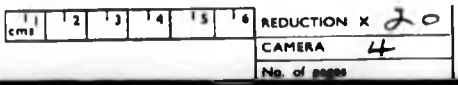


SECRETION OF EXTRACELLULAR PROTEINS BY *AEROMONAS SALMONICIDA* AND RELATED SPECIES

by

Stuart E. Wilson

Thesis submitted for the degree of
Doctor of Philosophy

Department of Biological & Molecular Sciences
University of Stirling
May 1992

Contents

Table of contents

List of tables

List of figures

Abbreviations

6-APA	6-aminopenicillanic acid
p-APMA	*p*-aminophenylmercuric acid
BSA	bovine serum albumin
Buffer I	PBS/ 0.2% gelatin
Buffer II	PBS/ 0.2% gelatin/ 0.1% Triton X-100
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazone
CF	culture filtrate
DAB	diaminobenzamidine tetrahydrochloride
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
E_a	activation energy
EDDA	ethylenediaminedi(*o*-hydroxyphenylacetic acid)
EDTA	ethylenediaminetetracetic acid
FCA	Freund's complete adjuvant
FICA	Freund's incomplete adjuvant
FPLC	fast protein liquid chromatography
GCAT	glycerophospholipid: cholesterol transferase
GDH	glutamate dehydrogenase

HPA	hide powder azure
HU	haemolytic units
KDO	2-keto-3-deoxyoctulosonic acid
LCAT	lecithin: cholesterol transferase
LCL	leucocytolytic factor
LD_{50}	50% lethal dose
LPS	lipopolysaccharide
MAC	membrane attack complex
MIC	minimum inhibitory concentration
M_r	relative molecular weight
NADH	nicotinamide adenine dinucleotide (reduced)
PBS	phosphate-buffered saline
pI	isoelectric point
PMSF	phenylmethanesulphonyl fluoride
RBC	red blood cells
REF	restriction endonuclease fingerprinting
RNA	ribonucleic acid
RNAse	ribonuclease
RS	regularly-structured
Sarkosyl	sodium lauryl sarcosinate
SDH	succinate dehydrogenase

SDS	sodium dodecyl sulphate
ssp.	subspecies
TCA	trichloroacetic acid
TEMED	tetraethylenemethylenediamine
Tris	2-amino 2(hydroxymethyl)1,3-propandiol
TSA	tryptone soy agar
TSB	tryptone soy broth

Acknowledgements

I would like to give special thanks to my supervisor, Dr. Lewis Stevens, for his guidance and support throughout my PhD and for helpful discussions in the preparation of this manuscript. I would also like to thank my secondary supervisor, Dr. Mick North, for additional advice and use of some of the technical apparatus required during the course of this project.

In addition I would like to express my appreciation to Lewis Taylor, Doris Duncan and Joan Barnet (Department of Biological and Molecular Sciences, University of Stirling) and Dr. Mary Tatner and Dr. Val Inglis (Institute of Aquaculture, University of Stirling) for their technical assistance during the course of this work.

I would also like to thank all my friends and colleagues at the university for contributing to a fun and memorable PhD. In particular, to all the girls who shared my life, well my office actually - Sharon, Morag, Jackie and Shauna; to my sports partners a big thank you, playing with you was good for my ego (apart from when I played Marcos, then my ego went into hiding); to the lunch crew over the years (Rob, Neil *et al.*); to Hazel, who when down at the NERC unit, made the long trip to the stores a pleasure rather than a chore; and to Annette for my almost weekly dinners over the past few months, which helped me to survive both my stomach's and wallet's winter of discontent while writing up. Last and least I wouldn't like to thank Derek and Andrew for anything, but I suppose I'd better; two finer friends I could find almost anywhere (only joking - I think). Thanks lads for all the good times. Cheers!

My biggest and most special thanks go to my parents who have always given great encouragement to my sister and myself in everything we have done, especially with respect to our education. Nothing is ever too much trouble for you. Thank you both for giving me the chance neither of you got, and to my dad who always says 'you only get one chance', well I hope I've taken it.

This work was carried out at the Department of Biological and Molecular Sciences, University of Stirling, and was supported by a University of Stirling studentship.

Abstract

This thesis describes the study of extracellular enzyme activities secreted by the Gram-negative bacterium *Aeromonas salmonicida* and related species. Initially, cell fractionation criteria were used to define operationally the cellular and extracellular location of proteins. It was demonstrated that proteins detectable in the extracellular medium of *A. salmonicida* were selectively secreted beyond the outer membrane and not present as a result of general non-specific release due to cell lysis. Several extracellular enzyme activities were detected in the culture filtrate of *A. salmonicida*, of which a number of protease and amylase activities were partially characterised. The principal extracellular protease of M, 70000 was purified to homogeneity by a combination of ion-exchange and gel filtration chromatography, while another major extracellular protein of M, 56000 was purified by preparative-SDS-polyacrylamide gel electrophoresis. Rabbit polyclonal antibodies were then raised against these purified extracellular proteins. In assays with trout erythrocytes, haemolytic activity in the culture filtrate was inhibited by pre-incubating with antibodies raised against the 56kDa protein, suggesting that this protein is in fact a haemolysin.

After the preliminary studies on the production of extracellular proteins by this organism, a series of experiments was carried out to compare the extracellular protein production of *A. salmonicida* and related species. The study of strain variation at species and sub-species level, with respect to extracellular protein production, was approached in several ways. Firstly, the strains were examined for the production of extracellular enzyme activity; secondly, polyclonal antibodies raised against the purified extracellular protease and haemolysin enzymes were used in Western blotting experiments to screen strains for cross-reactivity; and lastly, culture filtrates of all strains studied were applied to substrate-SDS-polyacrylamide gels to detect hydrolytic enzyme activity.

It was found that the extracellular protease and haemolysin enzymes were common to the majority of *A. salmonicida* ssp. *salmonicida* strains studied. Positive cross-reactivity was also observed with the extracellular fraction of *A. hydrophila* when probed with *A. salmonicida* antibodies against the protease and haemolysin activities.

Abstract

This thesis describes the study of extracellular enzyme activities secreted by the Gram-negative bacterium *Aeromonas salmonicida* and related species. Initially, cell fractionation criteria were used to define operationally the cellular and extracellular location of proteins. It was demonstrated that proteins detectable in the extracellular medium of *A. salmonicida* were selectively secreted beyond the outer membrane and not present as a result of general non-specific release due to cell lysis. Several extracellular enzyme activities were detected in the culture filtrate of *A. salmonicida*, of which a number of protease and amylase activities were partially characterised. The principal extracellular protease of M, 70000 was purified to homogeneity by a combination of ion-exchange and gel filtration chromatography, while another major extracellular protein of M, 56000 was purified by preparative-SDS-polyacrylamide gel electrophoresis. Rabbit polyclonal antibodies were then raised against these purified extracellular proteins. In assays with trout erythrocytes, haemolytic activity in the culture filtrate was inhibited by pre-incubating with antibodies raised against the 56kDa protein, suggesting that this protein is in fact a haemolysin.

After the preliminary studies on the production of extracellular proteins by this organism, a series of experiments was carried out to compare the extracellular protein production of *A. salmonicida* and related species. The study of strain variation at species and sub-species level, with respect to extracellular protein production, was approached in several ways. Firstly, the strains were examined for the production of extracellular enzyme activity; secondly, polyclonal antibodies raised against the purified extracellular protease and haemolysin enzymes were used in Western blotting experiments to screen strains for cross-reactivity; and lastly, culture filtrates of all strains studied were applied to substrate-SDS-polyacrylamide gels to detect hydrolytic enzyme activity.

It was found that the extracellular protease and haemolysin enzymes were common to the majority of *A. salmonicida* ssp. *salmonicida* strains studied. Positive cross-reactivity was also observed with the extracellular fraction of *A. hydrophila* when probed with *A. salmonicida* antibodies against the protease and haemolysin activities.

On the evidence of Western blotting, extracellular protein production by other *Aeromonas* species does not appear to be related to *A. salmonicida*. Detection of protease and amylase activity in substrate-SDS-polyacrylamide gels allowed a detailed comparison of the hydrolytic enzyme production by members of the *Aeromonas* genus. The results obtained in this way demonstrated striking homogeneity of protease and amylase secretion within *A. salmonicida* ssp. *salmonicida*, but substantial variation between species and subspecies of the *Aeromonas* genus.

1. Introduction

1.1 General Introduction

Aeromonas salmonicida is the causative agent of furunculosis and other associated diseases in salmonid (salmon and trout) and non-salmonid fish. The disease is named after the raised liquefactive muscle lesions (furuncles) which sometimes occur in chronically infected fish. Furunculosis was originally described from hatchery fish in Germany by Emmerich and Weibel (1894) and is now observed in most of the salmonid-producing countries in the world with notable exceptions being countries where fish are not heavily cultured, for example in Australia, New Zealand and the USSR. The transfer of fish stocks is most certainly the reason for the spread of the fish pathogen *A. salmonicida*.

The clinical and gross pathological features of furunculosis infection have been well documented (Herman 1968; McCarthy & Roberts 1980; Paterson 1983). Based on the variety of clinical symptoms associated with furunculosis, three main degrees of *A salmonicida* infection have been described: acute, peracute and chronic (Austin & Austin 1987). Acute furunculosis infection is characterised by the sudden increase in mortality with few or no external clinical symptoms. Peracute furunculosis infection is similar to the acute infection but is confined to juvenile fish. Development of furuncles, lethargy, darkening in colour and small haemorrhages at the base of the fins is indicative of chronic *A. salmonicida* infection and corresponds with a low steady state mortality. The disease affects many areas of the body and pathological features of the disease may include tissue necrosis in the liver, accumulation of bacteria in the glomeruli, a dark and enlarged spleen, the formation of lesions or furuncles in muscle tissue and a fluid inflamed intestine. In all forms of furunculosis, large numbers of *A salmonicida* cells are present in the blood and infected tissues (Austin & Austin 1987).

The host range of *A. salmonicida* also includes many non-salmonid freshwater and

marine species. Indeed, 'atypical' strains of *A. salmonicida* have been linked with serious ulcerative diseases in commercially raised carp (Bootsma *et al.* 1977), goldfish (Elliot & Shotts 1980; Whittington *et al.* 1987) and eels (Kitao *et al.* 1985) as well as salmonid fish (Paterson 1983).

1.1.1 Taxonomy of *Aeromonas* species

The genus *Aeromonas* (Stanier 1943) belongs to the family Vibrionaceae (Sebald & Veron 1963; Veron 1966): the differential characteristics of the genera are shown in Table 1.1. Within the genus *Aeromonas* there are two distinct groups (Fig.1.1): the first are psychrophylic and non-motile *A. salmonicida* and the second group are mesophilic and motile aeromonads which are generally divided into three species, namely *A. hydrophila*, *A. caviae* and *A. sobria* (Popoff & Veron 1976; Popoff *et al.* 1981). The phenotypic basis for the differentiation of the *Aeromonas* genus is shown in Table 1.2, while other biochemical and physiological features of motile and non-motile aeromonads are given in Table 1.3. *A. salmonicida* can be further subdivided into three subspecies: namely, *A. salmonicida* ssp. *salmonicida*, *A. salmonicida* ssp. *achromogenes* and *A. salmonicida* ssp. *masoucida*. These three subspecies can be distinguished by biochemical characters (Table 1.2), but have similar genotypic properties (see below).

However, the classification of the *Aeromonas* genus is unclear, with currently eight proposed or validated phenotypic species that reside among at least 12 DNA hybridization groups (Table 1.4) (Carnahan & Joseph 1991). The literature concerned with *A. salmonicida* classification contains a prevalence of suggested taxonomic revisions, including removing the type genus *Aeromonas* from the family Vibrionaceae and elevating it to family status, i.e., the Aeromonadaceae (Colwell *et al.* 1986). The question of *Aeromonas* genus classification is particularly relevant to the motile species (Colwell *et al.* 1986; Austin *et al.* 1989; Carnahan & Joseph 1991). As mentioned above, according to Bergey's Manual of Systematic Bacteriology (Popoff 1984) the genus *Aeromonas* contains three mesophilic species, *A. hydrophila*, *A. caviae* and *A.*

Table 1.1. Differential characteristics of the genus Vibrionaceae [a]

Characteristics	*Vibrio*	*Aeromonas*	*Plesiomonas*
Sheathed polar flagella	+	-	-
Na^+ is required for growth or stimulates growth	+	-	-
Production of lipase	[+]	+	-
Utilisation of D-Mannitol	[+]	[+]	-
% mol (G+C) of DNA	38-51	57-63	51

[a] Symbols: +, all species positive; [+], most species positive; -, all species negative

Figure 1.1. Taxonomy of the family Vibrionaceae

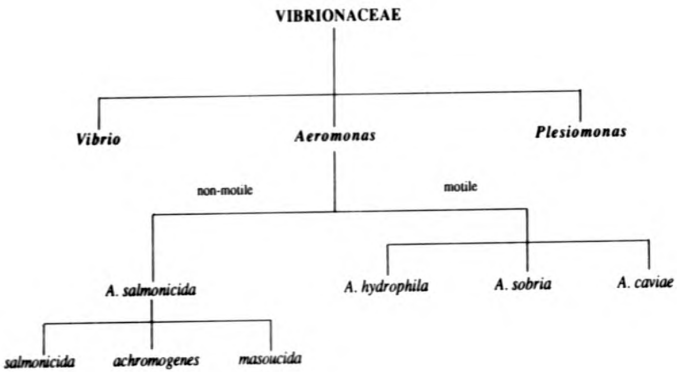


Table 1.2. Differentiation between *A. hydrophila*, *A. caviae*, *A. sobria*, *A. salmonicida* and *Plesiomonas* [a]

Characteristics	*A. hydrophila*	*A. caviae*	*A. sobria*	*A. salmonicida* subsp.			*P. shigelloides*
				salmonicida	*achromogenes*	*masoucida*	
Motility	+	+	+	-	-	-	+
Monotrichous flagellation in liquid medium	+	+	+	-	-	-	-
Lophotrichous flagellation in liquid medium	-	-	-	-	-	-	+
Coccobacilli in pairs, chains and clumps	-	-	-	+	+	+	-
Brown water-soluble pigment	-	-	-	+	-	-	-
Growth in nutrient broth at 37°C	+	+	+	-	-	-	+
Indole production in 1% peptone water	+	+	+	-	+	+	+
Esculin hydrolysis	+	+	-	+	-	+	n.d.
Growth in KCN broth	+	+	-	-	-	-	-
L-Histidine and L-arginine utilisation	+	+	-	-	-	-	-
L-Arabinose utilisation	+	+	-	-	-	-	n.d.
Fermentation of salicin	+	+	+	d	d	d	-
Fermentation of sucrose	+	+	+	-	+	+	-
Fermentation of mannitol	+	+	+	+	-	+	-
Breakdown of inositol	-	-	-	-	-	-	+
Acetoin from glucose	+	-	d	-	-	+	-
Gas from sucrose	+	-	+	+	-	+	-
H_2S from cysteine	+	-	+	-	-	+	n.d.

[a] Symbols: +, typically positive; -, typically negative; d, differs among strains; n.d., not determined

Table 1.3. Other characteristics of motile *Aeromonas* species and *Aeromonas salmonicida* [a]

Characteristics	Motile *Aeromonas* species	*A. salmonicida*
Oxidase	+	+
NO_3^- reduced to NO_2^-	+	+
Lysine decarboxylase	d	d
Ornithine decarboxylase	-	-
Arginine dihydrolase	+	+
Tryptophan & phenylalanine deaminases	-	-
Urease	-[b]	-
Starch, gelatin, DNA & RNA hydrolysis	+	+
Tween 80 esterase	+	+
Citrate (Simmons')	+	+
Growth in peptone water without NaCl	+	+
Fermentation of maltose, galactose & trehalose	+[b]	+[b]
Fermentation of cellobiose, lactose & sorbitol	d	-
Fermentation of dulcitol, rhamnose, inositol, xylose, raffinose & adonitol	-	-
Breakdown of malonate, mucate & D-tartrate	-	-
Fermentation of glycerol	d	d
Tetrathionate reductase	d	-

[a] For symbols see Tables 1 & 2

[b] Aberrant strains occur

Table 1.4 DNA hybridization groups of the *Aeromonas* genus*

DNA hybridization group (DHG)	Species
1	*A. hydrophila*
2	*A. hydrophila*
3	*A. hydrophila*/*A. salmonicida*
4	*A. caviae*
5A	*A. caviae*
5B	*A. caviae*/*A. media*
6	*A. eucrenophila*
7	*A. sobria*
8†	*A. sobria*
9Δ	*A. sobria*
10†	*A. veronii*
11	*A. veronii*
12	*A. schubertii*

† DHG's 8 and 10 share enough DNA homology to be included in the same DNA hybridization group, DHG 8/10

Δ Newly proposed as *A. jandaei* (Carnahan *et al.* 1991)

* Compiled from Carnahan & Joseph (1991) and Altwegg & Luthy-Hottenstein (1991)

sobria, however in recent years a number of new motile *Aeromonas* species have been proposed: *A. veronii* (Hickman-Brenner *et al.* 1987), *A. schubertii* (Hickman-Brenner *et al.* 1988), *A. eucrenophila* (Schubert & Hegazi 1988) and *A. jandaei* and *A. trota* (Carnahan & Joseph 1991). The distinctness of *A. hydrophila*, *A. caviae* and *A. sobria* was demonstrated by Austin *et al.* (1989), but significant phenotypic variation was recognised within the *A. hydrophila* and *A. sobria* groups. On the basis of this phenotypic variation, new species are being characterised and assigned to one of the DNA hybridization groups. For example, strains assigned to DNA hybridization groups 7-9 were formerly all phenotypically classed as *A. sobria*, but isolates associated with DNA group 9 are now proposed as *A. jandaei* on the basis of being 'esculin and sucrose-negative' *A. sobria* (Table 1.4).

Recent numerical taxonomic studies have confirmed the low phenotypic similarity of non-motile aeromonads with their motile counterparts (Bryant *et al.* 1986; Austin *et al.* 1989). In fact, because of the high degree of phenotypic dissimilarity between motile and non-motile aeromonads, it was previously suggested that *A. salmonicida* be transferred from the *Aeromonas* genus to the Pseudomonadaceae as a novel genus and species (Smith 1963). However, the genotypic similarities between motile and non-motile groups supports the retention of *A. salmonicida* within the *Aeromonas* genus (MacInnes *et al.* 1979; Bast *et al.* 1988; Belland & Trust 1988; McCormick *et al.* 1990).

The current differentiation of the *A. salmonicida* species appears to be more generally accepted. Numerical taxonomy (Austin *et al.* 1989), DNA homology (Belland & Trust 1988; McCormick *et al.* 1990), serological (Paterson *et al.* 1980; Popoff & Lallier 1984) and biochemical (Chart *et al.* 1984; Popoff & Lallier 1984) studies, as well as the evaluation of plasmid profiles (Bast *et al.* 1988), have demonstrated the marked homogeneity of *A. salmonicida* ssp. *salmonicida*. In fact, DNA hybridization studies using single strand-specific endonuclease S1 from *Aspergillus oryzae* to study polynucleotide sequence relatedness, indicate greater than a 97% level of relatedness.

However, there is some debate as to the correct subspecies differentiation, with Belland & Trust (1988) suggesting that ssp. *achromogenes* and *masoucida* be combined into a single group, and also that a new subspecies (epithet *nova*) be introduced for the atypical ssp. *salmonicida* strains (Belland & Trust 1988; Austin *et al.* 1989). The latter suggestion is clearly justified for *A. salmonicida* ssp. *salmonicida* strains which infect non-salmonid fish, since their phenotypic (Austin *et al.* 1989) and genotypic (Belland & Trust 1988) properties support the elevation to a full subspecies level. However, the suggestion by Belland & Trust (1988) of combining ssp. *achromogenes* and *masoucida* into a single group has been dismissed by the numerical taxonomic studies of Austin *et al.* (1989), which supported previous observations that these subspecies may occupy an intermediate position between *A. hydrophila* and *A. salmonicida* ssp. *salmonicida* (Paterson *et al.* 1980; Belland & Trust 1988).

A large range of species-specific bacteriophages have also been used in *A. salmonicida* typing studies. Bacteriophages acting on *A. salmonicida* may be isolated from hatcheries, water and sewage, or from lysogenic isolates (Christison *et al.* 1938; Paterson *et al.* 1969; Popoff & Vieu 1970). *A. salmonicida* phages can be divided into three morphological groups (Popoff 1971). The first group contains phages which resemble T-even phages of *Escherichia coli* in having a head (100nm), a long contractile tail (130nm), a collar and a base plate with fibres. Phages of the second group have a polyhedric head (65nm) and a long contractile tail (150nm). The third group contains phages which possess a polyhedric head (60nm), a short tail (90nm) with an anchor-like plate. Popoff (1971) investigated the resistance of *A. salmonicida* phages to physical factors. The phages are usually resistant to temperature of 60-65°C for 10 minutes and some phages also endure exposure to 75°C. All phages of *A. salmonicida* are stable at pH 6.5-11.0.

Phages of *A. salmonicida* have been used in the identification of isolates, in cell wall studies of this organism and in studies on the epidemiology of the disease (Paterson *et al.* 1969; Popoff 1971; Rodgers *et al.* 1981; Trust *et al.* 1980). The lipopolysaccharide (LPS) layer appears to possess the receptor for most *A. salmonicida* bacteriophage

(Rodgers *et al.* 1981), however Udey and Guzman (1980) have reported phage which only infect cells possessing an A-layer (cell envelope of *A. salmonicida* is discussed in Sections 1.2.3 & 3.1).

The bacteriophage typing analysis of *A. salmonicida* is one of only two methods capable of differentiating between typical *A. salmonicida* isolates (Popoff & Lallier 1984). The other method, the result of a more recent study, comprises a restriction endonuclease fingerprinting (REF) analysis of *A. salmonicida* cellular DNA visualised by silver staining (McCormick *et al.* 1990). Like previous techniques, REF indicated that *A. salmonicida* is a very homogenous pathogen, but in contrast to the earlier studies, REF analysis permitted the identification of subgroups within the subspecies which may be useful in epidemiological studies.

1.1.2 Morphology and growth characteristics

Members of the genus *Aeromonas* are Gram-negative, rod-shaped bacteria with rounded ends, measuring 1-3.5 by 0.3-1 µm. The cells can occur singly, in pairs or short chains. On complex media, cells of *A. salmonicida* appear as coccobacilli; the length is less than twice the width (Popoff 1984). This species commonly develops short chains and clumps (Smith 1963). The optimum growth temperature for *A. salmonicida* is 22-25°C with most strains able to grow at 5°C; *A. salmonicida* does not grow at all at 37°C (Popoff 1984). The majority of the typical *A. salmonicida* strains produce a brown water-soluble pigment; however, pigment production does not occur anaerobically (Williamson 1928).

1.1.3 Ecology of *A. salmonicida*

A. salmonicida is a parasite lacking the ability to exist as a saprophyte under natural conditions and so is considered to be a true pathogen (Popoff 1984). However, *A. salmonicida* has the ability to survive in the aquatic environment, but in an apparently non-culturable/dormant state (Allen-Austin *et al.* 1984). *A. salmonicida* cells were observed as microcolonies on particulates obtained from river water, and the presence of these microcolonies could serve as foci for infection. It is not known how long *A. salmonicida* can survive in the aquatic environment, but some studies have suggested that the bacterium may remain viable for several weeks in fresh and sea water (Austin & Austin 1987), and for many months in river sediments (Michel & Dubois-Darnaudpeys 1980).

A. salmonicida has the ability to cause latent infections; Paterson (1983) previously noted that little was known of the furunculosis carrier state, except that it existed. Asymptomatic carriers can be demonstrated by immunosuppression of the fish by cortico-steroids (Bullock & Stuckey 1975). Paterson (1983) suggested that the carrier state was of considerable importance as a focus of *A. salmonicida* infection. The carrier fish would function as a reservoir of infection within fish populations. However, great difficulty has been experienced in isolating *A. salmonicida* from anything other than infected fish (Paterson 1983). McIntosh and Austin (1991) suggested that the difficulty in recovering *A. salmonicida* from surface waters and carrier fish could be due to inadequacies of conventional culture media if the pathogen in the carrier fish occurred as an L-form; a cell-wall deficient bacterium capable of multiplication (Maxted 1972). Induction of *A. salmonicida* L-forms has been achieved by the action of lysozyme and antibiotics (McIntosh & Austin 1988), and by complement in combination with anti-*A. salmonicida* rabbit antiserum (McIntosh & Austin 1990). By developing specially formulated media for culturing *A. salmonicida* L-forms, McIntosh and Austin (1988) isolated 'natural' L-form colonies from diseased salmon. This suggests that *A. salmonicida* L-forms may be involved in the disease process (McIntosh & Austin 1991).

1.2 Biochemical characteristics of extracellular products of *A. salmonicida*

Protein secretion is a fundamental process that occurs in all living cells. Although it was known that Gram-negative bacteria exported proteins to various compartments and assembled proteins into the cell envelope, it was presumed that these organisms rarely secreted proteins beyond their outer membrane. Like most studies on Gram-negative bacteria, *E. coli* was used as a model for protein export and as a consequence of the general inability of this bacterium to secrete proteins into the external medium it was presumed that other members of this class of bacteria would behave similarly. However, recent evidence presented by a number of workers (Hirst *et al.* 1984; Poole & Hancock 1983; Mackman *et al.* 1986; Howard & Buckley 1985; Pohlner *et al.* 1987) has challenged this view and it has now been unambiguously shown that numerous Gram-negative species actively secrete proteins into the external milieu.

Proteins that are incorporated into the cell envelope or secreted into the culture medium are synthesised on ribosomes attached to the cytosolic side of the inner membrane and are then translocated across this membrane into the periplasm, outer membrane or medium. All periplasmic and outer membrane proteins so far studied appear to be exported by the same mechanism (Randall *et al.* 1987; Hirst & Welch 1988). In general, this process involves the synthesis of the protein as a precursor with an amino-terminal extension (signal sequence) and the removal of this sequence by signal peptidase in the cytoplasmic membrane. Energy is required in the form of high-energy phosphate and an export apparatus is necessary for the correct processing of the protein precursors.

The secretion of extracellular proteins, however, does not appear to be as straight-forward in comparison with their cell-bound counterparts (Pugsley & Schwartz 1985). Most extracellular proteins are synthesised and exported to the periplasm in a similar manner as periplasmic and outer membrane proteins (signal peptide-dependent pathway), but a number of important exceptions have been demonstrated, including the colicins and the -haemolysins of *E. coli* (Pugsley & Schwartz 1984; Felmlee *et al.*

1985). These extracellular proteins are secreted beyond the outer membrane in a signal peptide-independent manner, possibly through the interaction of long stretches of hydrophobic amino acids which could insert into the cytoplasmic membrane and initiate the secretion process (Pugsley 1988). The only extracellular protein of *A. salmonicida* to be studied in terms of protein secretion is the A-protein, although this protein is not normally referred to as truly extracellular since it is associated with the cell envelope (see below). A large number of extracellular products have been detected in the culture medium of *A. salmonicida* cultures. However, the mechanism by which these proteins are secreted has received little attention, apart from preliminary studies which correlate protein secretion with protein synthesis on membrane-bound polysomes (Campbell *et al.* 1990).

The range of extracellular enzymes detected in the culture medium of *A. salmonicida* include proteases, haemolysins and glycerophospholipid: cholesterol acyltransferase (GCAT) and leucocytolytic activities (Ellis 1991). In general, the only physical property which has been determined for most of these activities is the M_r of the enzymes, and the values displayed by these enzymes appear to be slightly different to the norm for bacterial extracellular proteins. In contrast to eukaryotic cells, which quite often secrete large glycoproteins, bacterial extracellular enzymes have relatively low molecular weights (20-40kDa) and are usually free of carbohydrate (Burns 1983). However, a number of extracellular proteins of *A. salmonicida* are either relatively large polypeptides such as the protease (Price *et al.* 1989), or large glycoproteins including haemolysin (Nomura *et al.* 1988) and leucocytolysin (Fuller *et al.* 1977) components. Early studies revealed a prominent characteristic of bacterial extracellular proteins being a low cysteine content which, in turn, means a low incidence of disulphide bridges in secreted proteins (Pollock 1962). Apart from the low occurrence of cysteine, bacterial extracellular enzymes do not have any obviously distinctive amino acid composition and their polarities do not differ markedly from those of intracellular proteins (Glenn 1976). Another feature of extracellular enzymes includes the ability to tolerate high temperatures and other extreme environmental situations, and so results in the relatively long half-lives of these enzymes in comparison with labile intracellular enzymes. Also,

in comparison with highly specific intracellular enzymes associated with metabolic regulation mechanisms, the extracellular counterparts such as proteases have, typically, low substrate specificities (Gibb & Strohl 1987).

1.2.1 Extracellular protease activity of *A. salmonicida*

There have been a number of studies investigating the extracellular proteolytic activities of *A. salmonicida* but there has been considerable variation in the reported sizes, ranging from 11 to 87.5kDa, and substrate specificities (Dahle 1971a; Shieh & Maclean 1975; Sheeran & Smith 1981; Mellergaard 1983; Tajima *et al.* 1984; Fyfe *et al.* 1986a). In another report Sheeran and Smith (1981) observed that strain 480 produced two distinct proteolytic activities, one a serine protease and the other a metalloprotease. Metalloprotease activity was also demonstrated in *A. salmonicida* culture medium by Rockey *et al.* (1988), although no detailed biochemical properties of the enzyme(s) were presented. Recently, Price *et al* (1989) attempted to clarify some anomalies in the literature concerning the proteases produced by *A. salmonicida*. The strains studied (MT004, 1102 and 480) were those used by previous workers. These strains showed a very similar pattern with two types of protease produced. The protease of molecular weight 70kDa was active against casein and gelatin and is most probably the same enzyme as that from strains Ar-4 and MT004, described by Tajima *et al.* (1984) and Fyfe *et al.* (1986a) respectively, whereas the protease(s) of approximate molecular weight 20kDa is (are) active against gelatin but not casein.

The 70kDa enzyme has been classified as a serine protease on the basis of its inhibition by phenylmethanesulphonyl fluoride (PMSF). However, less than 20% inhibition of this protease was observed on incubation with ovomucoid, ovoinhibitor, aprotinin, antipain or benzamidine, which distinguishes it from other serine proteases such as trypsin and chymotrypsin. Ellis (1987) identified $_2$-macroglobulin as the only serum protein capable of inhibiting the serine protease of *A. salmonicida*. The resistance to serum protease inhibitors may be correlated with the unusual nature of the active site to provide pathogenic adaption to host defence mechanisms.

Experiments with low molecular weight substrates (*p*-nitroanilides) revealed a degree of overlap between the 70kDa protease and thrombin (Price *et al.* 1990), however the 70kDa protease showed less discrimination between the substrates. A possible physiological role of the 70kDa protease was suggested as that of digestion of host proteins as a nutrient source for the bacterium. It was also noted that blood clots formed in the heart of rainbow trout injected with the extracellular products of *A. salmonicida*, presumed to be due to (thrombin-like) protease action (Ellis *et al.* 1981).

Further studies of substrate specificity of the purified serine protease of *A. salmonicida* have been reported (Price *et al.* 1990). The protease of molecular weight 70kDa degrades casein to fragments of less than 10kDa, bovine serum albumin (BSA) into two large fragments (60 & 25kDa) and appears hardly active against native ovalbumin. However, BSA which has been denatured by carboxymethylation of cysteine residues is degraded to small fragments.

The production of the 70kDa caseinase enzyme is affected by a number of factors, including temperature, oxygenation, pH, and composition of the growth medium. Lowering the incubation temperature to 10°C resulted in more than a 2-fold decrease in extracellular caseinase activity, compared with cells grown at 25°C (Fyfe *et al.* 1987b). Anaerobic growth was found to have an even more pronounced effect on protease production; only one-tenth of the protease activity was present in the culture filtrates of the anaerobic culture (Fyfe *et al.* 1986b). Dahle (1971a) demonstrated that protease production was dependent on the pH of the growth medium being raised above pH 7.0. A number of medium components also affected caseinase enzyme production; high molecular weight compounds (e.g., peptone) (Dahle 1971b) and certain concentrations of amino acids (Sakai 1985b) were found to be necessary for protease production. However, unpublished findings (Laverty, E., this laboratory) suggest that the presence of peptides [e.g., $(glycine)_3$ & $(leucine)_5$] are more important than either amino acids or proteins.

1.2.2 Extracellular phospholipase and haemolysin activities of *A. salmonicida*

The other major focus of interest on the extracellular products of *A. salmonicida* has been on membrane-damaging toxins. There have been several reports on enzymes of this type in the literature, including GCAT (Buckley *et al.* 1982; Lee & Ellis 1990), leucocytolysin (Fuller *et al.* 1977) and haemolysin activities (Titball & Munn 1981, 1983, 1985a; Fyfe *et al.* 1987a; Nomura *et al.* 1988). It was demonstrated that bacteria in the family Vibrionaceae release a glycerophospholipid: cholesterol acyltransferase (GCAT) which is active against both liposome substrates and human erythrocyte membranes (MacIntyre & Buckley 1978; MacIntyre *et al.* 1979). GCAT was found in outer membrane fragments, termed 'blebs', which were released during growth (MacIntyre *et al.* 1980). GCAT was purified to near homogeneity from the culture supernatant of *A. salmonicida* and was found to have a molecular weight of 23.6kDa (Buckley *et al.* 1982). The purified GCAT exhibits phospholipase, acyltransferase and lysophospholipase activities (Fig.1.2). Partial characterisation of GCAT from *A. salmonicida* distinguished it from bacterial phospholipases: however, it shares a number of characteristic features of mammalian lecithin: cholesterol acyltransferase (LCAT); for example, it has no divalent cation requirement and is stimulated both by albumin and by human apolipoprotein A-1. The enzyme exhibits 2-positional specificity as an acyltransferase and as a phospholipase which is unlike the 1-position specificity of nearly all bacterial phospholipases so far characterised.

A. salmonicida GCAT is much less specific than mammalian LCAT (Glomset & Norum 1973) in the phospholipids it uses. In contrast to LCAT, which is specific for phosphatidylcholine, the bacterial enzyme is capable of using a wide range of naturally occurring phospholipids as acyl donors. The results indicate that the enzyme has a preference for phospholipids carrying short-chain or unsaturated fatty acids and is less dependent on the nature of the phospholipid head group.

Buckley (1983) examined further the structural requirements of the bacterial acyltransferase for the acyl donor. The enzyme is able to use a variety of hydrophobic

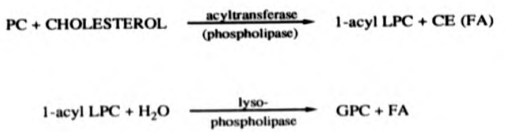


Figure 1.2. Proposed sequential reaction mechanism for the complete deacylation of PC by the bacterial GCAT enzyme. CE, cholesteryl ester; FA, fatty acid; GPC, glycerophosphorycholine; LPC, lysophosphatidylcholine; PC, phosphatidylcholine. Adapted from Buckley *et al.* 1982.

esters as acyl donors, yet acyl transfer is specifically from the 2-position when phosphatidylcholine is the substrate. This observation suggests that the enzyme-catalysed acyl transfer requires hydrogen bonding between donor and acceptor. Even though sphingomyelin is not a substrate for the enzyme it inhibits acyl transfer from phosphatidylcholine to cholesterol, indicating that microbial enzyme-catalysed acyl transfer depends on the formation of a complex between the acyl donor and acyl acceptor.

Recently the nucleotide sequence of a gene encoding *A. hydrophila* GCAT was determined (Thornton *et al.* 1988). Comparison of the amino terminal sequence with that of GCAT purified from *A. salmonicida* showed that only one of the first 18 amino acids is different in the enzymes from the two species. GCAT from *A. salmonicida* contains a threonine at position 3 rather than a serine. The DNA sequence of the GCAT gene of *A. hydrophila* encodes a protein of 281 amino acids with a molecular weight of 31303, which is significantly larger than the molecular weight of the protein from *A. salmonicida* as determined by SDS-polyacrylamide gel electrophoresis (Buckley *et al.* 1982). This may reflect major differences between the two proteins or, alternatively, GCAT may undergo post-translational modification.

Karlsson (1962) demonstrated the presence of a haemolysin among the extracellular products of *A. salmonicida*. Since then Titball and Munn (1981) have identified two distinct haemolytic activities. One, H-lysin, is a broad-spectrum haemolysin with maximum activity against horse erythrocytes, and the other, T-lysin, is only active against trout erythrocytes. H-lysin is only produced under restricted *in vitro* conditions (stationary culture). The purified H-lysin contained detectable levels of GCAT activity, but the results suggested that the two activities were due to different extracellular products since membrane filtration of the preparation removes H-lysin activity (Titball & Munn 1981) but fails to remove GCAT from the preparation (Titball & Munn 1985a). It has been reported that H-lysin is secreted as an inactive precursor which, on proteolytic cleavage, yielded an active haemolysin of M, 29.5kDa (Titball & Munn 1985a).

The haemolytic activity of partially purified T-lysin was suggested to be due to the combined effect of two components: an activity (designated T_1) which caused incomplete lysis of erythrocytes and a caseinase enzyme (Titball & Munn 1983). Caseinase-negative mutants of *A. salmonicida* produce an inactive precursor of haemolysin that is activated by an autogenous protease (Titball *et al.* 1985).

Other haemolytic activities have been reported to be released into the culture supernatant by *A. salmonicida* (Nomura & Saito 1982; Fyfe *et al.* 1987a). Ten different strains of *A. salmonicida* were grown under identical conditions and the pattern of extracellular proteins produced by each strain were compared. Two significant common components were observed; one with a molecular weight of 70kDa, found to be the serine protease mentioned previously (Price *et al.* 1989), and the other with a molecular weight of 56kDa that was haemolytic to trout erythrocytes. It was suggested that the 56kDa haemolysin may correspond to the haemolysin described by Karlsson (1962) and that the haemolysins observed by Titball and Munn (1981) may be less widely distributed.

Nomura *et al.* (1988) have recently purified and characterised a new extracellular haemolysin from *A. salmonicida*, termed 'salmolysin'. The production of an extracellular haemolytic toxin was previously reported (Nomura & Saito 1982) and the optimum pH range was from 7.5 to 8.0, and the optimum temperature was around 20°C. The molecular weight of the purified salmolysin was estimated to be approximately 200kDa by the sedimentation equilibrium method. Salmolysin is an acidic protein with an isoelectric point of 5.4. Carbohydrate and protein analyses indicated that salmolysin is a glycoprotein which contains approximately 62% carbohydrate and 38% protein.

A leucocytolytic (LCL) factor was demonstrated by Klontz *et al.* (1966) and later identified by Fuller *et al.* (1977) as a glycoprotein with a molecular weight in the range of 100-300kDa. The protein and carbohydrate composition was estimated as 35% and 55%, respectively.

Recently, Lee and Ellis (1990) identified a GCAT enzyme which forms a complex with LPS. The protein component of the complex comprised a single polypeptide chain of molecular weight 25kDa, but the whole toxin had a molecular weight of over 2000kDa, estimated by FPLC Superose gel filtration. The isoelectric point of free GCAT was determined to be pI 4.3, whereas GCAT/LPS produced an unfocused smear corresponding to pIs' from 5.0 to 5.8. The effect of LPS-complexing with free GCAT was determined by comparing its properties with the small amount of free GCAT also present in the culture medium. It was found that complexing of the LPS with GCAT conferred a number of advantages over the free GCAT. Free GCAT was found to be more susceptible to heat inactivation and possibly, of more significance *in vivo*, GCAT in the GCAT/LPS complex was more resistant to proteolysis and had enhanced haemolytic activity.

Lee and Ellis (1991) have demonstrated that the GCAT/LPS complex possesses a number of biochemical characteristics in common with several of the other previously identified extracellular proteins. It is suggested that all of these enzyme activities (phospholipase (GCAT), leucocytolysin, cytotoxin, T-lysin, H-lysin & salmolysin) are properties of the single entity GCAT/LPS (Ellis 1991).

1.2.3 Cell surface layer of *A. salmonicida*

A. salmonicida possesses a superficial coat called the A-layer (Fig.1.3) (Udey & Fryer 1978; Hamilton *et al.* 1981; Trust *et al.* 1980). This layer is composed of carbohydrate in the form of lipopolysaccharide (LPS) and a tetragonally arrayed protein of molecular weight 49kDa known as the A-protein (Kay *et al.* 1981). Regularly-structured (RS) layers are ubiquitous in nature and are found as part of the cell envelope of virtually every taxonomic group of walled eubacteria and archaebacteria (Sleytr & Messner 1983). In eubacteria, these RS layers are non-covalently associated with the underlying cell wall.

The amino acid composition of RS proteins of different eubacteria is very similar. They

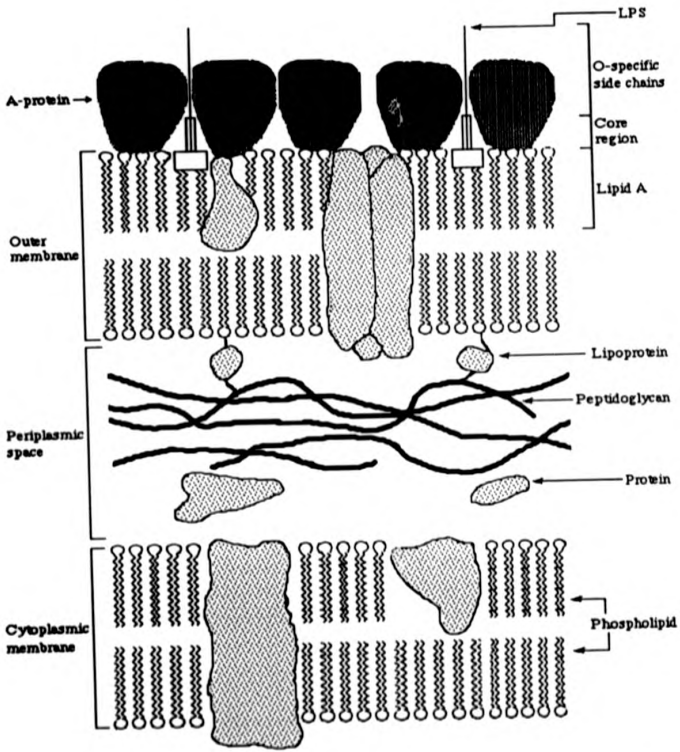


Figure 1.3. Model of the Gram-negative cell envelope (After Sleytr & Messner 1983)

are acidic proteins with a high proportion of non-polar and acidic amino acids and contain little or no sulphur-containing amino acids (Sleytr & Messner 1983; Kandler & Konig 1985; Konig & Stetter 1986; Sleytr *et al.* 1986; Bingle *et al.* 1986). The acidic nature of RS proteins has been confirmed by their low isoelectric points which are usually within the pH range 4-6 (Smit 1986). Isoelectric focusing of the purified A-protein from *A. salmonicida* demonstrated several distinguishable isoelectric forms, with pIs' ranging from 5.7 to 5.9. The appearance of multiple isoelectric forms is not unusual, particularly for cell surface localised proteins. The amino acid sequence has not been determined for any RS protein, but the amino acid composition of *A. salmonicida* A-protein was determined (Kay *et al.* 1981) and was found to be substantially hydrophobic (45%). The N-terminal sequence of the A-protein is exceptionally hydrophobic. Conformational studies have been done on very few RS proteins. So far, the secondary structure of RS proteins, as estimated by circular dichroism, shows a low level of -helical structure (2-14%), 20-35% B-structure and a high level of aperiodic structure (Baumeister *et al.* 1982; Bingle *et al.* 1986; Dooley *et al.* 1988). The secondary structure of *A. salmonicida* A-protein complies with these general limits, exhibiting 14% -helix and 19-28% B-structure.

Using computer-enhanced imaging techniques, it was found that the A-protein subunits formed a tetragonal array of alternating light and heavy domains, forming an open porous structure with concave depressions formed from four subunits. Stewart *et al.* (1986) have recently described two different square arrays present in a single RS layer of *A. salmonicida*, formed from the same polypeptide. They propose that the two patterns may reflect a structural transformation of the layer such that the permeability of the layer would be affected. An increase in permeability would facilitate release of possible virulence factors such as protease and haemolysin.

The A-protein of *A. salmonicida* strain 449 has been particularly well characterised biochemically. Belland and Trust (1985) were successful in obtaining mutants blocked in A-protein synthesis, export and assembly. Their results suggested that the A-protein is coded by a single chromosomal gene and it is exported through the periplasmic space

and outer membrane. Cloning studies originally suggested that the A-protein was synthesised without an amino-terminal signal sequence, since immunoblotting of the cloned A-protein was indistinguishable from the M_r 49kDa mature protein found in the assembled surface array (Belland & Trust 1987). However, sequencing of the A-protein gene revealed that the protein coded for did contain a typical signal sequence for protein secretion (Kay & Trust 1991).

The question of relatedness and taxonomic significance of RS proteins has been considered for selected strains of *A. salmonicida* (Kay *et al.* 1984). The RS proteins of three *A. salmonicida* strains shared similar molecular weights, amino acid compositions and N-terminal amino acid sequences. It was suggested that the differences in the tryptic peptides could be due to minor positional differences in certain amino acids within the RS proteins. As expected antigenic cross-reactivity was observed among the RS proteins of the three *A. salmonicida* strains.

Gram-negative bacteria are defined by an outer membrane containing lipopolysaccharide (LPS) (Fig.1.3). Chart *et al.* (1984) studied the lipopolysaccharides of typical and atypical strains of *A. salmonicida*. It was demonstrated that the O-polysaccharide strains were homogeneous with respect to chain length. Chemical analysis revealed that the smooth LPS of three typical strains had similar sugar compositions. Cellular LPS 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 (Shaw *et al.* 1986). Immunofluorescence analysis and phage binding studies demonstrated that a number of the O-polysaccharide chains traversed the surface protein array of *A. salmonicida* and were exposed on the cell surface (Chart *et al.* 1984). The synthesis of A-protein and LPS side chains may be coordinately regulated in this organism. Mutants lacking O-specific polysaccharides secreted A-protein into the culture medium (Belland & Trust 1985). It was suggested that the LPS may play an important role in assembly and maintenance of the A-layer, and such a structural role might account for the homogeneity in chain length of the LPS (Chart *et al.* 1984; Evenberg *et al.* 1985;

Belland & Trust 1985).

Growth of *A. salmonicida* at elevated temperatures (> 25°C) resulted in the production of A-layer-negative mutants which do not possess the ability to autoaggregate (Ishiguro *et al.* 1981). The attenuated derivatives appear to lose A-protein in an irreversible manner due to genetic deletion of the 5' region of the A-protein gene (Belland & Trust 1987).

1.3 Virulence factors of *A. salmonicida*

1.3.1 Introduction

The study of the origin and mechanisms of disease production is referred to as pathogenesis. This is derived from 'pathos' meaning disease and 'genesis' meaning to produce. The development of bacterial infectious disease is dependent on the interaction between host and bacterium. Therefore, there are two main aspects of disease production: firstly, the host has a number of defence mechanisms for eliminating infectious and foreign agents, and secondly, the disease-causing bacterium can evade the host defense by a number of its own mechanisms (Widders 1988). The diversity of mechanisms utilised for defence and infection is the reason for the enormous spectrum of the resulting diseases.

The host has numerous defence mechanisms that, used either singly or in combination, will normally provide protection against pathogens. Anatomic barriers such as skin and mucous membranes serve a primary protective function by preventing bacterial penetration. The regular cleansing activity of tears and secretory juices produced by some hosts aids the anatomic barriers. Other passive mechanisms of protection include the presence of normal flora which may compete for space, receptors and nutrients or even induce a protective environment (Arp 1988). Finally, the ultimate mechanisms of defence are the specific defence mechanisms of the host's immune system, involving both humoral and cellular components and other minor serum factors.

There is some debate as to the nature of the virulence mechanisms of *A. salmonicida* (Ellis *et al.* 1988). Numerous reports have described a variety of extracellular proteins secreted by *A. salmonicida* (see Section 1.2), and some of these activities have been associated with virulence (Cipriano *et al.* 1981; Sheeran *et al.* 1984; Sakai 1985a; Shieh 1985; Fyfe *et al.* 1986a). Several workers have also correlated the presence of A-layer with virulence (Ishiguro *et al.* 1981; Kay *et al.* 1981); and with autoaggregation (Kay *et al.* 1981; Evenberg *et al.* 1982).

1.3.2 A-layer and its possible role in virulence

Cell surface proteins represent the predominant interface between the cell and the environment. The bacterial cell surface must play a major defensive role in protecting the pathogen from the defence mechanisms of the host. Therefore, regularly structured (RS) layers must be important for the few bacterial pathogens that have been identified as possessing this type of surface component. Kay *et al.* (1981) suggested that if the A-layer plays a key role in the virulence of *A. salmonicida*, then the structure of the A-protein should be conserved even in bacterial strains from different locations and hosts. Studies on the surface antigens of *A. salmonicida* revealed that both LPS (Chart *et al.* 1984) and A-protein (Kay *et al.* 1981) display considerable antigenic conservation, implying important functions of these surface structures in the virulence of this pathogen.

A critically important first step for a number of bacterial pathogens is that of attachment to the mucosal surface (Reed & Williams 1978; Arp 1988), i.e., pathogenic bacteria must have the ability to successfully colonise the mucosal surface. The requirements for bacterial colonisation of the mucosal membrane are described by Arp (1988). The infecting bacteria must establish and maintain their position in close proximity to the mucosal membrane. This is achieved by establishing some form of surface interaction to avoid being swept away, obtaining nutrients in the host environment for growth, sufficient multiplication to maintain or expand the bacterial population, and evading the

host defence mechanisms.

In general, there are three ways in which bacteria interact with the mucosal surface, and the terms of interaction are based on the degree of intimacy between bacterial and mucosal surfaces. The bacterial-mucosal interactions can be loosely classed in increasing degrees of intimacy as association, adhesion and invasion. Association describes weak and reversible attachment to the mucosal membrane which may be sufficient for surfaces that are not subjected to physical shear forces. Adhesion is a relatively stable, irreversible interaction between the bacterial and mucosal surfaces and is a preferred mechanism of attachment (Jones & Isaacson 1983). A ligand-receptor type mechanism is the basis for the adhesion interaction between the bacterial and mucosal surfaces. The complementary molecules are typically bacterial surface proteins and carbohydrate-containing components (glycoproteins and glycolipids) of the epithelial cell membrane (glycocolyx) and mucous layer (Lark 1986).

Adhesion of *A. salmonicida* to host tissue cells may be an important initial stage in the infection process (Parker & Munn 1985; Johnson *et al.* 1985). The presence of an A-layer on *A. salmonicida* cells has been associated with an increased ability to adhere to various fish and non-fish cells (Udey & Fryer 1978; Parker & Munn 1985). Comparison of aggregating (A^+) and nonaggregating (A^-) isolates revealed that aggregating strains have a greater adhesive ability than nonaggregating strains. The binding ability of the aggregating cells was general rather than specific, and was most likely a result of the hydrophobic nature of the surface of these cells (Parker & Munn 1985). Recent studies have demonstrated that the A-layer is capable of binding a number of high molecular weight host basement membrane molecules such as fibronectin, laminin and collagen-IV (Kay & Trust 1991).

Once the invading organism has penetrated the anatomic barriers of the host, a number of specific immune defence mechanisms are activated to make conditions unfavourable for survival of the invader. The host's mechanisms of defence can be divided into two types, humoral and cellular defence mechanisms. The major components of the humoral defence mechanism are antibodies and complement.

Microbial binding of host cell constituents, in the form of nonimmune immunoglobulin binding, also limits induction of a specific antibody response (Widders 1988). This mechanism of effecting antibody synthesis is most commonly associated with Gram-positive organisms, e.g., protein A of *S. aureus*. However, Gram-negative bacteria including *Taylorella equigenitalis* (causative agent of equine metritis) and *Haemophilus somnus* (mucosal pathogen of cattle), express surface receptors which bind to the Fc portion of the immunoglobulin molecule (Widders *et al.* 1985, 1988; Yarnall *et al.* 1988). The A-layer of *A. salmonicida* has also been shown to bind specifically to immunoglobulins in serum (Phipps & Kay 1988), and so may have the effect of reducing a specific antibody response.

The complement system is the other major humoral factor in the host's repertoire of defence mechanisms. The complement system comprises upwards of 20 serum proteins which interact in a series of cleavage reactions by a cascade mechanism resulting in the formation of biologically active fragments. These fragments exert their effects in two major ways: firstly, by the deposition of an opsonic protein (C3b), which promotes phagocytosis of invading bacteria by interacting with specific receptors on phagocytic cells, and secondly, by the assembly of the membrane attack complex (MAC) capable of the direct killing of a wide variety of susceptible Gram-negative bacteria.

Complement activation may occur by either of two pathways, the classical (antibody-dependent) or alternative (antibody-independent) pathways. The alternative pathway is stimulated by surface components of the invading organism; these components include capsular polysaccharides, lipopolysaccharides (LPS), and other cell envelope constituents. However, specific antibody induces a more rapid activation and also provides direction to the complement attack mechanism (Frank *et al.* 1987). Both pathways result in the activation of C3, the vital component of complement, then converge and proceed to form the C5b-9 protein complex, the membrane attack complex. The deposition of as few as 50-100 C5b-9 complexes per target cell may be sufficient for killing to occur (Born & Bhakdi 1986).

The mechanism of complement-mediated killing is not precisely understood (Frank *et al.* 1987), but is known to depend on irreversible damage of the cytoplasmic membrane (Taylor 1988). The failure of complement-mediated killing may result either by preventing (or at least limiting) complement activation, or from failure of activated complement to exert its effect (Woolcock 1988). In general, rough strains of Gram-negative bacteria, which are devoid of O-specific side chains on the outer membrane-located LPS, are highly sensitive to complement-mediated killing, whereas smooth strains possessing intact LPS are usually resistant to the direct killing action of complement (Rowley 1956; Woolcock 1988). In addition to LPS, other cell surface components, including polysaccharide capsule components, and outer membrane proteins, may also reduce sensitivity to complement by diminishing its activation or affecting the normally stable insertion of the MAC into the bacterial surface (Joiner 1985; Taylor 1988).

The A-layer has been suggested to prevent attack by noxious enzymes and the interaction between serum complement components and the plasma membrane (Ishiguro *et al.* 1981; Kay *et al.* 1981). A number of studies have indicated that the A-layer and LPS of *A. salmonicida* play an important role in resisting the bactericidal action of serum (Munn *et al.* 1982; Johnson *et al.* 1985; Sakai & Kimura 1985). Using mutant derivative *A. salmonicida* strains deficient in A-layer and high molecular weight LPS, Munn *et al.* (1982) and Johnson *et al.* (1985) demonstrated that the presence of A-layer and the full complement of LPS conferred resistance to the bactericidal action of serum. A-layer-negative strains displayed an intermediate resistance, while strains deficient in both A-layer and high molecular weight LPS were the most susceptible to serum killing. Loss of the O-specific polysaccharide chains was the major contributing factor of LPS to serum resistance (Munn *et al.* 1982). The bactericidal activity of the serum was correlated with complement activity, since heat-treatment and addition of 10mM EDTA which inhibit complement activity (Nonaka *et al.* 1981; Sakai 1981) removed the serum bactericidal activity.

The cell envelope plays an important role in limiting the effect of the activated MAC

(C5b-9). Frank *et al.* (1987) have suggested that different bacterial species have evolved different mechanisms for dealing with lytic attack by the MAC. The A-protein may possibly act as a protective barrier to the cell-killing MAC components of complement. Joiner *et al.* (1986) demonstrated that C3b preferably associated with the longest O-polysaccharide chains of the LPS. Since bound C3b is the first step in the formation of the MAC, then it is clear that the complex will be generated at a considerable distance from the outer membrane. Thus the MAC is less likely to insert successfully into the outer membrane and as a result will increase the organism's chance of survival.

Phagocytes (neutrophils, macrophages and eosinophils) are important components of a host's defences against invading microorganisms. The complex interactions that regulate cellular defences usually provide a decisive advantage to the host. At the site of infection phagocytes attach to the invading bacteria by binding to either recognisable cell envelope moieties (Van Oss 1978), or to serum opsonins present on the bacterial surface (Horwitz 1982) via a wide array of phagocyte cell surface receptors. Once binding has occurred the phagocyte ingests the organism into a plasma membrane-lined compartment called a phagosome, in which the environment is extremely hostile to the ingested bacteria.

Despite the highly bacteriocidal action of the cellular defence system some bacteria are able to survive, or even thrive, in these apparently hostile conditions; a wide range of strategies for bacterial evasion of the host's cellular defence mechanisms have been identified. The bacteria that successfully overcome the cellular defence mechanisms do so either by avoiding phagocyte binding or adapting to life within phagocytes (Cross & Kelly 1990). Evidence is accumulating that indicates that fish pathogens display effective antiphagocyte strategies. In the case of *A. salmonicida*, evidence indicates possession of the A-layer confers resistance to macrophages through binding (Trust *et al.* 1983; Parker & Munn 1985) and infiltration (Kay & Trust 1991): once inside, *A. salmonicida* can somehow resist phagocytic killing mechanisms (Kay & Trust 1991).

In general, the A-protein of *A. salmonicida* is considered responsible for the autoaggregation of virulent strains (Evenberg *et al.* 1982), and autoaggregation of *A. salmonicida* cells is considered essential for virulence (Sakai & Kimura 1985). Several workers have correlated the presence of the A-layer on the outer surface of *A. salmonicida* with virulence (Kay *et al.* 1981; Munn *et al.* 1982). The most direct evidence for a role in bacterial pathogenesis has been reported for the A-layer of *A. salmonicida* (Ishiguro *et al.* 1981). These workers investigated the effect of growth temperature on virulence of *A. salmonicida*. Growth at higher than optimal temperatures resulted in the selection of spontaneous attenuated derivatives in the initial bacterial culture. Virulent strains autoaggregated during growth and possessed the A-layer, whereas attenuated strains did not aggregate and did not possess the A-layer. Ishiguro *et al.* (1981) suggested that attenuation of virulence was a consequence of the loss of the A-layer.

However, autoaggregating virulent strains which appear to lack the A-layer (A^-) have been described (Johnson *et al.* 1985; Ward *et al.* 1985). Strain CN 8060 (A^-, autoaggregating) was found to be virulent for Atlantic salmon, confirming the association between autoaggregation and virulence, but questioning the association between possession of an A-layer and virulence (Ward *et al.* 1985). The cells of CN 8060 still retained the capacity to adhere efficiently to tissue culture monolayers.

Recently, it has been demonstrated that MT004 (a non-autoaggregating A-layer-negative (A^-) strain) has an LD_{50} of 1.7×10^7 by intramuscular injection in rainbow trout, indicating that at least moderate virulence can be retained in the absence of the autoaggregating property (Ellis *et al.* 1988). Strains MT028 [possesses A protein (A^+), is protease negative (P^-) and haemolysin positive (H^+)] and MT048 (A^+, P^+ & H^+) exhibited lower LD_{50} values of 2.1×10^4 and 2.4×10^5, respectively, by the intramuscular route of injection which is consistent with the presence of A-layer contributing to virulence.

1.3.3 Iron-sequestering mechanisms

In addition to the specific immune responses of the host a number of other factors influence the success of invading bacteria, including temperature, pH, oxygen tension, and the availability of essential nutrients (Griffiths *et al.* 1988). At present the availability of iron is the most widely researched and best understood of these factors. Iron is widely recognised as an essential nutrient for proliferation and survival of the invading pathogen (Bullen *et al.* 1978; Weinberg 1978; Griffiths *et al.* 1988; Payne 1988). However, the amount of free iron present in the body and available to the bacteria is extremely small. Most iron in the body is found intracellularly, as haem or stored in ferritin and haemosiderin, while extracellular iron is attached to high-affinity iron-binding glycoproteins, transferrin in serum, and lactoferrin in external secretions (Weinberg 1978). The amount of free iron in equilibrium with serum transferrin is approximately 10^{-18} M (Bullen *et al.* 1978), and since most invading bacteria require approximately 10^{-6} M iron for maximal growth the concentration of free iron in blood is not sufficient to support bacterial growth (Payne 1988). However, pathogenic bacteria can replicate successfully in the low-iron environment of the host, implying that they have evolved mechanisms for acquiring iron in these iron-restricted conditions.

Pathogenic bacteria have successfully adapted to the iron-limited environment of the host by assimilating iron either from protein-bound sources or from haem-containing compounds. In general, there are two mechanisms by which bacteria acquire iron: (i) direct utilisation of host iron compounds, and (ii) synthesis of high-affinity iron chelators to dissociate the iron from host complexes. Some pathogens sequester iron by interacting directly with iron-binding proteins.

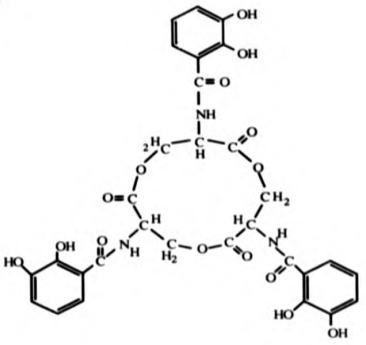
Apart from autoaggregation, colonies of A$^+$ bacteria can be directly distinguished from those of A$^-$ bacteria by growth on Congo red agar; the A-protein is the component responsible for binding Congo red (Ishiguro *et al.* 1985). A selective extraction procedure resulted in a fraction composed of 95% A-protein, and over 90% of the

bound Congo red was found in this fraction. This suggested that the A-protein was the major protein responsible for dye-binding activity. Confirmation of this was demonstrated by the binding of Congo red to pure A-protein. Further studies of dye-binding by *A. salmonicida* revealed that Congo red binding to the A-protein was structurally specific (Kay *et al.* 1985). Inhibitor studies demonstrated that Congo red binding was strongly inhibited by protoporphyrin and haemin. Kay *et al.* (1985) speculated that binding of these porphyrin-type compounds by the A-layer could represent some means of sequestering iron by *A. salmonicida*; this may be of particular importance in the acquisition of iron by atypical strains of *A. salmonicida*. In fact, when porphyrins such as haematoporphyrin and haemoglobin are added to the normal growth media of atypical strains, a significant improvement in their growth occurs (Ishiguro *et al.* 1986).

The most widely studied and best-understood mechanism for the assimilation of iron from iron-binding proteins is the production of low molecular weight, high-affinity iron-chelating agents, referred to as siderophores (Lankford 1973; Neilands 1981). Siderophore synthesis is usually derepressed in the low-iron environment of the host. The high-affinity siderophore compounds are secreted into the external milieu where they remove iron from lower affinity complexes and facilitate its transport back into the cell with the assistance of specific receptors present on the bacterial surface (Griffiths *et al.* 1988; Payne 1988). In general, there are two main classes of siderophore (Fig.1.4); the catechols and the hydroxamates, of which enterobactin and aerobactin are respective representatives. These siderophores have both been identified in enteric bacteria (the bacteria studied in most depth with respect to siderophore production). Enterobactin is the most commonly found catechol siderophore in enteric bacteria.

A. salmonicida is capable of growth in a low iron environment; this ability has been correlated with siderophore-mediated iron uptake and induction of iron-regulated outer membrane proteins (Chart & Trust 1983; Aoki & Holland 1985; Hirst *et al.* 1991). Hirst *et al.* (1991) demonstrated that typical *A. salmonicida* strains sequester iron via a siderophore-dependent mechanism. These workers could not isolate the siderophore-

(a)



(b)

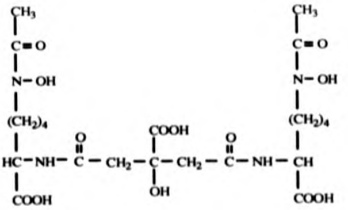


Figure 1.4. Structures of the siderophores (a) enterobactin and (b) aerobactin

like activity by the method used by Chart & Trust (1983), citing the insolubility of the siderophore in the organic solvent used in the extraction procedure as a possible reason for this failure. However, a catechol-type siderophore was detected in the culture filtrate of *A. salmonicida* and isolated by an alternative method. The partially purified siderophore was found to behave as a 2,3-diphenolate catechol. The *A. salmonicida* siderophore shared some common properties (2,3-diphenol catechol; insolubility in organic solvents) with siderophores from the related species, amonabactin and anguibactin produced by *A. hydrophila* and *V. anguillarium*, respectively. However, the siderophore of *A. salmonicida* was clearly quite different in structure, since *A. salmonicida* was unable to utilise either amonabactin or anguibactin.

An integral part of adaption to reduced iron levels through the siderophore high-affinity uptake system is the production of one or more proteins in the outer membranes of Gram-negative bacteria (Neilands 1982). Some of these outer membrane proteins are siderophore receptors (Griffiths 1987), and it is possible that the iron-regulated outer membrane proteins described by Chart & Trust (1983) and Aoki & Holland (1985) may have a siderophore receptor-type function in the iron uptake system of this organism.

1.3.4 Extracellular products as virulence factors

In addition to its putative role in protection, nutrient uptake and virulence, the A-layer has been shown to have a significant effect on the secretion of extracellular proteins by *A. salmonicida* (Titball & Munn 1985b). These workers demonstrated that the secretion of proteins into the external medium was growth phase-related, and the presence of the A-layer altered the secretory phase. A-layer-negative strains secreted proteins into the extracellular medium during late log phase of growth, whereas strains possessing the A-layer did not secrete proteins until the stationary phase. Titball & Munn (1985b) suggested that the impermeable nature of the A-layer may alter at the end of the log phase and so facilitate the release of extracellular proteins. Indeed, two conformations of the A-layer have been shown to be present on the surface of *A. salmonicida* (Stewart *et al.* 1986). Type II pattern appears to be a more open arrangement than the type I

pattern: if these patterns correspond to structural transformations, then the type II pattern will be of a more permeable nature and may correspond to the possible A-layer form associated with extracellular protein secretion.

Ellis *et al.* (1981) demonstrated that the extracellular products on injection into the fish were lethal and produced most of the pathological changes associated with furunculosis. In an attempt to identify the principal virulence factors in the culture filtrate, a number of workers have investigated the virulence of mutants or strains differing in their extracellular components (Hackett *et al.* 1984; Drinan & Smith 1985; Sakai 1985a) or the toxicity of purified or partially purified components of the culture filtrate (Cipriano *et al.* 1981; Hastings *et al.* 1981; Fyfe *et al.* 1986a). Various lines of evidence suggest that the extracellular protease of *A. salmonicida* is the major toxic factor. Sakai (1978) demonstrated that when injected intraperitoneally into Kokanee salmon, partially purified extracellular protease caused lesions and mortality. A protease-rich fraction of culture filtrate from preparative isoelectric focusing produced typical furuncular lesions on intramuscular injection into rainbow trout. The toxicity and pathogenicity of four fractions of culture filtrate were compared by Cipriano *et al.* (1981). Similar results were obtained with both properties associated with the fraction containing protease (fraction 2) which was also lethal for Atlantic salmon and brook trout. Two proteolytic enzymes were purified from the culture filtrate of *A. salmonicida* by Sheeran *et al.* (1984) and were found to be pathogenic in brown trout. Slight differences were observed in the pathology produced by the two proteases, with P1 (caseinase) causing greater liquefaction of muscle at the site of injection as compared with P2 (gelatinase).

Other workers have reported on the involvement of protease activity in the disease process. SDS-polyacrylamide gel electrophoresis analysis was used to demonstrate purity of the protease fraction (Fyfe *et al.* 1986a). The pure protease was shown to be less efficient in causing muscle pathogenesis by intramuscular injection compared with unfractionated culture filtrate, suggesting that other extracellular components may act synergistically with the protease. The synergistic effects of protease and haemolysin in

pathogenicity have been indicated in studies where the haemolytic activity was found to be due to the combined effect of T_1 activity which causes incomplete lysis, and the protease which is able to completely lyse the remaining erythrocyte ghosts (Titball & Munn 1983; Titball *et al.* 1985).

The extracellular proteolytic activity was strongly implicated as a principal virulence factor by the work of Sakai (1985a). This work demonstrated that a mutant defective in protease secretion, but resembling its parent wild type in many other biochemical characteristics, was able to survive for a period of time in fish tissues but was avirulent. The P^- mutant was unable to grow on selective media in the absence of amino acids whilst the parent strain was able to utilise casein as the sole amino acid source (Sakai 1985b). These results do not necessarily imply a virulence role for the protease; it may be that the protease is simply a growth-aiding factor, allowing virulent bacteria to obtain amino acids in the host environment. The recent observation that the P^- mutant MT028 was highly virulent in rainbow trout suggests that the extracellular protease does not have a major role in virulence (Ellis *et al.* 1988).

Hackett *et al.* (1984) came to similar conclusions on the role of extracellular protease and haemolysin. They found no difference in the LD_{50} when fish were exposed to a $A^+P^+H^+$ isolate or its $A^+P^-H^-$ variant indicating these extracellular activities were not necessary for virulence or pathogenicity in the acute form of furunculosis. Ellis *et al.* (1988) correlated the protease with certain pathogenic effects which may hasten the time to death but not with the ultimate lethal effect of unfractionated extracellular components. These workers also demonstrated a lack of relationship between haemolysin titres and minimal lethal dose of the extracellular fraction. It was concluded that the protease and haemolysin are involved in the pathogenesis of furunculosis but the lethal component is not the protease or haemolysin.

Of the other extracellular products reported, leucocytolysin (LCL) (Fuller *et al.* 1977), salmolysin (Nomura *et al.* 1988) and a possible enterotoxin (Jiwa 1983), the most

likely candidate seems to be salmolysin which has a high haemolytic activity against salmonid erythrocytes and is lethal to rainbow trout at low concentrations (48.5μg toxin/kg fish) when injected intramuscularly. Intravenous injection of high doses of partially purified LCL (1.13mg) into rainbow trout (2kg) only produced a mild and transient leucopaenia (Fuller *et al.* 1977) and this would suggest that LCL is not a likely candidate.

Recently, Lee and Ellis (1990) have indicated that an extracellular enzyme (GCAT/LPS) displaying a number of activities (see Section 1.2.2) is the major lethal toxin of *A. salmonicida*. The toxicity of this enzyme was specifically neutralised by rabbit anti-GCAT/LPS antiserum (Lee & Ellis 1990). The protease has been implicated in the pathogenesis of furuncle formation (Fyfe *et al.* 1986a) and the work of Lee and Ellis (1991) confirmed this observation. Injection of purified protease with GCAT/LPS resulted in liquefaction and haemorrhaging of muscle fibres, typical of that induced by unfractionated culture filtrate.

1.4 Aims of study

The volume of literature concerning the production of extracellular proteins by *A. salmonicida*, and their relationship with the virulence of this organism, is quite considerable (Ellis *et al.* 1988; Olivier 1990; Ellis 1991). However, the process by which proteins are released into the culture medium of *A. salmonicida* has not been empirically defined; i.e., are proteins detected in the culture filtrate present due to active secretion or as a result of general non-specific release due to cell lysis. Perhaps during infection some *A. salmonicida* cells lyse and release hydrolytic enzymes into the host environment to obtain nutrients for growth of the remaining cells, or alternatively, *A. salmonicida* specifically secretes these enzymes so as there is no reduction in bacterial numbers which would favour the host. The initial part of this project attempted to determine the manner in which these proteins are released into the extracellular medium. The strategy used for studying protein release by *A. salmonicida* was that of cell

fractionation which would determine the discreteness of the various intra- and extra-cellular fractions of *A. salmonicida*. If the proteins present in the extracellular medium were found to constitute a fraction distinct from the intracellular fractions, then it would indicate that the proteins were released via an active secretory mechanism and not as a result of cell lysis.

The major interest of this study was to gain a more detailed knowledge of the extracellular proteins of *A. salmonicida*. Since the extracellular proteins are thought to play a key role in the virulence of *A. salmonicida* then a better understanding of their production should help in the development of a vaccine for the eventual elimination of the disease. However, only a brief overview of the various reports on secreted enzyme activities of *A. salmonicida* is required to illustrate the substantial degree of variation in the biochemical characteristics attributed to the extracellular proteins by different research groups (Price *et al.* 1989; Lee & Ellis 1990). Since the development of a vaccine requires a detailed and accurate picture of the virulence factors involved in the disease process, one aim of this study was to attempt to determine the reason(s) for, and resolve, the widely differing and conflicting results prevalent in the literature concerning the extracellular proteins of *A. salmonicida*. For example, is strain variation a major factor in the observed heterogeneity of protein secretion, or are the methods of detection used to examine the extracellular enzyme activity at fault ?

The idea of strain variation was pursued further in this study, in an attempt to resolve the apparent contradiction of heterogeneous protein secretion by *A. salmonicida*, with taxonomic data which indicate a marked homogeneity in the phenotypic and genotypic properties of this organism (see Section 1.1.1). Since most work on *A. salmonicida* in our laboratory has been concerned with the extracellular 70kDa protease (Price *et al.* 1989; Campbell *et al.* 1990; Price *et al.* 1990), this enzyme, and other proteolytic and hydrolytic activities, were examined thoroughly as a basis for studying the apparent heterogeneity of the extracellular protein production of this organism. The scope of the research was extended and the methods used to analyse the culture filtrates of *A. salmonicida* were also employed to examine other members of the genera for a

comparative study of extracellular enzyme production by *Aeromonas* species.

A number of workers have reported on the comparative characteristics of extracellular protease and haemolysin production by motile aeromonads (Kozaki *et al.* 1989; Monfort & Baleux 1991; Nieto & Ellis 1991), but no comparison with the non-motile *Aeromonas* species is evident in the literature. However, it has been demonstrated that cloned extracellular enzymes of the motile *A. hydrophila* have been successfully secreted into the culture medium by *A. salmonicida* (Buckley 1989; Wong *et al.* 1989), indicating that these two relatively different bacteria must possess a similar secretory apparatus. Therefore, it may follow that if these bacteria are capable of secreting the others proteins, then perhaps there will be similarities in the type of extracellular proteins secreted by these organisms.

The results obtained from the comparative study of extracellular protein secretion would allow us to comment on the question of relatedness between strains of *A. salmonicida* ssp. *salmonicida* and also between *A. salmonicida* and other *Aeromonas* species.

2. Materials and methods

2.1 Materials

Aeromonas strains used in this study are given in Table 2.1. The strains were routinely subcultured on petri dishes containing 4% (w/v) tryptic soy agar (Gibco, Paisley, Scotland). Pure stock cultures were stored in tryptic soy broth (TSB) in 10% glycerol at -20°C.

Casein (light white soluble) was obtained from BDH, Poole, England. The following protease inhibitors were all obtained from Sigma: PMSF, iodoacetic acid, EDTA, chicken ovomucoid, chicken ovoinhibitor and benzamidine. Molecular weight standards for SDS-polyacrylamide gel electrophoresis were also obtained from Sigma. For immunochemical experiments, nitrocellulose was purchased from Anderman & Co. Ltd., Surrey; diaminobenzamidine tetrahydrochloride obtained from Sigma; and goat anti-rabbit horseradish peroxidase secondary antibody obtained from North East Biomedicals, Uxbridge.

2.2 Growth conditions of strains used in this study

All strains were grown in 50ml batches in medium containing 3% (w/v) TSB (Price *et al.* 1989) for 20h at 22-24°C in an orbital incubator (100rpm), except for *A. hydrophila* and *A. sobria* which were grown under the same conditions except at 30°C. Changes to growth conditions are detailed in the appropriate sections.

Table 2.1. *Aeromonas* strains and other bacterial isolates used in this study

Organism	Isolate	Host source	Country of origin
A. salmonicida ssp. *salmonicida*	MT004	*Salmo salar*	Scotland
	MT004rp*	-	-
	1102†	*Salmo salar*	Scotland
	184/86	"	Norway
	MT048	"	Scotland
	MT028	*Salmo trutta*	United States
ssp. *achromogenes*	MT535	*Cyprinus carpio*	Scotland
	MT365	*Salmo salar*	"
ssp. *masoucida*	2020†	*Oncorhynchus masou*	Japan
A. media	2237†	Fish farm effluent	England
A. hydrophila	9240†	Contaminated milk	-
A. sobria	12065†	Fish (unspecified)	-
V. anguillarium	8575*	-	-
	8587*	-	-
X. maltophilia	2225Δ	-	-

* MT004rp, derivative of MT004 with reduced protease (and other extracellular enzyme activities); obtained by continuous laboratory sub-culturing on 4% TSA

† Strains purchased from the National Collection of Industrial and Marine Bacteria Limited (NCIMB), Aberdeen

* Obtained from Wellcome (host and geographic origin unknown)

Δ *X. maltophilia*, isolated from an *A. salmonicida* culture grown on 4% TSA containing 4μg ampicillin per ml

Strains MT004, MT048, MT028, MT535 & MT365 were obtained from A.E. Ellis, DAFS Marine Laboratory, Aberdeen

2.3 Cell fractionation

2.3.1 Periplasmic extraction techniques

For periplasmic extractions, cells were harvested after 20h of growth. The periplasmic fraction was prepared by several methods; sphaeroplast formation (Neu & Heppel 1964), cold osmotic shock (Neu & Heppel 1965; Willis *et al.* 1974; Hazelbauer & Harayama 1979; Hirst & Holmgren 1987) and treatment with chloroform (Ames *et al.* 1984).

Periplasmic proteins were released on sphaeroplast formation by the EDTA-lysozyme method of Neu & Heppel (1964). The standard procedure was as follows: 1g (wet weight) of cells was suspended in 10ml of 20% (w/v) sucrose-Tris/HCl, pH8.0 at 24°C. The suspension was supplemented with EDTA to give a concentration of 1mM, followed immediately by sufficient 1% lysozyme to give 10μg per ml. The mixture was gently agitated on a shaker for 15 min and then centrifuged at 12000 *g* x 10 min. The resulting pellet was suspended in a volume of water equal to that of the original sucrose-Tris suspension.

Several cold osmotic shock procedures were examined to determine the most suitable for isolation of the periplasmic fraction of *A. salmonicida* . All of these 'shock' procedures were based on the original method of Neu & Heppel (1965). Periplasmic extraction by the cold osmotic shock method of Willis *et al.* (1974) was carried out as follows: 1g of cells was washed twice in 0.33M Tris/HCl buffer, pH7.3 and then suspended in 20ml of a solution containing 20% (w/v) sucrose and 1mM EDTA in 0.033M Tris/HCl buffer, pH8.0. Cells were shaken for 10 min at room temperature and collected by centrifugation at 12000 *g* x 20min, and the pellet was rapidly suspended in 2ml of ice-cold 0.5mM $MgCl_2$. The cells were stirred on ice for 10 min and then removed by centrifugation. The supernatant fraction ('shock fluid') was used as the periplasmic protein fraction.

The osmotic shock procedure of Hazelbauer & Harayama (1979) is a modified version of the original (Neu & Heppel 1965) in which smaller volumes of 'shock' solutions are used. In this 'mini-shock' procedure, cells were suspended in 0.2ml of 30mM Tris/HCl (pH7.3), 20% (w/v) sucrose, 0.1mM EDTA, centrifuged after standing for 10 min at room temperature and resuspended in 0.6ml of ice-cold 0.5mM $MgCl_2$. The supernatant after centrifugation of this suspension is the shock fluid. All centrifugations were for 2 min at maximum speed in a microcentrifuge.

The osmotic shock procedure of Hirst & Holmgren (1987) employed polymyxin B as the membrane perturbant. Cells were washed in ice-cold phoshate-buffered saline (PBS), pH7.2, suspended in the same volume of ice-cold 0.9% NaCl containing 2000 units of polymyxin B per ml and then incubated at 4°C for 15 min. The supernatant was separated from the cells by centrifugation at 12000 *g* x 20 min, and the cell pellet was resuspended in PBS.

For chloroform ($CHCl_3$) treatment the method of Ames *et al.* (1984) was followed. Cells were collected at 1100 *g* x 10 min after washing in 0.33M Tris/HCl buffer, pH8.0. The wash buffer was decanted thoroughly. The cell pellet was resuspended by brief vortexing in the residual medium and 10µl of $CHCl_3$ was then added. The tubes were vortexed briefly and maintained at room temperature for 15 min, before adding 2.5ml of 0.01M Tris/HCl buffer, pH8.0. The cells were separated by centrifugation at 12000 *g* x 20 min, and the supernatant fraction containing the periplasmic proteins was retained.

In all cases, the perturbed ('shocked') cells were resuspended in a small volume of distilled H_2O (2-5ml) and further disrupted by sonication (4 x 30s). The suspension was centrifuged at 12000 *g* x 20 min and the supernatant was collected. This fraction was considered to contain the cytoplasmic proteins of the cell.

2.3.2 Separation of cytoplasmic and outer membranes

The insoluble proteins of the inner and outer membranes were separated by the method of Filip *et al.* (1973). The standard procedure used was as follows: cells, from 20h cultures, were centrifuged at 12000 *g* x 20 min and then washed twice with 0.33M Tris/HCl buffer, pH 7.3. The cells were then suspended in 8ml of envelope buffer (0.01M Tris/HCl buffer, pH 7.3) and sonicated (6 x 30s). The perturbed cells were centrifuged at 10000 *g* x 20 min to remove unbroken cells and the supernatant centrifuged again at 75000 *g* x 90 min to obtain a pellet comprising both inner and outer membrane fractions.

This membrane pellet was resuspended in 1ml of 0.75% sodium lauryl sarcosinate (Sarkosyl) in envelope buffer and incubated at room temperature for 30 min. The membrane/detergent suspension was then centrifuged at 75000 *g* x 90 min and the resulting supernatant contained the detergent-soluble proteins of the inner membrane. The pellet containing the Sarkosyl-insoluble proteins was solubilised in 2% SDS and this fraction was used as the outer membrane preparation.

2.4 Cell marker assays

2.4.1 Glutamate dehydrogenase

Glutamate dehydrogenase (GDH) was assayed as a cytoplasmic marker enzyme. Activity was assayed using the method of Kinghorn & Pateman (1973) in which GDH activity was determined by following the oxidation of NADH at 30°C at 340nm. The reaction mixture contained 0.1mM mercaptoethanol and 0.5mM EDTA in 0.7ml of 0.05M sodium phosphate buffer, pH8.0, 0.05ml of 0.2M 2-oxoglutarate, pH7.0, 0.1ml of 0.4M ammonium chloride and 0.05ml of 2mg/ml NADH. 0.1ml of enzyme sample was added to the reaction mixture and the change in absorbance followed over time.

2.4.2 ß-lactamase

ß-lactamase activity was assayed as a periplasmic marker enzyme. Activity was assayed by the method of Nicas & Hancock (1983). 0.1ml of enzyme sample was added to 0.9ml of a solution of the chromogenic ß-lactam nitrocefin in 0.1M sodium phosphate buffer, pH7.0. The rate of conversion of nitrocefin to nitrocefoic acid at 30°C was followed over time by the change in the absorbance at 540nm.

2.4.3 Ribonuclease

Ribonuclease (RNAse) was assayed as a periplasmic marker enzyme. Activity was assayed by the method of Campbell *et al.* (1990). The assay mix contained 55µl 0.2M Tris/HCl, pH7.5, 10µl 0.02M EDTA, and 10µl of sample in an eppendorf tube. The reaction was started by the addition of 25µl of RNA (12mg/ml) and continued for 15 min at 30°C. The reaction was stopped by the addition of 15.4% perchloric acid/0.75% uranyl acetate. The reaction mixture was centrifuged at maximum speed in a microfuge for 2min and the A_{260} of the supernatant was measured.

2.4.4 Succinate dehydrogenase

Succinate dehydrogenase activity was assayed as a cytoplasmic membrane marker enzyme. Activity was assayed by the method of Pennington (1961). The reaction mixture contained 0.2ml of phosphate buffer, 0.04ml of 0.5% *p*-iodo-nitrotetrazolium violet (INT), 0.04ml of 0.25M sucrose, 0.04ml of 0.5M sodium succinate and a volume of water and enzyme sample to a total volume of 1ml. The assay mix was incubated for 20 min at 30°C with shaking and the reaction was stopped with the addition of 0.08ml of a 50% TCA solution. The chromogen was extracted with 1.6ml of ethyl acetate in glass stoppered tubes and the A_{490} measured. For blanks, sodium succinate was added after the TCA solution.

2.4.5 NADH oxidase

NADH oxidase was assayed as an inner membrane marker enzyme. Activity was measured by the method of Osborn *et al.* (1972). Incubation mixtures contained 0.05M Tris/HCl, pH7.5, 0.12mM NADH, 0.2mM dithiothreitol and the enzyme solution in a volume of 1.0ml. The rate of decrease in absorbance at 340nm (A_{340}) was measured at 30°C.

2.4.6 2-Keto-3-deoxyoctulosonic acid

Membrane fractions (1.0mg of protein) were precipitated with cold 10% TCA (5ml) and collected by centrifugation at 20000 *g* x 20 min. The precipitate was suspended in 0.5ml of 0.01M H_2SO_4 and hydrolysed at 100°C for 20 min to liberate 2-keto-3-deoxyoctulosonic acid (KDO) from lipopolysaccharide. KDO was determined directly on the hydrolysate by the thiobarbituric acid method of Osborn (1963), a modified procedure of Weissbach & Hurwitz (1959).

The sample in 0.2ml or less of solution was added to 0.25ml of 0.04M HIO_4 in 0.0625M H_2SO_4. After 20 min at room temperature, 0.5ml of 0.2mM $NaAsO_2$ in 0.5M HCl was added with shaking and the solution was allowed to stand for 2 min. 2ml of 0.3% thiobarbituric acid (pH2) was added and after stirring the mixture was heated at 100°C for 10 min. The mixture was cooled and the final chromogen was extracted into 1.5ml of cyclohexanone prior to measurement at 548nm. Under assay conditions, 1.0μmole of KDO gave an absorbancy of 19.0 at 548nm.

2.5 Assays for extracellular enzyme activity

2.5.1 Hide powder azure assay for proteolytic activity

Hide powder azure (HPA) (10mg/ml) was suspended in distilled H_2O and sonicated (8 x 15s) to produce a fine suspension. Assay mixtures routinely contained HPA (0.5ml), 0.1M sodium phosphate buffer, pH 7.0 (0.4ml) and sample (0.1ml) (North 1978). Incubations were carried out at 30°C for 30min and reactions were stopped by addition of ice-cold 50% (w/v) trichloroacetic acid (TCA) (0.2ml). Supernatants containing TCA soluble products were obtained by centrifugation at 1100 *g* x 10 min in a bench centrifuge. The absorbance at 595nm of the supernatants were then measured, and under these conditions an increase in absorbance of 1.0 was equivalent to the hydrolysis of 3.4mg HPA (North 1978). 1 unit corresponds to the hydrolysis of 1mg HPA over 30 minutes.

2.5.2 Caseinase assay for proteolytic activity

Protease activity against casein was assayed as described by Price *et al.* (1989): 0.25ml sample was incubated with 0.75ml H_2O and 1.0ml casein solution (1% casein in 0.2M glycine/NaOH buffer, pH 9.0) at 37°C for 30 min. The reaction was stopped by the addition of 3.0ml 0.11M TCA. The tubes were stood at 4°C for 10-15 min and the precipitate formed removed by centrifugation at 1100 *g* x 10 min in a bench centrifuge. The absorbance of the supernatant was determined at 280nm; 1 unit of activity is defined as that amount of enzyme required to digest 1mg of casein over a 30 min period. Under the conditions described, the digestion of 1mg casein gives rise to an A_{280} of 0.14 after the addition of the TCA solution and centrifugation.

2.5.3 Amylase activity assay

Amylase activity was assayed as described previously (Stevens & Relton 1981). Sample and distilled H_2O with a combined volume of 1.0ml was added to 1.0ml starch solution (0.75% starch, 0.044M KH_2PO_4 buffer, pH 7.5, 2mM$CaCl_2$) and incubated at room temperature for 2h. 5ml of distilled H_2O was then added and followed by the addition of I_2/KI reagent. For blanks the sample was added after the I_2/KI reagent. The reaction is measured at 620nm and the activity is obtained by subtracting the sample reading from the blank and is given as A_{620}/min/ml. 1 unit of amylase activity is the amount of enzyme that will hydrolyse 10mg starch/min/ml.

2.5.4 Haemolytic titration

Rainbow trout (*Salmo gairdneri*) blood was collected and red blood cells (RBCs) were washed three times in PBS, pH7.3, and resuspended at 1% (v/v) in PBS. Haemolytic activity was determined by diluting 100µl sample in 2-fold steps in PBS, adding 100µl RBC suspension, and incubating at 37°C for 1h. The dilution of sample which caused 50% haemolysis was defined as one haemolytic unit (HU).

2.6 Analytical and preparative SDS-polyacrylamide gel electrophoresis

2.6.1 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970). Solutions used in the preparation of the gels and protein samples are shown in Table 2.2. Samples were prepared by mixing with an equal volume of sample (solubilisation) buffer and boiling for 3 min. Electrophoresis was carried out at 10mA through the stacking gel and 30mA through the separating gel until the bromophenol blue tracking dye emerged from the bottom of the gel. Polyacrylamide gels were stained with 0.1% Coomassie blue R250 for 1h and destained in 10% acetic acid with several changes of destain solution.

Table 2.2. Solutions for SDS-polyacrylamide and substrate-SDS-polyacrylamide gels

Separating gel buffer:
1.5M Tris
0.4% SDS
pH to 8.7 with HCl

Stacking gel buffer:
0.5M Tris
0.4% SDS
pH to 6.8 with HCl

Sample buffer 2x conc.:
30ml 10% SDS
0.96M Glycine
0.5% SDS
pH is 8.3 when diluted

Reservoir buffer 5x conc.:
0.125M Tris
12.5ml Stacking gel buffer
10ml Glycerol
pH to 6.8 with HCl

Acrylamide stock solution:
30% Acrylamide
0.8% Bis-acrylamide

Staining solution:
0.1% Coomassie blue R250
in acetic acid, methanol, H_2O
(2:5:5)

Separating gel 7.5%:

Separating gel buffer	5.0ml
Distilled H_2O	10.0ml
Acrylamide stock solution	5.0ml
TEMED	15µl
10% Ammonium persulphate	70µl

Stacking gel buffer 4%:

Stacking gel buffer	2.5ml
Distilled H_2O	6.1ml
Acrylamide stock solution	1.34ml
TEMED	10µl
10% Ammonium persulphate	50µl

For other separating gel concentrations, the volumes of distilled H_2O and acrylamide stock solution were adjusted as required. Substrate-SDS-polyacrylamide gels were prepared in an identical manner, with the volume of distilled H_2O in the separating gel adjusted for the addition of the substrate stock solution.

Sample preparation:
Sample in H_2O
Dilute 1:1 with Sample buffer 2x containing 2µl 2-mercaptoethanol per ml. Heat in boiling water bath for 3 min
Add 1µl 1:1 mixture of 1% bromophenol blue/2-mercaptoethanol per 10µl boiled sample

2.6.2 Gelatin-SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gels were prepared as normal (Section 2.6.1) except gelatin was incorporated into the separating gel at a final concentration of 0.2%. This method was adapted from that of Heussen & Dowdle (1980). Samples were solubilised with an equal volume of sample buffer at room temperature and allowed to stand for 10 min and electrophoresis was conducted as above (Section 2.6.1). After electrophoresis, gels were incubated in 2.5% Triton X-100 in water for 30 min to displace SDS and then in 0.1M glycine/NaOH buffer, pH 8.0 for 5h at 30°C. Gels were stained with 0.1% Amido black. After the gel was destained with 10% acetic acid, proteolytic activity was visualised as a clear band against a dark background.

2.6.3 Starch-SDS-polyacrylamide gel electrophoresis

This method was adapted from that of Lacks & Springhorn (1980) with starch incorporated at 0.17% into the separating gel. Sample preparation and electrophoresis were carried out in an identical manner as in Section 2.6.2. For development of amylase activity, SDS was displaced (Section 2.6.2) and the gel was then incubated in 0.1M sodium phosphate buffer, pH 7.0 at 30°C for 16h. The gels were then stained with I_2/KI reagent for 1 min, and then destained with methanol: acetic acid: water (1:1:8).

2.6.4 Preparative SDS-polyacrylamide gel electrophoresis

Preparative SDS-polyacrylamide gel electrophoresis was used to obtain the final immunogen preparation for production of rabbit polyclonal antibodies against selected extracellular proteins. A stacking gel (4%) minus well former was poured onto an SDS-polyacrylamide gel (Section 2.6.1). Samples were prepared at a final volume between 0.5-1.0ml (containing ≈ 100µg of desired protein) and strip-loaded onto the stacking gel and run under the same conditions described in Section 2.6.1. The gel was lightly stained with 0.1% Coomassie blue R250 and the bands of interest were carefully excised. The excised bands were washed several times in phosphate-buffered saline

(PBS) and then cut into small pieces and suspended in 0.8ml PBS. The gel suspension was sonicated in an ice-bath until a completely homogeneous preparation was obtained; this preparation was the final highly pure immunogen used in subsequent antibody production regimes.

2.7 Characterisation of extracellular enzyme activities on substrate-SDS-polyacrylamide gels

2.7.1 Effect of inhibitors on extracellular protease and amylase activities

The effect of phenylmethanesulphonyl fluoride (PMSF) and 1,10-phenanthroline on the extracellular protease and amylase activities was determined by pre-incubating culture filtrate with these inhibitor compounds (1mM and 10mM respectively) for 30 min prior to sample preparation and electrophoresis. In the case of the reversible inhibitor 1,10-phenanthroline, it was also incorporated at the same concentration into the subsequent development buffers since omission resulted in the reactivation of some of the affected activities.

2.7.2 Heat stability of extracellular protease activities

Samples of culture filtrate were pre-incubated at 56°C for 30 min prior to solubilisation and electrophoresis. Gelatin-SDS-gels were developed and stained as described in Section 2.6.2.

2.7.3 Effect of pH on extracellular amylase activities

Sample preparation and electrophoresis was as described in Section 2.6.2. However, before development the gels were cut into strips and, thereafter, the gel strips were incubated at different pHs' all the way through the development procedure. The gel strips were then stained as described in Section 2.6.3.

2.8 Protein estimation

Protein concentrations were estimated by measurement of the change in absorption spectra when proteins bind Coomassie brilliant blue G250 according to the method of Sedmak & Grossberg (1977). BSA was used to produce a standard curve. This method has several advantages over other protein assays: it is simple and reproducible; it has good sensitivity, detecting as little as 1µg of protein (c.f. 5-10 µg by the Lowry method); absorbances of the solutions after addition of protein are stable for 60-90 minutes at room temperature; and the dye has a long shelf life. However, the dose response curve is not linear when plotted arithmetically, but it is rectlinear from 0.5-50 µg protein when the absorbance ratio at 620 and 465nm is plotted.

2.9 Purification of extracellular caseinase enzyme

2.9.1 Ammonium sulphate precipitation

Culture filtrate was 65% (w/v) saturated wuth ammonium sulphate [$(NH_4)_2SO_4$] and the solution was stirred at 4°C for 3h. The precipitated material was centrifuged at 20000 *g* x 30 min and the resulting pellet was redissolved in a small volume of distilled H_2O. The redissolved pellet was dialysed against distilled H_2O overnight at 4°C and centrifuged at 20000 *g* x 30 min and the supernatant retained.

2.9.2 Ion-exchange chromatography

A DEAE-Sephadex A-50 column (4.9cm^2 x 12cm) was equilibrated with 10mM sodium phosphate buffer, pH7.0. After washing to remove unbound material, 25ml portions of sodium phosphate solutions (pH 7.0) of increasing molarity (50, 100, 150, 200 and 250mM) were applied to the column, and 4ml fractions were collected for subsequent analysis.

2.9.3 Gel filtration chromatography

Chromatography was carried out on a column (6.2cm^2 x 35cm) of Sephacryl S-300. The column was eluted with 50mM sodium phosphate buffer, pH 7.5 and fractions of 6ml collected for analysis.

2.10 Characterisation of extracellular enzyme activity

2.10.1 Extracellular caseinase activity

(i) Optimum temperature for caseinase activity and heat stability

The optimum temperature for proteolysis was determined over the range 20 to 60°C. The heat stability of the caseinase enzyme was tested by pre-incubating the enzyme extract at the test temperature for 30 min. For both determinations the caseinase activity was assayed as described in Section 2.5.2.

(ii) Optimum pH for caseinase activity

The optimum pH for extracellular caseinase activity was examined over the range pH 5.8 to 10.5, using 0.1M sodium phosphate buffer (pH 5.8-7.0), 0.1M Tris/HCl buffer (pH 6.8-8.0) and 0.1M glycine/NaOH buffer (pH 7.8-10.5).

(iii) Effects of inhibitors on caseinase activity

The effect of protease inhibitors on protease actvitity was tested by pre-incubating inhibitor, at concentrations shown in section 4.2.1.3, with the enzyme extract for 30 min at room temperature. The inhibitor-protease mixture was then assayed for protease activity as described in Section 2.5.2.

2.10.2 Extracellular amylase activity

The effects of assay temperature, pH and heat stability on extracellular amylase activity were determined in the same manner as for caseinase activity (Section 2.10.1). The amylase activity was examined over the pH range 4.0 to 9.5, using 0.1M acetate buffer (pH 4.0-5.5), 0.1M sodium phosphate buffer (pH 5.0-8.0) and 0.1M Tris/HCl buffer (pH 8.0-9.5). The amylase activity was assayed as described in Section 2.5.3.

2.11 Growth of *A. salmonicida* under different physiological conditions

2.11.1 Effect of growth temperature

A. salmonicida was grown under the same conditions as described in Section 2.2, but over a range of temperatures from 24 to 36°C. Intracellular and extracellular fractions were prepared as previously described in Section 2.3.

2.11.2 Effect of iron-limitation

A. salmonicida was grown under iron (Fe^{3+})-restricted and -supplemented conditions for 20h at 22°C. Fe^{3+}-supplementation was achieved by adding $FeCl_3$ to the growth medium at a final concentration of 10mM. The low-Fe^{3+} environment was obtained by the addition of the specific iron chelator, ethylenediaminedi(*o*-hydroxyphenylacetic acid) (EDDA) into the growth medium at a final concentration of 100μM.

2.11.3 Effect of various ions and compounds on extracellular enzyme production

The effect of ammonium ions on extracellular enzyme production was determined by supplementing the standard TSB growth medium with ammonium ions by adding ammonium tartrate to a final concentration of 50mM. Glucose and starch were also added to the growth medium at concentrations stated in Section 5.5. The effects of these compounds on extracellular enzymes were determined by assaying selected activities as described in Section 2.5.

2.12 Antibody production and characterisation

2.12.1 Raising antibodies to selected extracellular proteins

A homogeneous immunogen preparation was obtained as described in Section 2.6.4. To the 0.8ml of immunogen, 1.4ml of Freund's complete adjuvant (FCA) was added and the mixture was then shaken vigorously for 3h, at which time one droplet of the preparation would not disperse when added onto the surface of water. The immunogen was then injected subcutaneously in four different sites (≈ 0.5ml per site) into female New Zealand white rabbits. At 4 and 6 weeks booster injections were administered; the same procedure was followed, except that Freund's incomplete adjuvant (FICA) was used in place of FCA. The rabbit was fully bled 8 weeks from the time of the first injection. Antiserum was prepared by standing the blood at room temperature for 3h then overnight at 4°C, and the red blood cells were pelleted by centrifugation at 12000 *g* x 10 min. The supernatant (antiserum) was immediately divided into 50µl aliquots and stored at -70°C until required; once thawed antiserum was not refrozen. Non-immune serum was obtained from the same rabbit by a small bleeding prior to injection of immunogen; antiserum was prepared in the same manner as the immune serum.

2.12.2 Western blotting

A. salmonicida extracellular proteins were separated by SDS-polyacrylamide gel electrophoresis using the discontinuous buffer system as described by Laemmli (1970) (see Section 2.6.1). After electrophoresis, the proteins were transferred to nitrocellulose filter by the methanol/Tris/glycine method of Towbin *et al.* (1979). The gel was immersed in transfer buffer [1.92M glycine, 0.25M Tris/methanol/water 1/2/7 (v/v/v)] and blotted onto nitrocellulose paper at 0.3A for 3h. After blotting, the nitrocellulose was incubated in Buffer I [PBS/ 0.2% gelatin (w/v)] for 2h. The nitrocellulose was incubated in fresh Buffer II [PBS/ 0.2% gelatin/ 0.1% Triton X-100 (w/v)] with shaking for 1h with primary antibody, rabbit immune serum (diluted as required in Buffer II). The nitrocellulose was washed 3 x 5 min in Buffer II and then

secondary antibody, goat anti-rabbit antibody (diluted 1:1000 in Buffer II) was added. After incubation with the secondary antibody for 1h in the dark, the nitrocellulose was washed for 4 x 5 min in PBS and stained with 0.05% diaminobenzamidine tetrahydrochloride (DAB) in 50mM Tris/HCl buffer, pH 7.6. After approximately 20sec, hydrogen peroxide (H_2O_2) was added (1µl H_2O_2 per ml DAB) and the colour development was stopped at the appropriate point by repeated washes with tap H_2O.

3. Cell fractionation studies on *A.salmonicida*

3.1 Introduction

In common with other members of the Vibrionaceae family, *A. salmonicida* is known to release a large number of enzymes having diverse activities into the external milieu (Ellis *et al.* 1981; Campbell *et al.* 1990). The proteins are considered to be secreted because they are found in the culture medium and because their presence there is compatible with their function and mode of action. However, although proteins are detected in the culture medium it is not always clear how this has arisen, whether it is through active secretion or as a result of cell lysis. Numerous organisms are capable of selective export and localisation of proteins. These processes of protein localisation are selective and efficient in that proteins are strictly compartmentalised to a particular cellular location (Silhavy *et al.* 1983) i.e., protein synthesis takes place on ribosomes in the cytoplasm, and the periplasmic proteins, outer membrane proteins and extracellular proteins are then transferred from ribosomes and pass across the cytoplasmic membrane to a specific location within, or outside, the cell.

A series of experiments was performed to determine whether the proteins present in the extracellular medium of *A. salmonicida* were released via active secretion or as a result of general non-specific release due to cell lysis. In order to verify that the proteins in the culture filtrate of *A. salmonicida* make up a discrete (extra)cellular fraction it was necessary to characterise the intracellular fractions and demonstrate that these fractions are distinct in composition from the extracellular medium of *A. salmonicida*. If a protein is truly secreted then the release of that protein should be efficient, i.e., the bulk of the particular protein is released into the medium and that release should occur without concomitant release of other proteins which are not normally extracellular.

The Gram-negative cell is comprised of the cytoplasm surrounded by a cell envelope (Fig.1.3). The cell envelope is a complex structure which consists of two membranes,

the cytoplasmic membrane and the outer membrane, enclosing the peptidoglycan layer and the periplasmic space. Precise partitioning mechanisms exist which ensure that each compartment in the envelope contains its own unique set of polypeptides. The cytoplasmic membrane is a typical plasma bilayer containing phospholipids and a large number of proteins involved in various cellular functions, including respiration, electron transport and membrane biogenesis (Pugsley & Schwartz 1985). The outer membrane consists of glycolipid (lipopolysaccharide [LPS]) with the polysaccharide side chains protruding from the cell surface. The general structure of LPS is illustrated in Figure 1.3. It consists of three regions: the O-polysaccharide, the core polysaccharide and a lipid complex (called lipid A). The O-polysaccharide side chain structures are extremely variable and characterise the LPSs' of different Gram-negative bacteria, whereas the core component (containing 2-keto-3-deoxyoctulosonic acid [KDO]) does not vary. In addition to LPS, the outer membrane contains approximately 50 proteins mainly involved in nutrient uptake and cell-wall integrity (Pugsley & Schwartz 1985). Some proteins of the outer membrane have specialised functions: one is a lipoprotein that covalently attaches to the peptidoglycan layer of the cell wall, while others, known as porins, function as diffusion channels for the movement of nutrients into the cell.

Between the two membranes lies the periplasmic space, the nature of which is the subject of debate both in terms of size (Ferguson 1990; Van Wielink & Duine 1990; Graham *et al.* 1991) and composition (Pugsley 1986). It was suggested that the periplasm was a water-filled compartment containing about 20% of the total cellular water (Stock *et al.* 1977). However, more recent evidence from diffusion of fluorescently-labelled proteins (Brass *et al.* 1986) and freeze-substitution experiments (Hobot *et al.* 1984) suggests the presence of a 'periplasmic gel' (i.e., murein in the form of a hydrated gel). However, the periplasmic gel is not present in all cases; some Gram-negative bacteria such as *Vibrio cholerae*, do not contain stainable material (Graham *et al.* 1991). The cell envelope of *A. salmonicida* also contains the additional proteinaceous surface layer called the A-layer consisting of a single protein with a subunit molecular weight of 49kDa (see Section 1.2.3). The O-specific polysaccharide

chains of the LPS are thought to protrude through the A-layer of *A. salmonicida*.

The subcellular location of a protein is usually operationally defined according to cell fractionation criteria (Pugsley & Schwartz 1985). To determine the protein composition of fractions, and how discrete each fraction is, for example, proteins may be identified by their characteristic migration in SDS-polyacrylamide gels and by biochemical examination for known characteristics of different cell components such as enzymes or cell envelope constituents.

Reliable methods of subcellular fractionation are required for the analysis of bacterial protein localisation (Silhavy *et al.* 1983). The development of techniques for separating cell components into relatively pure subfractions has greatly assisted studies on protein export and localisation. In general, the difference in solubility between cytosolic and membrane proteins is the principal criterion used when fractionating bacterial intracellular proteins, with membrane proteins remaining insoluble after disruption of the bacteria. Membrane and soluble fractions are separated by high-speed centrifugation of a bacterial lysate. The particulate fraction is composed of the bacterial cell envelope containing the cytoplasmic membrane, outer membrane, and cell wall components and the supernatant contains cytoplasmic and periplasmic components.

The differential release of intracellular marker enzymes and their mobility on SDS-polyacrylamide gel electrophoresis are the criteria by which the effectiveness of the separation techniques can be determined. The principle behind this system is that proteins with known enzyme activities and various other cellular components are localised in specific compartments of the Gram-negative cell (Table 3.1). Since these enzymes, along with most other cellular components, can be readily assayed they can be used to assess the purity of fractions obtained by the various physical fractionation methods employed. For example, when attempting to determine the purity of inner and outer membrane preparations, the distribution of succinate dehydrogenase (SDH), an integral cytoplasmic membrane protein (Hederstedt & Rutberg 1981), between the two fractions would give an indication of the purity of the preparations.

Table 3.1. Examples of enzymes and other cell constituents commonly employed as cellular markers to assess the effectiveness of cell fractionation techniques

Cell fraction	Marker	Reference
Cytoplasm	Catechol oxygenase	Hirst & Holmgren (1987)
	Chloramphenicol acetyltransferase	Ichige *et al.* (1988)
	ß-Galactosidase	Andro *et al.* (1984)
	Glucose-6-phosphate dehydrogenase	Jensen *et al.* (1980)
	Lactate dehydrogenase	Hancock & Nikaido (1978)
	Glutamate dehydrogenase	Howard & Buckley (1985)
Periplasm	Alkaline phosphatase	Jensen *et al.* (1980)
	ß-lactamase	Andro *et al.* (1984)
		Hirst & Holmgren (1987)
		Ichige *et al.* (1988)
	Ribonuclease (RNAse)	Howard & Buckley (1983)
Inner membrane	NADH dehydrogenase	Miyazaki *et al.* (1989)
	NADH oxidase	Thom & Randall (1988)
	Succinate dehydrogenase	Hancock & Nikaido (1978)
Outer membrane	2-keto-3-deoxyoctulosonic acid (KDO)	Hancock & Nikaido (1978)
		Thom & Randall (1988)
	L-glycero-D-mannoheptose	Hancock & Nikaido (1978)

It should be noted that cell fractionation data must be interpreted with caution, because although the techniques are generally adequate for defining the normal and permanent components of that fraction, errors can occur when defining the location of abnormal or transient components of the export pathway, by degradation and aggregation of proteins, and also when working on genetically altered systems (Tommassen 1986).

3.2 Separation of the soluble cytoplasmic and periplasmic compartments

Once cells are disrupted, cytoplasmic components cannot be distinguished from periplasmic components because both are soluble. Therefore, the separation of these two soluble compartments is dependent on a procedure which relies on a controlled disruption of the bacterial cell. The outer membrane and cell wall of the bacterium must be perturbed or removed without compromising the integrity of the inner membrane. A number of experiments were carried out in order to assess the effectiveness of a variety of treatments which are known to disrupt partially the outer membranes of Gram-negative bacteria, including preparing sphaeroplasts and using controlled osmotic shock. These methods release periplasmic components, whereas cytoplasmic components remain associated with the sphaeroplast.

The fractions obtained by these methods were analysed in order to assess the integrity of cytoplasmic and periplasmic marker enzymes. There are numerous enzymes suitable for use as cytoplasmic markers one of which, glutamate dehydrogenase (GDH), was used in this study. However, the selection of periplasmic marker enzymes was not as straightforward, because of the type of assay required for their detection, i.e., assaying for binding-proteins with radioactively labeled substrate and detecting by fluorography. The most commonly used periplasmic marker enzyme is ß-lactamase (Table 3.1), the enzyme responsible for the hydrolysis of ß-lactam antibiotics (Richmond & Sykes 1973; Bush 1989a). This enzyme can readily be detected using chromogenic substrates such as nitrocefin and PADAC [7-(thienyl-2-acetomido)-3-[2-*N*,*N*-dimethyl-aminophenylazo)-pyridinium methyl]-3-cephem-4-carboxylic acid]. However, in

Table 3.2. The effect of various treatments on the differential release of glutamate dehydrogenase and ribonuclease from *A. salmonicida* strain MT004

Treatment	Conditions	Enzyme	Shock fluid		Shocked cells		Ref.
			U/ml	%	U/ml	%	
Sphaeroplast formation	20% sucrose 1mM EDTA 10μg/ml lysozyme	GDH†	0.42	18	1.93	82	a
Osmotic shock	0.9% NaCl 1mM EDTA	GDH	0.34	15	1.92	85	b
Osmotic shock	20% sucrose 1mM EDTA	GDH	0.02	<1	2.32	>99	c
		RNAse^Δ	0.63	58	0.46	42	
Osmotic shock	20% sucrose 2mMEDTA	GDH	0.07	3	2.22	97	c
		RNAse	0.92	74	0.32	26	
Osmotic shock	0.9% NaCl Polymixin B	GDH	0.16	7	2.08	93	d
Chloroform extraction	$CHCl_3$	GDH	0.73	34	1.42	66	e

† GDH, glutamate dehydrogenase. Activity expressed as μmoles NADH oxidised/min/ml

^ΔRNAse, ribonuclease. Activity expressed as μmoles nucleotide released/min/ml

a-f refer to the treatments and conditions used by : a, Neu and Heppel (1964)
b, Hazelbauer and Harayama (1979)
c, Willis *et al.* (1974)
d, Hirst and Holmgren (1987)
e, Ames *et al.* (1984)

preliminary experiments with *A. salmonicida* strain MT004 using nitrocefin as the substrate, no ß-lactamase activity could be detected in the soluble cellular extracts and attempts to induce ß-lactamase activity in liquid culture were unsuccessful. Further details of work on ß-lactamase activity in *A. salmonicida* is shown in the Appendix. Ribonuclease (RNAse) is another enzyme suitable for differentiating between cytoplasm and periplasm, and it was found to be present at detectable levels in the *A. salmonicida* periplasm and so was used as the periplasmic marker enzyme in this study. The one disadvantage of RNAse over ß-lactamase is that it is commonly secreted by several Gram-negative organisms into the culture filtrate. It is, therefore, unsuitable for differentiating the periplasmic proteins from extracellular proteins and therefore cannot be used as a sensor of cell integrity.

Initially, the EDTA-lysozyme method for preparation of sphaeroplasts was examined (Neu & Heppel 1964). However, the preliminary experiments revealed that this method resulted in a significant amount of the cytoplasmic marker enzyme, GDH, being released into the supernatant fraction (Table 3.2). Adjustments in the concentrations of EDTA and lysozyme from 0.5-3.0mM and 1.0-10.0 µg/ml, respectively, did not result in a significant increase in the effectiveness of this procedure when applied to *A. salmonicida* (data not shown). No combination of these treatments reduced the level of GDH release to less than 15%.

The classical method for extracting periplasmic proteins from Gram-negative bacteria is the cold osmotic shock method of Neu and Heppel (1965). In this procedure the bacteria are first suspended in a concentrated solution of sucrose in the presence of EDTA and then suddenly shifted to a medium of low osmotic strength. A series of experiments were carried out using several modifications (Willis *et al.* 1974; Hazelbauer & Harayama 1979; Hirst & Holmgren 1987) of the original cold osmotic shock method. The main differences between these methods are the volume of sample, the osmotic stabiliser employed, or the type and concentration of membrane perturbant used for the selective release of the periplasmic proteins. The membrane perturbants investigated were EDTA, polymyxin B and chloroform. The release of periplasmic

proteins by chloroform was based on the method of Ames *et al.* (1984). The process by which proteins are released by chloroform treatment is not known but it is assumed to be unrelated to osmotic shock.

The majority of these procedures were unsuccessful when applied to the periplasmic extraction of *A. salmonicida*, as judged by the release of GDH (Table 3.2). The method of Hazelbauer and Harayama (1979), which simplifies the procedure by using smaller volumes, was shown to be unsatisfactory since 15% of the total GDH activity was released (Table 3.2). In this method sucrose was replaced as the osmotic stabiliser by sodium chloride and the latter may have affected the viability of the *A. salmonicida* cell suspension. The method of Hirst and Holmgren (1987) using polymyxin B as the outer membrane perturbant also employed sodium chloride as the osmotic stabiliser. The effect of combining sucrose and polymyxin B was not investigated, although this may have reduced the release of GDH from a reasonable 7% still further. The chloroform extraction procedure was found to be much too harsh a treatment for working with *A. salmonicida*, with 34% of the cytoplasmic marker enzyme being released (Table 3.2).

The most reliable periplasmic extraction procedure for *A. salmonicida* was found to be a cold osmotic shock method with 0.33M Tris/HCl, pH7.3 wash buffer and 1mM EDTA as the membrane perturbant (Willis *et al.* 1974). Using this treatment less than 1% of the total cellular GDH activity was released from the cytoplasm (Table 3.2). When the periplasmic marker enzyme, RNAse, was assayed it was found that 58% of the enzyme activity was released into the supernatant. Increasing the EDTA concentration to 2mM increased the amount of RNAse released to greater than 74%, but also caused greater disruption of the cytoplasmic membrane as shown by the release of 3% of the total GDH activity.

The cytoplasmic and periplasmic fractions obtained by the procedure of Willis *et al.* (1974) were analysed by SDS-polyacrylamide gel electrophoresis (Fig.3.1). Major differences were observed in the protein profiles of the two soluble fractions. Several intense bands observed in the cytoplasmic extract (lane 2) in the region of 70-110kDa

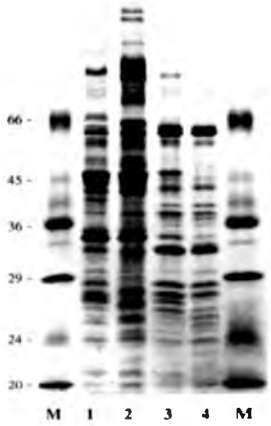


Figure 3.1. Separation of the soluble cytoplasmic and periplasmic proteins of *A. salmonicida* strain MT004 by the cold osmotic shock method of Willis *et al.* (1974). Lane 1, total soluble intracellular (cytoplasmic & periplasmic) fraction; lane 2, cytoplasmic fraction only; lanes 3 & 4, periplasmic fractions only (obtained from two separate preparations). Lanes 1 & 2 and lanes 3 & 4 were loaded with 20µg and 10µg of protein respectively. Lane M, molecular weight standards [bovine serum albumin (66kDa), ovalbumin (45kDa), glyceraldehyde-3-phosphate dehydrogenase (36kDa), carbonic anhydrase (29kDa), trypsinogen (24kDa), trypsin soyabean inhibitor (20kDa), lysozyme (14kDa)]

and proteins of lower molecular weights of 70, 63, 45, 36 and 35kDa were clearly not visible in the periplasmic fraction. Several proteins that were novel to the periplasm were also noted, including those with apparent molecular weights of 64, 60, 40 and 32kDa (lane 3). The reproducibility of this procedure is illustrated by comparing lanes 3 and 4. These periplasmic fractions, extracted in different experiments, are essentially the same apart from one noteable exception, that being the presence of a 45kDa band in lane 3 and the absence of this protein in lane 4.

The successful differential release of RNAse compared with GDH, in tandem with the findings of SDS-polyacrylamide gel electrophoresis, indicated that this was a reliable method for fractionating the two soluble intracellular compartments of *A. salmonicida*.

3.3 Proteins of the unfractionated cell envelope

Figure 3.2 (lane 1) shows an electrophoretic separation of total membrane proteins, prepared by sonic treatment and high-speed centrifugation of the resulting membrane suspension. Several prominent bands were observed divided between two regions of the gel (M_r 42-47kDa and 27-32kDa). These proteins will be shown to be components of the outer membrane.

3.3.1 Solubility of envelope proteins

The effect of solubilisation temperature of the membranes on the protein profile was determined (Fig. 3.2). The electrophoretic pattern obtained with membrane preparations heated at 100°C in SDS sample buffer for 3 min is shown in lane 1. Figure 3.2 also illustrates the effect of the time of heating: a minimum of 1 min was necessary to obtain the pattern identical to that of lane 1, and no further change in banding was observed by prolonging the heating up to 10 min (lanes 2-6). Therefore, samples were routinely treated for 3 min at 100°C in all other experiments. If the membranes were incubated at 37°C for 30min, one of the major bands (42kDa) was completely absent and two

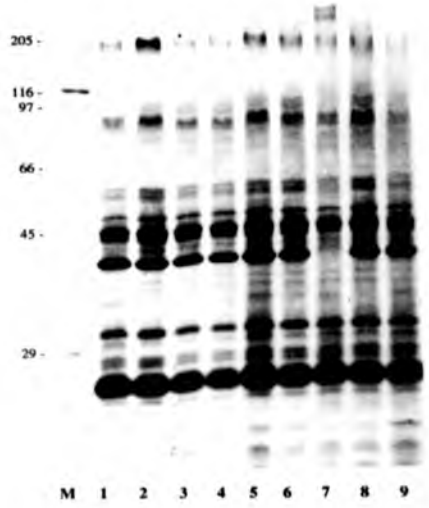


Figure 3.2. The effect of solubilisation temperature on the total membrane protein profile of *A. salmonicida* strain MT004. Total membrane (cytoplasmic & outer membranes) samples were incubated in SDS sample buffer at: 100°C for 3min (lanes 1 & 5), 15sec (lane 2), 30sec (lane 3), 1min (lane 4), 10min (lane 6), 37°C for 30min (lane 7), 37°C for 30min, then 70°C for 30min (lane 8), 37°C for 30min, then 100°C for 3min (lane 9). Approximately 30µg of protein was applied to each lane. Lane M, molecular weight standards [myosin (205kDa), β-galactosidase (116kDa), phosphorylase b (97kDa), bovine serum albumin (66kDa), ovalbumin (45kDa), carbonic anhydrase (29kDa)]

others (45 and 47kDa) were slightly reduced (lane 7). Heating the sample at 100°C after it had undergone the 37°C incubation produced a pattern identical to lane 1 (lane 9). Therefore, the absence of the 42kDa protein was due to insolubility at the lower temperature and not in anyway due to protein degradation.

3.3.2 Separation of the cytoplasmic and outer membranes

Separation of the outer and inner cell membranes was accomplished by the differential solubilisation of proteins in the ionic detergent, sodium lauryl sarcosinate (Sarkosyl) (Filip *et al.* 1973). This method relies on the finding that components of the outer membrane are not solubilised by this detergent while, in contrast, components of the cytoplasmic membrane are normally solubilised under these conditions.

The fractions obtained by solubilisation in Sarkosyl, were analysed for the inner membrane marker enzymes NADH oxidase and succinate dehydrogenase and for 2-keto-3-deoxyoctulosonic acid (KDO) a constituent of LPS which is attached to the outer membrane. The presence of the vast majority (all in the case of succinate dehydrogenase) of the two enzymic inner membrane markers in the fraction soluble in Sarkosyl (Table 3.3) demonstrates that a good separation, if not a complete separation, of the two membrane fractions has been achieved. The outer membrane marker KDO was found to be exclusively located in the detergent-insoluble fraction. These results indicating a high separation of membranes were confirmed by SDS-polyacrylamide gel electrophoresis analysis (Fig.3.3). Gel electrophoresis showed that the patterns of proteins in the Sarkosyl-insoluble fraction (lane 2) differed completely from the banding pattern of the detergent-soluble fraction (lane 1). The marker enzyme results, in combination with those of SDS-polyacrylamide gel electrophoresis, indicate that the soluble and insoluble fractions are relatively pure cytoplasmic and outer membrane fractions respectively.

In the inner membrane fraction (Sarkosyl-soluble fraction) two protein bands with apparent molecular weights of 75 and 48kDa were most prominent. However, the outer

Table 3.3. Distribution of membrane markers in the Sarkosyl-soluble and -insoluble membrane fractions

Fraction	Protein (mg)	SDH[a]		NADH oxidase[b]		KDO[c]
		U/ml	%	U/ml	%	
Total membranes	10.6	1.54	100	3.0	100	88
Sarkosyl-soluble	6.6	n.d.	100	n.d.	92	12.5
Sarkosyl-insoluble	4.4	0	0	0.27	8	188

[a] SDH, Succinate dehydrogenase. Activity expressed as ΔA_{490}/min/ml

[b] Activity expressed as μmoles NADH oxidised/min/ml

[c] Amount expressed as nanomoles per milligram of protein

n.d., not detectable due to inhibitory effect of 0.75% Sarkosyl

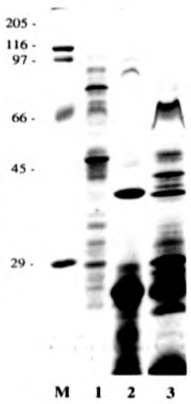


Figure 3.3. Separation of the cytoplasmic and outer membranes of *A. salmonicida* strain MT004 by differential solubilisation in the ionic detergent, Sarkosyl. The protein profiles of the cytoplasmic and outer membrane fractions are shown in lanes 1 and 2, respectively. Lane 3 shows the outer membrane profile obtained by cold osmotic shock. Approximately 10, 20 and 30μg of protein was applied to lanes 1, 2 & 3 respectively. Lane M, molecular weight standards (for full description see Fig.3.2)

membrane fraction (Sarkosyl-insoluble fraction) of *A. salmonicida* also contained two protein bands which could justifiably be labeled major bands. These were proteins of 42 and 27kDa. These bands were clearly evident in the total cell envelope fraction (Fig. 3.2, lane 1).

An attempt was made to separate the outer membrane from the other cellular fractions using the osmotic shock procedure mentioned previously. The supernatant produced by shocking the cells with ice-cold H_2O contained fragments of the disrupted outer membrane. These membrane fragments were separated from the periplasmic proteins by high-speed centrifugation and the protein profile of the membrane pellet is shown in lane 3. Several common bands can be identified between the two outer membrane preparations including the principal 42 and 27kDa proteins. However, it is clear that the osmotic shock procedure produces several additional bands such as the 48, 44 and 29kDa proteins which are most probably contaminating cytoplasmic membrane components.

The 42kDa outer membrane protein was previously shown to be insoluble at 37°C (Fig.3.2, absent in lane 7). Similar results were obtained with the isolated outer membrane fraction (Fig.3.4, lane 2). When the outer membrane fraction was incubated at 37°C for 30min in the presence of *A. salmonicida* culture filtrate containing protease activity, most proteins were degraded and were no longer visible on the gel (c.f. lanes 1 & 3). However, the 42kDa protein (and another minor outer membrane component of molecular weight 28.5kDa) were not affected by the protease activity.

The appearance of an intense band of molecular weight 24kDa (lanes 3 & 4) and the disappearance of the 27kDa outer membrane protein suggests that the 24kDa band is probably a breakdown product of the 27kDa protein. This is shown more clearly in lane 5 where a smaller amount of protease activity was incubated with the outer membrane proteins. The 27kDa protein is firstly reduced by approximately 0.5kDa, then another 2.5kDa fragment is cleaved to produce the 24kDa final product. Lane 5 also demonstrates that in common with the 42 and 28.5kDa proteins, the 45kDa component

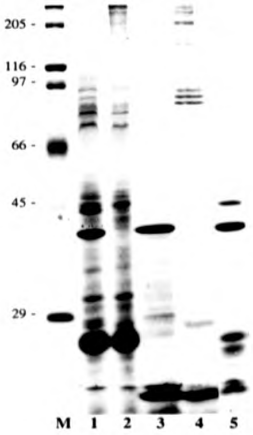


Figure 3.4. The effect of solubilisation temperature and protease action on the outer membrane protein of *A. salmonicida* strain MT004. Outer membrane samples were prepared by differential solubilisation in 0.75% Sarkosyl and solubilised at 100°C (lanes 1, 3 & 5) and 37°C (lanes 2 & 4), then culture filtrate containing 0.2 units (lanes 3 & 4) and < 0.02 units (lane 5) of 70kDa protease activity was added and incubated at 37°C for 30 min. Approximately 20μg of protein was applied to each lane, of which less approximately 0.5μg is culture filtrate protein. Lane M, molecular weight standards (for full description see Fig.3.2)

is stable to lower amounts of protease. It should be noted that the concentration of culture filtrate added to the outer membrane fraction was very low (50-fold lower than Fig.3.6, lane 5) and so the only bands visible on the gel were of the outer membrane proteins.

3.3.3 Major outer membrane proteins of *A. salmonicida* strains

A comparison of the outer membrane protein compositions of *A. salmonicida* strains revealed striking similarities in protein composition with respect to both major and minor proteins (Fig. 3.5). The principal outer membrane proteins of molecular weight 42 and 27kDa were present in each isolate. Minor bands of molecular weights 47, 45 and 28.5kDa were also present in the strains analysed. The outer membrane profiles from a parent strain and a protease-reduced derivative are shown in lanes 1 and 2, respectively. The mutant strain with reduced extracellular protease (caseinase) activity was obtained as a result of continual laboratory sub-culturing. The mutant derivative possesses two additional outer membrane proteins with apparent molecular weights of 43 and 35kDa. The other major difference between the strains tested is the presence of a major 49kDa band in the outer membrane profile of strain 184/86 (lane 4). This strain is autoagglutinating and is known to possess the 49kDa cell surface protein known as the A-protein, whereas the other strains analysed were all A-layer negative. Other strains used in this study (MT048 & MT028) also produced the A-protein (not shown).

3.4 Extracellular proteins of *A. salmonicida*

The extracellular proteins released by *A. salmonicida* are shown in Figure 3.6, lane 5. The intense band of molecular weight 70kDa has been previously identified as a serine protease capable of degrading casein (Price *et al* 1989). A number of other major bands were observed with molecular weights of 60, 56, 47, 36, 33 and 24kDa. Several other less prominent bands were also detected. The results obtained by SDS-poly-

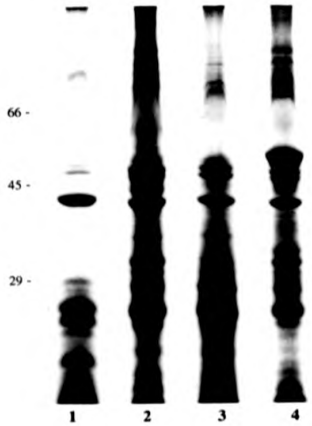


Figure 3.5. Comparison of the outer membrane protein profiles of four *A. salmonicida* strains. Outer membrane fractions from strains MT004 (lane 1), MT004rp (lane 2), 1102 (lane 3) and 184/86 (lane 4) were prepared by differential solubilisation in 0.75% Sarkosyl. 50μg of protein was applied to each lane, except lane 1 which contains 20μg of protein. Molecular weight standards are as described in Fig. 3.2.

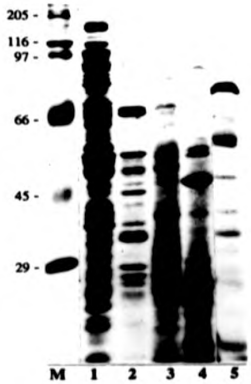


Figure 3.6. SDS-polyacrylamide gel electrophoresis of intracellular and extracellular fractions of *A. salmonicida* strain MT004. Cytoplasmic (lane 1), periplasmic (lane 2), cytoplasmic membrane (lane 3), outer membrane (lane 4) and extracellular (lane 5) fractions are shown. Approximately 10-15μg of protein were applied to each lane, except for lane 1 which contains 40μg. Lane M, molecular weight standards (for full description see Fig.3.2)

acrylamide gel electrophoresis indicate that at least 15 proteins are present in the extracellular medium of *A. salmonicida*.

By comparing the protein profiles of the various fractions by SDS-polyacrylamide gel electrophoresis it can be seen that, in general, there are very few common bands between the extracellular medium and the intracellular fractions (Figure 3.6, c.f. lanes 1-4 & 5). The proteins present in the extracellular medium are probably unique to that fraction. Glutamate dehydrogenase was exclusive to the intracellular extract with no activity detectable in the culture medium.

3.5 Discussion

A number of previous workers have reported on various enzymes released into the extracellular medium of *A. salmonicida* including proteases (Sheeran & Smith 1981; Fyfe *et al.* 1986a; Price *et al.* 1989), various haemolysins (Titball & Munn 1981, 1983, 1985a; Fyfe *et al.* 1987a; Nomura *et al.* 1988) and phospholipase and amylase activities (Campbell *et al.* 1990). Proteins are considered to be secreted if they are found in the culture medium and if their presence there is compatible with their function and their mode of action (Pugsley 1988). The enzymes mentioned previously fill these requirements because of their known toxicity towards the host (haemolysins) and their ability to facilitate the digestion of the host tissues (protease, phospholipase and amylase) (Campbell *et al.* 1990).

The presence of proteins in the culture medium of *A. salmonicida* was demonstrated to be due to a true secretion mechanism and not as a result of general non-specific release due to cell lysis. This conclusion was reached following a series of cellular fractionation experiments in which the extracellular medium was shown to be distinct in composition from the cellular fraction by the findings of SDS-polyacrylamide gel electrophoresis in conjunction with the distribution of cellular marker enzymes. The intracellular and extracellular fractions were shown to have very different protein

profiles by SDS-polyacrylamide gel electrophoresis, with the extracellular medium containing a very small number of proteins in proportion to the intracellular protein composition. The proteins in the extracellular medium proteins appeared, in the majority of cases, to be exclusive to that fraction.

The principal evidence for active secretion in *A. salmonicida* was the distribution of marker enzymes. The marker enzymes were located in fractions corresponding to the compartments where they carry out their cellular function. The 70kDa extracellular protease was used as a marker enzyme for the external medium, in which the caseinase enzyme exhibited a high specific activity. In contrast, the specific activity of the 70kDa protease in the soluble intracellular extract was very low, < 1% of that found in the culture filtrate. The situation was completely reversed when the specific activity of the cytoplasmic marker enzyme, glutamate dehydrogenase, was determined in the soluble intracellular and extracellular fractions. The specific activity of glutamate dehydrogenase in the soluble intracellular extract was reasonably high, while glutamate dehydrogenase activity was undetectable in the extracellular medium under the assay conditions employed. Glutamate dehydrogenase can be used as a sensor of cell integrity, and the absence of activity in the culture filtrate implies that cell structure has not been compromised under the growth conditions used in this study.

Therefore, from the combined results of SDS-polyacrylamide gel electrophoresis and the distribution of the glutamate dehydrogenase, along with the presence of enzymes in the culture medium being compatible with their function, it can be concluded that proteins are truly secreted into the external milieu by *A. salmonicida*.

In the last few years numerous reports have been concerned with the cell envelope of *A. salmonicida* (Kay *et al.* 1981; Evenberg *et al.* 1982; Nakajima *et al.* 1983; Belland & Trust 1985; Bernoth 1990). The interest in the *A. salmonicida* membrane is due to the presence of the tetragonally arrayed 49kDa A protein which forms the outermost layer of the cell envelope. The outer membrane of *A. salmonicida* has been prepared by a variety of methods and analysed electrophoretically. Some of the major protein bands

are characteristic of all these membrane preparations (e.g. A-layer protein), whereas others vary with membrane preparation and conditions of growth.

SDS-polyacrylamide gel electrophoresis and analysis of cellular markers indicated that differential solubilisation with sodium lauryl sarcosinate produced a good separation of inner and outer membranes. The results of this study on the outer membrane of *A. salmonicida* are consistent with a number of the previous reports with respect to the electrophoretic pattern of the major outer membrane proteins. The principal outer membrane proteins observed were assigned molecular weights of 49, 42 and 27kDa. These molecular weights correspond within reasonable limits to those published previously (Table 3.4).

Several reports have demonstrated that some membrane proteins of other genera have different mobilities on SDS-polyacrylamide gel electrophoresis, depending on the solubilisation procedure used prior to electrophoresis (Ames 1974; Ames *et al.* 1974; Russell 1975; Nakamura & Mizushima 1976). In the case of *A. salmonicida* no actual change in membrane protein mobility was observed, but instead one of the major outer membrane proteins (42kDa) could only be detected at the higher solubilisation temperatures of 70 and 100°C. At the lower solubilisation temperature of 37°C the 42kDa band was absent and this did not appear to be as a result of altered migration on the gel. The apparent resistance of the 42kDa protein to proteolytic digestion by protease(s) in the *A. salmonicida* culture medium (in contrast to the 27kDa band), suggests that it is either structurally resistant or it is in a state that is not accessible to the protease(s). The solubility results would tend to support the latter explanation (without ruling out the possibility of the former also being true).

The 42kDa outer membrane protein has been mentioned in previous studies on the A-layer protein (Kay *et al.* 1981; Nakajima *et al.* 1983), mainly because it appears to be the most difficult contaminating protein to separate from the A-protein (Evenberg & Lugtenberg 1982; Kay *et al.* 1984). Evenberg and Lugtenberg (1982) also noted that the 42kDa protein was extremely resistant to extraction. The 42kDa protein has been

Table 3.4. Major outer membrane proteins of *A. salmonicida* strains studied

Strain	Major outer membrane proteins	Reference
MT004	42, 27kDa	This study
1102	"	"
184/86	49, 42, 27kDa	"
A450	49, 42, 26kDa	Kay *et al.* (1981)
V76/135	54, 50, 42, 22kDa	Evenberg *et al.* (1982)
B105/78	"	"
153/69	50, 42, 22kDa	"
1102	44, 40, 30, 29kDa	Nakajima *et al.* (1983)
Ah440	42kDa	Darveau *et al.* (1983)

purified and shown to share several characteristics with enterobacterial porins (Darveau *et al.* 1983). Unlike the results of this study, the mobility of the 42kDa protein on SDS-polyacrylamide gel electrophoresis was shown to be dependent on the solubilisation temperature (Darveau *et al.* 1983). This discrepancy may be due to the solubilisation temperature effect being easier to demonstrate with a purified preparation of the 42kDa protein.

The periplasm of *A. salmonicida* was separated from the other cellular compartments by the cold osmotic shock treatment of Willis *et al.* (1974). EDTA perturbs the outer membrane of Gram-negative bacteria causing the release of lipopolysaccharides (LPS), ions and other outer membrane components (Leive 1974). Osmotic shock of the perturbed cells with an ice-cold solution of 0.5mM $MgCl_2$ causes the release of periplasmic components, while soluble constituents in the cytoplasm remain cell bound due to the cytoplasmic membrane remaining intact, with the Mg^{2+} ions possibly stabilising the structure by interacting with negatively charged phospholipids.

Marker enzyme assays revealed a good separation of periplasm and cytoplasm by the cold osmotic shock method, with less than 1% of the cytoplasmic marker enzyme, glutamate dehydrogenase, released into the shock fluid (periplasm). However, approximately 25% of the periplasmic marker enzyme, RNAse, was found to be in the shocked cells (cytoplasm). These results suggest that the periplasm of *A. salmonicida* has been isolated in a relatively pure state, whereas the cytoplasmic fraction contains a small amount of contaminating periplasmic protein.

SDS-polyacrylamide gel electrophoresis of the periplasmic and cytoplasmic extracts obtained by cold osmotic shock clearly shows a distinct difference in the protein composition of the two fractions. In comparison with the cytoplasm, the protein profile of the periplasm is much simpler, with approximately 20-25 proteins detectable by SDS-polyacrylamide gel electrophoresis. Apart from the outer membrane, the periplasm is thought to have the smallest subset of cellular proteins (approximately 100 proteins), while the cytoplasmic membrane contains up to 300 proteins (Pugsley & Schwartz 1985).

In general, the periplasm of Gram-negative bacteria contains two types of proteins: hydrolytic enzymes involved in nutrient metabolism (e.g. proteases, nucleases and phosphatases) and substrate binding proteins involved in transport (Heppel 1971). Although no experiments were carried out on periplasmic binding proteins, the periplasmic extract contained a number of bands in the molecular weight range 25-40kDa, characteristic for binding proteins (Heppel 1971; Adams & Oxender 1989), which appeared to be absent in the cytoplasmic fraction.

Data obtained on the protein content of the two fractions suggest that the periplasm of *A. salmonicida* comprises approximately 3.5% of the total cellular protein. This is a similar value to 4% of cell protein reported for other organisms (Heppel 1971). These results suggest that cold osmotic shock of *A. salmonicida* cells could be a potentially convenient and effective first step in the purification and study of periplasmic enzymes.

A recent study suggested using the periplasmic protein patterns of organisms as a marker system for epidemiological studies (Gargallo-Viola & Lopez 1990). These workers employed the chloroform shock method of Ames *et al.* (1984) to obtain the periplasmic extracts because it was both simple and rapid. However, of all the other treatments used to separate the periplasm of *A. salmonicida*, the chloroform shock procedure gave particularly poor results. Also, when this method was applied to *Vibrio cholerae* it caused total release of the cytoplasmic marker enzyme, catechol oxygenase (Hirst & Holmgren 1987). It may be that members of the Vibrionaceae family require gentler conditions for cell fractionation. These observations imply that osmotic shock techniques need to be empirically determined for each bacterial species under investigation. If this is found to the case, the idea of a typing system involving periplasmic protein profiles may not be so straightforward when put into practice.

4. Extracellular enzymes of *A. salmonicida*

4.1 Introduction

Both Gram-negative and Gram-positive organisms are known to secrete proteins beyond their respective cell membranes (Pugsley & Schwartz 1985; Hirst & Welch 1988). One source of the current interest in protein secretion by bacteria is the fact that many secreted proteins (toxins and degradative enzymes) play an important role in bacterial pathogenicity. Several Gram-negative organisms have been studied in relation to the pathogenic effects of their secreted enzymes (Andro *et al.* 1984; Hirst *et al.* 1984; Howard & Buckley 1985; Dow *et al.* 1989).

A number of workers have investigated the extracellular proteins of Gram-negative bacteria which have haemolytic activity (Mackmann & Holland 1984; Manning *et al.* 1984; Lory & Tai 1983) or possess the ability to form holes in membrane bilayers (Hirst *et al.* 1984; Howard & Buckley 1985). Degradative enzymes have been reported from organisms which are pathogenic to various host types. These organisms include human pathogens such as *Neisseria gonorrhoeae* and *Haemophilus influenzae* which are known to secrete a protease specific for human immunoglobulin IgA1 (Bricker *et al.* 1983; Pohlner *et al.* 1987) and *Vibrio cholerae* which secretes a deoxyribonuclease (Focareta & Manning 1987), fish pathogens including *Aeromonas hydrophila* and *Vibrio anguillarium* which produce proteases, amylases and chitinases (Howard & Buckley 1983; Inamura *et al.* 1985; Gobius & Pemberton 1988; Roffey & Pemberton 1990; Farrell & Cross 1991) and phytopathogens such as *Pseudomonas*, *Xanthomonas* and *Erwinia* which produce extracellular pectinases, cellulases, amylases and proteases (Collmer & Keen 1986; Kotoujansky 1987; Daniels *et al.* 1988; Dow *et al.* 1989; Debette 1991; Margesin & Schinner 1991).

In studying protein secretion by *A. salmonicida* with a view to determining the homogeneity (or otherwise) of the extracellular protein production within the species

and the genus, the most obvious extracellular products to study are those which are readily detectable in the culture filtrate. In the case of *A. salmonicida*, the enzymes most prominent in the culture medium are a number of hydrolytic enzymes (Section 4.2, Table 4.1). However, in addition to being present at sufficient levels for detection by conventional techniques, it would also be desirable to have alternative methods of study which would allow a more sensitive and detailed examination of some of these extracellular activities. This type of approach would give a better insight into the comparative properties of the extracellular proteins which, in turn, may yield some information on the degree of similarity between members of the *Aeromonas* genus. As mentioned previously, degradative enzymes are present at high levels in *A salmonicida*, and it is possible to detect these enzymes *in situ* in substrate-SDS-polyacrylamide gels after undergoing electrophoresis.

SDS-polyacrylamide gel electrophoresis incorporating potential substrates copolymerised into the resolving gel has greatly assisted in the study of organisms having complex hydrolytic systems. A number of enzyme activities have been visualised using this procedure, including proteases, amylases and DNAses (Heussen & Dowdle 1980; Lacks & Springhorn 1980). This method has proved particularly successful when applied to the analyses of multiple proteases in parasitic protozoa and cellular slime molds (Lockwood *et al.* 1987; North & Cotter 1991). It is a valuable technique for this type of study because it has several distinct advantages over the conventional assay methods. Firstly, it is an extremely sensitive technique enabling trace amounts of hydrolytic activity to be detected. For example, in this study samples containing protein at levels as low as 5ng, exhibited readily detectable gelatinase activity with this procedure, whereas samples being tested in the conventional manner contained a minimum of 5µg of protein. Secondly, and perhaps more importantly, it allows the rapid analyses and preliminary characterisation of individual SDS-stable proteases present in unfractionated culture filtrates, therefore dispensing with the need for often elaborate and time consuming purification procedures. The gelatin-SDS-gel method can even be used to demonstrate the substrate specificity of individual enzymes using peptidyl aminomethylcoumarins as potential substrates (Robertson *et al.* 1990).

Another practical advantage of this technique is that it is convenient for handling large numbers of samples, and it is easy to make direct comparisons between the activities of individual samples. The only slight disadvantages of this method are that it is purely qualitative and so accurate determination of protease activity is not possible, and the molecular weights obtained are only apparent molecular weights which may not reflect the monomeric forms of the enzymes, because the samples are not boiled in SDS before electrophoresis. However, in a previous study it was noted that, in contrast to membrane proteins, the temperature of solubilisation did not affect the electrophoresis pattern of soluble cell proteins (Ames 1974).

Therefore, this highly sensitive electrophoretic technique can be seen to offer several advantages in studying certain types of enzymes. With this in mind the extracellular enzymes of *A. salmonicida* strain MT004, in particular protease and amylase activities, were examined in an attempt to determine the complexity of hydrolytic enzyme production by this organism. Another reason for using substrate-SDS-polyacrylamide gel electrophoresis to study protease and amylase activity was to assess the suitability of this technique for the possible comparison of the extracellular hydrolytic enzymes of different *Aeromonas* strains and species.

4.2 Extracellular proteins of *A. salmonicida* strain MT004

A. salmonicida strain MT004 was grown at 22°C for 20h in TSB and the culture filtrate was obtained by centrifugation. The culture filtrate was tested for a range of typical extracellular enzyme activities. A number of products were readily detectable in the culture medium of *A. salmonicida* strain MT004, including protease, amylase, nuclease and haemolysin activities (Table 4.1). Other enzymes such as -glucosidase, -mannosidase and alkaline phosphatase were present at the lower limits of detection in the unfractionated culture filtrate by the methods used.

Table 4.1. Extracellular enzyme activities present in the culture medium of *A. salmonicida* strain MT004

Enzyme	Activity†
Protease	0.31 units
	24 units
Ribonuclease	0.011 units
Deoxyribonuclease	0.0057 units
Amylase	0.008 units
Haemolysin	64 units

† Activites are expressed as the units of activity per minute per ml of culture filtrate. See sections 2.4 and 2.5 for definitions of units

4.2.1 Extracellular proteases produced by *A. salmonicida* strain MT004

Protease activity was detected by assaying for casein and hide powder azure hydrolysing activity and by SDS-polyacrylamide gel electrophoresis with gelatin incorporated into the resolving gel. Preliminary assay results indicated that there was considerable protease activity present in the culture filtrate in the form of a caseinase enzyme(s).

4.2.1.1 Gelatin-SDS-polyacrylamide gel electrophoresis

(i) Protease profiles

A number of proteolytic bands were detected when gels were incubated for 15h at 30°C (Fig.4.1). The culture filtrate, which was concentrated by ammonium sulphate precipitation and redissolving in 1/5th of the original volume, contained five reproducible proteolytic bands with apparent molecular weights of M_r 75, 70, 66, 44 and 33kDa (lane 1). The gels were incubated for 15h at 30°C since this was found to give sharp, well-defined bands. Incubating the gels for a shorter time in the development buffer did not reduce the intensities of the main protease bands, but the minor bands were not easily visualised at the shorter incubation times. However, optimum resolution of the principal 70kDa protease band occurred when both the electrophoresis and the incubation times were reduced to a minimum (e.g.< 2h). This was because the 70kDa protease was present in much greater proportions in the culture supernatant than the other proteases and so longer incubation times appeared to allow diffusion of this enzyme through the gel making the band of digestion less distinct.

When azocasein replaced gelatin as the gel substrate the protease profile of the culture supernatant was significantly altered (Fig.4.1, lane 2). Instead of five proteolytic bands being detected, only a single band of activity was observed. This protease band corresponded to the 70kDa band present in the gelatin gels. No other bands of activity were observed even when more concentrated samples were electrophoresed. (N.B.

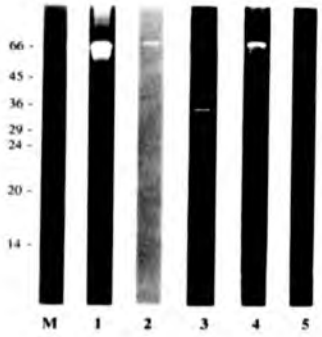


Figure 4.1. Protease activities present in the culture filtrate (CF) of *A. salmonicida* strain MT004 detected by gelatin- and azocasein-SDS-polyacrylamide gel electrophoresis. Lanes 1, 3-5; gelatin gels of CF (lane 1), CF+1mM PMSF (lane 3), CF+10mM 1,10-phenanthroline (lane 4) and heat-treated CF (lane 5). Lane 2; azocasein gel of CF. For protease inhibition studies, samples of CF were pre-incubated with the concentration of inhibitor stated for 30min prior to electrophoresis. 1,10-phenanthroline was also included in all subsequent development buffers. The heat-treated CF was maintained at 56°C for 30min prior to electrophoresis. Approximately 0.25µg of protein was applied to each lane. Lane M, molecular weight standards (for full description see Fig.3.1).

azocasein was preferred to casein as a potential substrate because activity could be observed without staining due to the colouration of azocasein).

If an appreciable difference in the extracellular protein profile of *A. salmonicida* was observed with different sample solubilisation temperatures, then it would suggest that molecular weight estimation by the gelatin gel method is not very reliable. Protein staining with Coomassie blue staining of the extracellular fraction solubilised at either 100°C or room temperature can be seen in Figure 4.2. The solubilisation temperature did not appear to affect the protein profile of the Coomassie blue stained extracellular fraction, in that the resulting banding patterns were almost identical (c.f. lanes 1 and 2), although solubilisation of the sample at room temperature resulted in less well defined and more diffuse bands than those observed when the sample was solubilised at 100°C. However, since there was no qualitative difference between the samples prepared at different solubilisation temperatures, the protease bands observed on gelatin-SDS-polyacrylamide gels are most probably displaying a good approximation of their molecular weights.

The two soluble intracellular fractions of *A. salmonicida* strain MT004 were also analysed for protease activity, in order to determine whether similar proteases existed inside the cell (Fig.4.3). No distinct proteolytic bands were observed in the cytoplasmic fraction, but protease activity of high molecular weight was detected just entering the resolving gel (lane 1). Two bands of proteolytic activity were observed in the periplasmic fraction; however, the molecular weights of these bands did not correspond to those of the extracellular proteases since the periplasmic bands migrated less quickly on SDS-polyacrylamide gel electrophoresis and were of apparent molecular weights of approximately 95 and 100kDa, much greater than any of the extracellular activities.

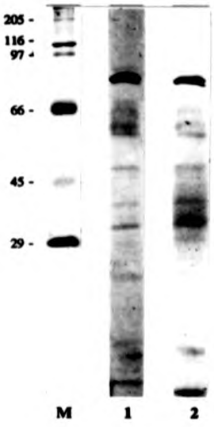


Figure 4.2. The effect of solubilisation temperature on the extracellular protein profile of *A. salmonicida* strain MT004. Extracellular samples were solubilised at 100°C (lane 1) and 25°C (lane 2) respectively. Approximately 10μg of protein was applied in each case. Lane M, molecular weight standards (for full description see Fig.3.2).

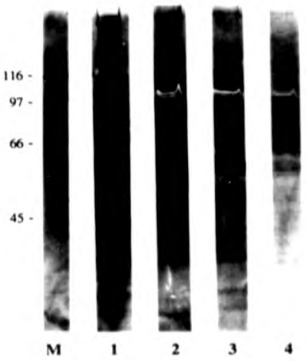


Figure 4.3. Protease activities present in the soluble intracellular fractions of *A. salmonicida* strain MT004 detected by gelatin-SDS-polyacrylamide gel electrophoresis. Cytoplasmic (lane 1) and periplasmic (lanes 2-4) fractions with 1mM PMSF (lane 3) and 1,10-phenanthroline (lane 4). For protease inhibition studies, samples were treated as in Fig.4.1. Lane 1 contains 30μg of protein, while lanes 2-4 contain approximately 5μg of protein. Lane M, molecular weight standards (for full description see Fig.3.2).

(ii) Inhibitors and heat stability of proteases

The effects of a number of proteolytic inhibitors on the protease profile was determined by incubating samples with putative inhibitor compounds at room temperature for 30 min prior to electrophoresis, and also by incubating the gelatin-SDS-gels with development buffer containing the compound of interest in the cases of the reversible inhibitors. The results of gelatin-SDS-gel inhibitor studies are shown in Figure 4.1 and are summarised in Table 4.2. All the extracellular protease activities were sensitive to either PMSF or 1,10-phenanthroline on the evidence of gelatin-SDS-polyacrylamide gel electrophoresis. The chelating agent 1,10-phenanthroline was employed as a substitute for the more extensively used metalloprotease inhibitor, EDTA, because EDTA caused a lightening of the amido black stain which made interpretation of the gels quite difficult. However, there was also a disadvantage in using 1,10-phenanthroline in this method since it seemed to form a coating on the surface of the gel which smeared on touching (see Section 7.2.3, Fig.7.7).

The extracellular proteases of apparent molecular weights 75, 70 and 44kDa were inhibited by PMSF (lane 3), implying that they were of the serine type, while the 66kDa protease was partially inhibited by this compound. The 33kDa protease was not inhibited by PMSF; instead it was found to be sensitive to the chelating agent 1,10-phenanthroline (lane 4) and EDTA (not shown), suggesting a requirement for metal ions which is indicative of metalloproteases. The periplasmic proteases were also shown to be sensitive to either PMSF or 1,10-phenanthroline (Fig.4.3). The protease of 100kDa was inhibited completely by 1,10-phenanthroline (lane 4), implying a metallo-type protease activity. The 95kDa protease was partially inhibited by PMSF (lane 3).

In general, the extracellular proteases were found to be heat labile, with only the 66kDa enzyme displaying significant residual activity after heat treatment at 56°C (Fig.4.1, lane 5).

Table 4.2. Locations, molecular weights and inhibition of proteolytic bands seen in gelatin gels[a]

Mol wt (kDa)	Located in:		Inhibited by[b]:		
	Supernatant	Periplasm	PMSF	EDTA	1,10-phe
100	–	+	–	+	+
95	–	+	(+)	–	–
80	+	–	+	–	–
70	+	–	+	–	–
66	+	–	+	–	–
44	+	–	+	–	–
33	+	–	–	+	+

[a] Symbols: +, inhibition; (+), partial inhibition; -, no inhibition

[b] PMSF (1mM); EDTA (10mM); 1,10-phenanthroline (10mM)

4.2.1.2 Extracellular caseinase production of *A. salmonicida* strain MT004

The presence of extracellular caseinase activity in the culture medium was found to be associated with growth phase. The production of the extracellular caseinase enzyme in relation to growth of *A. salmonicida* strain MT004 is shown in Figure 4.4. Caseinase activity in the culture supernatant increased significantly in the late-exponential growth phase until a maximum activity of 24 units/ml was observed between 18-20h of growth. Caseinase secretion ceased during the stationary phase of growth (Fig.4.4).

However, although the caseinase activity remained constant during the stationary growth phase, the specific activity of the protease decreased significantly during this period after reaching a maximum of 480 units/mg at 15h (Fig.4.5). The decrease in specific activity was due to an increase in the amount of protein present in the culture filtrate, perhaps as a result of cell lysis.

4.2.1.3 Characterisation of *A. salmonicida* extracellular caseinase protease

The biochemical and physical properties of the extracellular caseinase enzyme of *A salmonicida* strain MT004 were determined using a purified preparation of the protease. Details of the purification procedure are given in Section 6.2.

(i) Physicochemical properties

The caseinolytic activity emerged on gel filtration as a single peak (see Section 6.2, Fig. 6.2) which is in agreement with the results obtained by azocasein-SDS-polyacrylamide gel studies which indicated that only the 70kDa protease was capable of digesting azocasein/casein. The caseinase activity emerged at an elution volume, which from the behaviour of marker proteins corresponded to a molecular weight of 66000 ± 6000. Comparison of its electrophoretic mobility on SDS-polyacrylamide gel electrophoresis with those of standard proteins indicated that it had a molecular weight of 70000 ± 2000 (see Section 6.2, Fig.6.3). Taking the molecular weight data obtained by gel filtration and SDS-polyacrylamide gel electrophoresis, it can be concluded that the

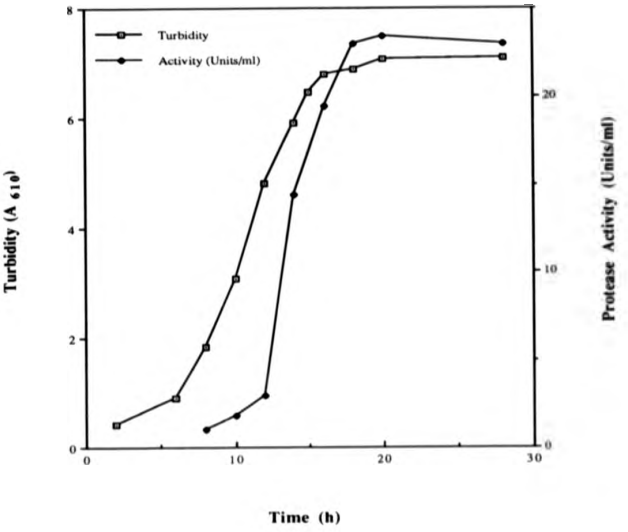


Figure 4.4. Growth-phase associated production of extracellular caseinase activity by *A. salmonicida* strain MT1004

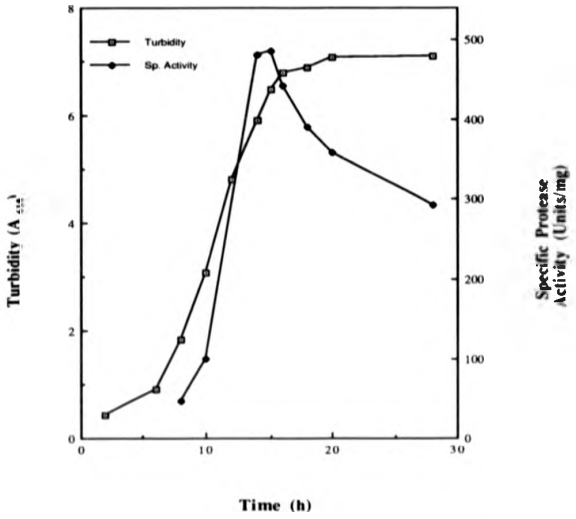


Figure 4.5. The relationship between growth phase of *A. salmonicida* strain MT004 and specific activity of the extracellular caseinase enzyme

protease displaying caseinolytic activity is monomeric with a molecular weight of 70kDa.

The effect of storage on the 70kDa extracellular caseinase was investigated over a one month period. The purified 70kDa protease was found to be quite unstable on storage even at -20°C (Fig.4.6). After 10 days of storage only 50% of the caseinase activity remained. This was in contrast to the caseinase activity in unfractionated culture filtrate in which 50% of the activity still remained after 1 month at -20°C.

(ii) Effect of pH and protease inhibitors

The effect of varying the pH on casein-degrading activity by the 70kDa protease was examined by using various buffers (Fig.4.7). The enzyme was active over a broad pH range, 6 to 10, without a sharp pH optimum. However, it was nearly inactive at values less than pH 5.

The effects of various inhibitors was examined by preincubating the enzyme with the test inhibitor for 30 min at room temperature and then measuring caseinase activity (Table 4.3). Phenylmethanesulphonyl fluoride (PMSF) at a concentration of 1.0mM strongly inhibited (>95%) the enzyme, while all the other inhibitors tested had no significant (< 10% inhibition) effect.

(iii) Temperature optimum and heat stability

The effect of assay temperature on the activity of the 70kDa protease was determined (Fig.4.8). The activity of the caseinase enzyme increased with temperature until it reached a maximum at 45°C. Above 45°C, the caseinase activity decreased with increasing temperature until at 60°C only a little proteolytic activity was detectable. The reduction in activity at the higher temperatures was most probably the result of thermal instability. The activation energy (E_a) of the 70kDa protease was determined using the Arrhenius equation and was calculated to be 37.0kJmol^{-1}. This is quite reasonable since

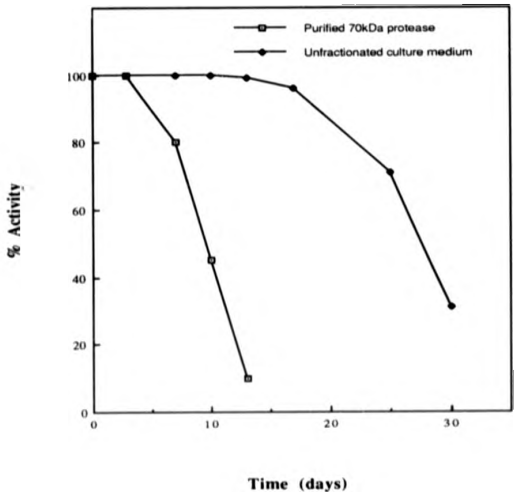


Figure 4.6. The effect of purification on the storage stability of the extracellular 70kDa protease of *A. salmonicida* strain MT004 when stored at -20°C

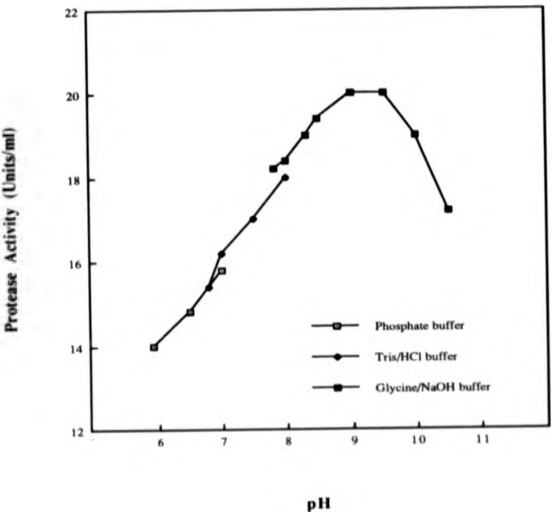


Figure 4.7. Optimum pH for the hydrolysis of casein by the purified extracellular 70kDa protease of *A. salmonicida* strain MT004. Experimental conditions are described in Section 2.10.1

Table 4.3. The effect of inhibitors on the activity of the extracellular 70kDa protease purified from *A. salmonicida* strain MT004[a]

Inhibitor	Concentration	Relative Activity (%)
None		100
PMSF[b]	0.1mM	18
	1.0mM	2
EDTA	10.0mM	102
1,10-phenanthroline	10.0mM	101
Ovomucoid	0.25mg/ml	98
Trypsin soybean inhibitor	0.25mg/ml	98
Antipain	0.25mg/ml	100
Aprotinin	0.25mg/ml	101
Benzamidine	25.0mM	96
Iodoacetate	1.0mM	94

[a] Experimental conditions are described in Section 2.10.1

[b] PMSF, phenylmethanesulphonyl fluoride

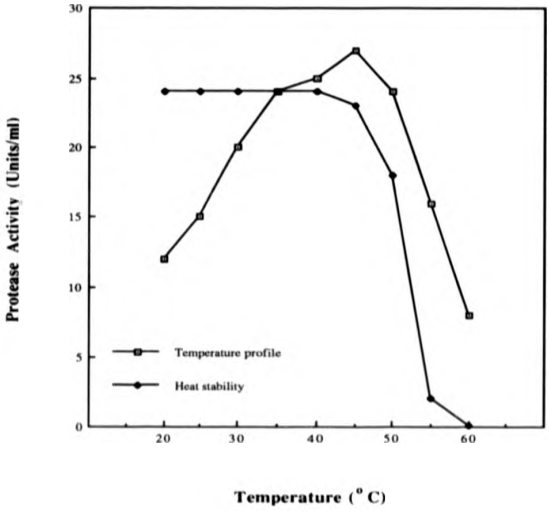


Figure 4.8. Optimum temperature for the hydrolysis of casein by the extracellular 70kDa protease, and the effect of temperature on the stability of the enzyme. Experimental conditions are described in Section 2.10.1

a reaction whose E_a is less than 51.0kJ mol^{-1} is less sensitive to temperature (Price & Dwek 1979), and so is consistent with the extracellular location of the protease requiring it to be active in more extreme conditions than those inside the cell.

The heat stability of the caseinase protease was determined by pre-incubating the enzyme solution at the test temperature for 30 min and then initiating the reaction by addition of the casein substrate. The activity of the protease was not significantly affected at temperatures up to, and including, 45°C (Fig.4.8). However, at higher temperatures the stability of the caseinase enzyme was greatly reduced, to the extent that at 60°C there was less than 5% of the activity observed at 45°C.

4.2.2 Extracellular amylases produced by *A. salmonicida* strain MT004

Amylase activity was detected by assaying for starch hydrolysing activity and by SDS-polyacrylamide gel electrophoresis with starch incorporated into the resolving gel at a final concentration of 0.17%.

4.2.2.1 Starch-SDS-polyacrylamide gel electrophoresis

(i) Amylase profile

The amylase profile of *A. salmonicida* strain MT004 culture medium is shown in Figure 4.9. A number of amylolytic bands were detected when the gels were incubated for 5h at 30°C. These conditions for developing starch gels were chosen because longer incubation times tended to produce quite diffuse bands of activity, probably due to diffusion of the amylases within the gel. Shorter incubation times on the other hand, did not always allow the development of minor bands of amylase activity. Thin gels (0.8mm thick) were always used since they allowed rapid diffusion of SDS out of the gel and development buffer into the gel, thus reducing the time between electrophoresis and development.

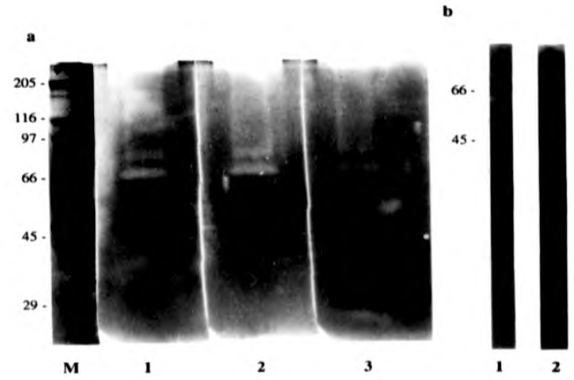


Figure 4.9. Amylase activities present in the culture filtrate (CF) of *A. salmonicida* strain MT004 detected by starch-SDS-polyacrylamide gel electrophoresis. Gel a; 7.5% gel strips of CF developed at pH 4.5 (lane 1), pH 7.0 (lane 2) and pH 9.5 (lane 3) respectively. Gel b; 12% gel strips of CF only (lane 1) and CF+10mM 1,10-phenanthroline (lane 2) respectively. For 1,10-phenanthroline inhibitor study, sample was treated as in Fig 4.1. Approximately 1.0μg of protein was applied to each lane. Lane M, molecular weight standards (for full description see Fig.3.2).

The culture medium, which was concentrated by ammonium sulphate precipitation and redissolving in 1/20th of the original volume, contained three amylolytic bands with apparent molecular weights of approximately >180, 80 and 63kD (Fig. 4.9a). The presence of these bands appeared to be slightly affected by the pH of the incubation buffer. At pH 7.0 the bands were at their most intense, while at pH 4.5 the high molecular weight amylase was much less intense, and at pH 9.5 the lower molecular weight bands were reduced (compare lanes 2, 1 & 3 respectively). Depending on the concentration of acrylamide used, an additional band running slightly slower than the 63kDa protein could sometimes be visualised (Fig.4.9b, lane 1). This amylase had an apparent molecular weight of 65kDa.

It is possible that the presence of starch in the resolving gel may affect the migration of the amylase enzymes by some sort of enzyme-substrate interaction. The incorporation of starch into the polyacrylamide gel was investigated in order to determine whether amylase mobility was affected. Two gels were poured, with water replacing starch in one of the gels, and a sample of extracellular medium was prepared for electrophoresis and divided into two aliquots with one applied to each gel. The gel with starch incorporated into the resolving gel was developed for amylase activity by the standard procedure. The second gel was incubated in development buffer containing 0.5% starch which had been prepared by autoclaving at 120 p.s.i. for 20 min. Autoclaving was carried out to cause a partial breakdown of the starch which, in turn, would allow greater penetration of the starch into the gel. After 5h incubation, the gel was washed in water and then stained with the KI/I_2 solution.

The migration of the amylase bands in the two gels were compared and the mobilities were found to be identical (not shown), demonstrating that starch incorporation into the resolving gel did not cause retention of the extracellular amylase enzymes. However, it should be noted that the method of incubating the gel in a solution of starch was not as sensitive as the standard procedure, with the main activities of molecular weights 80 and 63kDa appearing much less intense and the minor amylase band of apparent molecular weight 65kDa not detectable by this method.

(ii) Inhibitor of amylase activity

A number of bacterial amylases share many common properties, including a requirement for Ca^{2+} and sensitivity to EDTA (Vihinen & Mantsala 1989). The effect of 1,10-phenanthroline (used instead of EDTA) on the extracellular amylase activity is shown in Figure 4.9b (lane 2). The chelator appears to cause a partial inhibition of the amylase activity, particularly with the amylase of apparent molecular weight 63kDa. The 65kDa amylase activity does not appear to display the same sensitivity to 1,10-phenanthroline.

4.2.2.2 Extracellular amylase production of *A. salmonicida* strain MT004

As with the extracellular caseinase activity, amylase production by *A. salmonicida* strain MT004 appeared to be growth phase-associated. Growth and amylase production of strain MT004 is shown in Figure 4.10. Amylase activity appeared in the culture medium around the mid-exponential phase of growth. Maximum amylase activity of 9.8mg starch hydrolysed/l was observed in the extracellular medium 16h after inoculation.

(i) Effect of pH and inhibitors

The extracellular amylases of *A. salmonicida* were active over a broad pH range from 4.5 to 9.5 (Fig. 4.11). There was no apparent sharp peak of activity at any pH value, perhaps due to the fact that the amylase activity represented at least three separate enzymes with possibly quite different properties. Therefore, although the individual enzymes may be active over a narrow pH range their activities could overlap to give the impression of activity over a large pH range. In this respect, the pH profile displays a slight depression at pH 7.0, with twin 'peaks' either side of this pH value. This may indicate that the extracellular amylases have different pH optima. These results are consistent with the different pH dependencies of the amylases previously displayed by starch-SDS-polyacrylamide gel electrophoresis.

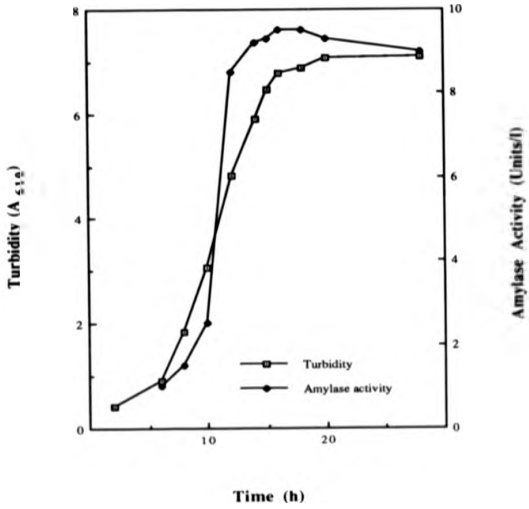


Figure 4.10. Growth-phase associated production of extracellular amylase activity by *A. salmonicida* strain MT004

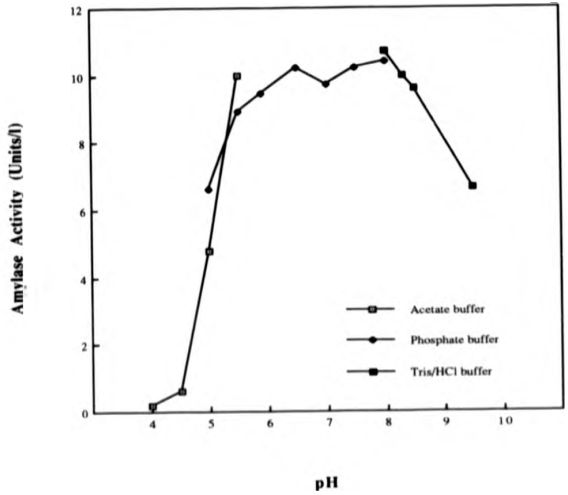


Figure 4.11. Optimum pH for the hydrolysis of starch by the extracellular amylase activity of *A. salmonicida* strain MT004. Experimental conditions are described in Section 2.10.2

Table 4.4. The effect of inhibitors on the extracellular amylase activity of *A. salmonicida* strain MT004 [a]

Inhibitor	Concentration	Relative Activity (%)
None		100
PMSF[b]	1.0mM	100
EDTA	10.0mM	28
1,10-phenanthroline	10.0mM	33
$HgCl_2$	0.1mM	0

[a] Experimental conditions are described in Section 2.10.2

[b] PMSF, phenylmethanesulphonyl fluoride

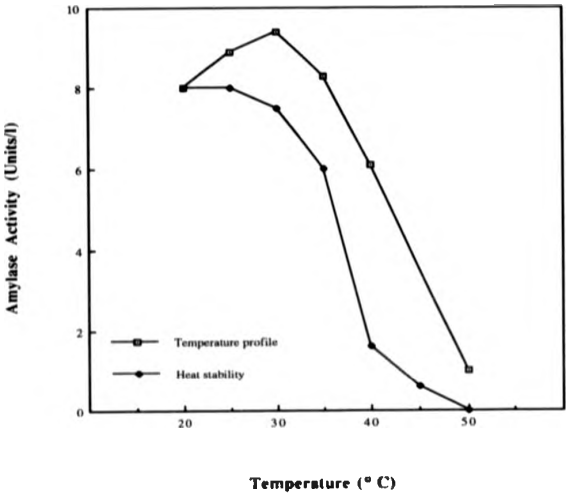


Figure 4.12. Optimum temperature for the hydrolysis of starch by extracellular amylases, and the effect of temperature on the stability of the enzymes. Experimental conditions are described in Section 2.10.2

Table 4.4 shows the effect of a number of compounds on the extracellular amylase activity. Less than 30% of the amylase activity remained after treatment with 10mM solutions of EDTA and 1,10-phenanthroline. However, this conflicted with the results obtained by starch-SDS-polyacrylamide gel electrophoresis which indicated that the extracellular amylases of *A. salmonicida* were relatively resistant to inhibition by 1,10-phenanthroline. It may be that the enzymes are inhibited to this degree, but that the remaining activity is capable of degrading similar amounts of incorporated starch due to the length of incubation (5h). The most effective inhibitor was $HgCl_2$ which caused complete inhibition of amylase activity at concentrations as low as 0.1mM. PMSF had no effect on the amylase activity.

(ii) Temperature optimum and heat stability

The profile of the extracellular amylase activity over a wide temperature range is shown in Figure 4.12. Maximum activity was observed at 30°C, while at temperatures only slightly higher (40 & 45°C) there was a significant reduction in the extracellular amylase activity. Heat stability values were obtained following the same procedure as with the caseinase enzyme. In comparison with the caseinase enzyme, the extracellular amylases were not as stable to heat treatment. Temperatures greater than 30°C caused a significant reduction in amylase activity, with pre-incubation of the extracellular medium at 50°C resulting in the complete loss of activity (Fig.4.12).

As with the results on the effect of pH, these results give no information on the individual properties of the amylases with respect to temperature. The activity of the amylases may be affected differently by temperature; for example, the lower amylase activity at 40°C may not be as a result of the amylase enzymes being similarly affected at this temperature, but that one (or more) of the amylases have lower optimum temperature values. Accurate determination of the physical properties of the extracellular amylase enzymes will not be possible until purified preparations of these activities are obtained.

4.3 Discussion

The results of this study confirm a number of previous reports on the extracellular products of *A. salmonicida*, and also add to the literature particularly with respect to the extracellular protease production of this organism. In general, the presence of protease, nuclease, amylase and haemolysin activities in the culture medium of *A. salmonicida* supported the findings of previous workers (Campbell *et al.* 1990; Ellis 1991). However, the use of SDS-polyacrylamide gel electrophoresis with potential enzyme substrates incorporated into the resolving gel, allowed a more detailed characterisation of some of the hydrolytic enzymes present in the culture supernatant of *A. salmonicida*.

Five proteolytic bands were detected in the culture medium by the gelatin gel technique. However, previous studies on the extracellular protease activity of *A. salmonicida* have, in the majority of cases, only identified a single proteolytic component (Shieh & Maclean 1975; Mellergaard 1983; Tajima *et al.* 1984; Fyfe *et al.* 1986a). More recent results suggested that these strains produced two proteases, differing in molecular weight and in substrate specificity (Price *et al.* 1989), the principal enzyme being a serine protease of molecular weight 70kDa active against casein and gelatin. The principal protease observed in this study also possessed an apparent molecular weight of 70kDa. Also in agreement with Price *et al.* (1989) was the observation that this was the only protease active against (azo)casein.

The 70kDa protease was found to be unstable on storage even at temperatures as low as -20°C. In comparison with the unfractionated culture filtrate where its half-life was extended three-fold, the caseinase enzyme was particularly unstable when stored in the purified form. Since the protease retains its stability better in the unpurified form, the loss of activity is most probably a result of autolysis rather than a simple denaturation effect. In the purified form the protease will be the only substrate available for itself and so will be degraded quicker than when other protein substrates are available, such as in the unfractionated culture filtrate.

Price *et al.* (1989) detected another minor (less well characterised) protease activity, which was active against gelatin but not casein, and was assigned a tentative molecular weight of approximately 20kDa. Although several minor proteolytic activities detected were active against gelatin but not casein in this study, no protease of apparent molecular weight 20kDa was observed. The gelatin-hydrolysing proteases of strain MT004 were assigned apparent molecular weights of 75, 66, 44 and 33kDa.

The 33kDa protease was demonstrated to be a metalloenzyme by its sensitivity to the chelator, 1,10-phenanthroline. Sheeran and Smith (1981) reported a second extracellular proteolytic activity associated with *A. salmonicida*. This protease was classified as a metalloprotease on the basis of its inhibition by EDTA and was active against gelatin, but did not hydrolyse casein. The presence of a metalloprotease was also noted by Rockey *et al.* (1988), however no indication of the molecular weights of these activities was given. It is possible that the 33kDa enzyme corresponds to both the metalloproteases reported by Sheeran and Smith (1981) and Rockey *et al.* (1988), and the low molecular weight protease detected by Price *et al.* (1989). The difference in the molecular weights may be a consequence of the different methods used for molecular weight estimation (i.e. SDS-polyacrylamide gel electrophoresis as opposed to gel filtration).

As previously mentioned (Section 4.1), the molecular weight exhibited on a gelatin gel is not regarded as a true estimate, and so should be verified by other means (e.g. purification and molecular weight estimation). However, it should be noted that the value of 70kDa obtained for the caseinase enzyme by gelatin-SDS-polyacrylamide gel electrophoresis is identical to that obtained by the more conventional methods (Tajima *et al.* 1984; Fyfe *et al.* 1986a; Price *et al.* 1989; this study, Sections 4.2.1.3 & 6.2), and that solubilisation temperature only affected the definition of the protein bands of the extracellular medium and not the actual profile. In fact, the temperature of solubilisation appears to have no qualitative effect on soluble protein fractions (Ames 1974). This is in direct contrast to bacterial membrane proteins which are sensitive to solubilisation temperature (Ames 1974; Ames *et al.* 1974). In the original SDS-

polyacrylamide gel electrophoresis studies, lower temperatures of solubilisation, such as 37 and 45°C, were standardly employed (Dunker & Rueckert 1969; Weber & Osborn 1969), and the change to higher solubilisation temperatures appeared to be a practical consideration of reducing the time of sample preparation and not because it was thought to give more accurate or reliable results.

In addition, previous literature suggests that molecular weights estimated from gelatin-SDS-gels are, in the main, consistent with the results of gel filtration, although discrepancies have been noted (North & Cotter 1991). In their studies, North & Cotter (1991) found that a protease of apparent molecular weight 18kDa on electrophoretic analysis behaved as a larger protein on gel filtration or ultrafiltration. However, they observed that this enzyme was an exceptional case, since all other *Dictyostelium discoideum* proteases examined in this way showed no discrepancies with respect to molecular weight. It is possible that artefacts can be caused by this method; for example a 68kDa protease from *Leishmania mexicana mexicana* was found to appear sometimes as a double band of activity, due to its mobility being dependent on conditions of solubilisation for electrophoresis (Lockwood *et al.* 1987).

With the exception of the 33kDa metalloenzyme, the extracellular proteases of *A. salmonicida* strain MT004 were of the serine type. Of the other proteases, it is possible that the 44kDa enzyme is the same as the 43kDa protease referred to by Dahle (1971a). However, until further work is carried out on these minor proteolytic components their relationship with proteases described in previous reports cannot be clarified.

On the basis of gelatin-SDS-polyacrylamide gel electrophoresis, the complexity of *A. salmonicida* extracellular protease production appears to be much greater than previous reports suggest (Price *et al.* 1989; Ellis 1991). In general, this current investigation provisionally confirms several findings of previous reports and perhaps clarifies further the anomalies of the literature with respect to the extracellular proteases of *A. salmonicida*.

5. Effect of growth conditions on *A. salmonicida* protein production

5.1 Introduction

The ability of microorganisms to survive, and even proliferate, in extreme environments is widely recognised (Roszak & Colwell 1987; Smith 1990). Microbial cells also must frequently experience changes of environment which they are powerless to control. Microorganisms have developed powers of adaptibility and if the external environment shifts (e.g., alterations in temperature, pH, oxygenation, salt balance, nutrient concentration, etc.) then microbial cells can readily and rapidly adapt to these changes. This quick accommodation to marked changes in the environment is effected by organisms changing themselves, structurally and functionally, i.e., phenotypic alterations. The nutrients and enviromental factors which are crucial to the growth of the organism are relatively unknown, with the notable exception of iron-limitation (Smith 1990). *In vitro* experiments of iron-limitation revealed the production of siderophores and new outer membrane proteins (Neilands 1981, 1982).

Perhaps the most obvious bacterial adaptations to environmental changes are alterations in the bacterial cell envelope (Stock *et al.* 1989). The outer membrane of a Gram-negative bacterium is a permeability barrier with at least three different types of proteins involved in the selective uptake processes of the cell. One class of proteins, termed general diffusion porins, form pores which allow non-specific, passive diffusion of hydrophilic solutes with molecular weights of below about 650 daltons (e.g., amino acids, small peptides, mono- and di-saccharides, inorganic salts), a second type of porins are channels used for the specific penetration of molecules such as maltose and nucleosides, and a third class of outer membrane proteins are involved in the transport of relatively high molecular weight substances such as vitamin B12 and iron-chelator complexes (Burns 1983; Benz & Bauer 1988). These outer membrane proteins may be of importance when nutrients are limited in the external medium (Stock *et al.* 1989). For example, as mentioned above alterations in the outer membrane protein profiles of a

number of bacteria incuding *E. coli* (McIntosh & Earhart 1977), *Salmonella typhimurium* (Neilands 1982), *Klebsiella* species (Williams *et al.* 1984; Shand *et al.* 1985), and *Yersinia* species (Carniel *et al.* 1987) have been found when these organisms were grown in a low iron environment. Most research has concentrated on iron-limitation but some attention has been given to the in vivo effects of carbon, Mg^{2+} and PO_4^{3-} limitation (Ellwood & Tempest 1972; Ellwood 1974; Brown & Williams 1985).

The external environment may also influence synthesis and secretion of bacterial extracellular proteins. Control of extracellular protein production occurs at the transcriptional level; in bacteria, the synthesis of most extracellular proteins is influenced by the level of catabolites present in the growth medium. The presence of catabolites (e.g., glucose) produces a reduction in the intracellular concentration of cyclic adenosine 3′5′-monophosphate (cAMP) which in turn causes a reduced formation of the cAMP-CRP (cAMP receptor protein) which initiates transcription. Another type of repression, end-product (feedback) repression, also affects the synthesis of extracellular proteins. In this case, intermediates or end-products of the enzyme reaction inhibit further enzyme synthesis, a widely reported example being the repression of extracellular proteases by amino acids.

Since in addition to the effects of nutrients on outer membrane and extracellular proteins, physical factors such as temperature, pH and oxygenation can affect bacterial protein production, the effect of culture conditions on the production of outer membrane and extracellular proteins of *A. salmonicida* was determined. The response of *A. salmonicida* to the altered culture conditions will reveal whether this organism shares similar strategies to cope with environmental changes as with other bacteria, and whether regulation of extracellular protein production is controlled by repression-type mechanisms.

5.2 Effect of incubation temperature on protein production

The optimum growth temperature of *A. salmonicida* is 22-25°C (Popoff 1984). The protein profiles of the soluble intracellular, outer membrane and extracellular fractions of *A. salmonicida* grown at 22°C are shown in Figure 5.1 (lanes 1, 3 & 5 respectively). The effect of increasing the incubation temperature to 32°C on these fractions is shown in lanes 2, 4 and 6 respectively. Over the range 24-36°C the A_{610} declined by approximately 25-30%.

5.2.1 Extracellular protein production

The most striking difference produced by increasing the growth temperature was the effect on the extracellular protein production by *A. salmonicida* (Fig.5.1, c.f. lanes 5 & 6). It is clearly evident that most extracellular proteins (especially the 70kDa protease) were not produced in as great amounts, if at all, at the higher growth temperature. This result was confirmed by the very low caseinase activity in the extracellular medium of cells incubated at 32°C ((0.24units/mg protein compared with 500 units/mg protein at 22°C). However, a greater number of bands was present in the extracellular medium at 32°C, and the protein content was also over 200-fold higher (10.6mg/ml compared with 0.05mg/ml at 22°C). This suggested that these proteins were released as a result of cell lysis, and SDS-polyacrylamide gel electrophoresis identified common bands in the soluble fraction (c.f. lanes 2 & 6). Conclusive proof of cell lysis was obtained by the detection of the intracellular marker enzyme, glutamate dehydrogenase, in the extracellular medium of *A. salmonicida* incubated at 32°C (20nmoles nucleotide released/ml).

Generally, all other extracellular enzymes examined appeared to exhibit marked similarities to the caseinase enzyme with respect to its production being dependent on the growth temperature (Fig.5.2). For example, both amylase and DNAse activities

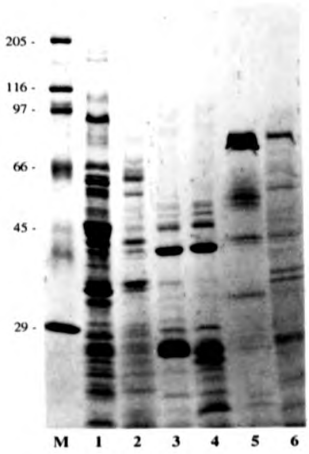


Figure 5.1. The effect of temperature on the intracellular and extracellular fractions of *A. salmonicida* strain MT004. Soluble intracellular (lanes 1 & 2), outer membrane (lanes 3 & 4) and extracellular (lanes 5 & 6) fractions from cultures incubated at 22°C (lanes 1, 3 & 5) and 32°C (lanes 2, 4 & 6) respectively. Similar volumes of cellular fractions from the two cultures were applied in each case, except lane 5 which contains approximately double the volume of lane 6. Lane M, molecular weight standards (for full description see Fig.3.2)

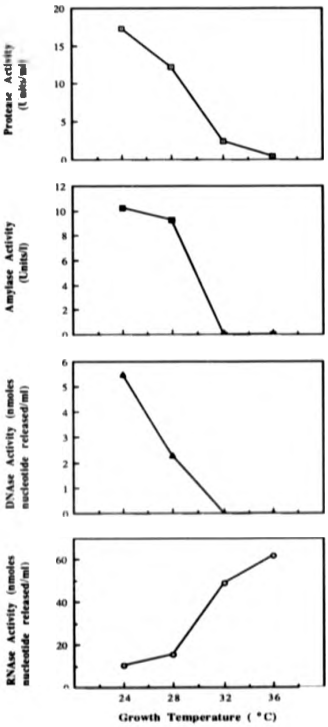


Figure 5.2. Effect of growth temperature on the production of selected activities of *A. salmonicida* strain MT004. Activities were determined as described in Section 2.5

were significantly reduced at temperatures greater than 25°C, with virtually no activity detectable in the culture medium of cells grown at 32°C. The suppressing effect of an increased temperature is not due to the inactivation of these enzymes, since they are relatively stable at temperatures in the region of 30°C (see Section 4.2). The suppression of extracellular production could not be relieved by adaptation to elevated temperature.

In one case, however, that of RNAse, the extracellular activity of this enzyme appeared to increase dramatically with an increase in the incubation temperature (Fig.5.2). At 32°C, RNAse activity was observed to be over four times greater than at 24°C. However, the specific activity of RNAse in the culture medium of cells grown at the higher temperature was much lower (4.5 compared with 220 nmoles nucleotide released/mg at 24°C), since the amount of protein present in the culture medium was considerably greater at the higher temperature. Again this is most probably due to cell lysis occurring at the higher incubation temperature. The increase in RNAse activity in the extracellular medium of cells grown at 32°C was probably a consequence of cell lysis causing the release of periplasmic RNAse activity into the medium.

One interesting observation was made on the effect of temperature on the extracellular protein production of *A. salmonicida*. Cells grown at the higher temperature, secreting low, or negligible, amounts of extracellular enzymes were, on sub-culturing into fresh TSB medium at 24°C, found to regain their protein-secreting capacity (Fig.5.3). This reversion is demonstrated clearly with the extracellular caseinase enzyme which, on transferring the cells to medium at 24°C, is produced at a similar level to that before the switch of temperature to 32°C. The other extracellular proteins were similarly affected, with all activities reaching their original levels (not shown).

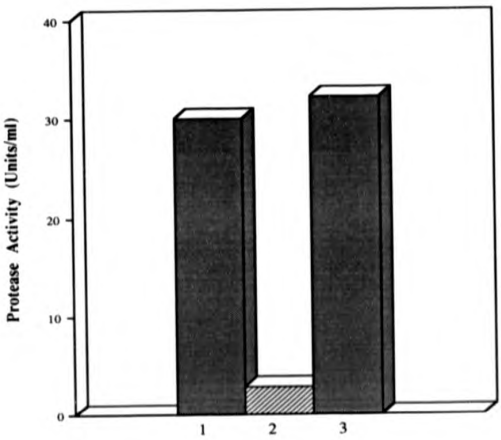


Figure 5.3. Effect of temperature shift on extracellular protease production. *A. salmonicida* strain MT004 was grown under (1) standard conditions (i.e., in TSB at 24°C for 20h), then (2) sub-cultured into fresh TSB medium and incubated at 32°C for 20h, and finally (3) transferred into fresh TSB and incubated at the original temperature of 24°C for 20h. Protease activity was determined as described in Section 2.5.2

5.2.2 Intracellular protein production

Increasing the growth temperature of *A. salmonicida* to 32°C also affected the intracellular soluble proteins (Fig. 5.1, c.f. lanes 1 & 2) and the outer membrane protein profile (c.f. lanes 3 & 4). Several prominent intracellular soluble proteins produced at 22°C are completely absent from the cells grown at 32°C. The major proteins absent from the cells incubated at 32°C have molecular weights of 60, 45 and 35kDa.

The only major difference between the outer membrane proteins produced at the two different temperatures is the presence of bands of molecular weight 26.5 and 24kDa found in the 32°C outer membrane fraction and their absence at the normal growth temperature (lane 4). The 26.5 and 24kDa bands correspond to those mentioned previously concerning the protease-susceptibility of the outer membrane proteins (see Section 3.3.2, Fig.3.4). The identical breakdown pattern of the 27kDa protein is in agreement with the conclusion that cell lysis occurs at 32°C, i.e., the cells lyse, disrupting the membranes and so making membrane proteins accessible to attack by the trace amount of extracellular protease present, resulting in a similar profile to Section 3, Fig.3.4, lane 5.

5.3 Effect of NH_4^+ ion concentration on extracellular enzyme production

Ammonium ion (NH_4^+) concentration in the growth medium of *A. salmonicida* was altered by the addition of ammonium tartrate to a final concentration of 50mM. The bacterial growth was slightly reduced, but not as dramatically as the extracellular caseinase activity (Fig.5.4). The activity of the caseinase protease was reduced approximately 5-fold (7 units/ml compared to 30 units/ml) when the NH_4^+ ion concentration of the medium was increased. The other extracellular enzymes examined did not appear to be as sensitive (< 5% decrease), indicating that NH_4^+ ions appear to have a specific effect on extracellular protease production by this organism.

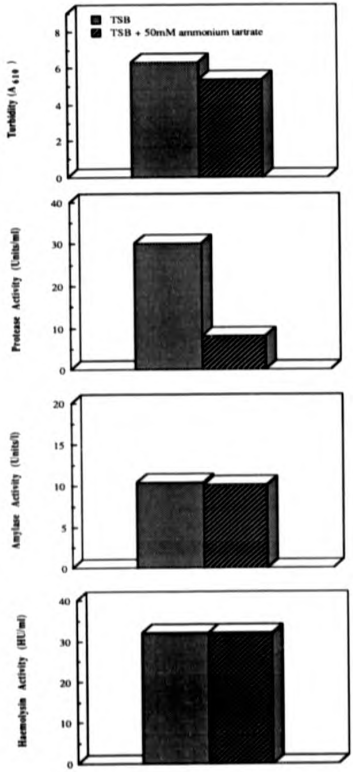


Figure 5.4. The effect of NH_4^+ ions on growth and the production of selected activities of *A. salmonicida* strain MT004. Activities were determined as described in Section 2.5

5.4 Effect of iron-limitation on protein production

The effect of iron-limitation on *A. salmonicida* strain MT004 was investigated by growing the cells in TSB containing ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDA) at a final concentration of 10μM. EDDA is a specific chelator of iron, therefore, any alteration in growth or protein production of *A. salmonicida* observed when EDDA is present in the growth medium should be as a direct consequence of iron-limitation.

5.4.1 Outer membrane proteins

The effect of iron limitation on the outer membrane proteins of *A. salmonicida* is shown in Figure 5.5. The outer membrane fraction of cells grown under normal conditions is shown in lane 1, while lane 2 shows the iron-limited outer membrane profile. The results demonstrate that under iron limitation, three (four in some SDS-polyacrylamide gel electrophoresis runs) outer membrane proteins with apparent molecular weights of 89, 79 and 77kDa were induced. All other outer membrane proteins visualised by SDS-polyacrylamide gel electrophoresis appear unaltered by growth in a low iron environment.

5.4.2 Extracellular enzyme activity

Iron-limitation had little effect on the growth of *A. salmonicida*, with only a slight decrease in the turbidity of the culture, while addition of iron to the culture medium in the form of iron chloride caused a slight increase in growth (Fig.5.6). The effect of iron-limitation and -supplementation on a number of extracellular enzymes of *A. salmonicida* strain MT004 is also shown in Figure 5.6. Addition of EDDA selectively induced caseinase activity, while other extracellular enzyme production was not affected. Caseinase activity in the culture medium was significantly increased, approximately 25% under iron-limiting conditions. The production of other extracellular enzymes, including haemolysin, amylase and RNAse activities, remained

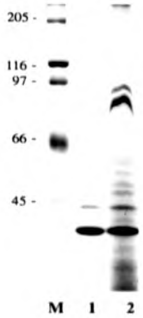


Figure 5.5. The effect of iron limitation on the outer membrane proteins of *A. salmonicida* strain MT004. Outer membrane fractions prepared from cells grown under normal (lane 1) and iron-limited (lane 2) conditions, respectively. Approximately 15μg of protein was applied in each case. Lane M, molecular weight standards (for full description see Fig.3.2)

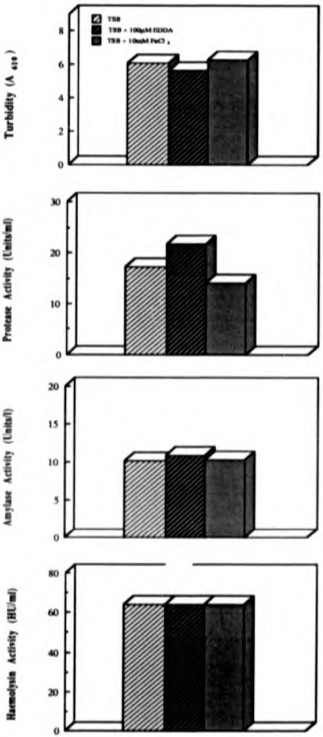


Figure 5.6. The effect of iron-limitation and -supplementation on growth and the production of selected activities of *A. salmonicida* strain MT004. Activities were determined as described in Section 2.5

at levels similar to those displayed under normal growth conditions. Supplementation of the medium with iron also affected the level of caseinase activity in the culture filtrate of *A. salmonicida*, however in this case the activity was reduced by approximately 20%. As with iron-limitation, the addition of iron to the culture medium did not alter the levels of other extracellular enzymes significantly.

5.5 Effect of starch and glucose on extracellular caseinase and amylase activity

Extracellular caseinase and amylase production by strain MT004 was affected by the carbohydrate composition of the growth medium (Fig. 5.7). Addition of glucose into the growth medium at a final concentration of 1% resulted in the complete repression of extracellular amylase production and a reduction of caseinase activity by approximately 75%. Although starch at the same concentration stimulated growth of *A. salmonicida*, it also caused repression of amylase activity, but to a lesser extent (20%) than glucose, while 0.1% starch appeared to both stimulate amylase production and greatly increase growth of *A. salmonicida*. However, the presence of 0.1% starch did not appear to affect the extracellular caseinase activity, but 1% starch reduced the caseinase activity by approximately 25%.

5.6 Discussion

The response of *A. salmonicida* to various environmental and nutritional factors revealed general similarities between this organism and other bacteria. Incorporating the iron chelator EDDA into the culture medium demonstrated the ability of *A. salmonicida* to survive and proliferate in a low iron environment. This ability of *A. salmonicida* to adapt to iron-restricted conditions confirms previous findings which relate survival of this organism to acquisition of iron by two different mechanisms (Hirst *et al.* 1991). One mechanism was inducible and appeared to involve production of low molecular weight siderophores (Chart & Trust 1983; Hirst *et al.* 1991) and induction of iron-

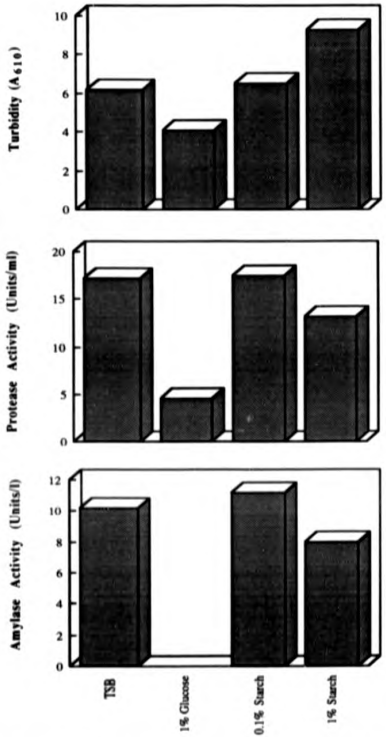


Figure 5.7. The effect of glucose and starch on growth and the production of selected activities of *A. salmonicida* strain MT004. Activities were determined as described in Section 2.5

repressible outer membrane proteins (Chart & Trust 1983; Aoki & Holland 1985), while the other mechanism required cell contact with Fe^{3+}-transferrin or -lactoferrin (Chart & Trust 1983).

In this study, the protein profiles of the outer membrane fraction from cells grown under iron-restricted conditions exhibited three iron-repressible outer membrane proteins: M_rs' 77, 79 and 89kDa. These M_r values compare reasonably with those previously published by Chart & Trust (1983) and Aoki & Holland (1985), which were 77, 78 and 83kDa and 72, 74 and 93kDa respectively. The variation is most probably due to the electrophoretic analysis of the outer membrane fraction rather than a qualitative difference in the iron-repressible outer membrane proteins. The values reported in this study were obtained using 7.5% acrylamide in the resolving gel of the SDS gels, compared with 11 and 12.5% acrylamide gels used by Aoki & Holland (1985) and Chart & Trust (1983) respectively. The reduction in acrylamide concentration in the resolving gel allows a better separation of higher molecular weight proteins, such as the iron-repressible outer membrane proteins, and as a consequence a more accurate molecular weight estimation of these proteins should be achieved. The production of normally repressed outer membrane proteins under conditions of iron-limitation by *A. salmonicida* is in line with the response of numerous other Gram-negative bacteria (Neilands 1982). The actual role of the iron-repressible outer membrane proteins of *A. salmonicida* remains unclear, but they may act as receptors for the Fe^{3+}-siderophore complex, or possibly they may directly bind to lactoferrin and transferrin as an alternative to the siderophore-mediated iron uptake mechanism.

In addition to previous studies on the effect of low Fe^{3+} levels on *A. salmonicida*, it has been demonstrated that the extracellular enzyme production of this organism is also altered. A reduction in free Fe^{3+} resulted in a decrease in extracellular caseinase activity of approximately 25%, while supplementation of the growth medium with $FeCl_3$ resulted in an increase in caseinase activity by approximately 20%. In contrast with *A. salmonicida*, the extracellular protease activity of *A. hydrophila* was unaffected by

$FeCl_3$, but protease secretion was significantly reduced by the addition of Fe^{2+} ions to the growth medium (Pansare *et al.* 1985).

In addition to Fe^{3+} ions, a number of other ions/compounds were observed to affect the production of extracellular caseinase. For example, the addition of NH_4^+ ions to the culture medium of *A. salmonicida* appeared to affect the organism in a similar, but more dramatic manner as Fe^{3+}-supplementation, i.e., the extracellular caseinase activity was once again selectively reduced but to a much greater extent (75% compared to 20%). Supplementation of the growth medium with glucose or starch also caused a decrease in the production of extracellular caseinase activity, but in these cases the effect was not specific since extracellular amylase activity was also suppressed. In fact, it has been demonstrated that the presence of glucose completely suppresses all extracellular protein production by this organism (Coleman *et al.* 1987).

A number of other factors have also been shown to affect extracellular protein production by *A. salmonicida*. For example, under anaerobic growth extracellular levels of protease were approximately only 10% of that when *A. salmonicida* was grown aerobically (Fyfe *et al.* 1986b). These results, together with previously published data, including the effect of pH (Dahle 1971b), complex substances (e.g., peptone & casein) (Dahle 1971b) and amino acids (Sakai 1985b), on *A. salmonicida* extracelluar caseinase production, indicate that the synthesis of this enzyme is subject to a multiple control mechanism.

One important aspect of these results which should be noted is the effect of elevated culture temperature on suppressing the production of extracellular enzymes by *A. salmonicida*. The suppressed extracellular protein production by this organism at higher growth temperatures has been correlated with a decrease in membrane-associated ribosomes at the higher temperatures (Campbell *et al.* 1990). An increase in incubation temperature has previously been associated with the loss of ability of typical strains to produce the A-layer (Ishiguro *et al.* 1981), and the presence of the A-layer has been

correlated with virulence of this organism (Udey & Fryer 1978; Ishiguro *et al.* 1981). Since hydrolytic enzyme secretion appears to be affected under the same conditions *in vitro* as the A-layer, it may indicate a similar type of control mechanism operating in both situations: in the case of the A-layer, this consists of a partial gene deletion affecting the amino terminal region of the A-protein (Belland & Trust 1987). However, this is unlikely since there is a major difference between the control of these virulence factors in that, unlike loss of ability to produce the A-layer, the effect on protein secretion is reversible.

Since most, if not all, extracellular proteins are affected by an increase in growth temperature it is quite likely that extracellular protein synthesis and secretion is under the control of a single regulator (called a regulon) which exerts its effect on unlinked genetic components of the extracellular proteins (Gottesman 1984; Griffiths 1989). Generally, the regulator, which may be an activator or a repressor, recognises a particular sequence common to all of the genes in the regulon and assures the coordinate expression of the regulon genes in response to a particular stimulus. This higher regulatory regulation network, termed global regulation, allows a concerted response to specific environmental stimuli including altered levels of nutrients (e.g., phosphate & nitrogen limitation) and aerobic/anaerobic, temperature and osmolarity changes, and has been demonstrated in a number of bacterial species (Gottesman 1984; Neidhardt *et al.* 1990). In the case of *A. salmonicida*, a number of factors may specifically regulate the individual genes encoding extracellular enzymes (e.g., NH_4^+ ions affect protease production; high starch levels reduce amylase production), but other stimuli (e.g., temperature & glucose availability) may operate at a higher regulatory level and involve the coordinate regulation of a number of extracellular protein operons.

6. Raising antibodies against selected extracellular proteins

6.1 Introduction

Antibodies are probably the most widely used tool in studying protein export and secretion. Immunoblotting (Western blotting) and immunoprecipitation are commonly used immunochemical techniques which have been successfully applied to protein export and secretion studies. When these techniques are coupled with SDS-polyacrylamide gel electrophoresis and the use of radioisotopes, a number of important characteristics of the antigen can be determined readily. These assays can be used to determine the presence, quantity, and specificity of an antigen, the relative molecular weight of the polypeptide chain, its rate of synthesis or degradation, and presence of certain post-translational modifications.

The availability of antibodies against extracellular proteins is clearly advantageous in the comparative study of protein secretion in *A. salmonicida* and related species. In addition to their use in identifying antigenically related molecules in the same, and between different, bacterial species of *Aeromonas*, the antibodies could be used in future studies, for example in determining the route and mechanism of export out of the cell.

In considering the preparation of an immunogen the major decision to be made is how pure does the immunogen need to be before starting an immunisation schedule. Immunoblotting is the main immunochemical technique employed in this study of protein secretion and is used for the purpose of comparing a range of *Aeromonas* species for cross-reactivity to specific extracellular components. Therefore, antibody specificity is obviously of paramount importance, and so the immunogen must be purified to homogeneity. Raising unispecific antibodies demands preliminary work in the purification of the proteins under examination. When using protein antigens, if the polypeptide of interest can be seen as a unique band on an SDS-polyacrylamide gel,

then the band from the gel can be used as the purified material against which to raise antibodies. Antigens purified this way often induce good antibody responses. Since the immunogen is denatured by this route of preparation, the resulting antibodies are usually particularly good for techniques that need or benefit from denaturation-specific antibodies, including Western blotting.

The antigens chosen for immunochemical study were the extracellular 70kDa serine protease and an extracellular protein of molecular weight 56kDa of undefined activity. The reason for selecting the 56kDa protein was that previous workers had indicated that a major extracellular component of this molecular weight was in fact a haemolysin (Fyfe *et al.* 1987a). Since significant haemolytic activity could be detected in the culture filtrate of the principal *A. salmonicida* strain used in this study (MT004, see Section 4.2) it was decided to undertake a more detailed examination of this activity. Any inhibitory effect of incorporating polyclonal antibodies raised against the extracellular 56kDa protein into the trout erythrocyte microtitre plate assay on the haemolysin activity would allow a more positive correlation between the 56kDa protein and the extracellular haemolytic activity of *A. salmonicida*. The haemolytic titration assay used in a number of previous studies on *A. salmonicida* (Titball & Munn 1981, 1983, 1985a; Hastings & Ellis 1985; Lee & Ellis 1990) and in this study only reveals the overall haemolytic activity present in the culture filtrate. Possessing an antibody against a specific haemolysin may allow a more detailed study of the extracellular haemolytic activities of *A. salmonicida* and related species. The presence of haemolytic activity in other *Aeromonas* species may be examined in relation to a specific extracellular haemolysin of *A. salmonicida*.

In the same way the 70kDa protease was selected for antibody studies to enable the differentiation, or otherwise, of this enzyme from other caseinase activities which may be present in the culture supernatants of other *Aeromonas* species. Any antigenic similarities observed between the culture filtrates of the *Aeromonas* species may indicate a possible relationship in the virulence mechanisms of these organisms.

6.2 Preparation of immunogens

6.2.1 Purification of the 70kDa extracellular serine protease

The purification steps for the 70kDa protease are summarised in Table 6.1. The procedure is based on that described by Fyfe *et al.* (1986a) and subsequently modified by Price *et al.* (1989). An additional 0-65% ammonium sulphate fractionation cut was included as the initial step in this purification procedure. This step was just as effective as lyophilisation for concentrating the culture filtrate, and in addition, whereas there was no selectivity in concentrating by lyophilisation, ammonium sulphate fractionation had the advantage of removing all the proteins which would only precipitate at concentrations of the salt greater than 65%. The caseinase activity was found to be exclusive to the 0-65% ammonium sulphate fraction, with no activity detectable in the supernatant. The precipitates were dissolved in 10mM sodium phosphate buffer (pH 7.0) and after dialysis against the same buffer, the crude protease was obtained. Approximately 75% of the total caseinolytic activity was recovered after the ammonium sulphate fractionation step.

The subsequent DEAE-Sephadex anion-exchange chromatography stage yielded one quite broad caseinolytic peak, which was eluted mainly in the 150 and 200mM portions of the sodium phosphate (pH 7.0) solutions (Fig.6.1). Fractions containing most activity were pooled, concentrated and applied to the Sephacryl S-300 gel filtration column. A typical elution profile from the Sephacryl column is shown in Figure 6.2. The pooled fractions of caseinolytic activity from the Sephacryl column contained about 0.6mg protein. The specific activity of the preparation (1983 units/mg) represented a 6.6-fold purification over the initial culture supernatant (Table 6.1).

Electrophoresis of the purified extracellular protease on SDS-polyacrylamide gels gave a single protein band (Fig.6.3), confirming the homogeneity of the preparation. Comparison of its electrophoretic mobility with those of standard proteins indicated that it had a molecular weight of 70kDa. This value for the molecular weight of the

Table 6.1. Purification of an extracellular caseinolytic protease from *A. salmonicida* strain MT004

	Volume (ml)	Protein (mg)	Activity (units)	Specific Act. (units/mg)	Yield (%)
Culture supernatant	225	11.25	3375	300	100
0-65% $(NH_4)_2SO_4$ cut	4	7.38	2534	343	75
DEAE-Sephadex pooled fractions	65	2.68	2326	868	68
Sephacryl S-300 pooled fractions	18	0.58	1150	1983	34

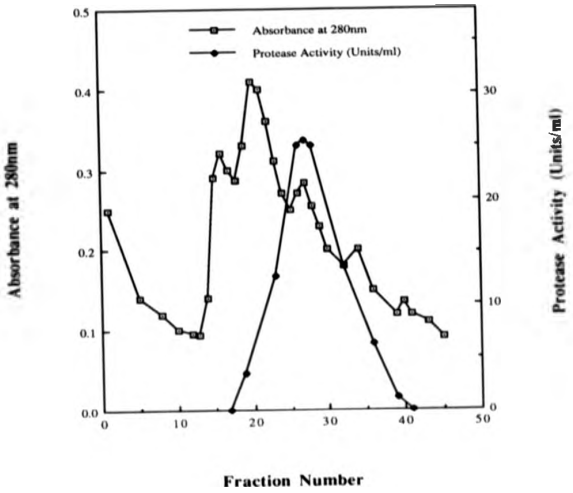


Figure 6.1. DEAE-Sephadex anion-exchange chromatography of extracellular products of *A. salmonicida* strain MT004. The purification of the 70kDa protease from the culture filtrate is described in Section 6.2. Fractions (4ml) were collected and analysed for protein by absorbance at 280nm (A_{280}) and caseinolytic activity. Fractions 22-33 were pooled for further purification

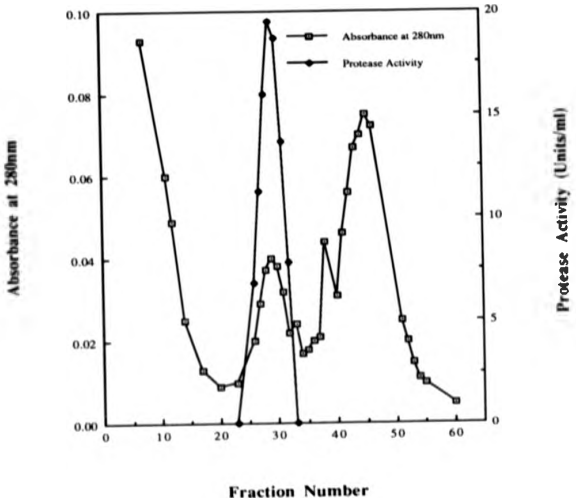


Figure 6.2. Sephacryl S-300 gel filtration chromatography of extracellular products of *A. salmonicida* strain MT004. The purification of the 70kDa protease from the culture filtrate is described in Section 6.2. Fractions (6ml) were collected and analysed for protein by absorbance at 280nm (A_{280}) and caseinolytic activity. Fractions 26-32 were pooled and used as the purified material

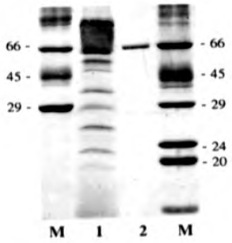


Figure 6.3. SDS-polyacrylamide gel electrophoresis of the purified 70kDa extracellular protease from *A. salmonicida* strain MT004. Unfractionated culture filtrate (lane 1) and purified 70kDa protease (lane 2) respectively. Lanes 1 and 2 contain approximately 15 and 2μg of protein respectively. Lane M, molecular weight standards (for full description see Fig.3.1)

caseinase enzyme is in accord with that reported previously (Fyfe *et al.* 1986a; Price *et al.* 1989).

As a final purification step the Sephacryl fraction was subjected to preparative SDS-polyacrylamide gel electrophoresis (Fig.6.4). The protease band was excised for immunogen preparation. This additional step was performed to reduce even further the presence of any low-level contaminants and also eliminate the possibility of high molecular weight aggregates in the purified sample.

6.2.2 Purification of a 56kDa extracellular protein

A 0-60% ammonium sulphate fractionation of culture filtrate was prepared. This procedure removed over 30% of the extracellular protein present in the culture medium. The pellet produced by precipitating with 60% ammonium sulphate was redissolved in distilled H_2O. The selectively concentrated sample was then applied to a preparative SDS-polyacrylamide gel (Fig.6.5). After electrophoresis, the protein band of interest was located in the gel by a light staining with Coomassie blue. The 56kDa protein was shown to be relatively abundant and reasonably separated from nearby contaminating bands. The desired gel slice containing the 56kDa protein band was carefully excised to obtain the pure polypeptide for immunisation purposes.

The final purified preparations of the extracellular 70kDa serine protease and 56kDa protein are shown in Figure 6.6. These samples were obtained by homogenising excised gel and boiling in SDS-containing sample buffer. Figure 6.6 demonstrates the homogeneity of the two immunogen preparations used in the immunisation regime.

6.3 Characterisation of the antisera

Analysis by Western blotting revealed that the antisera raised against the 70kDa protease and the 56kDa protein recognised specific proteins in the samples of medium from cultures of strain MT004 (Fig.6.7). Antibodies raised against the extracellular protease

Figure 6.4. Preparative SDS-polyacrylamide gel electrophoresis of the purified 70kDa extracellular protease of *A. salmonicida* strain MT004. Approximately 100µg of protein was applied to the gel. Direction of migration was from top to bottom (cathode to anode)

Figure 6.5. Preparative SDS-polyacrylamide gel electrophoresis of a 0-60% $(NH_4)_2SO_4$ fractionation of *A. salmonicida* strain MT004 culture filtrate. The band excised is arrowed. Approximately 500µg of protein was applied to the gel. Direction of migration was from top to bottom (cathode to anode)

Figure 6.6. The final purified preparations of the extracellular 70kDa and 56kDa proteins of *A. salmonicida* strain MT004. Purified 56kDa protein (lane 1) and 70kDa protease (lane 2) were used as immunogens for polyclonal antibody production. See text for sample preparation. Lane M, molecular weight standards (for full description see Fig.4)

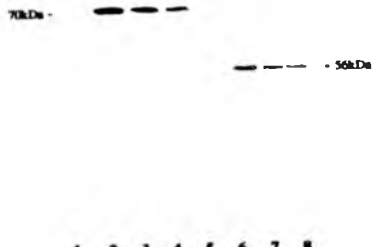


Figure 6.7. Western blot characterisation of the polyclonal antisera raised against the extracellular 56kDa protein and 70kDa protease. Samples of *A. salmonicida* strain MT004 culture filtrate were applied (lanes 1-8) and probed with pre-immune antiserum (lanes 1& 5), antisera raised against the 70kDa protease (lanes 2-4) and the 56kDa protein (lanes 6-8). Antibody dilutions used were 1: 250 (lanes 2 & 6), 1: 1000 (lanes 3 & 7) and 1: 5000 (lanes 4 & 8) respectively. Approximately 10μg of protein was applied in each case

recognised a single protein band with a molecular weight of 70kDa (lanes 2-4); the antibodies raised against the 56kDa extracellular protein also recognised a single band (molecular weight, 56kDa; lanes 6-8). These results, in conjunction with the absence of banding observed with pre-immune sera (lanes 1 & 5), suggest that the immunogen preparation and the immunisation regime have successfully produced unispecific antibodies to the desired extracellular proteins of *A. salmonicida*. The finding that the antibody binding can be visualised at high dilutions such as 1:5000 (lanes 4 & 8) indicates that the antisera will be of sufficient quality to allow the use of dilute primary antibody which will reduce non-specific binding of immunoglobulin to a minimum.

6.4 Is the 56kDa extracellular protein a haemolysin?

Antiserum raised against the purified 56kDa extracellular protein was added (1:100 dilution) to the trout erythrocyte suspension (see Section 2.5.4) in one row of wells in a microtitre plate. In another row of wells on the same microtitre plate pre-immune antiserum was incorporated into the reaction mixture (also 1:100 dilution) and used as a control. After 10min pre-incubation at room temperature culture filtrate was added to the wells and the reaction mixture was incubated for a further 60min at 37°C. The haemolytic activity was found to be significantly reduced by the addition of the antiserum raised against the 56kDa extracellular protein. Addition of the antiserum substantially lowered the haemolytic activity of the culture supernatant of *A salmonicida* from 64 HU in the control to 8 HU, but did not completely inhibit lysis of the red blood cells. The haemolytic activity did not appear to be affected by the presence of non-immune serum in the reaction wells. It is most probable that the reduction in haemolytic activity is due to the antibodies forming an immune complex with the 56kDa protein, and so inhibiting its activity. Therefore, this would imply that the 56kDa protein is an extracellular haemolysin of *A. salmonicida*.

6.5 Discussion

An important point in raising antibodies against desired antigens for the purpose of screening organisms for antigenically related components is the preparation of highly purified material for use as the immunogen. Injection of pure preparations of immunogen produce antibodies of high specificity which allows for a simple and unambiguous interpretation of the screening experiments. On the evidence of the Western blot analysis (Fig.6.7) it is apparent that the immunogens used in the immunisation regimes were of sufficient quantity and purity to ensure a good immune response resulting in the production of two unispecific polyclonal antibodies. The specificity of these antibodies will ensure that any cross-reactivity observed in the culture supernatants of *A. salmonicida* and related species will allow a confident prediction that the cross-reactive components are antigenically related to either the 70kDa protease or the 56kDa haemolysin. In possible future studies on the secretion of these extracellular proteins the specificity of the antibodies will be of particular importance for immunoprecipitation experiments using radiolabeled proteins, because the sensitivity of this technique would exaggerate any non-specific binding and, in turn, complicate the interpretation of the autoradiograms by having high non-specific background.

The importance of the specificity of the antibodies raised against the 56kDa extracellular protein was made apparent by their use in demonstrating the enzyme activity of this protein. The antibodies were demonstrated to inhibit the lysis of trout erythrocytes in the haemolytic microtitre plate assay; since the antibodies were known to be unispecific, this enabled a reasonably confident identification of the 56kDa extracellular protein as a haemolysin. The molecular weight determined for this protein is in accord with a previous report of a haemolysin enzyme (Fyfe *et al.* 1987a). Pre-incubation of the assay mix with antibodies against 56kDa extracellular protein did not, however, result in the complete inhibition of trout erythrocyte lysis. The most obvious explanation for this observation is that *A. salmonicida* secretes more than one haemolytic enzyme into the culture medium. However, it is also possible that *A. salmonicida* produces a single

haemolysin but that it is secreted in more than one form, with only one of these forms in a state open to antibody-binding. For example, Ellis (1991) has suggested that all of the haemolysin/GCAT-type enzymes so far reported in the literature (MacIntyre *et al.* 1979; Titball & Munn 1981, 1983; Fyfe *et al.* 1987a; Nomura *et al.* 1988; Lee & Ellis 1989) may only be different purified forms of a GCAT/LPS entity (see Section 1.2.2). Therefore, it may be that this enzyme is part of the GCAT/LPS entity and, as such, the proportion of the enzyme complexed with LPS may be sterically protected from antibody binding by the carbohydrate moieties of the LPS. This would result in only the soluble free form of the enzyme being inhibited by antibody binding and the complexed form retaining its activity, which would correspond with the partial inhibition of haemolysis observed in the microtitre plate assay. However, these results alone are not sufficient to allow a conclusion to be reached; it will be necessary to achieve at least a partial purification of the haemolytic activity to determine whether more than one haemolysin exists.

7. Comparative biochemistry of extracellular protein production by *A. salmonicida* and related species

7.1 Introduction

The classification of the *Aeromonas* genus is a matter of contention (Colwell *et al.* 1986), with the literature containing a number of suggested taxonomic revisions, particularly with respect to the motile species (Austin *et al.* 1989). There are several aspects of a cell that can be used to identify it and to determine its relationship with other organisms. Traditionally, morphological and physiological characteristics played key roles in bacterial taxonomy. In recent years, genetic relatedness has been introduced to the characterisation process through the development of methods for the determination of DNA base ratios and nucleic acid homologies.

Organisms are generally classified on the basis of their morphology, staining characteristics, and physiology, but may also be classified on the basis of growth, biochemical, and genetic characteristics. The shape of the cell is one of the first characteristics that is used in the identification of bacteria. Most bacteria can be characterised as spheres, rods, spirals or branches. The Gram stain divides bacteria into two groups based on the type of cell wall they have. The ability to ferment carbohydrates is another important aspect of bacterial identification. Many closely related organisms can be distinguished by their abilities to ferment various carbohydrates and metabolise assorted compounds (e.g., proteins, fats and starch). Growth on selective media often help in the identification of an organism.

Some bacteria can be distinguished on the basis of serological reactions, i.e., using antibodies against unique chemical properties of the bacterial cell surface. This is the basis of the slide agglutination test, in which bacteria clump together if antibodies recognise cell surface antigens. Antibodies raised against purified proteins of a bacterial species may also be used for serological studies. Closely related proteins will react in a

similar manner with antiserum, and so the extent of cross-reaction between proteins from different bacterial species will reflect the degree of relatedness between these organisms.

Various combinations of these methods have enabled the identification and characterisation of a very large number of bacteria. However, disagreement on the relative importance of the individual characteristics led to the development of numerical taxonomy by Sneath (1963) which groups organisms by quantifying the similarities and differences among them. The relationship is expressed mathematically as the ratio of the number of characteristics the organisms share to the total number of features compared; it is expressed as a percentage. A large number of tests must be conducted and double-negative tests are usually not included. Therefore, the similarity coefficient is a calculation of relatedness that does not include double negatives.

Methods based on genetic relatedness of organisms may also used to identify and classify organisms. Since the early 1960s evolutionary relationships can often be deduced by comparing the base composition of their DNA (Doty 1962). Organisms that are closely related have DNAs with very similar sequences of nucleotides. In the base-pairing of double-stranded DNA, the numbers of adenine (A) and thymine (T), and guanine (G) and cytosine (C), are equal. However, the ratio of (G+C) bases to (A+T) bases varies in different bacteria and the ratio is normally expressed as the % (G+C). Since the % (G+C) is very similar for a given species (≤5% variation between subspecies) and genus (≤10% variation between species) it has become an important measurement in genetic relatedness. Species having similar % (G+C) values may be closely related, but caution must be applied since bacteria with similar % (G+C) values are not necessarily closely related; therefore, this method must be used in conjunction with other taxonomic criteria.

The common technique for determining the % (G+C) content of DNA is to determine its melting temperature (T_m) by measuring its absorbancy at 260nm at various temperatures. The principle behind this method is that DNA with a higher % (G+C)

will exhibit a higher T_m. The GC base pairs are held together by three hydrogen bonds, whereas AT base pairs are linked by only two hydrogen bonds, so separating them will require a higher temperature.

In the early 1970s the development of the DNA:DNA hybridization technique by De Ley (1970) allowed the degree of hybridisation between bacterial DNAs and, in turn, the evolutionary relationships between organisms to be determined. Organisms that are closely related have similar proteins, and so, in turn, the DNA that codes for these proteins must also be similar. Consequently, if the complementary DNA strands from closely related organisms are allowed to reanneal, there should be a good match and the two strands will hybridize with each other. However, the more unrelated organisms are, the poorer their DNAs will hydrogen bond and so they will form poor DNA hybrids. Thus, the degree of relatedness can be determined by the extent of hybridization.

The development of polynucleotide sequencing techniques (Maxam & Gilbert 1977; Sanger *et al.* 1977) in the mid-1970s allowed protein sequences to be compared directly as a measurement of relatedness. Nucleotide sequencing of the 16S ribosomal RNAs has been the most common approach of this type. The base sequences of rRNAs are highly conserved, so they are accurate indicators of evolutionary relatedness. Perhaps once the technology involved in sequencing becomes more accessible and straightforward, the direct comparison methods may be a feasible procedure for everyday identification and classification.

Another commonly used identification system is that of phage typing. Bacteriophages (bacterial viruses) generally have a very narrow host range, that is they infect only very closely related bacteria. Thus, the ability of specific bacteriophage to grow on a bacterial population can be used to identify or type bacteria.

The identification and classification of *A. salmonicida* and the *Aeromonas* genus has been determined by a combination of these methods, and is discussed in some detail in

Section 1.1.1. However, the following study involves a different type of procedure for comparing relatedness of bacteria. Strains of *A. salmonicida* ssp. *salmonicida* have been found to be extremely homogeneous in their phenotypic and genotypic properties. Since members of the *Aeromonas* genus are known to secrete a number of enzymes into the culture medium, it was decided to examine the culture filtrates of these organisms to determine whether the high degree of homogeneity noted in other properties of *A. salmonicida* ssp. *salmonicida* is also displayed in its extracellular enzyme production. Examination of *A. salmonicida* ssp. *salmonicida* strain MT004 culture filtrate by *in situ* detection of protease and amylase activities on substrate-SDS-gels resulted in a detailed hydrolytic enzyme profile of this organism. This procedure is relatively straightforward and allows the direct comparison of a large number of bacterial strains with each gel run. Therefore, this method was used as the basis for determining any possible variation of extracellular hydrolytic enzyme production by *A. salmonicida* ssp. *salmonicida*, and also for comparing the hydrolytic secretory products of other members of the *Aeromonas* genus.

In addition to this novel comparative procedure, the relatedness of the extracellular 70kDa protease and 56kDa haemolysin of *A. salmonicida* ssp. *salmonicida* strain MT004 with other members of this subspecies and *Aeromonas* species was also examined. These techniques may add to the current state of knowledge on the question of classification of the *Aeromonas* genus.

7.2 Comparative study of extracellular protease production by *A. salmonicida* and related species

A number of *A. salmonicida* strains and a selection of other related species were investigated with respect to the secretion of extracellular proteases. The study of extracellular protease production was approached in three different ways; firstly, the strains were examined for the production of extracellular caseinase enzyme activity; secondly, polyclonal antibodies raised against the purified extracellular caseinase (see

Section 6) were used to probe the strains to reveal any cross-reactivity; lastly, culture filtrates of all the strains studied were applied to gelatin-SDS-gels and processed in the normal way to determine the variation, or otherwise, of the caseinase and non-caseinase proteolytic enzymes produced by these organisms.

7.2.1 Extracellular caseinase enzyme activity

The strains under investigation were tested for extracellular caseinase activity using the standard assay procedure used for *A. salmonicida* strain MT004 (described in Section 2.5.2). The results revealed that not all strains exhibited significant caseinase activity (Fig.7.1). Those strains which were caseinase-negative included *A. salmonicida* ssp. *masoucida*, *A. media* and *A. sobria*. With *A. salmonicida* ssp. *salmonicida* and *achromogenes* some differentiation in extracellular caseinase production of these strains was displayed by this assay procedure. *A. salmonicida* ssp. *salmonicida* strain MT028 displayed very little caseinase activity, in contrast to the other strains of this subspecies tested which all exhibited high activities for the extracellular caseinase enzyme(s). Similarly, for the two strains of ssp. *achromogenes* examined MT365 was found to be high in caseinase activity, whereas strain MT535 was caseinase-negative.

The assay procedure also revealed that *A. salmonicida* produced less caseinase activity as the growth temperature was increased (see Section 5.2.1). At a growth temperature of 32°C *A. salmonicida* produced only 10% the amount of protease compared with cultures incubated at 24°C. When the extracellular protein profiles of cultures grown at 22 and 32°C were compared by SDS-polyacrylamide gel electrophoresis it could clearly be seen that the intense band of M_r 70kDa present in the culture grown at 22°C was greatly reduced in the extracellular medium prepared from cultures grown at 32°C (see Section 5, Fig.5.1). The effect of growth temperature on caseinase production by *A. hydrophila* was examined to determine whether it followed the same pattern as *A salmonicida* (Fig.5.2). *A. hydrophila* produced approximately the same caseinase activity as *A. salmonicida* when grown at 22°C, however unlike that of *A*.

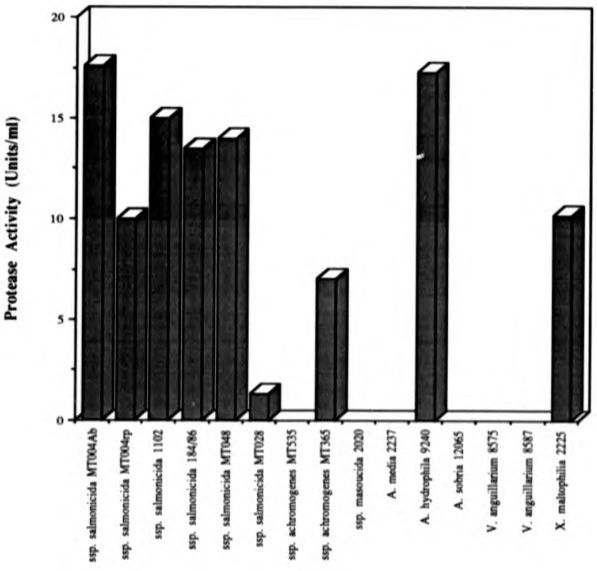


Figure 7.1. Extracellular protease activity of different *Aeromonas* strains and other isolates. Activity was determined as described in Section 2.5.2

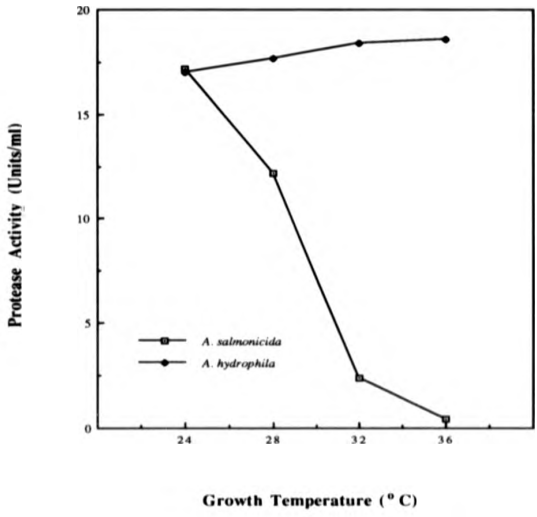


Figure 7.2. The effect of growth temperature on the production of extracellular caseinase enzymes by *A. salmonicida* and *A. hydrophila*. Activity was determined as described in Section 2.5.2

salmonicida the caseinase activity of *A. hydrophila* was not significantly altered at the higher growth temperatures (Fig. 7.2).

7.2.2 Western blot screening with polyclonal antibodies against the 70kDa protease

Western blotting experiments, using rabbit polyclonal antibodies raised against purified extracellular 70kDa protease, would reveal whether the caseinase-producing species were all secreting a caseinase enzyme related to the 70kDa protease or if antigenically different proteases were responsible for the extracellular caseinase activities of these organisms. The 70kDa antiserum was used to screen the strains for any possible cross-reactivity (Fig.7.3). The results obtained indicated that of the six *A. salmonicida* ssp. *salmonicida* strains (lanes 1-6) probed only MT028 (lane 6) did not display any sign of cross-reactivity. The other 5 strains all gave a positive result with a single band of M_r 70kDa observed. All other strains screened were negative for cross-reactivity with the exception of *A. hydrophila* (lane 11) which exhibited a single band at a slightly lower molecular weight (approx. 65kDa).

7.2.3 Gelatin-SDS-polyacrylamide gel electrophoresis

The previous experiments revealed that not all the organisms secreted caseinase-type enzymes and that the caseinases that were detected were not all antigenically related. It is most probable that the strains that were caseinase-negative would produce other types of proteases capable of hydrolysing other protein substrates.

Gelatin-SDS-gel electrophoresis was carried out in order to gain a further insight into the number and the properties of the extracellular proteases produced by these strains and also the individual properties of the caseinolytic enzymes detected by the caseinase assay. Gelatin-SDS-gel electrophoresis revealed a large and heterogeneous group of extracellular proteases produced by the genus *Aeromonas* and related species (Fig.7.4). The pattern of protease production amongst *A. salmonicida* ssp. *salmonicida* strains was quite consistent with two main bands of activity, the principal protease having an

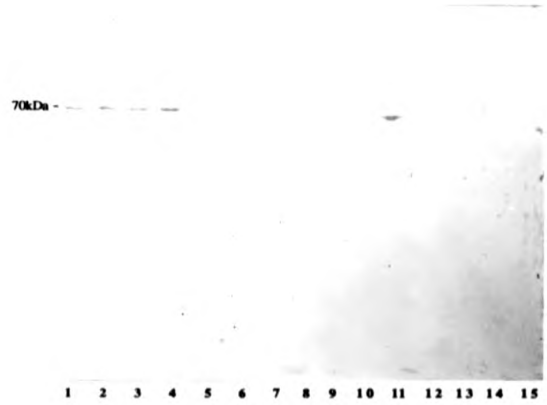


Figure 7.3. Western blot of the extracellular fractions of *A. salmonicida* ssp. *salmonicida* strains MT004 (lane 1), MT004rp (lane 2), 1102 (lane 3), 184/86 (lane 4), MT048 (lane 5), MT028 (lane 6), *A. salmonicida* ssp. *achromogenes* strains MT535 (lane 7), MT365 (lane 8), *A. salmonicida* ssp. *masoucida* 2020 (lane 9), *A. media* 2237 (lane 10), *A. hydrophila* 9240 (lane 11), *A. sobria* 12065 (lane 12), *Vibrio anguillarium* strains 8575 (lane 13), 8587 (lane 14) and *Xanthomonas maltophilia* 2225 (lane 15). Samples of culture filtrate were screened with polyclonal antiserum (1: 1000 dilution) unispecific to the 70kDa extracellular protease

ORIGINAL IN COLOUR

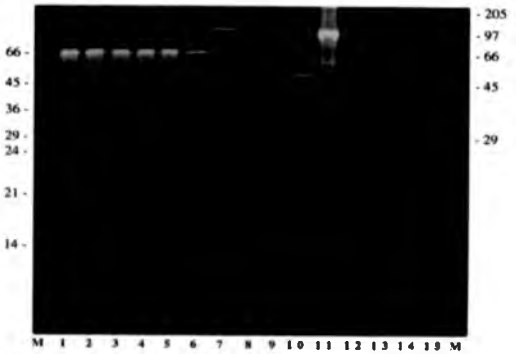


Figure 7.4. Gelatin-SDS-polyacrylamide gel electrophoresis of the extracellular fractions of *A. salmonicida* ssp. *salmonicida* strains MT004 (lane 1), MT004rp (lane 2), 1102 (lane 3), 184/86 (lane 4), MT048 (lane 5), MT028 (lane 6), *A. salmonicida* ssp. *achromogenes* strains MT535 (lane 7), MT365 (lane 8), *A. salmonicida* ssp. *masoucida* 2020 (lane 9), *A. media* 2237 (lane 10), *A. hydrophila* 9240 (lane 11), *A. sobria* 12065 (lane 12), *Vibrio anguillarium* strains 8575 (lane 13), 8587 (lane 14) and *Xanthomonas maltophilia* 2225 (lane 15). Approximately 0.25µg of protein was applied in each case. Lane M, molecular weight standards (for full description see Figs.3.1 & 3.2)

apparent M_r of 70kDa and the other M_r 33kDa (lanes 1-5). Strain MT028 had a similar band of activity in the 70kDa region (lane 6), but this strain was shown previously to be very low in caseinase activity and did not display any cross-reactivity with the antiserum against the 70kDa protease. This would suggest that the proteases were most probably quite different. Strain MT028 also produced a number of additional proteases that were not present in the other ssp. *salmonicida* culture filtrates. These proteases displayed apparent molecular weights of 105, 90, 66, 35 and 34kDa on gelatin-SDS polyacrylamide gels (Table 7.1).

Of the other two subspecies of *A. salmonicida*, namely *achromogenes* and *masoucida*, only ssp. *achromogenes* consistently displayed protease activity on gelatin gels (Fig. 7.4, lanes 7 & 8). Ssp. *achromogenes* exhibited a small degree of heterogeneity in its extracellular protease production. Both ssp. *achromogenes* strains MT535 and MT365 secreted four proteases with apparent molecular weights of 110, 95, 70 and 60kDa. However, strain MT365 also secreted an additional extracellular protease of apparent molecular weight 34kDa. This protease appears to present in substantial amounts in the extracellular medium of MT365 and so suggests that the variation between the two strains is not simply due to differences in sample loading. Little extracellular gelatinase activity was produced by ssp. *masoucida*, with only two very faint high molecular weight bands (110 & 95kDa) observed, even when samples high in protein were loaded.

Considerable variation in extracellular protease production was observed in the other three *Aeromonas* species examined, both with each other and with the *A. salmonicida* subspecies. *A. media* and *A. sobria* both produced proteases of apparent molecular weights 110 and 95kDa. These proteases may correspond to those previously mentioned being produced by *A. salmonicida* ssp. *achromogenes* and *masoucida*. *A. media* also secreted three other proteases into the external medium (lane 10); a major activity of apparent molecular weight 50kDa and two minor activities (45 & 44kDa). Two other minor activities with apparent molecular weights of 70 and 50kDa were also observed in the extracellular samples of *A. sobria*.

Table 7.1. Extracellular protease activities produced by *Aeromonas*, *V. anguillarium* and *X. maltophilia* isolates, and some characteristics of the enzymes [a]

Isolate	Strains	M_r (kDa)	Type	Heat stability
A. salmonicida				
ssp. *salmonicida*	MT004, 1102,	80	Serine	-
	184/86 & MT048	70	Serine	-
		60	Serine	-
		45	Serine	-
		33	Metallo	-
	MT028	105	Metallo	-
		90	Metallo	-
		70	Serine	[+]
		38	?	-
		34	Metallo	-
ssp. *achromogenes*	MT365 & 535	110	Serine	+
		95	Serine	+
		70	Metallo	-
		66	Metallo	-
		34[b]	Metallo	+
ssp. *masoucida*	2020	110[c]		
		95[c]		
A. media	2237	50	Metallo	+
		45	Metallo	+
		44	Metallo	+
A. hydrophilia	9240	110	Serine	-
		60	Serine	-
		49	Serine	-
		33	Metallo	-

cont. overleaf

Isolate	Strains	M_r (kDa)	Type	Heat stability
A. sobria	12065	110[c]		
		95[c]		
		70	?	+
V. anguillarium	8575 & 8587	60	Metallo	+
		56	Metallo	+
		55	Metallo	+
X. maltophilia	2225	>200	?	?
		70	Serine	-
		46	Serine	[+]
		45	Serine	-
		43	Serine	-

[a] Symbols: +, positive; [+], partially positive; -, negative; ?, unknown

[b] The 34kDa protease is not produced by strain MT535

[c] Only trace amounts of activity present

A number of proteolytic activities were also demonstrated in the culture filtrate of *A. hydrophila* strain 9240 (lane 11). The major band of activity displayed an apparent molecular weight of 90kDa. Other less prominent, but still quite significant, protease activities with apparent molecular weights of 60, 49 and 33kDa were also observed in the culture filtrate of strain 9240.

Two *Vibrio anguillarium* (serotype 2) strains, 8575 and 8587, and an unrelated *Xanthomonas maltophilia* strain were also analysed for extracellular protease activity. The two *V. anguillarium* strains displayed identical banding patterns with a main protease band of apparent molecular weight 60kDa (lanes 13 & 14). *X. maltophilia* produced several significant proteases with apparent molecular weights of >180, 70, 46, 45 and 43kDa (lane 15).

Caseinase assays identified strains with the capacity to hydrolyse casein (see Section 7.2.1). Figure 7.4 demonstrated the full range of SDS-stable extracellular proteolytic activities present in the culture filtrates of the respective strains. To determine which activities were the caseinase-type enzymes from the complex protease patterns, gels incorporating azocasein were used. The results obtained with azocasein-SDS-gels revealed a much simpler protease pattern (Fig.7.5), implying that only a relatively small number of the proteases have the capacity to hydrolyse casein-type substrates. In general, the presence of caseinase-type enzymes in the culture filtrates of certain strains (*A. salmonicida* ssp. *salmonicida* strains MT004, MT004rp, 1102, 184/86, MT048 & *achromogenes* strain MT365, and *A. hydrophila* strain 9240), as shown by azocasein-SDS-gels, correlates with the findings of the casein assays. The only discrepancies are with strains possessing low caseinase activity, according to assay determinations, which did not exhibit activity even when the gels were heavily overloaded. This may be a consequence of the casein-type gels appearing less sensitive to protease action. In other cases, these two approaches complement each other very well, particularly in the case of the ssp. *achromogenes* strains which differ in their ability to produce an extracellular caseinase enzyme. Assaying for casein-hydrolysing activity revealed strain MT365 to be caseinase-positive, whereas strain MT535 was caseinase-negative.

ORIGINAL IN COLOUR

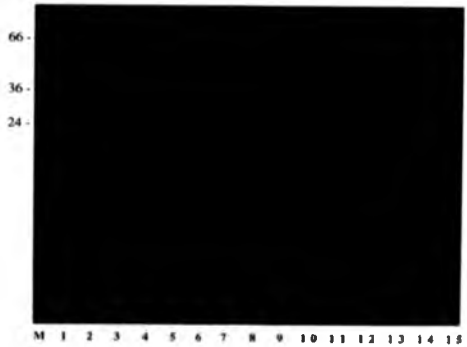


Figure 7.5. Azocasein-SDS-polyacrylamide gel electrophoresis of the extracellular fractions of *A. salmonicida* ssp. *salmonicida* strains MT004 (lane 1), MT004rp (lane 2), 1102 (lane 3), 184/86 (lane 4), MT048 (lane 5), MT028 (lane 6), *A. salmonicida* ssp. *achromogenes* strains MT535 (lane 7), MT365 (lane 8), *A. salmonicida* ssp. *masoucida* 2020 (lane 9), *A. media* 2237 (lane 10), *A. hydrophila* 9240 (lane 11), *A. sobria* 12065 (lane 12), *Vibrio anguillarium* strains 8575 (lane 13), 8587 (lane 14) and *Xanthomonas maltophilia* 2225 (lane 15). Approximately 0.25µg of protein was applied in each case. Lane M, molecular weight standards (for full description see Figs.3.1 & 3.2)

Gelatin-SDS-gels demonstrated that although most protease bands were common between the two strains, MT365 secreted one additional band of activity (M_r 34kDa), therefore this activity was most probably the caseinase enzyme. Subsequent studies confirmed this interpretation by the appearance of a 34kDa protease band on gels in which azocasein replaced gelatin as the gel substrate (Fig.7.5, lane 8).
A series of gelatin gels were run to obtain further information on the biochemical properties of the proteases revealed by gelatin-SDS-gel electrophoresis. The sensitivity to PMSF and 1,10-phenanthroline and the heat stability of the extracellular proteases were examined (Figs. 7.6, 7.7 & 7.8, respectively). In the majority of cases, the results of these treatments were quite straightforward to interpret on the gelatin gels, and a summary of these results is given in Table 7.1.

One of the main points to urise from these results was the difference in properties of proteases which appeared very similar on first inspection in Figure 7.4. For example, a number of strains including ssp. *salmonicida* and *achromogenes*, produced very similar looking extracellular 70kDa proteases. Gelatin-SDS-gel electrophoresis, after specific sample treatments, allowed the individual properties of these enzymes to be determined. The results demonstrated that, between the subspecies, these proteases are most probably quite different. The 70kDa proteases of ssp. *achromogenes* were shown to be metalloproteases by their sensitivity to 1,10-phenanthroline (Fig.7.7, lanes 7 & 8), whereas the 70kDa enymes of ssp. *salmonicida* were found to be of the serine-type since they were inhibited by PMSF (Fig.7.6, lanes 1-5). Heat stability experiments demonstrated a difference between the 70kDa serine protease of strain MT028 compared with the 70kDa serine-type enzyme of the other typical *A. salmonicida* strains. The 70kDa enzyme from strain MT028 appeared to be more heat stable (Fig. 7.8, c.f. lanes 5 & 6) and also did not cross-react with antibody raised against the 70kDa protease purified from the culture filtrate of strain MT004.

However, one major discrepancy was noted in the results concerning the extracellular protease production of the bacteria examined in this study. In the case of *X. maltophilia*, the relatively high caseinase activity detected by the standard assay

Figure 7.6. The effect of PMSF on the extracellular proteases of *A. salmonicida* ssp. *salmonicida* strains MT004 (lane 1), MT004rp (lane 2), 1102 (lane 3), 184/86 (lane 4), MT048 (lane 5), MT028 (lane 6), *A. salmonicida* ssp. *achromogenes* strains MT535 (lane 7), MT365 (lane 8), *A. salmonicida* ssp. *masoucida* 2020 (lane 9), *A. media* 2237 (lane 10), *A. hydrophila* 9240 (lane 11), *A. sobria* 12065 (lane 12), *Vibrio anguillarium* strains 8575 (lane 13), 8587 (lane 14) and *Xanthomonas maltophilia* 2225 (lane 15). Samples of culture filtrate were incubated with 1mM PMSF for 30 minutes at room temperature prior to electrophoresis on gelatin-SDS-polyacrylamide gels. Approximately 0.25µg of protein was applied in each case. Lane M, molecular weight standards (for full description see Figs.3.1 & 3.2)

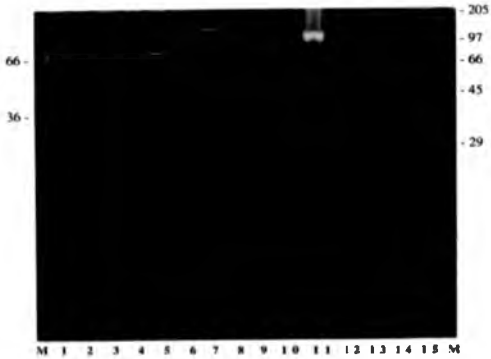


Figure 7.7. The effect of 1,10-phenanthroline on the extracellular proteases of *A. salmonicida* ssp. *salmonicida* strains MT004 (lane 1), MT004rp (lane 2), 1102 (lane 3), 184/86 (lane 4), MT048 (lane 5), MT028 (lane 6), *A. salmonicida* ssp. *achromogenes* strains MT535 (lane 7), MT365 (lane 8), *A. salmonicida* ssp. *masoucida* 2020 (lane 9), *A. media* 2237 (lane 10), *A. hydrophila* 9240 (lane 11), *A. sobria* 12065 (lane 12), *Vibrio anguillarium* strains 8575 (lane 13), 8587 (lane 14) and *Xanthomonas maltophilia* 2225 (lane 15). Samples of culture filtrate were incubated with 10mM 1,10-phenanthroline for 30 minutes at room temperature prior to electrophoresis on gelatin-SDS-polyacrylamide gels. Approximately 0.25µg of protein was applied in each case. Lane M, molecular weight standards (for full description see Figs.3.1 & 3.2)

Figure 7.8. Heat stability of the extracellular proteases of *A. salmonicida* ssp. *salmonicida* strains MT004 (lane 1), MT004rp (lane 2), 1102 (lane 3), 184/86 (lane 4), MT048 (lane 5), MT028 (lane 6), *A. salmonicida* ssp. *achromogenes* strains MT535 (lane 7), MT365 (lane 8), *A. salmonicida* ssp. *masoucida* 2020 (lane 9), *A. media* 2237 (lane 10), *A. hydrophila* 9240 (lane 11), *A. sobria* 12065 (lane 12), *Vibrio anguillarium* strains 8575 (lane 13), 8587 (lane 14) and *Xanthomonas maltophilia* 2225 (lane 15). Samples of culture filtrate were incubated at 56°C for 30 minutes prior to electrophoresis on gelatin-SDS-polyacrylamide gels. Approximately 0.25µg of protein was applied in each case. Lane M, molecular weight standards (for full description see Figs.3.1 & 3.2)

procedure (Fig.7.1) conflicts with the absence of protease bands on azocasein-SDS-gel electrophoresis (Fig.7.5). The reason for this ambiguity is not known, but the difference in substrate was found not to affect the banding pattern since replacement of azocasein with casein as the gel substrate did not allow the development of any protease bands (not shown). The most likely explanation for the conflicting results is that the enzyme responsible for the casein-hydrolysing activity may be particularly sensitive to the denaturing agent SDS, and so will not be able to refold into its correct conformation even after the removal of SDS by Triton X-100.

7.3 Comparative study of extracellular haemolysin production by *A. salmonicida* and related species

Haemolysin assays and immunoblotting experiments, using polyclonal antibodies raised against the 56kDa extracellular protein (see Section 6), were used to compare the haemolytic activities present in the culture filtrates of the various *Aeromonas* species examined in this study.

7.3.1 Extracellular haemolysin activity

Haemolytic activity of the extracellular medium of the species under study was determined by a microtitre double-dilution method (described in Section 2.5.4). The results obtained by this assay procedure revealed considerable variation between the extracellular haemolytic activities of the aeromonads (Fig.7.9). This variation is displayed at both the species and sub-species level. Of the six *A. salmonicida* ssp. *salmonicida* strains studied, only one did not produce significant haemolytic activity; the exception being the very weakly haemolytic strain, MT028. The other ssp. *salmonicida* strains all produced similar levels of haemolytic activity in the range of 16 to 64 HU/ml, considerably greater than MT028 which never produced haemolysin activity at levels greater than 4 HU/ml.

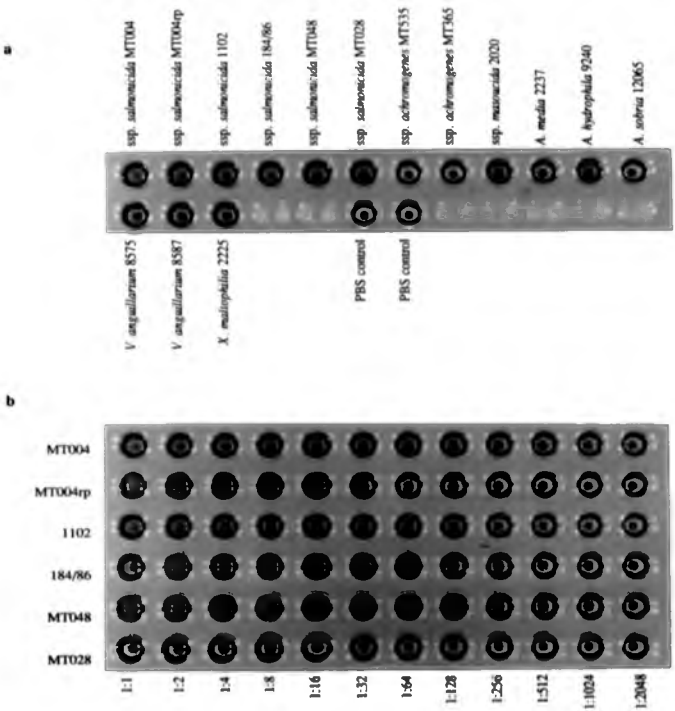


Figure 7. 9. Haemolytic activity present in the culture filtrates of *Aeromonas* strains and other isolates. The microtitre plate assay was used to: (a) screen culture filtrates of all strains for haemolytic activity, and (b) estimate the relative haemolytic activities of the ssp. *salmonicida* isolates. Haemolytic activity was determined as described in Section 2.5.4, except in the case of comparing the ssp. *salmonicida* isolates, in which the culture filtrates were diluted step-wise to the dilutions shown

Of all the other *Aeromonas* species screened, only *A. salmonicida* ssp. *masoucida* and *A. hydrophila* produced extracellular haemolytic activity at detectable levels. *A. salmonicida* ssp. *masoucida* and *A. hydrophila* exhibited haemolytic activities within the range of 8 to 16 HU/ml, slightly lower than the amounts produced by *A. salmonicida* ssp. *salmonicida* strains. *A. salmonicida* ssp. *achromogenes*, *A. media* and *A. sobria* were all found to be haemolysin-negative under the assay conditions employed in this study.

7.3.2 Western blot screening with polyclonal antibodies against the 56kDa haemolysin

Antiserum against the 56kDa haemolysin was used to screen *Aeromonas* strains for any possible cross-reactivity. The results obtained indicated that of the six ssp. *salmonicida* strains probed only one, MT028, did not display any sign of cross-reactivity (Fig.7.10). MT028 was the 'variant' *A. salmonicida* ssp. *salmonicida* strain with respect to haemolytic activity in that it secreted considerably less haemolysin into the culture medium compared with the other strains of the subspecies. All other ssp. *salmonicida* strains examined gave a positive cross-reaction when probed with a single band of M_r 56kDa present in each case (Fig.7.10, lanes 1-5). The other *Aeromonas* strains screened (*A. salmonicida* ssp. *achromogenes* & *masoucida*, *A. media* and *A. sobria*) were all negative for cross-reactivity with the antiserum against the 56kDa haemolysin, with the exception of *A. hydrophila* which reacted in an identical manner to that of the *A. salmonicida* ssp. *salmonicida* strains (lane 11).

7.4 Comparative study of extracellular amylase production by *A. salmonicida* and related species

The production of extracellular amylases by *Aeromonas* species was investigated by assaying for starch-hydrolysing activity and also by applying samples of the respective culture filtrates to starch-SDS-gels and processing them as described previously (see Section 4.2.2).

Figure 7.10. Western blot of the extracellular fractions of *A. salmonicida* ssp. *salmonicida* strains MT004 (lane 1), MT004rp (lane 2), 1102 (lane 3), 184/86 (lane 4), MT048 (lane 5), MT028 (lane 6), *A. salmonicida* ssp. *achromogenes* strains MT535 (lane 7), MT365 (lane 8), *A. salmonicida* ssp. *masoucida* 2020 (lane 9), *A. media* 2237 (lane 10), *A. hydrophila* 9240 (lane 11), *A. sobria* 12065 (lane 12), *Vibrio anguillarium* strains 8575 (lane 13), 8587 (lane 14) and *Xanthomonas maltophilia* 2225 (lane 15). Samples of culture filtrate were screened with polyclonal antiserum (1: 1000 dilution) unispecific to the 56kDa extracellular haemolysin

7.4.1 Extracellular amylase activity

Culture filtrates of the strains under study were assayed for starch-hydrolysing activity, and most were found to produce considerable extracellular amylase activity (Fig.7.11). However, there was a substantial difference in the amount of amylase activity from strain to strain of the *A. salmonicida* ssp. *salmonicida* cultures. Strain MT004 produced the highest levels of starch-hydrolysing activity (9.6 units/l), with the levels of activity in the culture filtrates of MT028 and 184/86 less than 50% of the MT004 value (3.2 & 4.2 units/l, respectively). The other *A. salmonicida* subspecies also secreted significant amylase activity into the extracellular medium, with the ssp. *masoucida* exhibiting the highest specific activity of all the isolates examined (10.7 units/l). The remaining *Aeromonas* species also produced extracellular amylase activity, but the levels detected were not as high in the cases of *A. media* and *A. sobria*. Amylase assays of the culture filtrates of the *V. anguillarium* and *X. maltophilia* strains suggested that these isolates only produced extracellular amylase at very low levels or not at all.

7.4.2 Starch-SDS-polyacrylamide gel electrophoresis

A sample of culture filtrate from each isolate was applied to starch-SDS-gels to gain more detailed information on the amylase activities detected by the standard assay procedure. The results obtained by this method are shown in Figure 7.12a and summarised in Table 7.2. Although the amount of extracellular amylase activity was found to vary between the *A. salmonicida* ssp. *salmonicida* strains (Fig.7.11), no qualitative difference in the amylase banding pattern was observed, with the exception of strain MT028 (Fig.7.12a, lanes 1-6). The majority of the ssp. *salmonicida* strains produced four identical bands of activity with apparent molecular weights of >200, 80, 65 and 63kDa (see Section 4.2.2). However, strain MT028 did not appear to produce any of these activities; instead it produced two amylases, a minor band which runs just behind the 80kDa amylase of the other ssp. *salmonicida* strains and a major band of activity of apparent molecular weight 35kDa. For some reason the 35kDa band did not develop on this gel but it can be seen in Figure 7.12b, with the extracellular fraction

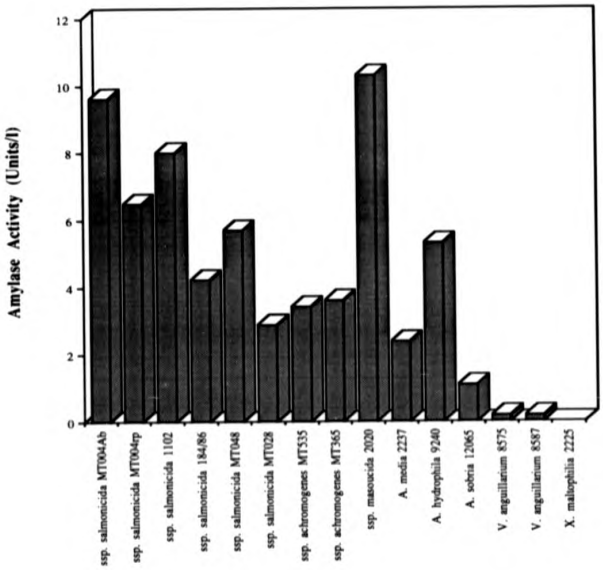


Figure 7.11. Extracellular amylase activity of different *Aeromonas* strains and other isolates. Activity was determined as described in Section 2.5.3

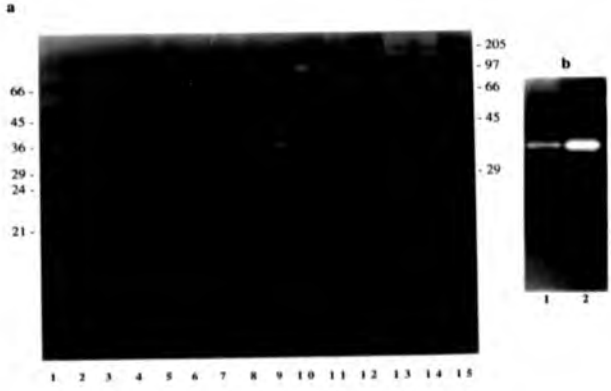


Figure 7.12. Starch-SDS-polyacrylamide gel electrophoresis of the extracellular fractions of (a) *A. salmonicida* ssp. *salmonicida* strains MT004 (lane 1), MT004rp (lane 2), 1102 (lane 3), 184/86 (lane 4), MT048 (lane 5), MT028 (lane 6), *A. salmonicida* ssp. *achromogenes* strains MT535 (lane 7), MT365 (lane 8), *A. salmonicida* ssp. *masoucida* 2020 (lane 9), *A. media* 2237 (lane 10), *A hydrophila* 9240 (lane 11), *A. sobria* 12065 (lane 12), *Vibrio anguillarium* strains 8575 (lane 13), 8587 (lane 14) and *Xanthomonas maltophilia* 2225 (lane 15), and (b) ssp. *salmonicida* strain MT028 (lane 1) and ssp. *masoucida* 2020 (lane 2). Approximately 1.0µg of protein was applied in each case. Lane M, molecular weight standards (for full description see Figs.3.1 & 3.2)

Table 7.2. Extracellular amylase activities produced by *Aeromonas*, *V. anguillarium* and *X. maltophilia* isolates, and their sensitivity to 1,10-phenanthroline [a]

Isolate	Strains	M_r (kDa)	Sensitivity to 1,10-phenanthroline
A. salmonicida			
ssp. *salmonicida*	MT004, 1102,	>200	?
	184/86 & MT048	80	[+]
		65	[+]
		63	[+]
	MT028	85	-
		35	[+]
ssp. *achromogenes*	MT365 & 535	90	-
		75	-
		66	-
		35	[+]
ssp. *masoucida*	2020	35	[+]
A. media	2237	150	-
		80	+
		70	+
A. hydrophilia	9240	100	-
		60	[+]
		45	[+]
V. anguillarium	8575 & 8587	multiple (50->200)	+
X. maltophilia	2225	80	+

[a] Symbols: +, sensitive; [+], partially sensitive; -, resistant; ?, unknown

from *A. salmonicida* ssp. *masoucida* along side for reference.

A. salmonicida ssp. *achromogenes* produced some minor activities in the molecular weight range 65 to 90kDa; however, the principal amylase secreted by these strains was of apparent molecular weight 35kDa (Fig. 7.12a, lanes 7 & 8). This activity appeared to be very similar to the 35kDa amylase detected in the culture filtrate of strain MT028, and also the single amylase produced by *A. salmonicida* ssp. *masoucida* (lane 9). *A. media* strain 2237 and *A. hydrophila* strain 9240 each displayed several bands of amylase activity on starch-SDS-gels (lanes 10 & 11), with the major activities having apparent molecular weights of 80kDa and 45 and 60kDa for *A. media* and *A. hydrophila*, respectively. Although *A. sobria* exhibited readily detectable levels of starch-hydrolysing activity by the standard assay procedure no actual band of amylase activity was observed on starch-SDS-polyacrylamide gel electrophoresis (lane 12). In contrast, multiple amylase banding patterns were displayed by the two *V. anguillarium* strains examined in this study (lanes 13 & 14), even though very little amylase activity (levels < 5% that of *A. salmonicida* MT004) was detected by the standard procedure (Fig.7.11). The absence of banding in the *A. sobria* culture filtrate may be explained in a similar way to that of the caseinase enzyme of *X. maltophilia*, i.e., the enzyme(s) is irreversibly denatured by SDS. However, the results relating to the extracellular amylase activity of *V. anguillarium* can not be explained in this way. It may be that although the total level of *V. anguillarium* amylase activity is relatively low, the length of gel incubation may be sufficient to allow the development of the bands.

The effect of the chelator, 1,10-phenanthroline, on the extracellular amylase activities is shown in Figure 7.13. Most of the enzymes were found to be at least partially sensitive to 1,10-phenanthroline, while a smaller number were either completely inhibited or resistant to this compound (Table 7.2). The vast majority of the major amylase activities of the *A. salmonicida* strains were affected to a certain degree (Fig.7.13, lanes 1-9), but some of the minor enzymes of ssp. *achromogenes* retained complete activity (lane 8). In contrast, the extracellular amylase enzymes of *A. media* were, with the exception of the high molecular weight activity, completely inhibited by 1,10-phenanthroline (lane

Figure 7.13. The effect of 1,10-phenanthroline on the extracellular amylases of *A. salmonicida* ssp. *salmonicida* strains MT004 (lane 1), MT004rp (lane 2), 1102 (lane 3), 184/86 (lane 4), MT048 (lane 5), MT028 (lane 6), *A. salmonicida* ssp. *achromogenes* strains MT535 (lane 7), MT365 (lane 8), *A salmonicida* ssp. *masoucida* 2020 (lane 9), *A. media* 2237 (lane 10), *A. hydrophila* 9240 (lane 11), *A. sobria* 12065 (lane 12), *Vibrio anguillarium* strains 8575 (lane 13), 8587 (lane 14) and *Xanthomonas maltophilia* 2225 (lane 15). Samples of culture filtrate were incubated with 10mM 1,10-phenanthroline for 30 minutes at room temperature prior to electrophoresis on starch-SDS-polyacrylamide gels. Approximately 0.25µg of protein was applied in each case. Lane M, molecular weight standards (for full description see Figs.3.1 & 3.2)

10). The most notable effect of 1,10-phenanthroline was observed with the culture filtrate of *V. anguillarium* in which every activity was dramatically affected with only the M_r 50kDa enzyme retaining any significant level of activity (lanes 13 & 14).

7.5 Discussion

The homogeneity of *A. salmonicida* ssp. *salmonicida* previously demonstrated by numerical taxonomy (Austin *et al.* 1989), DNA homology (Belland & Trust 1988; McCormick *et al.* 1990), serological (Paterson *et al.* 1980; Popoff & Lallier 1984) and biochemical (Chart *et al.* 1984; Popoff & Lallier 1984) studies, as well as the evaluation of plasmid profiles (Bast *et al.* 1988), is, in the most part, confirmed by the results of the SDS-activity gels. Of the six *A. salmonicida* ssp. *salmonicida* isolates examined by this procedure, all but one (MT028), displayed qualitatively identical extracellular hydrolytic (proteolytic & amylolytic) enzyme production. The overall pattern of extracellular enzyme activity was unique to ssp. *salmonicida* strains, however SDS-activity gels did indicate that similar protease and amylase activities may be present in culture filtrates of different species. For example, the high molecular weight double band of protease activity observed in the extracellular extract of the *A. salmonicida* ssp. *achromogenes* isolates is also present in the culture filtrates of ssp. *masoucida*, *A. media*, *A. sobria* and *A. hydrophila* (when samples of lower activity are loaded), and possibly a similar M_r 35kDa amylase appeared to be produced by ssp. *masoucida*, ssp. *salmonicida* strain MT028 and the ssp. *achromogenes* isolates. The suggestion of enzyme similarity between species of *Aeromonas* is only tentative and further characterisation (e.g., immunochemical) is necessary to confirm this possibility. In this context, it should be noted that on first inspection the 70kDa proteases of strain MT028 and ssp. *achromogenes* isolates MT535 and MT365 appeared to be very similar to the 70kDa enzyme of the *A. salmonicida* ssp. *salmonicida* isolates; however, closer examination by inhibitor, heat stability and immunoblotting studies, dismissed this idea.

Antigenically similar proteins were detectable in the culture filtrates of five ssp. *salmonicida* strains and also in the extracellular medium of *A. hydrophila*. Polyclonal antibodies unispecific for the extracellular 70kDa protease and 56kDa haemolysin of strain MT004, cross-reacted with single extracellular components of *A. hydrophila*, with M_rs' 65kDa and 56kDa respectively. Therefore, it is quite clear that *A. salmonicida* isolates share at least two common extracellular antigens with one group of motile aeromonads. Although the phenotypic characteristics of motile and non-motile bacteria are quite different (Allen *et al.* 1983), the antigenic similarity of these enzymes may indicate a degree of relatedness between the two groups of aeromonads, as suggested by genotypic studies (Belland & Trust 1988; Allen *et al.* 1989). Also, perhaps the ability of *A. salmonicida* ssp. *salmonicida* to secrete cloned extracellular proteins of *A. hydrophila* (Buckley 1989; Wong *et al.* 1989) is a consequence of the similarity of the mechanism of extracellular enzyme production and a similarity in the enymes they secrete.

Atypical isolates are considered to be a more heterogeneous group of organisms than their typical counterparts (Belland & Trust 1988),with mean sequence homologies of 77% and 98% respectively. Although the extracellular protease profile of isolate MT365 exhibited caseinase activity, while strain MT535 was caseinase-negative, these two *A. salmonicida* isolates displayed several common protease (gelatinase) bands. These strains also shared a major amylase activity, M_r 35kDa, and were both found to be haemolysin-negative under the assay conditions used. Thus, there is a relatively high degree of homogeneity between these two *A. salmonicida* strains, even though they were isolated from different fish species (MT535, carp; MT365, salmon).

This is the first demonstration of specific extracellular enzyme activities in the culture filtrates of *A. salmonicida* ssp. *masoucida* and *A. media*. Ssp. *masoucida* does not appear to secrete such high levels of protease in comparison to other members of the *A. salmonicida* species, but significant amylase activity was detected by the conventional assay method and this activity was demonstrated to be the property of a single enzyme, M_r 35kDa; this is similar to the principal *A. salmonicida* ssp.

achromogenes enzyme. Three bands of protease activity were detected in the extracellular medium of *A. media*, the major activity a Mr 50kDa metallo-type enzyme. This organism also produced a number of extracellular amylase activities which, like its protease composition, did not appear to resemble enzymes of the other species.

A number of workers have reported on the extracellular protease activities of *A. hydrophila* and *A. sobria* (Thune *et al.* 1982; Nieto & Ellis 1986; Leung & Stevenson 1988; Chabot & Thune 1991), but like the situation with *A. salmonicida*, the exact nature of the activity appears confused. The reason for the apparent disagreement in the literature concerning the proteolytic activity of these species is that, unlike *A. salmonicida*, there is considerable heterogeneity in extracellular enzyme production between strains (Nieto & Ellis 1991). Depending on the strain examined, between one and twelve proteases were present in the culture filtrates of these motile aeromonads. The dissimilarity in extracellular enzyme production by these motile *Aeromonas* species is consistent with the significant phenotypic variation (Allen *et al.* 1989) of these species.

In summary, this comparative study confirms previous findings, based on genotypic data, which indicated a certain degree of relatedness between *A. salmonicida* and at least one group of the motile aeromonads (*A. hydrophila*). Further studies with greater numbers and types of motile aeromonads are required before the relationship between *A. salmonicida* and *A. hydrophila* can be extended to other members of the motile *Aeromonas* species. The main conclusion to be drawn from these results and previously published data is that *A. salmonicida* ssp. *salmonicida* strains display a marked homogeneity in all characteristics examined to date, i.e., strain variation is not apparent in either phenotypic or genotypic properties of *A. salmonicida* ssp. *salmonicida* isolates.

8. General discussion

8.1 Protein secretion in *A. salmonicida*

Until the last decade the ability of Gram-negative bacteria to actively secrete proteins beyond their outer membrane was not widely recognised (Hirst & Welch 1988). In the mid 1980s proteins present in the culture medium of these bacteria were only considered to be extracellular because their presence there was compatible with their function and, therefore, would be beneficial to the bacteria (Pugsley 1988). However, recent evidence indicates that numerous Gram-negative bacteria actively secrete proteins into the external medium (Hirst & Welch 1988). For example, addition of the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) results in the accumulation of a precursor to the normally extracellular hole-forming cytolysin aerolysin (Howard & Buckley 1985); CCCP is thought to inhibit an energy-dependent translocation process at the cytoplasmic membrane (Enequist *et al.* 1981). In the case of *A. salmonicida*, a large number of enzyme activities have been detected in the extracellular medium (Ellis 1991), but no indication of the process by which these proteins were released has been presented.

A number of physical fractionation techniques commonly used with other Gram-negative bacteria (Pugsley & Schwartz 1985), including cold osmotic shock and differential solubilisation of membranes in non-ionic detergent, were applied to *A salmonicida* and the fractions were analysed to determine whether proteins present in the culture medium were true secretion products or were present as a result of non-specific release due to cell lysis. The degree of separation of the cellular compartments of *A. salmonicida* achieved by the various fractionation procedures employed was analysed with respect to the protein composition of the cell by migration of proteins on SDS-polyacrylamide gels and the distribution of cellular markers. Cellular fractionation of *A. salmonicida* and the subsequent analysis of the fractions indicated that this bacterium shared several characteristics with other Gram-negative bacteria.

The similarities between *A. salmonicida* and other Gram-negative bacteria are best illustrated by the following examples: (i) *A. salmonicida* releases proteins into the growth medium (Sections 3.2, 3.3 & 3.4) (Pugsley & Schwartz 1985); (ii) extracellular proteins of *A. salmonicida* are true secretory products and are not present as the result of cell lysis (Sections 3.2, 3.3 & 3.4) (Hirst & Welch 1988; Pugsley 1988); (iii) cell fractionation experiments revealed cellular compartmentation and proteins were demonstrated to be distributed in distinct subsets within and outside the cell (Sections 3.2, 3.3 & 3.4) (Pugsley & Schwartz 1985; Holland *et al.* 1989; von Heijne 1988; Trun & Silhavy 1989); (iv) the outer membrane contained the smallest complement of cellular proteins, with only a few major proteins visible on SDS-polyacrylamide gels (Sections 3.3.2 & 3.3.3) (Lugtenberg & Van Alphen 1983; Nikaido & Vaara 1985; Pugsley & Schwartz 1985); (v) one of the major outer membrane proteins, M_r 42kDa, was found to have distinctive extraction and solubilisation properties, a feature of pore-forming proteins called porins (Sections 3.3.1 & 3.3.2) (Benz & Bauer 1988); (vi) some strains of *A. salmonicida* produced a cell surface associated protein, M_r 49kDa (known to form a surface layer external to the outer membrane [Udey & Fryer 1978; Kay *et al.* 1981]) (Section 3.3.3) (Sleytr & Messner 1983; Koval & Murray 1986); (vi) a number of high molecular weight outer membrane proteins were influenced by the level of free Fe^{3+}-present in the growth medium (Section 5.4) (Neilands 1982; Griffiths 1987).

8.2 Bacterial extracellular proteins

In addition to these similarities the extracellular proteins of *A. salmonicida* have several features in common with other secreted proteins of Gram-negative bacteria and extracellular proteins in general. The main types of protein released into the extracellular medium by Gram-negative bacteria are hydrolytic enzymes and toxins (Pugsley 1988). A large number of these types of activities have been identified from a variety of bacterial species, including proteases (Plaut 1983; Inamura *et al.* 1985; Molla *et al.* 1986; Wandersman *et al.* 1986; Wandersman 1989), nucleases (Nestle & Roberts 1969;

Newland *et al.* 1985) and haemolysins (Howard & Buckley 1983; Mackman & Holland 1984; Manning *et al.* 1984). Several enzymes with similar activities to those mentioned above were detected in the culture medium of *A. salmonicida*, including proteases, amylases, RNAse, DNAse and haemolysin activities (see Section 4.2).

Very little is known about the mechanisms of secretion of extracellular proteins by *A. salmonicida*. The small amount of information available suggests that the mechanism of secretion used by *A. salmonicida* is consistent with most other secretory systems: (i) extracellular enzymes are synthesised on ribosomes associated with the cytoplasmic membrane (Randall & Hardy 1977; Campbell *et al.* 1990); (ii) A-protein, the only protein of *A. salmonicida* studied in any detail with respect to protein export, is initially synthesised with a typical signal peptide of approximate M_r 2kDa (Kay & Trust 1991) which is subsequently cleaved to produce the mature cell envelope associated protein of M_r 49kDa (Belland & Trust 1987). Apart from the A-protein, little else is known of the secretion mechanisms of individual *A. salmonicida* extracellular proteins. However, one observation that has been made is that the 70kDa caseinase enzyme is required for the activation of extracellular haemolysin (Titball *et al.* 1985), i.e., haemolysin is secreted into the culture medium as an inactive precursor and is then proteolytically processed by the 70kDa protease to produce the mature enzyme. This form of extracellular protein production has also been demonstrated for the activation of extracellular aerolysin produced by *A. hydrophila* (Howard & Buckley 1985).

Extracellular proteins are usually secreted as single polypeptides with relatively low molecular weights (Burns 1983), although a number of exceptions have been identified such as cholera toxin of *Vibrio cholerae* (A subunit & pentapeptide of B subunits), *E. coli* haemolysin (M_r 107kDa before processing to the 58kDa active form) (Felmlee *et al.* 1985) and *Neisseria gonorrhoeae* IgA protease (M_r 169kDa before a two-step proteolytic activation process to the 49kDa mature form) (Wandersman 1989). The majority of the extracellular proteins secreted by *A. salmonicida* appear to be monomeric with molecular weights in the range M_r 20-60kDa (Sections 3.4 & 4.2.1). However, preliminary studies of extracellular amylase activity using starch-SDS-

polyacrylamide gels revealed enzymes displaying apparent molecular weights greater than 60kDa (Section 4.2.2), and the molecular weight of the purified major extracellular protease was determined to be 70kDa by SDS-polyacrylamide gel electrophoresis and gel filtration (Sections 4.2.2 & 6.2). Several bacterial extracellular proteases have been identified and the mature forms of the enzymes normally exhibit molecular weights in the range M_r 30-60kDa (Wandersman 1989). The reason for the relatively large size of the principal *A. salmonicida* extracellular protease is not known but may possibly be due to an additional function of the enzyme, as yet unknown. The 70kDa protease was implicated in the activation of an extracellular haemolysin of *A. salmonicida* (Titball *et al.* 1985) and in this context, the enzyme which nicks cholera toxin (cholera lectin) (Booth *et al.* 1984) is also a haemagglutinin (Finklestein & Hanne 1982) and therefore has more than one function.

Proteases are divided into four classes: serine-, cysteine-, metallo- or aspartic-proteases (Webb 1984). Gelatin-SDS-polyacrylamide gel electrophoresis incorporating potential protease inhibitors indicated that the extracellular proteases were either of the serine- or metallo-type (Section 4.2.1). This is quite consistent with other bacteria which produce proteases belonging to three out of the four classes of activity (aspartate proteases have never been found in bacteria) (Bond & Butler 1987).

8.3 Comparative biochemistry of extracellular protein production in *Aeromonas*

8.3.1 Taxonomy of the *Aeromonas* genus

The definitive classification of the *Aeromonas* genus is still to be achieved; the most recent classification of the *Aeromonas* species in Bergey's Manual of Systematic Bacteriology (Popoff 1984) appears to be too simple a categorisation, particularly with respect to the motile species. The non-motile *A. salmonicida* species are differentiated into three subspecies, ssp. *salmonicida*, ssp. *achromogenes* and ssp. *masoucida*. However, although these subdivisions are generally accepted there are still some areas

of debate, with the classification of the atypical *A. salmonicida* subspecies currently under discussion (Belland & Trust 1988; Austin *et al.* 1989). Substrate-SDS-polyacrylamide gel electrophoresis confirmed previous taxonomic findings: (i) *A. salmonicida* ssp. *salmonicida* isolates display a high degree of homology (Sections 7.2.2, 7.3.2 & 7.4.2) (Paterson *et al.* 1980; Chart *et al.* 1984; Bast *et al.* 1988; Belland & Trust 1988; Austin *et al.* 1989; McCormick *et al.* 1990); (ii) ssp. *salmonicida* can be differentiated from ssp. *achromogenes* and ssp. *masoucida* (Sections 7.2, 7.3 & 7.4) (Belland & Trust 1988; Austin *et al.* 1989); (iii) non-motile *A. salmonicida* isolates are phenotypically quite distinguishable from their motile counterparts (Sections 7.2.2 & 7.4.2) (Bryant *et al.* 1986; Austin *et al.* 1989). However, it should be noted that screening of motile and non-motile *Aeromonas* species by Western blotting using rabbit polyclonal antiserum raised against selected *A. salmonicida* extracellular proteins, revealed a certain degree of relatedness between the two divisions, i.e., *A. salmonicida* and *A. hydrophila* (Sections 7.2.2 & 7.3.2) (MacInnes *et al.* 1979; Bast *et al.* 1988; Belland & Trust 1988; McCormick *et al.* 1990).

The motile aeromonad group is divided into three species, namely *A. hydrophila*, *A. sobria* and *A. caviae*, but at least 12 DNA hybridization groups are known to exist (Carnahan & Joseph 1991). The over-simplification of the classification of the motile group of *Aeromonas* species may have contributed quite significantly to the idea that these species display extreme variation in their phenotypic characters. Recently, species with different phenotypic characteristics have been identified and assigned to a specific hybridization group; for example, *A. veronii* (Hickman-Brenner *et al.* 1987), *A. schubertii* (Hickman-Brenner *et al.* 1988), *A. eucrenophilia* (Schubert & Hegazi 1988) and *A. jandaei* and *A. trota* (Carnahan & Joseph 1991). Perhaps recognition of the newly proposed species will result in a clearer picture of the relationships between the different hybridization groups, and possibly the phenotypic characteristics will be found to be less variable than the current literature would imply. In this context, the successful application of substrate-SDS-polyacrylamide gel electrophoresis to the study of phenotypic variability in *A. salmonicida* could be extended to the taxonomic characterisation of the motile *Aeromonas* species.

8.3.2 Substrate-SDS-polyacrylamide gel electrophoresis

Although the method of detecting hydrolytic enzyme activity after SDS-polyacrylamide gel electrophoresis is not in common laboratory use (as judged by the small number of publications referring to this procedure), its suitability in the study of extracellular proteins has several advantages; some benefits attributable to the actual technique and others due to the inherent properties of the extracellular proteins themselves. The methodology of the technique offers several advantages: (i) it allows the examination of complex hydrolytic systems without the requirement for purified material; (ii) it has high sensitivity enabling the detection of trace amounts of activity, not easily detectable by conventional assay procedures; (iii) the ability to examine multiple samples on each electrophoresis run allows rapid analysis and comparability of the samples; (iv) the procedure only requires apparatus that is affordable and easily accessible in most laboratories.

As mentioned above, the properties of the extracellular enzymes (see Section 1.1.4) under examination also contribute in their own detection and characterisation: (i) extracellular proteins are recognised as being relatively stable even when subjected to extreme environments; (ii) in contrast with their intracellular counterparts, extracellular enzymes usually have a low specificity towards substrates, e.g., most extracellular proteases are likely to act as scavengers with the ability to degrade a wide variety of different potential nutrient sources, whereas intracellular proteases normally have specific metabolic functions such as degradation of abnormal proteins (Waxman & Goldberg 1986) and modification of regulatory enzymes (Maurizi 1987). The fact that the majority of extracellular proteins are single polypeptides is probably quite significant in the success of this procedure, since a monomeric protein will be more likely to refold into the correct conformation to regain activity after the removal of the denaturing agent SDS. The low substrate specificity of most secreted enzymes ensures that activity will be detectable by the hydrolysis of substrates such as gelatin and starch. Therefore, it can be seen that this technique has several distinct advantages over conventional techniques in the study of certain groups of extracellular proteins.

8.4 How different is *A. salmonicida* in comparison with its motile counterparts ?

8.4.1 Psychrophile versus mesophiles

The rate of growth of a bacterium is linear only over a limited temperature range for growth, since the growth rate falls abruptly at both the upper and the lower limits of the temperature range (Stanier *et al.* 1977). The effect of temperature on the bacterial growth rate can be illustrated in the form of an Arrhenius plot (Fig.8.1). The linear portion of the plot extends over the normal range of growth temperature. In the low and high temperature ranges, the plot becomes vertical at the minimum and maximum temperatures for growth, respectively. The temperature at which growth rate is maximal is called the optimum temperature for growth. Although the numerical values of these cardinal temperatures (minimum, optimum and maximum), and the range of temperature over which growth is possible, vary widely among bacteria the general form of the Arrhenius plot of growth rates is typical for all bacteria studied (Neidhardt *et al.* 1990).

On the basis of the temperature range of growth, bacteria are divided into three broad groups: thermophiles, which grow at elevated temperatures (above 50°C); mesophiles, which grow best in the middle temperature range (20 to 45°C); psychrophiles, which can grow at low temperatures (5°C or below) (Stanier *et al.* 1977; Cano & Colome 1986; Neidhardt *et al.* 1990). The optimum temperatures for growth of *A. salmonicida* and the motile species are 22 to 25°C and 28°C, respectively (Popoff 1984). Although the optimum growth temperature of *A. salmonicida* is in the mesophile temperature range, *A. salmonicida* is classified as a psychrophile because of its ability to grow at 5°C; all other aeromonads are mesophiles although some are capable of growth at 5°C (Popoff 1984).

The factors that determine the temperature limits for growth have been revealed by two types of investigations: comparisons of the properties of organisms with widely

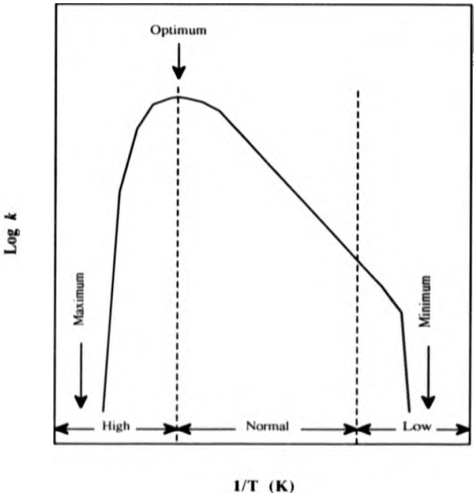


Figure 8.1. General form of an Arrhenius plot of bacterial growth. The cardinal temperatures (maximum, optimum & minimum for growth) and the growth temperature ranges (high, normal & low) are shown. (After Neidhardt *et al.* 1990)

different temperature ranges; and analyses of the properties of temperature-sensitive (*ts*) mutants. The *ts* mutants, termed heat-sensitve (*hs*), that have a decreased maximum temperature of growth do not have an altered minimum temperature of growth. Cold-sensitive (*cs*) mutants in which the minimum temperature of growth is increased do not have altered maximum temperature of growth.

The primary structure of a protein determines both its heat stability and its functional activity, be it catalytic or structural. It seems that mutations in the primary structure of a protein more readily affect the heat stability property of the protein compared to its functional property (Langridge 1968); the thermal stability of the protein is usually decreased. It follows that bacteria evolving in the absence of the challenge of elevated temperature would contain very few thermostable proteins. Indeed, Koffler & Gale (1957) found that bacteria that are incapable of growing at high temperatures contain very few thermostable proteins. The overall thermal stability of soluble cell proteins was estimated by measuring the rates at which the protein in a cell-free extract became insoluble as a result of heat denaturation at different temperatures. Most cytoplasmic proteins were precipitated by an 8 min heat treatment of 60°C, whereas only a very small percentage of the proteins from representative thermophiles were precipitated under the same conditions. Studies on the kinetics of thermal denaturation of enzymes and protein-containing structures (e.g., flagella & ribosomes) have shown that many proteins of thermophilic bacteria are considerably more heat-stable than their homologues from mesophilic bacteria (Stanier *et al.* 1977). Therefore, it is apparent that thermophiles have adapted to their thermal environment through mutational alterations to the primary structures of most (if not all) of their cellular proteins.

The chemical basis for loss of function at high temperature is self-evident: those chemical bonds that maintain the proper secondary and tertiary structure of proteins become weakened at elevated temperatures, resulting in denaturation and loss of function. However, loss of function at lower temperatures is more difficult to explain because most chemical bonds are strengthened as temperature decreases; but hydrophobic bonds weaken at low temperature. The selective pressures for the ability

to grow at low temperatures is quite different from that for growth at high temperature, in that challenge of low temperature does not seem essential for the ability of the organism to grow at lower temperatures (Ingraham 1973). Studies with *cs* mutants have demonstrated that two major classes of proteins are affected by low temperatures; allosteric proteins (O'Donovan & Ingraham 1965; Taketa & Pogell 1965; O'Donovan & Neuhard 1970) and proteins involved in the self-assembly of ribosomes (Tu *et al.* 1968; Guthrie *et al.* 1969). Cold-sensitive mutants of both the regulatory and assembly type probably share the same biochemical basis: a change in the primary structure plus the weakening of hydrophobic bonds causes the protein to have a slightly altered conformation at low temperatures (Ingraham 1973). Therefore, proteins such as allosteric and ribosomal proteins which are likely to be significantly affected by conformational changes will be particularly sensitive to cold inactivation. In turn, it is not suprising that cold-sensitive mutants are usually affected by mutations in the structural genes which code for these proteins (Stanier *et al.* 1977).

In the case of the *Aeromonas* species, the classification of *A. salmonicida* as a psychrophile and the motile *Aeromonas* species as mesophiles is consistent with the selection pressure of higher temperature. *A. salmonicida* is a specific pathogen of fish and as such will never, in its natural environment, be open to high thermal challenge, whereas motile aeromonads are known to have a broad range of hosts (frogs, fish & mammals [including humans]) (Freij 1984; Khardori & Fainstein 1988; Cahill 1990) so the likelihood of high thermal challenge is greatly increased. The probable result of the difference in host range of these organisms is that mutations in the structural genes of *A. salmonicida* proteins resulting in the production of less heat-stable proteins are unlikely to be counterselected and, as a consequence, the upper limit of temperature for growth of this bacterium will be lower in comparison with its motile counterparts.

The production of less heat-stable proteins by psychrophylic bacteria appears to be borne out by the observation that although both *A. salmonicida* and *A. hydrophila* produce antigenically similar extracellular proteins (see Section 7.2.2 & 7.3.2), the proteases of the mesophilic species, *A. hydrophila* and *A. sobria*, were observed to be

slightly more resistant to heat treatment than their *A. salmonicida* counterparts (Section 7.2.3, Fig.7.8). Other workers have also observed that some strains of *A. hydrophila* and *A. sobria* produce heat-stable extracellular proteases, particularly heat-stable metalloprotease enzymes (Nieto & Ellis 1986, 1991; Chabot & Thune 1991). However, the metalloprotease secreted by *A. salmonicida* ssp. *salmonicida* is clearly inactivated by heat treatment. However, it should be noted that one strain of *A. salmonicida* ssp. *achromogenes* produced a heat-stable extracellular protease, and that this enzyme is also of the metallo-type (Section 7.2.3, Figs.7.7 & 7.8). Perhaps the heat-stability of a number of the metalloproteases produced by motile *Aeromonas* species is a characteristic of this enzyme type within these species, and may be correlated with the observation that the metalloproteases are, in general, of a smaller size than their serine protease counterparts.

The model for temperature control of bacterial growth does not, however, explain the effect of a temperature only slightly higher than the optimal temperature suppressing the production of the extracellular proteins of *A. salmonicida* (Section 5.2.1). One possible explanation of this phenomenon may be that elevated temperature causes a conformational change in a ribosomal protein affecting the formation of polysomes, but this is contradictory to the observed effect of ribosomal self-assembly being sensitive to low temperatures. The association of the polysomes with the cytoplasmic membrane may be affected by a change in the composition of the membrane at the higher temperature, however this would also affect the export of outer membrane proteins, but this does not occur (Section 5.2.2). Perhaps the secretory apparatus of *A. salmonicida* is sensitive to the higher growth temperature, but even this would not fully explain the situation since some intracellular proteins also appear to be affected (Section 5.2.2).

As suggested in Section 5.6, the most probable explanation for this phenomenon is that temperature acts as an environmental stimulus, triggering a series of signals via a regulator which controls gene expression at a higher level than individual operons. In this case, the regulon, as it has been termed (Neidhardt *et al.* 1990), may coordinately regulate the expression of the genes encoding most, if not all, of the extracellular enzymes with temperature the over-riding control factor, while other environmental

factors are still capable of regulating gene expression at the lower operon level.

8.4.2 Obligate pathogen versus free living organisms

Among the many factors constraining growth (e.g., temperature, pH, etc.) nutrient availabilty is the one most likely to limit overall cell proliferation (Tempest *et al.* 1983). The ability to grow and multiply is essential to the survival of microorganisms and is linked to the efficiency with which they obtain nutrients. In most natural environments, free-living bacteria live in a state of chronic starvation because nutrients are limiting and competition for them is great (Smith 1990). Therefore, the environment will influence the physiological and biochemical characteristics required by an organism for growth in its particular habitat.

A. salmonicida is a strict parasite under natural conditions unable to survive in surface waters, whereas all other *Aeromonas* species are free-living organisms found in water and sewage (Popoff 1984; Popoff & Veron 1984). In the case of the *Aeromonas* species it would appear that the strictly parasitic nature of *A. salmonicida* resulting in a life within the host, will confer a great advantage over the other aeromonads. The availability of nutrients to *A. salmonicida* will be significantly greater than to the free living *Aeromonas* species because potential nutrients will be present in very low concentrations in the environment. Since there is such a difference in habitat between the two bacterial groups it is likely that they have evolved distinctive characteristics which enable them to adapt to their particular environment.

No matter what the natural environment of a microorganism is, the metabolic challenge facing the bacterial cell is how it can transport substrates from its growth medium into its cytosol. Transport systems are located in the cytoplasmic membrane, but to enter the cell nutrients must also cross the other layers of the cell envelope. Most essential nutrients are able to diffuse through the outer membrane by passing through holes in the membrane formed by porins. Some of the porins form channels specific for certain substrates such as sugars and nucleosides, and these channels pass their substrate much

faster than the general diffusion pores (Nikaido & Vaara 1985; Benz & Bauer 1988). Nutrients diffuse across the outer membrane as long as the concentration of unbound nutrient in the periplasm is lower than that in the external medium. To establish a concentration gradient, Gram-negative bacteria maintain very low concentrations of nutrients within their periplasm, by producing binding proteins that sequester certain nutrients and actively pumping these and other nutrients across the cytoplasmic membrane into the cytosol (Ames 1986).

Since free living microorganisms exist in environments with very low concentrations of nutrients, it may be that they will have evolved different or more efficient transport systems. For example, *A. salmonicida* is known to produce a single porin-type protein of M_r 42kDa, and this porin forms a general diffusion channel (Darveau *et al.* 1983); however, it may be that the motile *Aeromonas* species produce more than one type of porin or only a single porin but with a much higher copy number. Both these situations would allow a greater uptake of nutrients into the periplasm of the bacteria. Other bacteria are known to produce multiple species of porin in a single strain (Nikaido & Vaara 1985) so it is quite possible that motile aeromonads could produce more than one porin-type protein.

In this context, the difference in the outer membrane composition of *A. salmonicida* and *A. hydrophila* should be noted. In contrast with the relatively simple protein profile of the *A. salmonicida* outer membrane (Kay *et al.* 1981; Evenberg *et al.* 1982; this study, section 3.3), the protein compositions of outer membranes isolated from a number of *A. hydrophila* strains are much more complex (Aoki & Holland 1985). There are several proteins present in the molecular weight range of 30-50kDa normally associated with porins (Benz & Bauer 1988). In fact, while studying the surface array protein of *A. hydrophila*, Dooley and Trust (1988) demonstrated the presence of two peptidoglycan-associated proteins displaying molecular weights of 48 and 50kDa; however, no data was presented on any porin-type properties of the proteins. In addition to possessing more than one porin species, producing channels specific for selective substrates would also confer an advantage for nutrient uptake since the rate of

diffusion of the substates is much greater with specific porins. Most general diffusion porins have molecular weights in the range M_r 32-43kDa, whereas specific porins usually have slightly higher molecular weights in the range M_r 44-48kDa (Benz & Bauer 1988). The molecular weights of 48 and 50kDa for the *A. hydrophila* porins may indicate that at least one of these species is a substrate-specific porin.

One of the most obvious differences between *A. salmonicida* and the other *Aeromonas* species is that *A. salmonicida* is non-motile, while all the others are motile by a single polar flagellum (Popoff 1984). Motility is related to taxis and these properties are important in the natural environment, either to seek favourable locations with a better supply of nutrients or to avoid unfavourable conditions like the presence of toxic agents (Rowbury *et al.* 1983). However, *A. salmonicida* does not require flagella since the concentration of nutrients in the immediate vicinity reduces the need to move and seek a better nutrient supply. *A. salmonicida* has a smaller genome size than the *A. hydrophila* group (Belland & Trust 1988), which may be a result of its strictly parasitic nature making the requirement for genes coding for motility properties redundant. The loss of a large number of genes involved in flagella structure and function and the chemotactic response to stimuli, should contribute significantly to the approximate 25% reduction in the genome size of *A. salmonicida*.

Although there are some differences in the factors affecting transport and tactic responses between the *Aeromonas* groups, the production of extracellular enzymes for hydrolysis of potential substrates to sizes that allow uptake through the cell envelope, appears to be quite similar. Perhaps the free-living bacteria would be expected to produce greater numbers of extracellular enzymes to maximise the types of substrates utilisable by the cell. However, all *Aeromonas* species secrete a wide range of hydrolytic enzymes (Sections 4 & 7) (Popoff 1984; Austin *et al.* 1989), with a number of different enzymes of each type of activity present in the culture filtrates.

8.5 Future directions

8.5.1 Secretion pathway(s) of extracellular proteins of *A. salmonicida*

Extracellular proteins are known to be secreted by a number of different mechanisms (see Section 1.2), but with the exception of the A-protein little is known of the secretion pathways utilised by *A. salmonicida*. However, the availability of polyclonal antibodies to specific extracellular protease and haemolysin enzymes of *A. salmonicida* should allow the secretion mechanism(s) of these proteins to be studied in greater detail. The main questions to be answered on the secretion of the extracellular proteins are: how rapidly are the secreted proteins exported from the cell; are they synthesised as precursor proteins; and if they are, when during the export process are the proteins processed to their mature form ? The use of these antibodies, in conjunction with radiolabeling experiments and SDS-polyacrylamide gel electrophoresis, will provide a very sensitive approach for determining the export pathway of the extracellular proteins of *A. salmonicida*.

For example, to determine the structure of the initially synthesised form of a protein, it is necessary to prevent any possible processing reactions from occurring. Since a large number of exported proteins are proteolytically cleaved during translocation across the cytoplasmic membrane (Hirst & Welch 1988), it is essential to inhibit this step of the export pathway if a precursor form is to be identified. Addition of the energy-uncoupler, CCCP (see Section 8.1), results in the accumulation of normally exported proteins in the cytosol and spanning the inner membrane (Howard & Buckley 1985). Therefore, although the half-life of a precursor is very short (Pugsley & Schwartz 1985), immunoprecipitation of ^{35}S-methionine pulse-labeled cultures of *A. salmonicida* containing an energy-uncoupling agent such as CCCP may allow the detection of possible precursor forms of the 70kDa protease or 56kDa haemolysin within the cell.

8.5.2 Contribution to virulence and pathogenesis by specific extracellular enzymes

Since *A. salmonicida* is the causative agent of furunculosis, an economically important disease in the fish farming industry (Trust 1986), a considerable amount of work has involved the attempted elucidation of the virulence determinants of this pathogen, with research concentrated on the extracellular products of the bacterium (Ellis 1991) and its surface layer protein (Olivier 1990). In the case of the extracellular products, a number of activities have been implicated in the disease process, including protease and various haemolysin, phospholipase and GCAT activities (Ellis 1991; Section 1.3). Although the recent work of Lee and Ellis (1990) suggests that a GCAT/LPS complex is the most important extracellular virulence factor, there is still considerable evidence that other enzymes also contribute to the pathogenicity of the disease, particularly the 70kDa protease (Fyfe *et al.* 1988; Lee & Ellis 1989). However, the significance of the extracellular products of *A. salmonicida* in virulence and pathogenesis is still not known.

The major problem in the study of *A. salmonicida* virulence has been the experimental approach taken; most research has involved isolating strains of *A. salmonicida* differing in their ability to cause disease and correlating the degree of virulence with the levels of individual extracellular activities produced by the different isolates (Hackett *et al.* 1984; Ellis *et al.* 1988; Ellis 1991). However, this fails to take account of possible synergistic interactions between virulence factors, and also assumes that inability to produce (or production of low levels of) a particular enzyme *in vitro* also applies *in vivo*, which is not necessarily the case. One way of critically assessing the role of a putative virulence factor is to compare the virulence of specifically deficient mutants with that of the parent (Trust 1986). Chemical mutagens may be used to produce deficient mutants, for example protease-deficient mutants, but there may also be an effect on other extracellular activities, as in the case of the pleiotropic secretory mutants of *A. hydrophila* (Howard & Buckley 1983).

Mutagenesis with transposons provides a better approach to obtaining isogenic mutants

for virulence studies. For example, Tn5 mutagenesis has been used to generate cell-surface mutants of *A. salmonicida* (Belland & Trust 1985) and protease-deficient mutants of *A. hydrophila* (Leung & Stevenson 1988); in future, studies of the 70kDa protease may provide more definitive information on its role in the disease process. However, one difficulty encountered in selecting protease-deficient mutants of *A. hydrophila* was that this bacterium, like *A. salmonicida*, produces more than one extracellular protease (Nieto & Ellis 1986, 1991; Chabot & Thune 1991). In their study, Leung & Stevenson (1988) circumvented this problem by using a parent strain of *A. hydrophila* known to produce only a single extracellular protease. In studying the 70kDa protease of *A. salmonicida*, the similar problem of multiple extracellular protease production may be overcome since this enzyme is the only protease in the culture filtrate capable of hydrolysing casein (Section 4.2.1). Therefore, transposon-induced 70kDa protease-negative mutants of *A. salmonicida* may be selected on the basis of their inability to degrade casein. The inability of the selected mutants to produce the 70kDa protease may be confirmed by screening the culture filtrates of these mutants with the unispecific antibodies raised against this enzyme (see Section 6).

This type of approach may prove more successful in providing a better understanding of the role of the extracellular 70kDa protease, and other extracellular components, in the virulence of *A. salmonicida* and the pathogenicity of the disease it causes. Further studies using the transposon-generated mutants may involve the genetic characterisation of the mutations, which would provide information on the genes encoding the virulence factors of this economically important fish pathogen.

APPENDIX

ß-lactamase activity of *Xanthomonas maltophilia* strain 2225

A.1 Introduction

In the course of an experiment to induce ß-lactamase activity in *A. salmonicida* strain MT004, an organism having high activity was isolated. The ß-lactamase of this organism was characterised; however, subsequently, the organism was found to lack some of the characteristics of *A. salmonicida* and was identified as *Xanthomonas maltophilia*. The studies relating to this are described in the appendix.

A. 2 Materials and methods

A.2.1 Materials

Antibiotics including cephaloridine, cephalothin, cefoxitin, cloxacillin, benzylpenicillin and ampicillin were all purchased from Sigma Chemical Co., Poole. Nitrocefin was obtained from Oxoid Ltd., Basingstoke. Oxytetracycline, potassium sulphonamide, florfenicol, oxolinic acid and clavulanic acid were kindly provided by Dr. Val Inglis, Institute of Aquaculture, University of Stirling.

A.2.2 Antibiotic susceptibilities

Minimum inhibitory concentrations (MICs') were determined by a doubling dilution method. Doubling dilutions were prepared in a microtitre plate: 50µl per well from 80 to 0.005µg per ml with 0µg antibiotic control, with all antibiotics prepared immediately before use. 50µl inoculum of bacteria in the log phase was added to give 1×10^4 colony forming units per well and cultures were incubated for 2 days at 22°C.

A.2.3 Detection of β-lactamase activity in polyacrylamide gels.

Detection of β-lactamase activity *in situ* was achieved by a modified method of Sanders *et al.* (1986). After electrophoresis of β-lactamase-containing sample on either denaturing (SDS) or non-denaturing polyacrylamide gels, the gels (after removing SDS from denaturing gels as described previously in Section 2.6) were overlayed with 3% molten agarose containing 150μg nitrocefin per ml. Once the agar hardened, β-lactamase activity appeared as a pink band in a yellow background.

A.2.4 Determination of β-lactamase activity

The standard assay for β-lactamase activity was based on the method of Nicas & Hancock (1983). 0.1ml of enzyme sample was added to 0.9ml of a 50mM solution of the chromogenic β-lactam nitrocefin in 0.1M sodium phosphate buffer, pH7.0. The rate of conversion of nitrocefin to nitrocefoic acid at 30°C was measureded by monitoring the change in absorbance at 540nm over time.

A.2.5 Characterisation of β-lactamase activity

(i) Substrate specificity

The structures of the main β-lactam compounds used in this study are given in Figure A.1. The activity of β-lactamase towards five different substrates was tested to determine the substrate specificity of the enzyme. Activity towards nitrocefin (486nm), cephaloridine (255nm), cephalothin (265nm), benzylpenicillin (230nm) and ampicillin (240nm) was assayed spectrophotometrically at the wavelengths of maximum absorption. 0.1ml of enzyme sample was added to 0.9ml of substrate and the change in absorbance was followed over time at 30°C. The K_m and V_{max} values were obtained using at least six different concentrations for each substrate.

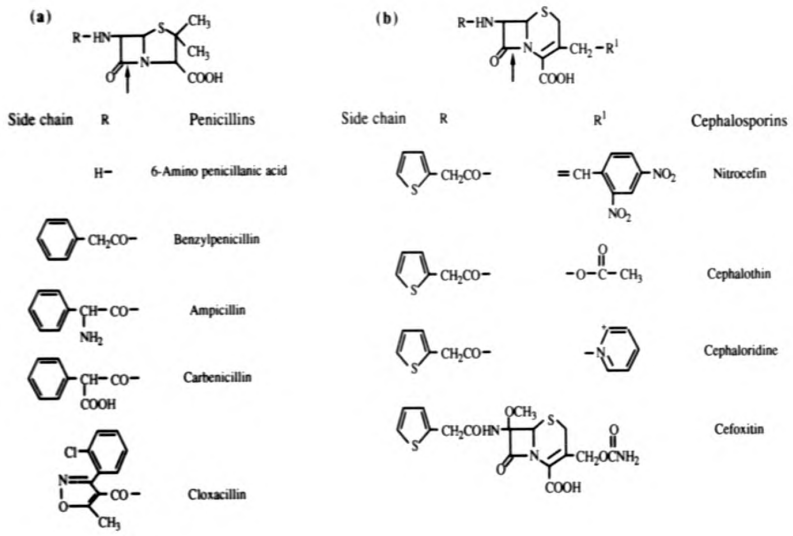


Figure A.1. Structures of (a) penicillin and (b) cephalosporin ß-lactams. Arrows indicate site of ß-lactamase action

(ii) Effect of inhibitors

The inhibitor profile of the ß-lactamase enzyme was determined using 50mM nitrocefin as substrate (see above). The effect of ß-lactam compounds (clavulanic acid, cefoxitin & cloxacillin) were tested without pre-incubation of inhibitor and enzyme, whereas non-ß-lactam compounds [EDTA, $HgCl_2$ & *p*-aminophenylmercuric acid (*p*-APMA)] were incubated for 10 min at 30°C and then the enzyme-inhibitor mixture was assayed for activity against nitrocefin.

A.3 Results

A.3.1 Isolation of ampicillin-resistant colonies from an *A. salmonicida* culture on TSA

Strains of different genera vary greatly in the amount of ß-lactamase that they produce when growing in the absence of antibiotic. Preliminary experiments were carried out on the ß-lactamase activity of *A. salmonicida* strain MT004 to assess its potential for use as a periplasmic marker enzyme. It was found that MT004 did not exhibit any detectable ß-lactamase activity when grown in liquid culture with TSB. However, the background level of ß-lactamase can be increased either by induction, or by selection of bacteria that have undergone a spontaneous mutation of the wild type to a stably derepressed state.

An attempt was made to induce ß-lactamase production in liquid culture by the addition of the penicillin derivative, 6-aminopenicillanic acid (6-APA), to the growth medium. Figure A.2 shows the effect of 6-APA on MT004 growth. MT004 displayed reasonable growth at concentrations of 6-APA up to 100$\mu g ml^{-1}$, however no observable induction of ß-lactamase activity was detected even at this high concentration of inducer.

Since induction of ß-lactamase activity was unsuccessful in liquid medium, an attempt was made to isolate mutants with a high level of antibiotic resistance on agar medium. This method is relatively straightforward as long as mutants exist within a sensitive population. *A. salmonicida* MT004 (1×10^8 cells) was surface inoculated onto TSA with ampicillin incorporated at a concentration of 4 $\mu g ml^{-1}$, well above the minimum

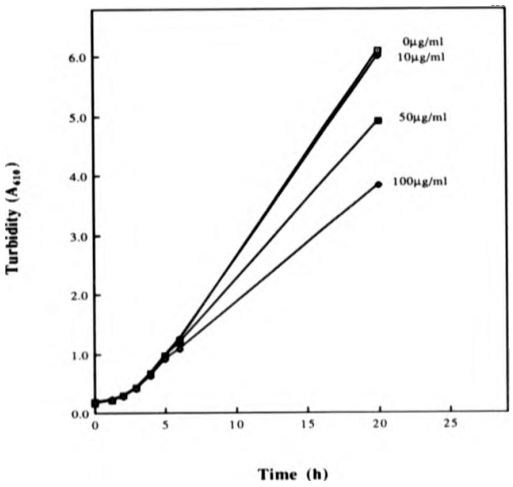


Figure A.2. Effect of 6-aminopenicillanic acid on the growth of *A. salmonicida* strain MT(K)4

inhibitory concentration of the strain ($0.3\mu gml^{-1}$ ampicillin, see Table A.2), therefore inhibiting the growth of sensitive bacteria. Ampicillin-resistant colonies were isolated at a level of 5-10 colony forming units (CFU) per 1×10^8 cells. Colonies were identified as ß-lactamase positive by observing colour change after the addition of a drop of nitrocefin solution.

A.3.2 Identification of ampicillin-resistant isolate

The procedure described in Section A.3.1 for the isolation of drug-resistant organisms does not yield any information about the organisms beyond their ability to grow at elevated drug levels. To ensure that a resistant strain isolated from a sensitive population is a mutant rather than a contaminant, microbiological tests that are used to identify the biotype of the parental strain need to be performed.

Since *Aeromonas* species identification was rejected due to lack of ability to ferment glucose (Table A.1), the ampicillin-resistant colonies isolated in this study were identified (National Collection of Industrial and Marine Bacteria) not as a mutant but as a contaminant. The contaminant was considered to be *Xanthomonas maltophilia*, a Gram-negative bacterium of the Pseudomonadaceae family. The biochemical and morphological characteristics of the ampicillin-resistant isolate are summarised in Table A.1.

A.3.3 Antibiotic susceptibilities of *A. salmonicida* and *X. maltophilia* strains

The two *A. salmonicida* strains examined displayed similar susceptibilities (within one dilution) to most of the antibiotics tested, with the noteable exception of potassium sulphonamide, with which MT004 showed significantly reduced susceptibility (Table A.2). *X. maltophilia* strain 2225 demonstrated lower susceptibilities to all antibiotics tested. All strains tested were most susceptible to oxolinic acid.

Table A.1. Biochemical and physiological properties of ampicillin-resistant isolate 2225

Gram stain	V
Motility	-
Growth at 37°C	+
41°C	-
Brown diffusable pigment	+ (37°C)
Oxidase	(+)
Catalase	+
O/F test	O
Arginine dihydrolase	-
Lysine decarboxylase	+
β-galactosidase	+
Citrate utilization	+
Nitrate reduction	+
Indole production	-
Acid from glucose	-
Acid from maltose	(+)
Urease	-
Hydrolysis of aesculin	+
Hydrolysis of gelatin	+
Hydrolysis of casein	+
Hydrolysis of starch	-
Hydrolysis of Tween 80	+
Production of DNAse	+
Alkilisation of Simmons citrate	+
Alkilisation of malonate	-
Alkilisation of tartrate	-
Egg yolk agar opacity	-
Assimilation of:	
Glucose	(+)
Maltose	+
Mannose	+
Mannitol	-
Arabinose	-
N-acetylglucosamine	+
Gluconate	-
Malate	+
Caprate	-
Adipate	-
Citrate	+
Phenylacetate	-

V, indicates a variable reaction

(+), indicates a weak positive

Table A.2. MICs' of antibiotics for *A. salmonicida* MT004 and *X. maltophilia* 2225

Isolate	MIC (μgml^{-1})						ß-lactamase activity[a]
	Ampicillin	Oxytetracycline	Sulphonamide[b]	Florfenicol	Oxolinic acid	Cephalothin	
A. salmonicida FCC	0.3	0.6	2.5	1.25	0.08	>80	n.d.
A. salmonicida MT004	0.3	1.25	>80	0.6	0.08	>80	<1
X. maltophilia 2225	>80	>80	>80	40	2.5	>80	1205

[a] ß-lactamase activity is expressed as nanomoles of nitrocefin hydrolysed min^{-1} (mg protein)$^{-1}$.

[b] Sulphonamide, 10% sulphachlorpyridazine . 2% trimethoprim

n.d. not determined

A.3.4 β-lactamase activity of *X. maltophilia* strain 2225

When *A. salmonicida* strain MT004 and *X. maltophilia* strain 2225 were tested for β-lactamase activity, only *X. maltophilia* strain 2225 displayed detectable β-lactamase activity in sonic extracts (Table A.2). Production of β-lactamase activity occurred in this isolate without the addition of inducers into the growth medium. No β-lactamase was found in sonic extracts of *A. salmonicida* strain MT004 even in the presence of inducer.

To detect the number of different β-lactamase enzymes produced by *X. maltophilia* strain 2225, β-lactamase activity was detected *in situ* on non-denaturing polyacrylamide gels by overlaying the gel with molten agar containing 150μg of nitrocefin per ml. Once the agar hardened, β-lactamase activity was visualised as a pink band in a yellow background and so allowed the number of distinct β-lactamase activities to be determined. A periplasmic extract of *X. maltophilia* strain 2225 produced a single band of β-lactamase activity after approximately 10 minutes with no other bands appearing even after 1 hour of staining, suggesting that either only one β-lactamase was produced by this strain or that any other type of β-lactamase produced was not capable of hydrolysing nitrocefin.

An attempt was made to determine the molecular weight of the *X. maltophilia* β-lactamase by using the same *in situ* technique as described previously, except overlaying an SDS-polyacrylamide gel after removing the SDS by incubating with 2.5% Triton X-100. However, no activity was detected on the SDS-polyacrylamide gel by this procedure, most probably due to irreversible denaturation of the β-lactamase by SDS. In an effort to circumvent this problem, a preparative non-denaturing polyacrylamide gel was run and the band of activity excised and homogenised in x2 SDS sample buffer and electrophoresed again on an SDS mini gel system. Coomassie blue staining of the gel for protein revealed a series of bands in a molecular weight range of 25-100kDa, with the principal band having a molecular weight of approximately 55kDa (not shown).

A.3.5 Enzymological properties of *X. maltophilia* ß-lactamase

A ß-lactamase can be fully characterised and classified only by combining information derived from a number of parameters, including kinetic analyses, specificity and inhibition of enzyme activity.

(1) Substrate specificity

The activity of the ß-lactamase with five different substrates and the K_m values for the substrates were determined in order to characterise the enzyme (Table A.3). The K_m and maximal velocity (V_{max}) values were obtained using at least six different concentrations for each substrate. The enzyme was found to be relatively non-specific, degrading both penicillins and cephalosporins, but it was slightly more active against benzylpenicillin and ampicillin than with cephaloridine or cephalothin.

(2) Inhibition studies

Inhibitory characteristics can be just as important as substrate activity profiles in distinguishing between ß-lactamases. Inhibitors of ß-lactamase activity can be divided into two types: (i) inhibitors with ß-lactam structure and (ii) non-ß-lactam inhibitory compounds.

(i) ß-lactam inhibitors

I_{50} values (50% inhibition) were determined for compounds that were resistant to hydrolysis by the enzyme (Table A.4). The enzyme was inhibited strongly by cefoxitin, but the most effective inhibitory activity was clavulanic acid with an I_{50} value of 0.16µM. Cloxacillin was not inhibitory at 10µM.

(ii) non-ß-lactam inhibitors

Table A.5 demonstrates the effects of various non-ß-lactam inhibitors on the ß-lactamase activity. The enzyme activity against nitrocefin was completely inhibited by treatment with $HgCl_2$ and partially by *p*-aminophenylmercuric acetate. EDTA showed no inhibitory effect.

Table A.3. Hydrolysis of ß-lactam antibiotics by *X. maltophilia* 2225 ß-lactamase

Substrate	Relative V_{max} [a]	K_m(μM)
Penicillin	100	116.5
Ampicillin	41	167.3
Nitrocefin	49	156.1
Cephaloridine	32	44.2
Cephalothin	10	23.0

[a] Relative rates of hydrolysis of substrates are expressed as percentages of penicillin hydrolysis.

Table A.4. Inhibitory effects of ß-lactam compounds on *X. maltophilia* 2225 ß-lactamase activity

Inhibitor	$I_{50}(\mu M)^a$
Clavulanic acid	0.16
Cloxacillin	>10
Cefoxitin	1.8

[a] I_{50}, inhibitor concentration required for 50% inhibition of enzyme activity. I_{50} values were determined with 50μM nitrocefin as substrate without preincubation of enzyme extract and inhibitor.

Table A.5. Effects of various inhibitors on *X. maltophilia* 2225 ß-lactamase activity[a]

Inhibitor	Concn (mM)	Inhibition(%)
EDTA	1.0	0
p-APMA[b]	0.5	88
$HgCl_2$	0.5	100

[a] The enzyme extract was preincubated in 0.1M phosphate buffer (pH 7.0) for 10 min at 30°C with each inhibitor at the indicated concentration, and the remaining activity was assayed with 50μM nitrocefin as the substrate

[b] *p*-APMA, *p*-aminophenylmercuric acetate.

A.4 Discussion

The physicochemical and enzymological properties of β-lactamase from *X. maltophilia* strain 2225 were examined. The *X. maltophilia* enzyme was shown to be a broad-spectrum β-lactamase, hydrolysing both penicillins and cephalosporins but with a slight preference for the former.

The activity of *X. maltophilia* β-lactamase was strongly inhibited by clavulanic acid, cefoxitin and *p*-aminophenylmercuric acetate, but not by EDTA. $HgCl_2$ and *p*-aminophenylmercuric acetate inhibitory activity indicates the presence of a catalytically important cysteine residue.

Previous reports on the β-lactamase activity of *X. maltophilia* suggest that most strains produce two distinct β-lactam hydrolysing enzymes (Saino *et al.* 1982, 1984). Another example of a Gram-negative organism known to express two distinct β-lactamase enzymes is *Yersinia enterocolitica* (Cornelis & Abraham 1975). Both *X. maltophilia* and *Y. enterocolitica* were shown to produce one enzyme with strong cephalosporinase activity (L-2 and B, respectively) and a second enzyme (L-1 and A, respectively) that hydrolyses a variety of penicillins.

However, strain 2225, the *X. maltophilia* isolate examined in this study produced only a single β-lactamase enzyme as demonstrated by activity staining on polyacrylamide gels. It is possible that a second enzyme is indeed present but that it is not very active against nitrocefin, the substrate used in the gel overlay technique. This is unlikely, however, since even if nitrocefin was only hydrolysed slowly its high molar absorption coefficient would still allow for the detection of very low levels of activity. The fact that both β-lactamases of *X. maltophilia* have previously been shown to hydrolyse nitrocefin (Saino *et al.* 1984; Iaconis & Sanders 1990) is further evidence for the presence of only one enzyme in this strain.

Attempts to determine the molecular weight of the enzyme by electrophoresing the

band containing activity were inconclusive because of the number of bands obtained on SDS-polyacrylamide gel electrophoresis. The most prominent Coomassie blue stained band was of approximate M_r 55kDa, which is greater than other ß-lactamases reported so far except for the L-1 (118kDa) and L-2 (56kDa) enzymes of *X. maltophilia* (Saino *et al.* 1984).

The substrate profile of the ß-lactamase enzyme produced by *X. maltophilia* strain 2225 does not correspond exactly to either of the previously identified ß-lactamases (L-1 and L-2) of this organism. It is most similar to the L-2 enzyme in that it displays a broad substrate range (Saino *et al.* 1984), hydrolysing both penicillins and cephalosporins, whereas the L-1 enzyme is unable to hydrolyse cephalosporins other than nitrocefin (Iaconis & Sanders 1990).

The effects of inhibitory ß-lactam compounds and various other low molecular weight inhibitors are also more consistent with the properties of the L-2 ß-lactamase, in that the enzyme from strain 2225 was inhibited by clavulanic acid, cefoxitin and *p*-aminophenylmercuric acetate, but not by EDTA. In this respect, strain 2225 enzyme acts like a typical cephalosporinase (Richmond & Sykes 1973; Sykes & Matthews 1976; Bush 1989c).

With respect to broad substrate specificity and sensitivity to inhibitors, the enzyme from *X. maltophilia* strain 2225 has general similarities to the group 2 ß-lactamases described by Bush (1989b, c). Further kinetic data, for carbenicillin and cloxacillin in particular, would allow for a more positive and accurate characterisation and classification of the ß-lactamase from *X. maltophilia* strain 2225.

References

Adams, M.D. & Oxender, D.L. (1989) *Journal of Biological Chemistry* **264,** 15739-15742

Allen-Austin, D.A., Austin, B. & Colwell, R.R. (1984) *FEMS Microbiology Letters* **21,** 143-146

Altwegg, M. & Lüthy-Hottenstein, J. (1991) *Experientia* **47,** 403-406

Ames, G.F.-L. (1974) *Journal of Biological Chemistry* **249,** 634-644

Ames, G.F-L. (1986) *Annual Review of Biochemistry* **55,** 397-426

Ames, G.F.-L.,Spudich, E.N. & Nikaido, H. (1974) *Journal of Bacteriology* **117,** 406-416

Ames, G.F-L., Prody, C. & Kustu, S. (1984) *Journal of Bacteriology* **160,** 1181-1183

Andro, T., Chambost, J-P., Kotoujansky, A., Cattaneo, J., Bertheau, Y., Barras, F., Van Gijsegem, F. & Coleno, A. (1984) *Journal of Bacteriology* **160,** 1199-1203

Aoki, T. & Holland, B.I. (1985) *FEMS Microbiology Letters* **27,** 299-305

Arp, L.H. (1988) In: *Virulence Mechanisms of Bacterial Pathogens* (ed. J.A. Roth), pp.3-27. American Society for Microbiology, Washington, DC

Austin, B. & Austin, D.A. (1987) In: *Bacterial Fish Pathogens: Disease in Farmed and Wild Fish*, pp.111-195. Ellis Horwood Ltd., Chichester

Austin, D.A., McIntosh, D. & Austin, B. (1989) *Systematic and Applied Microbiology* **11,** 277-290

Bast, L., Daly, J.G., DeGrandis, S.A. & Stevenson, R.M.W. (1988) *Journal of Fish Diseases* **11** , 135-145

Baumeister, W., Karrenberg, F., Rachel, R., Engel, A., ten Heggeler, B. & Saxton, W.O. (1982) *European Journal of Biochemistry* **125**, 535-544

Belland, R.J. & Trust, T.J. (1985) *Journal of Bacteriology* **163**, 877-881

Belland, R.J. & Trust, T.J. (1987) *Journal of Bacteriology* **169**, 4086-4091

Belland, R.J. & Trust, T.J. (1988) *Journal of General Microbiology* **134**, 307-315

Benz, R. & Bauer, K. (1988) *European Journal of Biochemistry* **176**, 1-19

Bernoth, E.-M. (1990) *Journal of Fish Diseases* **13**, 145-155

Bingle, W.H., Doran, J.L. & Page, W.J. (1986) *Canadian Journal of Microbiology* **32**, 112-120

Bond, J.S. & Butler, P.E. (1987) *Annual Review of Biochemistry* **56**, 333-364

Booth, B.A., Boesman-Finklestein, M. & Finklestein, R.A. (1984) *Infection and Immunity* **45**, 558-560

Bootsma, R., Fijan, N. & Blommaert, J. (1977) *Veterinarski Archives* **47**, 291-302

Born, J. & Bhakdi, S. (1986) *Immunology* **59**, 139-145

Brass, J.M., Higgins, C.F., Foley, M., Rugman, P.A., Birmingham, J. & Garland, P.B. (1986) *Journal of Bacteriology* **165**, 787-794

Bricker, J., Mulks, M.H., Plaut, A.G., Moxan, E.R. & Wright, A. (1983) *Proceedings of the National Academy of Science (USA)* **80**, 2681-2685

Brown, M.R.W. & Williams, P. (1985) *Annual Review of Microbiology* **39**, 527-556

Bryant, T.N., Lee, J.V., West, P.A. & Colwell, R.R. (1986) *Journal of Applied Bacteriology* **61**, 437-467

Buckley, J.T. (1983) *Biochemistry* **22**, 5490-5493

Buckley, J.T. (1989) *Biochemistry and Cell Biology* **68**, 221-224

Buckley, J.T. Halasa, L.N. & MacIntyre, S. (1982) *Journal of Biological Chemistry* **257**, 3320-3325

Bullen, J.J., Rodgers, H.J. & Griffiths, E. (1978) *Current Topics in Microbiology and Immunology* **80**, 1-35

Bullock, G.L. & Stuckey, H.M. (1975) *Progressive Fish Culturist* **37**, 237-239

Burns, R.G. (1983) In: *Microbes in their Natural Environments* (eds. J.H.Slater, R.Whittenbury & J.W.T.Wimpenny), pp.249-298. Cambridge University Press, Cambridge

Bush, K. (1989a) *Antimicrobial Agents and Chemotherapy* **33**, 259-263

Bush, K. (1989b) *Antimicrobial Agents and Chemotherapy* **33**, 264-270

Bush, K. (1989c) *Antimicrobial Agents and Chemotherapy* **33**, 271-276

Cahill, M.M. (1990) *Journal of Applied Bacteriology* **69**, 1-16

Campbell, C.M., Duncan, D., Price, N.C. & Stevens, L. (1990) *Journal of Fish Diseases* **13**, 463-474

Cano, R.J. & Colome, J.S. (1986) *Microbiology* West Publishing Company, St.Paul, Michigan

Carnahan, A.M. & Joseph, S.W. (1991) *Experientia* **47**, 402-403

Carniel, E., Mazigh, D. & Mollaret, H.H. (1987) *Infection and Immunity* **55**, 277-280

Chabot, D.J. & Thune, R.L. (1991) *Journal of Fish Diseases* **14**, 171-183

Chart, H., Shaw, D.H., Ishiguro, E.E. & Trust, T.J. (1984) *Journal of Bacteriology* **158**, 16-22

Chart, H. & Trust, T.J. (1983) *Journal of Bacteriology* **156**, 758-764

Christison, M.H., MacKenzie, J. & Mackie, T.J. (1938) *Fisheries Scotland Salmon Fish.*, **5**, H.M.S.O., Edinburgh

Cipriano, R.C., Griffin, B.R. & Lidgerding, B.C. (1981) *Canadian Journal of Fisheries and Aquatic Sciences* **38**, 1322-1326

Collmer, A. & Keen, N.T. (1986) *Annual Review of Phytopathology* **24**, 383-409

Coleman, G., Abbas-Ali, B., Sutherland, J., Fyfe, L. & Finley, A. (1987) In: *Extracellular Enzymes of Microorganisms* (eds. J. Chaloupka & V. Krumphanzl), pp. 13-21. Plenum Press, New York & London

Colwell, R.R., MacDonell, M.T. & DeLey, J. (1986) *International Journal of Systematic Bacteriology* **36**, 473-477

Cornelis, G. & Abraham, E.P. (1975) *Journal of General Microbiology* **87**, 273-284

Cross, A.S. & Kelly, N.M. (1990) *FEMS Microbiology and Immunology* **64**, 245-258

Dahle, H.K. (1971a) *Acta pathologica microbiologica Scandinavica Section B* **79**, 726-738

Dahle, H.K. (1971b) *Acta pathologica microbiologica Scandinavica Section B* **79**, 739-746

Daniels, M.J., Dow, J.M. & Osbourn, A.E. (1988) *Annual Review of Phytopathology* **26**, 285-312

Darveau, R.P., MacIntyre, S., Buckley, J.T. & Hancock, R.E.W. (1983) *Journal of Bacteriology* **156**, 1006-1011

Debette, J. (1991) *Current Microbiology* **22**, 85-90

DeLey, J., Cattoir, H. & Reynaerts, R. (1970) *European Journal of Biochemistry* **12**, 133-142

Dooley, J.S.G., McCubbin, W.D., Kay, C.M. & Trust, T.J. (1988) *Journal of Bacteriology* **170**, 2631-2638

Dooley, J.S.G. & Trust, T.J. (1988) *Journal of Bacteriology* **170**, 499-506

Doty, P. (1962) *Biochemical Society Symposia* **21** (eds. D.J. Bell & J.K. Grant), pp.8-28. Cambridge University Press, Cambridge

Dow, J.M., Daniels, M.J., Dums, F., Turner, P.C. & Gough, C. (1989) *Journal of Cell Science Suppl.* **11**, 59-72

Drinan, E. & Smith, P.R. (1985) In: *Fish and Shellfish Pathology* (ed. A.E. Ellis), pp.79-83. Academic Press, London

Dunker, A.K. & Rueckert, R.R. (1969) *Journal of Biological Chemistry* **244**, 5074-5080

Elliot, D.G. & Shotts, E.B. (1980) *Journal of Fish Diseases* **3**, 145-151

Ellis, A.E. (1987) *Microbial Pathogenesis* **3**, 167-177

Ellis, A.E. (1991) *Journal of Fish Diseases* **14**, 265-277

Ellis, A.E., Hastings, T.S. & Munro, A.L.S. (1981) *Journal of Fish Diseases* **4**, 41-51

Ellis, A.E., Burrows, A.S. & Stapleton, K.J. (1988) *Journal of Fish Diseases* **11**, 309-323

Ellwood, D.C. (1974) *Journal of Medical Microbiology* **7**, 391-393

Ellwood, D.C. & Tempest, D.W. (1972) *Advances in Microbial Physiology* **7**, 83-117

Emmerich, R. & Weibel, E. (1894) *Archives fur Hygiene und Bakteriologie* **21**, 1-21

Enequist, H.G., Hirst, T.R., Harayama, S., Hardy, S.J.S. & Randall, L.L. (1981) *European Journal of Biochemistry* **116**, 227-233

Evenberg, D., Van Boxtel, R., Lugtenberg, B., Schurer, F., Blommaert, J. & Bootsma, R. (1982) *Biochimica et Biophysica Acta* **684**, 241-248

Evenberg, D. & Lugtenberg, B. (1982) *Biochimica et Biophysica Acta* **684**, 249-254

Evenberg, D., Versluis, R. & Lugtenberg, B. (1985) *Biochimica et Biophysica Acta* **815**, 233-244

Farrel, D.H. & Crosa, J.H. (1991) *Biochemistry* **30**, 3432-3436

Felmlee, T., Pellet, S., Lee, E.Y. & Welch, R.A. (1985) *Journal of Bacteriology* **163**, 88-93

Ferguson, S.J. (1990) *Trends in Biochemical Sciences* **15**, 377

Filip, C., Fletcher, G., Wulff, J.L. & Earhart, C.F. (1973) *Journal of Bacteriology* **115**, 717-722

Finklestein, R.A. & Hanne, L.F. (1982) *Infection and Immunity* **36**, 1199-1208

Focareta, T. & Manning, P.A. (1987) *Gene* **53**, 31-40

Frank, M.M., Joiner, K. & Hammer, C. (1987) *Reviews of Infectious Diseases* **9** (Suppl.5), S537-S545

Freij, B.J. (1984) *Pediatric Infectious Disease* **3**, 164-175

Fuller, D.W., Pilcher, K.S. & Fryer, J.L. (1977) *Journal of the Fisheries Research Board of Canada* **34**, 1118-1125

Fyfe, L., Finley, A., Coleman, G. & Munro, A.L.S. (1986a) *Journal of Fish Diseases* **9**, 403-409

Fyfe, L., Coleman, G. & Munro, A.L.S. (1986b) *Annales de Institut Pasteur/ Microbiologie* **137 A**, 117-123

Fyfe, L., Coleman, G. & Munro, A.L.S. (1987a) *Applied and Environmental Microbiology* **53**, 722-726

Fyfe, L., Coleman, G. & Munro, A.L.S. (1987b) *Journal of Applied Bacteriology* **62**, 367-370

Fyfe, L., Coleman, G. & Munro, A.L.S. (1988) *Journal of Fish Diseases* **11**, 101-104

Gargallo-Viola, D. & Lopez, D. (1990) *Journal of Clinical Microbiology* **28**, 136-139

Gibb, J.D. & Strohl, W.R. (1987) *Canadian Journal of Microbiology* **34**, 187-190

Glenn, A.R. (1976) *Annual Review of Microbiology* **30**, 41-62

Glomset, J.A. & Norum, J.R. (1973) *Advances in Lipid Research* **11**, 1-65

Gobius, K.S. & Pemberton, J.M. (1988) *Journal of Bacteriology* **170**, 1325-1332

Gottesman, S. (1984) *Annual Review of Genetics* **18**, 415-441

Graham, L.L., Beveridge, T.J. & Nanninga, N. (1991) *Trends in Biochemical Sciences* **16**, 328-329

Griffiths, E. (1987) In: *Iron and Infection: Molecular, Physiological and Clinical Aspects* (eds. J.J. Bullen & E. Griffiths), pp. 69-137. John Wiley & Sons Ltd., Chichester

Griffiths, E. (1989) *Current Opinions in Infectious Diseases* **2**, 819-826

Griffiths, E., Chart, H. & Stevenson, P. (1988) In: *Virulence Mechanisms of Bacterial Pathogens* (ed. J.A. Roth), pp.121-137. American Society for Microbiology, Washington, DC

Guthrie, C., Nashimoto, H. & Nomuro, M. (1969) *Proceedings of the National Academy of Sciences (USA)* **63**, 384-392

Hackett, J.L., Lynch, W.H., Paterson, W.D. & Coombs, D.H. (1984) *Canadian Journal of Fisheries and Aquatic Sciences* **41**, 1354-1360

Hamilton, R.C., Kalnins, H., Ackland, N.R. & Ashburner, L.D. (1981) *Journal of General Microbiology* **122**, 363-366

Hancock, R.E.W. & Nikaido, H. (1978) *Journal of Bacteriology* **136**, 381-389

Hastings, T.S. & Ellis, A.E. (1985) In: *Fish and Shellfish Pathology* (ed. A.E. Ellis), pp.69-77. Academic Press, London

Hastings, T.S., Ellis, A.E. & Munro, A.L.S. (1981) *Society of General Microbiology Quarterly* **8**, 254-255

Hazelbauer, G.L. & Harayama, S. (1979) *Cell* **16**, 617-625

Hederstedt, L. & Rutberg, L. (1981) *Microbiological Reviews* **45**, 542-555

Heppel, L.A. (1971) In: *Structure and Function of Biological Membranes* (ed. L.I. Rothfield), pp. 224-247. Academic Press, New York

Herman, R.L. (1968) *Transactions of the American Fisheries Society* **97**, 221-230

Heussen, C. & Dowdle, E.B. (1980) *Analytical Biochemistry* **102**, 196-202

Hickman-Brenner, F.W., MacDonald, K.L., Steigerwalt, A.G., Fanning, G.R., Brenner, D.J. & Farmer III, J.J. (1987) *Journal of Clinical Microbiology* **25**, 900-906

Hickman-Brenner, F.W., Fanning, G.R., Arduino, M.J., Brenner, D.J. & Farmer III, J.J. (1988) *Journal of Clinical Microbiology* **26**, 1561-1564

Hirst, I.D., Hastings, T.S. & Ellis, A.E. (1991) *Journal of General Microbiology* **137**, 1185-1192

Hirst, T.R. & Holmgren, J. (1987) *Journal of Bacteriology* **169**, 1037-1045

Hirst, T.R., Sanchez, J., Kaper, J.B., Hardy, S.J.S. & Holmgren, J. (1984) *Proceedings of the National Academy of Sciences (USA)* **81**, 7752-7756

Hirst, T.R. & Welch, R.A. (1988) *Trends in Biochemical Sciences* **13**, 265-269

Hobot, J.A., Carlemalm, E., Villiger, W. & Kellenberger, E. (1984) *Journal of Bacteriology* **160**, 143-152

Holland, I.B., Wang, R., Seror, S.J. & Blight, M. (1989) *Symposia of the Society for General Microbiology* **44** (eds. S. Baumberg, I. Hunter & M. Rhodes) pp.219-254. Cambridge University Press, Cambridge

Horwitz, M.A. (1982) *Review of Infectious Diseases* **4**, 104-123

Howard, S.P. & Buckley, J.T. (1983) *Journal of Bacteriology* **154**, 413-418

Howard, S.P. & Buckley, J.T. (1985) *Journal of Bacteriology* **161**, 1118-1124

Iaconis, J.P. & Sanders, C.C. (1990) *Antimicrobial Agents and Chemotherapy* **25**, 362-365

Ichige, A., Oishi, K. & Mizushima, S. (1988) *Journal of Bacteriology* **170**, 3537-3542

Ingraham, J.L. (1973) In: *Temperature and Life* (eds. H. Precht, J. Christopherson, H. Hensel & W. Larcher), pp.60-77. Springer-Verlag, Berlin

Inamura, H., Nakai, T. & Muroga, K. (1985) *Bulletin of the Japanese Society of Scientific Fisheries* **51**, 1915-1920

Ishiguro, E.E., Ainsworth, T., Kay, W.W. & Trust, T.J. (1986) *Applied and Environmental Microbiology* **15**, 668-670

Ishiguro, E.E., Ainsworth, T., Trust, T.J. & Kay, W.W. (1985) *Journal of Bacteriology* **164**, 1233-1237

Ishiguro, E.E., Kay, W.W., Ainsworth, T., Chamberlain, R.A., Buckley, J.T., & Trust, T.J. (1981) *Journal of Bacteriology* **148**, 333-340

Jensen, S.E., Fecycz, I.T., Stemke, G.W. & Campbell, J.N. (1980) *Canadian Journal of Microbiology* **26**, 87-93

Jiwa, S.F.H. (1983) *Veterinary Microbiology* **8**, 17-34

Johnson, C.M., Tatner, M.F. & Horne, M.T. (1985) *Journal of Fish Biology* **27**, 445-458

Joiner, K.A. (1985) *Current Topics in Microbiology and Immunology* **121**, 135-158

Joiner, K.A., Grossman, N., Schmetz, M. & Leive, L. (1986) *Journal of Immunology* **136**, 710-715

Jones, G.W. & Isaacson, R.E. (1983) *Critical Reviews in Microbiology* **10**, 229-260

Kandler, O. & Konig, H. (1985) In: *The Bacteria*, vol.8 (eds. C.R. Woese & R.S. Wolfe), pp.413-457. Academic Press, New York

Karlsson, K.A. (1962) *Nordisk Veteriarmedicin* **14** (Suppl.2), 793-797

Kay, W.W., Buckley, J.T., Ishiguro, E.E., Phipps, B.M., Monette, J.P.L. & Trust, T.J. (1981) *Journal of Bacteriology* **147**, 1077-1084

Kay, W.W., Phipps, B.M., Ishiguro, E.E., Olafson, R.W. & Trust, T.J. (1984) *Canadian Journal of Biochemistry and Cell Biology* **62**, 1064-1071

Kay, W.W., Phipps, B.M., Ishiguro, E.E. & Trust, T.J. (1985) *Journal of Bacteriology* **164**, 1332-1336

Kay, W.W. & Trust, T.J. (1991) *Experientia* **47**, 412-414

Khardori, N. & Fainstein, V. (1988) *Annual Review of Microbiology* **42**, 395-419

Kinghorn, J.R. & Pateman, J.A. (1973) *Journal of General Microbiology* **78**, 39-46

Kitao, T., Yoshida, T., Aoki, T. & Fukudome, M. (1985) *Fish Pathology* **20**, 107-114

Klontz, G.W., Yasutake, W.T. & Ross, A.J. (1966) *American Journal of Veterinary Research* **27**, 1455-1460

Koffler, H. & Gale, G.O. (1957) *Archives of Biochemistry and Biophysics* **67**, 249-251

Konig, H. & Stetter, K.O. (1986) *Systematic and Applied Microbiology* **7**, 300-309

Kotoujansky, A. (1987) *Annual Review of Phytopathology* **25**, 405-430

Koval, S.F. & Murray, R.G.E. (1986) *Microbiological Sciences* **3**, 357-361

Kozaki, S., Asao, T., Kamata, Y. & Sakaguchi, G. (1989) *Journal of Clinical Microbiology* **27**, 1782-1786

Lacks, S.A. & Springhorn, S.S. (1980) *Journal of Biological Chemistry* **255**, 7467-7473

Laemmli, U.K. (1970) *Nature* **277**, 680-685

Langbridge, J. (1968) *Genetics* **108**, 116-126

Lankford, C.E. (1973) *CRC Critical Reviews in Microbiology* **2**, 273-331

Lark, D.L. (1986) In: *Protein-Carbohydrate Interactions in Biological Systems*. Academic Press, New York

Lee, K.-K. & Ellis, A.E. (1990) *Journal of Bacteriology* **172**, 5382-5393

Lee, K.-K. & Ellis, A.E. (1991) *Journal of Fish Diseases* **14**, 453-460

Leive, L. (1974) *Annals of the New York Academy of Science* **235**, 109-127

Leung, K.Y. & Stevenson, R.M.W. (1988) *Journal of General Microbiology* **134**, 151-160

Lockwood, B.C., North, M.J., Mallinson, D.J. & Coombs, G.H. (1987) *FEMS Microbiology Letters* **48**, 345-350

Lory, S. & Tai, P-C. (1983) *Gene* **22**, 95-101

Lugtenberg, B & Van Alphen, L. (1983) *Biochimica et Biophysica Acta* **737**, 51-115

McCarthy, D.H. & Roberts, R.J. (1980) *Advances in Aquatic Microbiology* **2**, 293-340

McCormick, W.A., Stevenson, R.M.W. & MacInnes, J.I. (1990) *Canadian Journal of Microbiology* **36**, 24-32

MacInnes, J.I., Trust, T.J. & Crosa, J. H. (1979) *Canadian Journal of Microbiology* **25**, 578-586

McIntosh, D. & Austin, B. (1988) *Journal of Diarrhoeal Disease Research* **6**, 131-136

McIntosh, D. & Austin, B. (1990) *Systematic and Applied Microbiology* **13**, 378-381

McIntosh, D. & Austin, B. (1991) *Journal of Applied Bacteriology Symposium Supplement* **70**, 1S-7S

McIntosh, M.A. & Earhart, C.F. (1977) *Journal of Bacteriology* **131**, 331-339

MacIntyre, S. & Buckley, J.T. (1978) *Journal of Bacteriology* **135**, 402-407

MacIntyre, S., Trust, T.J. & Buckley, J.T. (1979) *Journal of Bacteriology* **139**, 132-136

MacIntyre, S., Trust, T.J. & Buckley, J.T. (1980) *Canadian Journal of Biochemistry* **58**, 1018-1025

Mackman, N. & Holland, I.B. (1984) *Molecular and General Genetics* **196**, 129-134

Mackman, N., Nicaud, J.M., Gray, L. & Holland, I.B. (1986) *Current Topics in Microbiology and Immunology* **125**, 159-181

Manning, P.M., Brown, M.H. & Heuzenroeder, M.W. (1984) *Gene* **31**, 225-231

Margesin, R. & Schinner, F. (1991) *FEMS Microbiology Letters* **79**, 257-262

Maurizi, M.R. (1987) *Journal of Biological Chemistry* **262**, 2696-2703

Maxam, A.M. & Gilbert, W. (1977) *Proceedings of the National Academy of Sciences (USA)* **74**, 560-564

Maxted, W.B. (1972) *Methods in Microbiology* **7A**, 424-449

Mellergaard, S. (1983) *Journal of Applied Bacteriology* **54**, 289-294

Michel, C. & Dubois-Dumaudpeys, A. (1980) *Annales de Recherche Veterinaire* **11**, 375-386

Miyazaki, H., Yanagida, N., Horinouchi, T. & Beppu, T. (1989) *Journal of Bacteriology* **171**, 6566-6572

Molla, A., Matsumota, K., Oyamada, I., Katsuki, T. & Maeda, H. (1986) *Infection and Immunity* **53**, 522-529

Montford, P. & Baleux, B. (1991) *Research in Microbiology* **142**, 95-102

Munn, C.B., Ishiguro, E.E., Kay, W.W. & Trust, T.J. (1982) *Infection and Immunity* **36,** 1069-1075

Nakajima, K., Muroga, K. & Hancock, R.E.W. (1983) *International Journal of Systematic Bacteriology* **33,** 1-8

Nakamura, K. & Mizushima, S. (1976) *Journal of Biochemistry* **80,** 1411-1422

Neidhardt, F.C., Ingraham, J.L. & Schaechter, M. (1990) *Physiology of the Bacterial Cell*. Sinauer Associates, Inc., Massachusetts

Neilands, J.B. (1981) *Annual Review of Biochemistry* **50,** 715-731

Neilands, J.B. (1982) *Annual Review of Microbiology* **36,** 285-309

Nestle, M. & Roberts, W.K. (1969) *Journal of Biological Chemistry* **244,** 5213-5218

Neu, H.C. & Heppel, L.A. (1964) *Journal of Biological Chemistry* **239,** 3893-3900

Neu, H.C. & Heppel, L.A. (1965) *Journal of Biological Chemistry* **240,** 3685-3692

Newland, J.W., Green, B.A., Gray, L. & Holland, I.B. (1985) *Infection and Immunity* **47,** 691-696

Nicas, T.I. & Hancock, R.E.W. (1983) *Journal of Bacteriology* **153,** 281-285

Nieto, T.P. & Ellis, A.E. (1986) *Journal of General Microbiology* **132,** 1975-1979

Nieto, T.P. & Ellis, A.E. (1991) *Journal of Fish Diseases* **14,** 229-235

Nikaido, H. & Vaara, M. (1985) *Microbiological Reviews* **49,** 1-32

Nomura, S., Fujino, M., Yamakawa, M. & Kawahara, E. (1988) *Journal of Bacteriology* **170,** 3694-3702

Nomura, S. & Saito, H. (1982) *Bulletin of the Japanese Society of Scientific Fisheries* **48,** 1589-1597

Nonaka, M., Yamaguichi, N., Natsuume-Sakai, S. & Takahashi, M. (1981) *Journal of Immunology* **126**, 1489-1494

North, M.J. (1978) *Biochemical Society Transactions* **6**, 400-403

North, M.J. & Cotter, D.A. (1991) *Developmental Genetics* **12**, 154-162

O'Donovan, G.A. & Ingraham, J.L. (1965) *Proceedings of the National Academy of Sciences (USA)* **50**, 499-506

O'Donovan, G.A. & Neuhard, J. (1970) *Bacteriological Reviews* **34**, 278-343

Olivier, G. (1990) *Journal of Aquatic Animal Health* **2**, 119-127

Osborn, M.J. (1963) *Proceedings of the National Academy of Sciences (USA)* **50**, 499-506

Osborn, M.J., Gander, J.E., Parisi, E. & Carson, J. (1972) *Journal of Biological Chemistry* **247**, 3962-3972

Pansare, A.C., Venugopal, V. & Lewis, N.F. (1985) *Agricultural and Biological Chemistry* **49**, 2451-2452

Parker, N.D. & Munn, C.B. (1985) In: *Fish and Shellfish Pathology* (Ed. A.E. Ellis), pp.97-105. Academic Press, London

Paterson, W.D. (1983) In: *Antigens of Fish Pathogens* (eds. D.P. Anderson, M.Dorson & P.H.Dubourget) pp.119-137

Paterson, W.D., Douey, D. & Desautels, D. (1980) *Canadian Journal of Microbiology* **26**, 588-598

Paterson, W.D., Douglas, R.J, Grinyer, I. & MacDermott, L.A. (1969) *Journal of the Fisheries Research Board of Canada* **26**, 629-632

Payne, S.M. (1988) *CRC Critical Reviews in Microbiology* **16**, 81-111

Pennington, R.J. (1961) *Biochemical Journal* **80**, 649-654

Phipps, B.M. & Kay, W.W. (1988) *Journal of Biological Chemistry* **263**, 9298-9303

Plaut, A.G. (1983) *Annual Review of Microbiology* **37**, 603-622

Pohlner, J., Halter, R., Beyreuther, K. & Meyer, T.F. (1987) *Nature* **325**, 458-462

Pollock, M.R. (1962) In: *The Bacteria*, vol.4 (eds. I.C. Gunsalus & R.Y. Stanier), pp.121-178. Academic Press, New York

Poole, K. & Hancock, R.E.W. (1983) *FEMS Microbiology Letters* **16**, 25-29

Popoff, M. (1971) *Annales de Recherche Veterinaire* **2**, 33-45

Popoff, M. (1984) In: *Bergey's Manual of Systematic Bacteriology*, vol.1 (N.R. Kreig), pp. 545-548. Williams and Wilkins, Baltimore

Popoff, M. & Lallier, R. (1984) *Methods in Microbiology* **16**, 127-145

Popoff, M. & Véron, M. (1976) *Journal of General Microbiology* **94**, 11-22

Popoff, M. & Vieu, J.F. (1970) *C.R Acad Sci.* **270**, 2219-2222

Price, N.C., Banks, R.M., Campbell, C.M., Duncan, D. & Stevens, L. (1990) *Journal of Fish Diseases* **13**, 49-58

Price, N.C. & Dwek, R.A. (1979) *Principles and Problems in Physical Chemistry for Biochemists* (2nd ed.). Clarendon Press, Oxford

Price, N.C., Stevens, L., Duncan, D. & Snodgrass, M. (1989) *Journal of Fish Diseases* **12**, 223-232

Pugsley, A.P. (1986) *Microbiological Sciences* **3**, 362-364

Pugsley, A.P. (1988) In: *Protein Transfer and Organelle Biogenesis*. (eds. R.C. Das & P.W. Robbins) pp.607-652. Academic Press, London and New York

Pugsley, A.P. & Schwartz, M. (1984) *EMBO Journal* **3**, 2393-2397

Pugsley, A.P. & Schwartz, M. (1985) *FEMS Microbiology Review* **32**, 3-38

Randall, L.L. & Hardy, S.J.S. (1977) *European Journal of Biochemistry* **97**, 70-76

Rendall, L.L., Hardy, S.J.S. & Thom, J.R. (1987) *Annual Review of Microbiology* **41,** 507-541

Reed, W.P. & Williams, Jr., R.C. (1978) *Journal of Chronic Diseases* **31,** 67-72

Richmond, M.H. & Sykes, R.B. (1973) In: *Advances in Microbial Physiology* vol.9 (eds. A.H.Rose & D.W. Tempest), pp. 31-88. Academic Press, London

Robertson, C.D., North, M.J., Lockwood, B.C. & Coombs, G.H. (1990) *Journal of General Microbiology* **136,** 921-925

Rockey, D.D., Fryer, J.L. & Rohovec, J.S. (1988) *Diseases of Aquatic Organisms* **5,** 197-204

Rodgers, C.J., Pringle, J.H., McCarthy, D.H. & Austin, B. (1981) *Journal of General Microbiology* **125,** 335-345

Roffey, P.E. & Pemberton, J.M. (1990) *Current Microbiology* **21,** 329-337

Roszak, D.B. & Colwell, R.R. (1987) *Microbiological Reviews* **51,** 365-379

Rowbury, R.J., Armitage, J.P. & King, C. (1983) In: *Microbes in their Natural Environments* (eds. J.H. Slater, R. Whittenbury & J.W.T. Wimpenny), pp.299-350. Cambridge University Press, Cambridge

Rowley, D. (1956) *Annals of the New York Academy of Science* **66,** 304-311

Russell, R.R.B. (1975) *Canadian Journal of Microbiology* **22,** 83-91

Saino, Y., Inoue, M. & Mitsuhashi, S. (1984) *Antimicrobial Agents and Chemotherapy* **25,** 362-365

Saino, Y., Kobayashi, M., Inoue, M. & Mitsuhashi, S. (1984) *Antimicrobial Agents and Chemotherapy* **22,** 564-570

Sakai, D.K. (1978) *Scientific Reports of the Hokkaido Fish Hatchery* **33,** 55-73

Sakai, D.K. (1981) *Bulletin of the Japanese Society of Scientific Fisheries* **47,** 565-571

Sakai, D.K. (1985a) *Infection and Immunity* **48**, 146-152

Sakai, D.K. (1985b) *Applied and Environmental Microbiology* **50**, 1031-1037

Sakai, D.K. & Kimura, T. (1985) *Fish Pathology* **20**, 9-21

Sanders, C.C., Sanders, W.E, & Moland, E.S. (1986) *Antimicrobial Agents and Chemotherapy* **30**, 951-952

Sanger, F., Nicklen, S. & Coulson, A.R. (1977) *Proceedings of the National Academy of Sciences (USA)* **74**, 5463-5467

Schubert, R.H.W. & Hegazi, M. (1988) *Zentralblatt fur Bakteriologie und Hygiene A* **268**, 34-39

Sébald, M. & Véron, M. (1963) *Annales de Institut Pasteur* **105**, 897-910

Sedmak, J.J & Grossberg, S.E. (1977) *Analytical Biochemistry* **79**, 544-552

Shand, G.H., Anwar, H., Kadurugamuwa, J., Brown, M.R., Silverman, S.H. & Melling, J. (1985) *Infection and Immunity* **48**, 35-39

Shaw, D.H., Squires, M., Ishiguro, E.E. & Trust, T.J. (1986) *European Journal of Biochemistry* **161**, 309-313

Sheeran, B., Drinan, E. & Smith, P.R. (1984) In: *Fish Diseases*, Fourth Session of EIFAC/COPRAQ (ed. Acuigrup), pp. 89-100. Editora, Spain

Sheeran, B. & Smith, P.R. (1981) *FEMS Microbiology Letters* **11**, 73-76

Shieh, H.S. & MacLean, J.R. (1975) *International Journal of Biochemistry* **6**, 653-656

Shieh, H.S. (1985) *Microbios Letters* **28**, 69-72

Silhavy, T.J., Benson, S.A. & Emr, S.D. (1983) *Microbiological Reviews* **47**, 313-344

Sleytr, B.V. & Messner, P. (1983) *Annual Review of Microbiology* **37**, 311-339

Sleytr, B.V., Sara, M., Kupcu, Z. & Messner, P. (1986) *Archives of Microbiology* **146**, 19-24

Smit, J. (1986) In: *Bacterial Outer Membranes as Model Systems* (ed. M. Inouye), pp.343-376. John Wiley & Sons Ltd., Chichester

Smith, H. (1990) *Journal of General Microbiology* **136**, 377-383

Smith, I.W. (1963) *Journal of General Microbiology* **33**, 263-274

Sneath, P.H.A. (1963) *Principles in Numerical Taxonomy*. Freeman, New York

Stanier, R.Y. (1943) *Journal of Bacteriology* **46**, 213-214

Stanier, R.Y., Adelberg, E.A. & Ingraham, J.L. (1977) *General Microbiology* (4th ed.). MacMillan Publishers Ltd., Hampshire

Stevens, L. & Relton, J.M. (1981) *Transactions of the British Mycological Society* **77**, 637-639

Stewart, M., Beveridge, T.J. & Trust, T.J. (1986) *Journal of Bacteriology* **166**, 120-127

Stock, J.B., Ninfa, J. & Stock, A.M. (1989) *Microbiological Reviews* **53**, 450-490

Stock, J.B., Rauch, B. & Roseman, S. (1977) *Journal of Biological Chemistry* **252**, 7850-7861

Sykes, R.B. & Matthew, M. (1976) *Journal of Antimicrobial Chemotherapy* **2**, 115-157

Tai, P.-C., Kessler, D.P. & Ingraham, J.L. (1968) *Journal of Bacteriology* **97**, 466-468

Tajima, K., Takahashi, T., Ezura, Y. & Kimura, T. (1984) *Bulletin of the Japanese Society of Scientific Fisheries* **50**, 145-150

Taketa, K. & Pogell, M. (1965) *Journal of Biological Chemistry* **240**, 651-662

Taylor, P.W. (1988) In: *Virulence Mechanisms of Bacterial Pathogens* (ed. J.A. Roth), pp.107-120. American Society for Microbiology, Washington, DC

Tempest, D.W., Neijssel, O.M. & Zevenboom, W. (1983) In: *Microbes in their Natural Environments* (eds. J.H. Slater, R. Whittenbury & J.W.T. Wimpenny), pp.119-152. Cambridge University Press, Cambridge

Thom, J.R. & Randall, L.L. (1988) *Journal of Bacteriology* **170**, 5654-5661

Thornton, J., Howard, S.P. & Buckley, J.T. (1988) *Biochimica et Biophysica Acta* **959**, 153-159

Thune, R.L., Graham, T.E., Riddle, L.M. & Amborski, R.L. (1982) *Transactions of the American Fisheries Society* **111**, 749-754

Titball, R.W. & Munn, C.B. (1981) *FEMS Microbiology Letters* **12**, 27-30

Titball, R.W. & Munn, C.B. (1983) *FEMS Microbiology Letters* **20**, 207-210

Titball, R.W. & Munn, C.B. (1985a) *Journal of General Microbiology* **131**, 1603-1609

Titball, R.W. & Munn, C.B. (1985b) In: *Fish and Shellfish Pathology* (ed. A.E. Ellis), pp.61-68. Academic Press, London

Titball, R.W., Bell, A. & Munn, C.B. (1985) *Infection and Immunity* **49**, 756-759

Tommassen, J. (1986) *Microbial Pathogenesis* **1**, 225-228

Towbin, H.T., Staehelin, T. & Gordon, J. (1979) *Proceedings of the National Academy of Sciences (USA)* **76**, 4360-4354

Trun, N.J. & Silhavy, T.J. (1989) *Journal of Cell Science Suppl.* **11**, 13-28

Trust, T.J. (1986) *Annual Review of Microbiology* **40**, 479-502

Trust, T.J., Kay, W.W. & Ishiguro, E.E. (1983) *Current Microbiology* **9**, 315-318

Trust, T.J., Khouri, A.G., Austin, R.A. & Ashburner, L.D. (1980) *FEMS Microbiology Letters* **9**, 39-42

Udey, L.R. & Fryer, J.L. (1978) *Marine Fisheries Review* **40**, 12-17

Udey, L.R. & Guzman, G. (1980) *American Society for Microbiology Proceedings*, Abstracts of Paper P30, Abstract B78

Van Oss, C.J. (1978) *Annual Review of Microbiology* **32**, 19-39

Van Wielink, J.E. & Duine, J.A. (1990) *Trends in Biochemical Sciences* **15**, 136-137

von Heijne, G. (1988) *ISI Atlas of Science: Biochemistry*, 205-209

Véron, M. (1966) *Annales de Microbiologie* **III**, 671-709

Vihinen, M. & Mäntsälä, P. (1989) *Critcal Reviews in Biochemistry and Molecular Biology* **24**, 329-418

Wandersman, C. (1989) *Molecular Microbiology* **3**, 1825-1831

Wandersman, C., Andro, T. & Bertheau, Y. (1986) *Journal of General Microbiology* **132**, 899-906

Ward, P.D., Waters, C.A. & Sweeney, K.J. (1985) In: *Fish and Shellfish Pathology* (ed. A.E. Ellis), pp.107-117. Academic Press, London

Waxman, L. & Goldberg, A.L. (1986) *Science* **232**, 500-503

Webb, E.C. (1984) In: *Enzyme Nomenclature*. Academic Press, New York

Weber, K. & Osborn, M. (1969) *Journal of Biological Chemistry* **244**, 4406-4412

Weinberg, E.D. (1978) *Microbiological Review* **42**, 45-66

Weissbach, A. & Hurwitz, J. (1959) *Journal of Biological Chemistry* **234**, 705-709

Whittington, R.J., Gudkovs, N., Carrigan, M.J., Ashburner, L.D. & Thurstan, S.J. (1987) *Journal of Fish Diseases* **10**, 353-362

Widders, P.R. (1988) In: *Virulence Mechanisms of Bacterial Pathogens* (ed. J.A. Roth), pp.94-106. American Society for Microbiology, Washington, DC

Widders, P.R., Stokes, C.R., Newby, T.J. & Bourne, F.J. (1985) *Infection and Immunity* **48**, 417-421

Widders, P.R., Smith, J.W., Yarnall, M., McGuire, T.C. & Corbeil, L.B. (1988) *Journal of Medical Microbiology* **26**, 307-313

Williams, P., Brown, M.R.W. & Lambert, P.A. (1984) *Journal of General Microbiology* **130**, 2357-2365

Williamson, I.J.F. (1928) *Fishery Board for Scotland, Salmon Fisheries: No.* **5**,

Willis, R.C., Morris, R.G., Cirakoglu, C., Schellenberg, G.D., Gerber, N.H. & Furlong, C.E. (1974) *Archives of Biochemistry and Biophysics* **161**, 64-75

Wong, K.R. & Buckley, J.T. (1989) *Science* **246**, 654-656

Wong, K.R., Green, M.J. & Buckley, J.T. (1989) *Journal of Bacteriology* **171**, 2523-2527

Woolcock, J.B. (1988) In: *Virulence Mechanisms of Bacterial Pathogens* (ed. J.A. Roth), pp.73-93. American Society for Microbiology, Washington, DC

Yarnall, M., Widders, P.R. & Corbeil, L.B. (1988) *Scandinavian Journal of Immunology* **28**, 129-136

NUMEROUS ORIGINALS IN COLOUR