). Chromatograms were visualised as described in 3.2.4.

3.2.10 Siderophore bioassay

The bioassay measured the size of the growth halo around a 3mm diameter well containing 30µl of sample on plates containing TSA + 200µg EDDA ml^{-1}, which had been seeded with approximately 10^5 cfu's of one of the six *A.salmonicida* strains. Diameter of the halo was measured after growth for 48h at 22°C, and the value corrected by subtraction of the 3mm diameter of the sample well. The samples included culture supernatant of *A.salmonicida*, solvent extracts of *A.salmonicida* MT004 culture supernatant and peak column fractions of the XAD-7 column. Culture supernatants of *A.hydrophila*, *A.sobria*, *E.coli*, *Ent.cloacae* and *V.anguillarum* were also examined. In separate experiments, 0.1µg DNase ml^{-1} and 0.1µg RNase ml^{-1} were added to the culture supernatants of *A.salmonicida* in order to minimise the possibility of plasmid DNA transfer being implicated in bacterial growth in the bioassay.

Bioassays were also carried out using siderophore-deficient strains of *V.anguillarum* (anguibactin-deficient) and *S.typhimurium* (enterobactin-deficient) grown in iron-restricted M9 media as described

by Lemos *et al.*, (1988) to examine if the siderophore produced by *A.salmonicida* was chemically related to anguibactin or enterobactin. Bacterial strains examined are listed in Table 3.2.

3.3 RESULTS

3.3.1 Growth of *A.salmonicida* under conditions of iron-restriction

As determined in Chapter 2, with the exception of one non-autoagglutinating atypical strain, all twenty-nine typical and thirteen atypical strains of *A.salmonicida* were able to grow and multiply under these conditions.

3.3.2 Iron-binding activity of *A.salmonicida* culture supernatant

The iron-binding activity of culture supernatant was examined in four typical and two atypical strains of *A.salmonicida* (Figure 3.1). The presence of iron-binding activity was detected in the iron-restricted culture supernatant of the four typical strains by the ability of the culture supernatant to inhibit binding of Fe-NTA to iron-free bovine transferrin (BTf). This inhibition was seen only with iron-restricted culture supernatants. Culture supernatants of atypical strains failed to inhibit this reaction and showed no iron-binding activity.

3.3.3 Iron reductase activity of *A.salmonicida* culture supernatant

The iron reductase activity of culture supernatant was examined in two typical and one atypical strain of *A.salmonicida* grown under iron-replete and iron-restricted conditions (Table 3.3). Only the culture supernatants of typical strains contained a reductase activity capable of reducing ferric iron to ferrous iron, using NADH or NADPH as reductant. An increased reductase activity was detected in the culture supernatants of typical strains grown under iron-restricted conditions.

3.3.4 Siderophore production

Using CAS agar, siderophore production by *A.salmonicida* appeared to be confined to the typical strains. Culture supernatants of the twenty-nine typical and fourteen atypical strains were assayed colorimetrically for the presence of phenolate-catechol and hydroxamate siderophores. The iron-restricted culture supernatants of typical strains were found to contain small quantities of a phenolate-catechol siderophore by the methods of Arnow (1937) and Rioux *et al.*, (1983). Small amounts of catechol were also detected in non-iron-restricted culture supernatants of typical strains by the assay of Rioux *et al.*, (1983) but not by the assay of Arnow. These catechols were only detected in the culture supernatant of the typical strains and were shown to possess no siderophore activity in the siderophore bioassays. Culture supernatants of atypical strains were negative in the siderophore assays. The colorimetric assays of Csáky (1948), Arnold & Viswanatha (1983) and Atkin *et al.*, (1970) for the detection of

hydroxamate siderophores in iron-restricted and non-iron-restricted culture supernatant were negative for both typical and atypical strains of *A.salmonicida*.

Crude preparations of siderophore in iron-restricted culture supernatant were analysed by paper chromatography and TLC. Both failed to detect any siderophore activity in *A.salmonicida*, although 2,3-DHBA could be detected in the culture supernatants of the typical strains. TLC was, however, successful in detecting the siderophores enterobactin, anguibactin and amonabactin in the culture supernatant of the appropriate siderophore-producing bacteria.

3.3.5 Siderophore production in salmonid sera

A.salmonicida MT004 was shown in 2.3.4 to be capable of growth in heat-inactivated salmonid sera. In the experiment, siderophore production in salmonid sera was assessed after bacteria were removed by centrifugation (Figure 3.3). Using the assay of Arnow (1937) on non-solvent extracted sera, a phenolate-catechol siderophore was detected in amounts, and produced at a rate, comparable to that seen by the same strain growing in iron-restricted media *in vitro* (Figure 3.2).

3.3.6 Siderophore production *in vivo*

Internal organs (spleen, liver and kidney) of rainbow trout infected with a virulent strain of *A.salmonicida* were examined for the presence of a soluble siderophore. Sonicated organs were extracted with ethyl acetate and analysed by TLC. Using this technique, a phenolate compound which reacted with ferric chloride/potassium ferricyanide spray

was identified in infected organs, which was not present in uninfected control organs. Comparing the Rf value of the unknown with standard phenolates, the material present in organs infected with *A.salmonicida* was identified to be 2,3-dihydroxybenzoic acid (2,3-DHBA).

3.3.7 Citrate as a siderophore

The production of citrate by two typical and an atypical strain of *A.salmonicida* growing under iron-replete and iron-restricted conditions was examined (Table 3.4). Both typical and atypical strains produced citric acid and similar levels of citrate were produced under both iron-replete and iron-restricted conditions.

3.3.8 Extraction and partial purification of the *A.salmonicida* MT004 siderophore

Attempts to extract the siderophore of *A.salmonicida* MT004 were made by extraction of acidified culture supernatants with dichloromethane-acetic acid, ethyl acetate, diethyl ether, chloroform, methanol and chloroform-phenol-diethyl ether. The extraction procedures were unsuccessful as determined by colorimetric methods and bioassay. The phenolate-catechol siderophore could be detected in acidified culture supernatant after these extractions by the assays of Arnow (1937) and Rioux *et al.* (1983), indicating that the siderophore remains in the aqueous phase.

A phenolate-catechol siderophore was successfully extracted by adsorption onto XAD-7 resin. A comparison of the Arnow assay and siderophore bioassay before and after passage of the supernatant through

the XAD-7 resin indicated that the resin was effective at adsorbing the siderophore. Elution of the XAD-7 resin with pure methanol resulted in a well defined peak of siderophore bioassay activity which coincided with the Arnow peak (Figure 3.4). However, much of the Arnow positive material eluted earlier with a lower concentration of methanol indicating elution of catechols with no siderophore activity. TLC of the pooled peak fractions (fraction 14-35) using butanol:acetic acid:water revealed the presence of six components possessing phenolic groups (Rf 0.16, 0.33, 0.39, 0.46, 0.54 and 0.73). Treatment of the pooled peak fractions and 2,3-DHBA with ferric chloride/potassium ferricyanide produced an intense blue colour characteristic of phenols containing vicinal di-hydric groups (Dawson *et al.*, 1989). In an attempt to extract the siderophore from developed TLC plates, the six components were scraped from the plate, eluted with methanol and each component tested in the siderophore bioassay. All the components were negative in the bioassay, suggesting that the siderophore had undergone decomposition on the silica gel support medium.

3.3.9 Cross-feeding

The ability of siderophore(s) present in the iron-restricted culture supernatant of typical or atypical strains of *A.salmonicida* to cross-feed other strains was examined using a siderophore bioassay (Table 3.5). Non-iron-restricted culture supernatant or uninoculated medium failed to support the growth of any strain. The typical strains were able to utilise their own culture supernatant to stimulate growth under conditions of iron-restriction but only one strain (typical MT423) was able to stimulate other typical and also the atypical strains.

Culture supernatant of the atypical strains failed to stimulate their own growth or the growth of the typical strains under conditions of iron-restriction. The addition of DNase and RNase to the culture supernatant had no effect on the growth of the various strains under iron-restriction (data not shown). Culture supernatants of *E.coli* NCTC9001, NCTC50147, *Ent.cloacae* NCTC11580, *A.hydrophila* B32 and 80-A1 and *A.sobria* P-261 containing enterobactin were able to promote growth of typical and atypical strains of *A.salmonicida* to a small extent, although aerobactin present in the culture supernatant of *E.coli* NCTC50147 in addition to enterobactin failed to promote better growth than enterobactin alone (Table 3.6). Culture supernatants of *A.hydrophila* 1108 containing amonabactin and *V.anguillarum* 2286 (775 pJM1) containing anguibactin were unable to promote the growth of typical and atypical *A.salmonicida* (Table 3.7).

The ability of iron-restricted culture supernatants of typical and atypical strains of *A.salmonicida* to cross-feed siderophore-deficient mutants was also examined (Table 3.8). Neither culture supernatants of typical or atypical strains were able to cross-feed mutant strains of *V.anguillarum* 775-H3 (anguibactin$^-$ OMP receptor$^-$) or 775-Tn1::5 (pJHC91) (anguibactin$^-$ OMP receptor$^+$). The culture supernatant of *V.anguillarum* 775 (pJM1) containing anguibactin served as a positive control for strain 775-Tn1::5. Culture supernatants of typical strains of *A.salmonicida* but not atypical strains were able to partially cross-feed mutant strains of *S.typhimurium* LT-2 *enb-1* and *enb-7* (enterobactin$^-$ OMP receptor$^+$) suggesting a similarity between the *A.salmonicida* siderophore and enterobactin. Culture supernatants of *E.coli* NCTC9001 containing enterobactin served as a positive control for the *S.typhimurium* mutants.

A number of catechols and iron chelating compounds were tested in the same bioassays for their ability to promote growth of *A.salmonicida* under conditions of iron-restriction. The compounds included 3,4-dihydroxyphenylalanine and 2,4,5-trihydroxyphenylalanine (6-hydroxy-dopa), thought to be intermediates of pigment biosynthesis (Shieh & Maclean, 1974; Donlon *et al.*, 1983), catechol (1,2-dihydroxybenzene) and 2,3-DHBA. None of these compounds were able to promote the growth of *A.salmonicida* under conditions of iron-restriction (data not shown).

3.4 DISCUSSION

All of the forty-two strains examined in this chapter, with the exception of one non-autoagglutinating atypical strain, were able to grow and multiply in the presence of various high affinity iron chelators where the available iron was reduced to less than 20ngml^{-1}. As discussed in Chapter 2, this indicates that both typical and atypical strains of *A.salmonicida* possess effective high affinity iron uptake mechanism(s). The most common high affinity iron uptake mechanism possessed by pathogenic bacteria involves production of siderophores (Griffith *et al.*, 1986). Although Chart & Trust (1983) found the mechanisms by which the typical and atypical strains of *A.salmonicida* acquired iron under conditions of iron-restriction to be varied (See Chapter 1), a number of typical strains acquired ferric iron by the production of a siderophore-like activity. This siderophore-like activity produced by certain typical strains was characterised by its ability to sequester $^{59}Fe^{3+}$ (as $^{59}FeCl_3$) from lactoferrin (Lf) across a dialysis membrane with a 14kDa molecular weight exclusion limit,

suggesting that it was a siderophore is soluble and low MW. In the present study, a universal chemical assay (CAS agar) was used to assess siderophore production by typical and atypical strains of *A.salmonicida*. Using the assay, in which Fe^{3+} removed from a chrome azurol S/Fe^{3+}/hexadecyltrimethyl ammonium bromide (CAS/Fe^{3+}/HDTMA) dye complex by a siderophore, changes colour from blue to orange, it was found that only the typical strains produced a siderophore. An iron-binding activity able to chelate ferric iron and prevent binding to apo-Tf was also detected in the iron-restricted culture supernatant of the typical strains. No siderophore production or iron-binding activity was detected in the atypical strains, indicating that atypical strains of *A.salmonicida* acquire iron by a siderophore-independent mechanism.

In addition to the presence of a siderophore and an iron-binding activity present in iron-restricted culture supernatants of typical strains of *A.salmonicida*, a ferric iron reductase activity was also detected by its ability to reduce ferric iron (as ferric citrate) to ferrous iron using NADH or NADPH as reductant. Iron reductase enzymes have been shown to be important in the ability of several bacterial pathogens to acquire iron from siderophores and iron complexed to citrate (Cox, 1980; Huyer & Page, 1989; Johnson *et al.*, 1991), since reductase activity reduces iron in the Fe^{3+}-siderophore/citrate complex to Fe^{2+} which is more soluble and thus is released from the siderophore. An iron reductase enzyme produced by *Listeria monocytogenes* (Adams *et al.*, 1990) and the pyocyanin of *Ps.aeruginosa* (Cox, 1986) are also able to mediate removal of Fe^{3+} from Fe^{3+}-Tf complexes. Since the iron reductase activity was only detected in the siderophore-producing typical strains of *A.salmonicida*, it suggests that the ferric reductase activity may be involved in utilisation of the *A.salmonicida*

Fe^{3+}-siderophore complex.

The utilisation and production of citric acid as a siderophore has also been reported for the plant pathogen *Bradyrhizobium japonicum* (Guerinot *et al.*, 1990). Similarly, citrate has been shown to be involved in iron uptake mechanisms for a number of bacterial pathogens including *E.coli* (Hussein *et al.*, 1981), *N.menigitidis* (Archibald & DeVoe, 1980), *Ps.aeruginosa* (Cox, 1980; Harding & Royt, 1990) and *V.cholerae* (Sigel *et al.*, 1985). Citrate was shown to be produced by both typical and atypical strains of *A.salmonicida* in this study. Although its function *in vivo* is not clear, results of bioassays carried out in Chapter 2 indicate that both typical and atypical strains of *A.salmonicida* are able to utilise citrate in obtaining iron.

The siderophore-like activity described by Chart & Trust (1983) could not be detected by the assays used to detect phenolate-catechol and hydroxamate siderophores. They used the assay of Arnow (1937) to detect phenolate-catechol siderophores in ethyl acetate extracts of acidified culture supernatant. Although this procedure has been used to detect enterobactin (O'Brien *et al.*, 1970; O'Brien & Gibson, 1970), (the siderophore produced by *E.coli* and other members of the *Enterobacteriaceae*) the extraction depends upon the solubility of this siderophore, and most other phenolate-catechols, in organic solvents at acidic pH, due to the release of the chelated ferric iron (Bezkorovainy, 1980). However, in this study, the siderophore produced by *A.salmonicida* could not be extracted from culture supernatant into a number of organic solvents capable of extracting enterobactin (O'Brien *et al.*, 1970), multocidin (Hu *et al.*, 1986) and pyochelin (Ankenbauer *et al.*, 1985). Recently a phenolate-catechol siderophore, composed of 2,3-DHBA, lysine, glycine and either tryptophan or phenylalanine

(amonabactin) has been described in *A. hydrophila* (Barghouti *et al.*, 1989) which also could not be extracted into organic solvents (Liles *et al. 1985).* Similarly, anguibactin, a phenolate-containing siderophore from *V.anguillarum*, composed of *N*-hydroxyhistamine, cysteine and 2,3-DHBA (Jalal *et al.*, 1989) could not be isolated from culture supernatant by solvent extraction (Actis *et al.*, 1986). With these two siderophores in mind, the non-solvent extracted, iron-restricted culture supernatant of *A.salmonicida* was examined directly for the presence of a phenolate-catechol siderophore, and a phenolate-catechol was detected using the assays of Arnow (1937) and Rioux *et al.* (1983). The *A.salmonicida* siderophore was partially purified using hydrophobic chromatography by adsorption onto a XAD-7 resin. The siderophore was found to behave as a 2,3-diphenolate-catechol. Its phenolate-catechol nature was demonstrated by a positive reaction by the assay of Arnow (1937) and by the ferric catechol-phenanthroline assay of Rioux *et al.* (1983). The presence of a phenolic group was demonstrated by its blue fluorescence under ultraviolet light and by its reaction with ferric chloride/potassium ferricyanide spray, which indicated that the phenolate-catechol possessed vicinal di-hydric groups typical of diphenols (Dawson *et al.*, 1989). A 2,3-diphenol structure for the *A.salmonicida* siderophore is probable since aromatic vic diols with 4- or 5- substituents fail to react in the Arnow assay (Barnum, 1977). Negative reactions with the Csáky assay and ferric perchlorate assay of Atkin *et al.*, (1970) indicated that the siderophore contained no hydroxamate functional groups. Although similar in chemical nature to anguibactin and amonabactin in being a 2,3-diphenol-catechol and insoluble in organic solvents, the *A.salmonicida* siderophore has different chromatographic properties when analysed by TLC. Differences

in the chemical nature of the siderophores were also observed in the siderophore bioassays in which *A.salmonicida* was unable to utilise culture supernatant containing either anguibactin or amonabactin.

Small amounts of non-siderophore phenolate-catechols present in non-iron-restricted culture supernatant of the typical strains were detected by the assay of Rioux *et al.* (1983). Their detection was probably due to the assay being more sensitive than the Arnow assay which failed to detect their presence. Since both assays are selective for aromatic vic-diols, a wide variety of catechols may be detected such as catechol, 2,3-DHBA and 3,4-dihydroxyphenylacetic acid (Barnum, 1977). A number of the possible intermediates of pigment biosynthesis, characteristic of the typical strains, such as 3,4-dihydroxyphenylalanine and 2,4,6-trihydroxyphenylalanine (6-hydroxy-dopa) (Shieh & Maclean, 1974; Donlon *et al.*, 1983) are aromatic vic-diols. It is possible that the non-siderophore catechols detected in non-iron-restricted culture supernatant at lower concentrations than seen in iron-restricted culture supernatant are the intermediates of pigment biosynthesis. The presence of 2,3-DHBA was detected by TLC in the iron-restricted culture supernatant of typical strains of *A.salmonicida*. None was detected in the iron-restricted culture supernatant of the atypical strains. The presence of 2,3-DHBA is characteristic of bacteria which produce phenolate-catechol siderophores (Actis *et al.*, 1986) and enterobactin, since enterobactin contains a number of ester bonds which cleave to release 2,3-dihydroxybenzoyl serine and 2,3-DHBA. The latter was also detected in the organs of Atlantic salmon infected with *A.salmonicida* suggesting that the *A.salmonicida* siderophore, like anguibactin (Wolf & Crosa, 1986), enterobactin (Griffiths & Humphreys, 1980) and pyoverdin

(Haas *et al.*, 1991) is produced *in vivo* during infection.

The siderophore present in the iron-restricted culture supernatant of the typical strains of *A.salmonicida* was found to stimulate growth of homologous strains. An interesting feature of the siderophore produced by the typical strain MT423 was its ability to stimulate growth of heterologous strains including the atypical strains. This ability may be due to the presence of an unidentified growth-promoting factor induced by the iron-restricted conditions in addition to a strain specific siderophore or it may be due to a slight difference in the chemical nature of the siderophore compared with the siderophore of *A.salmonicida* MT004. The ability of the MT423 iron-restricted culture supernatant to cross-feed heterologous strains suggests a similarity to the cross-feeding activity described by Chart & Trust (1983) in which a typical strain possessing a siderophore-activity was found to be able to cross-feed a non-siderophore-producing typical strain. However, in the present study, the MT423 iron-restricted culture supernatant was also found to be able to cross-feed the atypical strains. This suggests that the atypical strains possess a receptor capable of utilising siderophores present in iron-restricted culture supernatant, possibly the iron-regulated outer membrane proteins described by Chart & Trust (1983) and Aoki & Holland (1985)(See Chapter 6). A similar situation is seen with non-siderophore producing pathogens such as *H.parainfluenzae* (Williams *et al.*, 1990) and *N.gonorrhoeae* (West & Sparling, 1985; 1987) which are able to utilise exogenous enterobactin and aerobactin respectively. DNA hybridisation studies with *H.parainfluenzae* DNA demonstrate hybridisation with *E.coli fepA* genes indicating possession of a functional enterobactin-mediated iron acquistion system (Williams *et al.*, 1990). In cross-feeding experiments using culture supernatants

of a number of bacterial species containing the siderophores, aerobactin, amonabactin, anguibactin or enterobactin to stimulate growth of *A.salmonicida*, none stimulated growth to a large extent, indicating that both typical and atypical strains of *A.salmonicida* are unable to utilise exogenous siderophores.

In the siderophore bioassays involving siderophore-deficient mutants of *V.anguillarum* and *S.typhimurium*, culture supernatants of *A.salmonicida* were unable to cross-feed *V.anguillarum* indicating differences between the *A.salmonicida* siderophore and anguibactin. Culture supernatants of *A.salmonicida* were however able to partially cross-feed *S.typhimurium*. This indicates a similarity between the *A.salmonicida* siderophore and enterobactin. However, the growth stimulation due to the *A.salmonicida* siderophore was less than the enterobactin-producing *E.coli* control. The growth stimulation may, therefore, not be due to the *A.salmonicida* siderophore, but due to the presence of 2,3-DHBA in the *A.salmonicida* culture supernatant, since *E.coli* has been shown to be able to use 2,3-DHBA as a siderophore (Hancock *et al.*, 1977; Hantke, 1990).

The *A.salmonicida* siderophore was also shown to be produced in salmonid sera at levels comparable to those produced under conditions of iron-restriction *in vitro* and produced *in vivo*. Thus, its role as a virulence determinant of *A.salmonicida* is further confirmed.

TABLE 3.1 Bacterial siderophores.

Bacteria	Siderophore	Class	Ref
A.hydrophila	Amonabactin	P	Barghouti *et al.*,(1989)
A.salmonicida		NC	Chart & Trust (1983)
B.pertussis	Bordetellin	H	Gorringe *et al.*,(1990) Agiato & Dyer (1992)
C.diphtheriae		N	Russell *et al.*,(1984) Tai *et al.*,(1990)
Edwardsiella tarda		NC	Kokubo *et al.*,(1990)
Ent.cloacae	Aerobactin	H	Crosa *et al.*, (1988)
E.coli	Enterobactin	P	Griffiths (1987b)
	Aerobactin	H	Griffiths (1987b)
K.aerogenes	Enterobactin	P	Williams *et al.*,(1987)
K.pneumoniae	Aerobactin	H	Nassif & Sansonetti (1986)
P.multocida	Multocidin	N	Hu *et al.*,(1986)
Ps.aeruginosa	Pyoverdin	H	Wedenbaum *et al.*,(1983)
Ps.cepacia	Cepabactin	HP	Meyer *et al.*, (1989)
	Pyochelin	P	Sokol (1986)
Ps.pseudomallei	Malleobactin	H	Yang *et al.*,(1991)
Salmonella spp.	Enterobactin	P	Visca *et al.*,(1991)
S.typhi	Enterobactin	P	Faundez *et al.*,(1990)
S.typhimurium	Enterobactin	P	Griffiths, (1987b)
S.wein	Aerobactin	H	Visca *et al.*,(1991)
S.flexneri	Aerobactin	H	Payne *et al.*,(1983) Griffiths *et al.*,(1986b)
	Enterobactin	P	Payne *et al.*,(1983)
Staph.hyicus	Staphyloferrin	N	Konetschny-Rapp *et al.*,(1990)
V.anguillarum	Anguibactin	P	Actis *et al.*,(1986) Jalal *et al.*, (1989)
V.cholerae	Vibriobactin	P	Sigel & Payne (1982) Griffiths *et al.*,(1984)
V.vulnificus		H+P	Andrus *et al.*,(1983) Simpson & Oliver (1983)
Yersinia spp.	Aerobactin	H	Stuart *et al.*,(1986)

H, hydroxamate; P, phenolate; N, novel structure; NC, not characterised; HP, siderophore composed of both hydroxamate and phenolate functional groups; H+P, produces both hydroxamates and phenolates

TABLE 3.2 Strains of bacteria used in this chapter.

Bacteria	Strain	Source[a]
Aeromonas hydrophila [1]	B32	L.A.Rodriguez
Aeromonas hydrophila [1]	B51	L.A.Rodriguez
Aeromonas hydrophila [2]	1108	NCIMB
Aeromonas hydrophila [1]	80-A1	L.A.Rodriguez
Aeromonas sobria [1]	P-261	L.A.Rodriguez
Escherichia coli [1]	9001	NCTC
Escherichia coli [1,3]	50147 (K12 pColV-K270)	NCTC
Enterobacter cloacae [1]	11580	NCTC
Salmonella typhimurium [4]	LT-2 *enb-1*	M.L.Lemos
Salmonella typhimurium [5]	LT-2 *enb-7*	M.L.Lemos
Vibrio anguillarum [6]	2286 (775 pJM1)	NCIMB
Vibrio anguillarum [7]	775-3	M.L.Lemos
Vibrio anguillarum [8]	775-Tn*1*::5 (pJHC91)	M.L.Lemos

[a] L.A.Rodriguez, Microbiologia, Colegio Universitario de Orense, Orense, Spain; National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland; National Collection of Type Cultures, PHLS, London; M.L.Lemos, Departamento de Microbiologia y Parasitologia, Facultad de Biologia, Universidad de Santiago, Santiago de Compostela, Spain.

[1] enterobactin synthesis - positive Arnow assay in ethyl acetate extract of culture supernatant.

[2] amonabactin synthesis - positive Arnow assay in culture supernatant only.

[3] Aerobactin synthesis - positive Csaky assay.

[4] enterobactin$^-$ OMP receptor$^+$

[5] enterobactin$^-$ OMP receptor$^+$.

[6] anguibactin synthesis - positive Arnow assay in culture supernatant only.

[7] anguibactin$^-$ OMP receptor$^-$.

[8] anguibactin$^-$ OMP receptor$^+$.

TABLE 3.3 Specific activity of iron reductase* in iron-replete and iron-restricted 48h culture supernatants of *A.salmonicida* using NADH or NADPH as reductant.

Culture supernatant	NADH Strain of *A.salmonicida* (MT)			NADPH		
	004	423	194	004	423	194
Iron-replete	0.05	0.04	0.00	0.05	0.01	0.00
Iron-restricted	0.45	0.64	0.00	0.00	0.12	0.00

* Expressed as $\frac{\text{nmol}}{\lambda}$ Fe^{2+} formed per mg of protein per 30 min

NADH, β-nicotinamide adenine dinucleotide; NADPH, β-nicotinamide adenine dinucleotide phosphate

TABLE 3.4 Citrate levels in the culture supernatant of two typical and an atypical strain of *A.salmonicida* grown under iron-replete and iron-restricted conditions. Samples were taken at the times indicated.

Growth conditions	MT004 Time (h)			MT423 Time (h)			MT194 Time (h)		
	24	48	72	24	48	72	24	48	72
Iron-replete	<5	75	81	<5	81	87	<5	89	88
Iron-restricted	<5	87	108	<5	87	97	<5	103	83

TABLE 3.5 Summary of the ability of iron-restricted culture supernatants to cross-feed four typical strains and two atypical strains of *A.salmonicida* using a siderophore bioassay. The bioassay involved measuring the zone of growth around a 3mm diameter well containing 30µl sample on a plate containing TSA + 200µg EDDAml^{-1} seeded with 10^5 cfu of the 6 *A.salmonicida* strains. Results are the mean of triplicate values (+/- SE of the mean).

Strain (MT)* incorporated into agar		Diameter of zone of growth (mm) stimulated by iron-restricted culture supernatant of strain					
		004	616	194	525	423	486
004	(T)	6±0.7	0±0.0	0	0	17±3.1	0±0.0
616	(T)	0±0.0	18±0.7	0	0	15±0.8	0±0.0
194	(A)	0±0.0	0±0.0	0	0	15±1.1	0±0.0
525	(A)	0±0.0	0±0.0	0	0	14±0.5	0±0.0
423	(T)	0±0.0	0±0.0	0	0	12±1.7	0±0.0
486	(T)	0±0.0	0±0.0	0	0	10±1.3	17±0.8

* (T), typical; (A), atypical.

TABLE 3.6 Summary of the ability of iron-restricted culture supernatants of *Escherichia coli* and *Enterobacter cloacae* to stimulate growth of six *A.salmonicida* strains growing under conditions of iron-restriction. Results are the mean of triplicate values +/- SE of the mean).

Strain (MT)* incorporated into agar		Diameter of zone of growth (mm) stimulated by iron-restricted culture supernatant of		
		E.coli NCTC9001 [1]	*E.coli* NCTC50147 [2]	*Ent.cloacae* NCTC11580 [1]
004	(T)	4±0.3	2±0.0	5±0.0
616	(T)	4±0.3	4±0.0	6±0.5
194	(A)	5±0.0	2±0.0	4±0.6
525	(A)	4±0.0	3±0.4	3±0.0
423	(T)	5±0.4	4±0.4	5±0.1
486	(T)	5±0.5	3±0.2	4±0.3

[1] Culture supernatant containing enterobactin

[2] Culture supernatant containing enterobactin and aerobactin

* (T), typical; (A), atypical.

TABLE 3.7 Summary of the ability of iron-restricted culture supernatants of *Aeromonas hydrophila*, *Aeromonas sobria* and *Vibrio anguillarum* to stimulate growth of 6 *A.salmonicida* strains grown under conditions of iron-restriction. Results are the mean of triplicate values (+/- SE of the mean).

Strain (MT)* incorporated into agar		Diameter of zone of growth (mm) stimulated by iron-restricted culture supernatant of				
		A.hydrophila			*A.sobria*	*V.anguillarum*
		B32	1108	80-A1	P-261	2286 (775 pJM1)
004	(T)	1±0.0	0	2±0.3	0	0
616	(T)	2±0.1	0	2±0.5	0	0
423	(T)	2±0.3	0	2±0.2	0	0
486	(T)	2±0.1	0	2±0.0	0	0
194	(A)	2±0.0	0	2±0.0	0	0
525	(A)	1±0.0	0	1±0.2	0	0

* (T), typical; (A), atypical.

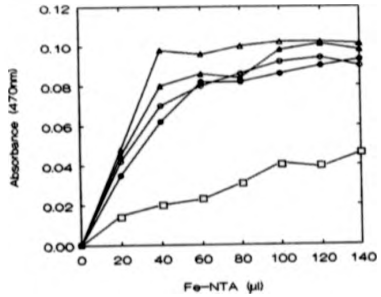
TABLE 3.8 Ability of iron-restricted culture supernatants of typical and atypical strains of *A.salmonicida* to cross-feed siderophore-deficient mutant strains of *Salmonella typhimurium* and *Vibrio anguillarum*.

	Cross-feeding			
	V.anguillarum		*S.typhimurium*	
Strain	H775-3	775::Tn1-5(pJHC91)	*enb-1*	*enb-7*
A.salmonicida MT004	-	-	+	+
A.salmonicida MT616	-	-	+	+
A.salmonicida MT194	-	-	-	-
A.salmonicida MT525	-	-	-	-
A.salmonicida MT423	-	-	+	+
A.salmonicida MT486	-	-	+	+
V.anguillarum 775	-	++	-	-
E.coli NCTC9001	-	-	++	++

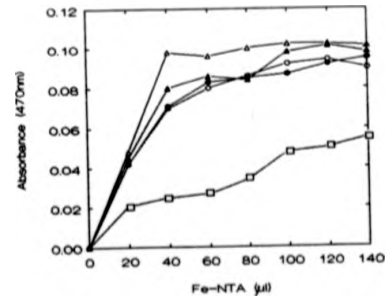
-, no growth; +, 0-5mm growth; ++, 5-10mm growth.

FIGURE 3.1 Effects of culture supernatant from *A.salmonicida* on the binding of iron by transferrin. Culture supernatant and iron-free transferrin were incubated for 1h at 22°C. The binding of iron by transferrin was measured at 470nm after each addition of 20ul Fe-NTA. (●) water; (△) TSB; (▲) TSB+EDDA; (○) culture supernatant from *A.salmonicida*; (□) iron-restricted culture supernatant from *A.salmonicida*.
A.salmonicida strains:
a) MT004, b) MT616, c) MT423, d) MT486, e) MT194, f) MT525.

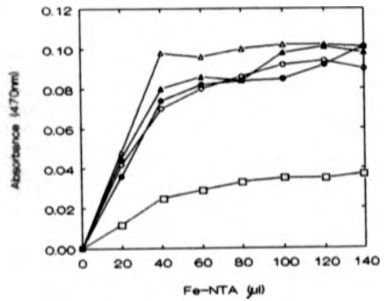
a)


0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance (470nm)
0
20
40
60
80
100
120
140
Fe-NTA (µl)

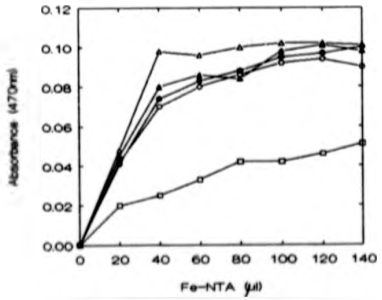
b)


0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance (470nm)
0
20
40
60
80
100
120
140
Fe-NTA (µl)

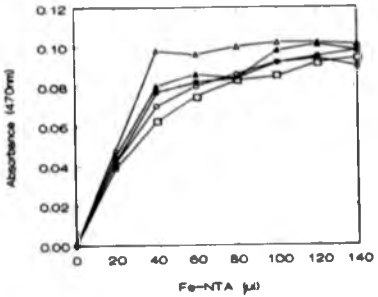
c)


0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance (470nm)
0
20
40
60
80
100
120
140
Fe-NTA (µl)

d)


0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance (470nm)
0
20
40
60
80
100
120
140
Fe-NTA (µl)

e)


0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance (470nm)
0
20
40
60
80
100
120
140
Fe-NTA (µl)

f)

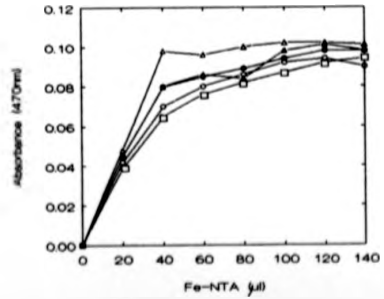

0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance (470nm)
0
20
40
60
80
100
120
140
Fe-NTA (µl)

FIGURE 3.2 Kinetics of siderophore production by *A.salmonicida* MT004 growing under iron-restricted conditions. Growth monitored by absorbance at 540nm (●) and siderophore production monitored by the Arnow assay (1937) (■).

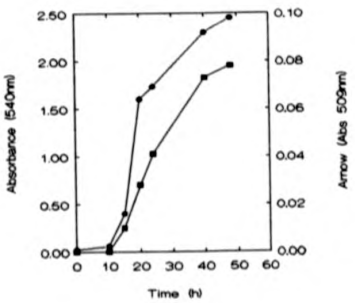
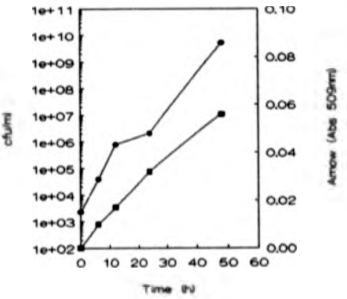

Absorbance (540nm)
Arrow (Abs 509nm)
Time (h)
2.50
2.00
1.50
1.00
0.50
0.00
0.10
0.08
0.06
0.04
0.02
0.00
0
10
20
30
40
50
60

FIGURE 3.3 Kinetics of siderophore production by *A.salmonicida* MT004 growing in heat-inactivated Atlantic salmon sera (a) and rainbow trout sera (b). Growth monitored by absorbance at 540nm (●) and siderophore production monitored by the Arnow assay (1937) (■).

a)


1e+11
1e+10
1e+09
1e+08
1e+07
1e+06
1e+05
1e+04
1e+03
1e+02
cfu/ml
0.10
0.08
0.06
0.04
0.02
0.00
Amow (Abs 509nm)
0 10 20 30 40 50 60
Time (h)

b)

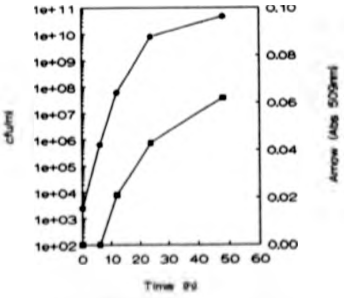
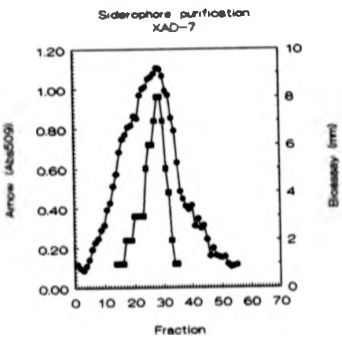

1e+11
1e+10
1e+09
1e+08
1e+07
1e+06
1e+05
1e+04
1e+03
1e+02
cfu/ml
0.10
0.08
0.06
0.04
0.02
0.00
Amow (Abs 509nm)
0 10 20 30 40 50 60
Time (h)

FIGURE 3.4 Partial purification of the *A.salmonicida* MT004 siderophore by adsorption chromatography using a XAD-7 column. Fractions of 2ml were collected, screened using the Arnow assay (1937) (●) and diluted five-fold for the bioassay (■). Fractions 1-10 were collected in 33% (v/v) methanol, fractions 11-21 in 66% (v/v) methanol and fractions 22-55 in 100% (v/v) methanol.


Siderophore purification
XAD-7
Amax (Abs509)
Bioassay (mm)
Fraction
1.20
1.00
0.80
0.60
0.40
0.20
0.00
10
8
6
4
2
0
0 10 20 30 40 50 60 70

CHAPTER 4:- UTILISATION OF TRANSFERRIN BY *AEROMONAS SALMONICIDA*

4.1 INTRODUCTION

Most pathogenic bacteria growing under conditions of iron-restriction *in vitro* possess high affinity iron uptake mechanisms capable of removing iron for growth from Tf and Lf (Griffiths *et al.*, 1988; Martinez *et al.*, 1990). In Chapter 3, it was reported that typical strains of *A.salmonicida* possess an inducible siderophore-mediated iron uptake mechanism and that atypical strains possess an unidentified siderophore-independent mechanism. A second iron uptake mechanism has been described by Chart & Trust (1983) for *A.salmonicida* A449, a typical strain which did not produce a siderophore. This strain, although able to acquire $^{59}Fe^{3+}$ from $^{59}Fe^{3+}$-EDDA by a constitutive mechanism was unable to obtain $^{59}Fe^{3+}$ from $^{59}Fe^{3+}$-Lf contained within a dialysis membrane with a 14kDa MW cut off. This suggests that the strain required a direct cell contact with the Fe^{3+}-Lf complex. The mechanism was constitutive and energy-dependent, in that it was strongly inhibited by the proton gradient uncoupler 2,4-dinitrophenol and was similar to the mechanism of iron uptake described for *N.meningitidis* (Simonson *et al.*, 1982) in that this pathogen also acquires iron by a direct cell contact with transferrin, with the mechanism being energy-dependent.

It is now known that *N.meningitidis* and other non-siderophore producing bacterial pathogens listed in Table 4.1 possess iron uptake mechanisms which enable them to utilise Tf and Lf-bound iron by a direct cell surface binding of Tf or Lf via iron-regulated outer membrane protein receptors which recognise the Fe^{3+}-Tf complex. These surface

receptors are essential in the uptake process since mutational loss of Tf-binding activity is correlated with the inability to acquire Tf-bound iron (Dyer *et al.*, 1987b;1988; Tsai *et al.*, 1988; Holland *et al.*, 1991).

An indication of the effectiveness of Tf-utilisation by direct interaction is demonstrated with the human respiratory pathogen *Bordetella pertussis*. The pathogen although able to utilise Tf and Lf via a direct cell surface interaction (Menozzi *et al.*, 1991; Agiato & Dyer, 1992)) also produces a siderophore (Gorringe *et al.*, 1990). Although both iron uptake mechanisms result in the internalisation of iron, the Tf-binding mechanism is more effective than siderophore-mediated uptake (Redhead & Hill, 1991).

In this chapter the ability of *A.salmonicida* to utilise Tf- and Lf-bound iron was examined and the possible mechanisms which may enable *A.salmonicida* to obtain iron from these sources were investigated.

4.2 MATERIALS AND METHODS

4.2.1 *A.salmonicida* and growth conditions

Four typical and two atypical strains of *A.salmonicida* were used in this study. For the identification of a putative Tf/Lf-binding protein, *A.salmonicida* was grown in TSB + 100µg EDDA ml^{-1}. Iron-replete bacteria were grown in TSB. *A.salmonicida* was incubated at 22°C for 48h on a rotary shaker.

4.2.2 Transferrins

Purified human and bovine Tfs and Lfs were obtained from Sigma and prepared in the iron-free apo-form by the method of Aisen *et al.*, (1978) as described in 2.2.4. ApoTf and Lf were saturated to 30% and 90% by the addition of a calculated amount of ferric citrate and the percentage iron saturation measured by the method of Graham & Bates (1976).

4.2.3 Utilisation of Tf-bound iron

A plate bioassay was used to determine which Tf/Lf sources could supply iron to typical and atypical strains of *A.salmonicida*. Approximately 10^8cfu of bacteria ml^{-1} were seeded into TSA containing 200μg EDDA ml^{-1}. Thirty μl of a 1mgml^{-1} solution of 30% and 90% iron-saturated Tf or Lf in 10mM Tris-HCl (pH7.4) were added to 3mm diameter wells punched in the agar. Plates were incubated for 48h at 22°C and were then examined for stimulation of growth around the wells. ApoTf and Lf and 100μM $FeCl_3$ were included as negative and positive controls respectively.

4.2.4 Siderophore-mediated utilisation of Tf-bound iron

Experiments to determine whether Tf-iron acquisition by typical and atypical strains of *A.salmonicida* was mediated by a siderophore-dependent mechanism or required direct cell surface contact were carried out as described by Morton & Williams (1990). *A.salmonicida* was inoculated into TSB containing 200μg EDDA ml^{-1} with or without 30% iron-saturated bovine or human Tf (1mgml^{-1}). To some

broths, a dialysis bag (Medicell; molecular weight cutoff approximately 10 kDa) containing 1ml bovine Tf (5mg in 1ml) or TSB were added. Bacteria were incubated at 22°C on a rotary shaker and growth monitored by measuring absorbance at 540nm.

4.2.5 Proteolytic degradation of Tf

Experiments to determine whether Tf-iron acquisition by both typical and atypical strains of *A.salmonicida* was mediated by a process involving the proteolytic degradation of Tf were carried out by two methods. One, using dialysis bag experiments, and the second, determining the proteolytic degradation of Tf, by SDS-PAGE using a partially purified preparation of an extracellular protease isolated from *A.salmonicida* MT004.

In the dialysis bag experiments, extracellular products (ECP) of the atypical *A.salmonicida* strain MT525 were prepared by a cellophane overlay technique, and proteolytic and inhibition assays were performed by the methods described by Gudmundsdottir *et al.*, (1990) in order to identify the nature of the proteolytic enzymes present in the ECP. One ml of ECP was incubated with 30% iron-saturated bovine Tf (5mgml^{-1}) at 22°C for 2h and then placed within a dialysis bag and the effects on bacterial growth monitored by measuring absorbance at 540nm.

In experiments to study the proteolytic degradation of Tf by proteolytic enzymes, a partially purified extracellular protease of the typical *A.salmonicida* strain MT004 (fraction 16 FPLC (Pharmacia-LKB) anion exchange chromatography; Lee & Ellis, 1989), (approximately 100ug ml^{-1}) was incubated, volume for volume, with bovine Tf (1mgml^{-1}) at 22°C for 2h and samples examined by SDS-PAGE as

described in 4.2.10.

4.2.6 Preparation of horseradish peroxidase-conjugated Tf/Lf

Horseradish peroxidase-conjugated Tf and Lf (HRP-Tf/Lf) from human and bovine sources was prepared by a two-step glutaraldehyde coupling procedure described by Avrameas *et al.*, (1978). HRP (17mg) was dissolved in 0.34ml 1% (v/v) glutaraldehyde solution in 0.1M phosphate buffer (pH6.8). The preparation was incubated at 22°C for 18h and then filtered through a Sephadex G-25M (PD-10; Pharmacia-LKB)) column equilibrated with 0.15M NaCl. The resultant fractions were collected and pooled in a total volume of 2.4ml. Tf or Lf (1.7ml of a 5mgml^{-1} solution) was added to the peroxidase solution and dialysed against 0.15M NaCl overnight at 4°C. Carbonate-bicarbonate buffer (0.34ml, 0.5M, pH9.5) was added and the preparation stored at 4°C. After 24h, 0.17ml 0.1M lysine (pH7.0) was added. After a further 2h incubation, the preparation was dialysed against PBS overnight at 4°C. The conjugate was then filter-sterilised using a Millipore 0.22µM filter and diluted with an equal volume of glycerol.

4.2.7 Preparation of biotinylated Tf/Lf

Biotinylated Tf and Lf (bio-Tf/Lf) were prepared as described by Morton & Williams (1990). Human and bovine Tf and Lf (1mgml^{-1}) were incubated with 250µg *N*-hydroxysuccinimido-biotin dissolved in 10µl dimethylformamide. After 2h incubation at 4°C, the biotinylation reaction was stopped by the addition of 100µl glycine (10mgml^{-1}). The solution was dialysed and lyophilised. Biotinylated human and bovine Tf

and Lf were used at a concentration of 50ngml^{-1} in the Tf-binding assays.

4.2.8 Tf/Lf-binding assays

A solid-phase dot blot enzyme assay was used to examine the binding of Tf or Lf to *A.salmonicida* as described by Schryvers & Morris (1988a,b). Two ul of a 48h broth culture of iron-replete or iron-restricted whole cells or isolated OMPs (10μgml^{-1}) were spotted onto nitrocellulose membranes (0.45μM;BioRad). Membranes were air-dried at room temperature for 10min, blocked with 3% (w/v) gelatin in Tris-buffered saline (TBS; 50mM Tris-HCl, 150mM NaCl, pH 7.5) and probed with either HRP-Tf/Lf or bio-Tf/Lf followed by peroxidase-labelled streptavidin (1:1000;Sigma). HRP-Tf/Lf or bio-Tf/Lf immobilised on nitrocellulose in place of whole cells or OMPs served as positive controls. Dot-blots were developed with a 4-chloro-1-naphthol/H_2O_2 substrate kit (BioRad).

4.2.9 OMP preparation

Outer membrane proteins were prepared from *A.salmonicida* grown in iron-restricted and iron-replete media.. Cells were harvested from 500ml broth by centrifugation (8000g, 20min at 4°C), washed with 20mM Tris-HCl (pH 7.2), resuspended in 20mM Tris-HCl, 10mM EDTA (pH 7.2) containing 0.1μg DNase and RNase ml^{-1} and phenylmethylsulphonyl fluoride (PMSF; 50μgml^{-1}) and sonicated on ice (6 x 30s). Whole cells and debris were removed by centrifugation (20,000g for 20min at 4°C). Inner membranes were solubilised by addition of Sarkosyl (sodium *N*-lauroyl

sarcosinate) to 1.5% (v/v) as described by Filip *et al.*, (1973) and incubated for 30min at 22°C. Sarkosyl-insoluble outer membrane proteins were collected by centrifugation at 100,000g for 1h at 4°C, washed and suspended in 20mM Tris-HCl and stored at -20°C.

4.2.10 Electrophoresis

Outer membrane proteins and proteolytic digests of Tf (20ug protein ml^{-1}) were incubated with sample buffer (1:2,v/v) comprising 4% (w/v) sodium dodecyl sulphate (SDS) and 5% (v/v) 2-mercaptoethanol in 0.01M Tris-HCl (pH 8.0) for 5min at 100°C. 20µl aliquots were subjected to polyacrylamide gel electrophoresis (PAGE) using a GE-2/4 gel electrophoresis apparatus (Pharmacia-LKB) in 4-30% polyacrylamide gradient gels (PAA 4/30;Pharmacia-LKB) at 150V for 170 min at 10°C. Electrophoresis buffer (pH 7.4) comprised 0.04M Tris, 0.02M sodium acetate, 0.002M EDTA and 0.2% (w/v) SDS.

4.2.11 Western blotting

Outer membrane proteins fractionated by SDS-PAGE were transferred to nitrocellulose (0.45um; BioRad) by electrophoresis at 30V for 16h using a BioRad Trans-blot cell. Transfer buffer comprised 25mM Tris-HCl (pH 8.3), 192mM glycine and 20% (v/v) methanol as described by Towbin *et al.*, (1979). After transfer nitrocellulose membranes were either stained for total protein with colloidal gold (Aurodye, Amersham) or blocked with 1% (w/v) gelatin in TBS and developed as for the Tf-binding assay described in 4.2.8.

4.3 RESULTS

4.3.1 Utilisation of Tf-bound iron

The ability of *A.salmonicida* to obtain iron bound to Tf and Lf was examined using a plate bioassay. Both typical and atypical strains were able to utilise iron bound to human and bovine sources of Tf and Lf at 30% iron saturation, the physiological % saturation of Tf (Bezkorovainy, 1987), and at 90% iron-saturation (Table 4.2). None of the strains examined were able to utilise the iron-free, apo-forms of Tf and Lf, although all of the strains were able to utilise 100μM $FeCl_3$ (data not shown).

4.3.2 Siderophore-mediated utilisation of Tf-bound iron

Growth kinetics of four typical and two atypical strains of *A.salmonicida* in the presence of an inhibitory concentration of EDDA (200μgml^{-1}) are shown in Figure 4.1. In both typical and atypical strains, inhibition of growth imposed by EDDA was reversed to normal by the addition of 100μM $FeCl_3$ (data not shown). The inhibition of growth was also reversed upon the addition of a 30% iron-saturated bovine Tf solution (1mgml^{-1}). However, when the 30% iron-saturated Tf added was contained within a dialysis bag with a 10kDa molecular weight exclusion limit, the growth inhibition imposed on typical strains was reversed while the growth of atypical strains remained inhibited. The presence of the dialysis bag alone containing only growth medium had no effect on the growth of *A.salmonicida* in either iron-replete or iron-restricted media (data not shown). Results of the dialysis bag experiments suggest

that a small MW soluble factor was responsible for the utilisation of Tf-bound iron by typical strains. In contrast, it is possible that atypical strains lacking this soluble factor utilise Tf-bound iron by a mechanism requiring a high MW soluble factor or direct contact between the bacterial cell surface and Tf.

4.3.3 Proteolytic degradation of Tf by ECP and extracellular proteases

The effects of atypical *A.salmonicida* ECP on 30% bovine Tf was examined in order to determine whether Tf-iron acquisition by atypical strains was mediated by a proteolytic degradation of Tf. ECP of *A.salmonicida* MT525 was found to possess proteolytic activity due to the presence of a metalloprotease (Gudmundsdottir *et al.*, 1990). ECP incubated with bovine Tf for 2h and then added to a dialysis bag was found to be capable of reversing the growth inhibition imposed on *A.salmonicida* by the addition of EDDA (Figure 4.2). The ECP of *A.salmonicida* MT525 alone had no effect on growth (data not shown).

Similar experiments to determine whether the proteolytic degradation of Tf by ECP of typical strains of *A.salmonicida* was capable of reversing the growth inhibition imposed by EDDA were not possible due to the presence of soluble siderophores in iron-restricted ECP, capable of reversing growth inhibition. The effects of iron-replete ECP was not examined. As an alternative approach, a partially purified preparation of an extracellular 70 kDa serine protease isolated from *A.salmonicida* MT004 (Lee & Ellis, 1989) was incubated with bovine Tf and then analysed by SDS-PAGE. Although the preparation had undergone partial auto-digestion due to prolonged storage, all the proteolytic activity could be attributed to the serine protease because of total

inhibition by PMSF. Analysis of the digest by SDS-PAGE indicated total digestion of Tf into a number of low molecular weight components (Plate 4.1).

4.3.4 Tf-binding assays

Six strains of *A.salmonicida* expressing the additional iron-regulated outer membrane proteins (IROMPs) described by Chart & Trust (1983) and Aoki & Holland (1985) were used to determine whether Tf or Lf could interact directly with the cell surface of *A.salmonicida* via the IROMPs. A solid phase dot blot enzyme assay was used in which either iron-replete and iron-restricted whole cells or their respective OMPs were immobilised onto nitrocellulose. Membranes were then probed with either HRP-Tf/Lf or bio-Tf/Lf followed by peroxidase-labelled streptavidin to detect either Tf- or Lf-binding activity. Using these binding assays on the four typical and two atypical strains identified in Table 4.2 it was not possible to detect the binding of Tf or Lf to whole cells or OMPs even when the IROMPs of *A.salmonicida* were shown to be present by SDS-PAGE in the preparations (the IROMPs of *A.salmonicida* are studied in further detail in Chapter 6). Similar results were obtained in separate experiments in which iron-replete or iron-restricted OMPs were subjected to SDS-PAGE followed by electroblotting onto nitrocellulose. No Tf- or Lf-binding activity was detected by probing membranes with either HRP-Tf/Lf or biotinylated Tf/Lf followed by streptavidin-peroxidase. HRP-Tf/Lf and biotinylated Tf/Lf immobilised onto nitrocellulose as positive controls gave a positive reaction when stained with 4-chloro-1-naphthol/H_2O_2.

4.4 DISCUSSION

Work confirming an earlier report that certain strains of *A.salmonicida* were able to grow and multiply under conditions of iron-restriction by the production of siderophores was presented in Chapters 2 and 3. Typical strains of *A.salmonicida* were found to possess an inducible siderophore-mediated iron uptake mechanism involving the production of a 2,3-diphenol catechol siderophore. Atypical strains of *A.salmonicida* were found to possess an unidentified siderophore-independent iron uptake mechanism. Chart & Trust (1983) found one typical strain of *A.salmonicida* (A449) to possess a constitutive mechanism not involving the production of a siderophore. The strain was unable to remove $^{59}Fe^{3+}$ from Lf-bound $^{59}Fe^{3+}$ across a dialysis membrane and suggested a direct interaction between Lf and the bacterial cell surface. Since a number of bacterial pathogens (listed in Table 4.1) are able to acquire iron bound to Tf via direct cell surface interactions without the production of siderophores, the aim of the present study was to examine the ability of typical and atypical strains to utilise Tf or Lf and to determine the mechanism of Tf-utilisation.

Using a plate bioassay it was demonstrated that both typical and atypical strains of *A.salmonicida* were able to utilise Tf- and Lf-bound iron from a variety of mammalian sources and to utilise the iron-binding proteins at physiological levels of iron-saturation found *in vivo*. The ability of *A.salmonicida* to utilise Tf and Lf from diverse mammalian sources differed from many of the bacterial pathogens capable of utilising Tf via direct cell surface interactions in that they are only able to effectively utilise host Tf as a source of iron for growth

(Schryvers & Gonzalez, 1990). This indicates that *A.salmonicida* has little or no species specificity in the source of Tf utilised.

Using dialysis bag experiments, the mechanism of Tf utilisation by *A.salmonicida* was examined. The inhibition of growth imposed on typical and atypical strains by the addition of 200µg EDDA ml^{-1} to the growth medium could be reversed by the addition of a 30% iron-saturated bovine Tf solution (1mgml^{-1}). When the same solution was contained within a dialysis bag with a molecular weight exclusion limit of 10kDa, the growth inhibition of typical strains was still reversed, but growth of atypical strains remained inhibited. This observation confirms the ability of typical strains to utilise Tf-bound iron by a siderophore-mediated mechanism and is similar to the mechanism of Tf-iron acquisition described for motile *Aeromonas* species (Massad *et al.*, 1991). Siderophores being soluble and of low molecular weight (500-1000Da) have the ability to pass through a dialysis membrane to form complexes with iron. The inability of atypical strains to utilise Tf contained within a dialysis bag suggests a mechanism involving direct cell surface contact or some other mechanism. A solid phase dot blot enzyme assay was used to determine if Tf utilisation by atypical strains involved Tf-binding. Whole cells or isolated OMPs immobilised onto nitrocellulose were tested for their ability to bind HRP-Tf/Lf or bio-Tf/Lf from different mammalian sources. Neither typical nor atypical strains (iron-replete or iron-restricted) were found to bind labelled Tf/Lf indicating lack of a cell surface receptor specific for mammalian Tf/Lf. In addition, OMPs subjected to SDS-PAGE and Western blotting and then probed with conjugated Tfs were unable to bind labelled conjugates. These techniques, used to identify the specific cell surface Tf/Lf receptors in *H.influenzae*, *H.pleuropneumoniae*,

N.gonorrhoeae, *P.haemolytica* and *P.multocida*, failed to identify such a receptor in *A.salmonicida*. The IROMPs of *A.salmonicida* were shown by SDS-PAGE to be present in the iron-restricted OMP and whole cell preparations examined for the ability to bind Tf and Lf. This indicates that the IROMPs are not involved in the binding of Tf or Lf.The inability of *A.salmonicida* to specifically bind Tf via a cell surface interaction suggests another similarity with the motile *Aeromonas* species which lack an uptake sytem for the direct use of Tf-bound iron (Massad *et al.*, 1991) although recent reports by Kishore *et al.*, (1991) and Ascencio *et al.*, (1992) indicate that *A.hydrophila* is able to bind human Lf via a specific interaction with an OMP receptor.

An additional mechanism involved in the utilisation of Tf-bound iron by pathogenic bacteria has been described by Griffiths (1987b) which involves the proteolytic degradation of Tf and disruption of the iron-binding site to release Fe^{3+}. Such a mechanism has been described by Döring *et al.*, (1988) for *Pseudomonas aeruginosa* in which only in the presence of the secreted proteolytic enzyme elastase can the *Ps.aeruginosa* siderophore, pyoverdin, facilitate iron release and uptake from Tf. Since both typical and atypical strains of *A.salmonicida* produce extracellular proteases which are regarded as important virulence factors in the pathogenesis of *A.salmonicida* infection (Ellis, 1991), the possibility that proteolytic degradation of Tf may enable typical and atypical strains to utilise Tf-bound iron was investigated.

Experiments examining this ability of extracellular products of a typical strain, and of a partially purified extracellular protease of a typical strain to degrade Tf, indicated that proteolytic degradation of Tf as a mechanism of Tf-iron acquisition by *A.salmonicida* was possible. In atypical strains, ECP containing a metalloprotease was able to

interact with Tf contained within a dialysis bag and reverse the growth inhibition imposed on atypical *A.salmonicida* by the addition of EDDA; Tf or ECP alone contained within the dialysis bag did not reverse the growth inhibition. Similarly, an extracellular serine protease produced by typical strains was found to be capable of degrading Tf, resulting in several low molecular weight components. It is not known whether the proteolytic degradation described in this study resulted in the disruption of iron-binding sites with the release of Fe^{3+}. It may be the case, as with the elastase produced by *Ps.aeruginosa*, that the serine protease facilitates the release of Fe^{3+} from Tf, enabling uptake by the siderophore produced by typical strains of *A.salmonicida*. However, the experiments with atypical strains suggest that factors in the ECP can release iron from Tf making it available for uptake in the absence of siderophores.

TABLE 4.1 Transferrin utilisation by pathogenic bacteria.

Bacteria		Ref
A.hydrophila	Lf-binding	Kishore *et al.*,(1991) Ascencio *et al.*,(1992)
B.bronchiseptica	Tf/Lf-binding	Menozzi *et al.*,(1991)
B.pertussis	Tf utilisation	Redhead & Hill (1991)
	Tf/Lf-binding	Redhead *et al.*,(1987)
	Tf/Lf-binding*	Menozzi *et al.*,(1991)
Branhamella catarrhalis	Tf/Lf-binding*	Schryvers & Lee (1989)
H.influenzae	Tf/Lf-binding*	Schryvers (1988;1989)
	Tf-binding*	Morton & Williams (1990)
H.pleuropneumoniae	Tf utilisation	Niven *et al.*,(1989) Morton & Williams (1989) Morton & Williams (1990)
	Tf-binding*	Gonzalez *et al.*,(1990) Ricard *et al.*,(1991) Gerlach *et al.*,(1992)
H.somnus	Tf-binding*	Ogunnariwo *et al.*,(1990)
Mycoplasma pneumoniae	Lf utilisation	Tryon & Baseman (1987)
N.gonorrhoeae	Tf/Lf-binding*	Lee & Schryvers (1988) Blanton *et al.*,(1990)
N.meningitidis	Tf-binding*	Schryvers & Morris (1988a)
	Lf-binding*	Schryvers & Morris (1988b)
P.haemolytica	Tf-binding*	Ogunnariwo & Schryvers (1990)
P.multocida	Tf-binding*	Ogunnariwo *et al.*,(1991)
Staph.aureus	Lf-binding	Naidu *et al.*,(1991)
Staph.epidermidis	Lf-binding	Naidu *et al.*,(1990)

* putative Tf/Lf receptor identified and characterised in these species.

TABLE 4.2 Utilisation of transferrins by *Aeromonas salmonicida*.

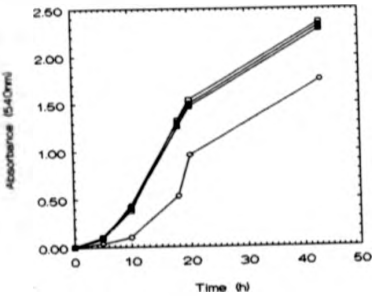
Strain**	Utilisation of*							
	HTf		HLf		BTf		BLf	
	% saturation							
	30	90	30	90	30	90	30	90
MT004 (T)	6±0.8	22±1.3	6±0.5	23±2.0	7±0.7	24±0.8	5±0.4	23±2.0
MT616 (T)	7±0.2	21±1.7	6±0.0	22±1.6	8±1.0	24±0.9	6±0.2	21±2.3
MT423 (T)	7±0.3	21±1.8	6±0.2	23±1.6	8±0.9	23±1.1	6±0.3	20±0.9
MT486 (T)	9±1.0	23±2.3	6±0.3	22±1.9	8±0.9	19±1.1	7±0.0	21±1.4
MT194 (A)	4±0.0	23±1.9	5±0.3	23±1.7	5±0.2	20±1.2	4±0.0	20±1.6
MT525 (A)	4±0.2	21±2.0	4±0.2	23±1.7	6±0.3	18±1.4	4±0.4	17±1.8

* Utilisation of transferrins was determined by measuring the zone of growth around a 3mm diameter well containing 30µl Tf or Lf on a plate containing TSA+200µg EDDA ml^{-1} seeded with 10^8 cfu s of 1 of 6 *A.salmonicida* strains; H/BTf30, H/BTf90, human/bovine transferrin saturated to 30% and 90% with FeCl3 respectively; H/BLf30, H/BLf90, human/bovine lactoferrin saturated to 30% and 90% with FeCl3 respectively. Results are the means of triplicate values (+/- SE of the mean).

** (T), typical; (A), atypical

FIGURE 4.1 Growth kinetics of *A.salmonicida* in Tryptone Soya Broth (TSB)(●), TSB+ethylene diamine di (*o*-hydroxyphenylacetic acid)(EDDA) (200μgml^{-1}) (○), TSB+EDDA+30% iron-saturated bovine transferrin (30% BTf) (1$mgml^{-1}$)(■), TSB+EDDA+30% BTf in dialysis bag (5$mgml^{-1}$)(□). Samples were taken at the times indicated and absorbance at 540nm measured.
A.salmonicida strain: a) MT004, b) MT616, c) MT423, d) MT486, e) MT194, f) MT525.

a)


Absorbance (540nm)
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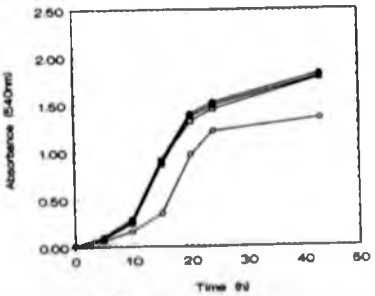
1.50
1.00
0.50
0.00
0
10
20
30
40
50
Time (h)

b)


Absorbance (540nm)
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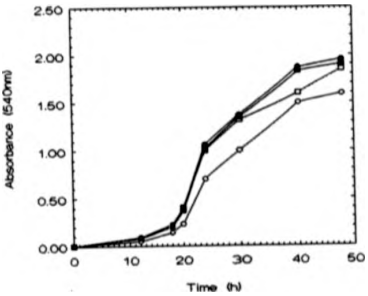
2.00
1.50
1.00
0.50
0.00
0
10
20
30
40
50
Time (h)

c)


Absorbance (540nm)
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10
20
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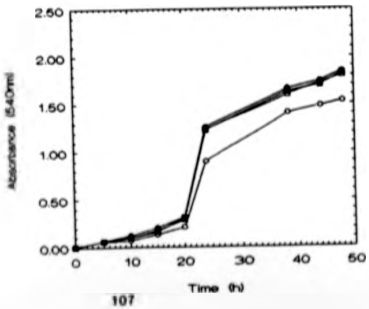
40
50
Time (h)

d)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20
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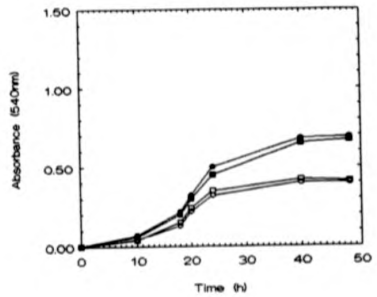
40
50
Time (h)

e)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Absorbance (540nm)
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Time (h)

f)

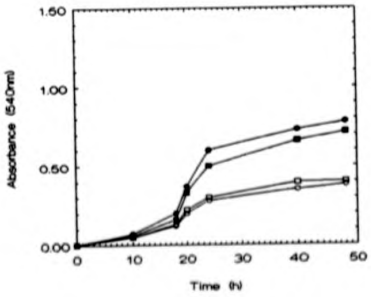

1.50
1.00
0.50
0.00
Absorbance (540nm)
0
10
20
30
40
50
Time (h)

FIGURE 4.2 Growth kinetics of *A.salmonicida* MT525 in Tryptone Soya Broth + ethylene diamine di (*o*-hydroxyphenylacetic acid) (TSB+EDDA) (200μgml^{-1}) (○), TSB+EDDA+30% iron-saturated bovine transferrin (30% BTf) (1mgml^{-1}) (■), TSB+EDDA+30% BTf in dialysis bag (5mgml^{-1})(□), TSB+EDDA+30% BTf+extracellular products (ECP) in dialysis bag (▲). Samples were taken at the times indicated and absorbance at 540nm measured.

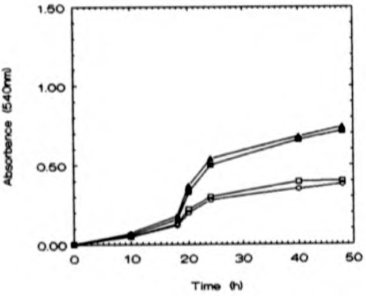
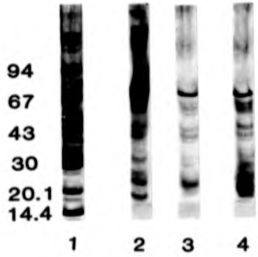

1.50
1.00
0.50
0.00
Absorbance (540nm)
0
10
20
30
40
50
Time (h)

PLATE 4.1 SDS-PAGE of proteolytic digest of transferrin. Proteins were detected by Western blotting and colloidal gold stain. Lane 1, low molecular weight protein standards in kDa; Lane 2, bovine transferrin; lane 3, partially purified 70kDa protease; lane 4, proteolytic digestion of transferrin.


94
67
43
30
20.1
14.4
1
2
3
4

CHAPTER 5:- UTILISATION OF HAEM COMPOUNDS BY *AEROMONAS SALMONICIDA*

5.1 INTRODUCTION

Typical and atypical strains of *A.salmonicida* have developed a variety of high affintity iron uptake mechanisms, such as the production of siderophores and the utilisation of transferrin-bound iron by proteolytic degradation to ensure survival under iron-restricted conditions. However, much of the vertebrate iron is found intracellularly as haem and haemoglobin and is only accessible through tissue damage and cell lysis by bacterial cytolysins and haemolysins. The production of haemolysins and the utilisation of haem sources of iron have been shown to contribute to virulence for a number of bacterial pathogens (Waalwijk *et al.*, 1983; Lebek & Greunig, 1985; Stoebner & Payne, 1988). When released, free haemoglobin is rapidly complexed with the circulating serum protein haptoglobin and quickly taken up by the reticuloendothelial system. Free haem is likewise rapidly bound to the serum protein haemopexin and cleared from the circulation by hepatic parenchymal cells. Excess haem is bound to serum albumin to form methaemalbumin which then becomes complexed with apohaemopexin. All of these proteins function in host defence by withholding iron (Bullen, 1981) although haemoglobin or haem may provide iron for bacterial growth if present in concentrations which exceed the binding capacity of haptoglobin, haemopexin or albumin.

A number of pathogens have an absolute requirement for haem, for example, *H.influenzae* (Coulton & Pang, 1983) and many bacterial pathogens (See Table 5.1) possess the ability to utilise haem sources of

iron, though the mechanisms of haem-utilisation are largely unknown. Although the formation of haem-complexes prevents the use of haem compounds by certain pathogens (Eaton *et al.*, 1982; Dyer *et al.*, 1987a), a number of bacteria are able to utilise haem complexed with host haem-binding proteins as a source of iron when growing under conditions of iron-restriction (Helms *et al.*, 1984; Stull, 1987; Zakaria-Meehan *et al.*, 1988; Lee, 1991; Massad *et al.* 1991).

It is well established that *A.salmonicida* is a successful systemic pathogen of salmonid fish, capable of rapid growth *in vivo* producing an acute haemorrhagic septicaemia often associated with liquefaction of host tissues. A lethal toxin of *A.salmonicida* has recently been identified with haemolytic, leukocytolytic and cytotoxic activity (Lee & Ellis, 1990) which acts in concert with a 70kDa protease (Lee & Ellis, 1989) to produce much of the pathology associated with *A.salmonicida* infection. However, another virulence determinant is the 49kDa A-layer surface protein (Munn *et al.* 1982; Trust *et al.* 1983) which among other properties, is capable of binding the aromatic sulphonated diazo dye, congo red and a variety of haem compounds (Ishiguro *et al.* 1985; Kay *et al.* 1985). The association of a congo red binding phenotype (crb^+) and virulence has been described for a number of pathogens including *E.coli* and *Shigella flexneri* (Stugard *et al.* 1989) and is often associated with the ability to use haem as a source of iron (Daskaleros & Payne, 1987; Deneer & Potter, 1989a). The aim of the present work was to study the ability of *A.salmonicida* to utilise haem sources of iron and to investigate the role of the A-layer protein and the iron-regulated outer membrane proteins (IROMPs) of *A.salmonicida* in haem utilisation.

5.2 MATERIALS AND METHODS

5.2.1 *A.salmonicida* and growth conditions

Two typical strains of *A.salmonicida* (MT004 & MT423) and two atypical strains (MT194 & MT534) were used in this study. *A.salmonicida* MT004 and MT534 are non-autoagglutinating, lacking the 49kDa A-layer protein (A^-) as determined by SDS-PAGE of outer membrane protein (OMP) preparations (See 6.3.1) and *A.salmonicida* MT423 and MT194 were autoagglutinating and possessed the A-layer protein (A^+). Bacteria were grown on Tryptone Soya Agar (TSA;Oxoid) at 22°C. Iron-restricted bacteria were grown on TSA + 100µg iron-free EDDA ml^{-1}.

5.2.2 Preparation of haem compounds

Atlantic salmon haemoglobin (prepared by lysis of Atlantic salmon erythrocytes using 8mM $MgCl_2$, 5mM imidazole at pH 8.0), haem (equine heart microperoxidase), haemoglobin (sheep and human), myoglobin (horse skeletal muscle), bovine haemin, protoporphyrin IX (P_{IX}) and cytochrome c (all from Sigma) used at a concentration of $1mgml^{-1}$ in phosphate-buffered saline (PBS). Haem, haemin, P_{IX} and cytochrome c were dissolved in a minimal volume of 0.2N NaOH prior to use. Haem-albumin was prepared by addition of bovine serum albumin (BSA)($1mgml^{-1}$) to haem ($0.5mgml^{-1}$) and human haemoglobin-haptoglobin was prepared by the addition of haptoglobin ($1mgml^{-1}$) to haemoglobin ($0.5mgml^{-1}$). The complexes were saturated at 50% capacity by incubation at 22°C for 20min, assuming a 1:1 stoichiometry of binding of haem to BSA, and of haemoglobin to haptoglobin (Lee, 1991).

5.2.3 Determination of iron present in haem compounds

The amount of iron present in the haem compounds was determined by the method of Cameron (1970). The sample or standard (200µl) was reduced by evaporation and then digested twice with 200µl 70% (v/v) perchloric acid for 30min at 100°C, followed by 200µl 30% (v/v) hydrogen peroxide for 30min at 100°C. The mixture was cooled to 22°C and iron reduced to the ferrous form by the addition of 100µl 10% (w/v) hydroxylammonium chloride. After 15min, 1ml 0.5% (v/v) 1,10 phenanthroline in 50% (v/v) ethanol and 1ml pyridine were added and the reaction volume made up to 10ml with ddH_2O and the absorbance at 510nm was compared to that of the iron standard.

5.2.4 Haem source assays

A plate bioassay was used to determine which haem sources could supply iron to typical and atypical strains of *A.salmonicida* grown under iron-replete or iron-restricted conditions. Approximately 10^5 cfu s iron-restricted bacteria ml^{-1} were seeded into TSA or TSA containing 100µg EDDA ml^{-1}. The haem sources (30µl, 1 $mgml^{-1}$) were added to 3mm diameter wells punched in the agar. Plates were incubated for 48h at 22°C and were then examined for stimulation of growth around the wells.

5.2.5 Haem-binding assays

48h cultures of iron-replete and iron-restricted *A.salmonicida* were washed and resuspended in PBS to an absorbance of 1.0 at 540nm (approximately 10^8cfuml^{-1}). Thirty μgml^{-1} congo red (CR), haem, Prx, haemoglobin or 40μgml^{-1} haemin were added separately to the bacterial suspensions and incubated at 22°C for 60-80min. At 20min intervals, bacteria were pelleted by centrifugation for 1min in an Eppendorf centrifuge. Binding of CR and haem compounds was determined by measuring the absorbance of the supernatant spectrophotometrically at 480nm for CR, 580nm for haem, 460nm for Prx, 430nm for haemoglobin and 400nm for haemin.

5.2.6 Competition binding assays

Competition binding assays were performed by incubating bacteria in the presence of 30μg CR ml^{-1} for 1h at 22°C. Bacteria were then pelleted by centrifugation and resuspended in PBS to which 30μg haem source or 40μg haemin were added. The amount of haem source bound over a 60-80min incubation period was determined spectrophotometrically. The binding specificity of the haem compounds was examined by incubation of bacteria in 1mgml^{-1} bovine transferrin (Tf) in PBS for 1h at 22°C prior to incubation with haem compounds.

5.2.7 Effect of trypsin on haem-binding

To determine whether CR or haem-binding was cell-surface associated, bacterial suspensions were incubated with 50μgml^{-1} trypsin

before or after the binding assays. Trypsin inhibited by phenylmethylsulphonyl fluoride (PMSF;50μgml^{-1} in propan-2-ol) was added as a negative control.

5.3 RESULTS

5.3.1 Haem utilisation

The ability of A$^+$ and A$^-$ strains of *A.salmonicida* grown under iron-replete or iron-restricted conditions to utilise a variety of haem sources and haem-complexes containing similar amounts of iron (Table 5.2) were examined using a plate bioassay (Table 5.3 & 5.4). Only A$^+$ strains possessing the 49kDa A-layer protein were able to utilise haem sources as a source of iron both under iron-replete and iron-restricted conditions, though iron-restricted bacteria utilised haem compounds to a greater extent (Table 5.3). In addition, both typical and atypical strains (regardless of culture conditions) were able to utilise haem to a similar extent, indicating that haem utilisation appears to be independent of siderophore production, since siderophore production can only be demonstrated in typical strains of *A.salmonicida* (See Chapter 3).

5.3.2 Haem-binding

The ability of A$^+$ and A$^-$ strains of *A.salmonicida* to bind haem was examined using the binding assay for CR described by Kay *et al.* (1985), replacing CR with a variety of haem sources tested in the plate bioassays. The haem-binding assays confirmed the results of the plate

bioassays in that only A* strains (both typical and atypical) were capable of binding haem sources of iron (Figure 5.1).

5.3.3 Effect of iron-restriction on haem-binding

Outer membrane protein profiles of *A.salmonicida* grown under iron-replete or iron-restricted conditions were examined by SDS-PAGE to confirm the presence or absence of the 49kDa A-layer protein (See Chapter 6). OMPs prepared from iron-restricted bacteria contained additional iron-regulated outer membrane proteins (IROMPs) described by Chart & Trust (1983) and Aoki & Holland (1985) and indicated that the A-layer protein was not an iron-regulated protein (See Chapter 6). Haem-binding by A* strains grown under iron-replete or iron-restricted conditions were identical, indicating that the IROMPs of *A.salmonicida* were not involved in haem-binding (Figure 5.1).

5.3.4 Inhibition of haem-binding by CR

Competition binding assays were performed with A* strains of *A.salmonicida* to examine if the sources of haem examined in this study shared the same binding site or receptor as the haems and porphyrins studied previously (Kay *et al.* 1985) (Figure 5.2). A* strains pre-incubated with 30μg CR ml^{-1} for 1h were subsequently unable to bind any of the haem sources used in this study, indicating that these compounds shared the same binding sites as CR. Pre-incubation of *A.salmonicida* cells with Tf prior to haem-binding assays indicated that *A.salmonicida* was able to specifically bind haem compounds (data not shown).

5.3.5 Effect of trypsin on haem-binding

A-layer-proficient (A$^+$) strains of *A.salmonicida* pre-treated with trypsin prior to the haem-binding assays were unable to bind either haem or CR in the subsequent binding assay (Figure 5.3). With strains pre-incubated with haem or CR followed by trypsin digestion there was a concomitant release of approximately 80% of the pre-bound haem or CR. Cells treated with trypsin+PMSF were able to bind haem or CR normally, indicating haem and CR binding is mediated by a cell-surface exposed binding site or receptor.

5.4 DISCUSSION

Haemolytic and proteolytic damage of host tissue may play an important role in the pathogenesis of *A.salmonicida* infection by the liberation of intracellular iron stores for use by the organism during growth *in vivo*. Although *A.salmonicida* is known to possess several iron uptake mechanisms capable of utilising extracellular iron sources (See Chapters 3 & 4) its ability to utilise intracellular sources of iron such as haem and haemoglobin has not been hitherto reported. However, it is known that the fastidious growth of atypical strains on normal laboratory media can be improved when a source of haem is added (Ishiguro *et al.*, 1986). Since typical strains of *A.salmonicida* are known to produce a potent cytolysin capable of lysing salmonid erythrocytes (Lee & Ellis, 1989; 1990), and previous studies have demonstrated the ability of *A.salmonicida*, possessing the 49kDa A-layer protein, to bind congo red, and a number of haem

compounds (Kay *et al.*, 1985), the ability to utilise haem sources of iron under conditions of iron-restriction was investigated.

In common with a number of bacterial pathogens listed in Table 5.1, *A.salmonicida* was able to utilise a variety of haem sources representative of the differing physiological functions of haem, for example, oxygen and electron transport. In addition, *A.salmonicida* was able to utilise Pix, which lacks an iron atom, for growth indicating that *A.salmonicida* is capable of incorporating iron, present as contaminating iron present in the iron-restricted media, into Pix to form haem. This ability is similar to *H.influenzae* which possesses the enzyme ferrochelatase which is capable of haem formation from Pix (White & Granick, 1963). In addition, *A.salmonicida* was found to be able to use haem complexed to the serum proteins albumin and haptoglobin, an important iron witholding defence mechanism against a number of pathogens, for instance, *E.coli* (Eaton *et al.*, 1982) but not for pathogens such as *Vibrio vulnificus* whose virulence is increased in the presence of haem-complexes (Helms *et al.*, 1984; Zakaria-Meehan *et al.*, 1988). Although the mechanism of haem and haem-complex utilisation is not clear, it is known that *Porphyromonas gingivalis* is capable of degrading haem-complexes by a trypsin-like enzyme activity (Carman *et al.*, 1990). Since *A.salmonicida* is able to utilise Tf-bound iron by a process involving the proteolytic degradation of Tf (See Chapter 4) it is interesting to speculate that the proteases of *A.salmonicida* may also be involved in the utilisation of haem compounds. It is possible that the extensive proteolyic capability of *A.salmonicida* may contribute to the degradation of these host haem-sequestering proteins and the liberation of haem compounds.

The ability of *A.salmonicida* to bind haem sources of iron was found

to be related to the possession of the 49kDa A-layer surface array protein, confirming a previous report (Ishuiguro *et al.*, 1985). In the present study only A$^+$ autoagglutinating strains were able to bind and utilise haem sources. The A-layer is considered to be an important virulence factor in conferring resistance to serum killing (Munn *et al.*, 1982). The present work suggests that a further role of the A-layer may be the utilisation of intracellular stores of iron in the fish host. Trypsin treatment of whole cells essentially eliminated the ability of A$^+$ cells to bind haem sources suggesting that a cell-surface associated protein is involved as a receptor or binding site in haem uptake. Competition binding experiments using a number of haem compounds indicated that the haem compounds share the same receptor/binding protein as CR (Ishiguro *et al.*, 1985; Kay *et al.*, 1985) and that haem-binding was via a specific process.

Comparison of haem-binding by iron-replete and iron-restricted cells indicated no differences in the quantity of haem bound suggesting that haem-binding is via a constitutive mechanism and that the additional iron-regulated outer membrane proteins (IROMPs) expressed by iron-restricted cells are not involved in haem-binding. This is in contrast to the iron-regulated haem-binding OMPs of *S.flexneri* and *E.coli* involved in the uptake and binding of haem (Stugard *et al.*, 1989) and a number of putative haem-binding proteins induced under conditions of haem- or iron-restriction (Coulton & Pang, 1983; Daneer & Potter, 1989a; Carman *et al.*, 1990; Otto *et al.*, 1990; Lee, 1992). In addition, SDS-PAGE of OMP preparations of iron-replete and iron-restricted cells confirmed the earlier work of Kay *et al.*, (1985) in that the 49kDa A-layer surface array protein is not an iron-regulated protein.

Lack of siderophore production by atypical strains of *A.salmonicida*

allowed a comparison of haem utilisation by siderophore-positive typical strains and siderophore-negative atypical strains in order to determine if haem-utilisation was siderophore-mediated. As with previous studies using siderophore-deficient mutants of *V.anguillarum* (anguibactin-negative) (Mazoy & Lemos, 1991), *V.cholerae* (vibriobactin-negative)(Stoebner & Payne, 1987), motile *Aeromonas* species (amonabactin-negative)(Massad *et al.* 1991) and *S.flexneri* (aerobactin-negative)(Lawlor *et al.*, 1987), utilisation of haem was independent of siderophore production.

In conclusion, *A.salmonicida* was found in this study to be able to overcome conditions of iron-restriction induced *in vitro* by the utilisation of various haem sources of iron such as haem, haemin and haemoglobin, and by utilising haem complexes such as haemoglobin-haptoglobin and haem-albumin. The mechanism of haem-binding was constitutive and was related to the possession of the 49kDa A-layer protein. Competitive binding studies showed the same receptor to be involved in binding all the haem compounds tested. The haem-binding mechanism was not iron-regulated and was found to operate independently of the siderophore-mediated iron acquisition mechanism possessed by the typical strains of *A.salmonicida*.

TABLE 5.1 Haem-utilisation by bacterial pathogens.

Bacteria	Reference
A.hydrophila	Massad *et al.*, (1991)
B.gingivalis	Barua *et al.*, (1990)
*E.coli**	Stugard *et al.*, (1989)
H.ducreyi	Lee (1991)
H.influenzae	Coulton & Pang (1983)
*H.pleuropneumoniae**	Deneer & Potter (1989a)
P.gingivalis	Bramanti & Holt (1991)
*S.flexneri**	Stugard *et al.*, (1989)
V.anguillarum	Mazoy & Lemos (1991)
V.cholerae	Stoebner & Payne (1988)
V.vulnificus	Helm *et al.*, (1984)

* Haem-utilisation associated with congo-red binding phenotype (crb+)

TABLE 5.2 Iron content of haem compounds determined by the assay of Cameron (1970) expressed as μg Fe^{3+} per mg haem compound. Results are the mean of triplicate values (+/- SE of the mean).

Haem source	μg Fe^{3+} per mg haem
Haemin	8.75±1.3
Cytochrome c	11.25±2.7
Haemoglobin (Sheep)	4.75±2.0
Haemoglobin (Human)	13.00±3.4
Haem	4.00±1.6
Protoporphyrin IX	0.00±0.0
Myoglobin	4.50±1.2
Human Haemoglobin: Haptoglobin Haem:Albumin	6.00±0.8 4.75±0.6

TABLE 5.3 Utilisation of haem compounds by *Aeromonas salmonicida* growing under iron-restricted conditions.

	Utilisation of*							
Strain	Hm	H	H-Alb	Myo	Hb**	Hb-Hp	Pix	Cytc
MT004 (A⁻T)	0	0	0	0	0	0	0	0
MT534 (A⁻A)	0	0	0	0	0	0	0	0
MT423 (A⁺T)	26±2.3	24±1.8	23±2.6	11±0.9	20±1.5	17±2.0	30±2.3	14±1.1
MT194 (A⁺T)	27±2.2	27±1.6	21±0.6	9±0.2	26±1.7	21±1.5	24±2.2	7±0.2

* Utilisation of haem compounds was determined by measuring the zone of growth around a 3mm diameter well containing 30µl haem compound on a plate containing TSA + 100µg EDDA ml^{-1} seeded with 10^5 cfu each of four *A.salmonicida* strains. Results are the means of triplicate values (+/- SE of the mean).

** Human, sheep and salmon haemoglobin were all utilised by *A.salmonicida* to the same extent.

A⁻, non-autoagglutinating, A-layer negative; A⁺, autoagglutinating, A-layer positive; T, typical strain; A, atypical strain; Hm, haemin; H, haem; H-Alb, haem-albumin; Myo, myoglobin; Hb, human, sheep or salmon haemoglobin; Hb-Hp, human haemoglobin-haptoglobin; Pix, protoporphyrin IX; Cytc, cytochrome c.

TABLE 5.4 Utilisation of haem compounds by *Aeromonas salmonicida* growing under iron-replete conditions.

	Utilisation of*							
Strain	Hm	H	H-Alb	Myo	Hb**	Hb-Hp	Pix	Cytc
MT004 (A^-T)	0	0	0	0	0	0	0	0
MT534 (A^-A)	0	0	0	0	0	0	0	0
MT423 (A^+T)	20±2.0	18±2.5	19±1.5	10±2.0	15±1.0	13±2.0	26±2.3	10±0.3
MT194 (A^+A)	22±1.0	23±1.9	20±0.4	8±0.3	22±1.1	19±1.0	20±1.4	5±0.1

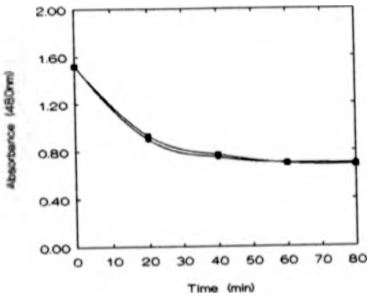
* Utilisation of haem compounds was determined by measuring the zone of growth around a 3mm diameter well containing 30µl haem compound on a plate containing TSA seeded with 10^5 cfu each of four *A.salmonicida* strains. Results are the means of triplicate values (+/- SE of the mean).

** Human, sheep and salmon haemoglobin were all utilised by *A.salmonicida* to the same extent.

A^-, non-autoagglutinating, A-layer negative; A^+, autoagglutinating, A-layer positive; T, typical strain; A, atypical strain; Hm, haemin; H, haem; H-Alb, haem-albumin; Myo, myoglobin; Hb, human, sheep or salmon haemoglobin; Hb-Hp, human haemoglobin-haptoglobin; Pix, protoporphyrin IX; Cytc, cytochrome c.

FIGURE 5.1 Binding of congo red by A$^+$ and A$^-$ strains of *A.salmonicida* grown under iron-replete conditions (●) or iron-restricted conditions (■) measured spectrophotometrically (absorbance at 480nm) as a function of time. Uptake of congo red expressed as the depletion of the dye from solution. Similar binding patterns were obtained for haem-, haemin-, protoporphyrin IX- and haemoglobin-binding. a) *A.salmonicida* MT423 (A$^+$); b) *A.salmonicida* MT004 (A$^-$).

a)


2.00
1.60
1.20
0.80
0.40
0.00
Absorbance (480nm)
0
10
20
30
40
50
60
70
80
Time (min)

b)

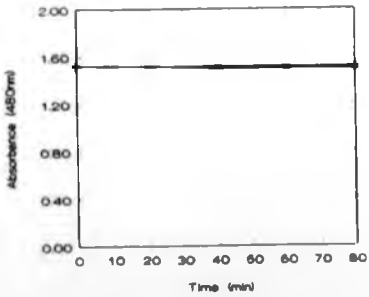

2.00
1.60
1.20
0.80
0.40
0.00
Absorbance (480nm)
0
10
20
30
40
50
60
70
80
Time (min)

FIGURE 5.2 Competition binding between haem and congo red by *A.salmonicida* MT423. Cells were allowed to pre-bind congo red for 1h and the haem-binding activity determined as above. (●) cells pre-bound with congo red; (O) control.

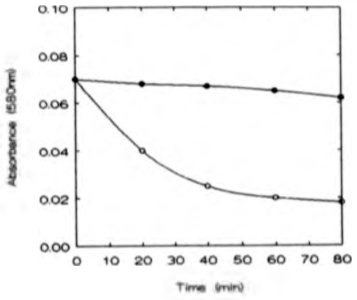
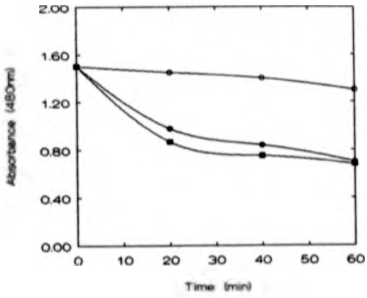

0.10
0.08
0.06
0.04
0.02
0.00
Absorbance (550nm)
0 10 20 30 40 50 60 70 80
Time (min)

FIGURE 5.3 Effect of trypsin on congo red binding by *A.salmonicida* MT423. Binding of congo red measured spectrophotometrically by absorbance at 480nm as a function of time after whole cells were pre-treated with trypsin (O) or trypsin + phenylmethylsulphonyl fluoride (●); Control cells (■).


2.00
1.60
1.20
0.80
0.40
0.00
Absorbance (480nm)
0
10
20
30
40
50
60
Time (min)

CHAPTER 6:- IRON-REGULATED OUTER MEMBRANE PROTEINS OF *AEROMONAS SALMONICIDA*

6.1 INTRODUCTION

The Gram-negative bacterial cell envelope is a complex structure which consists of two membranes separated by a layer of peptidoglycan. This peptidoglycan forms a macromolecular network in which linear amino sugar chains consisting of alternating residues of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) which are cross-linked by small peptides, attached to the NAM residues.

External to the peptidoglycan layer and the cytoplasmic membrane is the outer membrane (OM), a planar lipid bilayer composed of protein, phospholipid (PL) and lipopolysaccharide (LPS) which in turn is composed of three covalently linked regions: lipid A, core polysaccharide (low MW LPS) and the O antigen side chains (high MW LPS). The PL in the OM occupies the inner face, with LPS occupying the outer face. The LPS appears to be anchored in the OM by binding to outer membrane proteins (OMPs) possibly through hydrophobic interactions with its lipid A component.

Bacteria have a remarkable ability to alter their metabolism in reponse to changing environmental conditions such as temperature, pH, osmolarity, growth in biofilms, nutrient availability etc. The bacterial OM is recognised as the prime structure through which these adaptations are mediated (Brown & Williams, 1985; Williams, 1988).

The ability to phenotypically alter OM components provides pathogenic bacteria with the means for rapid adaptation to different host environments and for escape from host defence mechanisms. The role

of *in vivo* induced phenotypic OM alterations in pathogenic bacteria, in nutrient acquisition and on the susceptibility to serum killing and phagocytosis, for instance, have only begun to be examined (Smith, 1990).

It is, however, well established that bacteria growing *in vivo* during infection manufacture envelopes with characteristics specifically adapted to survival in that environment. This is particularly the case with respect to iron deprivation within the host. A number of bacterial pathogens express iron-regulated OMPs (IROMPs) under these conditions, which are expressed *in vivo* during infection (Table 6.1). These IROMPs are known to function as receptor proteins (Table 6.2) for a number of factors involved with the high affinity iron uptake mechanisms of these pathogenic bacteria. The IROMPs have been demonstrated to be immunogenic for many bacteria and have been identified as potential protective antigens for a number of pathogens (Sokol & Woods, 1984; 1986; Bolin & Jensen, 1987; Gilmour *et al.*, 1991).

Attempts to characterise the OM components of *A.salmonicida* have concentrated on the 49kDa A-layer protein and LPS (Chart *et al.*, 1984a; Evenberg *et al.*, 1985). Additional IROMPs have been identified in *A.salmonicida* grown under conditions of iron-restriction (Chart & Trust, 1983; Aoki & Holland, 1985). Studies indicating that the IROMPs of *A.salmonicida* are capable of binding and internalising $^{59}FeCl_3$ would indicate that the proteins play an important role in the iron uptake mechanisms of *A.salmonicida* although their exact function is not known (Chart & Trust, 1983). Since little is known regarding the immunogenicity, antigenic variation between strains and expression *in vivo* of these IROMPs, it was the aim of this study to examine the role of the IROMPs of *A.salmonicida* as potential protective antigens.

6.2 METHODS AND MATERIALS

6.2.1 *A.salmonicida* and *in vitro* growth conditions

Sixteen typical and two atypical strains of *A.salmonicida* and a number of other bacterial pathogens were used in this chapter and are listed in Table 6.3. For identification of IROMPs, *A.salmonicida* was grown in TSB + 100μg iron-free EDDA ml^{-1} or 2,2'-dipyridyl (100μM). Iron-replete bacteria were grown in TSB lacking an iron chelator. *A.salmonicida* was incubated at 22°C for 48h on a rotary shaker.

6.2.2 *In vivo* material

Furuncle material and peritoneal fluid was obtained aseptically from Atlantic salmon showing clinical signs of furunculosis during a natural outbreak on a farm in Scotland. The material was centrifuged at 800g for 10min at 4°C to remove tissue and cell debris and then further centrifuged at 4000g for 20min at 4°C to pellet bacteria. The bacterial pellet was then extracted with 2M guanidine-HCl as described in 6.2.3.

6.2.3 Guanidine-HCl extraction of *in vivo* material

Whole cell extracts of *A.salmonicida* isolated directly without subculture from *in vivo* material were prepared as described by Johnson *et al.*, (1985). Pelleted cells were washed in phosphate buffered saline (PBS) and resuspended to a density of approximately 10^8 cells ml^{-1} in 2M guanidine-HCl (GHCl) and incubated for 30min at 22°C. The extract was

centrifuged at 2000g for 20min at 4°C, and the supernatant dialysed overnight against PBS at 4°C and examined by SDS-PAGE and Western blotting.

6.2.4 OMP preparation

Outer membrane proteins were prepared from *A.salmonicida* grown under iron-replete and iron-restricted conditions as described in 4.2.9.

6.2.5 Kinetics of IROMP induction

To measure the kinetics of IROMP induction, *A.salmonicida* MT004 was grown in TSB overnight. A cell sample was removed and 2,2'-dipyridyl was added to the remainder of the culture to a concentration of 100μM. Growth was continued at 22°C and samples were removed at 20min intervals, chilled on ice and collected by centrifugation (8000g for 20min at 4°C). Cell pellets were then used for OMP preparation as described in 4.2.9.

6.2.6 Sensitivity to membrane antagonists (detergents)

Membrane antagonist sensitivities were determined on TSA seeded with 10^8 cells of three typical and an atypical strain of *A.salmonicida* using sterile filter discs (6mm) on which 50μl of a 10% w/v solution of antagonist was dried (Thornton *et al.*, 1991). Sensitivities were expressed as the radius of the zone of growth inhibition minus the radius of the disc.

6.2.7 Electrophoresis

Outer membrane proteins (20μg protein ml^{-1}), low molecular weight protein standards (Pharmacia-LKB) and biotinylated molecular weight markers (Pierce) were incubated with sample buffer and subjected to SDS-PAGE as described in 4.2.10.

6.2.8 Antiserum against OMPs

Atlantic salmon (approximately 1kg in weight) were held in seawater tanks supplied with fresh running seawater at 10-13°C. Fish were anaesthetised with ethyl *p*-aminobenzoate (benzocaine; 25ppm in ethanol) during immunisation and bleeding procedures. Immunisation regimes lasted for 42 days after primary injections, with fish receiving booster injections identical in composition to primary injection after 28 days. Fish were bled from the caudal vein, the blood allowed to clot at 4°C overnight and the serum removed, pooled and aliquots stored at -20°C. Antigen preparations and immunisation regimes are summarised in Table 6.4.

6.2.9 Rabbit antiserum against IROMPs

OMPs *of A.salmonicida* NT004 grown in the presence of 100μM 2,2'-dipyridyl were separated by SDS-PAGE. Strips of the polyacrylamide gel were stained with Coomassie brilliant blue R-250 to visualise the IROMP-containing region which was excised, washed in ddH_2O and emulsified in PBS by repeated passage through a 26-gauge needle and syringe. An adult New Zealand White rabbit (Serotec) received several

subcutaneous injections of the IROMP antigen (50μg) in Freund's complete adjuvant (FCA). The rabbit was boosted on day 28 with the IROMP antigen (50μg) in Freund's incomplete adjuvant. On day 42 the rabbit was exsanguinated and antiserum collected. Pre-immune serum collected at the time of primary immunisation served as a control.

6.2.10 Absorption of rabbit antiserum

Rabbit antiserum against the IROMPs of *A.salmonicida* NT004 was absorbed to remove antibodies against LPS and OMPs other than the IROMPs by the method of Meyer *et al.*, (1990). The antiserum was mixed with a sonicated extract (6 x 30s on ice) from 48h *A.salmonicida* NT004 cells grown at 22°C in TSB supplemented with 100μM $FeCl_3$ for 2h at 37°C. Absorbed antiserum was obtained by centrifugation of the mixture at 10,000g for 10min in order to remove bacteria.

6.2.11 Purification of rabbit IgG

Rabbit immunoglobulin G (IgG) was affinity-purified from rabbit immune serum using an ImmunoPure A/G column (Pierce). Protein concentration of the rabbit IgG was determined by the method of Bradford (1976) using rabbit gamma-globulin (Sigma) as standard using a BioRad protein assay kit.

6.2.12 Western blotting

Outer membrane proteins fractionated by SDS-PAGE were transferred to nitrocellulose as described in 4.2.11. After transfer, nitrocellulose

membranes were either stained for total protein with colloidal gold (Aurodye, Amersham) or blocked for 1h with 10% (v/v) normal goat serum (SAPU) in Tris-buffered saline (pH 7.5) containing 0.05% (v/v) Tween 20 (TTBS), prior to immunostaining. For detection of Atlantic salmon antibody, normal goat serum-blocked, TTBS-washed membranes were immersed for 1h in control or immune Atlantic salmon serum (1:250). Antibody dilution buffer was comprised of TTBS plus 10% (v/v) normal goat serum. Membranes were washed again and then incubated for 1h in Rabbit anti-salmon immunoglobulin M (R anti-SIgM; 1:1000)(Supplied by Dr T.S. Hastings, SOAFD, Marine Laboratory, Aberdeen). After further washing, membranes were incubated with goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma; 1:3000). Biotinylated molecular weight markers were visualised by the addition of avidin-conjugated alkaline phosphatase (Pierce; 1:1000) to the first antibody solution. Immunoreactive bands were then visualised by incubating membranes in 0.1M carbonate buffer containing 0.3mg tetrazolium blue ml^{-1} and 0.15mg 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt) ml^{-1}.

For detection of rabbit antibody, normal goat serum-blocked, TTBS-washed membranes were immersed for 1h in pre-immune or immune rabbit anti-IROMP. After further washing, membranes were incubated for 1h in goat anti-rabbit IgG-alkaline phosphatase (Sigma; 1:3000). Immunoreactive bands were visualised as described above.

6.2.13 Cross-reactivity of anti-IROMP antisera

Antigenic cross-reactivity of *A.salmonicida* IROMPS was examined using eighteen strains by immunoblotting techniques using salmon and rabbit anti-IROMP sera. Cross-reactivity of the rabbit anti-IROMP

antisera was also examined using iron-restricted OMP preparations of *A.hydrophila* 67-P-24, *A.hydrophila* 80-A1, *E.coli* Y1090, *V.anguillarum* MT323 and *Yersinia ruckeri* MT130 (Plates 6.5 & 6.6).

6.3 RESULTS

6.3.1 Effects of iron-restriction on the OMP composition of *A.salmonicida*

To determine the effects of iron-restriction on the OMPs of *A.salmonicida*, sixteen typical and two atypical strains (Table 6.3) were grown in TSB containing the high affinity iron chelator EDDA (100μgml^{-1}). OMPs were compared by SDS-PAGE with the OMPs from cells grown in TSB alone and from cells grown in TSB with EDDA and supplemented with $FeCl_3$ (0.54mM)(Chart & Trust, 1983) to overcome the iron-restricted conditions. Growth under iron-restricted conditions resulted in the expression of four novel OMPs of apparent molecular weight of 82,77,72 and 70 kDa (Plates 6.1 and 6.2) which were not present when the same strains were grown under iron-replete conditions or iron-restricted conditions supplemented with $FeCl_3$.

To ensure that the changes in the OMPs were not the result of poor growth conditions, or of the iron chelator used, *A.salmonicida* MT004 was grown under several different conditions and temperatures and the OMP profiles examined as before. *A.salmonicida* MT004 was cultured in TSB at 18°C and 22°C, with and without shaking, and under anaerobic conditions. None of the growth conditions examined resulted in the induction of the 82,77,72 or 70kDa IROMPs (data not shown). Finally, an additional iron chelator 2,2-dipyridyl (100μM) was used to induce iron-restricted

conditions and *A.salmonicida* cultured as before. In this case, the induction of the four IROMPs was identical qualitatively, however, greater amounts IROMPs were produced using 2,2'-dipyridyl as chelator (Plate 6.1).

6.3.2 Kinetics of IROMP induction

An overnight 2l culture of *A.salmonicida* MT004 grown under iron-replete conditions was made iron-restricted by the addition of 2,2'-dipyridyl. Aliquots (200ml) were removed at 20min intervals and examined for OMP composition. Analysis of the OMPs by SDS-PAGE indicated that IROMPs were induced between 60-80min after the addition of 2,2'-dipyridyl (data not shown).

6.3.3 Sensitivity to membrane antagonists (detergents)

The sensitivity of *A.salmonicida* to membrane antagonists was examined in order to determine the integrity of *A.salmonicida* cell envelopes grown under iron-replete and iron-restricted conditions. The three typical and the atypical strain were generally insensitive to the non-ionic detergent, nonidet P40, more sensitive to the anionic detergent, SDS, and slightly more sensitive to the cationic detergent, HDTMA (Table 6.6). The strains examined were generally more sensitive to the detergents when grown under iron-restricted conditions. This suggests changes in the integrity of the cell surface and/or membrane under iron-restricted conditions.

6.3.4 Immunogenicity of IROMPs

The immunogenicity of OM components of *A.salmonicida* has been reported previously (Chart *et al.*, 1984; Hastings & Ellis, 1990; Lund *et al.*, 1991) with the 49kDa A-layer and both low MW LPS (core polysaccharide) and high MW LPS (O-antigen) being immunogenic in Atlantic salmon. However, these studies used either whole cells or OMPs of *A.salmonicida* grown under iron-replete conditions as antigen. To determine the immunogenicity of the IROMPs of *A.salmonicida*, OMPs obtained from *A.salmonicida* grown under conditions of iron-restriction were examined by Western blotting techniques. Results indicate that in addition to the A-layer protein and LPS, the IROMPs of *A.salmonicida* were immunogenic in Atlantic salmon, with fish immunised with OMPs of *A.salmonicida* grown under iron-restricted conditions reacting strongly with the IROMPs of *A.salmonicida* (Plate 6.3).

The IROMPs of *A.salmonicida* were also found to be immunogenic in rabbits, and thus facilitated the production of a rabbit anti-IROMP antisera which reacted with one or more of the IROMPs. As with all attempts to produce a rabbit antisera against a OMP component partially purified by excision from SDS polyacrylamide gels, the OMP antigen preparation had a high LPS content due to LPS being intrinsically associated with the OMPs. However, in this study, extensive adsorption of the rabbit antisera with a sonicated extract of iron-replete *A.salmonicida* cells to removed antibodies to the LPS and OMPs other than the IROMPs, to produce a specific rabbit anti-IROMP antiserum.

6.3.5 Antigenic cross-reactivity of IROMPs

Although *A.salmonicida* is considered to be a homogeneous species, heterogeneity in LPS has been recently reported (Rockey *et al.*, 1991). Therefore, strain differences in IROMP expression and antigenicity between typical and atypical isolates were investigated. Sixteen typical and two atypical strains of *A.salmonicida*, many of which were recent clinical isolates collected by the Marine Laboratory, were grown under iron-replete and iron-restricted conditions and their OMP profiles were examined by SDS-PAGE. The 82,77,72 and 70 kDa IROMPs were found to be expressed by all of the strains examined (Plate 6.2) and antigenic homogeneity of one or more of the IROMPs was confirmed using a specific rabbit anti-IROMP antiserum (Plate 6.4). A number of other gram-negative bacterial pathogens were examined for the presence of the four IROMPs (Plate 6.5). Although all the bacteria examined produced IROMPs under conditions of iron-restriction, none had the same molecular weight as *A.salmonicida* although iron-restricted OMPs of *E.coli* Y1090 and *V.anguillarum* MT323 cross-reacted immunologically with the rabbit antiserum (Plate 6.6).

6.3.6 Expression of IROMPs *in vivo*

Outer membrane proteins of *A.salmonicida* isolated directly without subculture from furuncle material and peritoneal fluid of two Atlantic salmon showing clinical signs of *A.salmonicida* infection were examined in guanidine-HCl extracts of whole cells by SDS-PAGE. OMP profiles were then compared with OMP profiles of a laboratory strain of *A.salmonicida* (MT004) and the strain subsequently isolated from the infected Atlantic

salmon cultured under iron-restricted conditions (MT1057)(Plate 6.7). Growth *in vivo* resulted in the expression of four OMPs of apparent molecular weight of 82,77,72 and 70 kDa which were not present when the same strain was grown under iron-replete conditions (Plate 6.7). When compared with OMPs from *A.salmonicida* grown under conditions of iron-restriction *in vitro*, OMPs of *in vivo* cultured bacteria were identified as the iron-regulated OMPs of *A.salmonicida* and their identity confirmed by Western blot analysis using a rabbit antiserum specific for the IROMPs of *A.salmonicida* (Plate 6.8), despite the antisera cross-reacting with material of approximately 38kDa thought to be of host origin.

6.4 DISCUSSION

Bacterial pathogens which invade and colonise an animal host are exposed to an iron-restricted environment due to the sequestration of iron to transferrin, lactoferrin, ferritin, haemoglobin and other compounds. The ability of a bacterial pathogen to successfully compete for the sequestered iron is considered to be an important aspect of its virulence and pathogenicity. The iron uptake mechanisms of *A.salmonicida* have been described in previous chapters: a common feature of the mechanisms described is the possession of specific OMPs which are induced under conditions of iron-restriction which may function as receptors for siderophores, transferrin/lactoferrin or haem (Table 6.2). It has been demonstrated in this chapter that *A.salmonicida* responds to an iron-restricted environment by alterations in the protein composition of its outer membrane with the induction of four iron-regulated outer membrane proteins (IROMPs) with an apparent molecular weight of 82,77,72

and 70kDa. The IROMPs were not expressed under iron-replete conditions or under iron-restricted conditions where the iron chelator had been saturated with $FeCl_3$. *Aeromonas salmonicida* has previously been shown to express additional IROMPs (Chart & Trust, 1983; Aoki & Holland, 1985), with the reported MW of the proteins similar to those reported in this study. Chart & Trust (1983), using EDDA as iron chelator, found three typical strains of *A.salmonicida*, A449, A450 and a LPS (O-antigen)-deficient mutant A450-3R to express three IROMPs of 83.2,77.7 and 76.6kDa. Similarly Aoki & Holland (1985), using 2,2'-dipyridyl as iron chelator, found eight strains to express three IROMPs of 93,74 and 72kDa. In previous studies, the choice of iron chelator has been shown to influence the number of IROMPs induced in strains of *E.coli* (Chart *et al.*, 1986), with an 81kDa IROMP lacking in *E.coli* grown in the presence of 2,2'-dipyridyl. However, in this study it was found that the strains of *A.salmonicida* examined in this study produced identical IROMP profiles when grown in the presence of EDDA or 2,2'-dipyridyl, although there were quantitative differences in the amounts produced.

Induction of IROMPs by *A.salmonicida* was detectable within 60-80min of the nutrient shift imposed by 2,2'-dipyridyl. This induction period is similar to that described for *A.salmonicida* using $^{59}Fe^{3+}$ binding/uptake assays (Chart & Trust, 1983). They found IROMPs isolated from *A.salmonicida* A450, a strain possessing an inducible iron uptake mechanism, to bind $^{59}Fe^{3+}$ (as $FeCl_3$) after a 90min exposure to iron-restricted conditions. This 90min induction period was absent in *A.salmonicida* A449, which possessed a constitutive iron uptake mechanism, and was capable of binding $^{59}Fe^{3+}$ within 10min of exposure. OMPs isolated from iron-replete cells failed to bind $^{59}Fe^{3+}$. The

induction period for the IROMPs of *A.salmonicida* is similar to that recorded for the IROMPs of *E.coli* (Klebba *et al.*, 1982) in which the first of six IROMPs was induced within 24-30min and complete by 90min.

Rapid induction of IROMPs is necessary to render bacterial iron uptake mechanisms functional before the intracellular iron stores are depleted. Although no function has been ascribed to the IROMPs of *A.salmonicida*, they are able to bind $^{59}Fe^{3+}$ (Chart & Trust, 1983) but do not function in the direct binding of Tf or Lf or in the binding of haem compounds (see results presented in Chapters 4 and 5). The ability of non-siderophore-producing atypical strains of *A.salmonicida* to utilise siderophores produced by the typical strains and the fact that they share antigenically related IROMPs suggests that the IROMPs may function as a receptor protein for a Fe^{3+}-siderophore complex.

Results of the membrane antagonist experiments suggests that the cell integrity of *A.salmonicida* is altered in strains of *A.salmonicida* growing under conditions of iron-restriction. Similar cell surface disorganised mutants of *A.salmonicida* with weakened cell integrity have been described by Thornton *et al.*, (1991). These mutants possessed an incomplete A-layer protein and exhibited an increased sensitivity to both membrane antagonists and to the bactericidal action of serum. The authors suggest that these features are similar to the characteristics created by depletion of Ca^{2+} which interferes with normal A-layer subunit assembly. Since the iron-chelator EDDA was used to induce conditions of iron-restriction in the membrane antagonist assays it is possible that EDDA non-specifically bound Ca^{2+} or that restriction of Fe^{3+} may have had the same effect. An interesting feature of the surface-disorganised *A.salmonicida* mutants was their increased sensitivity to the bactericidal action of serum since the A-layer

protein normally confers resistance to this (Munn *et al.*, 1982). In addition, these mutants are effective as attenuated live *A.salmonicida* vaccines against furunculosis in salmonids. It has been proposed that such mutants may expose critical cell surface protective antigens normally shielded by the A-layer protein (Kay & Trust, 1991). These may include the IROMPs described in this study.

Although the IROMPs of *A.salmonicida* have been described previously (Chart & Trust, 1983; Aoki & Holland, 1985), their immunogenicity has not been established. Previous work has demonstrated that of the many cell surface and OM components examined, only the A-layer protein and LPS have been shown to be immunogenic in salmonids (Chart *et al.*, 1984a,b; Hastings & Ellis, 1990; Lund *et al.*, 1991) when using iron-replete whole cells or OMPs as antigen, although upto twelve components of the OM are immunogenic in the rabbit (Lund *et al.*, 1991). In this study, the immunogenicity of the IROMPs of *A.salmonicida* was examined by Western blotting techniques using Atlantic salmon immunised with OMPs of *A.salmonicida* grown under iron-restricted conditions. Under these conditions we found two of the four IROMPs of *A.salmonicida* to be immunogenic in Atlantic salmon. In addition, the IROMPs of *A.salmonicida* were found to be expressed *in vivo* during infection, in common with the IROMPs of a number of other bacterial pathogens (Table 6.1), since *A.salmonicida* isolated directly without subculture from furuncle material and peritoneal fluid of infected Atlantic salmon was found to express IROMPs (Plates 6.7 and 6.8).

All of the IROMPs of *A.salmonicida* showed antigenic cross-reactivity between typical and atypical strains, indicating homogeneity of the IROMPs and that specific differences in the pathogenic mechanisms possessed by typical and atypical strains is

unrelated to the expression of the IROMPs. Antigenic cross-reactivity of IROMPs from a number of bacterial pathogens has been described, for example, *E.coli* (Chart & Griffiths, 1985), *K.pneumoniae* (Williams *et al.*, 1987) *Neisseria* species (Mietzner *et al.*, 1986) and *P.multocida* (Ikeda & Hirsh, 1988). In addition, the 81kDa fepA enterobactin receptor protein has been identified immunologically in a panel of Gram-negative bacteria comprising of thirty-nine strains representing eighteen genera (Rutz *et al.*, 1991). The cross-reactivity of IROMPs in *A.salmonicida* and other bacterial pathogens implies that iron uptake mechanisms are conserved within a bacterial species and that these proteins serve a common function in pathogenicity.

The results presented in this chapter indicate that the IROMPs of *A.salmonicida* are expressed *in vivo* during infection of Atlantic salmon, are immunogenic in Atlantic salmon and are antigenically homogeneous between strains. In this respect, the IROMPs of *A.salmonicida* might offer effective targets for vaccine development by inducing antibodies which may prevent uptake of exogenous iron by *A.salmonicida*.

TABLE 6.1 Expression of the iron-regulated outer membrane proteins (IROMPs) of pathogenic bacteria *in vivo* by direct culture or immunoblotting.

Bacterium	Host	Site of infection	Reference
Bacteroides fragilis	Rat peritonitis		Otto *et al* (1991)
Escherichia coli	Guinea pig	peritoneal cavity	Griffiths *et al* (1983)
	Human urine		Lam *et al* (1984)
	Human antisera		Griffiths *et al* (1985b)
Haemophilus influenzae	Human sputum		van Alphen *et al* (1990)
	Rat	peritoneal cavity	
non-typable *H. influenzae*	Human antisera (porphyrin-inducible OMPs)		MacIver *et al* (1990)
Haemophilus pleuropneumoniae	Pig antisera		Deneer & Potter (1989a)
Klebsiella pneumoniae	Human urine		Lam *et al* (1984)
			Shand *et al* (1985)
	Rabbit	peritoneal cavity	Kadurugamuwa *et al* (1988)
Neisseria gonorrhoeae	Human antisera		Fohn *et al* (1987)
Neisseria meningitidis	Human antisera		Black *et al* (1986)
			Ala'Aldeen *et al* (1990)
Pasteurella haemolytica	Bovine antisera		Deneer & Potter (1989b)
	Ovine pleural fluid*		Donachie & Gilmour (1988)
	Ovine antisera		Lainson *et al* (1991)
	Ovine chamber implant*		Sutherland *et al* (1990)
	Rabbit chamber implant*		Morck *et al* (1991)
Porphyromonas gingivalis	Human antisera		Chen *et al* (1991)
Proteus spp.	Human urine		Lam *et al* (1984)
			Shand *et al* (1985)

TABLE 6.1 Continued.

Bacterium	Host	Site of infection	Reference
Pseudomonas aeruginosa	Human	sputum	Anwar *et al* (1984)
			Brown *et al* (1984)
	Rat lung*		Cochrane *et al* (1988)
	Mice & Rat chamber implant		Kelly *et al* (1989)
	Human antisera		Shand *et al* (1991)
Salmonella typhi	Human antisera		Fernandez-Beros *et al* (1989)
Staphylococcus epidermidis	Human peritoneal dialysate (PD)		Williams *et al* (1988b)
	Human PD		Smith *et al* (1991)
	Human PD*		Wilcox *et al* (1991)
Vibrio cholerae	Rabbit intestinal fluid		Sciortino & Finkelstein (1983)
	Rabbit ileal loop*		Richardson *et al* (1989)
			Jonson *et al* (1989)

* immunogenicity of IROMPs confirmed by Western blotting with convalescent sera.

TABLE 6.2 Receptor functions of the iron-regulated outer membrane proteins (IROMPs) of pathogenic bacteria.

Bacterium	IROMP	MW (kDa)	Ligand	Ref
E.coli	fepA	84	enterobactin	Hollifield & Neilands (1978)
	IutA (cir)	74	aerobactin	Binderelf *et al*(1982) Grewal *et al* (1982)
	Fhu	78	ferrichrome	Fecker & Braun (1983)
	FecA	80.5	Fe^{3+}-citrate	Wagegg & Braun (1981)
Ps.aeruginosa		14	pyoverdin	Sokol(1984)
		80	pyochelin	Meyer *et al* (1990)
V.anguillarum	OM2	84	anguibactin	Actis *et al* (1985)
S.flexneri		101	Haem/CR	Stugard *et al* (1989)
E.coli		101	Haem/CR	Stugard *et al* (1989)
B.bronchiseptica/		27	Lf	Manozzi *et al* (1991)
B.pertussis		32	Tf	
N.meningitidis		105	Lf	Schryvers & Morris (1988a)
		71	Tf	Schryvers & Morris (1988b)
A.pleuropneumoniae		105	Tf/CR	Deneer & Potter (1989a)
		64,99	Tf	Ricard *et al* (1991)
		56,105	Tf	Gonzalez *et al* (1990)
		60	Tf/haemin	Gerlach *et al* (1992)
H.influenzae		94-106	Lf	Schryvers (1989)
		68	Tf	Schryvers (1989)
		72	Tf	Morton & Williams (1990)
		39	haemin	Lee (1992)
H.somnus		73,85,105	Tf	Ogunnariwo *et al* (1990)
P.haemolytica		100	Tf	Ogunnariwo & Schryvers (1990)
P.multocida		82	Tf	Ogunnariwo *et al* (1990)

Lf, lactoferrin; Tf, transferrin; CR, congo red.

TABLE 6.3 Strains of bacteria used in the the study of iron-regulated outer membrane proteins.

Bacterium	Strain	Source*
Aeromonas hydrophila	67-P-24	L.A.Rodriguez
Aeromonas hydrophila	80-A1	L.A.Rodriguez
Aeromonas salmonicida	MT004	Marine Laboratory
Aeromonas salmonicida	MT194	Marine Laboratory
Aeromonas salmonicida	MT423	Marine Laboratory
Aeromonas salmonicida	MT525	Marine Laboratory
Aeromonas salmonicida	MT616	Marine Laboratory
Aeromonas salmonicida	MT1057	Marine Laboratory
Aeromonas salmonicida	MT1058	AVL
Aeromonas salmonicida	MT1059	Marine Laboratory
Aeromonas salmonicida	MT1060	Marine Laboratory
Aeromonas salmonicida	MT1061	Marine Laboratory
Aeromonas salmonicida	MT1077	Marine Laboratory
Aeromonas salmonicida	MT1078	AVL
Aeromonas salmonicida	MT1080	Marine Laboratory
Aeromonas salmonicida	MT1143	Marine Laboratory
Aeromonas salmonicida	MT1144	NVI
Aeromonas salmonicida	MT1186	Marine Laboratory
Aeromonas salmonicida	MT1423	Marine Laboratory
Aeromonas salmonicida	MT1429	Marine Laboratory
Escherichia coli	Y1090	Aberdeen University
Vibrio anguillarum	MT323	Marine Laboratory
Yersinia ruckeri	MT130	Marine Laboratory

* L.A.Rodriguez, Microbiologia Colegio Universitario de Orense, Orense, Spain; AVL, Aquaculture Vaccines Limited, Saffron Walden , Essex, UK; Norwegian Veterinary Institute, Oslo, Norway.

TABLE 6.4 Immunisation regime used to develop antibodies against outer membrane proteins of *Aeromonas salmonicida* in Atlantic salmon. Fish were inoculated intraperitoneally with outer membrane proteins of *A.salmonicida* NT004 and NT423 grown under iron-replete conditions or with outer membrane proteins of *A.salmonicida* NT004 and NT423 grown under iron-restricted conditions. Fish inoculated with PBS alone or in combination with FCA served as controls.

Antigen	Dose/fish	Adjuvant	Booster* (days post-immunisation)	Serum designation
PBS			24	S anti-PBS
PBS		FCA	24	S anti-FCA
Fe^{+}OMP	40µg	FCA	24	S anti-Fe^{+}OMP
Fe^{-}OMP	40µg	FCA	24	S anti-Fe^{-}OMP

* Boosted fish received Freund's incomplete adjuvant

TABLE 6.5 Sensitivity of iron-replete and iron-restricted *A.salmonicida* to the membrane antagonists: NP40, nonidet P40; SDS, sodium dodecyl sulphate; HDTMA, hexadecyltrimethyl ammonium bromide. Results are expressed as the zone of inhibition (in mm) around wells containing the membrane antagonist and are the mean of triplicate values (+/- SE of the mean).

Strain	Growth condition	NP40 (non-ionic)	SDS (anionic)	HDTMA (cationic)
MT004	iron-replete	0±0.0	6±0.2	2±0.1
	iron-restricted	0±0.0	5±0.6	2±0.1
MT423	iron-replete	0±0.0	5±0.3	9±0.3
	iron-restricted	4±0.9	8±0.2	14±1.0
MT486	iron-replete	0±0.0	6±0.0	9±0.5
	iron-restricted	0±0.0	6±0.4	10±0.6
MT194	iron-replete	0±0.0	5±0.7	0±0.0
	iron-restricted	2±0.1	7±0.2	3±0.5

PLATE 6.1 SDS-PAGE of iron-replete and iron-restricted outer membrane proteins (OMPs) of *Aeromonas salmonicida* MT004 and MT423. Proteins were detected by Western blotting and colloidal gold stain. Lane *, low molecular weight protein standards in kDa. Lane 1, *A.salmonicida* grown in Tryptone Soya Broth (TSB); Lane 2, *A.salmonicida* grown in TSB + 100µg ethylene diamine di (*o*-hydroxyphenylacetic acid)(EDDA)ml^{-1}; Lane 3, *A.salmonicida* grown in TSB + 2,2'-dipyridyl (100µM).

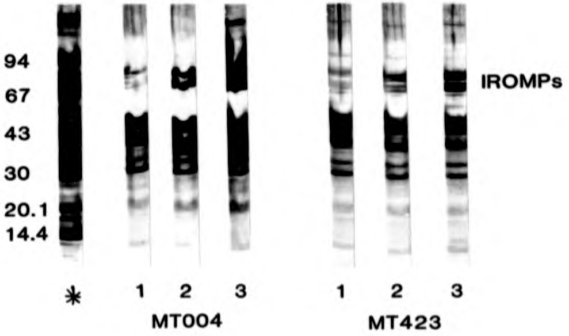
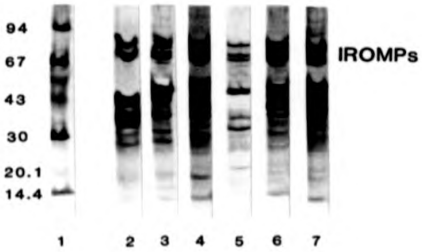

94
67
43
30
20.1
14.4
IROMPs
*
1
2
3
1
2
3
MT004
MT423

PLATE 6.2a SDS-PAGE of the iron-restricted outer membrane proteins (IROMPs) of *A.salmonicida* strains grown in TSB + 2,2'-dipyridyl (100μM). Proteins were detected by Western blotting and colloidal gold stain. Lane 1, low molecular weight protein standards in kDa; Lane 2, MT004; Lane 3, MT423; Lane 4, MT486; Lane 5, MT1058; Lane 6, MT194; Lane 7, MT525. Position of IROMPs are indicated.

PLATE 6.2b SDS-PAGE of the IROMPs of *A.salmonicida* strains grown in TSB + 2,2'-dipyridyl (100μM). Proteins were detected by Western blotting and colloidal gold stain. Lane 1, low molecular weight protein standards in kDa; Lane 2, MT1060; Lane 3, MT1061; Lane 4, MT1077; Lane 5, MT1078; Lane 6, MT1080; Lane 7, MT1143; Lane 8, MT1144; Lane 9, MT1186; Lane 10, MT1423; Lane 11, MT1429. Position of IROMPs are indicated.


94
67
43
30
20.1
14.4
IROMPs
1
2
3
4
5
6
7

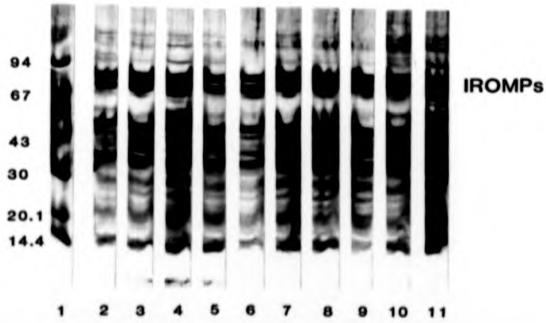
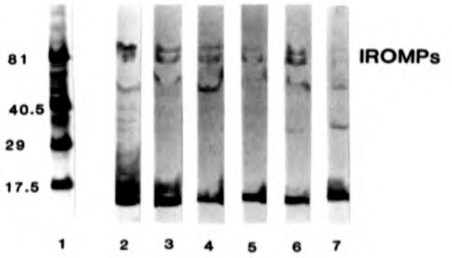

94
67
43
30
20.1
14.4
IROMPs
1
2
3
4
5
6
7
8
9
10
11

PLATE 6.3 Western blot of the IROMPs of *A. salmonicida* strains stained with an Atlantic salmon antisera raised against the IROMPs of *A.salmonicida*. Lane 1, biotinylated molecular weight standards in kDa; Lane 2, MT004; Lane 3, MT423; Lane 4, MT486; Lane 5, MT1058; Lane 6, MT194; Lane 7, MT525. Position of IROMPs are indicated.

PLATE 6.4 Western blot of the IROMPs of *A.salmonicida* strains stained with a rabbit antisera specific for the IROMPs of *A.salmonicida*. Lane 1, biotinylated molecular weight standards in kDa; Lane 2, MT1060; Lane 3, MT1061; Lane 4, MT1077; Lane 5, MT1078; Lane 6, MT1080; Lane 7, MT1143; Lane 8, MT1144; Lane 9, MT1186; Lane 10, MT1423; Lane 11, MT1429. Position of IROMPs are indicated.


81
40.5
29
17.5
IROMPs
1
2
3
4
5
6
7

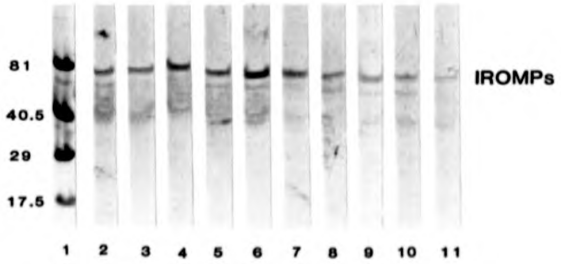
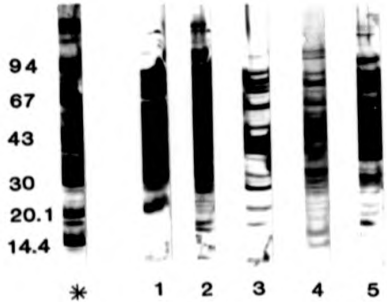
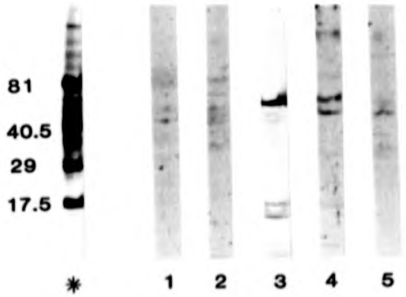

81
40.5
29
17.5
IROMPs
1
2
3
4
5
6
7
8
9
10
11

PLATE 6.5 SDS-PAGE of the OMPs of a number of bacterial pathogens grown in TSB + 2,2'-dipyridyl (100μM). Proteins were detected by Western blotting and colloidal gold stain. Lane *, low molecular weight protein standards in kDa; Lane 1, *Aeromonas hydrophila* 87-P-24; Lane 2, *Aeromonas hydrophila* 80-A1; Lane 3, *Escherichia coli* Y1090; Lane 4, *Vibrio anguillarum* MT323; Lane 5, *Yersinia ruckeri* MT130.

PLATE 6.6 Western blot of the OMPs of a number of bacterial pathogens stained with a rabbit antisera specific for the IROMPs of *A.salmonicida*. Lane *, biotinylated molecular weight standards- in kDa; Lane 1, *Aeromonas hydrophila* 87-P-24; Lane 2, *Aeromonas hydrophila* 80-A1; Lane 3, *Escherichia coli* Y1090; Lane 4, *Vibrio anguillarum* MT323; Lane 5, *Yersinia ruckeri* MT130.


94
67
43
30
20.1
14.4
*
1
2
3
4
5

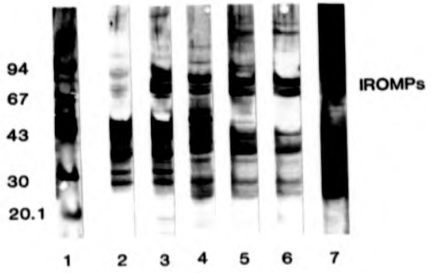

81
40.5
29
17.5
*
1
2
3
4
5

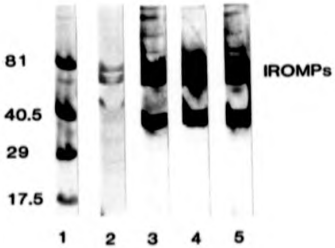
Lane 2. OMPs of iron-replete *A. salmonicida*

PLATE 6.7 SDS-PAGE of OMPs of *A.salmonicida* and GHCl extracts of *in vivo* material. Proteins were detected by Western blotting and colloidal gold stain. Lane 1, low molecular weight protein standards in kDa; Lane 3, OMPs of iron-restricted *A.salmonicida* MT004; Lane 4, OMPs of iron-restricted *A.salmonicida* (*in vivo* isolate); Lane 5 and 6, GHCl extract of peritoneal fluid; Lane 7, GHCl extract of furuncle muscle extract.

Lane 2 OMPs of iron-restricted *A. salmonicida*

PLATE 6.8 Western blots of *in vivo* material stained with a rabbit antisera specific for the IROMPs of *A.salmonicida*. Lane 1, biotinylated molecular weight standards in kDa; Lane 3 and 4, GHCl extract of peritoneal fluid; Lane 5, GHCl extract of furuncle muscle extract.


94
67
43
30
20.1
IROMPs
1
2
3
4
5
6
7


81
40.5
29
17.5
IROMPs
1
2
3
4
5

CHAPTER 7:- OUTER MEMBRANE PROTEIN VACCINES AGAINST *AEROMONAS SALMONICIDA*

7.1 INTRODUCTION

Currently available commercial *A.salmonicida* vaccines consist of chemically-inactivated, aluminium adjuvanted whole cell bacterin preparations. Experimental data and field usage indicates that although these bacterins meet with some success in the prevention of furunculosis in Atlantic salmon, an effective vaccine against *A.salmonicida* containing defined antigens that will elicit a protective response needs to be developed.

The low levels of protection induced by existing *A.salmonicida* vaccines and many killed bacterial vaccines in general is thought to be due to a number of reasons (Ellis, 1988): (i) destruction of protective antigens during inactivation of the bacterin; (ii) antigenic competition as a result of the complex mixture of microbial products present in the bacterin and (iii) the correct immune response may not be induced. This often results in minimal responses to most of the protective antigens Ellis, 1988). It is therefore important to identify which antigens stimulate protective immunity. These protective antigens are probably those which have an important role as virulence determinants.

Although many of the bacterial vaccines in current use are killed or attenuated whole-cell vaccines such as *Bordetella pertussis* and bacillus Calmette-Guerin (BCG) or toxoids (chemically inactivated toxin vaccines) such as diphtheria and tetanus vaccines, there is a move toward defined subunit vaccines such as capsular polysaccharides chemically conjugated to proteins ie conjugate vaccines (Donnelly *et*

al., 1990; Lifely *et al.*, 1991; Vella & Ellis, 1991). Further subunit vaccine development is being carried out to use other bacterial surface components such as LPS, OMPs, flagellae and pili or fimbriae (Dougan *et al.*, 1989)

For an OMP to serve as a protective antigen and be useful as a vaccine candidate, the protein must be immunogenic and be exposed on the bacterial cell surface (Brown *et al.*, 1988). In addition, antibody induced against the OMP must be biologically active ie must have bactericidal and/or opsonic activity to be protective. A number of experimental OMP vaccines have been evaluated using passive and active immunisation studies, substantiating the role of OMPs of bacterial pathogens as protective antigens (Table 7.1). Studies of the role of the cell surface components of *A.salmonicida* have been restricted to LPS and the A-layer protein (Chart *et al.*, 1984a; Evenberg *et al.*, 1985; Olivier *et al.*, 1985). The IROMPs of a number of bacterial pathogens have been identified as potential protective antigens. Sokol & Woods (1988) found an anti-14kDa ferri-pyochelin receptor of *Ps.aeruginosa* to passively immunise mice in a burned mouse model. In addition, the antisera significantly enhanced phagocytosis by human PMN's as well as inhibiting the binding and uptake of $^{59}Fe^{3+}$-pyochelin. Similarly, Bolin & Jensen (1987) found that a rabbit antiserum raised against the IROMPs of *E.coli* passively protected turkeys from experimental colisepticaemia. The role of IROMPs as protective antigens in active immunisation studies has recently been described by Gilmour *et al.*, (1991) for *P.haemolytica* in lambs. Sodium salicylate extracts (SSE) of *P.haemolytica* cultured under iron-restricted conditions, to induce expression of three iron-regulated proteins (IRPs) significantly protected lambs from experimental pasteurellosis when compared with SSE

extracts of iron-replete bacteria. Induction of the protective immunity was associated with the presence of antibody against the 35, 70 & 100kDa IRPs.

Since the IROMPs of *A.salmonicida* were shown to expressed *in vivo* and immunogenic in Atlantic salmon in Chapter 6, the aim of this chapter was to evaluate their protective capacity and ability to elicit a specific immune response in Atlantic salmon.

7.2 METHODS AND MATERIALS

7.2.1 *A.salmonicida* and growth conditions

Three typical strains of *A.salmonicida* (MT004, MT423 & MT1057) were used in this study. For preparation of IROMPs, *A.salmonicida* was grown in TSB + 2,2'-dipyridyl (100μM). Iron-replete bacteria were grown in TSB. *A. salmonicida* was incubated at 22°C for 48h on a rotary shaker.

7.2.2 OMP preparation

Outer membrane proteins were prepared from *A.salmonicida* grown under iron-replete and iron-restricted conditions as described in 4.2.9.

7.2.3 Protein assay

Protein concentation of the OMP preparations was determined by the method of Bradford (1976) using BSA as standard using a BioRad protein assay kit.

7.2.4 Carbohydrate assay

Total carbohydrate content of the OMP preparations was determined by the phenol-sulphuric acid method of Dubois *et al.*, (1956) using glucose as standard.

7.2.5 Limulus amoebocyte assay

A limulus amoebocyte lysate (LAL) assay kit (E-toxate;Sigma) was used to determine lipopolysaccharide (LPS) present in the OMP preparations using *E.coli* 055:B5 LPS as standard.

7.2.6 LPS Assay

Lipopolysaccharide content of the OMP preparations was determined by the method of Keler & Nowotny (1986) using *A.salmonicida* MT004 cell wall LPS extracted by the method of Westphal & Jann (1965) as standard.

7.2.7 2-keto-3-deoxyoctanate (KDO) Assay

The presence of 2-keto-3-deoxyoctanate (KDO) in the OMP preparation was determined by the thiobarbituric acid method described by Karkhanis *et al.*, (1978) after hydrolysis of samples in 0.2N sulphuric acid for 30min.

7.2.8 Electrophoresis

Outer membrane proteins (20µg protein ml^{-1}), low molecular weight protein standards (Pharmacia-LKB) and biotinylated molecular weight markers (Pierce) were subjected to SDS-PAGE as described in 4.2.10.

7.2.9 Passive immunisation

Atlantic salmon parr (about 10-20g) were held in a 1 m fresh water tank (approximate volume 300-350l) supplied with running freshwater from a local loch and heated to 14°C. All tanks were contained within the freshwater high risk unit at SOAFD's Fish Cultivation Unit, Aultbea, Wester Ross. Fish were starved for a 24h period prior to immunisation in order to reduce gut contents. Fish were anaesthetised with ethyl *p*-aminobenzoate (benzocaine; 25ppm in ethanol) during immunisation procedures. Fish were passively immunised intaperitoneally (ip) with either: (i) 100µl salmon anti-IROMP immune serum; (ii) control (anti-PBS) serum; (iii) rabbit anti-IROMP antisera; (iv) or 50µl purified rabbit anti-IROMP immunoglobulin G (IgG) plus 50µl normal rabbit serum (SAPU) as a source of complement or control rabbit serum (NRS). Fish were group marked using a Panjet (FH Wright Ltd., Dundee, Scotland) with a saturated solution of Alcian blue in PBS. Serum samples were collected from five fish per group before passive immunisation to determine background antibody levels. Details of immune and control sera used to passively immunise Atlantic salmon are summarised in Table 7.2.

7.2.10 Dot-blot immunoassay

Sera from rainbow trout (20-100g) passively immunised ip with 200µl normal rabbit serum (SAPU) was collected periodically and analysed using a dot-enzyme immunoassay (dot-blot) to measure persistence of circulating rabbit antibody. Two µl of serially diluted rabbit, normal rainbow trout or passively immunised rainbow trout sera were spotted on to TBS-washed nitrocellulose (0.45µm, BioRad) and blocked for 1h in 1% BSA-TBS. After two 5min washes with TTBS, dot-blots were incubated for 1h with 1% BSA-TBS containing donkey anti-rabbit IgG-horseradish peroxidase (SAPU; 1:1000). After a further two 5min washes with TTBS, bound donkey anti-rabbit IgG-HRP was visualised using 4-chloro-1-naphthol/H_2O_2 (BioRad), and rabbit antibody expressed as the reciprocal dilution giving a positive reaction.

7.2.11 Active immunisation

Atlantic salmon parr (about 10-20g) maintained as described in 7.2.9 were immunised with OMP preparations described in Table 7.3 (prepared as described in 4.2.8). Fish were challenged with virulent *A.salmonicida* (MT423 or MT1057) 42 days after immunisation. Each fish received a single ip injection of antigen (100µl). Fish injected with phosphate-buffered saline (PBS) alone served as controls. For numbers of fish in each trial, tank distribution, and number of replicate tanks see Results.

7.2.12 *A.salmonicida* challenge

Forty-two days post-active immunisation or 2h post-passive immunisation, fish were subjected to a 24h bath challenge exposure of approximately $1x10^8$ cfuml^{-1} of *A.salmonicida* MT423 (Trial I and passive immunisation trial) or MT1057 (Trial III). Fish in Trial II experienced a natural outbreak of furunculosis and the *A.salmonicida* strain isolated was designated MT1057. *A.salmonicida* MT423 or MT1057 were obtained from liquid N_2 stock and cultured on TSA at 15°C for 72h prior to use. For the challenge procedure, plates were washed with PBS and *A.salmonicida* resuspended to a density of approximately $1x10^9$ cells ml^{-1} (Absorbance at 540nm = 1.0) and added to the tanks to a final density of approximately $1x10^8$ cells ml^{-1} (ie approximately 30ml in 300l). Prior to challenge fish were starved for 24h in order to reduce the organic content of the water and prevent *A.salmonicida* from adsorbing to organic matter and preventing biofilm formation (Bricknell, SOAFD Marine Laboratory, unpublished observations). At the time of challenge, water flow to the tanks was stopped and the tanks aerated with compressed air for the 24h challenge period. After the 24h challenge period, the water flow and feed was restored. Mortalities were recorded daily and *A.salmonicida* infection confirmed bacteriologically by inoculation of dissected kidney material onto TSA plates followed by incubation at 22°C for 48-96h. The relative percentage survival (RPS) of each vaccinated group was determined by the method of Amend (1981) as follows:-

$$RPS = 100 \times 1 - \frac{\text{(\% specific vaccinate mortality)}}{\text{(\% specific control mortality)}}$$

7.2.13 Enzyme-linked immunosorbent assay (ELISA)

Titertek PVC immunoassay plates were coated with 3μg OMP antigen (30μgml^{-1} in 0.05M carbonate/bicarbonate buffer, pH9.6) and incubated overnight at 22°C. Antigen coated immunoassay plates were washed twice for 5 min with Tris-buffered saline (50mM Tris-HCl, 150mM NaCl, pH 7.5) containing 0.05% Tween 20 (TTBS) and blocked with 5% normal goat serum (SAPU) in TTBS for 1h at 22°C. Immune salmon serum (100μl) diluted 1/10 in 5% NGS/TTBS was added to the immunoassay plates and incubated for 1h at 22°C. After two further 5 min washes with TTBS, immunoassay plates were incubated for 90 min at 37°C with 100μl undiluted mouse anti-rainbow trout IgM monoclonal antibody (mAb4C10)(supplied by Dr G Houghton, SOAFD Marine Laboratory, Aberdeen) which cross-reacts with salmon IgM (Thuvander *et al.*, 1990). After two further 5 min washes with TTBS, immunoassay plates were incubated for 1h at 37°C with 100μl goat anti-mouse IgG-alkaline phosphatase (Sigma; 1:1000 or BioRad; 1:3000) followed by one 5 min TTBS wash and the two TBS washes. Plates were then washed with 200μl 10mM diethanolamine (pH9.5) containing 0.5mM $MgCl_2$ followed by 100μl enzyme substrate (1mg *p*-nitrophenyl phosphate ml^{-1} in 10mM diethanolamine, 0.5mM $MgCl_2$ solution) for 45 min at 22°C. The reaction was terminated by the addition of 50μl 0.1M EDTA solution and the absorbance of the bound conjugate measured at 405nm using a Titertek Multiscan MC plate reader.

7.2.14 Western blotting

Outer membrane proteins fractionated by SDS-PAGE were transferred to nitrocellulose as described in 4.2.11. After transfer, nitrocellulose membranes were either stained for total protein with colloidal gold (Aurodye, Amersham) or blocked for 1h with 10% (v/v) normal goat serum (SAPU) in Tris-buffered saline (pH 7.5) containing 0.05% (v/v) Tween 20 (TTBS).

Atlantic salmon antibody was detected in Western blots as described in 6.2.16.

7.2.15 Bactericidal assay

A bactericidal assay was used to measure serum killing of *A.salmonicida* in salmon anti-IROMP and rabbit anti-IROMP antisera as described by Sutherland (1988). In triplicate assays, 20µl immune serum was mixed with 100µl of a bacterial inoculum ($2x10^4$ $cfuml^{-1}$). The assay suspensions were incubated for 15min at 22°C to allow antibody-bacteria interaction to occur. Heat-inactivated normal rainbow trout serum or normal rabbit serum served as the respective controls. Eighty µl fresh rainbow trout or rabbit serum (SAPU) was added as a complement source, except when evaluating the effect of antibody on *A.salmonicida* in the absence of complement, in which case 80µl of heat-inactivated rainbow trout (45°C, 30min) or rabbit serum (56°C, 30min) was added. Assay mixtures were incubated at 22°C for 2h. After incubation, 50µl samples of each triplicate suspension were removed, diluted in ten-fold dilutions and inoculated onto TSA plates. After incubation at 22°C for 48h, the mean number of $cfuml^{-1}$ were calculated.

The mean percent of bacterial inoculum which was killed (%K), in each suspension was calculated from the formula:-

$$\%K = 100 \times 1 - \frac{\text{(mean number of cfuml}^{-1}\text{ after incubation)}}{\text{(mean number of cfuml}^{-1}\text{ before incubation)}}.$$

In separate experiments, salmon immune serum was selectively absorbed with LPS-sensitised sheep red blood cells (SRBC)(Deb & Harry, 1976) to remove antibodies against LPS or absorbed with OMPs of *A.salmonicida* grown under iron-replete conditions to remove all antibodies except those directed against IROMPs. Absorbed sera was tested in an attempt to identify target antigens implicated in serum killing.

7.3 RESULTS

7.3.1 Composition of OMP vaccine

The *A.salmonicida* OMP vaccines used in this study were analysed by number of chemical assays (Table 7.4) and also examined by SDS-PAGE (Plate 7.1). The OMP nature of the preparations was confirmed by their insolubility in the detergent sarkosyl (sodium *N*-lauroyl sarcosinate) and by the presence of 2-keto-3-deoxyoctanate (KDO), characteristic of the LPS intrinsically associated with the Gram-negative OM. The OMP nature of the preparations was confirmed by SDS-PAGE, with the profiles being similar to those described by others (Chart & Trust, 1983; Aoki & Holland, 1985). At the vaccine protein concentration of 200μgml^{-1} used in this study, high levels of LPS were

detected by the Limulus amoebocyte assay and by the assay of Keler & Nowotny (1986), as well as a high carbohydrate content. The protein content of the OMP vaccine with respect to the IROMPs in the OMP vaccines prepared from *A.salmonicida* grown under conditions of iron-restriction was not determined. OMPs prepared from *A.salmonicida* MT423, an A-layer proficient strain, contained lower amounts of both LPS and carbohydrate than OMPs prepared from *A.salmonicida* MT004, an A-layer deficient mutant. This was due to the presence of the additional A-layer protein, since OMPs of A-layer-positive cells possess a higher protein:LPS/carbohydrate ratio. Therefore standardisation of the protein concentration of the OMP vaccines resulted in less LPS and carbohydrate compared with OMPs of A-layer-negative strain.

7.3.2 Passive immunisation

A salmon antiserum raised against the OMPs of *A.salmonicida* MT004 & MT423 grown under conditions of iron-restriction was used to assess the potential of IROMPs as protective antigens. This antiserum, shown to contain antibodies directed against the IROMPs by Western blotting techniques, was used to passively immunise Atlantic salmon against a bath challenge exposure to a virulent strain of *A.salmonicida*. The results presented in Table 7.5 indicate that passive protection was achieved using this antiserum with a RPS of 77%. Since the antisera raised against OMPs of two strains of *A.salmonicida* grown under conditions of iron-restriction contained additional antibodies, other than to the IROMPs, such as those against low MW LPS and the 49kDa A-layer protein, the contribution of the anti-IROMP antibody in this serum to the protective capacity could not be established.

To overcome this situation, a monospecific anti-IROMP antiserum prepared in rabbits against the gel-isolated IROMPs of *A.salmonicida* MT004 was evaluated for the ability to passively immunise Atlantic salmon against a heterologous bath challenge exposure to *A.salmonicida*. Again the results presented in Table 7.5 indicate that antisera raised against the IROMPs of *A.salmonicida* passively protected Atlantic salmon against a bath challenge exposure with a RPS of 60%. The importance of Ig in passively protecting Atlantic salmon was confirmed when passive protection of Atlantic salmon was achieved using an affinity-isolated rabbit anti-IROMP IgG with a RPS of 83%. The IgG gave better passive protection due to a higher concentration of IgG in the affinity-purified preparation.

Analysis of serum obtained from rainbow trout passively immunised with 100µl normal rabbit serum administered ip, was carried out using a dot-blot immunoassay in order to determine the time of the appearance of NRS in the circulation and to determine the duration of circulating Ig. Results of the immunoassay (Table 7.6) indicated that the rabbit Ig was detected within 1h of ip administration, and thus present at the time of bath challenge exposure (ie 2h). The rabbit Ig reached a maximum titre of 1/32 which lasted for a duration of 10 days, indicating that the rabbit Ig was in circulation and capable of reaching fish tissues in which *A.salmonicida* may grow and multiply.

7.3.3 Active immunisation

The results of the passive immunisation experiments indicated that anti-IROMP Ig has the potential to confer protection against bath challenge exposure to *A.salmonicida*, establishing the role of IROMPs as

potential protective antigens in the vaccination of Atlantic salmon against furunculosis.

Active immunisation of Atlantic salmon with OMPs of *A.salmonicida* grown under iron-replete and iron-restricted conditions at a dose of 20μg OMP fish^{-1} were compared by homologous and heterologous bath challenge exposure 42 days post vaccination in order to establish the role of IROMPs of *A.salmonicida* as protective antigens. Results of three separate immunisation trials (Table 7.7, 7.8, & 7.9) using both homologous and heterologous challenge with *A.salmonicida* NT423 & NT1057, both natural and experimental challenge, indicated that RPS values of 36.9% (55% homologous challenge), 69.6% and 100% were achieved using OMPs of *A.salmonicida* grown under iron-restricted conditions, shown to contain the 82,77,72 and 70kDa IROMPs by SDS-PAGE; these were higher than RPS values achieved by OMPs of iron-replete *A.salmonicida*. In addition, when RPS values were calculated using iron-replete OMPs as control, again a high level of protection was induced by IROMP antigens with RPS's of 66.7%, 53.3% and 100%.

Although the OMP preparations contained LPS and a number of other OM components, SDS-PAGE analysis of OMP vaccines indicated that the only difference between iron-replete and iron-restricted OMPs was the presence of the IROMPs. Therefore the high levels of protective immunity induced by iron-restricted OMPs was not based on the LPS content or the presence of other OM components. Immunisation of Atlantic salmon with iron-restricted OMPs of an A-layer positive strain gave a similar degree of protection (Table 7.7) indicating that the A-layer was not an important component of the *A.salmonicida* OMP vaccine.

7.3.4 Antibody response to IROMPs in immunised Atlantic salmon

Active immunisation of Atlantic salmon with 20µg OMP fish^{-1} resulted in detectable levels of anti-OMP antibody in pre-challenge sera at the time of challenge (ie 42 days post-immunisation) when analysed by Western blotting and by ELISA (Plate 7.2; Table 7.10). In addition to antibodies against low MW LPS and a number of other OM components, sera of fish immunised with iron-restricted OMPs contained antibodies reactive with the IROMPs, and gave higher ELISA values when reacted with iron-restricted OMP antigens compared with iron-replete OMP antigens. Pre-vaccination sera contained no detectable antibodies to OM components or IROMPs. However, comparison of pre- and post-challenge control serum obtained from Trial III by ELISA indicated that an anti-OMP Ig response had developed during a 46 day post-challenge period in control fish. The anti-OMP Ig response was stronger against iron-restricted OMP antigens in the ELISA (Table 7.10) and indicated that anti-IROMP Ig was present in convalescent sera of fish experimentally exposed to *A.salmonicida*, confirming the immunogenicity of the IROMPs in Atlantic salmon and their expression *in vivo* during infection.

7.3.5 Bactericidal capacity of immune sera

The bactericidal action of salmon anti-OMP antisera containing antibodies against the IROMPs of *A.salmonicida* was examined against three typical and an atypical strain of *A.salmonicida* in an attempt to determine the functional mechanisms of immunity operating in the protection induced by the OMP vaccines. All of the strains examined were capable of growth in heat-inactivated normal fish serum; in

addition all but *A.salmonicida* MT004, an avirulent mutant lacking the 49kDa A-layer protein, were also capable of growth in the presence of complement. Thus serum complement was not bactericidal for virulent strains of *A.salmonicida* in the absence of antibody, indicating that these strains were not capable of activating the alternative complement pathway (Brown *et al.*, 1983; Taylor, 1983).

In the presence of complement, immune salmon serum was found to be bactericidal with 45.0-84.0% of *A.salmonicida* being killed in the assay. When the complement source was heat-inactivated, the bactericidal capacity of the immune serum was reduced to 15.0-20.0%.

Since the immune salmon serum contained antibodies against LPS and a number of OM components, in addition to antibodies against the IROMPs, the serum was selectively absorbed in order to determine which antibodies were responsible for the bactericidal activity (Table 7.11). Absorption of the immune serum with LPS sensitised SRBC failed to abolish the bactericidal activity, as did absorption of the immune serum with iron-replete OMPs. Only absorption of the immune serum with iron-restricted OMPs abolished the bactericidal activity of the immune serum, indicating that the IROMPs of *A.salmonicida* are the target for antibody in the bactericidal complex.

The bactericidal activity of the rabbit anti-IROMP antiserum was examined in order to confirm that the IROMPs were a target in the bactericidal complex (Table 7.12). Again, the four strains of *A.salmonicida* studied were able to grow in the presence of heat-inactivated normal rabbit serum, and complement was not bactericidal in the absence of specific antibody for the three virulent strains of *A.salmonicida*. In the presence of complement, immune rabbit serum was found to be bactericidal with 83.2-85.7% of *A.salmonicida*

being killed in the assay. When the complement source was heat-inactivated, the bactericidal capacity of the immune serum was reduced to 15.4-60.0%. In this case, immune rabbit serum was bactericidal in the absence of complement suggesting that bactericidal activity was partly due to the monospecific rabbit anti-IROMP antisera.

7.4 DISCUSSION

Results presented in Chapter 6 indicate that the IROMPs of *A.salmonicida* may serve as potential protective antigens in the vaccination of Atlantic salmon against furunculosis.

Firstly, it was demonstrated that immune salmon serum containing antibodies against the IROMPs of *A.salmonicida* was able to passively protect Atlantic salmon against challenge with virulent *A.salmonicida*. It was also demonstrated that a rabbit anti-IROMP antiserum and affinity-isolated IgG from this antiserum, was also able to passively immunise Atlantic salmon. Thus, it was confirmed that protective immunity was transferrable using antibodies raised against the IROMPs of *A.salmonicida*. These antibodies in passively immunised fish were in circulation for approximately 10 days post-challenge, suggesting that the presence of specific antibody may mediate protection. Although in several instances, agglutinating antibodies in actively immunised fish were not found to be correlated with protection (Olivier *et al.*, 1986b), this study confirms the importance of antibody in protection induced by IROMPs. The role of cell-mediated immunity in the protection afforded by the IROMPs of *A.salmonicida* was not investigated in this study.

Secondly, in order to examine their role as protective antigens, fish were immunised with iron-restricted OMPs of *A.salmonicida*

containing the 82,77,72 and 70kDa IROMPs and compared to fish immunised with iron-replete OMPs. Results of three immunisation trials indicate that IROMP vaccines conferred significant protection giving RPS's of 56.7%, 53.3% and 100% compared with OMP vaccines. In addition, active immunisation with iron-restricted OMPs elicited a serum anti-IROMP antibody response, measured by Western blotting and ELISA, at the time of challenge.

An interesting feature when examining the specific immune response of immunised Atlantic salmon was the finding that surviving post challenge control fish (ie convalescent fish) had developed an anti-IROMP antibody response, indicating expression of the IROMPs of *A.salmonicida in vivo* during infection. This confirms the immunogenicity of the IROMPs and their expression *in vivo* during infection. An interesting question arising from these observations is why aren't surviving fish immune to further infection? Although it is known from work carried at the Marine Laboratory that antibody levels of immune Atlantic salmon generally decrease after challenge (Ellis, unpublished observations), it is possible that subsequent challenge decreases antibody present in convalescent fish. However, at present the question remains largely unanswered.

In order to determine the functional mechanisms of immunity, the effects of the anti-IROMP antisera on *A.salmonicida in vitro* were investigated. Antisera raised against a number of potentially protective OMP antigens have been demonstrated to be bactericidal eg *H.influenzae* (Munson *et al.*, 1983; Loeb, 1987; Rubin *et al.*, 1991) and *N.mengitidis* (Brodeur *et al.*, 1985; Saukkonen *et al.*, 1987). Recently antibodies raised against the 70kDa Tf-binding protein of *N.meningitidis* were found to bactericidal (Bhanerjee-Bhatnagar & Frasch, 1991;

Pettersson *et al.*, 1991). Although virulent strains of *A.salmonicida* are not normally susceptible to the bactericidal activity of serum (containing antibodies against A-layer and LPS) and complement (Munn *et al.*, 1982), antibody and complement-mediated bacterial killing may occur when antibodies are directed against the IROMPs or additional antigens expressed *in vivo*. This may be a possible mechanism contributing to the prevention of *A.salmonicida* infection in Atlantic salmon. Recent work by Thornton *et al.*, (1991), using surface-disorganised, live, attenuated mutants of *A.salmonicida* reported that these strains possessed increased sensitivities to membrane antagonists and to the bactericidal action of serum compared to the wild-type parent strain. It was suggested that these features may be due to incomplete assembly of the 49-kDa A-layer protein. It has been proposed by Kay & Trust (1991) that such strains may expose important cell surface antigens, normally shielded by the A-layer structure. In Chapter 6, it was found that *A.salmonicida* grown under conditions of iron-restriction also possessed increased sensitivities to membrane antagonists and thus may have an incomplete A-layer and expose important cell surface proteins which may be the target of bactericidal antibody.

In this study, it was found that immune salmon serum containing antibodies against the IROMPs was bactericidal in the presence of complement, although antibody was slightly bactericidal in the absence of complement. Absorption of the immune serum to remove antibodies against the IROMPs abolished the bactericidal activity of the serum but absorption with LPS or iron-replete OMPs had no effect. To confirm that the IROMPs were the target antigen in the bactericidal complex, the assays were repeated using a monospecific rabbit anti-IROMP antiserum. This was also bactericidal in the presence of complement against both

typical and atypical strains. Again, as with the immune salmon serum, antibody was bactericidal in the absence of complement. It is possible that antibodies against the IROMPs in some way interfered with the iron uptake mechanisms of *A.salmonicida*. It is well recognised that antisera raised against certain bacterial IROMPs have the potential to inhibit the biological function of the receptor protein. For instance, antisera against the 78kDa fhuA ferrichrome receptor protein in *E.coli* K12 partially inhibited ferrichrome-iron uptake (Coulton, 1982) and antisera against the 14kDa pyochelin receptor protein of *Ps.aeruginosa* was found to inhibit binding and uptake of $^{59}Fe^{3+}$-pyochelin (Sokol & Woods, 1984;1986) In addition, antisera raised against 80kDa pyoverdin receptor protein inhibits uptake of $^{59}Fe^{3+}$-pyoverdin (Meyer *et al.*, 1990) and a polyclonal rabbit antisera against the 74kDa IutA aerobactin receptor of *E.coli* K12 was found to be able to inhibit the binding of aerobactin, cloacin DF13 and bacteriophage B74K (Roberts *et al.*, 1989). Although the opsonic activity of anti-IROMP antibody in contributing to the mechanisms of protective immunity were not examined in this study, it is possible that these antibodies also enhance the bactericidal activities of the fish's phagocytes.

The results of this study have important implications for the formulation of a successful furunculosis vaccine against both typical and atypical strains of *A.salmonicida*. The poor efficacy of existing furunculosis vaccines may be explained by the limited immune response of Atlantic salmon to antigens expressed on the cell surface of *A.salmonicida* grown in iron-replete media. In contrast, the increased number of antigens seen in *A.salmonicida* grown under iron-restricted conditions and their immunogenicity in Atlantic salmon may be essential for an effective furunculosis vaccine. It is now known that the IROMPs

of *A.salmonicida* are immunogenic, expressed *in vivo*, and are antigenically homogeneous. Antibody against IROMPs is bactericidal and protective and results reported here indicate that it is beneficial to include these antigens in a furunculosis vaccine. The protective efficacy of furunculosis vaccines containing IROMPs of *A.salmonicida* are assessed in Chapter 8.

TABLE 7.1 Summary of outer membrane protein (OMP) vaccines.

Bacterium	OMP	Reference
Bordetella bronchiseptica	mAb anti-68kDa OMP passively protects mice	Montarez *et al* (1985)
	Affinity-purified 68kDa OMP protective in mice	Kobisch & Novotny (1990)
Bordetella pertussis	69kDa OMP protects against lethal challenge in mice mAb passively protects	Shahin *et al* (1990)
	69kDa OMP protective	Novotny *et al* (1991)
Borrelia burgdorferi	anti-outer surface protein A (OspA) passively protects mice	Simon *et al* (1991)
Chlamydia trachomatis	mAb recognise epitopes of OMP - protective	Zhang *et al* (1987)
	OMPs - protective antigen mAb anti-OMP neutralise chlamydial toxicity in mouse model	Zhang *et al* (1989)
	Ab to major OMP neutralises infectivity for hamster kidney cells	Su & Caldwell (1991)
Haemophilus influenzae type b	46kDa OMP (protein a) protects rats from bacteraemia	Loeb (1987)
	anti-98kDa OMP Ab passively protects infant rats	Kimura *et al* (1985)
	mAb anti-39kDa OMP passively protects infant rats	Hansen *et al* (1982)
	anti-P2 porin passively protects infant rats	Munson *et al* (1983)
	anti-145kDa OMP Ab passively protects rats	Rubin *et al* (1991)
Haemophilus pleuropneumoniae	OMPs protective in pigs	Rapp & Ross (1988)
	proteinase K-treated OM vaccine protective in swine	Chiang *et al* (1991)
Haemophilus somnus	anti-78 & 40kDa OMP passively protects cattle	Gogolewski *et al* (1987; 1988)

TABLE 7.1 Continued.

Bacterium	OMP	Reference
Neisseria meningitidis	anti-class 2 OMP passively protects mice	Brodeur *et al* (1985)
	anti-class 1 OMP (1.16) passively protects rats	Saukkonen *et al* (1987)
	serotype 2b OMP vaccine protective	Wang & Frasch (1984)
Pasteurella haemolytica	Ab against OMPs associated with resistance	Mosier *et al* (1989)
	SSE extract of iron-restricted cells protects lambs against pasteurelosis	Gilmour *et al* (1991)
Pasteurella multocida	anti-50kDa antiphagocytic OMP factor passively protects turkeys	Truscott & Hirsh (1988)
	mAb anti-37.5 surface exposed OMP passively protects rabbits & mice	Lu *et al* (1991a)
	Rabbit anti-OMP inhibits proliferation in mouse lung	Lu *et al* (1991b)
	OMP significant protection in rabbits against homologous challenge	Lu *et al* (1991c)
Proteus mirabilis	OMP protect mice from pyelonephritis	Moayeri *et al* (1991)
Pseudomonas aeruginosa	purified 35-37kDa porin F OMP protects actively immunised mice - immunogenic rabbit anti-porin F passively protects	Gilleland *et al* (1984)
	mAb anti-porin F passively protects	Hancock *et al* (1985)
	Purified porin F protects mice	Matthews-Greer & Gilleland (1987)
Salmonella typhiumurium	LT2 porin protects mice	Matsui & Arai (1989; 1990)

TABLE 7.2 Immunisation regimes used to determine efficacy of immune Atlantic salmon and rabbit sera containing antibodies against IROMPs of *A.salmonicida* by passive immunisation of Atlantic salmon.

Antiserum*	Specificity	Dose fish^{-1}
NSS	anti-PBS control	100µl
ISS	anti-IROMP	100µl
NRS	control	100µl
IRS	anti-IROMP	100µl
NRIgG**	control IgG	50µl
IRIgG**	anti-IROMP IgG	50µl

NSS - PBS injected Atlantic salmon serum
ISS - immune Atlantic salmon serum
NRS - normal rabbit serum
IRS - immune rabbit serum
NRIgG - normal rabbit immunoglobulin G
IRIgG - immune rabbit immunoglobulin G

* - Immune Atlantic salmon serum was prepared as described in 6.2.11.
- Immune rabbit serum was prepared as described in 6.2.12.
** - Atlantic salmon immunised with rabbit IgG serum received 50µl NRS as a source of complement.

TABLE 7.3 Immunisation regimes used to determine efficacy of outer membrane protein (OMP) vaccines of *A.salmonicida* in Atlantic salmon.

Antigen*	Dose $fish^{-1}$
PBS	—
MT004 Fe^{+}OMP	20μg
MT004 Fe^{-}OMP	20μg
MT423 Fe^{-}OMP**	20μg

* Fe^{+}OMP, OMPs prepared from iron-replete *A.salmonicida* MT004 grown in Tryptone Soya Broth (TSB) as described in 4.2.9; Fe^{-}OMP, OMPs prepared from iron-restricted *A.salmonicida* MT004 or MT423 grown in TSB + 2,2'-dipyridyl as described in 4.2.9.

** Trial 1 only

TABLE 7.4 Biochemical analysis of *A.salmonicida* outer membrane protein (OMP) vaccines.

OMP antigen	protein $\mu g ml^{-1}$	LPS $\mu g ml^{-1}$	LAL $EU ml^{-1}$	KDO μgLPS mg protein ml^{-1}	Carbohydrate $mg ml^{-1}$
MT004 Fe^+	200	230	>268	5.5	4.3
MT004 Fe^-	200	285	>268	5.0	9.0
MT423 Fe^-	200	54.4	>268	0.2	0.38

LPS, lipopolysaccharide; LAL, limulus amoebocyte lysate; KDO, 2-keto-3-deoxyoctanate.

TABLE 7.5 Efficacy of the passive immunisation of Atlantic salmon with antibodies against IROMPs of *A.salmonicida*.

Treatment	n=	% Mort	RPS v PBS control	RPS v control serum
PBS	20	40	—	—
NSS	7	43	-7.5	—
ISS	10	10	75	77
NRS	20	20	50	—
IRS	20	10	75	50
NRIgG	20	30	25	—
IRIgG	20	5	88	83

NSS - normal Atlantic salmon serum
ISS - immune Atlantic salmon serum
NRS - normal rabbit serum
IRS - immune rabbit serum
NRIgG - normal rabbit immunoglobulin G
IRIgG - immune rabbit immunoglobulin G

TABLE 7.6 Duration of circulating rabbit immunoglobulin in passively immunised Rainbow trout, analysed by dot-blot.

Time (days)	Rabbit Ig titre
0.04	0
0.08	4
0.25	8
0.50	8
1	32
2	32
4	32
6	32
8	16
10	32
12	16
14	4
16	0
18	0
20	0

TABLE 7.7 Efficacy of active immunisation of Atlantic salmon with outer membrane proteins of *A.salmonicida* challenged with an experimental infection (Trial I), using strain MT423. Fish were distributed in 5 tanks ie 11 fish group^{-1} tank^{-1} where numbers allowed.

Antigen	n=	%mort	RPS	RPS v vaccine control
PBS	54	44.4	—	—
MT004 Fe^{+}OMP	17	64.7	-45.7	—
MT004 Fe^{-}OMP	25	28.0	36.9	56.7
MT423 Fe^{-}OMP	50	20.0	55.0	—

TABLE 7.8 Efficacy of active immunisation of Atlantic salmon with outer membrane proteins of *A.salmonicida* naturally challenged (Trial II) by strain MT1057. Fish were distributed in 7 tanks ie 11 fish group^{-1} tank^{-1} where numbers allowed.

Antigen	n=	%mort	RPS	RPS v vaccine control
PBS	77	29.9	—	—
MT004 Fe^{+}OMP	77	19.5	34.8	—
MT004 Fe^{-}OMP	77	9.1	69.6	53.3

TABLE 7.9 Efficacy of active immunisation of Atlantic salmon with OMPs of *A.salmonicida* challenged with an experimental infection (Trial III) by strain MT423. Fish were distributed in 8 tanks ie 4 fish group^{-1} tank^{-1} where numbers allowed.

Antigen	n=	%mort	RPS	RPS v vaccine control
PBS	32	65.6	—	—
MT004 Fe^{+}OMP	32	6.3	90.4	—
MT004 Fe^{-}OMP	32	0.0	100	100

TABLE 7.10 Serum antibody response of Atlantic salmon immunised with OMPs of *A.salmonicida* examined by IROMP ELISA (Trial III).

Vaccine	n=	Antigen	ELISA antibody response mean OD_{405} (+/-SE) 1	2	3
PBS	5	Fe^+	0.000	0.000±0.000	0.000±0.000
		Fe^-	0.000	0.000±0.000	0.157±0.053
MT004 Fe^+OMP	5	Fe^+	0.000	0.285±0.097	0.018±0.000
		Fe^-	0.000	0.208±0.071	0.016±0.000
MT004 Fe^-OMP	5	Fe^+	0.000	0.266±0.142	0.130±0.024
		Fe^-	0.000	0.306±0.162	0.116±0.039

1 pre-vaccination sera
2 pre-challenge sera
3 post-challenge sera

Fe^+, iron-replete outer membrane protein antigen; Fe^-, iron-restricted outer membrane protein antigen.

TABLE 7.11 Bactericidal activity of Atlantic salmon serum immunised with Fe⁻OMPs expressed as %K. Results are the mean of triplicate values (+/- SE of the mean).

Strain (MT)	Control	Immune - C	Immune + C	Abs LPS	Abs Fe⁺OMP	Abs Fe⁻OMP
004 A⁻T	0	15.8±2.6	65.8±0.5	68.4±0.3	71.1±0.8	0
423 A⁺T	0	25.0±2.5	84.0±1.0	82.5±0.5	81.0±0.5	0
486 A⁺T	0	20.0±2.0	52.0±2.1	52.0±4.6	36.0±2.0	0
194 A⁺A	0	15.0±1.5	45.0±1.5	36.0±2.0	39.0±1.9	0

-C, heat-inactivated sera; +C, normal sera; Abs LPS, sera absorbed with lipopolysaccharide (LPS) to remove anti-LPS antibodies; Abs Fe⁺OMP, sera absorbed with iron-replete OMPs to remove anti-Fe⁺OMP antibodies; Abs Fe⁻OMP, sera absorbed with iron-restricted OMPs to remove anti-Fe⁻OMP antibodies.

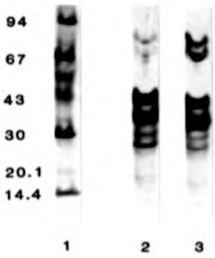
TABLE 7.12 Bactericidal activity of rabbit antisera raised against the IROMPs of *A.salmonicida* expressed as %K. Results are the mean of triplicate values (+/- SE of the mean).

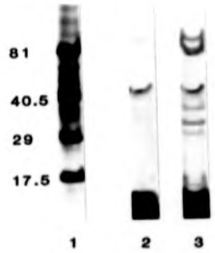
Strain (MT)	Control	Immune - C	Immune + C
004 A⁻T	0	42.1±0.0	63.2± 2.6
423 A⁺T	0	60.0±5.0	85.7± 1.0
486 A⁺T	0	15.4±3.8	66.9±10.0
194 A⁺A	0	44.0±2.0	73.6± 2.4

-C, heat-inactivated sera; +C, normal sera.

PLATE 7.1 SDS-PAGE of iron-replete and iron-restricted outer membrane protein (OMP) antigens of *A.salmonicida* MT004. Lane 1, low molecular weight protein standards in kDa; Lane 2, OMPs of iron-replete *A.salmonicida* MT004 grown in Tryptone Soya Broth (TSB); Lane 3, OMPs of iron-restricted *A.salmonicida* MT004 grown in TSB + 2,2'-dipyridyl (100μM).

PLATE 7.2 Western blot of iron-replete and iron-restricted outer membrane protein (OMP) antigens of *A.salmonicida* MT004 stained with antisera from Atlantic salmon immunised with iron-restricted OMP antigens of *A.salmonicida*. Lane 1, biotinylated molecular weight standards in kDa; Lane 2, OMPs of iron-replete *A.salmonicida* MT004 grown in Tryptone Soya Broth (TSB); Lane 3, OMPs of iron-restricted *A.salmonicida* MT004 grown in TSB + 2,2'-dipyridyl (100μM).


94
67
43
30
20.1
14.4
1
2
3


81
40.5
29
17.5
1
2
3

CHAPTER 8:- EVALUATION OF AN IRON-RESTRICTED BACTERIN OF *AEROMONAS SALMONICIDA*

8.1 INTRODUCTION

Furunculosis is the most economically important disease of cultivated Atlantic salmon in Scotland. Its association with serious economic loss and the increasing prevalence of multi-antibiotic-resistant strains has led to many attempts to develop an *A.salmonicida* vaccine. The first reports of attempts to vaccinate fish against *A.salmonicida* were by Duff (1942) using a chloroform-inactivated *A.salmonicida* whole cell bacterin, administered orally to brook trout. However, the success of Duff has not been repeated by others and the efficacy of the many attempts to develop vaccines against the disease to date (see Michel, 1982; Munro, 1984; Hastings, 1988 for reviews) has been variable and much lower when compared to vaccines developed against other important bacterial septicaemic diseases of fish such as *Y.ruckeri* (enteric redmouth), *V.anguillarum* and *V.ordalii* (vibriosis)(Ellis, 1988) and *V.salmonicida* (Hitra disease)(Holm & Jorgensen, 1987). These successful vaccines are based upon inactivated whole cell bacterins, containing both cell-associated and extracellular antigens, where the protective antigen is thought to be LPS (Ellis, 1988).

Bacterial vaccines are generally developed to combat bacterial disease by raising specific antibody that reacts with or neutralises the virulence determinants of the bacterial pathogen. Such antigens are by definition termed protective antigens. Attempts to identify protective

antigens of *A.salmonicida* have generally taken two approaches. The first of these examined the ability of crude *A.salmonicida* bacterin vaccines to confer protection against experimental challenge. Such work has generally been unsuccessful probably because of the lack of appropriate protective antigens in the bacterin. The second has been to determine the immunogenicity and protective ability of specific virulence factors. A number of components such as the 49kDa A-layer and LPS are known to be immunogenic but generally not protective (Chart *et al.*, 1984b) although there is some evidence of the A-layer providing protective immunity (McCarthy *et al.*, 1983; Olivier *et al.*, 1985b). A number of other factors important in virulence such as haemolysin and protease are generally poor immunogens in salmonids (Hastings & Ellis, 1988;1990) although passive immunisation studies identified protease as a potential protective antigen (Ellis *et al.*, 1988a). Although agglutinating antibody levels are recognised by some workers as not being correlated with protection (Michel & Faivre, 1982; Cipriano, 1982a; McCarthy *et al.*, 1983; Olivier *et al.*, 1985b), passive immunisation studies have demonstrated that an antibody response in fish can be protective if important antigens can be identified and their immunogenicity enhanced by chemical or physical modification (Hastings & Ellis, 1990).

Recently the use of attenuated bacterial vaccines using metabolic and regulatory mutants has recieved much attention for *Salmonella* and other species and evaluated as live vaccine candidates (Levine *et al.*, 1987; Cooper *et al.*, 1990; Jones *et al.*, 1991; Verma & Lindberg, 1991). This strategy for vaccine construction has also been applied to the development of a furunculosis vaccine. The use of live, attenuated strains of *A.salmonicida* has been examined using two approaches.

Firstly, avirulent *aroA* mutants of *A.salmonicida* have been constructed and found to induce effective protection against experimental furunculosis in brown trout (Vaughan, Trinity College, Dublin, personal communication). Secondly, avirulent, surface-disorganised, slow-growing aminoglycoside-resistant mutants of *A.salmonicida*, and a rapidly growing pseudorevertant strain of *A.salmonicida* have been found to be capable of tissue persistence (Thornton *et al.*, 1991). In addition, the pseudorevertant strain was found to be capable of effectively protecting salmonid fish against lethal challenge when administered either by ip or immersion, indicating the role of the mutant as a live, attenuated furunculosis vaccine candidate. It is possible that the tissue persistence of these attenuated *A.salmonicida* mutant strains enables them to grow and multiply slowly and to adapt to the *in vivo* environment, and may enable them to express a full array of virulence factors.

Results presented in Chapters 6 and 7 identified the IROMPs of *A.salmonicida* as protective antigens in the vaccination of Atlantic salmon against furunculosis. Although the use of a pure IROMP vaccine would be appropriate in human medicine and certain veterinary animals, the cost of manufacture would not justify its usage as a mass-administered vaccine in the aquaculture industry. Another approach may involve the use of recombinant DNA technology in which the *A.salmonicida* genes involved in the synthesis of the IROMPs could be cloned and then expressed in large quantities in other non-pathogenic microorganisms. An alternative approach is to produce an iron-restricted *A.salmonicida* bacterin in which the production of IROMPs is induced, but without the need for purification.

The aim of this study was to evaluate the potential of an

iron-restricted bacterin containing the protective IROMP antigens of *A.salmonicida* and to compare the efficacy of the whole cell bacterin with that of the partially purified IROMP vaccine.

8.2 METHODS AND MATERIALS

8.2.1 *A.salmonicida* and growth conditions

Two strains of *A.salmonicida* (MT004 & MT423) were used in this study Iron-restricted bacteria were grown in TSB + 100μM 2,2'-dipyridyl. Iron-replete bacteria were grown in TSB supplemented with FeCl$_3$ (100μM). *A.salmonicida* was incubated at 22°C for 48h on a rotary shaker.

8.2.2 Production of an iron-restricted vaccine

The experimental vaccine consisted of an iron-restricted bacterin of *A.salmonicida* MT004 and was compared to an identical bacterin grown under iron-replete conditions. After 48h incubation at 22°C, 10ml aliquots were removed for biochemical analyses described below. Formalin (40% (w/v) formaldehyde solution) was added to a final concentration of 0.5% (v/v) and broths reincubated at 22°C for a further 48h. Cell counts of the bacterins were performed using a bacterial counting chamber and the iron-restricted bacterin adjusted to 10^8, 10^9 and 10^7 cellsml^{-1} either by dilution of the bacterin with 48h iron-restricted ECP or concentrated ten-fold by centrifugation. Sterility of the vaccine was confirmed by inoculating 100μl aliquots of the vaccine on TSA and incubating at 22°C for 48h. The vaccine was

stored at 4°C until use.

8.2.3 Biochemical analyses

Cellular components

8.2.4 Guanidine-HCl extraction of whole cells

Whole cell extracts of *A.salmonicida* were prepared using guanidine-HCl (GHCl) as described by Johnson *et al.*, (1986). *A.salmonicida* grown under iron-replete or iron-restricted conditions were washed in PBS and resuspended to a density of approximately 10^9 cellsml^{-1} in 2M guanidine-HCl and incubated for 30min at 22°C with mixing. The mixture was centrifuged at 2000*g* for 30min, and the supernatant dialysed overnight against PBS at 4°C and examined by SDS-PAGE.

8.2.5 OMP preparation

Outer membrane proteins were prepared from *A.salmonicida* grown under iron-replete and iron-restricted conditions as described in 4.2.9.

8.2.6 Determination of IROMPs in the iron-restricted bacterin

The presence of IROMPs in the iron-restricted *A.salmonicida* vaccine was determined by ELISA. Titertek PVC immunoassay plates were pre-coated with poly-L-lysine ($1mgml^{-1}$) for 15min prior to being coated with 10^9 *A.salmonicida* cells ml^{-1} in 0.05M carbonate/bicarbonate buffer

and incubated overnight at 22°C. Antigen coated immunoassay plates were washed twice for 5min with TTBS and blocked with 5% normal sheep serum (NSS; SAPU) in TTBS for 1h at 22°C. Rabbit anti-IROMP antibody (1:100, 100ul) in 5% NSS/TTBS were added to the immunoassay plates and incubated for 1h at 37°C. After two further 5min washes with TTBS, immunoassay plates were incubated for 1h at 37°C with 100µl sheep anti-rabbit IgG-alkaline phosphatase (Sigma; 1:1000) followed by one 5min TTBS wash and two TBS washes. Plates were then washed with 100µl 10mM diethanolamine (pH9.5) containing 0.5mM $MgCl_2$ followed by 100µl substrate (1mg *p*-nitrophenyl phosphate ml^{-1} in 10mM diethanolamine, 0.5mM $MgCl_2$ solution) for 30min at 22°C. The reaction was terminated by the addition of 50µl 0.1M EDTA solution and the absorbance of the bound conjugate measured at 405nm using a TiterTek Multiscan MC plate reader.

Extracellular components (ECP)

8.2.7 ECP preparation

Extracellular components (ECP) were obtained from *A.salmonicida* MT004 & MT423 grown under iron-replete and iron-restricted conditions by centrifugation (8000*g* for 30min at 4°C) and analysed in an attempt to characterise components of the experimental vaccine.

8.2.8 Protein assay

Protein concentration of extracellular products (ECP) was determined by the method of Bradford (1976) using BSA as standard with a BioRad protein assay kit.

8.2.9 Protease assay

Proteolytic activity of ECP was determined by measuring hide powder azure (HPA) or azocoll digestion as described by Hastings (1986). ECP (100µl) was added to HPA/azocoll (10mgml^{-1} in PBS) and incubated at 37°C for 15min. The reaction was terminated by the addition of an equal volume of 10% (w/v) trichloroacetic acid (TCA) to the reaction mixture. The resulting precipitate was then pelleted by centrifugation at 3000*g* for 15min and the absorbance at 600nm was measured. The control was prepared by incubating ECP in the presence of TCA and HPA/azocoll substrate. One unit of enzyme activity was defined as the amount which yielded an increase in absorbance of 0.01.

8.2.10 Haemolytic assay

Haemolytic activity of ECP was determined by the method described by Hastings (1986). Blood from rainbow trout (*Oncorhynchus mykiss*) was collected in Alsever's solution (Gibco) and red blood cells (RBC) washed three times in PBS by centrifugation at 1000*g* for 10min at 4°C. The packed RBC s were resuspended to 1% (v/v) in PBS. ECP (100µl) was then diluted serially with PBS in a microtitre plate to which 100µl 1% (v/v) red blood cell suspension was added. The final well, lacking ECP served as control. The plates were incubated at 22°C for 2hr and the haemolytic titre expressed as haemolytic units ml^{-1} (HU ml^{-1}) obtained from the reciprocal of the dilution producing a visually determined 50% endpoint of haemolysis.

8.2.11 LPS assay

Endotoxin (LPS) present in ECP was assayed by the method of Keler & Nowotny (1986).

8.2.12 Electrophoresis

Outer membrane proteins (20μg protein ml^{-1}), low molecular weight protein standards (Pharmacia-LKB) and biotinylated molecular weight markers (Pierce) were incubated with sample buffer and subjected to SDS-PAGE as described in 4.2.10

8.2.13 Active immunisation

Atlantic salmon parr (about 10-20g) maintained as described in 7.2.9 were immunised with the vaccines described in Table 8.1. Immunisation regimes lasted for 42 days after primary injection of 100μl vaccine/fish. Fish injected with phosphate-buffered saline (PBS) alone served as controls. For numbers of fish, tank distribution, and number of replicate tanks see Results (8.3).

8.2.14 *A.salmonicida* challenge

Forty-two days post-immunisation, fish were subjected to a 24h bath exposure of approximately 1x10^{8} cfuml^{-1} of *A.salmonicida* NT423 as described in 7.2.12. The relative percentage survival (RPS) of each vaccinated group was determined by the method of Amend (1981) as described in 7.2.12.

8.2.15 ELISA

Antibody responses against IROMPs of *A.salmonicida* in immunised fish were analysed by an ELISA as described in 7.2.13.

8.2.16 Western blotting

Whole cell extracts, OMPs and ECP fractionated by SDS-PAGE were transferred to nitrocellulose as described in 4.2.11. After transfer, nitrocellulose membranes were either stained for total protein with colloidal gold (Aurodye, Amersham) or blocked for 1h with 10% (v/v) normal goat serum (SAPU) in Tris-buffered saline (pH 7.5) containing 0.05% (v/v) Tween 20 (TTBS).

Atlantic salmon antibody was detected in Western blots as described in 8.2.16.

8.3 RESULTS

8.3.1 Composition of the iron-restricted bacterin

The cell-associated and extracellular components of the iron-replete and iron-restricted bacterins of *A.salmonicida* were analysed by a number of chemical assays (Table 8.2). The iron-replete bacterin grew to a cell density of 8.5×10^7 cellsml^{-1} yielding a vaccine which was administered at a dose of approximately 10^7 cells $fish^{-1}$. By comparison, the iron-restricted bacterin grew to a cell density of 9.8×10^7 cellsml^{-1}. In this instance, the vaccine was administered at a

dose of approximately 10^8, 10^7 and 10^6 cells $fish^{-1}$. Comparison of the extracellular products of iron-replete and iron-restricted bacterins indicated generally higher levels of protease and haemolytic activity and lower ECP protein content in the iron-restricted bacterin. SDS-PAGE analysis of ECPs revealed no qualitative differences in the protein composition of the extracellular components of *A.salmonicida* (Plate 8.1). Interestingly, the LPS content of the iron-restricted ECP was found to be approximately 25% of that of the iron-replete ECP.

Comparison of the cellular components of the iron-replete and iron-restricted bacterins were made by SDS-PAGE analysis of guanidine-HCl whole cell extracts and OMP preparations (Plate 8.1). The only qualitative differences detected by SDS-PAGE were the presence of the 82,77,72 & 70kDa IROMPs of *A.salmonicida* in the OMP preparations prepared from the iron-restricted bacterin. The IROMPs were not detected in the iron-replete bacterin.

The chemical analyses determined above were carried out on the extracellular products and whole cells from the bacterins before the addition of formalin. Since the IROMPs of *A.salmonicida* are important protective antigens, it was important to determine their presence in the inactivated formalinised whole cell bacterins. This was performed using an ELISA. IROMP antigens were found to be present in the iron-restricted bacterin after the addition of formalin (data not shown).

8.3.2 Efficacy of the iron-restricted bacterin

Active immunisation of Atlantic salmon with an iron-restricted bacterin of *A.salmonicida* MT004 at a dose of 10^8, 10^7 and 10^6

cells $fish^{-1}$ and with an iron-replete bacterin at a dose of 10^7 cells $fish^{-1}$ was compared by heterologous bath challenge in order to determine the effectiveness of the IROMP antigens in an iron-restricted whole cell *A.salmonicida* bacterin (Table 8.3). The challenge was severe with 86.4% mortality in control fish. All the vaccines examined performed well with RPS's of 89.8, 89.8, 71.8 and 87.2% for the iron-restricted bacterin (10^8, 10^7 & 10^6) and the iron-replete bacterin (10^7) respectively. This is compared to a RPS of approximately 60% achieved using a commercially available furunculosis vaccine (Furovac, AVL) used in all immunisation trials as an internal control (SOAFD Marine Laboratory, unpublished observations). Little in the way of a dose-response to the vaccine was observed, although the bacterins at lower cell densities performed marginally better. Surprisingly, the iron-replete bacterin performed as well, dose for dose, as the iron-restricted bacterin in acheiving a RPS of 87.2%

8.3.3 Antibody response to the iron-restricted vaccine

The antibody response of Atlantic salmon immunised with the iron-replete and iron-restricted bacterins of *A.salmonicida* (both at 10^8 $cells ml^{-1}$) were examined 42 days post-vaccination (ie pre-challenge) by Western blotting and ELISA to determine the presence of an anti-IROMP antibody response. Antisera were either reacted against both whole cell GHCl extracts and OMP preparations prepared from iron-replete and iron-restricted *A.salmonicida* in the immunoblots, or reacted against iron-replete and iron-restricted OMP preparations in the ELISA. As expected, only the antisera from fish immunised with the iron-restricted bacterin reacted with the IROMPs of *A.salmonicida* present in the

iron-restricted OMP preparations (Plate 8.2 & Table 8.4). In addition to an anti-IROMP response identified in the appropriate fish, the only other cell-associated component of *A.salmonicida* present in both iron-replete and iron-restricted bacterins found to be immunogenic by Western blotting in the immunised fish was low MW LPS (Plate 8.2).

In an attempt to partly explain the success of the iron-replete bacterin of *A.salmonicida*, lacking the IROMP antigens, fish antisera from immunised fish was reacted against extracellular components of the iron-replete and iron-restricted bacterin, to determine the presence of an additional protective antigen. Results of the Western blots (Plate 8.2), indicate that the only immunogenic component of both iron-replete and iron-restricted extracellular products was the 70kDa protease of *A.salmonicida*. No additional immunogenic components were identified in the extracellular products of either the iron-replete or iron-restricted bacterins.

8.4 DISCUSSION

Results presented in previous chapters (6 and 7) indicated the role of the IROMPs of *A.salmonicida* as important protective antigens in the development of a furunculosis vaccine. However for the purpose of an economically viable *A.salmonicida* vaccine, acceptable to the aquaculture industry, an iron-restricted *A.salmonicida* bacterin produced to include the IROMP antigens was prepared. The results of this study are the first to show induction of protective immunity in Atlantic salmon against furunculosis by administration of an iron-restricted bacterin of *A.salmonicida*. Such a vaccine would be feasible to manufacture cheaply with little modification to existing fermentation

facilities. The vaccine was found to elicit protective immunity, since an anti-IROMP antibody response could be detected at the time of challenge. Fish were also shown to be protected against lethal challenge, with RPS values of 71.8-89.8% being recorded. Results of the dose response in fish to the vaccine indicated that doses of 10^6-10^7 cells $fish^{-1}$ were successful at inducing immunity and such cell densities could be readily achieved in the bacterin grown under normal laboratory conditions.

The success of the iron-replete bacterin in inducing protective immunity in Atlantic salmon against furunculosis was surprising although results of the active immunisation of Atlantic salmon with iron-replete OMP conferred a significant degree of protection. The iron-replete bacterin was similar with respect to protein content, cell mass and cell number. It was however, lacking the IROMP antigen(s) and was found to contain four times the extracellular LPS content of the iron-restricted bacterin. It is becoming apparent from immunisation studies carried out at SOAFD Marine Laboratory that a form of secreted soluble extracellular LPS (sLPS), of differing sugar composition to that of the cell wall LPS is important as a protective antigen at levels of approximately $2mgml^{-1}$ (Ellis, unpublished observations). Since iron-replete bacterin had LPS levels approaching $1mgml^{-1}$ this may possibly explain the success of this vaccine in the absence of the IROMP antigen. However, when determining the antibody responses of immunised fish to this vaccine we were only able to detect antibody to the low MW LPS component. It is known, however, that high MW LPS is immunogenic in Rainbow trout (Hastings & Ellis, 1991) and in Atlantic salmon (Lund *et al.*, 1991). In addition to the immunogenicity of LPS and IROMPs in Atlantic salmon, we were also able to detect an antibody response in fish immunised with the

iron-replete or iron-restricted bacterin against the 70kDa protease of *A.salmonicida*. Although a previous passive immunisation study by Ellis *et al.*, (1988a) identified the protease as a potential protective antigen when comparing antisera raised against the ECP of protease-deficient and protease-proficient strains, the lack of protection observed in fish passively immunised with a rabbit antiserum specific for the 70kDa protease discounted the protective nature of this antigen (Lee, 1990).

The role of cell-mediated immunity induced in Atlantic salmon by immunisation with either the iron-replete or iron-restricted bacterin was not determined. It is possible that factors present in either the iron-replete or iron-restricted bacterin (or both) are responsible for the induction of cell-mediated immunity and thus contribute to the protective immunity observed with both *A.salmonicida* bacterins.

It appears that inclusion of the two protective antigens (ie IROMPs and sLPS) in an iron-restricted bacterin will be necessary in future immunisation studies. At present, in order to achieve expression of the IROMP antigens, the bacterin needs to be produced under iron-restricted conditions. These growth conditions, however, do not favour the production of high levels of the extracellular LPS antigen. Thus to achieve these high levels of LPS, the iron-restricted bacterin must be supplemented with purified extracellular LPS to the desired concentration of $2mgml^{-1}$. Immunisation of Atlantic salmon with this LPS supplemented iron-restricted bacterin of *A.salmonicida* NTD04 is currently underway and the subject of extensive field trial investigations.

TABLE 8.1 Immunisation regimes used to determine efficacy of iron-replete (Fe^+) and iron-restricted (Fe^-) bacterin vaccines of *A.salmonicida* in Atlantic salmon.

Antigen	Dose $fish^{-1}$
PBS	100µl
$Fe^+ 10^8$	100µl
$Fe^- 10^9$	100µl
$Fe^- 10^8$	100µl
$Fe^- 10^7$	100µl

TABLE 8.2 Analysis of *A.salmonicida* MT004 iron-replete (Fe^+) and iron-restricted (Fe^-) vaccines.

Vaccine	Cell no.	Cell mass $mgml^{-1}$	Total protein $mgml^{-1}$	ECP protein $mgml^{-1}$	Protease $unitsml^{-1}$	Haemolysin $HUml^{-1}$	LPS μgml^{-1}
Fe^- 10^7	$9.8x10^6$	9	0.4	ND	ND	ND	ND
Fe^- 10^8	$9.8x10^7$	20	0.6	0.15	433	63	280
Fe^- 10^9	$9.8x10^8$	142	2.1	ND	ND	ND	ND
Fe^+ 10^8	$8.5x10^7$	27	1.2	0.43	67	16	960

ND, not determined.

TABLE 8.3 Efficacy of active immunisation of Atlantic salmon with an iron-restricted vaccine of *A.salmonicida* challenged with an experimental infection. Fish were distributed in 5 tanks ie 9 fish group^{-1} tank^{-1} where numbers allowed.

Antigen	n=	%mort	RPS v PBS control
PBS	44	86.4	—
Fe^{-}10^{7}	45	8.8	89.8
Fe^{-}10^{8}	45	8.8	89.8
Fe^{-}10^{9}	45	24.4	71.8
Fe^{+}10^{8}	45	11.1	87.2

TABLE 8.4 Serum antibody response of Atlantic salmon immunised with iron-replete and iron-restricted bacterin vaccines of *A.salmonicida* examined by IROMP ELISA.

Vaccine	n=	antigen	ELISA antibody response mean OD_{405} (+/-SE)		
			1	2	3
PBS	5	Fe^+	0.000	0.000±0.000	0.070±0.010
		Fe^-	0.000	0.000±0.000	0.105±0.023
Fe^+10^8	5	Fe^+	0.000	0.261±0.032	0.125±0.053
		Fe^-	0.000	0.250±0.035	0.110±0.040
Fe^-10^8	5	Fe^+	0.000	0.165±0.050	0.121±0.022
		Fe^-	0.000	0.200±0.064	0.160±0.036

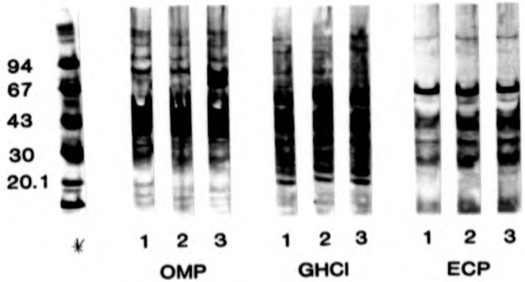
1 pre-vaccination sera
2 pre-challenge sera
3 post-challenge sera

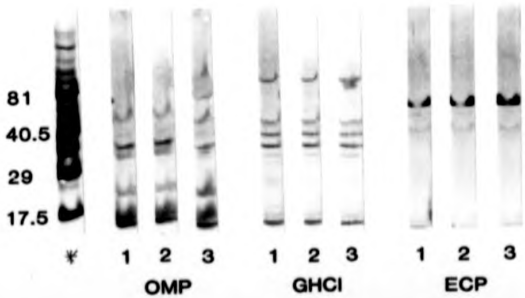
Fe^+, iron-replete outer membrane protein antigen; Fe^-, iron-restricted outer membrane protein antigen.

PLATE 8.1 SDS-PAGE of vaccine antigens of *A. salmonicida* MT004. Proteins were detected by Western blotting and colloidal gold stain. OMP, outer membrane proteins; GHCl, guanidine hydrochloride whole cell extract; ECP, extracellular products. Lane *, low molecular weight protein standards in kDa; Lane 1, *A.salmonicida* antigens grown in Trytone Soya Broth (TSB); Lane 2, *A.salmonicida* antigens grown in TSB + ferric chloride ($FeCl_3$;100μM); Lane 3, *A.salmonicida* antigens grown in TSB + 2,2'-dipyridyl (100μM).

Lane 1 A.salmonicida antigens grown in Tryptone Soya Broth (TSB)

PLATE 8.2 Western blots of vaccine antigens of *A.salmonicida* MT004 stained with antisera from Atlantic salmon immunised with an iron-restricted *A.salmonicida* MT004 bacterin. OMP, outer membrane protein; GHCl, guanidine hydrochloride whole cell extract; ECP, extracellular products. Lane *, biotinylated molecular weight standards in kDa; Lane 2, *A.salmonicida* antigens grown in TSB + ferric chloride ($FeCl_3$;100μM); Lane 3, *A.salmonicida* antigens grown in TSB + 2,2'-dipyridyl (100μM).


94
67
43
30
20.1
1 2 3
1 2 3
1 2 3
OMP
GHCl
ECP


81
40.5
29
17.5
1 2 3
1 2 3
1 2 3
OMP
GHCl
ECP

CHAPTER 9:- GENERAL DISCUSSION

The interest in the topic of iron and infection originates from the work of Schade & Caroline who discovered that the bacteriostatic properties of blood and egg white, capable of inhibiting the growth of certain bacteria (Schade & Caroline, 1944;1946), were due to the presence of specific iron-binding proteins, later identified to be transferrin in blood and ovotransferrin in egg white. It was thought that the iron-binding proteins bound iron so tightly that the bacteria were unable to obtain sufficient amounts for growth. The addition of iron was found to overcome the resulting bacteriostasis. These observations were however largely ignored for the following 20 years and was then renewed with the interest of the role of iron in bacterial virulence and host defence, with the discovery that animals injected with iron were generally more susceptible to bacterial infection. This enhanced susceptibility caused by iron, and the ability of iron to abolish the antibacterial effects of body fluids is now well recognised (Griffiths, 1987b).

It is now established that many bacterial pathogens are capable of successful growth and multiplication *in vivo*, in spite of the virtual absence of freely available iron in the normal body fluids and the hypoferraemia of infection (Griffiths, 1987b). All known bacterial pathogens, with the exception of the lactobacilli (Archibald, 1983), require iron for growth and multiplication and they must, initially, be able to adapt to the iron-restricted environment found *in vivo* and develop mechanisms for the assimilation of protein-bound iron or for acquiring it from liberated haem. The study of bacterial iron uptake

mechanisms is made possible with the use of high affinity iron chelators, both natural and synthetic, such as Tf, Lf, EDDA and 2,2'-dipyridyl, to induce conditions of iron-restriction *in vitro* in order to mimic the environment encountered *in vivo* during infection. In addition, it is well known that bacteria can undergo rapid phenotypic changes in both their metabolism and in the composition of their outer membranes when growing *in vivo* during infection (Neilands, 1982; Brown & Williams, 1985; Williams, 1986). Thus, studies of bacterial pathogens growing under conditions of iron-restriction has led to an increased understanding of the mechanisms of microbial virulence and pathogenicity.

A number of high affinity iron uptake mechanisms possessed by bacterial pathogens in order to acquire iron and overcome the severely iron-restricted environment encountered *in vivo* have been identified in recent years (Griffiths, 1987b; Martinez *et al.*, 1990). These mechanisms are usually siderophore-mediated, involve the direct utilisation of host iron-binding proteins such as Tf or Lf or involve the utilisation of haem compounds or haem complexes liberated from the lysis of host cells by the action of cytolysins and haemolysins. Recently, a mechanistically novel iron transport system has been identified in *Serratia marcescens* (Zimmerman *et al.*, 1989; Angerer *et al.*, 1992) which does not involve siderophores or receptor proteins.

The initial aim of the studies presented in this thesis was to examine the ability of *A.salmonicida* to grow under conditions of iron-restriction and to characterise the iron uptake mechanisms of *A.salmonicida*. The ability of typical strains of *A.salmonicida* to produce a rapid haemorrhagic septicaemic disease in salmonids and their capacity for rapid growth in body organs *in vivo* is well acknowledged.

In contrast, atypical strains of *A.salmonicida* produce a more superficial, localised disease with little systemic involvement. The role of the iron uptake mechanisms in the pathogenesis of furunculosis was initially studied by Chart & Trust (1983). They found typical and atypical strains to be able to grow under conditions of iron-restriction *in vitro*, however, typical strains were more efficient under these conditions and it was suggested that this may explain the differences in pathogenesis. They found typical strains to acquire iron by two mechanisms; one was an inducible mechanism involving the production of a siderophore-like activity, ie a soluble, low MW iron chelator; the second was a constitutive mechanism apparently involving a direct interaction between the bacterial cell surface and the host iron-binding protein. Growth under conditions of iron-restriction also resulted in the appearance of three additional iron-regulated OMPs (IROMPs) which were found to be involved in the binding of $^{59}Fe^{3+}$, though their function was not determined. The iron uptake mechanisms of atypical strains were not characterised.

Later studies by Kay *et al.*, (1985) and Ishiguro *et al.*, (1985) examining the ability of strains of *A.salmonicida* possessing the 49kDa A-layer protein to bind the sulphonated diazo dye congo red, found that these strains were also capable of binding a number of haem compounds such as haemin and protoporphyrin IX, suggesting a possible role in iron uptake processes of *A.salmonicida*. Additional studies by Kay *et al.*, (1985) suggested that *A.salmonicida* was capable of utilising haem compounds for growth since the normally fastidious growth of atypicals strains on conventional laboratory media could be enhanced by the presence of haem compounds, in particular haemin and haemoglobin in the growth media.

During the studies presented in this thesis, it was found that all of the forty-two strains of *A.salmonicida* examined, with the exception of one non-autoagglutinating atypical strain, were capable of growth under conditions of iron-restriction induced *in vitro* by the presence of the high affinity iron chelators, Tf, EDDA and 2,2'-dipyridyl, to restrict the availability of free iron in the growth media. A number of typical and atypical strains examined were also capable of growth in heat-inactivated salmonid serum in the presence of host iron-binding proteins where the available free iron was virtually zero. Growth under conditions of iron-restriction was found to result in the production of siderophores by all of the typical strains of *A.salmonicida*. Siderophore production was not detected in atypical strains of *A.salmonicida*. The siderophore was partially purified by hydrophobic chromatography using XAD-7 resin, used to partially purify anguibactin (Actis *et al.*, 1986) and found to be by the chemical assay of Arnow (1937), a 2,3-diphenol catechol siderophore with an aromatic structure based upon 2,3-DHBA, as determined by TLC. The siderophore, unlike most phenolate siderophores eg enterobactin, was found to be insoluble in organic solvents at acidic pH, which initially suggested a similarity with the *V.anguillarum* siderophore, anguibactin, and the *A.hydrophila* siderophore, amonabactin. However, the behaviour of the *A.salmonicida* siderophore in bioassays with mutant strains of *S.typhimurium* and *V.anguillarum* which lacked the ability to produce siderophores but which possessed the necessary OMP receptor capable of utilising exogenous enterobactin or anguibactin, suggested the *A.salmonicida* siderophore was not chemically related to either of these phenolate siderophores. Similarly, the inability of the *A.salmonicida* siderophore to cross-feed iron-restricted motile *Aeromonas* species suggested that the siderophore

was not chemically related to amonabactin either. Results of bioassays performed with iron-restricted cells of *A.salmonicida* to examine the ability of *A.salmonicida* to utilise enterobactin, anguibactin and amonabactin suggested that both typical and atypical strains of *A.salmonicida* were unable to utilise exogenous siderophore and that the IROMPs of *A.salmonicida* were not involved in functioning as receptor proteins for these siderophores. A degree of strain-specificity was observed with the siderophore in that the ability of the *A.salmonicida* siderophore to cross-feed iron-restricted cells of *A.salmonicida* was only observed with one strain (typical strain NT423). Interestingly, it was found that this strain was also able to cross-feed the non-siderophore producing atypical strains of *A.salmonicida*. Since atypical strains also possess IROMPs of identical molecular weight and which exhibit antigenic homogeneity with the typical strains, it is possible that they function as receptors for the Fe^{3+}-siderophore complex. The *A.salmonicida* siderophore was also found to be produced during growth in heat-inactivated salmonid serum and was found to be associated with an iron-binding activity ie in the presence of the siderophore and iron-free apo-Tf, iron preferentially bound to the siderophore. Typical strains were also found to produce an iron reductase enzyme activity whose role it may be to solubilise iron bound to the siderophore for use by the bacterial cell. Alternatively the iron reductase activity may serve to solubilise iron bound to Tf as with the pyocyanin of *Ps.aeruginosa* (Cox, 1986). Evidence that 2,3-DHBA, not present in mammalian tissues, was produced *in vivo* in the organs of rainbow trout infected with *A.salmonicida* suggests that the *A.salmonicida* siderophore is produced *in vivo* during infection as are the siderophores of *E.coli* (Griffiths & Humphreys, 1980), *Ps.aeruginosa*

(Haas *et al.*, 1991) and *V.anguillarum* (Wolf & Crosa, 1986). The expression of the *A.salmonicida* siderophore *in vivo* suggests its role as an important virulence factor in the pathogenesis of furunculosis.

Growth of *A.salmonicida* in heat-inactivated salmonid serum where the only available iron was bound to serum Tf indicated the ability of the pathogen to utilise salmonid Tf sources of iron. In addition, all of the strains of *A.salmonicida* examined in this study were found to be able to utilise Tf and Lf sources of iron at 30 and 90% of iron-saturation, from bovine and human sources. This ability to utilise various sources of Tf and Lf, though not in the iron-free, apo-form, indicates that *A.salmonicida* possesses no species specificity in its ability to utilise Tf and Lf, as with other pathogens which utilise Fe-Tf by direct interaction, in the absence of siderophores (Schryvers & Gonzalez, 1989). This may possibly explain the wide host range of *A.salmonicida* and its ability to infect fish other than salmonids. Chart & Trust (1983) found one typical strain to obtain iron from Lf by direct interaction between the cell surface and the iron-binding protein. Such mechanisms are common in a number of bacterial pathogens such as *H.influenzae*, *A.pleuropneumoniae*, *N.gonorrhoeae*, *N.menigitidis* and *P.multocida* which lack siderophore-mediated iron uptake mechanisms. The human respiratory pathogen, *B.pertussis* appears to be an exception in possessing both a siderophore-mediated iron uptake mechanism (based on the hydroxamate siderophore, bordetellin) and the ability to utilise Tf via direct cell surface interaction (Redhead, 1991; Ascencio *et al.*, 1992). In this study we were unable to detect Tf/Lf binding via cell surface receptor protein in either typical or atypical strains of *A.salmonicida* using a dot-blot assay and iron-restricted whole cells or OMPs prepared from iron-restricted bacteria containing the IROMPs of

A.salmonicida, a method used successfully to identify putative Tf or Lf receptors in other bacterial pathogens. Using dialysis bag experiments to determine the mechanism of Tf utilisation by *A.salmonicida* it was found that typical strains could acquire Fe from Tf by a soluble low MW siderophore-mediated mechanism. In the absence of a siderophore, atypical strains were unable to utilise Tf-bound iron contained within a dialysis bag. However, when Tf was incubated with the ECP of an atypical strain containing a metalloprotease (presumably the 20kDa metalloprotease described by Gudmundsdottir *et al.*, 1990) and then placed in the dialysis bag with a 10kDa MW cutoff, the atypical strain was able to utilise Tf-bound iron, suggesting that the extracellular metalloprotease of the atypical strain was capable of digesting Tf to release iron which could then be utilised by the atypical strain. Similar experiments were not possible with the iron-restricted ECP of a typical strain due to the presence of the soluble low MW siderophore in the ECP. Alternatively, a partially purified preparation of the 70kDa serine protease of *A.salmonicida* MT004 was obtained which has been shown previously to be important in the pathogenesis of furunculosis (Lee & Ellis, 1990; Ellis, 1991). This 70kDa serine protease was then incubated with bovine Tf and then examined by SDS-PAGE. Using this technique it was found that the 70kDa protease was in fact capable of the proteolytic degradation of Tf into a number of low MW components. Thus, this study identified a second iron uptake mechanism of *A.salmonicida*; a mechanism seen only previously with the elastase enzyme of *Ps.aeruginosa* (Döring *et al.*, 1987). Thus an additional role for the extracellular 70kDa serine protease of the typical strains of *A.salmonicida* and the metalloprotease of the atypical strains of *A.salmonicida* was identified. In addition, the growth of *A.salmonicida*

under conditions of iron-restriction results in increased extracellular proteolytic activities of both typical and atypical strains and the protease of typical strains is known to be produced *in vivo* (Ellis, 1991); Rockey *et al.*, 1988).

A third potential iron uptake mechanism has been demonstrated in both typical and atypical strains which possess the 49kDa crystalline virulence surface array A-layer protein. The A-protein, a well established virulence determinant of *A.salmonicida* which is conserved amongst both typical and atypical strains of diverse geographical origin (Chu *et al.*, 1992), has previously been shown to bind congo red dye and a variety of haem compounds (Kay *et al.*, 1985). Results of this study demonstrated the binding of additional haem compounds eg haemoglobin and the haem-sequestering-complexes such as haemoglobin-haptoglobin and haem-albumin. These haem-complexes serve an important role in host defence by removal of haem sources resulting from the lysis of host erythrocytes through the action of cytolysins and haemolysins (Griffiths, 1987b). The mechanism of utilisation of these haem-complexes is not understood. However, it is known that *P.gingivalis* is capable of proteolytic digestion of these complexes by trypsin-like enzyme activity (Carmen *et al.*, 1990). Since the 70kDa protease was found to be capable of the degradation of Tf, it may be feasible that the enzyme is also capable of degrading these haem-complexes in order to enable for them to be utilised by the cell as an iron source. Bioassays indicated that strains possessing the 49kDa A-layer protein were, in addition to being able to bind haem compounds, were also able to utilise these compounds as an iron source. Interestingly strains were also able to utilise the porphyrin ring structure of protoporphyrin IX which lacks the iron atom of haem. This

indicated that *A.salmonicida* possesses an enzyme activity similar to the ferrochelatase of *H.influenzae* which is capable of incorporating the iron atom, present as contaminating iron in the iron-restricted media, into the porphyrin ring structure to form haem. The utilisation of haem compounds by *A.salmonicida* as with other pathogens such as the motile *Aeromonas* species (Hassad *et al.*, 1991), *S.flexneri* (Lawlor *et al.*, 1987), *V.anguillarum* (Mazoy & Lemos, 1991) and *V.cholerae* (Stoebner & Payne, 1988) was found to be independent of siderophore production. Competition binding studies indicated that the haem compounds shared the same binding protein as congo red and that this binding activity could be abolished by trypsinisation of whole cells, indicating that the binding protein was exposed on the cell surface. Although the A-layer protein has recently been shown to be resistant to the action of trypsin (Doig *et al.*, 1992) it is possible that trypsinisation of whole cells disrupts the CR binding site without affecting the structural integrity of the A-layer protein. The IROMPs of *A.salmonicida* were shown not to be involved since no increase in the binding of haem compounds was observed with iron-restricted cells of *A.salmonicida*.

The ability of both typical and atypical strains of *A.salmonicida* to utilise haem compounds may be related to the fact that furunculosis is typically a haemorrhagic disease associated with liquefactive necrosis due to the action of the QCAT enzyme and the 70kDa protease acting in concert to reproduce much of the pathology associated with furunculosis (Lee & Ellis, 1989; Ellis, 1991). Since the expression of both these extracellular toxins is influenced by the level of iron in the environment, a further role which they play in the pathogenesis of furunculosis may be as components of the iron uptake mechanisms of the

pathogen. It is known that the protease decreases clotting time of rainbow trout blood (Price *et al.*, 1990) and that QCAT is capable of lysis of salmonid erythrocytes (Lee & Ellis, 1991b), though this activity is rarely detected within the general vasculature *in vivo* during infection (Ellis, 1991). It is possible that the bacteria are able to survive in areas of haemorrhage resulting from the action of the protease and through the action of QCAT upon the clot causing localised haemolysis from which sufficient iron may be released for utilisation by the bacterium.

Furunculosis due to typical strains of *A.salmonicida* continues to be a serious economic threat to the successful rearing of farmed Atlantic salmon in Scotland. The emergence and spread of multi-antibiotic-resistant strains of *A.salmonicida* to the three antibiotics licenced for use in the Scottish aquaculture industry has led to a determined effort to produce an *A.salmonicida* vaccine against furunculosis in recent years. However, over fifty years of attempts to vaccinate fish have been unsuccessful in the development of an effective and reliable vaccine. At present, only two commercial furunculosis vaccines (Furogen; Aquahealth and Furovac; AVL) are licenced for use in Scotland. Both vaccines are chemically-inactivated, aluminium-adjuvanted whole cell bacterins with variable efficacy in the protection of Atlantic salmon against furunculosis. The lack of success of many of the earlier attempts to vaccinate fish against furunculosis appears to be due to failure to induce immunity against the virulence determinants of *A.salmonicida*. Many of the virulence determinants of *A.salmonicida* are known to be immunogenic in rabbit, but are non-immunogenic in Atlantic salmon which appear to possess only a limited immune response to the cellular and extracellular antigens of

A.salmonicida. This has led a number of workers to investigate ways of increasing immunogenicity of these antigens by physical and chemical ways eg adsorption to latex beads (Tatner, 1990) and chemical conjugation to carriers such as BSA and keyhole limpet haemocyanin (KLH), by the use of adjuvants (Hastings & Ellis, 1990) and by the use of immunostimulants such as 1,3 β-glucan (Nikl *et al.*, 1991). There are only limited reports of protection studies using these modified antigens of *A.salmonicida* (Nikl *et al.*, 1991).

It is well recognised that bacterial vaccines work by raising antibody or inducing cell-mediated immunity which react against bacterial virulence factors ie those antigens which enable the pathogen to infect the vertebrate host and cause disease such as LPS, fimbriae etc. This implies that the virulence determinants must be expressed by the pathogen *in vivo* for the vaccine to be effective. To date, most studies on the antigenic composition of bacterial pathogens has been based on bacteria grown *in vitro* on conventional laboratory media. The surface and virulence properties of cells cultivated on conventional laboratory media may be very different to those occurring *in vivo* during infection (Brown *et al.*, 1988). In many cases it remains to be established that putative virulence-associated antigens are produced *in vivo* during infection. A number of extracellular and cellular antigens of *A.salmonicida* have been identified in this study as being iron-regulated and thus have the potential to be expressed *in vivo* during infection. In addition, it is well recognised that the exposed outer surface of a bacterial cell plays a crucial role in its survival in the vertebrate host (Williams, 1988). It mediates adhesion of the cell to the mucosal surfaces and also its interaction with host defences. The components of the surface are therefore obvious molecular

targets for humoral defence and are of interest to those involved in vaccine research and development. In common with the iron uptake mechanisms examined in other bacterial pathogens, the IROMPs of *A.salmonicida* appear to be a component of these mechanisms. Growth under conditions of iron-restriction in this study resulted in the expression of four novel IROMPs identified previously by Chart & Trust (1983) and Aoki & Holland (1985) which possibly function as receptors for the components of the iron uptake mechanisms described eg for a Fe^{3+}-siderophore complex. In this study, it was established that the IROMPs of *A.salmonicida* are expressed *in vivo* during infection, since bacteria isolated directly without subculture from furuncle material and peritoneal fluid of infected Atlantic salmon expressed IROMPs. Results of this study also confirmed by immunological methods, that convalescent fish exposed to sublethal *A.salmonicida* challenge possessed an anti-IROMP antibody response. Thus this observation confirms their importance as a virulence determinant of *A.salmonicida*. In previous studies, the immunogenicity of the OMPs of *A.salmonicida* have used OMPs isolated from iron-replete bacteria and have concentrated on the immunogenicity of the A-layer and LPS components of the OM. Therefore, it was not known if these additional IROMP antigens of *A.salmonicida* expressed *in vivo* and under conditions of iron-restriction *in vitro*, were immunogenic in Atlantic salmon. In these studies, it was found that the IROMPs of *A.salmonicida* were immunogenic in Atlantic salmon and also antigenically homogeneous in all typical and atypical strains of *A.salmonicida* strains examined, including a number of recent clinical isolates and were thus identified as potential protective antigens in the vaccination of Atlantic salmon against furunculosis. Immunisation studies were performed using the partially purified IROMP antigen to

assess the protective capacity of the antigen. Passive immunisation studies, using anti-IROMP antisera raised in Atlantic salmon and a monospecific rabbit anti-IROMP serum and affinity-isolated rabbit ant-IROMP IgG, passively protected Atlantic salmon against bath challenge exposure to *A.salmonicida*. Thus, antibody against the IROMPs of *A.salmonicida* was found to be important in protection against furunculosis. Active immunisation studies were also performed in order to determine the efficacy of immunising Atlantic salmon with the IROMPs of *A.salmonicida* and also to determine the ability of the IROMP antigen to elicit a protective anti-IROMP antibody response. Results of three experimental immunisation trials indicated protective immunity was induced in Atlantic salmon which were protected against lethal heterologous bath challenge exposure to *A.salmonicida* and that vaccination led to the induction of an anti-IROMP antibody response measured by ELISA and Western blotting at the time of challenge.

In an attempt to determine the mechanism of immunity observed in immunisation studies, normally by the induction of bactericidal or opsonising antibody, bactericidal assays were performed to examine whether, as with other bacterial pathogens, anti-IROMP antisera was capable of preventing uptake of iron by the bacterium. In addition bactericidal assays were performed in order to determine which antigens were the potential targets of bactericidal antibody. Virulent strains of *A.salmonicida* possessing the 49kDa A-layer protein are normally resistant to the bactericidal action of immune and non-immune serum. The A-layer protein and LPS apparently shield the bacterial OM from attack from components of the complement cascade. The cell surface exposure of the *A.salmonicida* IROMPs was not determined in this study, however, it is possible that they are cell-surface exposed, external to

the A-layer protein and potentially accessible to antibody. However, results of membrane antagonist experiments confirm the results of Thornton *et al.*, (1991) which suggest that growth of *A.salmonicida* under conditions of Ca^{2+}-limitation or under conditions of iron-restriction (this study) may result in the incomplete assembly of the A-layer protein and thus may expose important cell surface structures such as the IROMPs normally shielded by the complete A-layer protein structure (Kay & Trust, 1991). In the bactericidal assays, both salmon anti-IROMP and the monospecific rabbit anti-IROMP antisera were bactericidal against both typical and atypical strains of *A,.salmonicida* grown under conditions of iron-restriction, in the presence of complement, though the antisera was also bactericidal in the absence of complement. Absorption of the salmon antisera with LPS and iron-replete OMPs failed to abolish this bactericidal activity, though the activity was abolished by absorption with iron-restricted OMPs, indicating that the IROMP antigens were the target of the bactericidal complex.

In an attempt to produce a vaccine containing the IROMP antigens of *A.salmonicida* in a preparation easily manufactured with existing production facilities and which is affordable to the aquaculture industry, an iron-restricted, formalinised whole cell bacterin of *A.salmonicida* was evaluated for its ability to confer protection in Atlantic salmon against furunculosis. Such an iron-restricted bacterin was produced on a laboratory scale by culturing *A.salmonicida* in the presence of the iron chelator, 2,2'-dipyridyl. Analysis of the vaccine by an immunoassay confirmed the presence of the IROMP antigen in the preparation. Active immunisation of Atlantic salmon with this iron-restricted bacterin of *A.salmonicida* conferred a significant level of protection against lethal bath challenge. The degree of protection

was greater than that induced by an existing commercial furunculosis vaccine licenced for use in the Scottish aquaculture industry. The vaccine also induced an anti-IROMP antibody response in immunised fish at the time of challenge. A vaccine similar in composition except grown under iron-supplemented conditions and consequently contained higher levels of soluble LPS surprisingly gave similar levels of protection. These apparent contradictions may be explained with the knowledge that the soluble LPS component of the *A.salmonicida* has recently been identified has an additional protective antigen of *A.salmonicida* (Ellis, unpublished observations).

At present, the practical problems associated with cultivation of bacterial pathogens under iron-restricted conditions on an industrial scale arise due to the use of stainless steel fermentors from which contaminating iron compounds may leach to produce iron-replete growth conditions. The alternative is the use of glass or ceramic fermentors, however, these are of generally smaller capacity. An alternative approach is to involve the use of recombinant DNA technology. This approach will no longer require growth of bacteria under conditions of iron-restriction and instead involves the cloning of genes encoding the iron-regulated virulence determinants into a non-pathogenic microorganism such as *E.coli*. Work currently underway at SOAFD Marine Laboratory is examining the potential of DNA technology to clone and express in large quantities the *A.salmonicida* IROMP antigen in *E.coli* (McGillivray, personal communication).

Results presented in this thesis have identified an important protective antigen of *A.salmonicida* which is immunogenic, antigenically cross-reactive, expressed *in vivo* during infection and protective. In addition, antibodies raised against the protective antigen are known to

be bactericidal *in vitro*. Thus, a protective antigen of *A.salmonicida* was identified and its contribution to mechanisms of immune protection were elucidated. Partially purified preparations of the antigen and an iron-restricted whole cell *A.salmonicida* bacterin produced to express the antigen, can be effectively used to induce protective immunity in Atlantic salmon against furunculosis. Current work carried out in collaboration with SOAFD, Institute of Aquaculture, University of Stirling, the Scottish Salmon Growers Association and the Crown Estate Commission is investigating the effectiveness of the iron-restricted *A.salmonicida* bacterin containing the second protective antigen whilst the importance of the role of cell-mediated immunity in the immune protection observed in these studies is being evaluated. This furunculosis vaccine (manufactured on a commercial scale by AVL (ATC No 4964/0009)) is currently undergoing a second year of extensive field trials in Scotland with the view to gaining a product licence and being manufactured commercially.

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