

***Aeromonas hydrophila* Vaccine Development Using
Immunoproteomics**

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by

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**UNIVERSITY OF
STIRLING**

To my brother
Radjassegarane Poobalane

DECLARATION

I declare that this thesis has been compiled by myself, and is the result of my own investigations. It has not been submitted for any other degree and all sources of information have been duly acknowledged.

Saravanane Poobalane

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ABBREVIATIONS

| | |
|----------------|--|
| 1D | one dimensional |
| 2D | two dimensional |
| µg | microgram |
| µl | microlitre |
| APW | alkaline peptone water |
| a_w | water activity |
| BSA | bovine serum albumin |
| CFU | colony forming unit |
| DNA | deoxyribonucleic acid |
| ECP | extracellular products |
| e.g. | example |
| ELISA | enzyme linked immunosorbent assay |
| <i>et. al.</i> | <i>"et alia"</i> : and others |
| EUS | epizootic ulcerative syndrome |
| FCA | Freund's complete adjuvant |
| G | gramme(s) |
| × g | multiples of gravity |
| h | hour(s) |
| HAKI | Research Institute for Fisheries, Aquaculture and Irrigation |
| HRP | horseradish peroxidase |
| HSWB | high salt wash buffer |
| Ig | immunoglobulin |
| IMP | inner membrane protein |
| <i>i.e.</i> | <i>"id est"</i> : that is |
| IP | intraperitoneal(ly) |
| IPTG | isopropyl-beta-d-thiogalactopyranoside |
| IROMP(s) | iron regulated outer membrane protein(s) |
| kb | kilo base |
| kDa | kilo Dalton(s) |
| kg | kilo gramme |
| L | litre(s) |
| LB | Luria Bertani |
| LD | lethal dose |
| LPS | lipopolysaccharide |
| LSWB | low salt wash buffer |
| M | molar |
| mA | milliampere(s) |
| MAb(s) | monoclonal antibody (antibodies) |
| MALDI-TOF MS | matrix-assisted laser desorption ionisation time-of-flight mass spectrometry |
| MAS | motile aeromonad septicaemia |
| min | minute(s) |
| ml | millilitre |
| MW | molecular weight |
| NCBI | National Centre for Biotechnology Information |
| NCIMB | National Collection of Industrial and Marine Bacteria |
| OD | optical density |
| OMP | outer membrane protein |

| | |
|----------|--|
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| pI | Isoelectric point |
| RNA | ribonucleic acid |
| RPS | relative percentage survival |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| sec | second(s) |
| sp. | species |
| TBS | tris buffered saline |
| TEM | transmission electron microscopy |
| TSA | tryptone soy agar |
| TSB | tryptone soy broth |
| TTBS | tris buffered saline with Tween-20 |
| UV | ultraviolet |
| V | volt |
| v/v | volume/volume |
| WC | whole cell |
| w/v | weight/volume |

ABSTRACT

Aeromonas hydrophila is an opportunistic pathogen that causes a wide range of symptoms and diseases in fish. Development of a commercial vaccine has been problematic due to the heterogeneity between isolates of *A. hydrophila*. A new approach using immunoproteomics was used in this study to try to develop a vaccine that would protect against a wide range of *A. hydrophila* strains.

The virulence of 14 isolates of *A. hydrophila* from different geographical regions was determined in common carp (*Cyprinus carpio*) indicating that 6 isolates were virulent, while 8 isolates were avirulent. Expression of cellular and extracellular products (ECP) of six of these isolates (4 virulent and 2 avirulent isolates) were examined following culture of the bacterium *in vitro*, in tryptic soy broth, and *in vivo*, in dialysis tubing placed within the peritoneal cavity of carp. Two types of molecular weight cut off tubes (25 and 100 kDa) were used for the implants. Whole cell (WC), outer membrane protein (OMP) and ECPs of the bacteria grown *in vitro* and *in vivo* were analysed by 1 dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (1D SDS-PAGE). Additionally, 2D SDS-PAGE was used to analyse WC preparations of *A. hydrophila* grown *in vitro* and *in vivo*. The production of unique proteins and up and down-regulation of protein expression were observed in all the preparations of bacteria grown *in vitro* and *in vivo*. Unique bands were seen in the 1D SDS-PAGE at 58 and 55 kDa for WC and OMP preparations, respectively, for all the isolates cultured *in vivo*. Bands of increased intensity were observed at 70, 55, 50 and 25 kDa with WC preparations for the virulent isolates cultured *in vivo*. Analysis of WC preparations by 2D SDS-PAGE indicated differences in the expression of spots between bacteria cultured

in vitro and *in vivo*. A number of unique spots, mostly between 30 and 80 kDa with pI values ranging from 5.0-6.0 were observed in the bacteria grown *in vivo*.

The protein profiles of different preparations (WC, OMP, ECP) of bacteria cultured *in vitro* and *in vivo* were screened by 1D Western blot using antibodies from carp artificially infected with different isolates of *A. hydrophila* to identify potential vaccine candidates. The WC preparations of *A. hydrophila* (T4 isolate) grown *in vitro* were also analysed by 2D Western blot. A 50 kDa protein of *A. hydrophila* was found to be the most immunogenic molecule in both WC and OMP of bacteria grown both *in vitro* and *in vivo*. The protection efficacy of this protein was determined in goldfish by vaccinating fish with electro-eluted 50 kDa protein then challenging the fish with *A. hydrophila*. Fish were also passively immunised with fish sera raised to the 50 kDa protein and then challenged. The relative percentage survival (RPS) was 67 % in the vaccination trial, while the results were inconclusive for the passive immunisation trial.

The 50 kDa protein was confirmed to be the S-layer protein of *A. hydrophila* following identification using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Recombinant S-layer protein was then produced and the cross-protection efficacy of this protein against six virulent isolates of *A. hydrophila* was confirmed in a large scale vaccination trial using carp. The RPS value for the 6 isolates of *A. hydrophila* ranged from between 56 and 87 %. The results of this project suggest that the immunogenic S-layer protein of *A. hydrophila* could be used as a common antigen to protect fish against infection by different isolates of this pathogenic bacterium.

TABLE OF CONTENTS

| | |
|--|-----------|
| Declaration | iii |
| Acknowledgements | iv |
| Abbreviations..... | v |
| Abstract | vii |
| Chapter 1. Introduction..... | 1 |
| 1.1. Introduction | 2 |
| 1.2. Historical background and taxonomy | 3 |
| 1.3. Characteristics of <i>A. hydrophila</i> | 4 |
| 1.3.1. Growth characteristics | 4 |
| 1.3.2. Biochemical Characteristics..... | 5 |
| 1.4. Epizootiology..... | 7 |
| 1.4.1. Distribution of <i>A. hydrophila</i> and susceptible fish species | 7 |
| 1.4.2. Pathogenicity | 10 |
| 1.4.2.1. Clinical signs of infection | 11 |
| 1.4.3. Factors involved in pathogenicity..... | 13 |
| 1.4.3.1. Extracellular products | 15 |
| 1.4.3.2. The outer membrane and other surface components..... | 18 |
| 1.5. Diagnostics | 22 |
| 1.6. Treatments and control | 24 |
| 1.6.1. Antibiotics | 24 |
| 1.6.2. Immunostimulants..... | 27 |
| 1.6.3. Vaccines | 29 |
| 1.6.3.1. History of <i>A. hydrophila</i> vaccine development..... | 29 |
| 1.6.3.2. Approaches for developing an effective <i>A. hydrophila</i> vaccine | 33 |
| 1.7. Aims of the study | 35 |
| Chapter 2. Characterisation of <i>A. hydrophila</i>: Biochemistry, virulence and protein expression following culture <i>in vitro</i> and <i>in vivo</i>..... | 37 |
| 2.1. Introduction | 38 |
| 2.2. Materials and methods..... | 41 |
| 2.2.1. Biochemical characterisation of the <i>A. hydrophila</i> collection | 41 |
| 2.2.1.1. Bio-chemical characterisation..... | 43 |

| | |
|---|-----------|
| 2.2.1.2. Gram staining | 43 |
| 2.2.1.3. Motility test | 43 |
| 2.2.1.4. Standard concentration curve..... | 44 |
| 2.2.2. Virulence of <i>A. hydrophila</i> in common carp, <i>Cyprinus carpio</i> | 44 |
| 2.2.3. Culture of <i>A. hydrophila in vitro</i> and <i>in vivo</i> | 45 |
| 2.2.3.1. Culture of bacteria <i>in vitro</i> | 45 |
| 2.2.3.2. Culture of bacteria <i>in vivo</i> | 47 |
| 2.2.4. SDS-PAGE | 51 |
| 2.2.4.1. 1D SDS-PAGE | 51 |
| 2.2.4.2. 2D SDS-PAGE | 52 |
| 2.2.5. Ultra structure of outer membrane | 53 |
| 2.3. Results | 54 |
| 2.3.1. Biochemical characterisation | 54 |
| 2.3.1.1. Standard concentration curve..... | 56 |
| 2.3.2. Virulence of <i>A. hydrophila</i> | 57 |
| 2.3.3. Protein profiles of bacteria grown <i>in vitro</i> and <i>in vivo</i> | 58 |
| 2.3.3.1. Protein expression <i>in vitro</i> analysed by 1D SDS-PAGE | 58 |
| 2.3.3.2. Protein expression <i>in vivo</i> in 1D SDS-PAGE..... | 61 |
| 2.3.3.3. Protein expression of <i>A. hydrophila</i> grown <i>in vitro</i> and <i>in vivo</i> analysed by 2D SDS-PAGE | 64 |
| 2.3.3.4. TEM studies of <i>A. hydrophila</i> cultured <i>in vitro</i> and <i>in vivo</i> | 74 |
| 2.4. Discussion..... | 76 |
| Chapter 3. Identification of potential vaccine antigens on <i>A. hydrophila</i> | 87 |
| 3.1. Introduction | 88 |
| 3.2. Materials and methods..... | 91 |
| 3.2.1. Antibody response of common carp to <i>A. hydrophila</i> | 91 |
| 3.2.2. Polyclonal antibody production against <i>A. hydrophila</i> in rabbits | 92 |
| 3.2.3. Enzyme linked immunosorbent assay | 93 |
| 3.2.4. Western blot..... | 94 |
| 3.2.4.1. Western blot using common carp antibody..... | 95 |
| 3.2.4.2. 2D SDS-PAGE Western blot using common carp antibody.. | 96 |
| 3.2.4.3. Western blot using rabbit antibody | 96 |
| 3.2.5. Assessing the levels of protection of a 50 kDa protein in goldfish against <i>A. hydrophila</i> challenge | 97 |

| | |
|--|------------|
| 3.2.5.1. Preparation of the antigen | 97 |
| 3.2.5.2. Determination of the LD ₅₀ of <i>A. hydrophila</i> T4 isolate in goldfish | 98 |
| 3.2.5.3. Immunisation of goldfish with the electro-eluted 50kDa protein..... | 99 |
| 3.2.5.4. Passive immunisation of goldfish with anti-50kDa protein serum raised in goldfish..... | 99 |
| 3.3. Results..... | 100 |
| 3.3.1. Antibody response of common carp infected with different isolates of <i>A. hydrophila</i> | 100 |
| 3.3.1.1. Antibody response of common carp infected with <i>A. hydrophila</i> | 100 |
| 3.3.1.2. Western blot analysis of common carp serum..... | 101 |
| 3.3.1.3. 2D SDS-PAGE Western blot | 105 |
| 3.3.2. Response of rabbit antibody against <i>A. hydrophila</i> | 105 |
| 3.3.3. Vaccination and Passive immunisation of goldfish with an electro-eluted 50 kDa protein from <i>A. hydrophila</i> | 107 |
| 3.4. Discussion..... | 108 |
| Chapter 4. Production and efficacy testing of an <i>A. hydrophila</i> recombinant S-layer protein vaccine | 115 |
| 4.1. Introduction | 116 |
| 4.2. Materials and methods..... | 118 |
| 4.2.1. Identification of the 50 kDa protein of <i>A. hydrophila</i> by peptide mass fingerprinting..... | 118 |
| 4.2.2. Recombinant 50 kDa protein production..... | 118 |
| 4.2.2.1. DNA extraction from <i>A. hydrophila</i> T4 isolate..... | 119 |
| 4.2.2.2. Polymerase chain reaction (PCR) of <i>A. hydrophila</i> 50 kDa protein gene..... | 120 |
| 4.2.2.3. Preparation of PCR products for transformation into <i>E. coli</i> | 121 |
| 4.2.2.4. Transformation of vectors carrying 50 kDa protein gene into <i>E. coli</i> | 121 |
| 4.2.2.5. Expression of the recombinant 50 kDa protein in <i>E. coli</i> | 122 |
| 4.2.2.6. Large scale production of recombinant 50 kDa protein | 123 |

| | |
|--|------------|
| 4.2.3. Sequencing of the <i>A. hydrophila</i> T4 isolate 50 kDa protein gene | 124 |
| 4.2.4. Vaccination of common carp with recombinant 50 kDa protein ... | 124 |
| 4.2.4.1. Standardising the <i>A. hydrophila</i> challenge | 124 |
| 4.2.4.2. Vaccination | 125 |
| 4.2.4.3. Challenge studies | 126 |
| 4.2.4.4. Statistical analysis | 126 |
| 4.3. Results | 127 |
| 4.3.1. Peptide Mass Fingerprinting of the 50 kDa protein from <i>A. hydrophila</i> | 127 |
| 4.3.1.1. Sequence of the S-layer protein gene isolated from <i>A. hydrophila</i> T4 isolate | 129 |
| 4.3.2. Production of a recombinant protein for the S-layer of <i>A. hydrophila</i> isolate T4..... | 130 |
| 4.3.2.1. Large scale production of S-layer recombinant protein | 132 |
| 4.3.3. Efficacy of recombinant S-layer protein against <i>A. hydrophila</i> in common carp..... | 134 |
| 4.3.3.1. Standardisation of the challenge of common carp with <i>A. hydrophila</i> | 134 |
| 4.3.3.2. Vaccination of common carp with recombinant S-layer protein of <i>A. hydrophila</i> | 135 |
| 4.4. Discussion..... | 139 |
| Chapter 5. Discussion and final conclusions | 148 |
| References | 162 |
| Appendix I. Buffers and reagents | 195 |
| Appendix II. Mixtures and vector used for recombinant protein production | 200 |
| Appendix III. Publications and presentations from the project..... | 202 |

LIST OF TABLES

| | |
|---|-----|
| Table 1.1: Fish and shellfish species susceptible to <i>A. hydrophila</i> infection..... | 9 |
| Table 2.1: <i>A. hydrophila</i> strains used in this study | 42 |
| Table 2.2: Biochemical characterisation of <i>A. hydrophila</i> type strain 1134 and T4 using API 20E strips | 55 |
| Table 2.3: Rod morphology of different <i>A. hydrophila</i> strains | 56 |
| Table 2.4: No. of spots detected in 2D SDS-PAGE with WC preparations of bacteria grown <i>in vitro</i> and <i>in vivo</i> | 65 |
| Table 2.5: MW and pI of unique spots seen in 2D SDS-PAGE analysis for <i>A. hydrophila</i> strains grown <i>in vivo</i> in both MW cut off tubes compared with the same strains grown <i>in vitro</i> | 71 |
| Table 2.6: MW and pI of the most up and down-regulated spots in 2D SDS-PAGE of <i>A. hydrophila</i> grown either <i>in vitro</i> or <i>in vivo</i> (25 and 100 kDa MW cut off tube)..... | 72 |
| Table 2.7: MW and pI of the most up and down-regulated spots in 2D SDS-PAGE of <i>A. hydrophila</i> grown within the 25 and 100 kDa MW cut off tubes <i>in vivo</i> (up-regulated <i>in vivo</i> compared to <i>in vitro</i>)..... | 73 |
| Table 4.1: The consequences of the nucleotide/amino acid changes between sequence of <i>A. hydrophila</i> TF7 and T4 isolates | 130 |
| Table 4.1: The LD ₅₀ values of <i>A. hydrophila</i> strains for common carp..... | 134 |
| Table 4.2: Percentage mortality and relative percentage survival of carp vaccinated with recombinant S-layer protein of <i>A. hydrophila</i> and then challenged against the bacteria 35 days post-vaccination..... | 138 |

LIST OF FIGURES

| | |
|--|-----|
| Figure 1.1: Structure of the outer membrane..... | 19 |
| Figure 2.1: Implantation of dialysis tubing containing <i>A. hydrophila</i> for culture of the bacterium <i>in vivo</i> | 50 |
| Figure 2.2: Standard concentration curve for <i>A. hydrophila</i> | 57 |
| Figure 2.3: Mortalities of Common carp, <i>C. carpio</i> during challenge with different isolates of <i>A. hydrophila</i> | 58 |
| Figure 2.4: SDS-PAGE of <i>A. hydrophila</i> grown <i>in vitro</i> , stained with Coomassie blue | 60 |
| Figure 2.5: SDS-PAGE of <i>A. hydrophila</i> grown <i>in vivo</i> , stained with Coomassie blue | 62 |
| Figure 2.6a: 2D SDS-PAGE of <i>A. hydrophila</i> strains grown <i>in vitro</i> | 66 |
| Figure 2.6a: 2D SDS-PAGE of <i>A. hydrophila</i> strains grown <i>in vitro</i> | 67 |
| Figure 2.6b: 2D SDS-PAGE of <i>A. hydrophila</i> strains grown <i>in vitro</i> and <i>in vivo</i> ... | 68 |
| Figure 2.6b: 2D SDS-PAGE of <i>A. hydrophila</i> strains grown <i>in vitro</i> and <i>in vivo</i> ... | 69 |
| Figure 2.6b: 2D SDS-PAGE of <i>A. hydrophila</i> strains grown <i>in vitro</i> and <i>in vivo</i> ... | 70 |
| Figure 2.7: Transmission electron micrograph of <i>A. hydrophila</i> cultured <i>in vitro</i> and <i>in vivo</i> | 75 |
| Figure 3.1: Antibody response of common carp against different isolates of <i>A. hydrophila</i> determined by ELISA expressed as an absorbance at 450 nm | 101 |
| Figure 3.2: Western blot analysis of different whole cell preparations of <i>A. hydrophila</i> against pooled serum from common carp infected with 6 different <i>A. hydrophila</i> isolates | 102 |
| Figure 3.3: Western blot analysis of different preparations of 6 <i>A. hydrophila</i> isolates screened with serum raised against corresponding <i>A. hydrophila</i> isolates in common carp | 104 |
| Figure 3.4: 2D Western blot analysis of whole cell preparation of <i>A. hydrophila</i> T4 isolate screened with antibody from common carp infected with <i>A. hydrophila</i> T4 isolate | 106 |
| Figure 3.5: Western blot analysis of different whole cell preparation of <i>A. hydrophila</i> against rabbit anti- <i>A. hydrophila</i> serum | 106 |
| Figure 3.6: Cumulative percentage of goldfish mortality in preliminary vaccination trial..... | 108 |

| | |
|---|-----|
| Figure 4.1: Primers and restriction enzymes used for amplification of the 50 kDa protein gene | 120 |
| Figure 4.2: MALDI-TOF MS spectrum showing the peptide profiles of 50 kDa band | 127 |
| Figure 4.3: Nucleic and amino acid sequences of <i>A. hydrophila</i> S-layer protein | 128 |
| Figure 4.4: Sequence of amplified gene encoding the S-layer protein from <i>A. hydrophila</i> T4 isolate | 129 |
| Figure 4.5: Amplification of the S-layer gene of <i>A. hydrophila</i> isolate T4 shown on a 1 % agrose gel | 131 |
| Figure 4.6: Expression of S-layer protein of <i>A. hydrophila</i> with <i>E. coli</i> WC protein | 133 |
| Figure 4.7: Recombinant S-layer protein of <i>A. hydrophila</i> purified from <i>E. coli</i> | 133 |
| Figure 4.8: Cumulative percentage mortality of carp vaccinated with recombinant S-layer protein and challenged with <i>A. hydrophila</i> isolates | 136 |

Chapter 1. Introduction

1.1. Introduction

Aeromonas hydrophila is a primary (Esteve *et al.*, 1993), secondary (Joice *et al.*, 2002) and opportunistic pathogen (Dooley and Trust, 1988; Lio-Po *et al.*, 1996) of a variety of aquatic and terrestrial animals, including humans. It is a ubiquitous, free living, Gram-negative bacterium, mainly found in water and water-related environments and causes a wide variety of symptoms (Hazen *et al.*, 1978a). The disease caused by *A. hydrophila* is called motile aeromonad septicaemia (MAS) and this pathogen is associated with number of other diseases in fish, for example, epizootic ulcerative syndrome (EUS) as a secondary pathogen (Roberts, 1993; Pathiratne *et al.*, 1994; Lio-Po *et al.*, 1998). The clinical signs in fish vary from tissue swelling, necrosis, ulceration and haemorrhagic septicaemia (Hazen *et al.*, 1978a; Karunasagar *et al.*, 1986; Angka, 1990; Aguilar *et al.*, 1997; Azad *et al.*, 2001).

Aeromonas hydrophila is associated with disease in humans and domestic animals including sheep, dogs and cats, especially when exposed to periods of stress (Burke *et al.*, 1984; Howard and Buckley, 1985a; Janda and Duffey, 1988; Ghenghesh *et al.*, 1999; Ilhan *et al.*, 2006). It is reported to contribute to intestinal and extra-intestinal infections including diarrhea in humans and other animals (Agarwal *et al.*, 1998; Guimaraes *et al.*, 2002). It has also been found in a variety of food products producing a range of toxins such as haemolysin, enterotoxin and cytotoxin (Callister and Agger, 1987; Yucel *et al.*, 2005; Daskalov, 2006).

1.2. Historical background and taxonomy

The genus *Aeromonas* was first described by Zimmermann (1890), who isolated the bacterium from the drinking water supply of Chemnitz in Germany using gelatine agar. He named the bacterium "*Bacillus punctatus*". Sanarelli (1891) isolated a similar bacterium from the blood and lymph of frogs, which he called "*Bacillus hydrophilus fuscus*", but in 1901 Chester proposed a name change to "*Bacterium hydrophilium*" (Caselitz, 1966). In the first edition of the Bergey's manual, this species was erroneously designated as "*Proteus hydrophilus*". However, in the Sixth Edition the genus *Proteus* was reclassified as *Pseudomonas* (Speck and Stark, 1942; Rustigan and Stuart, 1943). The genus *Aeromonas* was finally adopted in the Seventh Edition of *Bergey's* manual (Stainer, 1943), and this particular organism was classified as *A. hydrophila*.

According to molecular genetic studies, Messner and Sleytr (1992) proposed that the genus *Aeromonas* might be placed in a new family, the *Aeromonadaceae*. This genus was previously placed in the family, *Vibrionaceae* (Farmer, 1992) based on its phenotypic expression. Sakazaki & Shimada (1984) serotyped strains of *Aeromonas* on the basis of its O-antigen lipopolysaccharide (LPS). The genus *Aeromonas* has been shown to be antigenically diverse with over 90 established or possible serogroups described within this genus (O'Farrell, 1975; Frerichs, 1989).

The family is sub-divided into psychrophilic and mesophilic species. The psychrophilic group is non-motile, does not grow at 37°C and is therefore

unimportant to clinical microbiology. Members of the mesophilic group grow at 37°C and are motile using polar flagella. This group is divided into three principal groups, *A. hydrophila*, *A. caviae* and *A. sobria* (Korbsrisate *et al.*, 2002).

1.3. Characteristics of *A. hydrophila*

1.3.1. Growth characteristics

Aeromonas hydrophila is a fermentative rod approximately $0.8-1.0 \times 1.0-3.5 \mu\text{m}$ in size, that is motile via a single polar flagella (Austin and Austin, 1999). It is able to produce two distinct types of flagella; polar flagella for swimming in liquids and lateral flagella for swarming over surfaces (Altarriba *et al.*, 2003). The bacterium can be isolated on non-selective media such as nutrient agar or tryptone soy agar (TSA), or on selective media such as Rimler-Shotts medium (Zimmermann, 1890) or peptone beef-extract glycogen agar (Sanarelli, 1891) by incubating at 20-30°C for 18-36 h. Colonies of *A. hydrophila* that are grown on TSA at 28°C for 18-24 h usually appear round, creamy to light yellow in colour, raised, and 2-3 mm in diameter. Most selective media use carbohydrate and ampicillin or penicillin as the selective agent (Palumbo *et al.*, 1985). Kay *et al.* (1985) recommended using sheep blood agar with $10 \mu\text{g ml}^{-1}$ ampicillin preceded by overnight enrichment in alkaline peptone water (APW) for the isolation of *A. hydrophila* from humans. Cephalothin has been reported as the best enrichment agent in APW for *A. hydrophila* isolation owing to its greater selectivity and efficiency in recovering stressed or low concentrations of bacteria (Sachan and Agarwal, 2000).

The culture environment plays a major role in the growth and virulence of the bacterium, especially with respect to available nutrients, temperature and pH.

Sautour *et al.* (2003) proposed a model for describing the effects of temperature, water activity (a_w) (ratio between vapour pressure of water and that of pure water at the same temperature) and pH on the growth of *A. hydrophila*, and showed that temperature and a_w are the main influences on the bacterium's growth, while no significant influence of pH was seen. Incubation of *A. hydrophila* at different pH values, *i.e.* 6.0, 6.5, 7.0 and 7.5 did not significantly affect the growth rates, but lag phases were shorter at pH 6.0 than pH 7.0 (Buncic and Avery, 1995).

Although *A. hydrophila* can be grown at a wide range of temperatures, many researchers claim that the most suitable temperature for culturing the bacterium is between 25 and 35°C, while some researchers have found 20°C as an optimal temperature for its growth (Popoff, 1984). However, Uddin *et al.* (1997) found that the optimum temperature for growth of *A. hydrophila* was $34.5 \pm 1.0^\circ\text{C}$, while protease production was greatest at $27.6 \pm 4.9^\circ\text{C}$. It is neither salt (<5%) nor acid (min. pH~6) tolerant, and has the ability to grow at temperatures as low as -0.1°C for some strains (Daskalov, 2006). The growth of *A. hydrophila* at different temperatures ranging from 4 to 42°C and 5 to 35°C have been reported by Palumbo *et al.* (1985) and Callister and Agger (1987), respectively.

1.3.2. Biochemical Characteristics

The biochemical characteristics of the *A. hydrophila* are very complex and researchers have found it hard to relate these activities with a particular function. For example, biochemical reactions of *A. hydrophila* have not been correlated with either toxin production or plasmid profiles of the bacteria (Kindschuh *et al.*, 1987; Noterdaeme *et al.*, 1991). However, a high degree of similarity was found in the

biochemical traits of *A. hydrophila* isolated from water and sediment at different sites and during different seasons along the river Porma, in Spain, although the isolates displayed different degrees of virulence in fish (Paniagua *et al.*, 1990). De Figueiredo and Plumb (1977) previously reported that *A. hydrophila* isolated from water was not as virulent as those isolates obtained from fish, despite isolates from both sources having similar biochemical characteristics.

Biochemical factors such as production of high affinity iron uptake systems (e.g. siderophores) have been correlated with virulence, although the bacteria have evolved many other factors for infecting fish (Ellis, 1999). There are two iron-transporting siderophores, aconitabactin and enterobactin in mesophilic *Aeromonas*, but production of aconitabactin is predominant in *A. hydrophila*. The type of siderophore produced by different strains might be a useful tool for subdividing the motile aeromonads (Barghouthi *et al.*, 1989). Barghouthi *et al.* (1991) suggested that aconitabactin could be a virulence factor of aeromonads. This was supported by the work of Naidu and Yadav (1997), in which production of aconitabactin was found to be greater in clinical isolates than in environmental isolates of *A. hydrophila*.

Merino *et al.* (2001) suggested that Mg^{2+} and possibly Co^{2+} have some role in *A. hydrophila*'s ability to swarm and may be related to processes such as adherence and biofilm formation. *N*-acylhomoserine lactone-dependent quorum sensing has also been reported to be involved in biofilm formation by *A. hydrophila* (Lynch *et al.*, 2002). Quorum sensing is a form of cell-to-cell communication employed by many Gram-negative bacteria to regulate a diverse array of characteristics

including bioluminescence, antibiotic production, swarming motility and production of extracellular virulence factors (Kirke *et al.*, 2004). *Aeromonas hydrophila* from a variety of different sources have been shown to have different chemotactic responses to the mucus of freshwater fish, amino acids and carbohydrates (Hazen *et al.*, 1982 and 1984). The authors found a significantly higher chemotactic index with clinical isolates compared to the environmental isolates, and suggested that this factor might contribute to the virulence of the pathogen.

1.4. Epizootiology

1.4.1. Distribution of *A. hydrophila* and susceptible fish species

The distribution of *A. hydrophila* in many aquatic systems globally indicates the successful adaptation of the bacterium to such environments (Hazen *et al.*, 1978b; Williams and LaRock, 1985). It is a common contaminant of fresh foods, including fish and other seafood (Rustigan and Stuart, 1943; Amend and Fender, 1976; Daskalov, 2006). The occurrence of *A. hydrophila* is higher in lotic systems compared with lentic systems, and saline environments have higher levels of *A. hydrophila* than fresh water environments (Hazen *et al.*, 1978b).

Fish disease is a major risk factor in commercial aquaculture with millions of dollars lost annually (Boulanger *et al.*, 1977; Fang *et al.*, 2000 and 2004). *Aeromonas hydrophila* infection is the scourge of fresh and warm water fish farming worldwide and is considered as a significant economic problem (Torres *et al.*, 1990; Rahman *et al.*, 2001a; Hu *et al.*, 2005), particularly in China and India over the past decade (Karunasagar *et al.*, 1989; Chang *et al.*, 1992). It is also

believed to be a pathogen of emerging importance for humans through consuming fish and shellfish contaminated with *A. hydrophila* (Vivekanandhan *et al.*, 2005).

Aeromonas hydrophila are psychrotrophic in nature with a multiplicity of virulence factors. They are commonly isolated from normal healthy fish, with only certain strains possessing the virulence factors necessary to induce disease (Cahill, 1990; Vivekanandhan *et al.*, 2005). There are many combinations of factors involved in host susceptibility to *A. hydrophila* infections. For example, environmental stress and injury, which precipitate infection, have been reported in common carp (*Cyprinus carpio*) (Pai *et al.*, 1995). Similarly, warmer water temperatures in the summer have been shown to increase the susceptibility of goldfish (*Carrassius auratus*) and koi (*C. auratus* × *C. carpio*) to infection by *A. hydrophila*, with secondary infections resulting from high parasite loads (Dixon and Issvoran, 1993).

A wide variety of fish and shellfish has been reported to be susceptible to *A. hydrophila* by a number of authors (Table 1.1). Esteve *et al.* (1994) reported that European eel, *Anguilla anguilla* were particularly susceptible and might be specific hosts for *A. hydrophila* due to dominance of this pathogen in diseased eel at different eel farms in Valencia (Spain) during various seasons. Rainbow trout (*Oncorhynchus mykiss*), on the other hand, were shown to be less susceptible than European eel to *A. hydrophila* (Esteve *et al.*, 1993).

Table 1.1: Fish and shellfish species susceptible to *A. hydrophila* infection

| Fish species | | Authors |
|----------------------------------|------------------------|----------------------------------|
| Scientific name | Common name | |
| <i>Anabas testudineus</i> | Climbing perch | Ansary <i>et al.</i> , 1992 |
| <i>Anguilla anguilla</i> | Eel | Esteve <i>et al.</i> , 1993 |
| <i>Argopecten purpuratus</i> | Scallops | Riquelme <i>et al.</i> , 1996 |
| <i>Carrassius auratus</i> | Goldfish | Maji <i>et al.</i> , 2006 |
| <i>Carassius carassius</i> | Crucian carp | Llobrera and Gacutan, 1987 |
| <i>Channa striatus</i> | Chevron snakehead | |
| <i>Clarias batrachus</i> | catfish | |
| <i>Clarias gariepinus</i> | African catfish | Angka <i>et al.</i> , 1995 |
| <i>Cyprinus carpio</i> | Common carp | Farkas and Olah, 1982 |
| <i>Etroplus suratensis</i> | Pearl spot | Pathiratne <i>et al.</i> , 1994 |
| <i>Glossogobius giurus</i> | goby | Llobrera and Gacutan, 1987 |
| <i>Ictalurus punctatus</i> | Channel catfish | Ventura and Grizzle, 1988 |
| <i>Macrobrachium rosenbergii</i> | Giant freshwater prawn | Tonguthai, 1992 |
| <i>Megalobrama amblycephala</i> | Wuchang bream | Nielsen <i>et al.</i> , 2001 |
| <i>Micropterus salmoides</i> | Largemouth bass | Huizinga <i>et al.</i> , 1979 |
| <i>Neoceratodus forsteri</i> | Australian lungfish | Kemp, 1994 |
| <i>Ophiocephalus striatus</i> | Snakehead | Llobrera and Gacutan, 1987 |
| <i>Oreochromis niloticus</i> | Nile tilapia | Yambot, 1998 |
| <i>Osphronemus gourami</i> | Giant gourami | Supriyadi, 1986 |
| <i>Pangasius pangasius</i> | Catfish | Tanasomwang and Saitanu, 1979 |
| <i>Puntius sarana</i> | Barb | Pathiratne <i>et al.</i> , 1994 |
| <i>Rasbora daniconius</i> | <i>Rasbora</i> | |
| <i>Silurus glanis</i> | Sheatfish | Farkas and Olah, 1982 |
| <i>Trichogaster pectoralis</i> | Snakeskin gourami | Pathiratne <i>et al.</i> , 1994 |
| <i>Tor khudreelongispinnis</i> | Mahseer | |
| <i>Wallago attu</i> | Catfish | |

1.4.2. Pathogenicity

Aeromonas hydrophila mainly causes motile aeromonad septicemia (MAS) and has also been reported to cause secondary infections associated with EUS outbreaks (Roberts, 1993). The disease caused by *A. hydrophila* has also been called 'Red-sore' disease (Huizinga *et al.*, 1979). It does not usually cause problems in fish populations under normal conditions, but when fish are under environmental or physiological stress or infected by other pathogens, *A. hydrophila* is a potential pathogen (Plumb *et al.*, 1976; Fang *et al.*, 2000). Generally, it has become an increasingly important pathogen in intensive fish culture due to increased environmental and physiological stress experienced by reared fish (Shaw and Squires, 1984). Under favourable environmental conditions, this pathogen seems to multiply and produce higher levels of ECP toxins in fish, which can cause sudden disease outbreaks and mortalities (Allan and Stevenson, 1981; Yadav *et al.*, 1992; Vivas *et al.*, 2004a).

Several studies have described a wide variation in the pathogenicity of *A. hydrophila* in different fish species. This is mainly due to the heterogeneity of strains and differences in the adhesive and enterotoxic mechanisms responsible for causing infection in fish (Fang *et al.*, 2004). A natural infection model (immersion challenge) in a genetically stable inbred strain of southern platyfish, *Xiphophorus maculatus*, was used to study the pathogenesis of the bacterium (Kawula *et al.*, 1996). They showed that the mortality was dependent on the concentration of bacteria and the appearance of clinical signs in fish that eventually died. In another study, haemolytic toxin of *A. hydrophila* reported to be

a major virulent factor, when its pathogenicity was studied using a suckling mice model infection (Heuzenroeder *et al.*, 1999).

1.4.2.1. Clinical signs of infection

The clinical signs of disease caused by *A. hydrophila* have been classified into four categories; acute, rapidly fatal septicaemia, with few gross symptoms; an acute form with dropsy, blisters, abscesses and scale protrusion; chronic ulcerous form with furuncles and abscesses; and a latent form with no symptoms (Karunasagar *et al.*, 1989). Clinical signs of infection have also been classified into three groups by other workers using an artificial challenge model in channel catfish: viz (1) MAS (systemic infection and signs of disease), (2) cutaneous (infection limited to skin and the underlying muscle) and (3) latent (systemic infection but no external signs of disease) (Grizzle and Kiryu, 1993).

The basic clinical signs of the pathogenesis of *A. hydrophila* are the presence of small surface lesions (which lead to the sloughing-off of scales), local haemorrhages particularly in the gills and vent, ulcers, abscesses, exophthalmia and abdominal distension, and moreover, it is often associated with abdominal oedema or dropsy (Jeney and Jeney, 1995). Azad *et al.* (2001) observed external signs, such as necrotic and oedematous changes in tilapia, *Oreochromis niloticus* infected with *A. hydrophila*. As well as the external clinical signs described, various signs of disease have been reported in the internal organs of different fish species. For example, liver and kidneys were found to be completely destroyed in largemouth bass, *Micropterus salmoides* infected with *A. hydrophila* (Huizinga *et al.*, 1979). Similarly, *A. hydrophila* infection in goldfish resulted in liver

degeneration and mild necrosis of the kidney (Tafalla *et al.*, 1999). Diffused necrosis in several internal organs and the presence of melanin-containing macrophages in the blood was also observed during a systemic *A. hydrophila* infection in channel catfish (Ventura and Grizzle, 1988).

Apart from external and internal clinical signs, immunological and biochemical parameters are also affected by *A. hydrophila* infection. For example, septicaemia in Indian major carp, catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) has been reported by Karunasagar *et al.* (1986 and 1989). Similarly, an increase in plasma glucose and leucocyte volume was noted in subordinate fish compared with dominant fish when juvenile rainbow trout were exposed to social stress following challenge with *A. hydrophila* (Peters *et al.*, 1988). Biochemical signs, especially accumulation of lipofuscin (a peroxidation product of lipid catabolism) together with tissue destruction in the fish, have also been observed (Ventura and Grizzle, 1988).

Toxin induced changes in the host have been reported by Rodriguez *et al.* (1993), who demonstrated that the acetylcholinesterase of *A. hydrophila* was found in the brain of infected rainbow trout which caused the fish to lose their “flight” reaction and then their equilibrium, leading to spasmodic swimming followed by death. The symptoms suggest that the toxin may be acting on the central nervous system. Endotoxins produced by *A. hydrophila* have been found to initiate febrile responses in the fish and other animals (Reynolds *et al.*, 1978). According to Ko *et al.* (2005), mice artificially infected with *A. hydrophila* can evoke a pronounced pro-inflammatory cytokine response.

In vitro assays have also been used as models to predict clinical changes in fish caused by *A. hydrophila*. In these experiments, the pathogen was seen to produce severe morphological changes in the first 2 h of incubating common carp epithelial monolayers *in vitro* with the bacterium, and changes could even be seen after 30 min incubation with one strain (Leung *et al.*, 1996). Apoptosis of lymphocytes by *A. hydrophila* in goldfish has also been reported *in vitro* (Shao *et al.*, 2004).

1.4.3. Factors involved in pathogenicity

It has been reported that environmental factors (Pansare *et al.*, 1985; Kobayashi *et al.*, 1993; Rahman *et al.*, 1997; Tsai *et al.*, 1997) such as culture temperature, time of incubation (Khalil and Mansour, 1997) and medium (Vivas *et al.*, 2005) can alter the expression of different virulence factors of *A. hydrophila*. For example, the expression of surface haemagglutinin (s) appears to be medium-dependent, as *A. hydrophila* strains grown in liquid medium show enhanced haemagglutination activity compared to those grown on solid medium (Del Corral *et al.*, 1990). Increased/decreased water temperatures have been reported to cause increased pathogenicity of the bacterium in cultured fish (Ventura and Grizzle, 1987; Yambot, 1998). These authors found that changes in water temperature, along with the increased stocking density, act as major factors in the occurrence of disease outbreaks by *A. hydrophila* in fish. Other possible factors reported to contribute to outbreaks of *A. hydrophila* infections in cultured fish include overfeeding and a drop in salinity (Doukas *et al.*, 1998). Carriers of *A. hydrophila* has also been reported to play a role in infection, when Gatesoupe (1991) observed the infection in fish fed with rotifers carrying the bacteria. In addition, starved *A. hydrophila* (bacteria cultured in distilled water containing 0.60 % NaCl,

0.50 % KCl, 0.10 % CaCl₂ 2H₂O and 0.20 % MgCl₂ 6H₂O) were shown to be highly virulent in cyprinids (common carp and goldfish) compared to bacteria cultured in nutrient broth (Rahman *et al.*, 1997).

With regard to its pathogenesis, differing views have been expressed on whether *A. hydrophila* is a primary or secondary opportunistic pathogen of compromised or stressed hosts (Jeney and Jeney, 1995). Primary infection by other pathogens seems to assist in the infection of fish by *A. hydrophila* as a secondary pathogen. For example, mechanical trauma caused by *Ichthyophthirius multifiliis* acts as a portal entry for *A. hydrophila* present in the water, thereby facilitating the invasion of the bacterium (Liu and Lu, 2004). Similarly, when channel catfish (*Ictalurus punctatus*) were artificially infected with *Edwardsiella ictaluri*, the infection by *A. hydrophila* as a secondary pathogen was enhanced. In this example, *A. hydrophila* was present in the water, although the fish were capable of clearing *E. ictaluri* (Nusbaum and Morrison, 2002).

Other factors have been reported to be involved in the pathogenicity of the bacterium. For example, serum resistance is considered a good indicator of the virulence of *A. hydrophila* associated with the pathogenicity (Leung *et al.*, 1995). It seems virulent strains of *A. hydrophila* are able to survive within macrophages even when they are treated with specific antiserum (Yin *et al.*, 1996). It had been previously suggested that the resistance of *A. hydrophila* to intracellular enzymatic digestion by macrophages of channel catfish may be due to an intrinsically resistant character of the bacterial envelope (Bach *et al.*, 1978). This resistance is achieved by *A. hydrophila in vivo* by producing a serum-resistant capsule,

however, it has been shown that the bacterium can also produce such capsules when grown in either synthetic medium or an autolysate of fish viscera (Merino *et al.*, 1994; Aguilar *et al.*, 1999).

1.4.3.1. Extracellular products

Extracellular products of *A. hydrophila* are considered to be major virulent factors of the bacterium (Allan & Stevenson, 1981; Ruangapan, 1986). However, the importance of each individual virulence factor in the pathogenicity of the bacterium in the various stages of infection remains unclear (Handfield *et al.*, 1996). *Aeromonas hydrophila* is very well known for producing a wide range of extracellular toxins such as enterotoxin (Ljungh *et al.*, 1981; Chakraborty *et al.*, 1984), aerolysin (Howard and Buckley, 1985c; Chakraborty *et al.*, 1986), cytotoxin (Boulanger *et al.*, 1977), haemolysin (Allan and Stevenson, 1981; Rodriguez *et al.*, 1992), protease (Leung and Stevenson, 1988a; Rodriguez *et al.*, 1992), amylase (Gobius and Pemberton, 1988), acetylcholine esterase (Nieto *et al.*, 1991), lipase/acyltransferase (Munn *et al.*, 1982; Buckley, 1982), leucocidins (Caselitz, 1966; Scholz *et al.*, 1974), enolase (Sha *et al.*, 2003), nucleases (Chang *et al.*, 1992; Favre *et al.*, 1993), chitinases (Ueda *et al.*, 1994) and unknown heat-stable virulence factors (Khalil and Mansour, 1997). Strains of *A. hydrophila* also produce gelatinase, caseinase, elastase, lipase, lecithinase and deoxyribonuclease (Favre *et al.*, 1993). The role of these enzymes is to provide nutrients to the bacterium by breaking down host proteins into smaller molecules which are then capable of entering into the bacterial cell (Cicmanec and Holder, 1979; Sakai, 1985).

Although *A. hydrophila* tend to produce a wide range of toxins, proteases are considered to be the main virulence factors in ECPs implicated in the pathogenicity of fish. For example, it has been suggested that production of proteases by *A. hydrophila* may initiate the disease by degenerating tissues/organs in fish (Howard and Buckley, 1982; O'Reilly and Day, 1983; Sakai, 1985). This was confirmed when elevated pathogenicity appeared to correlate with elevated protease activity (Khashe *et al.*, 1996; Vivas *et al.*, 2004a). Similarly, a higher proteolytic activity was found in *A. hydrophila* derived from humans, fish and other animal sources than those from water environments, confirming the involvement of the proteases in the pathogenicity of the bacteria from different hosts (Shotts *et al.*, 1985). In contrast, Allan and Stevenson (1981) observed increased toxic effects in fish by the ECP of a protease-negative mutant of *A. hydrophila* in comparison to the parent strain (with protease activity), and suggested that proteases may not be contributing to virulence.

The haemolysin toxin of *A. hydrophila* has been considered as one of the major virulence factors in the ECP of this bacterium. For example, Khalil and Mansour (1997) observed that the virulence of ECPs was closely associated with haemolysin activity, although Lallier *et al.* (1984) had previously suggested that haemolysis is not the principle toxic effect of the bacterium in fish. The response of speckled trout, *Salvelinus fontinalis* injected intraperitoneally (IP) with ECP from *A. hydrophila* suggested that the haemolytic activity is a significant lethal factor of the bacterium (Allan and Stevenson, 1981). This was confirmed when breakdown of haemoglobin (hemosiderosis) was observed in channel catfish infected by *A. hydrophila*, due to the haemolytic activity of virulent *A. hydrophila* (Ventura and

Grizzle, 1988). However, the activity of hemolysin produced by different *A. hydrophila* isolates is variable due to the heterogeneity of isolates. For example, differences were found in the activities of haemolysins expressed from cloned genes of two different *A. hydrophila* isolates in medium containing various erythrocytes from sources such as bovine, horse, rabbit and sheep (Hirono *et al.*, 1992). Authors have also shown that the haemolytic activity of *A. hydrophila* has antibacterial activity against some other bacterial species (e.g. *Staphylococcus spp*) (Messi *et al.*, 2003).

One other extracellular toxin thought to be involved in the virulence of *A. hydrophila* is aerolysin. It binds to specific glycoprotein receptors on the surface of eucaryotic cells, and inserts itself into the lipid bilayer forming holes approximately 3 nm in diameter (Karunasagar *et al.*, 1986). This in turn leads to destruction of the permeability membrane barrier and ultimately cell death (Howard and Buckley, 1985c). Thus, aerolysin kills cells by forming discrete channels in their plasma membranes (Buckley and Howard, 1999). Sirirat *et al.* (1999) found that aerolysin is expressed in greater amounts in the ECP of virulent isolates *A. hydrophila* than in avirulent isolates.

Expression of specific virulence factors, in the ECP, seems to be dependent on the availability of nutrients (Gonzalez-Serrano *et al.*, 2002). For example, Esteve and Birbeck (2004) showed that the amount of haemolytic and proteolytic activity of *A. hydrophila* is dependent on the culture media used, as differences in the expression of these activities was noted between bacteria grown in brain-heart infusion broth or tryptone soya yeast extract broth. Similarly, the differential up-

regulation of the enolase enzyme of *A. hydrophila* was observed with bacteria grown *in vivo* in mice compared with those grown *in vitro* in broth (Sha *et al.*, 2003).

The production of components within the ECP have been shown to be affected by culture temperature, as Merino *et al.* (1992) found *A. hydrophila* to be more virulent for fish and mice when grown at 20°C than 37°C due to increased levels of ECP activity at lower temperature. O'Reilly and Day (1983) found higher proteolytic activity at 28°C than at 37°C, while Khalil and Mansour (1997) found increased production of protease at 30°C compared to 25°C in strains of *A. hydrophila*. Similarly, the highest production of the haemolysin was noted when *A. hydrophila* was grown at 35°C rather than 25 or 30°C (Khalil and Mansour, 1997). *Aeromonas hydrophila* can produce haemolysin and cytotoxin at 37, 28 and 5°C, however, toxins are produced faster and are more stable at 28°C than at 37°C (Tsai *et al.*, 1997).

1.4.3.2. The outer membrane and other surface components

The outer membrane is the outermost structure on the surface of Gram-negative bacteria. Generally, Gram-negative bacteria are characteristically surrounded by a double membrane: the cytoplasmic or inner membrane, which is a phospholipid bilayer, and the asymmetrical outer membrane, which holds phospholipids and lipopolysaccharides (LPS) in its inner and outer layers, respectively (Figure 1.1) (Bos and Tommassen, 2004). Phospholipids are major structural and functional components of the cell envelope of all bacteria (Howard and Buckley, 1985b). The outer membrane assembly and the extracellular protein secretion are reported to

be regulated by the expression of many genes such as *exe* genes (Howard *et al.*, 1993). Proteins present in the outer membrane are composed of two classes: lipoprotein, which are anchored into the outer membrane via a N-terminal lipid tail, and integral proteins that contain membrane-spanning regions. All proteins destined for the outer membrane are synthesized in the cytoplasm as precursors with N-terminal signal sequences, which are essential for translocation across the inner membrane (Bos and Tommassen, 2004).

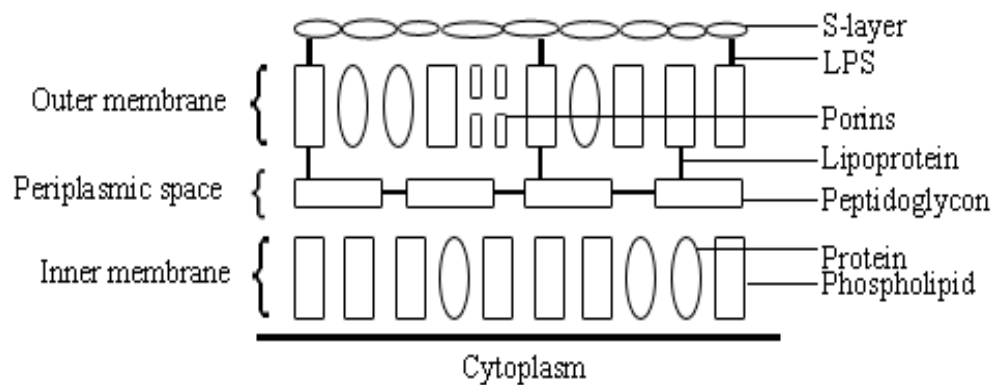


Figure 1.1: Structure of the outer membrane

The OMP are believed to be a primary factor involved in the attachment (adhesion) of *A. hydrophila* to various host tissues, as this is a prerequisite to initiate infection (Del Corral *et al.*, 1990). Quinn *et al.* (1994) isolated pore-forming OMP from *A. hydrophila* and suggested that it may be involved in the initial colonization of the bacterium on its host. Similarly, it was suggested that the 43 kDa porin may be an important adhesin with regard to entry into common carp epithelial cells because of an abundance of this particular receptor on the cell surface (Lee *et al.*, 1997).

The LPS is an important component of the outer membrane of Gram-negative bacteria (Howard and Buckley, 1985b), which consists of a hydrophobic membrane anchor, lipid A, substituted with an oligosaccharide core region that can be extended in some bacteria by a repeating oligosaccharide, the O-antigen (Bos and Tommassen, 2004). It plays an important role in the pathogenesis of the bacterium including having a role in adhesion and its ability to cause gastroenteritis (Merino *et al.*, 1996a; Knirel *et al.*, 2002). Shaw and Squires (1984) found an involvement of *A. hydrophila* LPS in bacteriophage attachment and other virulence properties such as serum resistance. It has been confirmed that *A. hydrophila* strains lacking a defined LPS are susceptible to killing by human serum (Janda *et al.*, 1994a). Similarly, Mittal *et al.* (1980) reported that virulent strains of *A. hydrophila* express a unique O-antigen, and were able to differentiate between virulent and less virulent strains on the basis of serogrouping and cell surface characteristics.

Many of the properties which facilitate the colonisation of the bacterium on its host are associated with the cell surface of *A. hydrophila*, and are very important in host-pathogen infection (Dooley and Trust, 1988). Cell surface structures enable this pathogen to bind to a large number of cells and biomolecules in host tissues (Janda and Duffey, 1988; Ascencio *et al.*, 1991a and 1998). These cell surface receptors can bind with iron-containing proteins of the host and may be involved in the acquisition of iron by the bacterium (*i.e.* the siderophores mentioned earlier) (Ascencio *et al.*, 1992). However, environmental factors such as temperature play a significant role in regulating virulent factors, including the biochemistry of the cell surface of *A. hydrophila* (Merino *et al.*, 1992).

The S-layer proteins are considered to play a major role in infection for a number of bacteria (Boulanger *et al.*, 1977; Dooley and Trust, 1988; Messner and Sleytr, 1992). The S-layer or paracrystalline surface layer was first described in *Spirillum* using electron microscopy (EM) by Houwink in 1953. Thereafter it has been reported in nearly every taxonomic group of walled eubacteria, and is a feature of most archaeobacterial cell envelopes (Sleytr and Messner, 1983 and 1988; Messner and Sleytr, 1992). Pathogenic bacteria such as *Clostridium botulinum*, *A. salmonicida*, *Campylobacter fetus* subsp. *fetus*, *C. rectus* and *Mycobacterium bovis* have all been reported to possess S-layers (Boulanger *et al.*, 1977; Messner and Sleytr, 1992).

Using a laboratory challenge model, Thune *et al.* (1986) showed the S-layer of *A. hydrophila* to correlate with virulence. It is believed to influence the interaction between the bacterial cell and its environment (Austin and Austin, 1999). The localisation of the S-layer on the surface of the cell suggests it has an important role in the growth and survival of bacteria, and is the site of interaction between the bacteria and the external environment. It possesses anti-phagocytic activity which may aid in the systemic dissemination of bacteria once invasion through the gastrointestinal mucosa has occurred (Janda *et al.*, 1994a). However, the degree of antigenicity of S-layer proteins varies among bacterial species (Kobayashi *et al.*, 1993). The S-layer in conjunction with LPS has been shown to play an integral part in the overt resistance of *A. salmonicida* to complement-mediated lysis (Chang *et al.*, 1992).

1.5. Diagnostics

Diagnosing disease and identifying the infectious agents included are important for managing any disease. Visible internal and external clinical signs indicating disease caused by *A. hydrophila* are discussed in the Section 1.4.2.1. However, diagnosis can be very difficult, since the progress of infection by *A. hydrophila* is rapid in the fish under favourable conditions for the bacterium (Jeney and Jeney 1995), and other pathogens such *A. salmonicida* and *Vibrio* species could also possibly cause similar signs. Confirming the association of *A. hydrophila* with disease/infection is important before treating the disease. A number of methods have been reported for the detection and identification of this pathogen, including traditional (phenotypic and biochemical characteristics), immunological and molecular techniques.

Traditional methods to detect and identify *A. hydrophila* include examination of the shape and colour of colonies on nutrient agar, Gram staining, morphology and motility of the bacterium and various biochemical analyses (Altwegg *et al.*, 1990; Chaudhury *et al.*, 1996; Yambot, 1998). A rapid method based on biochemical analysis using an API strip containing premixed chemicals has been routinely used for the identification of *A. hydrophila* (Dixon *et al.*, 1990; Gatesoupe, 1991; Hettiarachchi and Cheong, 1994; Noterdaeme *et al.*, 1991). However, traditional methods are not fully reliable for the identification of *A. hydrophila* to species level due to similarity in some phenotypic and biochemical characters with other species (*A. caviae* and *A. sorbia*). In addition, the biochemical characteristics expressed between different isolates of *A. hydrophila* are not always same (De Figueiredo and Plumb, 1977).

Immunological detection methods such as enzyme linked immunosorbent assay (ELISA) were developed for the detection of *A. hydrophila* by Merino *et al.* (1993) and Sendra *et al.* (1997). Korbsrisate *et al.* (2002) produced polyclonal antibodies against *A. hydrophila* for use in a specific direct agglutination test to identify *A. hydrophila*. Monoclonal antibodies (Mabs) have also played a vital role for the identification of fish pathogens (Adams and Thompson, 2006), and Mabs that recognise a particular serotype O side-chain core oligosaccharide of *A. hydrophila* have been developed (Cartwright *et al.*, 1994). Chanphong *et al.* (1999) developed Mabs against a 41 kDa protein of *A. hydrophila*, while Mabs were also produced against *A. hydrophila* that recognised a 110 kDa polypeptide on the bacterium (Delamare *et al.*, 2002). Although immunological methods have been useful in the identification of *A. hydrophila*, it might only be possible to identify particular isolates/serotypes with the specific antibodies developed due to heterogeneity of isolates (Merino *et al.*, 1993).

Molecular methods have been recommended for the identification *A. hydrophila* to overcome possible problems encountered with traditional or immunological methods. Sugita *et al.* (1994) suggested a deoxyribonucleic acid (DNA) based hybridisation method for the identification of *A. hydrophila*, while amplification of specific genes (e.g. haemolysin) of *A. hydrophila* by polymerase chain reaction (PCR) has been recommended for detection of the bacterium (Xia *et al.*, 2004). In addition, a rapid identification method was developed by sequencing 16s ribosomal DNA (rDNA) regions of *A. hydrophila* (Dorsch *et al.*, 1994). More recently, Chu and Lu (2005) developed a multiplex PCR method to amplify the 16s rDNA gene and the aerolysin gene of *A. hydrophila* to detect pathogenic strains of

A. hydrophila. A method combining immunological and molecular techniques (immuno-capture assay with PCR) has also been developed to provide a quick, sensitive and reproducible way of detecting *A. hydrophila* (Peng *et al.*, 2002).

1.6. Treatments and control

1.6.1. Antibiotics

Antibiotics are the major agents for controlling *A. hydrophila* (Fang *et al.*, 2004). Those described as being effective include furance (Mitchell and Plumb, 1980), sulfonamide (Bowser *et al.*, 1987), chloramphenical, neomycin, sulfamethoxazole-trimethoprim, streptomycin, naladixic acid, oxolinic acid, neomycin and sarafloxacin (Krovacek *et al.*, 1989; Dixon *et al.*, 1990), rifampicin (Ansary *et al.*, 1992), oxytetracycline (Tafalla *et al.*, 1999), cephamycins and moxalactam (Zervosen *et al.*, 2001), ciprofloxacin (Ko *et al.*, 2003), amoxycillin and enrofloxacin (Ilhan *et al.*, 2006). *Aeromonas hydrophila* is also found to be sensitive to other chemotherapeutants such as amino acid-derived hydroximates (Walter *et al.*, 1999) and hydrogen peroxide (H₂O₂) (Landre *et al.*, 2000).

Although antibiotics control *A. hydrophila* to a certain extent, it has been observed that bacterial pathogens become resistant to chemotherapeutants when they are used over an extended period of time (Mitchell and Plumb, 1980; Vivekanandhan *et al.*, 2002). Different isolates of *A. hydrophila* are reported to have resistance to the following antibiotics; ampicillin, carbenicillin, erythromycin, gentamicin, penicillin, tetracycline, nitrofuradantoin, ormetoprim-sulfadimethoxine, sulfamethoxazole-trimethoprim and triple sulfa (Dixon *et al.*, 1990; Ansary *et al.*, 1992; Dixon and Issvoran, 1993; Ilhan *et al.*, 2006).

The high incidence of antibiotic resistance in *A. hydrophila* isolated from cultured fish is assumed to be due to selective pressure exerted by the use of chemotherapeutics in aquaculture, since such resistance has not been found in *A. hydrophila* isolated from wild fish (Aoki *et al.*, 1971; Radu *et al.*, 2003). Moreover, the majority of researchers have found that resistance plasmids play a major role in the development of antibiotic resistant strains (Chang and Bolton, 1987; Choudhury *et al.*, 1996; Son *et al.*, 1997). It has also been found that the molecular size of the drug resistance plasmids in *A. hydrophila* range from approximately 3 to 150 kb (Chang and Bolton, 1987; Choudhury *et al.*, 1996; Son *et al.*, 1997). Restriction in the use of drugs to bacterial pathogens of fish will aid in minimising the development and spread of resistance plasmids in bacteria (Son *et al.*, 1997). However, some other factors are suspected of playing a role in the antibiotic resistance, since Ansary *et al.* (1992) found no plasmids in *A. hydrophila* which are resistant to antibiotics (ampicillin and carbenicillin).

Due to high resistance to some antibiotics applied in clinical practice, it is difficult to control *A. hydrophila* present in aquaculture systems (Riquelme *et al.*, 1996; Daskalov, 2006). The development of antibiotic resistant strains is making the use of drugs more and more ineffective. In addition, transmission of resistance from resistant bacteria from aquaculture farms to bacteria of human and/or veterinary significance remains a major public health concern (Shariff, 1998). Thayumanavan *et al.* (2003) warned that the increasing population of multiple drug-resistant *A. hydrophila* in fish and prawns may become a potential human health hazard. Also the use of antibiotics can considerably increase the production cost to the

aquaculture industry, so, it is essential that losses caused by diseases and the use of antibiotics be kept at a minimum (Gudding *et al.*, 1999).

Apart from antibiotic resistance, a number of authors have reported that the use of antibiotics has adverse effects, such as accumulation of tissue residues and immunosuppression of natural immunity in fish (Van Muiswinkel *et al.*, 1985; Ellis, 1988; Thompson and Adams, 2004). In contrast, other researchers reported that the use of certain antibiotics, such as sulfonamide and oxytetracycline, does not have adverse effects in fish (Bowser *et al.*, 1987; Tafalla *et al.*, 1999). The use of antibiotics and chemotherapeutics have been shown to evoke toxic effects in animals (Martin, 1973), and can interfere with certain steps in protein synthesis (Watson, 1975). It is also believed that mitochondrial protein synthesis might be inhibited by some antibiotics (De Vries and Kroon, 1970). Humoral and cellular immune responses of common carp have been shown to be suppressed during treatment with oxytetracycline, and an increased number of granulocytes were observed in the spleen of treated fish (Rijkers *et al.*, 1980).

The use of other chemicals in the aquaculture industry to kill ectoparasites, insects and weeds could also be immunosuppressors in fish. The reduction of several non-specific immune responses and serum enzymes were found, when Indian major carp (rohu) were exposed to the pesticide, α -permethrin (Nayak *et al.*, 2004a). These fish were found to be more susceptible to *A. hydrophila* challenge. Fish exposed to high levels of copper were also immunosuppressed, with a change in various immunological parameters occurring (Ellis, 1981; Shariff *et al.*, 2001).

1.6.2. Immunostimulants

A variety of immunostimulants have been reported to enhance immunity against *A. hydrophila*, these being mainly herbal extracts, glucans and vitamin C, although a number of other immunostimulants also have been reported to have promising effects. For example, increased immunity in blue gourami, *Trichogaster trichopterus* against a virulent strain of *A. hydrophila* was reported, when the fish was treated IP with an extract from marine algae, laminaran (Samuel *et al.*, 1996). An increase in serum glucose, cholesterol, total protein, red blood cell counts, hemoglobin and hematocrit/packed cell volume was found in *A. hydrophila*-infected common carp after treating them by immersion with a leaf extract of *Azadirachta indica* (Harikrishnan *et al.*, 2003). Similarly, herbal seed powder (*Achyranthes aspera*) in the diet increased innate immunity and resistance of rohu to *A. hydrophila* when experimentally infected with the bacterium (Vasudeva Rao *et al.*, 2006).

Other extracts from marine animals such as tunicate (sea squirt), *Ecteinascidia turbinata* enhanced both the humoral and the cellular immunity of American eel, *A. rostrata* against *A. hydrophila* (Davis and Hayasaka, 1984). It has been reported that glucans could also increase the immunity of fish against *A. hydrophila* as increased survival of tilapia and grass carp infected with *A. hydrophila* was observed when the fish were injected with glucans such as bar, krestin, scleroglucan and zymosan (Wang and Wang, 1997). Similarly, common carp injected IP with glucan showed an increase in their leucocyte counts (neutrophils and monocytes) and resistance to *A. hydrophila* challenge (Selvaraj *et al.*, 2005).

An adjuvant effect by the glucan was observed, since fish injected with glucan resulted in the highest antibody titer against *A. hydrophila* following vaccination.

Humoral and cell mediated immune responses were found to be increased in vitamin C supplemented catfish, *Mystus gulio* (Anbarasu and Chandran, 2001). The authors found a higher relative percentage survival (RPS) value in the vitamin C supplemented fish compared with the non vitamin C supplemented fish, when challenged with *A. hydrophila* following vaccination. Sobhana *et al.* (2002) found the infiltration of phagocytic cells into the injection site in mrigal (*i.e.* fish were injected with *A. hydrophila* IP) was quicker when their diet were supplemented with vitamin C, and the bacteria were cleared from the fish by Day 9 post-infection compared with vitamin C non-supplemented group.

Hormones such as human lactoferrin (HLF) have been found to have immunomodulatory activities, as Weifeng *et al.* (2004) observed the development of resistance to *A. hydrophila* in HLF-transgenic grass carp, *Ctenopharyngodon idella*. Supplementation of diets with yeast ribonucleotides (RNA) also resulted in an improved resistance of rohu to *A. hydrophila* infection (Choudhury *et al.*, 2005). Probiotics have been reported to increase the specific immune response, for example Ramadan *et al.* (1994) noted that supplementing the diet of tilapia with ascogen resulted in an increased antibody response in fish vaccinated with a formalin-killed preparations of *A. hydrophila*. Also, the dietary administration of hormones (triiodothyronine) in rohu enhanced the immune response of the fish vaccinated with formalin-killed *A. hydrophila*, which in turn conferred protection against an *A. hydrophila* challenge (Sahoo, 2003).

1.6.3. Vaccines

Vaccination of humans and other animals, including fish, is one of the major methods for preventing infectious disease (Potter and Baiuk, 2001). Immunisation primes the immune system of the host against pathogens encountered during infections (Thompson and Adams, 2004). Fish vaccination in the aquaculture industry has been considered to be very important in reducing economic losses caused by disease (Ellis *et al.*, 1997; Rahman and Kawai, 2000; Ebanks *et al.*, 2004). Several different kinds of vaccines have been investigated/developed against *A. hydrophila* including whole cell (WC), OMPs, ECPs, LPS and biofilms, although currently no commercial vaccine exists.

1.6.3.1. History of *A. hydrophila* vaccine development

Aeromonas hydrophila is an important pathogen that has caused major loss in the aquaculture industry for decades (Shotts *et al.*, 1972; Olivier *et al.*, 1981; Esteve *et al.*, 1995). Many attempts have been made to develop an effective vaccine against *A. hydrophila* (Lamers *et al.*, 1985; Baba *et al.*, 1988b; Leung *et al.*, 1997; Rahman and Kawai, 2000). The effects of number inactivated WC vaccines have been reported. For example, an increase in serum antibody levels against *A. hydrophila* was showed in common carp immersed in a preparation of heat inactivated *A. hydrophila* (Lamers *et al.*, 1985). Later, Kusuda *et al.* (1987) also found an increase in the concentration of total serum proteins, when common carp were immunised with formalin killed *A. hydrophila*. Rainbow trout, immunised by injection, immersion and oral administration of killed *A. hydrophila*, have been shown to produce antibodies in their serum, bile, skin and gut mucus, and skin and muscle extracts (Loghothetis and Austin, 1994). A polyvalent vaccine

containing heat killed WC and formalin inactivated ECP of *A. hydrophila* has also been tested in two Indian major carp species (rohu and mrigal), but it failed to protect the fish against bacterial challenge (Chandran *et al.*, 2002a). However, these authors observed relatively high antibody titres in immunised fish suggesting that the low survival in the vaccinated group may be due to an impact of unknown stress on the fish in the pond, or maybe the antibodies induced by the vaccine were not protective.

Thin layers of bacteria (biofilms) grown on the surfaces of nutrient flakes (chitin) have been used as heat inactivated biofilm vaccines against *A. hydrophila*, by oral administration. For example, bacteria harvested from chitin flakes suspended in TSB have been reported to elicit a protective response in Indian major carp (catla and rohu) and common carp (Azad *et al.*, 1999). These biofilm vaccines have been found to be retained for longer than free cell vaccines in the tissues of gut, spleen and kidney in Indian major carp (Azad *et al.*, 2000a). Catfish, fed with such biofilm vaccines, showed significantly higher serum agglutinating antibody titres and RPS compared to those fed with free cells, after subsequently challenging fish with *A. hydrophila* (Nayak *et al.* 2004b). A change in antigenic expression was noticed in *A. hydrophila* grown on a biofilm (Asha *et al.*, 2004). The authors found that the S-layer proteins were lost and the LPS of the bacteria contained an additional high molecular weight band in biofilm-cultured cells compared with planktonic cells. Asha *et al.* (2004) suggested that this high molecular weight LPS band might elicit a protective immune response when the biofilm was administrated as an oral vaccine.

Considerable interest has been shown in bacterial OMP vaccines since these were thought to contain some of the major antigens responsible for inducing an immune response in the host (Aoki and Holland, 1985; Fang *et al.*, 2004). Rahman and Kawai (2000) found that the OMPs of *A. hydrophila* elicited protection against an *A. hydrophila* challenge, and suggested that a vaccine based on selected OMP antigens may be effective. Munn (1994) suggested outer membrane components such as LPS could represent protective vaccine candidates for Gram-negative bacteria, while Dooley *et al.* (1986) reported that LPS of *A. hydrophila* possess highly immunogenic O polysaccharide chains of homogeneous length, and these were conserved both morphologically and antigenically in virulent isolates. The role of LPS in protection was also shown, in common carp vaccinated with crude LPS compared with a formalin-killed WC vaccine (Baba *et al.*, 1988b). This protection appears to be based on cellular immunity, particularly thymus cells and macrophages rather than humoral immunity (Baba *et al.*, 1988a). Similarly, Loghothetis and Austin (1996b) reported that LPS could be a major antigenic component of *A. hydrophila*.

Live WC cell vaccines have also been found to increase antibody responses in fish (Loghothetis and Austin, 1994). Other live vaccines, such as live attenuated (mutant) vaccines have also been explored for *A. hydrophila*. For example, growth-deficient mutants of *A. hydrophila* have been found to be promising live vaccine candidates in fish (Leung *et al.*, 1997). An AroA mutant *A. hydrophila* strain was also investigated and found to be protective in rainbow trout (Moral *et al.*, 1998). The aromatic amino acid pathway is blocked in this mutant with the result that its growth in fish tissues is prevented. The vaccine was also found to

elicit significant protection against *A. salmonicida* (Vivas *et al.*, 2004b). Vivas *et al.* (2004c) suggested that this live *aroA* attenuated vaccine had a high level of safety compared with normal strains as it has a lower potential to survive in water. Other live vaccines have also been investigated, for example Catfish, *Clarias batrachus* immunised with plasmid free *A. hydrophila* mutants, showed an increased survival rate following challenge with wild bacteria compared to the control group (Majumdar *et al.*, 2006). Mutant strains of *A. hydrophila* with a highly attenuated exoenzyme were also shown to confer protection in swordtail fish, *Xiphophorus helleri* (Liu and Bi, 2006).

Although all the vaccines reported have shown varying degrees of increased immunity and protection, no commercial vaccine is available for *A. hydrophila* (Loghothetis and Austin, 1996b; Rahman and Kawai, 2000; Fang *et al.*, 2004; Vivas *et al.*, 2005). This could be due to reduced efficacy of the vaccines against different isolates or serogroups of *A. hydrophila*, due to the heterogeneity of this bacterium (Stevenson, 1988; Janda *et al.*, 1996; Merino *et al.*, 1997; Aguilar *et al.*, 1999; Chandran *et al.*, 2002a). Over 90 established or provisional serogroups within the genus *Aeromonas* have been described, and the heterologous nature of *A. hydrophila*, both biochemically and serologically are still the greatest concern for developing an effective vaccine against *A. hydrophila* (Sakazaki and Shimada 1984; Stevenson, 1988; Khashe *et al.*, 1996; Newman, 1993; Janda *et al.*, 1994b; Leung *et al.*, 1995).

1.6.3.2. Approaches for developing an effective *A. hydrophila* vaccine

Many factors must be considered for developing an effective vaccine. The vaccine produced should be protective and should not cause any adverse effect in the host (Makela, 2000; Potter and Babiuk, 2001; Schuijffel *et al.*, 2005). In addition, the vaccine should be cost effective for global use in the aquaculture industry (Leong and Munn, 1991; Munn, 1994; Naidu and Yadav, 1997). The vaccines already developed by a number of research groups for *A. hydrophila* do not appear to be fully effective due to heterogenicity of the challenge isolates. Therefore, authors have recommended identifying common antigens between different isolates of *A. hydrophila* as vaccine candidates (Dooley *et al.*, 1988; Leung *et al.*, 1997). The protective nature of such antigens then needs to be established.

Understanding host-pathogen interactions, especially the host immune response to the pathogen, might offer important clues about potential protective antigens for vaccine development (Ellis, 1999). Such molecules tend to be ECP toxins and proteins located on the surface of pathogens. For example, the ECP of *A. hydrophila* has been reported to contain the antigens necessary for a successful vaccine against MAS (Allan and Stevenson, 1981). Loghothetis and Austin (1996b) suggested LPS as a vaccine candidate of *A. hydrophila* as they found increased antibody responses against this component in rainbow trout infected with live *A. hydrophila*. Similarly, other surface components of this bacterial pathogen, such as OMPs have been widely suggested as attractive targets for vaccines due to their involvement in the infection process (Esteve *et al.*, 1994; Zhang *et al.*, 2000). Recently, Maji *et al.* (2006) suggested using a 57 kDa and a 23 kDa protein found in OMP preparations of *A. hydrophila*, since they appear to

be highly immunogenic when screened with rabbit serum raised against crude OMP.

Proteomics combined with Western blot (immunoproteomics) and mass spectrometry techniques have been recognised as useful tools for identifying proteins of interest for vaccine development. The separation and characterisation of complex proteins by 2 dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (2D SDS-PAGE) has been used to acquire information about the expression of proteins in fish bacterial pathogens grown in different culture conditions. For example, the OMPs of *A. salmonicida*, grown *in vitro* in TSB or iron-restricted TSB, and *in vivo* within chambers implanted in the abdominal cavity of Atlantic salmon, *Salmo salar* have been compared for their differential expression (Ebanks *et al.*, 2004). After having obtained information on the expression of proteins, 2D SDS-PAGE Western blot allows identification of those antigens recognised in the host (Chen *et al.*, 2004). The application of matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) with 2D SDS-PAGE based techniques is another powerful tool for understanding the functions of the proteins (Guerrera and Kleiner, 2005). Overall, these techniques can provide crucial information about proteins of interest leading to the identification of an effective candidate for vaccine development (Chakravarti *et al.*, 2000). For example, recently, potential vaccine candidate proteins were identified using immunoproteomics for important human and animal pathogens, such as *Helicobacter pylori* (Krah *et al.*, 2004), *Neospora caninum* (Shin *et al.*, 2004), *Shigella flexneri* (Ying *et al.*, 2005) and *Klebsiella pneumoniae* (Kurupati *et al.*, 2006).

Another important factor in vaccine development is the ability to produce sufficient quantities of the protective proteins for commercialisation of the vaccine. Recombinant DNA technology enables rapid production of large quantities of protein in comparison to the traditional method, where purifying proteins from the pathogen might yield low quantities as well as involving time consuming processes (Munn, 1994; Chakravarti *et al.* 2000; Potter and Babiuk, 2001; Van den Bergh and Arckens, 2005). Recombinant protein vaccines have been reported to have conferred protection for a variety of human and animal pathogens (including fish) such as *Yersinia pestis* (Williamson *et al.*, 1995), *Ichthyophthirius multifiliis* (He *et al.*, 1997), rabies virus (Rupprecht *et al.*, 2005), *Plasmodium falciparum* (Saul *et al.*, 2005), and *Piscirickettsia salmonis* (Wilhelm *et al.*, 2006).

1.7. Aims of the study

The development of an effective vaccine against *A. hydrophila* has proven difficult due to the heterogeneity of isolates. The main aim of this project was to develop an *A. hydrophila* vaccine that would protect against different strains of *A. hydrophila* isolated from various geographical regions of the world. The different specific objectives of the project are summarised below:-

- Classification of different *A. hydrophila* isolates mainly on the basis of virulence using artificial infections in common carp.
- Comparison of protein profiles from different preparations (WC, ECP and OMP) of *A. hydrophila* isolates grown *in vitro* and *in vivo* for the identification of common and unique proteins.

- Identification of the immunogenic proteins of *A. hydrophila* grown *in vitro* and *in vivo* in the host (common carp) to determine vaccine candidates.
- Determination of the protection efficacy of the immunogenic proteins identified.
- Sequencing of the immunogenic protein and production of recombinant proteins.
- Efficacy testing of a recombinant vaccine against different isolates of *A. hydrophila* in common carp.

**Chapter 2. Characterisation of *A. hydrophila*:
Biochemistry, virulence and protein expression
following culture *in vitro* and *in vivo***

2.1. Introduction

Aeromonas hydrophila can be found in a wide range of different environments, and is able to adapt, survive and grow at a range of different pH, temperature, salt and dissolved oxygen concentrations, and when nutrients are limited (Karem *et al.*, 1994; Tsai *et al.*, 1997). The biochemical properties of bacteria are considered to be essential for differentiating them phenotypically, and in most cases *A. hydrophila* isolates are consistent with the expected expression of their biochemical characteristics (Altwegg *et al.*, 1990; Janda *et al.*, 1996). However, it has also been reported that the biochemical characteristics of *A. hydrophila* can be variable and highly temperature dependent (Lallier and Higgins, 1988; Kuijper *et al.*, 1989). The majority of *A. hydrophila* isolates examined by Janda *et al.* (1996) were shown to be able to ferment salicin, utilise DL-lactate and oxidise gluconate and very few isolates were able to ferment L-Rhamnose and utilise urocanic acid.

This bacterium has the potential to infect humans and other animals, however, not all the strains of *A. hydrophila* are virulent, moreover heterogeneous protein profiles are expressed between different virulent strains (Merino *et al.*, 1992; Aguilera-Arreola *et al.*, 2005). Nieto and Ellis (1991) concluded that there were significant qualitative as well as quantitative differences in the protein components of the extracellular products (ECP) of different *Aeromonas* species and isolates, even though they were grown under the same growth conditions. The ECP of *A. hydrophila* is considered to be a major virulent factor for permitting bacterial growth within the host and in its development of further infection (Kanai and Wakabayashi, 1984).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has been extremely useful as an analytical tool for the separation and quantification of proteins from complex mixtures (O' Farrell, 1975). Computer analysis of SDS-PAGE protein profiles of whole cell preparations has been widely used in the classification and identification of different species of lactic acid bacteria (Piraino *et al.*, 2006). The SDS-PAGE profile of whole cell (WC) preparations of *A. hydrophila* can provide information on the proteins expressed by the bacterium, and is a useful tool for studying differences between strains and can be used as a diagnostic and epidemiological tool (Haenninen, 1994).

As a technique, 2D SDS-PAGE provides much more information than 1D SDS-PAGE as it is capable of resolving thousands of proteins in a single procedure (O'Farrell, 1975). It has been traditionally used for separating complex mixtures of proteins and thereby to identify and characterize individual peptides/proteins (Chakravarti *et al.*, 2000; Khouldi *et al.*, 2004). It can also be used to compare quantities of proteins in related samples, some of which may have been altered by environmental conditions, or to compare mutant and wild type bacteria (Munn, 1994). Recently, it has been used for identification of potential vaccine candidates when applied in conjunction with Western blotting (Chen *et al.*, 2004).

The protein profiles of both the outer membrane protein (OMP) and ECP appear to change between isolates or within an isolate when *A. hydrophila* is cultured under different environmental conditions (Merino *et al.*, 1992; Rahman *et al.*, 2001a; Esteve and Birbeck, 2004; Imbert and Gancel, 2004). This heterogeneity is of particular interest when comparing the growth of the bacterium *in vivo* and *in vitro*,

since differential up regulation of genes and changes in the surface composition of the bacterium has been reported following culture *in vitro* and *in vivo* (Rahman and Kawai, 2000; Sha *et al.*, 2003). Considerable differences in protein profiles and ECP expression between bacteria cultured *in vitro* and *in vivo* have also been shown for a number of other pathogens, including *Streptococcus suis* (Quessy *et al.*, 1994), *Vibrio salmonicida* (Colquhoun and Sorum, 1998), *A. salmonicida* (Ellis *et al.*, 1997; Dacanay *et al.*, 2003; Wang *et al.*, 2004), *Photobacterium damsela* subsp. *piscicida* (Bakopoulos *et al.*, 2004), *Staphylococcus aureus* (Arbutnott *et al.*, 1992; Allard *et al.*, 2006), *Mycobacterium tuberculosis* (Akhtar *et al.*, 2006) and *Francisella tularensis* (Twine *et al.*, 2006).

Pansare *et al.* (1985) reported that certain amino acids, such as alanine and arginine, could be used as the sole source for nitrogen to support the growth of *A. hydrophila in vitro*, and suggested that the sarcoplasmic fraction of muscle in fish and shellfish is an ideal growth medium for the bacterium *in vivo*. True antigen expression occurs when the bacterium is grown *in vivo* compared to growth on artificial media, *in vitro* (Garduno *et al.*, 2000). In most cases, *A. hydrophila* tends to produce a capsule *in vivo* (Mateos and Paniagua, 1995).

Many authors have tried to mimic “near” *in vivo* conditions for culturing bacteria to determine the changes in protein profiles that occur when the bacteria are cultured under such conditions. The expression of these differences on bacteria may be important to incorporate into the vaccine to produce a protective vaccine. Changes in the OMP profiles have been found for a number of major OMPs isolated from different strains of *A. hydrophila* (Aoki and Holland, 1985) and *A. salmonicida*

(Hirst and Ellis, 1994) when cultured under iron-limited conditions. For most bacteria, the outer membrane is very important in protection from its host. A capsule, for example, helps the pathogen to survive in unfavourable environments, such as increased pH and temperature. The outer membrane also plays a significant role in virulence, since the outermost surface of the bacterium is in direct contact with host cells and immune defences (Ebanks *et al.*, 2005; Murakami *et al.*, 2002), and therefore outer membrane components have been considered as potentially important vaccine candidates (Vazquez-Juarez *et al.*, 2003).

The main objective of this chapter was to compare the biochemistry and the virulence of 14 isolates of *A. hydrophila* and then to determine differences between WC protein, OMP and ECP preparations of *A. hydrophila* that had been grown *in vitro* and *in vivo*. Analysis was performed using one dimensional (1D) and two dimensional (2D) SDS-PAGE to examine the profiles between the bacteria grown *in vitro* and *in vivo* and to identify common proteins between bacterial isolates. Changes in the morphology of the bacteria were also investigated by electron microscopy.

2.2. Materials and methods

2.2.1. Biochemical characterisation of the *A. hydrophila* collection

Biochemical and growth profiles, Gram staining, and motility tests were performed to characterise the *A. hydrophila* collection held at the Institute of Aquaculture. In total, fourteen *A. hydrophila* isolates from different geographical regions were used in this work (Table 2.1). The strains were revived from frozen stocks and

maintained on slopes of tryptone soy agar (TSA) (Oxoid, UK). The slopes were sub-cultured every six months, and bacteria were regularly sub-cultured on plates to check their purity. Gram staining and motility tests were carried out before sub-culturing onto new slopes.

Table 2.1: *A. hydrophila* strains used in this study

| Isolate | Host species | Country/date | Comments |
|---------|---|---|---|
| T4 | Rohu (<i>Labeo rohita</i>) | Bangladesh (1994) | From an Epizootic Ulcerative Syndrome (EUS) lesion |
| 98141 | Black shark (<i>Morulus Chrysophekadion</i>) | Ayuthaya Province, Thailand (1998) | From a Haemorrhagic lesion |
| 98140 | | | |
| 98139 | | | |
| Hh | Hedgehog (<i>Erinaceus Europaeus</i>) | Institute of Aquaculture, Scotland | - |
| Vds | Catfish (<i>Ictalurus punctatus</i>) | India | From an EUS lesion |
| Catla | Catla (<i>Catla catla</i>) | India | From heart blood |
| C24li | African catfish, (<i>Clarias gariepinus</i>) | Aquatic Animal Health Research Institute, Thailand | - |
| 2D20 | Frog (<i>Rana rugulosa</i>) | Asia | - |
| 3D14 | | | - |
| 2N14 | | | - |
| F1d 75 | | | - |
| B2/12 | unknown | Bangladesh | - |
| Calf | unknown | Institute of Aquaculture, Scotland | - |

All the above isolates were obtained from Institute of Aquaculture bacterial collection.

2.2.1.1. Bio-chemical characterisation

The bio-chemical characteristics of *A. hydrophila* T4 strain, before and after passaging once and twice through fish, and comparison with a type strain from the National Collection of Industrial and Marine Bacteria (NCIMB) was performed using API 20E (Biomérieux, France) strips, according to the manufacturer's instructions.

2.2.1.2. Gram staining

Gram staining was performed according to Gram (1884). The composition of the reagents used for Gram staining is described in Appendix I. A smear of bacteria was placed onto a slide and heat-fixed. Crystal violet was used to stain the smear for 1 min, and washed with tap water after pouring off the stain. Gram's iodine was then flooded onto the slides and left for 1 min. This was poured off and the smear was destained with acetone for 2-3 sec. The slides were washed with water, then flooded with safranin for 2 min. The slides were washed with tap water, air dried and examined under oil immersion at 100x.

2.2.1.3. Motility test

A small amount of paraffin wax was placed on four corners of a coverslip and a small loopful of sterile saline (0.85 %) was placed into the middle of the coverslip (Collins and Patricia, 1976). Bacteria from a fresh culture were resuspended in the saline solution. A microscope slide was carefully placed onto the coverslip, taking care not to lower the slide onto the suspension, in order to have a hanging drop from the coverslip and the motility observed under 40x magnification.

2.2.1.4. Standard concentration curve

A standard concentration curve was prepared for *A. hydrophila* isolate T4 by using viable drop counts technique (Miles and Misra, 1938). The strain was grown overnight in tryptic soy broth (TSB) (Oxoid, UK) at 28 °C and the pellets harvested and washed twice with phosphate buffered saline (PBS) (Appendix I) by centrifuging at 1600 × g for 15 min. Pellets were resuspended in PBS and dilutions of the bacteria prepared with an absorbance at 610 nm of 0.2, 0.4, 0.8, 1.0, 1.2. Dilutions were prepared from all these different concentrations in PBS to give a final dilution of 10⁻⁶ and 20 µl of this dilution for each absorbance was spotted onto six segments of a TSA plate. The plates were incubated overnight at 28 °C and an average colony forming unit (CFU) calculated for each plate. The number of CFU ml⁻¹ was calculated using the following formula, the average number of CFU in a segment × 50 × dilution factor (10⁶). A standard concentration curve of optical density (OD) at 610 nm vs the average number of CFU ml⁻¹ was then constructed.

2.2.2. Virulence of *A. hydrophila* in common carp, *Cyprinus carpio*

This experiment was conducted using the 14 strains of *A. hydrophila* shown in Table 2.1. The bacteria were grown and adjusted to an OD of 1.0 at 610 nm, equivalent to 1 × 10⁸ ml⁻¹ CFU, as described in Section 2.2.1.4. From this suspension, concentrations of 10⁶ and 10⁴ bacteria ml⁻¹ were prepared. Common carp, weighing 30-40 g, were obtained from the indoor fish culture system of Research Institute for Fisheries, Aquaculture and Irrigation, Hungary (HAKI). The fish were initially anaesthetised for 1 min in a 0.004 % (w/v) benzocaine solution. Six fish were used for each of the three bacterial dilutions described above and a

total of eighteen fish were used for each bacterial strain. Fish were injected intraperitoneally (IP) with 0.1 ml of the bacterial suspension. Each group, injected with one of the *A. hydrophila* isolates, was maintained in individual glass tanks, and clipped to identify the different bacterial concentrations injected. The tanks were supplied with water, which was passed through a biological recirculatory system and ultraviolet (UV) irradiation. The experiment was conducted in a temperature controlled indoor aquarium at a water temperature of 20-22°C. The fish were monitored three times a day and dead fish removed from the tanks. Kidney swabs were taken from dead fish and streaked onto TSA to confirm that mortality was due to *A. hydrophila*.

2.2.3. Culture of *A. hydrophila* *in vitro* and *in vivo*

The protein profiles of WC, OMP and ECP from bacteria grown *in vitro* and *in vivo* were examined by 1 and 2D SDS-PAGE. The protein concentration of each sample applied to the gels was determined using a protein concentration determination kit (Pierce scientific company, Rockford, USA) according to the manufacturer's instructions.

2.2.3.1. Culture of bacteria *in vitro*

All 14 strains shown in Table 2.1 were grown in TSB overnight at 28°C for WC and OMP preparations, while culture of the bacterium on TSA with a cellophane overlay was used to collect the ECP (Liu, 1957).

To prepare WC, the strains of *A. hydrophila* were grown and the concentration of the bacterial suspension adjusted to 1.0 at 610 nm as described in Section

2.2.1.4. The bacteria were pelleted by centrifugation at $1600 \times g$ for 10 min and the bacteria were then resuspended in sample buffer (Appendix I) at a concentration of $1-2 \text{ mg ml}^{-1}$ for SDS-PAGE analysis.

The OMPs from the bacteria were prepared using the method of Osborn *et al.* (1972), with modifications. All 14 strains were grown and harvested, as described in Section 2.2.1.4, but the pellets were washed twice with sterile 10 mM Tris-HCl, pH 7.8 instead of PBS. The pellets were then resuspended in lysis solution [10 mM Tris – HCl, 10 mM ethylene diamine tetra acetic acid (EDTA) + $50 \mu\text{g ml}^{-1}$ Phenylmethylsulphonylfluoride (PMSF)] using a volume approximately twice the size of the bacterial pellet. The suspension was incubated for 30 min at 45°C and then cooled quickly on ice. Two to three volumes of glass beads ($150-212 \mu\text{m}$) were added to the bacteria and vortexed 3 times for 60 sec at 10 sec intervals. DNase and RNase were each added at $2 \mu\text{g ml}^{-1}$ and incubated 30 min at $20-22^\circ\text{C}$. Unbroken cells and debris were removed by centrifuging at $2000 \times g$ for 10 min. The supernatant containing both OMP and inner membrane protein (IMP) were collected and centrifuged at $88,000 \times g$ for 1 h at 4°C to collect the OMP and IMP. The supernatant was removed and pellets resuspended in approximately 4 ml of 1.5 % (v/v) sodium lauryl sarcosinate and vortexed slowly before incubating for 30 min at 25°C to solubilise the IMPs (Filip, 1973). The suspension was centrifuged at $91,300 \times g$ for 40 min at 4°C to collect the insoluble OMPs. The pellet was dissolved and adjusted to a final protein concentration of $1-2 \mu\text{g } \mu\text{l}^{-1}$ in sample buffer for SDS-PAGE analysis.

To prepare the ECP from 4 virulent (T4, 98141, Hh and Vds) and 2 avirulent isolates (Catla and C24li), a 5 ml aliquot of bacterial culture, grown overnight as described in Section 2.2.1.4, was placed onto Cuprophan sheets (Medicell international ltd, UK) [with a 10 kilo Dalton (kDa) molecular weight (MW) cut off], which has been placed onto TSA within a large Petri dish (14 cm dia) (Liu, 1957). The membrane had first been boiled in 5 mM EDTA, 200 mM sodium bicarbonate, and autoclaved before placing on the TSA medium. The Petri dish was incubated overnight at 28°C, after which the bacterial culture was harvested from the membrane and suspended in sterile PBS. The suspension was centrifuged at 1600 × g for 15 min to remove the bacteria. The supernatant was collected and concentrated using 10, 000 MW cut off spin concentrators (Vivo Science, UK). The concentrate was filtered using a 0.45 µm filter, and the protein concentration of the filtrate adjusted to 1-2 mg ml⁻¹ in sample buffer for SDS-PAGE analysis.

2.2.3.2. Culture of bacteria *in vivo*

Four virulent strains (T4, 98141, Hh and Vds) and 2 avirulent strains (Catla and C24li) of *A. hydrophila* were grown *in vivo* for five days within dialysis tubing placed inside the abdominal cavity of common carp. The virulence of these isolates had previously been assessed, as described in Section 2.2.2.

Dialysis tubing with two different MW cut off values, 25 and 100 kDa, obtained from Medicell, UK, were used in the study. The tubing was pre-irradiated by the manufacturer for sterility, and one end of the tubing came sealed, while the other end was closed with a lid after filling the tubing with bacteria suspension. The two types of tubing were distinguished from each other by red and blue coloured lids.

Bacteria were cultured in TSB overnight at 28°C. The pellets were harvested by centrifuging at 1600 × g for 15 min and washed twice with the PBS at 1600 × g for 15 min. The pellets were resuspended in PBS and the concentration of the bacteria adjusted to 1 ×10⁷ bacteria ml⁻¹. Two implants for each molecular weight cut off were filled with 2 ml bacteria (T4 isolate) and placed in a tube containing TSB to check that the implants were leak-proof before implanting them into the fish.

After establishing that the implants did not leak, three tubes were prepared for each MW cut off, so that 6 tubes were prepared for each *A. hydrophila* strain. Each tube contained 2 mls of bacterial suspension at a concentration of 1 ×10⁷ bacteria ml⁻¹. One control tube filled with PBS was also prepared for each MW cut off. Preparation of the implants was carried out in a sterile cabinet and care was taken during the implantation of the tube to maintain as sterile conditions as possible. The operating area was first treated by UV light for 1 h prior to performing the surgical procedure, wiped with 70 % alcohol, and then covered with sterile aluminium foil.

The fish, obtained from the HAKI aquarium, were approximately 1-1½ kg in weight. They were individually transported to the laboratory in a 50 L bucket and anaesthetised for 1-2 min in a 0.004 % w/v benzocaine solution. Throughout the procedure approximately 1 ml of 0.001 % w/v benzocaine solution was pipetted onto the fish's gills (Colquhoun and Sorum, 1998). The surface of the fish's abdomen was swabbed with 70 % ethanol (v/v), and a 3-4 cm incision was made in the abdomen area between the pelvic fin and the anus. The area within the

incision was cleared with a sterilised cotton bud in order to avoid damaging the internal organs while implanting the dialysis tube into the abdominal cavity of the fish.

The dialysis tubing was folded in half and placed carefully into the abdominal cavity of the fish in order to avoid any physical injury. The incision was closed with 4-5 continuous stitches using 910 sutures (Ethicon Ltd, Edinburgh, UK), and a disinfectant cum sealant (Orahesive protective powder, Conva tec Ltd, UK) was then applied to the incision (Figure 2.1). The fish were marked by pectoral and ventral fin clipping to identify the strains and the molecular weight of the tubing implanted. They were then placed into two round plastic tanks 1 m × 1 m (diameter × depth) with aeration, and were swimming normally within 2-3 min of returning them to the tank. The fish were frequently observed over a five day period before retrieving the tubes from the fish on the fifth day.

To recover the implants, the fish were sacrificed by giving them an overdose of anaesthetic and the sutures carefully removed. The tubes were removed and placed in sterile tubes before pooling the contents of the respective molecular weight cut off tubes for analysis. After pooling the samples, the CFU of the suspension was determined, and the purity of the suspension was confirmed by streaking it out onto TSA and performing Gram staining on it. The pooled samples were briefly stored at 4°C before splitting the bacterial suspension for the different analyses to be carried out. Samples from tubes found to be broken in the fish abdominal cavity were not used in the analysis.

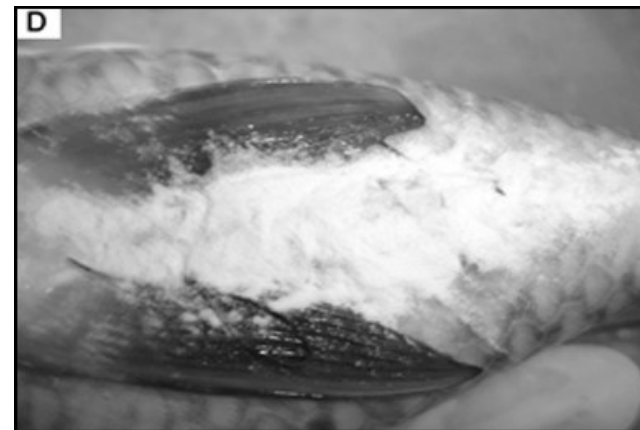
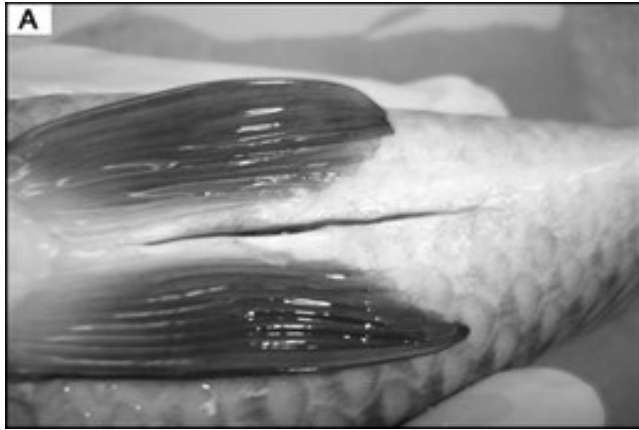


Figure 2.1: Implantation of dialysis tubing containing *A. hydrophila* for culture of the bacterium *in vivo*

(A) Incision, (B) Placing dialysis tubing into the incision, (C) Closing incision by stitching, (D) Covering incision with disinfectant cum sealant powder.

The WC, OMP, and ECP fractions were prepared from the bacteria for 1D SDS-PAGE. Only the WC was examined by 2D SDS-PAGE due to the amount of sample available. The samples were centrifuged at $1600 \times g$ for 15 min to separate the pellets from the ECP. The pellets were washed twice with PBS at $1600 \times g$ for 15 min and WC and OMP prepared for 1D and 2D SDS-PAGE as described in Sections 2.2.3.1 and 2.2.4 below. The ECP was concentrated using 10,000 MW cut off spin concentrators (Vivo science, UK) by centrifuging at $2000 \times g$ for 2 h. It was then filtered through a $0.45 \mu\text{m}$ filter and prepared for 1D SDS-PAGE as described in Section 2.2.3.1.

2.2.4. SDS-PAGE

2.2.4.1. 1D SDS-PAGE

The samples were boiled for 5 min and centrifuged at $2300 \times g$ for 2 min from which $15 \mu\text{l}$ of sample was loaded into the wells of a 4 % SDS-PAGE stacking gel (Appendix I) and run through a 12 % separating gel (Appendix I) according to Laemmli (1970). Broad range MW protein markers (Biolabs, UK) were run alongside the samples to serve as MW references. The gels were run between 45 and 60 min at 200 V and were then stained overnight in Coomassie brilliant blue R-250 (0.25 % w/v) in 50 % (v/v) methanol and 10 % (v/v) acetic acid and destained with 40 % (v/v) methanol and 10 % (v/v) acetic acid. Gels were scanned and the MW of bands was determined using TotalLab v2002.03 software program (Nonlinear Dynamics Ltd).

2.2.4.2. 2D SDS-PAGE

Whole cell preparations of *A. hydrophila* grown *in vitro* and *in vivo* were analysed by 2D SDS-PAGE as recommended by Berkelman and Stenstedt (2002). One ml of 1×10^8 ml⁻¹ bacterial suspension was centrifuged at $2300 \times g$ for 10 min and the pellet resuspended in 1 ml lysis solution (9 M urea, 4 % chaps, 40 mM Trisma base and 17 mM SDS). Ten μ l of nuclease mix (Amersham bioscience) was added and vortexed briefly, then left for 30 to 45 min at 20-22°C. Destreak solution (Amersham Bioscience) (125 μ l) was loaded in to the strip holder channel together with 0.6 % of Immobilized pH Gradients (IPG) buffer and 15 μ l of sample. A 7 cm immobiline drystrip gel (3-10 NL pH range, Amersham Bioscience) was rehydrated for 15 h by placing it into a strip holder channel with the gel side facing down taking care not to introduce air bubbles. Isoelectric focusing was achieved using 500 V for 30 min, 1000 V for 30 min and 8000 V for 1 h. The strip was placed in a 15 ml centrifuge tube containing 10 ml equilibration solution (50 mM Tris HCl (pH 8.8), 6 M urea, 30 % v/v glycerol, 2 % w/v SDS and 0.002 % w/v bromophenol blue) and the strip was equilibrated in the buffer for 20 – 30 min on a rocker. The strip was then placed on a 12.5 % SDS-PAGE gel, sealed with agarose sealant (0.5 % agarose (w/v) and few grains of bromophenol blue). The gel was subjected to 20 mA for 15 min, followed by 40 mA for approximately 90 min and then stained with a silver staining kit (Amersham Bioscience), according to the manufacturer's instructions. The spots on the stained gel were analysed using image master 2D platinum 0.6 software (Amersham bioscience).

2.2.5. Ultra structure of outer membrane

The difference in the ultrastructure of the outer membrane of bacteria grown *in vitro* and *in vivo* was examined by transmission electron microscopy (TEM) (Hayat, 1986). The bacterial pellets were fixed for 2-4 h in 2.5 % gluteraldehyde in 100 mM sodium cacodylate buffer (pH 7.2), then centrifuged at 2000 × g for 10 min and the supernatant removed. Fresh fixative was added and the pellets were gently loosened from the bottom of the centrifuge tube after 30 min using a sharpened wooden applicator. After 2 h the pellets were rinsed twice in buffer rinse (0.1 M sucrose in 100 mM sodium cacodylate), 5 min each time, in order to prevent osmotic shock to the cells.

The pellets were post-fixed in 1 % osmium in sodium cacodylate buffer in a closed vial for 1 h, then washed for three times with distilled water for 5 min on each wash. The samples were placed in *en-bloc* in 2 % (v/v) uranyl acetate in 30 % acetone and kept in the darkness for 1 h. They were then dehydrated through an acetone series: 60 % (v/v) for 20 min, 90 % for 20 min, 100 % for 30 min and 100 % for 50 min at 20-22°C. The samples were then infiltrated with Spurr's resin in 1 acetone: 1 Spurr's resin for 1 h and in 100 % Spurr's resin for 2×1 h. The material was then embedded in Beem capsules and polymerised for 16 h at 75°C.

All steps except polymerisation and *en-bloc*, were carried out with continuous agitation. Complete processing was carried out in a fume cupboard. Ultra-thin gold sections of approximately 90 nm thickness were cut and placed on 200 mesh formvar-coated copper grids. Once on the grids, the sections were stained with 4 % (v/v) uranyl acetate in 50 % (v/v) ethanol for 4 min and 7 min in Reynold's

(1963) lead citrate. The grids were finally examined under a TEM (FEI TECNAI G2 Spirit Bio Twin electron microscopy, Holland).

2.3. Results

2.3.1. Biochemical characterisation

Basic biochemical analysis including Gram staining, motility test, and CFU were carried out on each *A. hydrophila* isolate used in the study together with protein analysis by 1 and 2D SDS-PAGE. The colonies of *A. hydrophila* appeared milkish white to yellow in colour, and were circular and opaque in shape on TSA plates. The bio-chemical characteristics of *A. hydrophila* (T4 isolate) were similar before and after passaging through goldfish (*Carassius auratus*), and with the reference strain obtained from NCIMB, except for some differences in H₂S, indole, sorbitol and rhamnase production (Table 2.2).

All strains were Gram-negative and had rod shape morphology, but there were considerable differences in the lengths of the rod, and chains of rods were found with some strains. The morphology seen with each isolate is shown in Table 2.3 All strains were very active and appeared motile under the microscope.

Table 2.2: Biochemical characterisation of *A. hydrophila* type strain 1134 and T4 using API 20E strips

| Biochemical test | Result | | | |
|-------------------------------------|--------------|-----------------------|---|---|
| | 1134 (NCIMB) | T4 (before passaging) | T4 (after 1 st passaging in gold fish) | T4 (after 2 nd passaging in gold fish) |
| O-nitrophenyl-β-D-galactopyranoside | + | + | + | + |
| Arginine dehydrolysis | + | + | + | + |
| Lysine decarboxylase | + | + | + | + |
| Ornithine decarboxylase | - | - | - | - |
| Citrate utilization | + | + | + | + |
| H ₂ S production | - | + | + | - |
| Urease production | - | - | - | - |
| Tryptophane deaminase | - | - | - | - |
| Indole production | + | + | + | - |
| Acetoin production | + | + | + | + |
| Gelatin hydrolysis | + | + | + | + |
| Glucose | + | + | + | + |
| Mannitol | + | + | + | + |
| Inositol | - | - | - | - |
| Sorbitol | - | - | + | - |
| Rhamnose | + | + | - | - |
| Sucrose | + | + | + | + |
| Melibiose | - | - | - | - |
| Amygdalin | + | + | + | + |
| Arabinose | + | + | + | + |

Table 2.3: Rod morphology of different *A. hydrophila* strains

| Name of strains | Small/long rod | Chain Presence |
|------------------------|-----------------------|-----------------------|
| T4 | Small | × |
| 98141 | Long | √ |
| Hh | Small | × |
| Vds | Long | × |
| Catla | Long | √ |
| C24li | Long | × |
| 2D20 | Small | × |
| 3D14 | Long | √ |
| 2N14 | Long | √ |
| 98140 | Long | √ |
| 98139 | Long | √ |
| B2/12 | Long | × |
| F1d75 | Long | × |
| Calf | Long | × |

2.3.1.1. Standard concentration curve

The growth of the *A. hydrophila* was rapid reaching approximately 1×10^8 colonies in 1 ml after 18 h of culture. The standard concentration curve of *A. hydrophila* is shown in Figure 2.2.

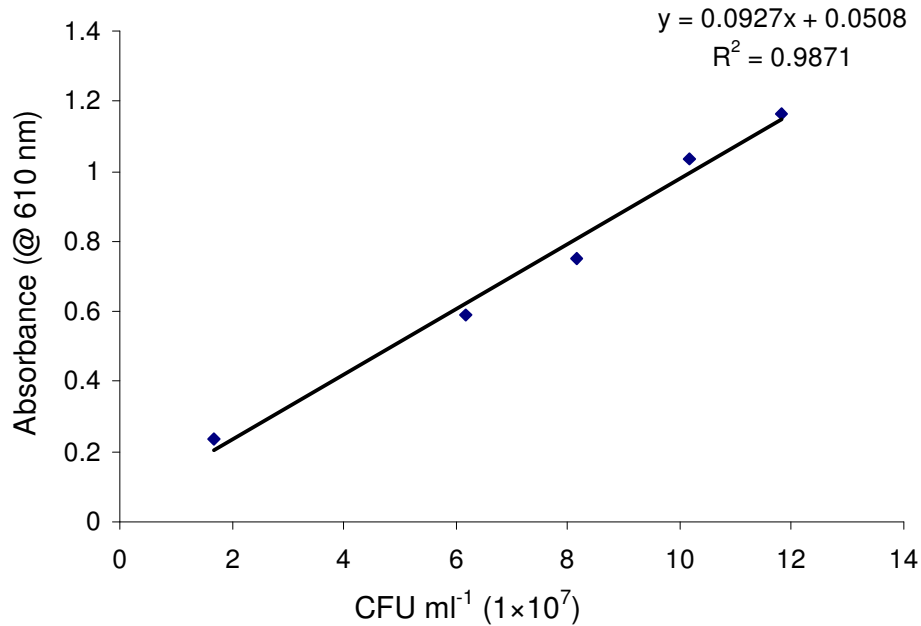


Figure 2.2: Standard concentration curve for *A. hydrophila*

2.3.2. Virulence of *A. hydrophila*

Of the 14 isolates tested, 6 isolates were found to be virulent, while the remainder were avirulent causing no mortalities. Mortality only occurred when 1×10^7 fish⁻¹ was injected into the fish with all six strains described as virulent, while no mortalities were recorded at doses of either 1×10^5 or 1×10^3 fish⁻¹. However, reddening and moderate swelling of the muscles were noted in all the fish from these six groups irrespective of the concentration of bacterial cells injected. Clinical signs appeared from the first day after injection with the highest concentration of the bacteria used, and after 3 days in the groups of fish injected with 1×10^5 and 1×10^3 bacteria ml⁻¹. Mortalities were observed from the first day post-injection until the ninth day after injection, and the experiment was stopped on Day 15 post-injection. The highest percentage mortality was observed with strain B2/12 (66.6 %) and the lowest with strains 98140 and Hh (Figure 2.3).

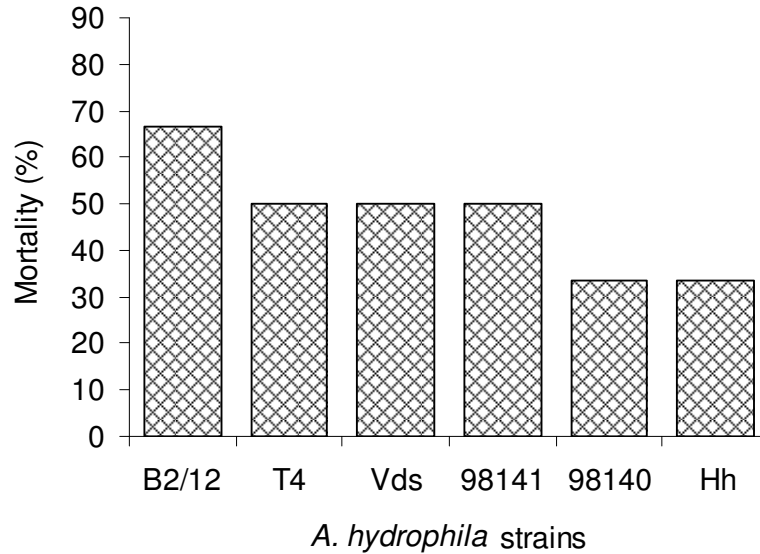


Figure 2.3: Mortalities of Common carp, *C. carpio* during challenge with different isolates of *A. hydrophila*
(N=6 fish per group).

2.3.3. Protein profiles of bacteria grown *in vitro* and *in vivo*

Considerable differences were found in the profiles of WC, OMP and ECP from the different bacteria grown *in vitro* and *in vivo*, when examined by 1 and 2D SDS-PAGE gel electrophoresis.

2.3.3.1. Protein expression *in vitro* analysed by 1D SDS-PAGE

The WC, OMP and ECP preparations of *A. hydrophila* isolates grown *in vitro* expressed different profiles by SDS-PAGE analysis for each preparation as well as each isolate.

Very different protein profiles were obtained with the WC preparations between the fourteen strains examined including the number of bands exhibited between the different isolates of *A. hydrophila* (Figure 2.4a). The number of bands

observed was greater with isolates 98141, Catla and C24li (lanes 3, 6 and 7) compared with the other isolates examined. Three high intensity bands were found at 45, 50, 55 kDa with isolate T4 (lane 2), which were not seen with rest of the isolates. It was very difficult to place the isolates in groups from the profiles obtained. However, 15 bands were found in common with all of the isolates compared to 5-10 unique bands found with individual isolates. Major bands in common between the fourteen strains were located at around 212, 97, 70, 50, 32 and 25 kDa. The majority of bands were expressed between 50 and 100 kDa with all the strains. Expression of bands with high intensity was also noticed between 40 and 50 kDa with most of the strains examined.

The OMP profiles showed far less bands than the WC profiles. Between 4 and 6 bands were observed with each strain between 25 and 66 kDa (Figure 2.4b). A major band was seen at approximately 50 kDa with all isolates except 2D20, Calf and 2N14 (lanes 8, 15 and 10), with darker bands noted for all the six virulent strains (lanes 2, 3, 4, 5, 11 and 13). A common band was also observed at around 27 kDa with all strains except T4 and 2D20 (lanes 2 and 8). Only the profiles of isolates 98140 and 98141 (lanes 3 and 11) looked identical, with no other similar pattern observed between strains.

The bands expressed in the ECP preparations of the bacteria were mostly between 27 and 70 kDa with all six (4 virulent and 2 avirulent) strains examined (Figure 2.4c). A common band was found at 55 kDa with all the isolates, but it was weakly expressed with all the avirulent isolates examined. A band at 70 kDa was found only in ECP from isolates T4 and Hh (lanes 2 and 4). Bands at 37, 40, and

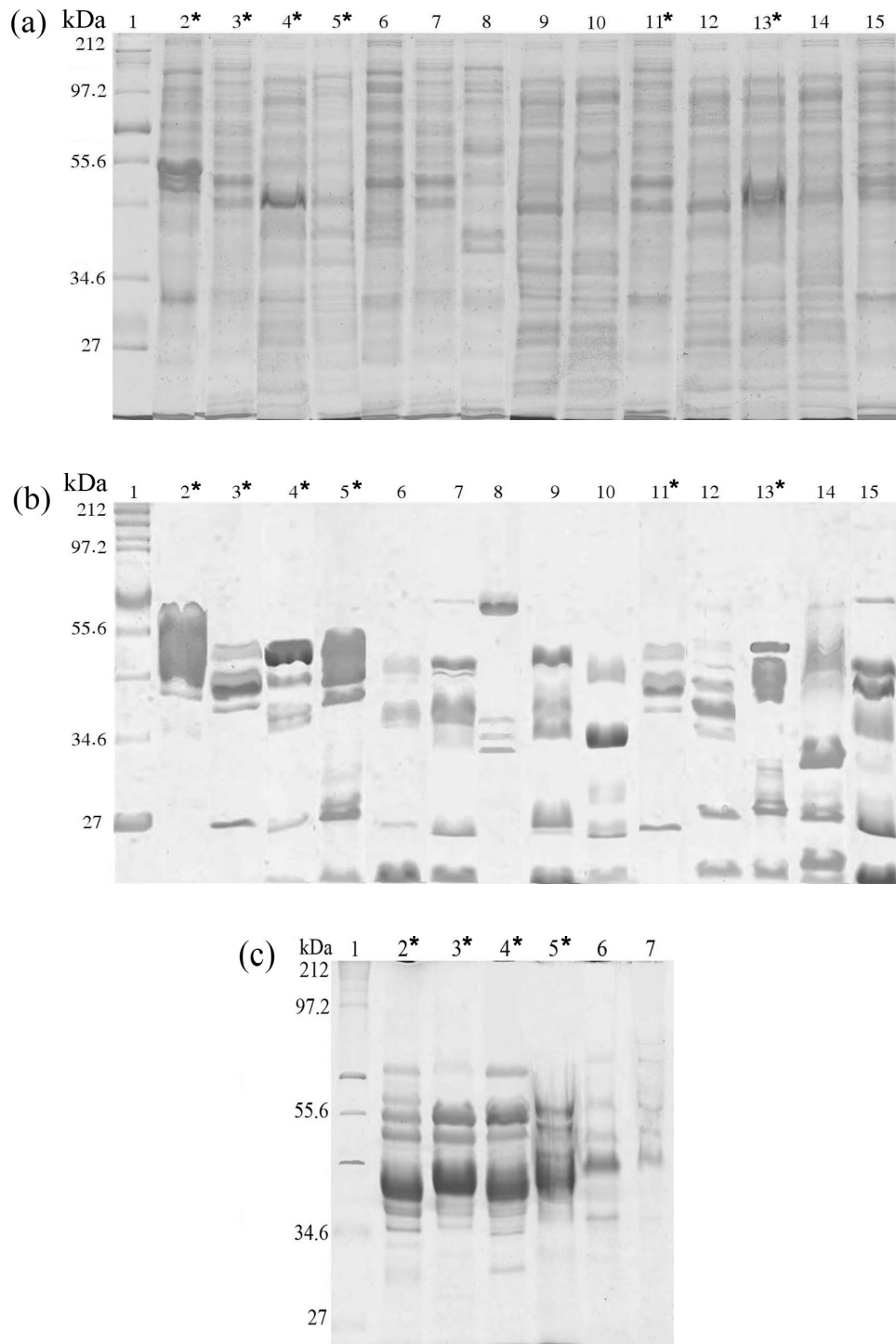


Figure 2.4: SDS-PAGE of *A. hydrophila* grown *in vitro*, stained with Coomassie blue

(a) Whole cell, (b) Outer membrane protein, (c) Extracellular products.
Lanes: (1) Standard marker; (2) T4; (3) 98141; (4) Hh; (5) Vds; (6) Catla; (7) C24li; (8) 2D20; (9) 3D14; (10) 2N14; (11) 98140; (12) 98139; (13) B2/12; (14) F1d75; (15) Calf. * Virulent isolates.

50 kDa were noted in all the virulent strains with high intensity at 40 kDa. Two weakly expressed bands were also observed below 37 kDa with most of the isolates. All the bands were expressed weakly with the avirulent strains compared with the virulent strains, but a moderate intensity band was found at 42 kDa in both avirulent strains examined.

2.3.3.2. Protein expression *in vivo* in 1D SDS-PAGE

The fish with the implants remained alive and healthy over the duration of the experiment and their internal organs also looked healthy when examined at the time of sacrifice. No growth of *A. hydrophila* was obtained from kidney swabs made from the implanted fish except for one fish implanted with the 98141 isolate and one with the Catla isolate as the dialysis tubes were damaged in the abdominal cavity of these two fish. Bacteria grown within the implants gave a CFU count of 1×10^8 bacteria ml^{-1} compared to 1×10^7 bacteria ml^{-1} before implanting the bacteria, which was more than a 9 fold increase in bacterial concentration. No bacteria growth was obtained from the control implants containing PBS.

Few bands were seen above 90 kDa, while an increase in the number of bands was observed below 90 kDa in WC preparations made from the bacteria cultured *in vivo* (Figure 2.5a), compared with the same isolates cultured *in vitro* (Figure 2.4a). Isolates grown *in vivo* expressed a unique band at 58 kDa, however three bands were seen in common at around 25, 50, 70 kDa between bacteria grown *in vitro* and grown *in vivo* within both MW cut off tubes. A faint band at 40 kDa was found with all the isolates grown *in vitro* and in the 100 kDa MW cut off tube, but was not seen with bacteria grown in 25 kDa MW cut off tube. In addition,

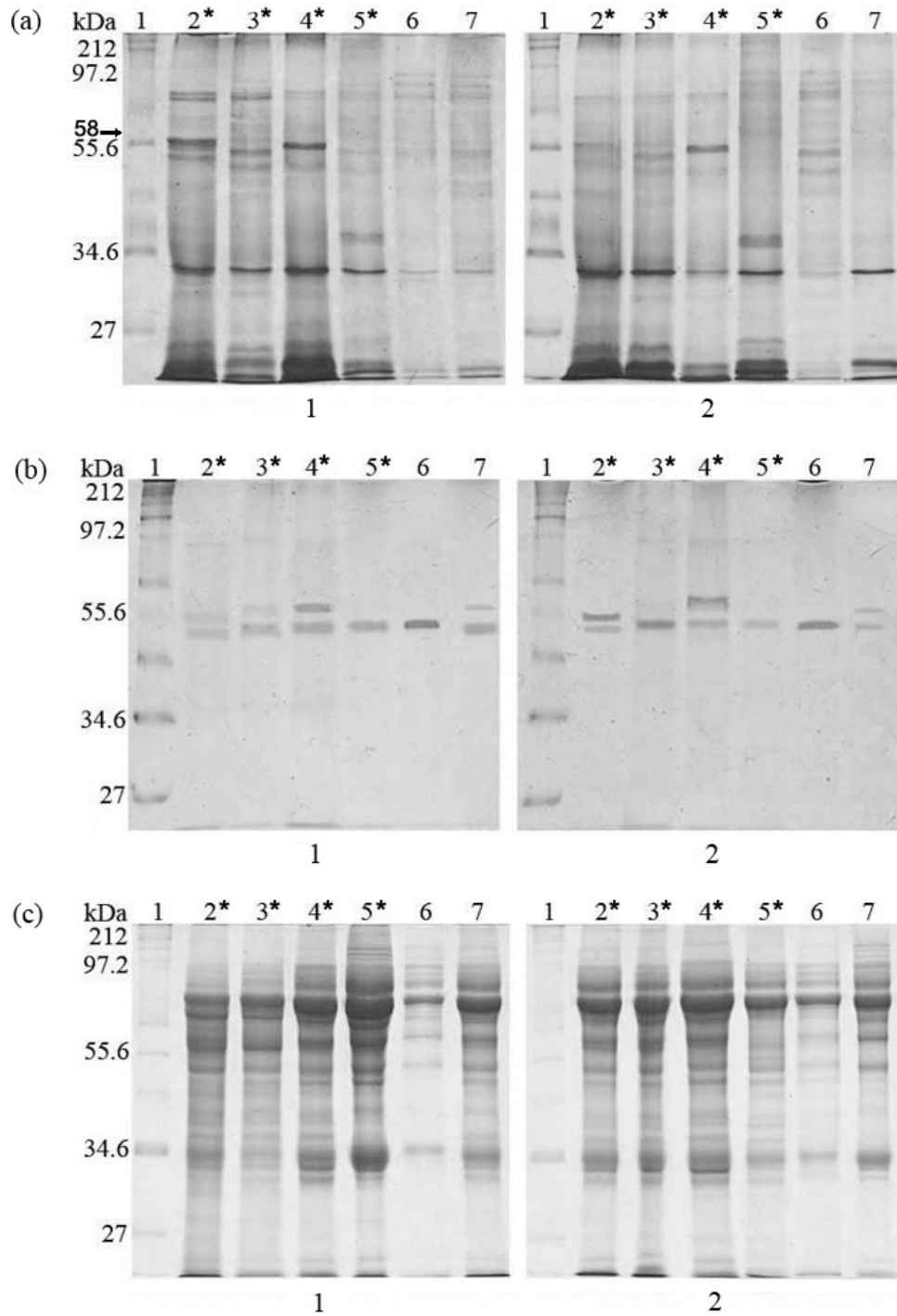


Figure 2.5: SDS-PAGE of *A. hydrophila* grown *in vivo*, stained with Coomassie blue

(a) Whole cell, (b) Outer membrane protein, (c) Extracellular products.

1. Profiles of strains from 25 kDa MW cut off dialysis tube.

2. Profiles of strains from 100 kDa MW cut off dialysis tube.

Lanes: (1) Standard marker; (2) T4; (3) 98141; (4) Hh; (5) Vds; (6) Catla; (7) C24li. * Virulent isolates.

another faint band at 43 kDa was present in all the isolates except the Hh isolate (lane 4) grown in the 25 kDa MW cut off tube *in vivo*. Bands were of greater intensity at approximately 25, 50, 55 and 70 kDa with all virulent strains grown *in vivo* in both MW cut off tubes compared with avirulent strains.

There were no differences between the OMP profiles of bacteria grown in the two different MW cut off tubes (Figure 2.5b). Differences were, however, evident in the OMP profiles of bacteria cultured *in vitro* and *in vivo*. The intensity of the bands was less in bacteria grown *in vivo* compared with *in vitro*. Common bands were seen at 50 and 55 kDa with all isolates grown in both MW cut off tubes *in vivo*, but were only weakly expressed at 55 kDa with Vds & Catla isolates (lane 5 and 6).

Fifteen to twenty bands were observed in the ECP profiles for bacteria grown *in vivo* (Figure 2.5c), however most of the bands seen with isolates grown *in vitro* were also seen in ECP from bacteria grown *in vivo*. A wide distribution of bands was seen in the ECP especially between 25 and 120 kDa in both of the MW cut off tubes, however there was little difference in the profiles of ECP recovered from the two different tubes. Bands were expressed most strongly at 35, 50, 60 and 80 kDa with all isolates grown in both MW cut off tubes. A band at 37 kDa was found with all the virulent isolates grown *in vitro* and *in vivo*, although it was not found with avirulent isolates. A greater number of bands were present in virulent isolates compared with avirulent isolates in bacteria grown in both MW cut off tubes. The total number of bands in the ECP was relatively low for bacteria cultured *in vitro* compared to when cultured *in vivo*.

2.3.3.3. Protein expression of *A. hydrophila* grown *in vitro* and *in vivo* analysed by 2D SDS-PAGE

Further differences were determined in the WC profiles between different isolates by 2D SDS-PAGE analysis, both in the apparent molecular mass of the expressed proteins and their pI value. The majority of protein spots were found between 30 and 80 kDa, between pI 4.5 and 8.5 with all of the isolates examined. Most of these spots were observed between 43 and 50 kDa, with pI values between 4.5 and 5.5 with all isolates analysed. The greatest number of spots in common between the isolates was found between the isolates T4 and F1d75 with 32 % similarity.

The greatest difference in the number of spots expressed between bacteria grown *in vitro* and *in vivo* was found with isolate 98141, followed by Catla and Vds isolates. An average of 37.5 and 40 % fewer spots were seen with the 98141 isolate grown *in vivo* in the 25 and 100 kDa MW cut off tubes respectively, compared to the same isolate grown *in vitro*. Around 29 and 36 % fewer spots were seen with the Catla isolate and 14 and 16 % fewer with Vds isolate, while a similar number of spots were expressed under the different growth conditions for isolate T4, Hh and C24li. There was little difference in the number of spots between the bacteria grown *in vivo* within the two different MW cut off tubes. Around 9 % of spots were not expressed in the Catla isolate grown *in vivo* in the 100 kDa MW cut off tube compare to the 25 kDa MW cut off tube.

The highest percentage of spots in common amongst all 3 growth conditions was with the Catla isolate (55.1 %) and the lowest was 29.7 % with 98141 (Table 2.4).

A notable number of unique spots were observed for all of the isolates grown *in vivo*, in both MW cut off tubes (Table 2.5). Approximately, 20-25 % of matching spots were noted between all the isolates cultured in each of the growth conditions. In general, up-regulation of the spots was found to be greater in bacteria grown *in vivo* compared with bacteria grown *in vitro* (Table 2.6). In addition, between the bacteria grown *in vivo* (Table 2.7), the up-regulation was found more in bacteria grown in the 100 kDa MW cut off tube compared to the 25 kDa MW cut off tube (Figure 2.6).

Table 2.4: No. of spots detected in 2D SDS-PAGE with WC preparations of bacteria grown *in vitro* and *in vivo*

| <i>A. hydrophila</i> isolates | No. of spots detected | | | % common spots ^a | No. of unique spots <i>in vivo</i> |
|-------------------------------|-----------------------|----------------|--------------|-----------------------------|------------------------------------|
| | <i>In vitro</i> | <i>In vivo</i> | | | |
| | | 25 kDa tube | 100 kDa tube | | |
| T4 | 115 | 113 | 115 | 55.1 | 4 |
| 98141 | 240 | 150 | 145 | 29.7 | 5 |
| Hh | 152 | 147 | 149 | 42.8 | 5 |
| Vds | 122 | 105 | 102 | 46.5 | 2 |
| Catla | 239 | 170 | 154 | 54.9 | 4 |
| C24li | 122 | 118 | 123 | 52 | 9 |

^a % common spots between *in vitro* and *in vivo*.

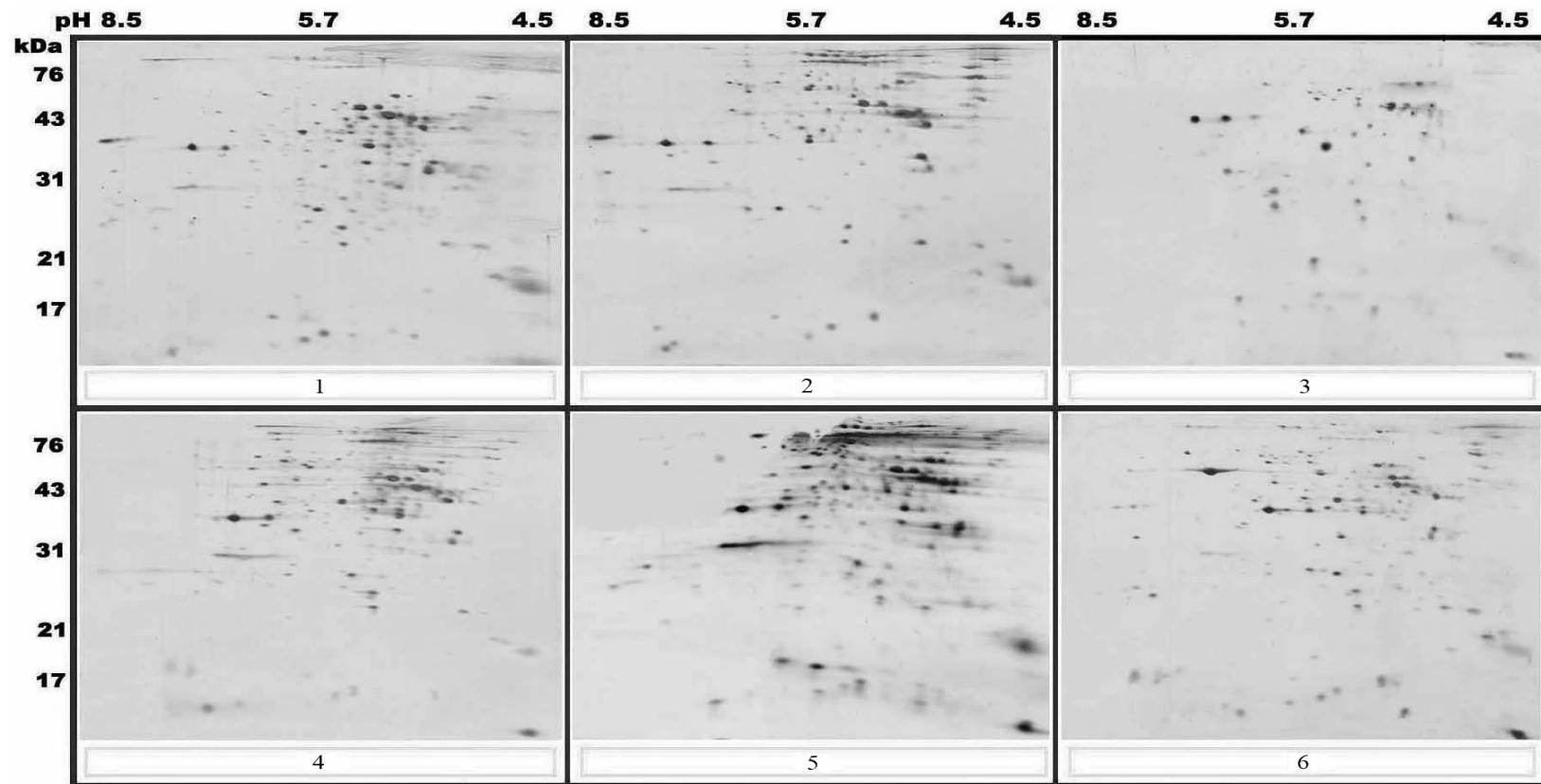


Figure 2.6a: 2D SDS-PAGE of *A. hydrophila* strains grown *in vitro*

(1) 2D20, (2) 3D14, (3) 2N14, (4) 98140, (5) 98139, (6) B2/12.

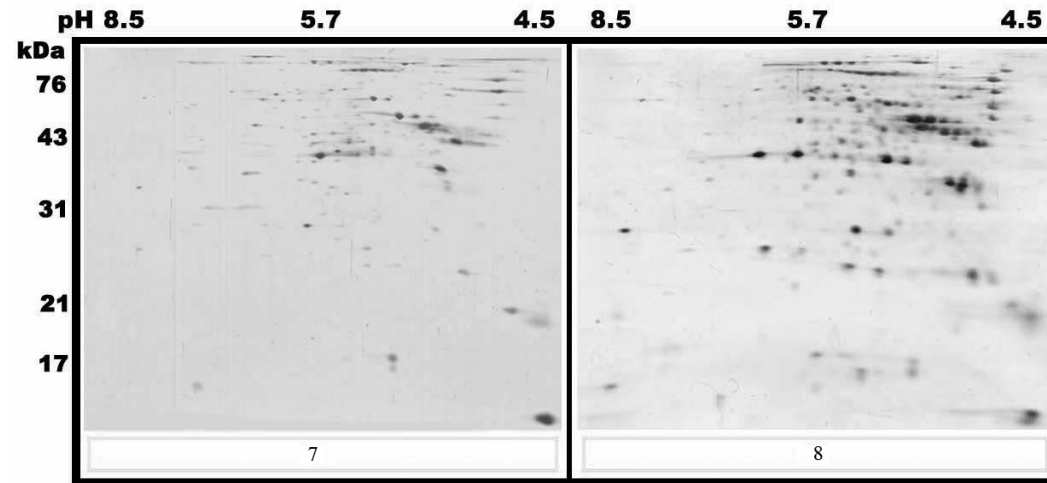


Figure 2.6a: 2D SDS-PAGE of *A. hydrophila* strains grown *in vitro*
(7) F1d75, (8) Calf.

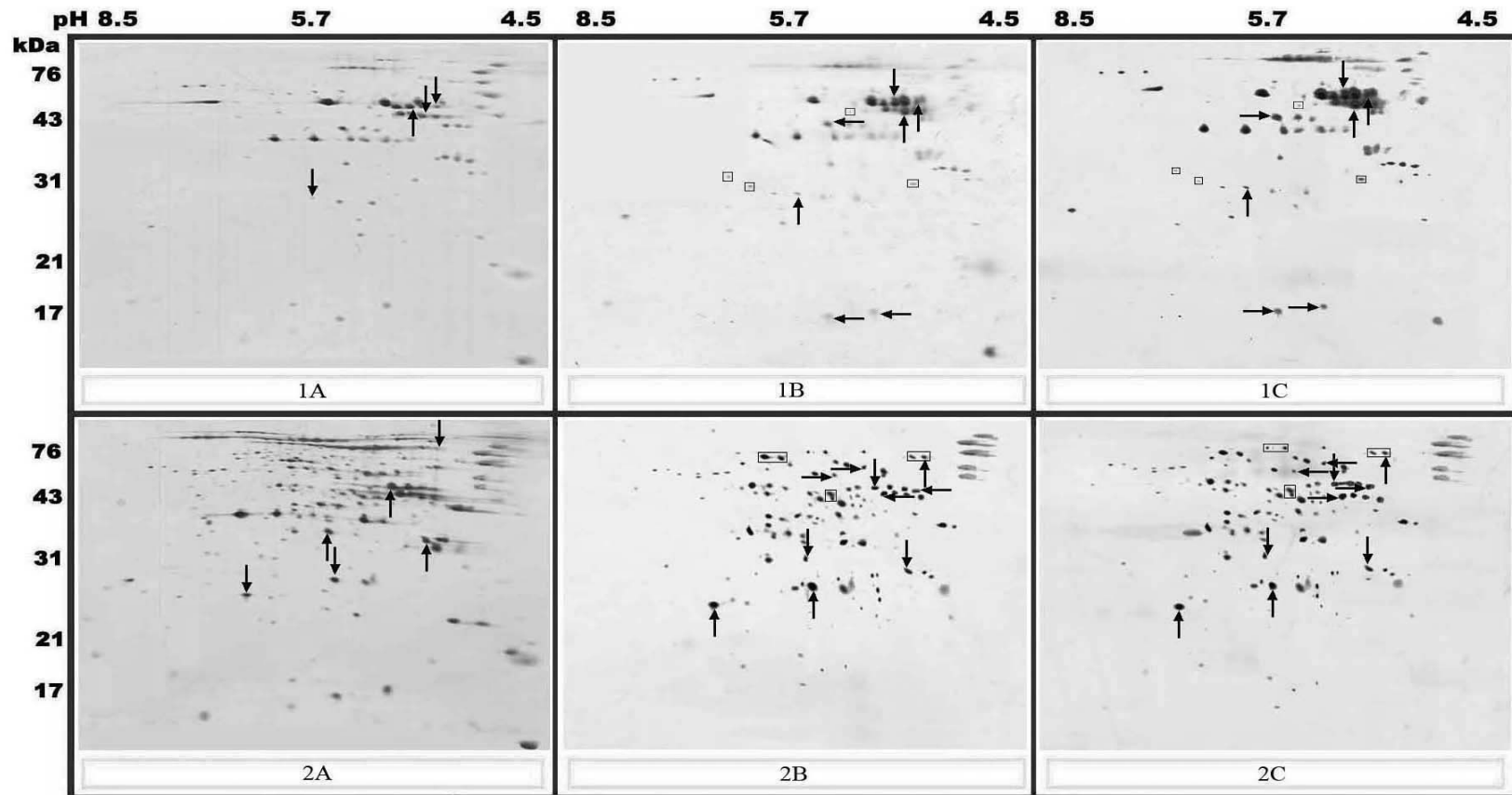


Figure 2.6b: 2D SDS-PAGE of *A. hydrophila* strains grown *in vitro* and *in vivo*

(A) Bacteria grown *in vitro*, (B) Bacteria grown in 25 kDa MW cut off tube *in vivo*, (C) Bacteria grown in 100 kDa MW cut off tube *in vivo* (1) T4, (2) 98141.

↑ up and ↓ down-regulated compared to either *in vivo* or *in vitro*. → up and ← down regulated compared to bacteria grown *in vivo* in 100 kDa MW cut off tubes. □ spots seen only in bacteria grown *in vivo*.

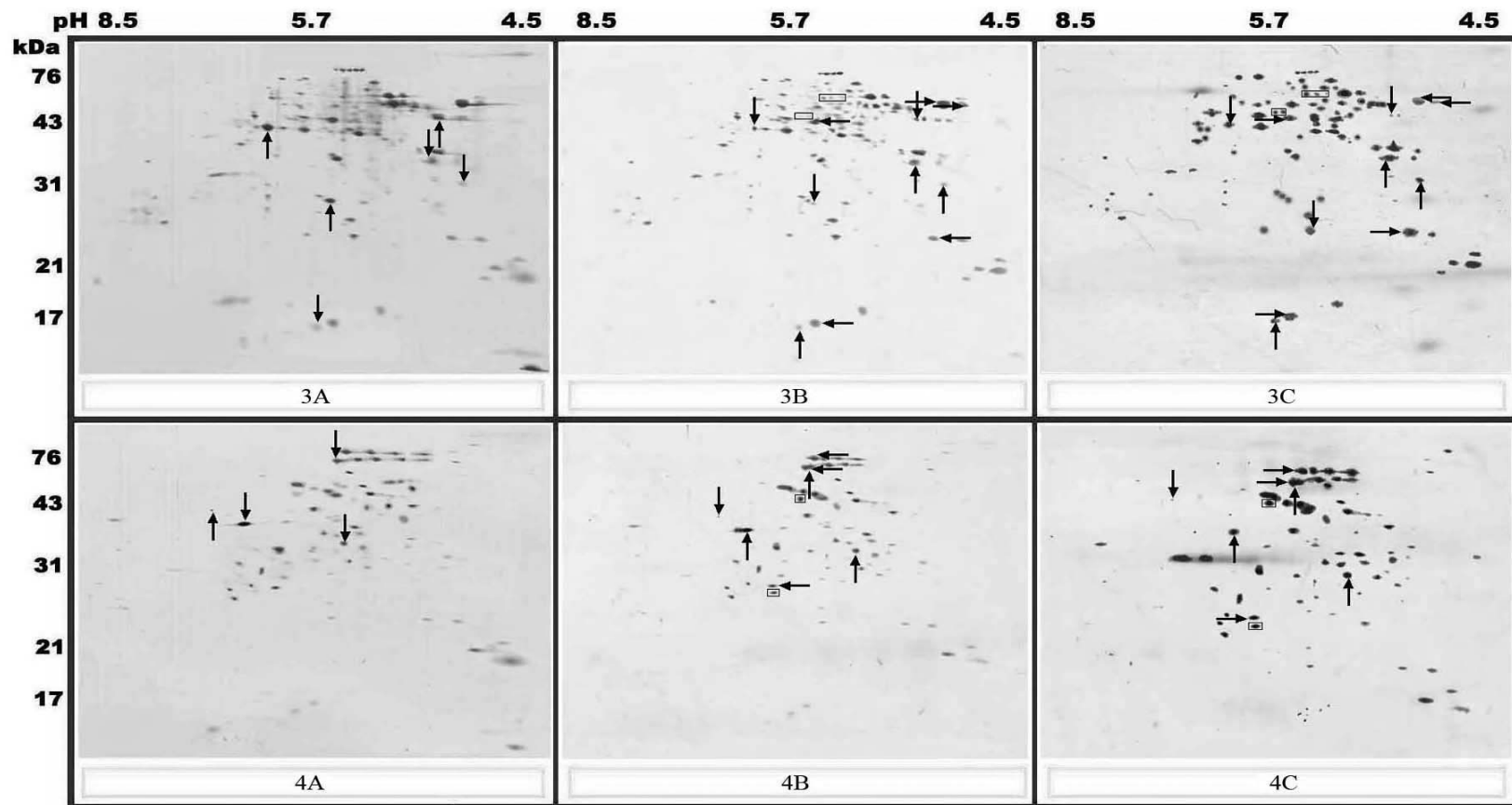


Figure 2.6b: 2D SDS-PAGE of *A. hydrophila* strains grown *in vitro* and *in vivo*

(A) Bacteria grown *in vitro*, (B) Bacteria grown in 25 kDa MW cut off tube *in vivo*, (C) Bacteria grown in 100 kDa MW cut off tube *in vivo* (3) Hh, (4) Vds.

↑ up and ↓ down-regulated compared to either *in vivo* or *in vitro*. → up and ← down regulated compared to bacteria grown *in vivo* in 100 kDa MW cut off tubes. □ spots seen only in bacteria grown *in vivo*.

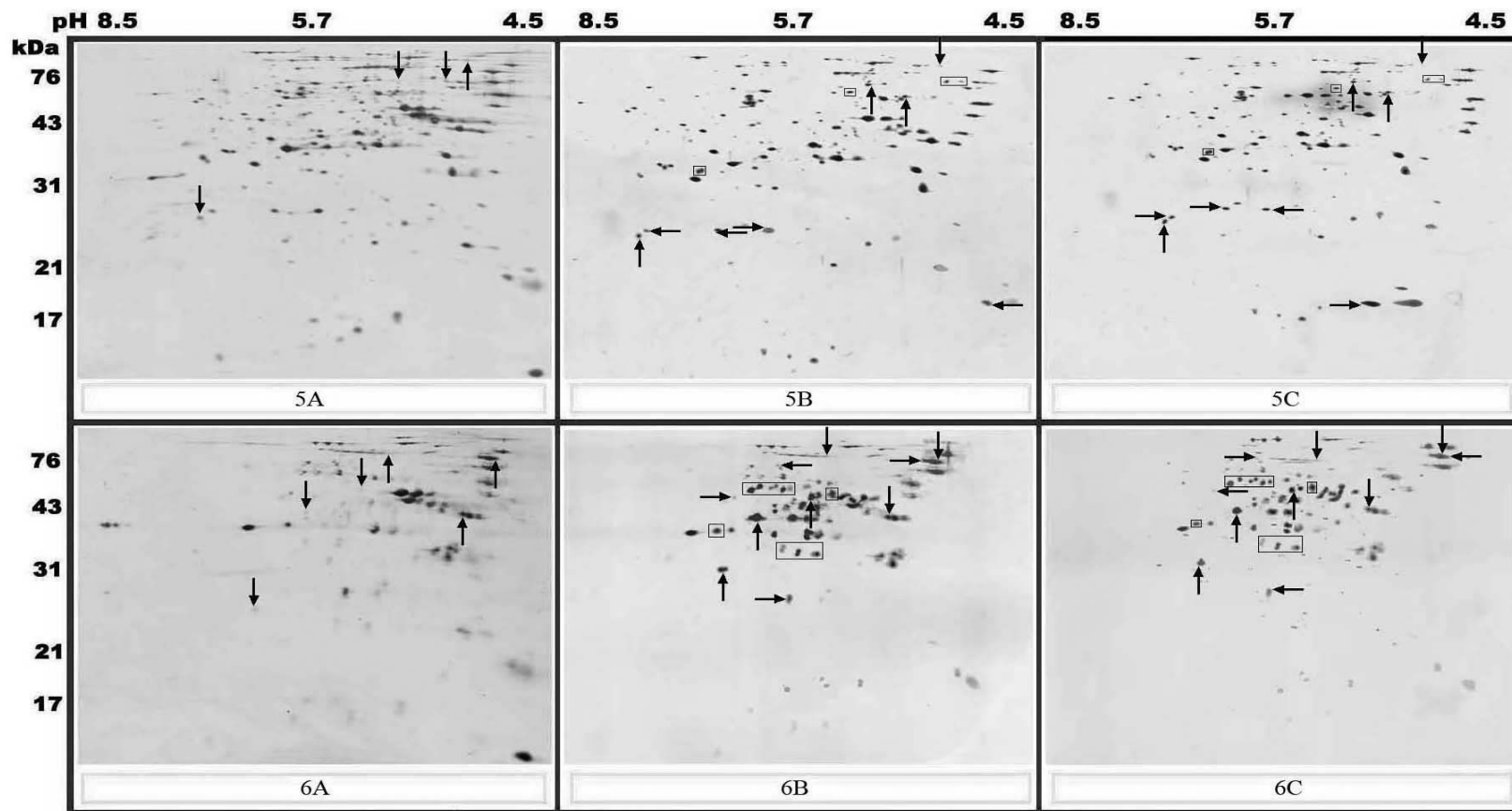


Figure 2.6b: 2D SDS-PAGE of *A. hydrophila* strains grown *in vitro* and *in vivo*

(A) Bacteria grown *in vitro*, (B) Bacteria grown in 25 kDa MW cut off tube *in vivo*, (C) Bacteria grown in 100 kDa MW cut off tube *in vivo* (5) Catla, (6) C24li.

↑ up and ↓ down-regulated compared to either *in vivo* or *in vitro*. → up and ← down regulated compared to bacteria grown *in vivo* in 100 kDa MW cut off tubes. □ spots seen only in bacteria grown *in vivo*.

Table 2.5: MW and pI of unique spots seen in 2D SDS-PAGE analysis for *A. hydrophila* strains grown *in vivo* in both MW cut off tubes compared with the same strains grown *in vitro*

| <i>A. hydrophila</i> isolates | MW | pI |
|-------------------------------|----|------|
| T4 | 51 | 5.4 |
| | 31 | 5.9 |
| | 30 | 5.1 |
| | 30 | 5.8 |
| 98141 | 77 | 5.5 |
| | 76 | 5.5 |
| | 72 | 5.1 |
| | 72 | 5.0 |
| | 47 | 5.4 |
| Hh | 61 | 5.5 |
| | 61 | 5.45 |
| | 61 | 5.4 |
| | 49 | 5.6 |
| | 48 | 5.7 |
| Vds | 56 | 5.6 |
| | 28 | 5.7 |
| Catla | 77 | 5.6 |
| | 76 | 5.5 |
| | 54 | 5.5 |
| | 27 | 5.7 |
| C24li | 60 | 5.2 |
| | 59 | 5.9 |
| | 59 | 5.8 |
| | 56 | 6.0 |
| | 54 | 5.5 |
| | 37 | 6.2 |
| | 34 | 5.7 |
| | 33 | 5.6 |
| | 33 | 5.5 |

Table 2.6: MW and pI of the most up and down-regulated spots in 2D SDS-PAGE of *A. hydrophila* grown either *in vitro* or *in vivo* (25 and 100 kDa MW cut off tube)

| <i>A. hydrophila</i> isolates | MW | pI | <i>in vitro</i> | <i>in vivo</i> |
|-------------------------------|----|------|-----------------|----------------|
| T4 | 57 | 5.2 | ↑ | ↓ |
| | 54 | 5.1 | ↓ | ↑ |
| | 50 | 5.15 | ↓ | ↑ |
| | 29 | 5.7 | ↓ | ↑ |
| 98141 | 29 | 5.15 | ↑ | ↓ |
| | 31 | 5.57 | ↑ | ↓ |
| | 50 | 5.3 | ↑ | ↓ |
| | 19 | 5.9 | ↓ | ↑ |
| | 22 | 5.5 | ↓ | ↑ |
| | 72 | 5.0 | ↓ | ↑ |
| Hh | 47 | 5.0 | ↑ | ↓ |
| | 42 | 5.85 | ↑ | ↓ |
| | 28 | 5.6 | ↑ | ↓ |
| | 35 | 5.0 | ↓ | ↑ |
| | 31 | 4.9 | ↓ | ↑ |
| | 16 | 5.7 | ↓ | ↑ |
| Vds | 40 | 6.0 | ↑ | ↓ |
| | 69 | 5.75 | ↓ | ↑ |
| | 38 | 5.86 | ↓ | ↑ |
| | 35 | 5.78 | ↓ | ↑ |
| Catla | 83 | 5.0 | ↑ | ↓ |
| | 64 | 5.12 | ↓ | ↑ |
| | 64 | 5.29 | ↓ | ↑ |
| | 26 | 6.86 | ↓ | ↑ |
| C24li | 75 | 5.45 | ↑ | ↓ |
| | 69 | 5.01 | ↑ | ↓ |
| | 41 | 5.20 | ↑ | ↓ |
| | 50 | 5.49 | ↓ | ↑ |
| | 41 | 5.71 | ↓ | ↑ |
| | 30 | 6.37 | ↓ | ↑ |

↑ up and ↓ down-regulated spots.

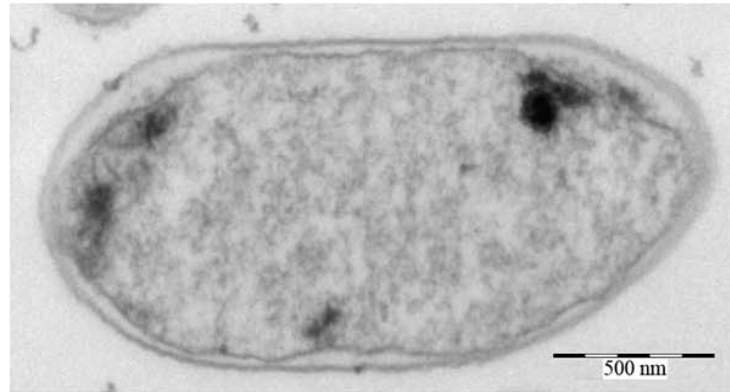
Table 2.7: MW and pI of the most up and down-regulated spots in 2D SDS-PAGE of *A. hydrophila* grown within the 25 and 100 kDa MW cut off tubes *in vivo* (up-regulated *in vivo* compared to *in vitro*)

| <i>A. hydrophila</i> isolates | MW | pI | MW of implants | |
|-------------------------------|----|------|----------------|---------|
| | | | 25 kDa | 100 kDa |
| T4 | 33 | 4.89 | → | ← |
| | 43 | 5.50 | ← | → |
| | 18 | 5.29 | ← | → |
| | 18 | 5.49 | ← | → |
| 98141 | 64 | 5.33 | → | ← |
| | 58 | 5.46 | → | ← |
| | 49 | 5.12 | ← | → |
| | 47 | 5.25 | ← | → |
| Hh | 55 | 4.89 | → | ← |
| | 55 | 5.33 | → | ← |
| | 46 | 5.60 | ← | → |
| | 21 | 4.95 | ← | → |
| | 16 | 5.60 | ← | → |
| Vds | 76 | 5.55 | ← | → |
| | 71 | 5.59 | ← | → |
| | 30 | 5.73 | ← | → |
| Catla | 26 | 5.75 | → | ← |
| | 26 | 6.74 | ← | → |
| | 26 | 6.16 | ← | → |
| | 20 | 4.90 | ← | → |
| C24li | 70 | 5.08 | → | ← |
| | 49 | 5.89 | → | ← |
| | 28 | 5.55 | → | ← |
| | 66 | 5.70 | ← | → |

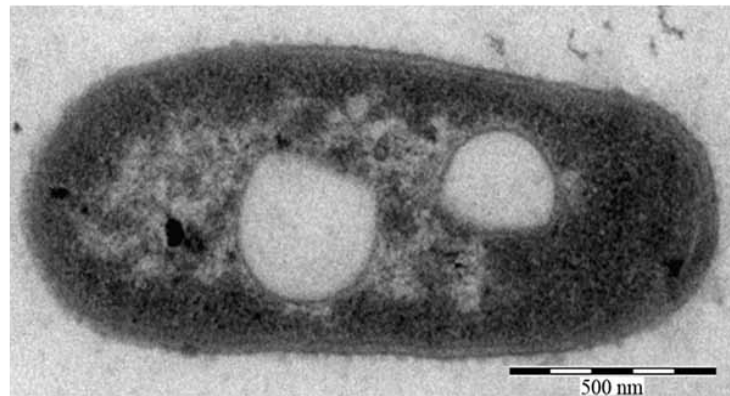
→ up and ← down-regulated.

2.3.3.4. TEM studies of *A. hydrophila* cultured *in vitro* and *in vivo*

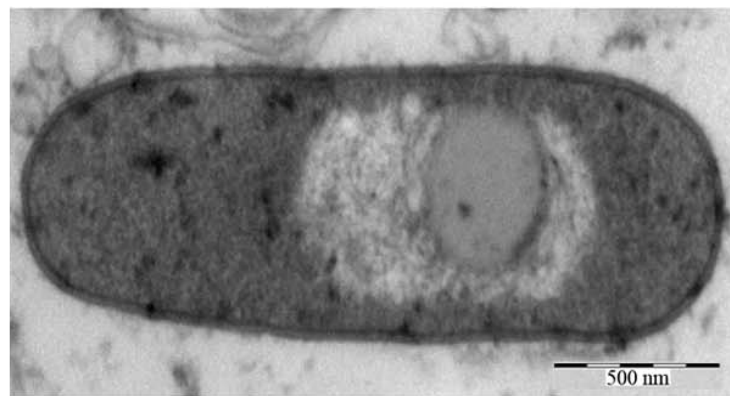
A difference was observed in the morphology of the bacteria grown *in vitro* and *in vivo* (Figure 2.7). A distinct gap was seen between the inner and outer membrane layers of *A. hydrophila* cultured *in vitro*. Whereas, in case of bacteria grown *in vivo*, the presence of an extracellular capsular layer was evident and the membranes were more compact compared with the bacteria grown *in vitro*. However, the thickness of the layer did not appear to vary between bacteria grown *in vitro* and *in vivo* and between the bacteria grown in the two different tubes *in vivo*, and in most cases the thickness of the outer membrane layer was around 13-25 nm.



A



B



C

Figure 2.7: Transmission electron micrograph of *A. hydrophila* cultured *in vitro* and *in vivo*

(A) Bacteria grown *in vitro*, (B) Bacteria grown *in vivo* with 25 kDa cut off tube, (C) Bacteria grown *in vivo* with 100 kDa cut off tube.

2.4. Discussion

Lallier and Higgins (1988) showed all 65 *A. hydrophila* strains in their study isolated from either diseased mammals or healthy and diseased fish, to be positive for O-nitrophenyl- β -D-galactopyranoside, arginine dehydrolyase, glucose, mannitol and saccharose. They also reported that all these isolates were negative for ornithine decarboxylase, H₂S, urease and inositol, and other biochemical characteristics were variable between the isolates. The biochemical results obtained with the API 20E microbial identification kit in this study were in agreement with the results of Lallier and Higgins (1988). However, minor differences were observed between the NCIMB reference strain and isolate T4, and with isolate T4 when it had been passaged once and twice through a goldfish. For example, isolate T4 was positive for H₂S before and after passaging once through a fish, but negative after passaging it a second time through fish. De Figueiredo and Plumb (1977) also observed variations in the biochemistry between the different isolates of *A. hydrophila* they screened, but they found these differences to be insufficient to separate the isolates into virulent and avirulent groups.

Several studies are reported in the literature relating to artificial challenge with various doses of *A. hydrophila* using different isolates in a range of fish species. The clinical signs which occurred were all similar at the time of mortality, although the time it took for these to appear varied depending on the concentration of *A. hydrophila* used. For example, when catfish (*Clarias batrachus*) were injected with 1×10^7 ml⁻¹ *A. hydrophila* clinical signs became apparent by three days post-injection, with petechiae and reddening of the abdomen, and similar clinical signs

were apparent by Day four post-injection in groups of fish injected with 10^3 , 10^4 , 10^5 and 10^6 ml⁻¹ bacteria by Angka (1990). Similar clinical signs were reported by Azad *et al.* (2001) who observed melanisation followed by reddening and swelling at the site of injection in tilapia, *Oreochromis mossambicus* 1 h post-injection, when *A. hydrophila* was administered intramuscularly using 10^7 and 10^8 CFU fish⁻¹. The authors found dermal necrosis and muscular degeneration at 12 h post-injection, followed by ulceration at Day 2 post-injection. In the present study, in which common carp were experimentally challenged with 14 different isolates of *A. hydrophila*, the same clinical signs were observed in fish injected with 1×10^7 ml⁻¹ the day following injection, and three days post-injection in fish injected with 1×10^3 and 1×10^5 ml⁻¹. Very clear symptoms of infection were seen externally, including reddening around the body, erosion of the tail and fins, and swelling and ulceration of muscles.

Mortality occurred from the first day post-injection until the ninth day in the present study. Six out of the fourteen isolates tested were pathogenic, and mortality was greatest in fish injected with 1×10^8 ml⁻¹ CFU. Santos *et al.* (1988), on the other hand, demonstrated that out of the 56 isolates of *A. hydrophila* they tested, 36 (64.3 %) were pathogenic to fingerling rainbow trout, *Oncorhynchus mykiss* using a lethal dose, *i.e.* 50 % (LD₅₀) between 1×10^4 and 1×10^7 bacteria ml⁻¹. *Aeromonas hydrophila*, injected into European eel, *Anuguilla anuguilla* by IP injection, were 100 % pathogenic at LD₅₀ doses of between $10^{5.4}$ and $10^{7.5}$ CFU fish⁻¹ depending on the isolates (Esteve *et al.*, 1993). Mortalities began at 18 h post-challenge, and 'red fin disease' was evident in elvers during the experiment.

Similarly, mortalities were seen on Day 1 post-injection in the group injected with 10^8 CFU fish⁻¹, and continued up to 8 days post-injection (Azad *et al.*, 2001).

The variation in the mortality caused by different *A. hydrophila* isolates might be due differences in the ECP composition of isolates used for infection. Sirirat *et al.* (1999) found 100 % mortalities in hybrid catfish, (*Clarias gariepinus* × *C. macrocephalus*) injected with ECP from highly virulent isolates of *A. hydrophila* within 18 h post-injection compared with the same mortality in fish injected with ECP from low virulent isolates within 96 h post-injection. Environmental factors, such as temperature, have been shown to affect the expression of different toxic components of the ECP, such as haemolysin, cytotoxin and enterotoxin of *A. hydrophila*, and the impact of temperature on ECP expression appears to be strain dependent (Gonzalez-Serrano *et al.*, 2002).

Researchers have also examined a number of other factors, which could possibly influence the infections caused by *A. hydrophila* in the fish. It was found, for example, that viable but non-culturable *A. hydrophila* (incubated in 0.35 % NaCl solution for 50 days) and bacteria cultured for a long time in nutrient broth (*i.e.* 28 days) greatly decreased in virulence compared with 1-day-cultured *A. hydrophila* when injected into goldfish (Rahman *et al.*, 2001b). In addition, the virulence of *A. hydrophila* in goldfish was significantly greater at water temperatures of 17°C and 25°C than that of 10°C and 32°C (Rahman *et al.*, 2001a). The LD₅₀ of bacteria delivered by subcutaneous injection in fish held at different temperatures were $10^{7.06}$ CFU fish⁻¹ at 10°C, $10^{6.03}$ CFU fish⁻¹ at 17°C, $10^{6.53}$ CFU fish⁻¹ at 25°C and $10^{7.28}$ CFU fish⁻¹ at 32°C.

Pai *et al.* (1995) found that abraded common carp exposed to *A. hydrophila* experienced no mortality at low stocking densities, but at higher stocking densities, mortalities occurred, and these increased with stocking density. Fish without abrasions that were exposed to this pathogen showed no mortality, even at highest stocking density, and thereby suggested that crowding alone was not sufficient to make the fingerlings susceptible to *A. hydrophila* (Pai *et al.*, 1995). A wide variation in mortality (0 to 100 %) among different sub species of rohu, *Labeo rohita* to *A. hydrophila* was reported and this was attributed to differential bactericidal activity in the fish (Sahoo *et al.*, 2004).

It is well known that most bacterial pathogens are able to change their proteins and ECP expression under different environmental conditions, and can modulate the expression of multiple virulence factors (Griffiths, 1989; Vivas *et al.*, 2005). Key factors such as temperature, nutrients, pH, salt, dissolved oxygen, osmolarity (Khalil and Mansour, 1997; Tsai *et al.*, 1997; Aguilar *et al.*, 1997; Rahman *et al.*, 2001a) and modified N₂, O₂ and CO₂ atmospheres (McMahon, 2000) influence bacteria growth and expression of their proteins and virulence factors. The following authors studied differences between bacteria grown *in vitro* and *in vivo* in attempt to identify virulence factors. Colquhoun and Sorum (1998) cultured *V. salmonicida* in dialysis tubing implanted into rainbow trout, while Jung (1999) and Bakopoulos *et al.* (2004) cultured *P. damselae* subsp. *piscicida* inside dialysis tubing implanted into the peritoneal cavity of sea bass, *Dicentrarchus labrax*. This method has also been used in other animals. For example, Morck *et al.* (1991) cultured *Pasteurella haemolytica* within a chamber implanted in the peritoneal cavity of a rabbit, while Davies *et al.* (1994) implanted *P. haemolytica* in chambers

into the peritoneal cavities of cattle. Similarly, mice were implanted with dialysis tubing filled with *Yersinia pestis* (Ferreira *et al.*, 1998) and *A. hydrophila* (Sha *et al.*, 2003).

In this study, the concentration of the different *A. hydrophila* suspensions recovered from the 25 and the 100 kDa MW cut off tubing implanted into the peritoneal cavity of common carp increased approximately 9 to 14 fold compared with the concentration used for implantation. Similarly, a 6 to 8 fold increase was reported in *P. damsela* subsp. *piscicida*, when implanted into seabass, irrespective of the pore size of the bags used (2 and 12 kDa MW cut off) (Bakopoulos *et al.*, 2004).

The current study identified differences in the protein profiles between *A. hydrophila* grown *in vitro* and *in vivo*. Approximately, 15-25 bands were observed with all the fourteen *A. hydrophila* isolates cultured *in vivo* as well as 5-10 unique bands with individual isolates. The number of bands expressed between 90 and 212 kDa was greater with *A. hydrophila* cultured *in vitro* than *in vivo*. In contrast, the number of bands expressed below 90 kDa increased with bacteria grown *in vivo* compared with *in vitro*. A unique band at 58 kDa was observed with all the isolates grown *in vivo*, however common bands were seen at around 25, 50 and 70 kDa between all the isolates of *A. hydrophila* grown *in vitro* and *in vivo*. The expression of total proteins has been found to be variable for *A. hydrophila*. Kokka *et al.* (1990) reported at least 30 resolvable proteins using Coomassie blue staining of WC protein profiles for eight *A. hydrophila* isolates (five serogroup 0:11 and three serogroup 0:22) and two isolates of *A. sorbia* (one serogroup 0:36 and

one rough strain) cultured *in vitro*. However, among five isolates of serogroup O:11, a predominant protein in the MW range of 52 kDa to 54 kDa was consistently observed on 10 % SDS-PAGE gels. Variations in the protein expression between bacteria grown in two different MW cut off tubing has also been reported for other bacteria. For example, a band at approximately 52 kDa was seen in *P. damsela* subsp. *piscicida* grown in a 300 kDa dialysis bag in seabass, while a band at 45 kDa was associated with bacteria grown in 25 kDa dialysis bag (Jung, 1999). Similar differences also found in the current study, as a faint band at 40 kDa observed in bacteria grown within the 100 kDa MW cut off tube was not expressed in bacteria grown in the 25 kDa MW cut off tube.

Different environmental conditions used for culturing *A. hydrophila*, mainly iron limited conditions (Aoki and Holland, 1985), and temperature (Merino *et al.*, 1992; Rahman *et al.*, 2001a) have been reported to influence the OMP profiles obtained. The 14 isolates of *A. hydrophila* examined in this study had 4 to 6 bands in their OMP, which varied considerably from strain to strain. A major band was seen at approximately 50 kDa in 11 of the 14 isolates. The OMP profiles of bacteria grown *in vitro* contained more bands than bacteria grown *in vivo*, as reported by Colquhoun and Sorum (1998) for *V. salmonicida*, and by Rahman and Kawai (2000) for *A. hydrophila* (where bacteria had been recovered from goldfish artificially infected with *A. hydrophila* by IP injection). Although the numbers of bands were not the same between bacteria grown *in vitro* and *in vivo*, some bands with the same molecular weight were found between the two sets of bacteria. Some of the weakly expressed bands in the OMP profiles of bacteria grown *in vitro* did not appear *in vivo*, as seen with *V. salmonicida* (Colquhoun and Sorum,

1998), *V. cholerae* (Jonson *et al.*, 1989) and *Yersinia enterocolitica* (Carniel *et al.*, 1987).

In another study, the OMP profiles of *A. hydrophila* from different origins contained 5 or 6 major proteins in each strain, but the profiles were different for each strain, although most of these strains had a major band at 36 kDa (Aoki and Holland, 1985). They found that the heterogeneity of OMPs of different *A. hydrophila* isolates was greater than that of the heterogeneity of various strains of *A. salmonicida*. The OMP prepared from a group of virulent *A. hydrophila* grown *in vitro* produced very similar electrophoretic profiles on SDS-PAGE, and major proteins were observed at 30 kDa and 45 to 55 kDa (Dooley and Trust, 1988). When partially purified OMPs were analysed, 2 out of 5 of *A. hydrophila* 0:11 serogroups grown *in vitro* had additional major proteins at 18 and 20 kDa, which had not been observed in the analysis of WC preparations of the bacteria (Kokka *et al.*, 1990). The OMP profiles of *A. hydrophila*, *A. sobria* and *A. caviae* cultured *in vitro* showed a common band at 52 kDa on an 11 % discontinuous SDS-PAGE (Maruvada *et al.*, 1992). The difference in the OMP expression of bacteria has been shown to be affected by number of factors, such as temperature, incubation period and nutrients. For example, OMP profiles of different *A. hydrophila* serotype 0:34 strains grown at 20°C had a decrease in a 41 kDa band and an increase in a 24 kDa band compared with bacteria grown at 37°C, although no other major differences were observed (Merino *et al.*, 1992). Rocha-de-Souza *et al.* (2001) observed difference in OMP profiles of *A. caviae* grown at 22°C and 37°C for 48 h and 24 h respectively. Growth of 18 isolates of *A. salmonicida* under iron-restricted

conditions showed four novel OMPs of apparent MW of 70, 72, 77, and 82kDa, which were not present in the iron-replete conditions (Hirst and Ellis, 1994).

The differences seen in the expression of ECP between bacteria grown *in vitro* and *in vivo* were extensive. It has already been reported that *A. hydrophila* is able to change its ECP expression when grown in different culture medium or at different temperatures (Merino *et al.*, 1992; Tsai *et al.*, 1997; Vivas *et al.*, 2004a). Differential expression of ECP has been reported for *P. damsela* subsp. *piscicida* when it was grown *in vitro* and *in vivo* (Bakopoulos *et al.*, 2004). It was also reported that growth of the pathogen *in vivo* induced the synthesis of more toxic ECPs in comparison with growth *in vitro*. In this study, the expression of the ECP was greater from bacteria cultured *in vivo* rather than *in vitro*, and this could be due to the bacteria having to obtain nutrients in the *in vivo* environment, where as they are already available in the *in vitro* environment. Therefore this increase in the ECPs of bacteria grown *in vivo* might act as an additional virulence factors in fish. Although, the variation in the expression of the ECP was greater from bacteria cultured *in vivo* compared with *in vitro*, there was little difference in the bands between samples recovered from the different MW cut off dialysis tubes *in vivo*, as also seen for *P. damsela* subsp. *piscicida* by Jung (1999). This may reflect the availability of similar nutrients and environmental conditions within both MW cut off dialysis tubes.

In the work by Imbert and Gancel (2004), where WC preparations were analysed by 2D SDS-PAGE, the lower the temperature used to culture *A. hydrophila* the greater the number of new proteins seen (from at least 22 proteins at 20 °C to 30

at 5°C), and some proteins were over expressed when the bacterium was subjected to cold shocks after culturing at 30°C. They also found that most of the house keeping proteins was consistently expressed at 30°C and 5°C. Approximately 54 protein spots were detected in Commassie blue stained gels of *A. hydrophila* OMP in the work by Chen *et al.* (2004), and most of the spots were in the pI 5.0-8.0 range and had molecular masses from 10-140 kDa.

Ebanks *et al.* (2004) found a 49 kDa VapA protein (S-layer protein) which is visible as multiple isoelectropherotypes in 2D SDS-PAGE profiles of OMP from *A. salmonicida* grown both in implants in abdominal cavity of the Atlantic salmon, *Salmo salar* and under iron-replete conditions *in vitro*. A similar type of expression of spots around 45 to 50 kDa range was also seen in *A. hydrophila* grown in both *in vitro* and *in vivo* in this study. Ebanks *et al.* (2004) also found proteins at 73, 76 and 85 kDa by 2D SDS-PAGE in the OMPs of the bacterium grown under iron-restriction as well as grown *in vivo*. However, these proteins were not present when the bacterium was grown under iron-replete conditions *in vitro*. They suggested that iron-restricted growth *in vitro* is a model to mimic *in vivo* growth conditions as it largely reproduces the same proteins obtained from *A. salmonicida* grown in implants within the peritoneal cavity of salmon, at least with respect to the OMPs. In the present study, different *A. hydrophila* isolates grown *in vivo* appeared to have unique spots mostly between 30 and 80 kDa compared with bacteria cultured *in vitro*. Moreover, 2D SDS-PAGE analysis showed further differences in the number of up and down regulated spots in bacteria grown *in vitro* or *in vivo* compared with 1D SDS-PAGE analysis. The number of bands and spots expressed by all the isolates in 1 and 2D SDS-PAGE respectively, was less

in bacteria grown *in vivo* compared to *in vitro*. However, the intensity of the spots was up regulated in bacteria grown *in vivo* compared to *in vitro*, and the diversity of the expression of the spots has found to be only 20-25 %.

Aeromonas salmonicida grown in media containing yeast extract, peptone, glucose and mineral salts, and another media comprising fish viscera produced a capsular polysaccharide that was not produced when the bacteria were grown on TSA (Merino *et al.*, 1996b). Capsule formation was also evidenced in glucose rich medium and in an autolysate of fish viscera for *A. hydrophila* serogroup 0:34 strains (Martinez *et al.*, 1995; Aguilar *et al.*, 1999). An extracellular capsular layer, however, was not seen either in *V. salmonicida* grown in implants in the peritoneal cavity of the rainbow trout or bacteria isolated from peritoneal fluid of the rainbow trout or bacteria grown *in vitro* (Colquhoun and Sorum, 1998). Repeated sub-culturing of certain capsular *Pasteurella multocida* serotype A strains in brain heart infusion agar resulted in the capsulated bacterium becoming non-capsulated, with an associated loss of virulence (Watt *et al.*, 2003). Wang *et al.* (2004), on the other hand, found novel capsular polysaccharide and lipopolysaccharide O-chain polysaccharide from *A. salmonicida* grown *in vivo* compared with the bacteria grown *in vitro*. In this study, the capsule was apparent in bacteria grown *in vivo*. It has been suggested that it has an important role in protecting the bacterium from the host immune system as it can confer serum resistance (Aguilar *et al.*, 1999).

The differences found in the protein profile analysis during this study suggest that the growth conditions experienced by the bacterium, especially the availability of nutrients, may play a major role in the up and down-regulation of proteins in *A.*

hydrophila. A number of new proteins and some others with increased expression were seen in bacteria grown *in vivo* and these might be involved in the virulence of the bacterium. Such proteins could be mostly surface proteins, which could be more easily recognised by the host immune system, and therefore these proteins may represent potential vaccine candidates.

In the current study, the difference in the expression of OMP profile bands between bacteria grown *in vitro* and *in vivo* suggests that different growth conditions can modify the OMP profiles of *A. hydrophila*, as reported previously by Rahman and Kawai (2000). Overall, the protein expression in WC and OMP preparations of *A. hydrophila* grown *in vivo* was reduced compared with bacteria grown *in vitro*, however, proteins between 45 and 50 kDa were observed for both *in vitro* and *in vivo* cultured bacteria. In contrast, more bands were expressed in the ECP for bacteria grown *in vivo* compared with *in vitro*.

Collectively, the results of the current study show the difference in the protein expression between the bacteria grown *in vivo* and *in vitro*. The immunogenicity of these proteins in fish was then determined (Chapter 3) to identify potential antigens for the development of an *A. hydrophila* vaccine.

Chapter 3. Identification of potential vaccine antigens on *A. hydrophila*

3.1. Introduction

Control of diseases caused by *A. hydrophila* can be difficult because of the existence of antibiotic resistance and the heterogeneity between strains of this bacterium (Loghothetis and Austin, 1996a; Janda *et al.*, 1996; Daskalov, 2006). It is important to minimise the use of anti-bacterial drugs in fish rearing not only because of antibiotic resistance, but also due to the risk of drug residues in fish and contamination of the aquatic environment (Rijkers *et al.*, 1980). Suppression of the host immune system can also occur following improper use of antibiotics and chemicals (Heppell and Davis, 2000; Hu *et al.*, 2005). It has been suggested that the use of vaccines in intensive fish culture systems is much more desirable for controlling fish diseases than using antibiotics and other therapeutic agents (Ellis, 1989; Leong, 1993; Samuel *et al.*, 1996; Lin *et al.*, 1996; Adams and Thompson, 2006), and there has been an increase in the use of vaccines since the late 1970s (Munn, 1994).

Most commercially available vaccines are bacterins or formalin-inactivated whole cell suspensions, for example *Aeromonas salmonicida*, *Vibrio anguillarum*, *V. ordalii*, *V. salmonicida*, and *Yersinia ruckeri* (Newman, 1993; Munn, 1994; Gudding *et al.*, 1999). However, as technologies have progressed and whole cell (WC) vaccines have failed to be developed for some pathogens, researchers have investigated the potential of different protective components (subunits) of pathogens as vaccines (Potter and Babiuk, 2001). These subunit vaccines contain only the important components required to induce immunity in the host, and they have no ability to infect the host or to replicate in the aquatic environment (Clark and Cassidy-Hanley, 2005).

The development of a successful vaccine requires antigen identification, analysing the efficacy of the protection and finally cost-effective vaccine production (Khushiramani *et al.*, 2007a; Chen *et al.*, 2004; Thompson and Adams, 2004). The identification of protective antigens has posed a great hurdle in the development of an *A. hydrophila* vaccine due to the diversity of the different strains in this bacterium, and at present, no vaccine against this pathogen is commercially available (Shotts and Rimler, 1973; McCoy and Pilcher, 1974; Fang *et al.*, 2004; Tatner, 1993). The problem of *A. hydrophila* heterogeneity could be overcome if common antigens between strains, which induce protection, could be identified (Stevenson, 1988; Fang *et al.*, 2004). Thus, it has been suggested that a component of *A. hydrophila*, which is immunogenic and will cross-protect against all isolates could be a possible vaccine candidate against this pathogen rather than using live, attenuated or inactivated bacterial WC vaccines (Dooley *et al.*, 1988; Leung *et al.*, 1997; Rahman and Kawai, 2000). Some of the surface proteins of *A. hydrophila* are thought to be important in protection against this pathogen (Karunasagar and Karunasagar, 1996), mainly O-antigens (Dooley *et al.*, 1985), lipopolysaccharide (LPS) (Loghothetis and Austin, 1994), S-layers (Leung *et al.*, 1997) and outer membrane proteins (Rahman and Kawai, 2000).

The ability to produce a specific immune response, including cellular and humoral responses, is characteristic of the vertebrate immune system and has been demonstrated in numerous fish species (Pilstrom *et al.*, 2005; Lund *et al.*, 2006). Production of specific antibodies against bacterial pathogens is dependent on the characteristics of the pathogen as well as the immune system of the host (Lund *et al.*, 1991 and 2006; Stromsheim *et al.*, 1994). These antibodies could aid in the

identification of antigens for the production of vaccines (Hirst and Ellis, 1994; Ellis, 1999; LaFrentz *et al.*, 2003). Immunological methods such as enzyme linked immunosorbent assay (ELISA) and Western blot are useful for identifying immunogenic antigens of a bacterium in the sera of fish which have survived an infection by the same bacterium (Merino *et al.*, 1993; Sendra *et al.*, 1997). Although it could possible to identify various immunogenic antigens for a bacterium, a particular immunogenic antigen needs to be protective against a wide range of strains in order to be an effective vaccine candidate (Hirst and Ellis, 1994; Makela, 2000). The protection of the target antigen for vaccine development can be evaluated by vaccination as well as passive immunisation of fish and subsequently challenging them with live pathogen (Azad *et al.*, 1999; Irie *et al.*, 2005).

The main aim of this Chapter was to identify potential vaccine antigens for *A. hydrophila*. Common carp (*Cyprinus carpio*) were infected with 6 isolates of *A. hydrophila* and sera collected from these fish. The titre values of antibodies elicited were measured by ELISA, and the antibodies were screened against WC, outer membrane protein (OMP) and extracellular product (ECP) preparations of 14 different isolates of *A. hydrophila*, by Western blot to identify any common antigens. The protective immunity elicited by a common antigen (50 kilo Dalton (kDa) protein) of *A. hydrophila* was then evaluated by vaccination and passive immunisation. This protein was electro-eluted from a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel containing proteins of *A. hydrophila* T4 isolate, and this was subsequently used to immunise fish. Levels of

protection to this protein were assessed by artificially challenging the fish with *A. hydrophila*.

3.2. Materials and methods

3.2.1. Antibody response of common carp to *A. hydrophila*

Four virulent strains; T4, 98141, Hh, Vds and two avirulent strains; Catla and C24li of *A. hydrophila* (described in Section 2.3.2) were used to determine the antibody response of common carp to *A. hydrophila*. Common carp (average weight 30 g) were obtained from Research Institute for Fisheries, Aquaculture and Irrigation, Hungary (HAKI) freshwater aquarium and maintained in 6 separate glass tanks. The fish were anesthetized with benzocaine, as described in Section 2.2.2, and injected intraperitoneally (IP) with 0.1 ml PBS containing 1×10^6 viable *A. hydrophila*. Each strain was injected into 24 fish and an additional 24 fish were injected with phosphate buffered saline (PBS) to serve as controls. After injection, the fish were placed back into a glass tank aquarium supplied with recirculating water that had been passed through a sedimentation tank, drum filter, biofilter and ultraviolet (UV) radiation. The temperature of the tank water was maintained at $20 \pm 1^\circ\text{C}$ throughout the experiment. The fish were observed regularly for any changes in behaviour. Blood samples were taken 3, 9, 12 and 21 days post-injection. Fish were scarified with an overdose of anesthetic before taking the blood samples. Pre-injection bleeds were also taken from six fish just prior to injection. The blood was collected and stored overnight at 4°C and the serum collected the next day by centrifuging at $2000 \times g$ for 5 min. The serum was stored at -20°C until further analysis.

3.2.2. Polyclonal antibody production against *A. hydrophila* in rabbits

Strain T4 was used to raise polyclonal rabbit serum against *A. hydrophila*. The bacterium was grown in tryptone soy broth (TSB) overnight and harvested, as described in Section 2.2.1.4. The cells were adjusted to a concentration of 1×10^8 ml⁻¹ in PBS and were heat-killed by incubating in a water bath at 60°C for 1 h. The effectiveness of heat killing was determined by looking for growth on tryptone soy agar (TSA) (Schneider and Rheinheimer, 1988). After confirmation that there were no live bacteria in the suspension, it was emulsified with Hunter's Titermax adjuvant (Cytrx, Georgia, USA) in equal volume. About 1ml of blood (pre-immune) was collected from the ear vein of a female New Zealand White rabbit (Charles River UK Ltd.), and then the rabbit was injected with 0.4 ml of the bacterial suspension subcutaneously into four sites using a 21g needle. Booster injections were given once a month for a three month period. Test bleeds were collected 2 weeks after each injection and stored overnight at 4°C. The blood was centrifuged at $2000 \times g$ for 5 min and the serum collected and stored at -20°C for analysis. The antibody titre of the serum and the antigens recognised by the serum were analysed by ELISA and Western blot, respectively, for each serum sample. The fourth and final immunisation was carried out with the same amount of the antigen diluted in PBS and administered by an intravenous injection into the ear vein. The animal was bled after 10 days by cardiac puncture after anaesthesia with pentobarbitonesodium (Sagatal) 60mg ml⁻¹ using a dose of 0.5 ml kg⁻¹ bodyweight. Serum was prepared as described above and stored at -20 °C for further analysis.

3.2.3. Enzyme linked immunosorbent assay

An indirect ELISA was used to measure the antibody titre in serum samples from both fish and rabbit according to Adams and Thompson (1990). A 96 well micro titre ELISA plate (Immulon™, Dynatech) was coated with 0.05 % (w/v) poly-L-lysine in coating buffer (Appendix I), 50 µl well⁻¹ for 60 min at 20-22°C. The plate was washed twice with low salt wash buffer (LSWB) (Appendix 1), and a suspension of *A. hydrophila* (corresponding isolates used for raising serum) with a concentration of 1 × 10⁸ ml⁻¹ was added to the plate at 100 µl well⁻¹. The plate was incubated overnight at 4°C, after which the bacterial cells were fixed to the bottom of the well using 50 µl well⁻¹ of 0.05 % (v/v) glutaraldehyde in PBS incubating for 20 min at 20-22°C. The plate was washed three times with LSBW. The plate was coated with 250 µl well⁻¹ 3 % (w/v) of casein (skimmed milk) (Marvel, UK) in H₂O, and incubated for 2 h at 20-22°C. The plate was washed three times with LSBW. The remainder of the procedure was slightly different depending on whether fish or rabbit serum was being used.

For the fish serum, two fold dilutions of the serum from 1/8-1/512 were made in antibody buffer (Appendix 1) and 100 µl well⁻¹ was added to the ELISA plate. The serum from the control fish was added to the last row as a negative control. The plate was incubated overnight at 4°C, after which the plate was washed 5 times with high salt wash buffer (HSWB) (Appendix 1), incubating for 5 min on last wash. Anti-common carp and crussian carp (*Carassius auratus*) monoclonal antibodies (MAbs, Aquatic Diagnostics Ltd., Stirling, UK) were used to detect common carp and goldfish (*C. auratus*) IgM, respectively following the manufacture's instructions. The plate was incubated for 2 h, and then washed with HSWB as

described above. Goat anti-mouse IgG-HRP (Sigma, Missouri, USA) diluted 1/1000 in conjugate buffer (Appendix I) was added to the plate at 100 $\mu\text{l well}^{-1}$, and incubated for 1 h at 20-22°C. The plate was again washed with HSWB as described above and 100 $\mu\text{l well}^{-1}$ chromogen in substrate buffer (Appendix I) was added to the wells. This was incubated for 10 min at 20-22°C and the reaction stopped with 50 μl of 2 M H_2SO_4 well⁻¹. Finally, the plate was read in an ELISA plate reader (Dynex technologies, UK) and the results were compared between control and sample wells. Values three times higher than the negative control value were considered positive. The positive values at the lowest dilution were considered as a titre value for the serum examined.

For the rabbit serum samples, 100 $\mu\text{L well}^{-1}$ of serum was added at 10 fold dilutions (1×10^{-1} to 1×10^8) made in antibody buffer. Then PBS was added to the last row at 100 $\mu\text{l well}^{-1}$ as a negative control. The plate was incubated for 1h at 20-22°C, before washing with 5 washes of HSWB, incubating for 5 min on the last wash. Goat anti-rabbit immunoglobulin conjugated to Horseradish Peroxidase (IgG-HRP) (Sigma, Missouri, USA) diluted 1/1000 in conjugate buffer was then added at 100 $\mu\text{l well}^{-1}$ and incubated for 1h at 20-22°C. The plate was washed again with HSWB as described above. The rest of the procedure was the same as that used for the fish serum above.

3.2.4. Western blot

Western blot analysis was performed using rabbit and fish anti-sera to identify which *A. hydrophila* antigens were recognised by the antibodies, using the method outlined by Wiens *et al.* (1990) with modifications. All the corresponding SDS-

PAGE gels for the Western blots reported in this Chapter can be found in Chapter 2.

3.2.4.1. Western blot using common carp antibody

Western blot analysis was carried out for all 14 isolates of *A. hydrophila* (described in Table 2.1) grown *in vitro* using pooled common carp serum raised against six isolates of *A. hydrophila* strains (T4, 98141, Hh, Vds, Catla and C24li). Different preparations of the bacterium (*i.e.* WC, OMP and ECP) prepared from these 6 isolates of *A. hydrophila* grown either *in vitro* or *in vivo* were screened in the Western blot with each of the 6 anti-sera raised against different isolates of *A. hydrophila*.

The bacterial preparations above described were run on a 12 % SDS-PAGE gel, as described in the Section, 2.2.4.1 using rainbow molecular weight (MW) marker (Amersham Biosciences, UK) as a reference. The gels were equilibrated in the transblot buffer (Appendix 1) and transferred on to nitrocellulose membranes according to the manufacture's instructions. The transfer was made using 60 V for 1 h. The nitrocellulose membranes were removed from the transfer apparatus and placed in 2 % (w/v) casein for 1 h at 20-22°C to block non-specific binding sites on the membranes. The membranes were washed three times with Tris buffered saline containing 0.1 % (v/v) Tween-20 (TTBS) (Appendix 1) for 5 min on each wash. After washing, the membranes were incubated overnight in the common carp anti-serum diluted 1/10 in Tris buffered saline (TBS) (Appendix 1). Membranes were washed with TTBS as described, before incubating the membranes with an anti-carp IgM monoclonal antibody (Aquatic Diagnostics Ltd,

Stirling, UK) for 2 h. The membranes were again washed with TTBS as described, before incubating with anti-mouse IgG-HRP (Sigma, Missouri, USA) for 1 h. They were then washed three times as described, including a fourth rinse of 1 min with TBS. The blots were developed by adding chromogen and substrate solution (2 ml of 4-chloro-naphthol solution (Appendix I) with 10 mls of PBS and 10 μ l of H₂O₂) and incubating at 20-22°C until bands were observed. The reaction was stopped by soaking the membranes in distilled water for 10 min. The MW of bands was determined using the rainbow molecular weight markers in TotalLab v2002.03 software (Nonlinear Dynamics Ltd).

3.2.4.2. 2D SDS-PAGE Western blot using common carp antibody

The antigenic profile of WC preparation of isolate T4 grown *in vitro* was screened using 2D SDS-PAGE Western blotting with anti-serum (raised for T4 isolate) from common carp. The 2D SDS-PAGE and Western blotting procedures were performed as described in Sections 2.2.4.2 and 3.2.4.1, respectively.

3.2.4.3. Western blot using rabbit antibody

All 14 isolates of *A. hydrophila* described in the Table 2.1 were analysed by Western blotting using the rabbit antibody. The procedure described in Section 3.2.4.1 was followed until blocking non-specific binding sites with casein. The membrane was then washed three times with TTBS for 5 min on each wash. It was placed in the anti-*A. hydrophila* rabbit serum 1/100 dilution in TBS and incubated for 1 h at 20-22°C. It was washed again as described above and then incubated in anti-rabbit IgG-HRP (Sigma, Missouri, USA) using a 1/100 dilution at

20-22°C for 1 h. The remaining of the procedure was the same as outlined in Section 3.2.4.1.

3.2.5. Assessing the levels of protection of a 50 kDa protein in goldfish against *A. hydrophila* challenge

3.2.5.1. Preparation of the antigen

The 50 kDa protein was eluted from 12 % SDS-PAGE profiles of *A. hydrophila* T4 strain. WC preparations of the bacterium were prepared as described in Section 2.2.3.1. An SDS-PAGE gel (16 × 18 cm size) was prepared as outlined in Section 2.2.4.1, and 100 µl of sample was loaded in to each well except the first and the last well, to which were added 20 µl of rainbow MW markers. The gels were run for between 5 and 6 h in a Hoefer SE 600 apparatus at 250 V with 130 mA. On completing the electrophoresis, the first two lanes of the gel were sliced off and stained and destained for 30 min each as described in Section 2.2.4.1. After confirming the location of the 50 kDa band, it was excised from the unstained gel and was finely chopped into small pieces. These were placed in elution tubes (Amersham Bioscience) containing 300 µl of SDS-PAGE reservoir buffer (Appendix I). The tubes were filled with the gel and a blotting paper disk (Amersham Bioscience) followed by a porous polyethylene plug (Amersham Bioscience) placed on top of the gel. The tip of the tube was cut and placed into a 1.5 ml centrifuge tube containing 300 µL of 4x SDS-PAGE reservoir buffer. The tubes were then placed into an electroeluter (Hoefer, San Francisco, USA) and subjected to 50 V at 0.5 mA and a reverse run at 50 V for 5 sec at the end. The eluted protein was collected and the reservoir buffer was removed from the samples by applying a buffer change with PBS using a 10,000 MW cut-off spin

concentrator (Vivo Science, UK) at $2000 \times g$ for 90 min. The concentration of protein was determined as described in Section 2.2.3. SDS-PAGE and Western blot were performed with the eluted protein to confirm the presence of the 50 kDa protein in the elutant prior to using the antigen in the immunisation trials.

3.2.5.2. Determination of the LD₅₀ of *A. hydrophila* T4 isolate in goldfish

Aeromonas hydrophila isolate T4 was randomly chosen for this study from one of the virulent isolates described in the Chapter 2. It was passaged twice through goldfish to check the virulence of the isolate before using it for the challenge. One fish weighing 30 g was injected with 0.1 ml of $1 \times 10^8 \text{ ml}^{-1}$ of bacteria. Three days after injection, the fish was sacrificed overdosing with benzocaine (0.01 % w/v) and the kidney was sampled by streaking a loopful of the organ onto TSA. The next day, colonies of T4 were identified morphologically, bio-chemically and using SDS-PAGE profiles. The isolate was re-cultured in TSB and injected into another goldfish. The next day, the fish was found dead and samples were taken from its kidney and streaked onto TSA. Colonies which grew were identified as *A. hydrophila* as described above, and this isolate was used for challenging both vaccinated and passively immunised fish.

Determination of the LD₅₀ of *A. hydrophila* T4 isolate in goldfish was carried out using three groups of fish with 4 fish per group. One group was injected with 0.1 ml of 1×10^8 bacterium, the second group with 0.1 ml of 1×10^7 bacterium and the third group with 0.1 ml of 5×10^7 bacterium ml^{-1} . The fourth group of fish (2 fish)

were injected with 0.1 ml of 2.5×10^7 bacterium ml^{-1} . All the fish which survived were sacrificed at the end of the experiment and sampled as described above.

3.2.5.3. Immunisation of goldfish with the electro-eluted 50kDa protein

For preliminary vaccination, four goldfish weighing around 30-40g were injected IP with 200 μL of suspension having 12.3 μg of 50 kDa protein in 60 μL of PBS and 140 μL of montanide adjuvant. Another four fish were also injected with PBS to serve as controls. All the fish were challenged with *A. hydrophila* 31 days post-vaccination and sacrificed 21 days after challenge as described above. Samples were taken from their kidneys as previously described. The relative percentage survival (RPS) was calculated using the following formula (Ellis, 1988).

$$\text{RPS} = 1 - \frac{\% \text{ vaccinated mortality}}{\% \text{ control mortality}} \times 100$$

3.2.5.4. Passive immunisation of goldfish with anti-50kDa protein serum raised in goldfish

Two goldfish weighing 30-40 g were injected IP with 200 μL of antigen (*i.e.* 12.3 μg 50 kDa protein) emulsified with Freund's complete adjuvant (FCA). Thirty four days later, both fish were re-vaccinated with the same suspension as described above except Freund's incomplete adjuvant was used in place of FCA. Seventeen days after the booster injection, blood was collected from one fish after sacrificing it as described above, while the other fish died 2 weeks after the booster injection. The anti-serum was collected from the blood as described in the Section 3.2.2 and an ELISA performed before using it for passive immunisation to establish its titre.

Three goldfish weighing between 30-40 g were immunised by IP injection with 0.1 ml of goldfish sera raised against the 50 kDa protein electro-eluted from *A. hydrophila* in Section 3.2.5.3, and 3 fish were injected with control serum collected from non-vaccinated goldfish. After 24 h all the fish were challenged with 0.1 ml of $2.5 \times 10^7 \text{ ml}^{-1}$ *A. hydrophila* T4 isolate in PBS by IP injection, but on the opposite side to the site where they had been injected with the antiserum (LaFrentz *et al.*, 2003). Kidney samples from fish which died during the experiment and surviving fish at Day 21 post-challenge were streaked on TSA to confirm specific mortality.

3.3. Results

3.3.1. Antibody response of common carp infected with different isolates of *A. hydrophila*

3.3.1.1. Antibody response of common carp infected with *A. hydrophila*

The antibody levels increased after Day 9 and a positive response was observed on Day 12 post-infection with all the isolates, except for isolate 98141. By Day 21 post-infection, this response had increased further for 3 of the isolates, with the highest antibody response recorded against isolate T4 followed by isolates C24li and Vds (Figure 3.1). In case of the Catla and Hh isolates, the antibody response began to fall after Day 12, while very little antibody response was observed against isolate 98141.

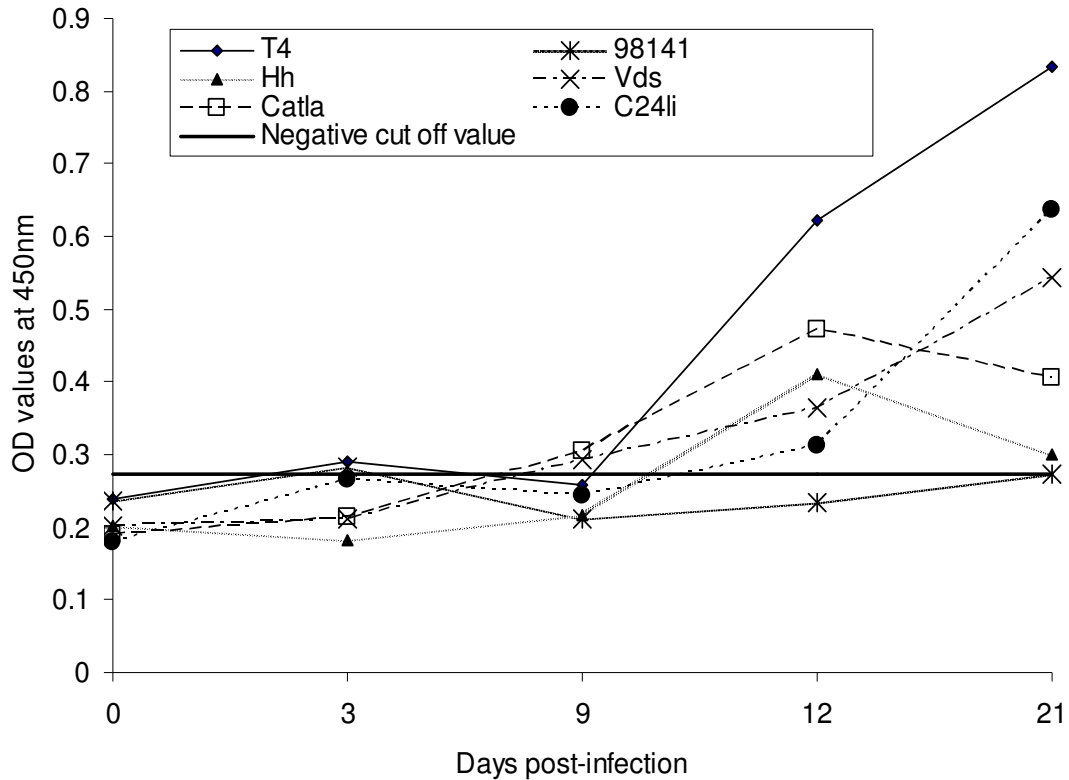


Figure 3.1: Antibody response of common carp against different isolates of *A. hydrophila* determined by ELISA expressed as an absorbance at 450 nm

All sera were diluted 1:512 and ELISA conditions are outlined in section 3.2.3.

3.3.1.2. Western blot analysis of common carp serum

The WC preparations of *A. hydrophila* isolates grown *in vitro*, screened with the anti-sera from infected common carp by Western blotting, exhibited a distribution of bands between 20 and 160 kDa (Figure 3.2). Carp antibodies bound to antigens ranging from 30-50 kDa for 3 of the virulent isolates, T4, Hh and B2/12. One band was observed at approximately 75 kDa except for isolates 2D20, 98140, 98141 and 98139, while another band was located at around 50 kDa except for isolate 2D20. Only one band could be observed with isolate 2D20 at around 33 kDa, whereas the greatest number of bands was observed with the Calf isolate.

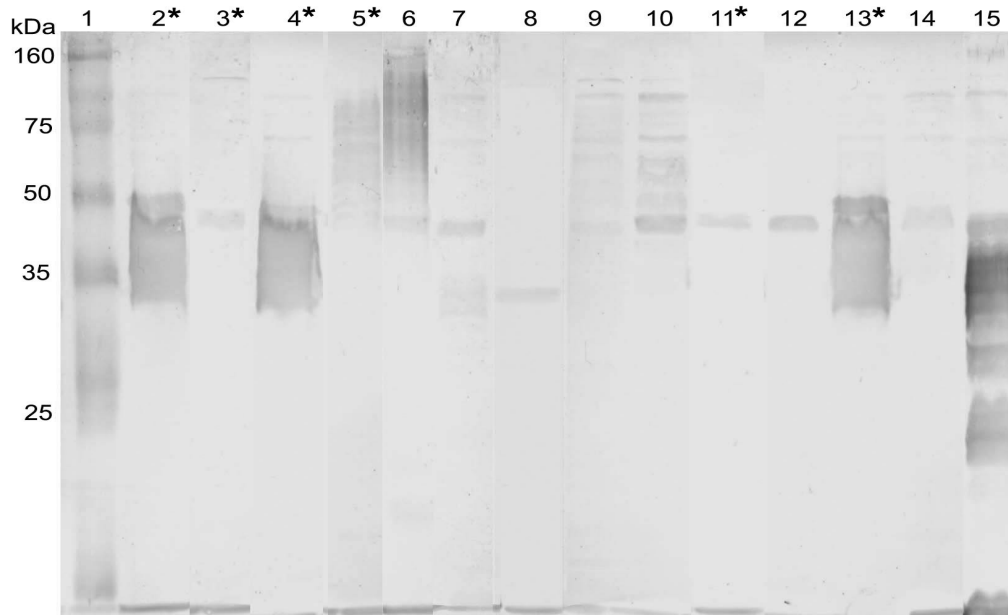


Figure 3.2: Western blot analysis of different whole cell preparations of *A. hydrophila* against pooled serum from common carp infected with 6 different *A. hydrophila* isolates

Lanes: (1) Standard marker; (2) T4; (3) 98141; (4) Hh; (5) Vds; (6) Catla; (7) C24li; (8) 2D20; (9) 3D14; (10) 2N14; (11) 98140; (12) 98139; (13) B2/12; (14) F1d75; (15) Calf.

The corresponding SDS-PAGE gel for this blot is shown in Chapter 2 (Figure 2.4). * Virulent isolates.

The antibody response against WC, OMP and ECP preparations of *A. hydrophila* grown *in vitro* and *in vivo*, showed similar profiles among isolates T4, 98141 and Hh with a little variation between them (Figure 3.3). With all the virulent isolates (T4, Hh, 98141 and Vds), a band was evident at around 50 kDa in WC and OMP preparations with bacteria grown both *in vitro* and *in vivo*. This band was also present in ECP preparations from bacteria cultured *in vitro* but not *in vivo*. The ECP preparations from all the virulent isolates grown in both MW cut off tubes exhibited 1-4 bands between 52 and 100 kDa.

A weakly stained band was also seen at 75 kDa and 32 kDa with the WC and ECP preparations, respectively, with isolate T4 grown *in vitro*. Isolate 98141 exhibited a band at 130 kDa with WC preparation and bands at 35 and 27 kDa with OMP preparations. Two bands at 40 and 20 kDa were seen in the ECP preparations of bacteria grown *in vitro*. Another virulent isolate, Hh, exhibited a band at 43 kDa in ECP preparations of bacteria grown *in vitro*. In the case of isolate Vds, expression of bands were very similar between 30 and 100 kDa with WC preparations from bacteria grown *in vitro* and *in vivo*. The OMP preparations from isolate Vds grown *in vitro* showed 6 bands between 25 and 50 kDa.

No bands were identified in the ECP preparations of the profiles of the avirulent isolates (Catla, C24li) cultured *in vitro*. The WC preparations from both avirulent isolates grown *in vitro* and *in vivo* showed a band at 92 kDa. A band at 50 kDa was observed in WC and OMP preparations of isolate Catla grown *in vitro*, while a band at 37 kDa was only seen with OMP preparations from the bacteria grown *in vitro*. A weakly stained band at 92 kDa was seen with ECP from isolate Catla

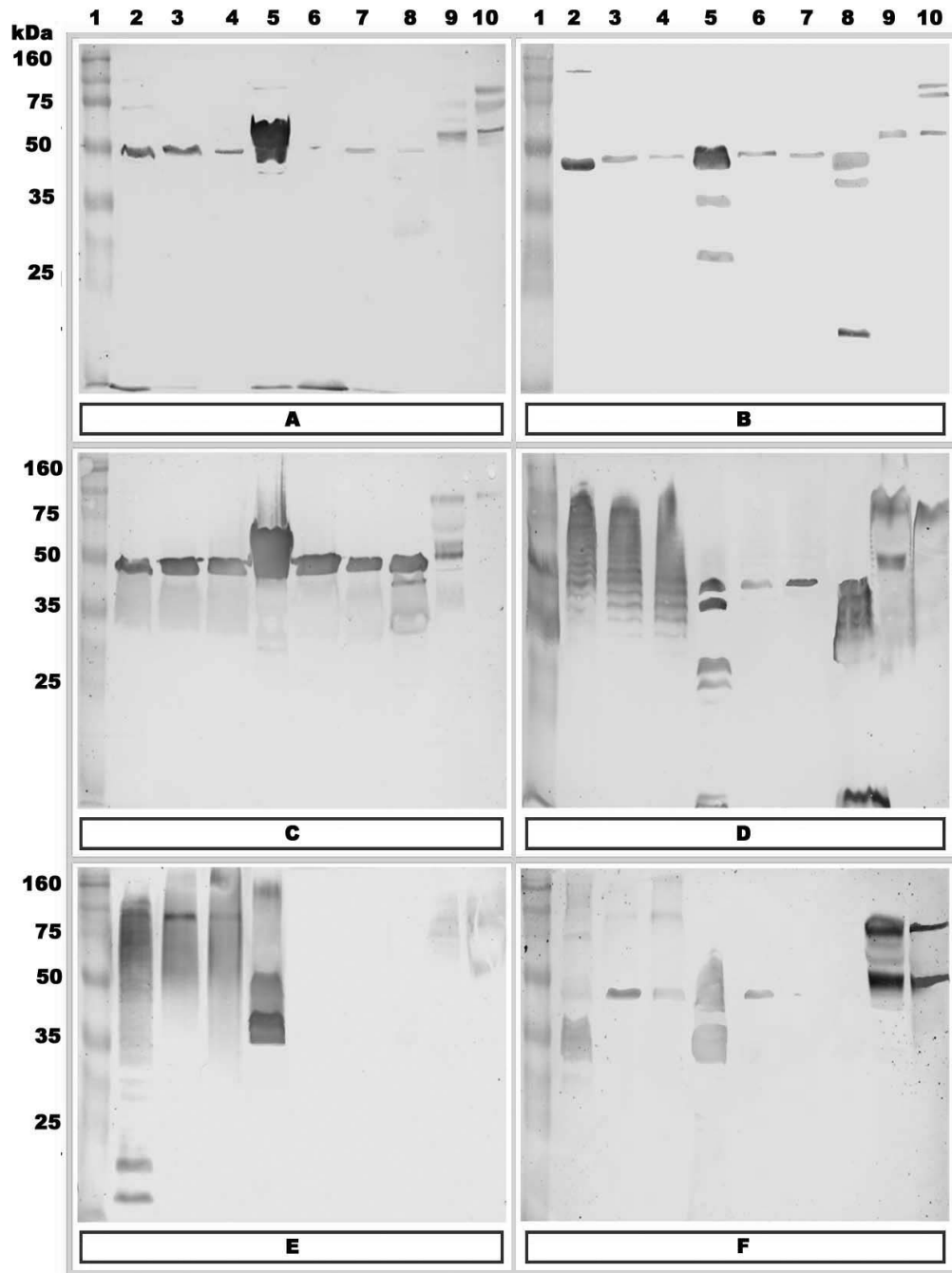


Figure 3.3: Western blot analysis of different preparations of 6 *A. hydrophila* isolates screened with serum raised against corresponding *A. hydrophila* isolates in common carp

(A) T4, (B) 98141, (C) Hh, (D) Vds, (E) Catla, (F) C24li.

Lanes: (1) Standard marker; (2) WC *in vitro*; (3) WC *in vivo* 25 kDa; (4) WC *in vivo* 100 kDa; (5) OMP *in vitro*; (6) OMP *in vivo* 25 kDa; (7) OMP *in vivo* 100 kDa; (8) ECP *in vitro*; (9) ECP *in vivo* 25 kDa; (10) ECP *in vivo* 100 kDa.

The corresponding SDS-PAGE gels for these blots are shown in Chapter 2 (Section 2.3.3).

grown in both 25 and 100 kDa MW cut off tube. Another weakly stained band at 75kDa was only seen in ECP from the bacteria cultured in the 25 kDa MW cut off tube. A band at around 50 kDa was seen in both WC and OMP preparations from isolate C24li grown *in vitro* and *in vivo*. Six bands were seen between 35 and 100 kDa with ECP preparations from bacteria grown in both MW cut off tubes but the bands were weakly stained in the case of bacteria grown in the 100 MW cut off tube. WC and OMP preparations from isolate C24li cultured *in vitro* exhibited a band at 35 kDa.

3.3.1.3. 2D SDS-PAGE Western blot

The 2D Western blot for *A. hydrophila* T4 isolate using the antibody raised against the isolate in common carp expressed three spots at approximately 50 kDa with pI values between 5 and 5.7 (Figure 3.4).

3.3.2. Response of rabbit antibody against *A. hydrophila*

The end-point titre of the rabbit anti-*A. hydrophila* serum was 1×10^{-7} as determined by ELISA. In Western blot, different banding profiles were exhibited with all 14 isolates used, using antibodies raised against heat-killed *A. hydrophila* (T4 isolate) in rabbit. A strong response was seen in the region between 15 to 75 kDa with all the isolates (Figure 3.5). Similar results were seen for the virulent isolates (T4, 98141, Hh, Vds, B2/12) analysed, except 98140 and the same result was also reproduced for the two avirulent isolates, 98139 and F1d75. Five major bands were seen at around 26, 28, 37, 50 and 75 kDa in seven of the isolates.

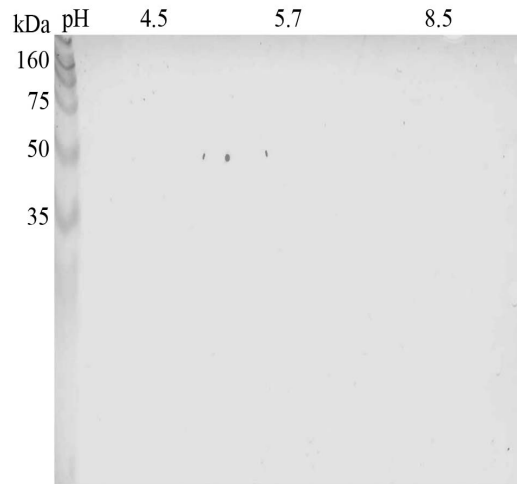


Figure 3.4: 2D Western blot analysis of whole cell preparation of *A. hydrophila* T4 isolate screened with antibody from common carp infected with *A. hydrophila* T4 isolate

The corresponding SDS-PAGE gel for this blot is shown in Chapter 2 (Figure 2.6).

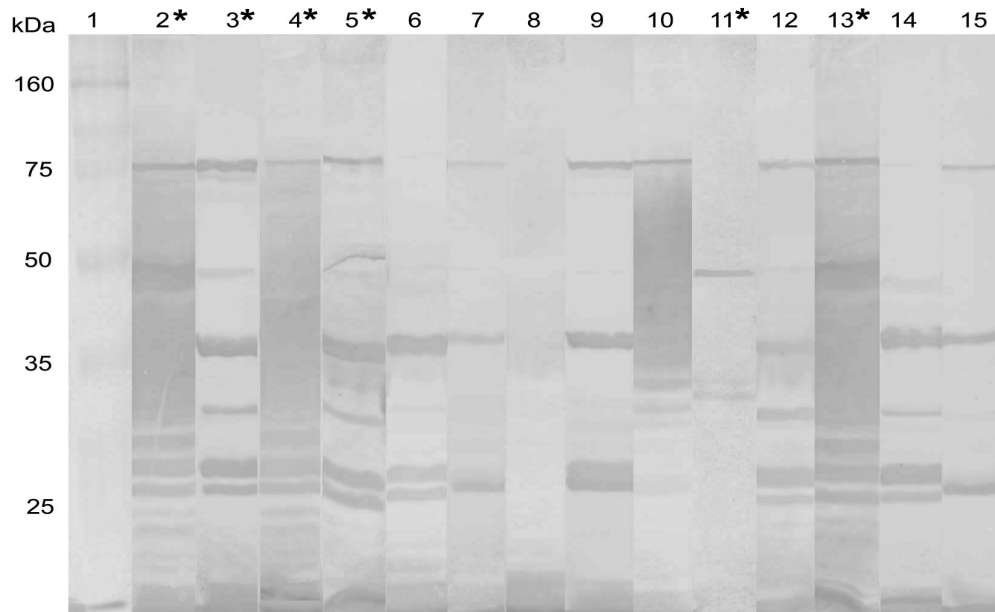


Figure 3.5: Western blot analysis of different whole cell preparation of *A. hydrophila* against rabbit anti-*A. hydrophila* serum

Lane: (1) Standard marker; (2) T4; (3) 98141; (4) Hh; (5) Vds; (6) Catla; (7) C24li; (8) 2D20; (9) 3D14; (10) 2N14; (11) 98140; (12) 98139; (13) B2/12; (14) F1d75; (15) Calf.

The corresponding SDS-PAGE gel for this blot is shown in Chapter 2 (Figure 2.4). * Virulent isolates.

3.3.3. Vaccination and Passive immunisation of goldfish with an electro-eluted 50 kDa protein from *A. hydrophila*

Aeromonas hydrophila strain T4, which had been maintained in the laboratory was of low virulence as fish did not die when injected with 0.1 ml of the bacterium IP at a concentration of 1×10^8 ml⁻¹. However, after the first passage, the strain appeared to have increased its virulence as fish died the day after re-injection into goldfish at the same concentration of bacteria. The LD₅₀ for 0.1 ml of *A. hydrophila* isolate T4 was determined to be 2.5×10^7 ml⁻¹ for goldfish weighing around 30-40g and this dose was subsequently used in the challenges performed in goldfish.

In the vaccination experiment, two control and one vaccinated fish died due to unknown causes before challenging them with *A. hydrophila* isolate T4. The two fish remaining in the control group died on Day one and Day 4 post-challenge (Figure 3.6). One fish from the vaccinated group was also sacrificed one week post-challenge as it was suffering from a severe lesion and *A. hydrophila* was isolated from swabs taken from the lesion and kidney of the sacrificed fish. The remaining two fish in the vaccinated group were healthy and sacrificed at the end of the experiment, at 21 days post-challenge. All kidney swabs taken from dead fish were positive for *A. hydrophila* while the samples taken from two vaccinated fish at the end of experiment were negative. Though the numbers of fish used in the experiment were low, the RPS value was 66.7 %.

The fish serum raised against the 50 kDa protein of *A. hydrophila* used to passively immunise fish, had a titre of 1/16. In the trial with this serum, one fish from the control group died two days post-infection and the presence of *A.*

hydrophila in its kidney was confirmed using an API 20E strip. No other fish died and no kidneys were positive for the bacteria when remaining fish were sampled at the end of the trial on Day 21.

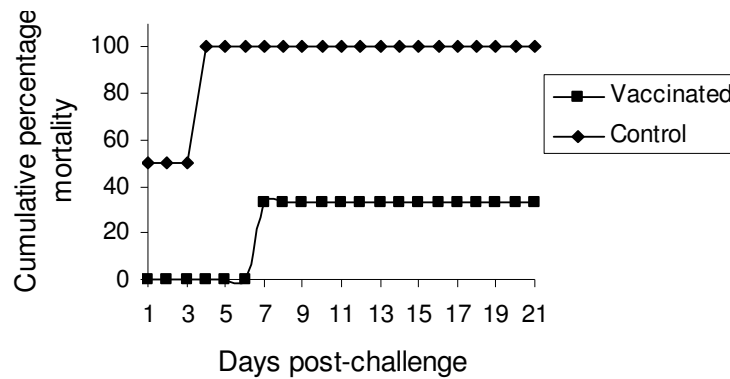


Figure 3.6: Cumulative percentage of goldfish mortality in preliminary vaccination trial

3.4. Discussion

It is difficult to prevent outbreaks of diseases caused by *A. hydrophila* due to the heterogenicity of isolates (*i.e.* it has been difficult to develop a successful vaccine) and there is increasing resistance of the bacterium to a wide range of antibiotics (Janda *et al.*, 1996; Dixon *et al.*, 1990; Daskalov, 2006). Use of antibiotics has been reported to stop the growth of favourable bacterial communities in aquaculture systems, leading to deterioration of systems, as well as an accumulation of antibiotic residues in fish tissues (Kulp and Borden, 1942; Martin, 1973; Van Muiswinkel *et al.*, 1985; Ellis, 1988; Ilhan *et al.*, 2006). Considering the disadvantages of using antibiotics discussed in Chapter 1, immunoprophylaxis is a safe method for preventing diseases in aquaculture rather than treating fish with antibiotics (Samuel *et al.*, 1996). A number of vaccine preparations containing

different components of *A. hydrophila* have been tried in an attempt to protect fish from diseases caused by this bacterium (Baba *et al.*, 1988b; Loghothetis and Austin, 1994; Rahman and Kawai, 2000; Perez *et al.*, 2002; Vivas *et al.*, 2004a; Rodrigues *et al.*, 2006). However, no commercial vaccine is yet available for *A. hydrophila* (Fang *et al.*, 2004). This may be because of insufficient ability to meet the efficacy, safety or cost effectiveness required for the vaccine preparations which have been tried to date (Clark and Cassidy-Hanley, 2005).

In this study, common carp were infected with *A. hydrophila* and the anti-sera produced were used to identify immunogenic components of the bacterium using Western blotting. The different *A. hydrophila* isolates examined elicited a variety of responses in common carp, as determined by ELISA. An increase in antibody response against *A. hydrophila* was seen after Day 9 post-infection for all the isolates except one isolate (98141). Antibody response peaked on Day 12 post-infection for two isolates (Hh and Catla) and was high on Day 21 post-infection for three isolates (T4, Vds and C24li). Authors have observed that the immune responses against *A. hydrophila* are variable according to the nature of isolates and fish species used for the infection (Loghothetis and Austin, 1996a; Sahoo *et al.*, 2004). For example, tilapia (*Oreochromis aureus*) artificially infected with *A. hydrophila* exhibited an increase in antibody response from 48 h, and a maximum titre (1:512) was observed by Day 30 post-infection (Prieto *et al.*, 1992). A similar titre was observed on Day 21 post-infection for all the isolates in the current study. Moreover, the antibody response of common carp did not show any differentiation between virulent and avirulent isolates of *A. hydrophila* in the ELISA. This may be due to differences in the ability of the immune system of the host to respond to

foreign agents. However, variation in individual immune responses against a specific isolate of *A. hydrophila* was seen to differ in rainbow trout, *Oncorhynchus mykiss*, as not all the fish developed the same levels of antibody response against a specific isolate (Loghothetis and Austin, 1996a).

Choosing the protective antigen(s) is an important basic step in establishing a suitable vaccine (Potter and Babiuk, 2001). The host immune response plays a vital role in the identification of antigens for producing effective vaccines (Ellis, 1999). In this study, Western blot analysis using the anti-sera produced on Day 21 post-infection, against different strains of *A. hydrophila*, showed differences in the profiles between the isolates. However, when pooled sera (from common carp infected with 6 different isolates) were used to examine the response against the 14 isolates of *A. hydrophila* (described in Table 2.1), a band at around 50 kDa was observed in all the isolates grown *in vitro*, except for isolate 2D20. Moreover, bands from 30-50 kDa were stained in the profiles of 3 of the virulent isolates, T4, Hh and B2/12 grown *in vitro*. The response seen with the fish serum in Western blotting against samples prepared from bacteria grown *in vitro* and *in vivo* was variable. However, the response seen against the virulent strains was quite similar. A band at around 50 kDa was seen with all the preparations (WC, OMP, ECP) from the virulent isolates grown both *in vitro* and *in vivo* compared with avirulent isolates, with the exception of the ECP from bacteria grown *in vivo*, which did not contain the band.

The 2D Western blot analysis is useful to identify all of the immunogenic components of a pathogen for diagnostic and vaccine production, as 2D SDS-

PAGE can separate even very complex mixtures of proteins to allow easier identification of individual proteins by Western blot (Klade, 2002; Chen *et al.*, 2004). The 2D Western blot analysis of *A. hydrophila* (T4 isolate) WC revealed 3 spots at approximately 50 kDa between 5 and 5.7 pl range when serum raised against T4 isolates was used. Spots in this particular region were darkly stained with all the isolates in 2D SDS-PAGE (Figure 2.6) suggesting that these spots contain particularly immunogenic proteins.

The results of the 1D and 2D Western blot analysis suggest that a molecule at approximately 50 kDa (ranging between 47 and 51 kDa) might be one of the major immunogenic components of *A. hydrophila*. Although the molecule varies in size with isolate and type of preparations, for the purpose of this thesis we will refer to this as the 50 kDa molecule. This similar 50 kDa protein of *A. hydrophila* has already been reported by number of authors to be a major virulence surface factor (Dooley and Trust, 1988; Janda and Duffey, 1988; Ascencio *et al.*, 1991b and 1998; Sendra *et al.*, 1997).

Antibodies raised against *A. hydrophila* (heat-killed T4 isolate) in rabbits were also analysed. The heavily stained band observed at 50 kDa with most of the isolates against pooled common carp antibody was found to be weakly stained with rabbit antibody. This indicates a difference in the host response, which, might be due to the fact that heat-killed cells were used in rabbits to elicit the antibody response, while live cells were used in fish. Other researchers have reported that the 50 kDa protein is recognised by antibodies raised against heat-killed *A. hydrophila* in rabbits (Sendra *et al.*, 1997).

Other components of *A. hydrophila* have also been reported as immunogenic when screened with antibodies from different sources by Western blotting. For example, antibodies raised in rainbow trout against live and killed *A. hydrophila* by immersion, injection or oral administration, reacted with formalised cells and LPS of the bacterium, and to a lesser extent with exopolysacchride, flagella, S-layer protein and a haemolytic component in the ECP of *A. hydrophila* (Loghothetis and Austin, 1994). The same authors (1996b) also suggested that the LPS of *A. hydrophila* was the major antigenic component of WCs, as strong agglutination of formalised and live cells of *A. hydrophila* was seen with serum from rainbow trout compared with extremely weak agglutination with boiled and LPS deficient *A. hydrophila*. Antibodies raised against formalin killed *A. hydrophila* in rabbit showed a response to LPS in Western blot and also showed that O polysaccharide was the immunodominant region of the LPS molecule (Dooley *et al.*, 1986).

Researchers have used direct immunisation as well as passive immunisation as a tool to evaluate the immune response and protection produced by a specific antigen/pathogen. For instance, carp, *C. carpio* injected IP with formalin killed *A. hydrophila* showed an increased agglutinating antibody titre one week after immunisation, and the peak response ranging from 1:2,048 to 8192 were observed 4 weeks after immunisation (Kusuda *et al.*, 1987). A specific immune response in catfish, *Clarias gariepinus* at 4 weeks post-immunisation with formalin-killed *A. hydrophila* was significantly greater than that measured in control catfish (Yin *et al.*, 1997). Indian major carp, rohu and mrigal (*Cirrhinus. mrigala*) had a detectable antibody response against all components of a polyvalent

vaccine (composed of whole cell and ECP of *A. hydrophila*) on Day 7 after primary immunisation, which peaked at 14 days post-immunisation (Chandran *et al.*, 2002b). A significant increase in antibody response in common carp against *A. salmonicida* was also observed at Day 14 post-immunisation when the fish were immunised with liposomes containing antigens of the bacteria (Irie *et al.*, 2005).

In vaccine development, fish immunised with various pathogens have been shown to elicit different levels of protection following challenge with the corresponding pathogens. It has been reported, for example, that an RPS value of 86.6 % was obtained in trout immunised with formalin-treated *V. anguillarum* for one month before challenging them with bacteria (Akhlaghi, 1999). The author observed that fish passively immunised with serum against formalin killed *V. anguillarum* raised either in trout, sheep and rabbits appeared only weakly protected by the fish serum (RPS of 40.0 %) compared with either the sheep or the rabbit serum (RPS of 93.3 and 86.6 %, respectively). In another trial, tilapia, *O. niloticus* which were challenged with *Streptococcus iniae* after immunising them with live bacteria had an 18 % cumulative mortality, while only a 0 and 3.3 % cumulative mortality was seen in fish passively immunised with serum raised against live or heat inactivated bacteria respectively (Shelby *et al.*, 2002). Tilapia, vaccinated with formalin-treated *S. agalactiae* showed 40 % mortality when challenged with the bacteria compared with 60 % mortality in the control group (Pasnik *et al.*, 2006). These authors also noted significantly less mortality ($P < 0.0001$) in the passively immunised group using fish serum raised against *S. agalactiae* compared with the control group after challenging them with the bacterium.

In the current study, the 50 kDa protein of *A. hydrophila* was considered to be the most immunogenic protein which is present in most isolates. The 50 kDa protein conferred protection in goldfish against *A. hydrophila* in the direct immunisation trial, however, the results of passive immunisation trial are inconclusive due to low mortality after challenge. The titre of the fish serum raised against the electro-eluted 50 kDa protein was also very low 1/16. Although both trials were carried out with a limited number of fish, the protection seen in goldfish against *A. hydrophila* in direct immunisation trial suggests that the 50 kDa protein may be a suitable vaccine candidate against this bacterium.

To conclude, the antibody responses in common carp following *A. hydrophila* infection were evaluated, and the anti-sera collected was used to identify common antigens between isolates to be used as possible vaccine candidates. The results suggested that a 50 kDa protein was the most common immunogenic antigen on the isolates. This particular protein was expressed by all the virulent isolates, in the WC and OMP preparations from bacteria grown both *in vitro* and *in vivo*. This molecule only appeared to be present in the ECP of bacteria grown *in vitro*, but not *in vivo*. Moreover, no apparent difference was seen in the response between bacteria grown in 25 and 100 kDa MW cut off tubes except with the ECP preparations. The efficacy of this 50 kDa protein in goldfish following vaccination and experimental challenge against *A. hydrophila* suggests that this protein may indeed be a protective vaccine candidate.

**Chapter 4. Production and efficacy testing of an
A. hydrophila recombinant S-layer protein
vaccine**

4.1. Introduction

Vaccination of humans and other animals induces specific immunity to assist in the elimination of microbes, neutralisation of microbial toxins and prevention of further microbial invasion (Minichiello, 2002). Recently, the expression of proteins in recombinant systems has become a widely used technique for vaccine production in mammals (Andersen and Krummen, 2002; Jenny *et al.*, 2003). This method enables rapid production of large quantities of specific protein (He *et al.*, 1997). Researchers are using recombinant DNA technology to develop protein vaccines for the aquaculture industry because it provides a means to produce sufficient quantities of the immunoprotective vaccine antigen inexpensively (Leong *et al.*, 1997; Wilhelm *et al.*, 2006). Such vaccines have enormous potential in the aquaculture industry as they are safe and efficient compared with live or attenuated vaccines (Clark and Cassidy-Hanley, 2005). They also provide an alternative approach to traditional formalin-killed whole cell (WC) vaccines that are not always efficacious.

Recombinant protein production involves a series of stages including DNA amplification, expression in host cells, purification and confirmation of protein immunogenicity in the host (Murthy *et al.*, 2004). Quality and yield of recombinant protein are dependent on the protein gene sequence, the vector, host cell and culture conditions used (Stevens, 2000). The primary microbial host for producing recombinant therapeutic proteins has been *Escherichia coli*, although many alternative organisms and expression systems are now being used (Baneyx, 1999; Swartz, 2001). The use of affinity tags (e.g. histidine tags) simplifies the purification of the recombinant fusion proteins (Nygren *et al.*, 1994).

Sequencing, identification and characterisation of the protein can be carried out either by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Wang *et al.*, 2003) or N-terminal sequencing (Prasad *et al.*, 2005). The MALDI-TOF MS is a superior method to identify all kinds of proteins between 1 and 300 kilo Dalton (kDa) with high accuracy and sensitivity (Bonk and Humeny, 2001). This method comprises digestion of the target protein, typically by trypsin and the resulting peptides are mass analysed and these data are compared with those held in a sequence database (Egelhofer *et al.*, 2002). Reverse translation of the sequence identified protein generates the sequence of the gene responsible for encoding the protein, which in turn facilitates primer design for DNA amplification.

Recombinant proteins have been used as vaccine candidates for a variety of fish pathogens, for example, the recombinant immobilization antigen of *Ichthyophthirius multifiliis* protected goldfish (*Carassius auratus*) against the parasite (He *et al.*, 1997). A recombinant protein from *Piscirickettsia salmonis* has been shown to elicit protection against salmonid rickettsial septicaemia (SRS), in coho salmon (*Oncorhynchus kisutch*) (Kuzyk *et al.*, 2001). Recombinant protein vaccines are, however, not always successful with regard to protection. Maurice *et al.* (2004) found increased antibody titres in goldfish immunised with recombinant A-layer proteins of *A. salmonicida* entrapped in alginate beads, however, vaccinated fish did not show any resistance to the bacterium. This was also the case for an 85a (31 kDa) recombinant protein antigen of *Mycobacterium marinum* as no protection was observed in striped bass, *Morone saxatilis*, when fish were challenged with the bacterium 70 days post-vaccination (Pasnik *et al.*, 2003).

The aim of the present Chapter was to identify the 50 kDa protein of *A. hydrophila* which exhibited immunogenic and protective properties in Chapter 3. Following sequencing by MALDI-TOF MS, production of a recombinant 50 kDa protein was planned to ensure a sufficient quantity of protein for large scale vaccination efficacy testing. A vaccination trial was designed using the recombinant protein in common carp (*Cyprinus carpio*) to assess firstly the level of protection elicited by this recombinant protein, and secondly to see, if the antigen cross-protected against different isolates of *A. hydrophila*.

4.2. Materials and methods

4.2.1. Identification of the 50 kDa protein of *A. hydrophila* by peptide mass fingerprinting

A whole cell preparation of *A. hydrophila* T4 isolate in Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was prepared as described in Section 2.2.3.1 and sent to University of Dundee, Scotland, UK for sequencing and identification of the 50 kDa protein by MALDI-TOF MS. Samples were in-gel reductively alkylated prior to staining with colloidal Coomassie blue, then digested in 0.1 % of n-octyl glucoside/20mM ammonium bicarbonate plus 12.5 µg ml⁻¹ trypsin, and the sample (1.5 µl) was spotted from the extract (30 µl) after adding an equal volume of acetonitrile for performing MALDI-TOF MS analysis.

4.2.2. Recombinant 50 kDa protein production

The protective nature of the 50 kDa protein from *A. hydrophila* was previously confirmed in goldfish using a small number of fish (Chapter 3). It was, therefore, of

interest to confirm this protection in a large scale trial using common carp. Thus recombinant protein was produced in order to have a sufficient quantity of protein for a large scale vaccination trial. All the recombinant protein work was conducted at the Genomic Laboratory, Tokyo University of Marine Sciences and Technology, Japan.

4.2.2.1. DNA extraction from *A. hydrophila* T4 isolate

Aeromonas hydrophila T4 isolate was grown overnight as described in Section 2.2.1.4, after which it was transferred to 1.5 ml centrifuge tubes and centrifuged at $5000 \times g$ for 5 min at 4°C . The pellet was resuspended in 567 μl Tris ethylenediaminetetraacetic acid (EDTA) (TE) buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8), 30 μl of 10 % (w/v) SDS and 3 μl of 20 mg ml^{-1} proteinase K. The bacteria were thoroughly mixed and incubated for 1 h at 37°C before adding 100 μl of 5 M NaCl and was again thoroughly mixing, after which, 80 μl cetyltrimethylammonium bromide (CTAB) in NaCl solution (10 % v/v CTAB in 0.7 M NaCl) was added, mixed and incubated for 10 min at 65°C . DNA was extracted from the sample with an equal volume (780 μl) of chloroform: isoamyl alcohol (24:1 ratio). The tube was inverted a couple of times and centrifuged at $5000 \times g$ for 5 min at 4°C . The aqueous phase was transferred to a fresh tube and extracted with phenol: chloroform: isoamyl alcohol (25:24:1 ratio). The contents of the tube was thoroughly mixed and centrifuged at $5000 \times g$ for 10 min at $20\text{-}22^{\circ}\text{C}$. The aqueous phase was transferred to a fresh tube, and the DNA precipitated with an equal volume of isopropanol and the contents of the tube were thoroughly mixed by inverting the tube a couple of times and centrifuged at $5000 \times g$ for 10 min at 4°C . The precipitate was washed with 70 % ethanol by centrifuging at $5000 \times g$ for

10 min at 4°C. The supernatant was removed and the pellet briefly dried at 20-22°C for 10 min. The pellets were resuspended in 100 µl TE buffer and stored at -20°C until used.

4.2.2.2. Polymerase chain reaction (PCR) of *A. hydrophila* 50 kDa protein gene

Specific primers were designed to amplify the full length of the 50 kDa protein gene based on the sequence data for S-layer gene of *A. hydrophila* published by Thomas and Trust (1995a). Restriction sites Nco I and Bgl II were added to the forward and reverse primers respectively (Figure 4.1) to assist its cloning into the expression vector pQE 60 (Appendix II). The PCR mixture (Appendix II) was prepared for 40 µl reactions and each reaction included 32 cycles using the following conditions; preheating to 95°C/5 min; denaturation for 95°C during 30 sec; annealing at 55°C/30 sec; elongation at 72°C/1min and a final elongation step at 72°C/5 min.

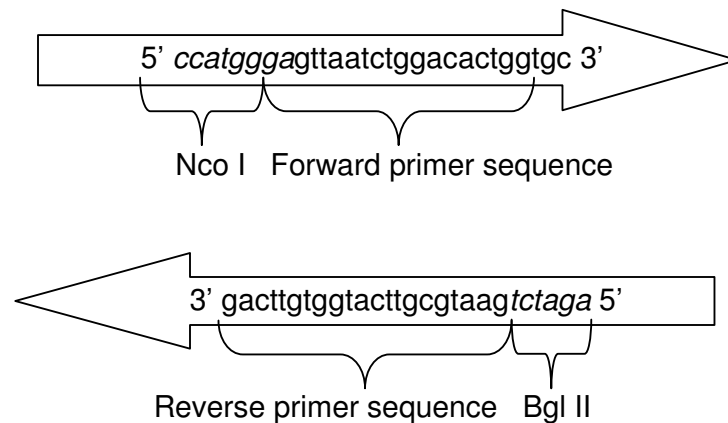


Figure 4.1: Primers and restriction enzymes used for amplification of the 50 kDa protein gene

4.2.2.3. Preparation of PCR products for transformation into *E. coli*

The PCR products were run on a 1 % agarose gel for 30 min at 100V. The target bands were identified by ultraviolet (UV) light and were cut from the gel using a scalpel and chopped into small pieces. The DNA were extracted from the gel using a DNA purification kit from Amersham Bio-Science. Digestion of the PCR products and the pQE 60 vector (Qiagen) were carried out overnight at 37°C with a digestion mixture described in Appendix II. Both pQE 60 vectors and PCR products were purified after the digestion process as described above. The ligation was attained by mixing 2 µl of vector with 8 µl of PCR products and adding 10 µl ligation high (Cosmo Bio Co Ltd, Tokyo) before incubating it overnight at 16°C.

4.2.2.4. Transformation of vectors carrying 50 kDa protein gene into *E. coli*

Escherichia coli, M15 (Quiagen, Tokyo, Japan) was used for transforming pQE 60 vectors carrying the amplified 50 kDa protein gene of *A. hydrophila*. Initially, 100 µl of cells were thawed from storage at -70°C. The pQE 60 vectors were added to the cells and incubated for 30 min on ice. The mixture was given a heat shock at 45°C for 45 sec before adding 800 µl of SOC medium (Sigma) and incubating at 37°C for 1 h with vigorous shaking. The cells were centrifuged at 2000 × g for 3 min and resuspended in 100 µl 2x yeast tryptone broth (2xYT). The cell suspension was transferred (spread plate) onto Luria Bertani (LB) agar plates containing ampicillin and kanamycin. The plate was incubated overnight at 37°C and approximately 10 colonies plated from the LB-agar plate and transferred to a fresh LB-agar plate. The fresh plate was incubated for approximately 4 h at 37°C, and then PCR was performed on colonies taken from the plate using colony PCR mixture (Appendix

II) to confirm the presence of the 50 kDa protein gene insert in the bacterial colonies. The PCR conditions described in Section 4.1.2.2 were applied using *E. coli* colonies having recombinant pQE60 as the template to the PCR carried out here.

4.2.2.5. Expression of the recombinant 50 kDa protein in *E. coli*

The clones containing the 50 kDa protein gene insert identified by PCR, were inoculated into LB broth containing ampicillin ($100 \mu\text{g ml}^{-1}$) and kanamycin ($25 \mu\text{g ml}^{-1}$), and incubated overnight at 37°C . The expression of the recombinant protein was achieved by adding 1mM isopropyl- β -thiogalactoside (IPTG) to the medium and incubating for 4 h. The *E. coli* cells were harvested and total proteins of IPTG induced and non-induced recombinant bacteria were separated on a 12 % SDS-PAGE and subjected to Western blot as outlined in Sections 2.2.4.1 and 3.2.4.2. After electroblotting onto a nitrocellulose membrane (ATTO Co., Tokyo, Japan), the membranes were blocked as outlined in Section 3.2.4 and incubated with an anti-histidine-tag antibody (Amersham Biosciences Buckinghamshire, UK) diluted 1:6000 in TBS for 1 h. As a second antibody, mouse anti-rabbit conjugated to IgG-alkaline phosphatase (Promega, Madison WI, U.S.A) was used at a concentration of 1:7500 and incubated for 1 h. The reaction was developed using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP-NBT) alkaline phosphatase substrate (1 tablet dissolved in 10 ml of double distilled water, Sigma-Aldrich Co, St Louis MO, U.S.A).

4.2.2.6. Large scale production of recombinant 50 kDa protein

Positive clones were cultured in 50 ml of LB broth with antibiotics, ampicillin (100 $\mu\text{g ml}^{-1}$) and kanamycin (25 $\mu\text{g ml}^{-1}$) overnight at 37°C with vigorous shaking. This culture was transferred to 1 L fresh LB broth and cultured at 37°C with vigorous shaking. The absorbance of the culture broth at 600nm was measured every hour until it reached 0.6, after which, the culture was induced to express the recombinant proteins by adding 1 mM IPTG. Bacterial growth was stopped exactly 4 h after inducement and the bacterial pellets were harvested at 4000 \times g for 30 min at 4°C. The pellets were resuspended in phosphate buffered saline (PBS) and stored at -80°C.

The bacterial pellet was subjected to 3 rounds of freeze-thawing before resuspending in sterile PBS and sonicating 60 times at 150 Watts for 20 sec with 10 sec intervals. After sonication, the soluble (native protein) and insoluble materials (inclusion bodies) were separated by centrifugation at 4000xg for 30 min at 4°C. Inclusion bodies were solubilised in 250 ml denaturing solution (8 M urea, 0.1 % (w/v) SDS and 100 mM Tris-HCl) with 10 mM imidazole, while imidazole was added to the supernatant at final concentration of 10 mM. Inclusion bodies and the denaturing solution mentioned above were mixed together and 20 ml of nickel beads (Ni Sepharose 6 fast flow, Amersham bioscience) was added to bind the target proteins. Both pellet and supernatant were placed on ice and shaken overnight.

A column (XK 16/20 empty lab scale column, Amersham Bio-Science) was filled with beads and washed three times with 20 mM washing buffer (Imidazole 20 mM,

NaH₂PO₄ 50 mM and NaCl 300 mM) pH 8. The proteins were eluted from the beads using elution buffer (Imidazole 250 mM, NaH₂PO₄ 50 mM and NaCl 300 mM) pH 8.5 and collected in fractions, the OD of which were measured at 280 nm. Fractions containing proteins were pooled together before concentrating using a Millipore membrane (10,000 MW cut off, Amicon), and dialysed overnight in sterile PBS using seamless cellulose tubing (12,000 MW cut off, Union Carbidge Corporation, Tokyo). The protein concentration of the suspension was measured using a Pierce protein determination kit and the protein was stored at -20°C until further use. SDS-PAGE described in Section 2.2.4.1 and Western blot described in Section 3.2.4.2 were performed with serum raised in common carp against *A. hydrophila* isolate T4 to confirm the immunogenicity of the recombinant 50 kDa protein.

4.2.3. Sequencing of the *A. hydrophila* T4 isolate 50 kDa protein gene

The 50 kDa protein gene of *A. hydrophila* isolate T4 was sequenced at the Genomic Laboratory, Tokyo University of Marine Sciences and Technology with primers (Figure 4.1) to compare the 50 kDa protein genome with *A. hydrophila* isolate TF7 S-layer protein genome reported by Thomas and Trust (1995a).

4.2.4. Vaccination of common carp with recombinant 50 kDa protein

4.2.4.1. Standardising the *A. hydrophila* challenge

The six virulent isolates (T4, 98140, 98141, Hh, B2/12 and Vds) described in Chapter 2, were passaged twice through common carp (30-40g). Bacterial

suspensions of these strains were prepared at a concentration of 1×10^8 bacteria ml^{-1} , as described in Section 2.2.1.4, and 0.1 ml of these were injected intraperitoneally (IP) into two fish for each strain. Samples were taken from the kidney of dead fish 24 h post-injection, and streaked onto tryptone soy agar (TSA) plates which were incubated overnight at 28°C . Colonies on the plate were confirmed as *A. hydrophila* as described in Section 2.2.1. The bacteria were re-passaged through fish using a bacterial suspension for each isolate at 5×10^7 bacteria ml^{-1} and 0.1 ml injected as described above.

After passaging the bacteria through the fish, the LD_{50} was determined for the six isolates in common carp (30-40 g). Initially, three doses of bacteria were selected; 2×10^7 , 5×10^7 and 2.5×10^7 bacteria ml^{-1} . The fish were injected IP with 0.1 ml of these suspensions and placed in a separate glass tank for each strain. The fish were maintained in a recirculatory water system with aeration. The concentrations of bacteria were modified and injected to a fresh group of fish until obtaining LD_{50} values for all the isolates.

4.2.4.2. Vaccination

Recombinant 50 kDa protein of *A. hydrophila* diluted in PBS was mixed with montanide adjuvant (Schering-Plough Aquaculture, Saffron Walden, UK) at a ratio of 30:70 (v/v) to give a final antigen concentration of $300 \mu\text{g ml}^{-1}$. Buffer (PBS) mixed with the adjuvant was also prepared at the same ratio as the antigen to serve as a negative control. Mixing was achieved by vortexing until the antigen was emulsified, and this was stored overnight at 4°C to ensure that the antigen did not separate from the adjuvant.

One hundred and fifty common carp (30-40 g) were vaccinated by IP injection with 0.1 ml of the vaccine preparation, and another 150 fish were injected with the PBS adjuvant mixture. The right side pectoral fins of control fish were clipped for identification. All the fish were maintained for 35 days in 1 × 1 m (Diameter × depth) tanks with recirculating water before challenging them with six different isolates of *A. hydrophila*.

4.2.4.3. Challenge studies

Each of the six virulent isolates described in Section 5.2.1 were used to challenge vaccinated fish. Twenty vaccinated and 20 control fish were injected IP with each strain after anaesthetising them as described in Section 2.2.2. The concentrations of the bacteria used in the challenge were 1×10^8 , 2×10^7 , 2×10^7 , 5×10^7 , 7.5×10^6 and 2×10^7 bacteria ml⁻¹ for T4, 98140, 98141, Hh, B2/12 and Vds respectively. All 40 fish within each group were placed in separate glass tank (90 cm length × 47 cm height × 40 cm depth) with aeration and recirculating water. The fish were maintained for 16 days post-challenge and dead fish were removed 3 times a day. Samples from the kidney of dead fish and also from surviving fish at the end of the experiment on Day 16 post-challenge were streaked onto TSA plates. Six surviving fish (3 vaccinated and 3 control fish) per bacterial strain were sacrificed with an overdose of benzocaine (0.01 % w/v) and their kidneys sampled.

4.2.4.4. Statistical analysis

The results obtained were analysed statistically using Chi-square test for survival, comparing the mortality of vaccinated fish with the control group fish after challenging with bacteria.

4.3. Results

4.3.1. Peptide Mass Fingerprinting of the 50 kDa protein from *A. hydrophila*

Sequencing of 50 kDa protein of *A. hydrophila* isolate T4 was carried out by MALDI-TOF MS and the spectrum of peptide profiles obtained can be seen in Figure 4.2. After matching the peptide mass fingerprint with that obtained by Thomas and Trust (1995a), the protein was identified as a 47.6 kDa S-layer protein. The whole amino acid and genomic sequence of the S-layer protein of *A. hydrophila* reported by these authors were obtained from the National Centre for Biotechnology Information (NCBI) database (Figure 4.3).

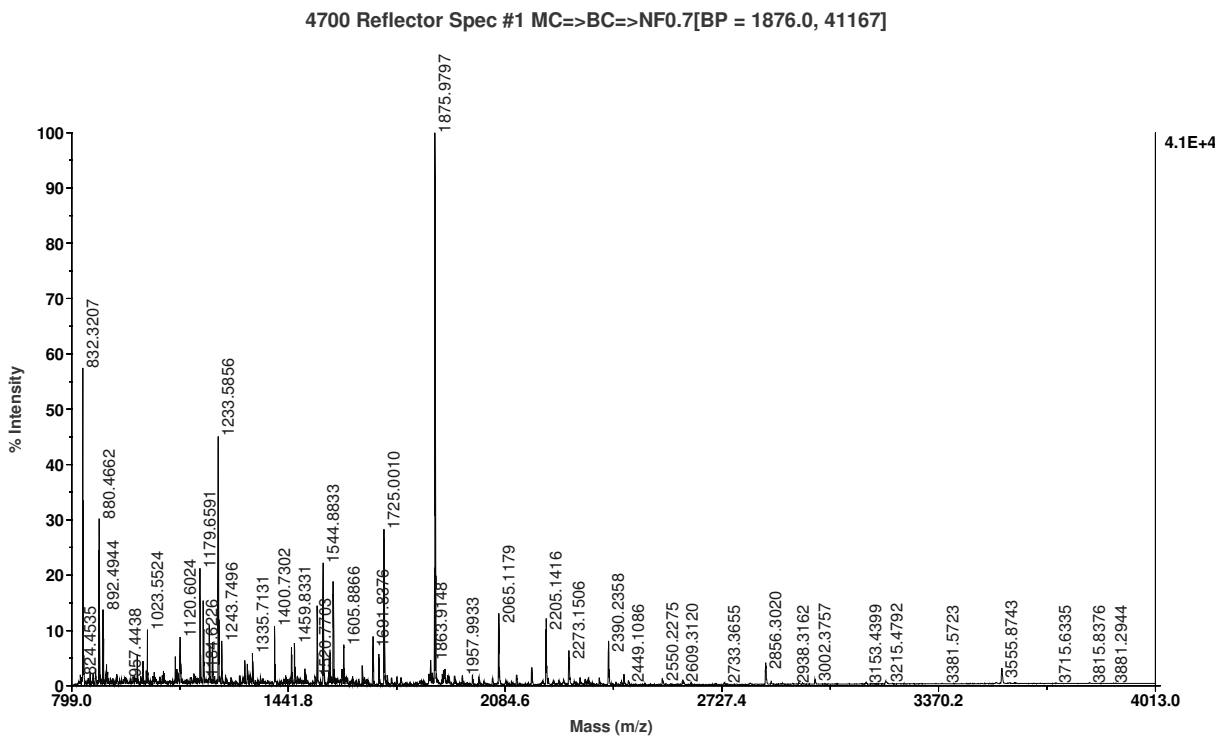


Figure 4.2: MALDI-TOF MS spectrum showing the peptide profiles of 50 kDa band

1 atgattctaataaaaaagacactgattgcactggccgttgcggctgagctttaacgtgctgca gttaatctggacactggctgctggc
1 M I L M K K T L I A L A V A G L S F N A A A V N L D T G A G
91 gtttctaagttgctagcgaatcaaaagtgatggcggcaggtactacttgggtaccgcagccgggtgctgctatgaatgcagtgagc
31 V S K F A S E I K V D G A A G T T L G T A A G A A M N A V S
181 aagctgggttctctatttctaccgtaacaagcgttacatcgttacgatgtaactgggtggtcactggctgggtgctgctgttgcggac
61 K L G F S I S T G N K R Y I R Y D V T G G S L A G V A V A D
271 ttgacctgggtgggtgactcctgttctgtagttgcagctgatagctccttggttatctctcagaccgccgctgatggtagctttgtg
91 L T L V G G T P V A V V A A D S S F V I S Q T A A D G S F V
361 atcgttgaagttgttctaagaaagacatccctgctgatgcagtgatgacctcaaaagccgatggctgtggaacgtaagacaaaaaac
121 I V E V V A K K D I P A D A V M T S K A D G R V N V K T K N
451 ggctgtagctatcagctatcgcctgttcgagactgctctggatgccgttgcctaacgatccagctaacacctggccaaggcaaatggctcaa
151 G V A I S Y R L F E T A L D A V A N D P A K T L A K A N G Q
541 ctgctgactttctcccagctatcctcgcctcaagttgagaagaagggttctgccgacaagatcgacgtgaccgagcttccatgaagttt
181 L L T F S P A I L A K V E K K G S A D K I D V T E S S M K F
631 gttaccaatgcgaatgtaaaagctactgataccatcctgggtcaagtaagcatcactgcagacgtaaacactctttggctaacggctact
211 V T N A N V K A T D T I L G Q V S I T A D V N T L L A N G T
721 cccgtgctgctaccagtgatattctgaatgcaagcaaaactgggtgtaagtgtgatttctctgcaggtgcagtagacgccgatacaaaa
241 P V A A T S D I L N A S K L V V N G D F S A G A V D A D N K
811 ctggttctgggtaccgtcaagctgaatgctccaatgctactaaagtgaagccgcgaaagctgagctggctgtggcagatgcaggtatt
271 L V L G T V K L N A A N A T K V E A A K A E L A V A D A G I
901 ggtgcagcagctccagcaggtaacatcagctactttgttgggtggcaaaagctcctatcgtcctccgaggtgtaactgctacttctgttccg
301 G A A A P A G N I S Y F V G G K A P I A P Q A V T A T F V P
991 gttgtaaaaagctggttatgagttggctgatgtaaatctgggcgaaattggtgctgaacaaaaatggtccaccaagaagtaacctg
331 V V K A G Y E L A D V N L G E I G V L N K N G S T K E A N L
1081 gtgctgctccagatattcttacaccaactggtgctgctatcctcaaacctccaacatcgtgtaagttctttgactgcttatgct
361 V L A P D T S Y T N L V R I S N T S N I A G K F F V T A Y A
1171 gatgatgtaagtctgtaagcttcgcactgtctgatgttctgctgagccggctgttctggaagctggcgcctccaccaagcagatgaaa
391 D D G K S V S F A L S D V A G Q P A V L E A G A S T K Q M K
1261 gtggctgatatctatgctgctgccaagccaaaggtctgctctgactggtgacaagaaactgcgtctgaaagttgaaggtgaagtgct
421 V A D I Y A A A Q A K G L A L T G D K K L R L K V E G E V A
1351 tcctgagcctgcagaactacaccgtctccaagacggtaacgctctgaacacatgaacgcattctaa
451 S L S L Q N Y T V S K D G N A L N T M N A F *

Figure 4.3: Nucleic and amino acid sequences of *A. hydrophila* S-layer protein

Signal peptide sequences are highlighted (Nucleic acid in green and amino acid in yellow).

NCBI accession number – AAA67043 (Thomas and Trust, 1995a).

4.3.1.1. Sequence of the S-layer protein gene isolated from *A. hydrophila* T4 isolate

The isolated gene encoding the S-layer protein from *A. hydrophila* T4 isolate differed by six bases from the published nucleotide sequence of the S-layer protein from *A. hydrophila* TF7 isolate (Thomas and Trust, 1995a), see Figure 4.4 and Table 4.1.

```
1 atgattctaataaaaaagacactgattgcactggccgttgctggctgagcttaacgctgctgcagttaatctggacactggctgctgg
91 gtttctaagtttgctagcgaatcaaaagttgatggcgcggcaggtactacctgggtaccgcagccgggtgctgctatgaatgcagtgagc
181 aagctgggtttctatttctaccggtaacaagcgttacatcgtacgatgtaactgggtgctcactggctggtgctgctggtcggac
271 ttgacctgggtgggtgactcctgtgctgtagttgcagctgatagctcctttgtatctctcagaccgccgctgatggtagctttgtg
361 atcgttgaagttggtgctaagaaagacatccctgctgatgcagtgatgacctccaagccgatggctggtgtaacgtaagaacaaaaat
451 ggcgtagctatcagctatcgcctgttcgagactgctctggatgccgttgtaacgatccagctaaagacctggccaaggcaaatggcaaa
541 ctgctgactttctccccagctatcctcgccaaagttgagaagaagggttctgccgacaagatcgcagtgaccgagcttccatgaagttt
631 gttaccaatgcgaatgttaaagctactgataccatcctgggtcaagtaagcatcactgcagacgtaaacactcttttgctaacggctact
721 cccgtggctgctaccagtgatattctgaatgcaagcaaacctgggtgtaatggtgatttctctgcaggtgcagtagacgccgataacaaa
811 ctggttctgggtaccgtcaagctgaatgctgcaaatgctactaaaagttgaagccgcgaaagctgagctggctgtggcagatgcaggtatt
901 ggtgcagcagctccagcaggtaacatcagctactttgttggtggcaagctcctatcgctccgcagctgtaactgctactttgctccg
991 gttgtaaaagctggttatgagttggctgatgtaaatcgggcgaaattggtgctgaacaaaaatggtccaccaaaagaagctaacctg
1081 gtgctggctccagatacctcttacaccaacctgggtcgtatctccaacacctccaacatcgctgtaagttctttgtgactgcttatgct
1171 gatgatgtaagctgtaagcttcgactgtctgatgttctggtcagccggctgttctggaagctggcgcctccaccacgcagatgaaa
1261 gtggctgatatctatgctgctgccaagccaaggtctggctctgactggtgacaagaaactgcgtctgaaaagttgaaggtgaagtgct
1351 tcctgagcctgcagaactacaccgtctccaagacggtaacgctctgaacccatgaacgattctaa
```

Figure 4.4: Sequence of amplified gene encoding the S-layer protein from *A. hydrophila* T4 isolate

Bases different to that genome reported for the S-layer protein of *A. hydrophila* isolate TF7 by Thomas and Trust (1995a) have been highlighted.

Gene encoding S-layer protein from *A. hydrophila* T4 isolate was amplified as outlined in Section 4.2.2.2.

Table 4.1: The consequences of the nucleotide/amino acid changes between sequence of *A. hydrophila* TF7 and T4 isolates

| Nucleotide position | TF7 published sequence (Nucleotide/amino acid) | Amplified T4 sequence (Nucleotide/amino acid) |
|---------------------|---|--|
| 90 | ggc/G | ggt/G |
| 442/3 | aca/T | aac/N |
| 967 | gct/A | tct/S |
| 1233 | gaa/E | gac/D |
| 1249 | aag/K | acg/T |

4.3.2. Production of a recombinant protein for the S-layer of *A. hydrophila* isolate T4

The production of a 1353 bp PCR product suggested that the specific amplification of the gene encoding S-layer protein from *A. hydrophila* T4 isolate was successful (Figure 4.5). The digestion of the purified 1353 bp fragment and the pQE60 vector by enzymes Nco I and Bgl II, and ligation of the digested genomic DNA within the pQE-60 vector was confirmed from the band obtained at around 4.8 kb on the 1 % agrose gel shown in Figure 4.5 (Lane 4).

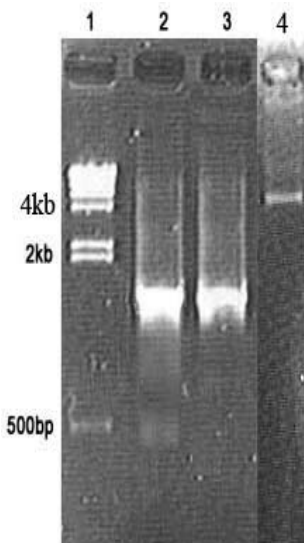


Figure 4.5: Amplification of the S-layer gene of *A. hydrophila* isolate T4 shown on a 1 % agrose gel

Lanes: (1) Standard marker; (2) S-layer protein gene; (3) purified S-layer protein gene; (4) pQE60 vector carrying S-layer protein gene.

After transformation of vectors into *E. coli* cells, the presence of the pQE60 having 1353 bp fragment constructs were confirmed by PCR. The SDS-PAGE analysis of *E. coli* transformed with pQE60 having 1353 bp fragment constructs indicated the presence of an abundant protein of molecular mass 45.5 kDa (Figure 4.6a). The protein also gave a positive reaction in Western blot against an anti-histidine antibody (Figure 4.6b).

4.3.2.1. Large scale production of S-layer recombinant protein

The concentration of bacteria in the culture media was 0.65 at OD₆₀₀ before IPTG induction. A final yield of 15 mg of purified protein was recovered from the nickel bead-packed column from 1 litre culture. The SDS-PAGE analysis revealed the elution step yielded a 45.5 kDa protein (Figure 4.7a). Western blotting using serum raised against the WC *A. hydrophila* T4 isolate in common carp (Figure 4.7b) exhibited a cross reaction with this overexpressed His-tagged protein.

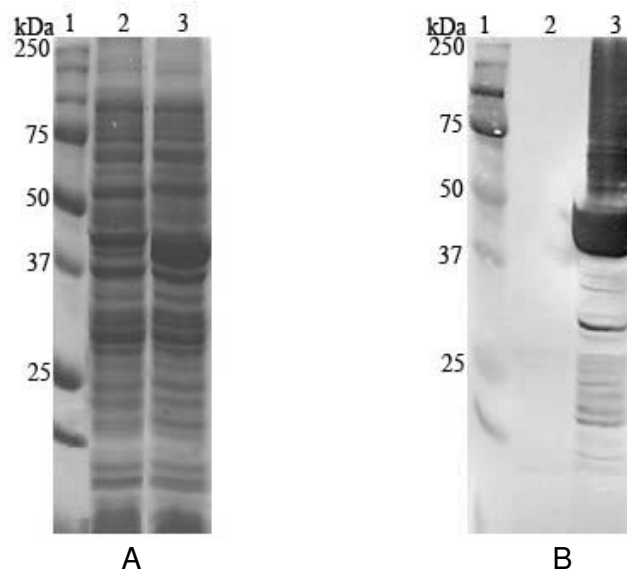


Figure 4.6: Expression of S-layer protein of *A. hydrophila* with *E. coli* WC protein

(A) 12 % SDS-PAGE stained with Coomassie blue, (B) Western blot of protein using an anti-histidine tag antibodies.
 Lanes: (1) Standard protein marker; (2) WC preparation of recombinant *E. coli* without IPTG induction; (3) WC preparation of recombinant *E. coli* with IPTG induction showing S-layer protein.

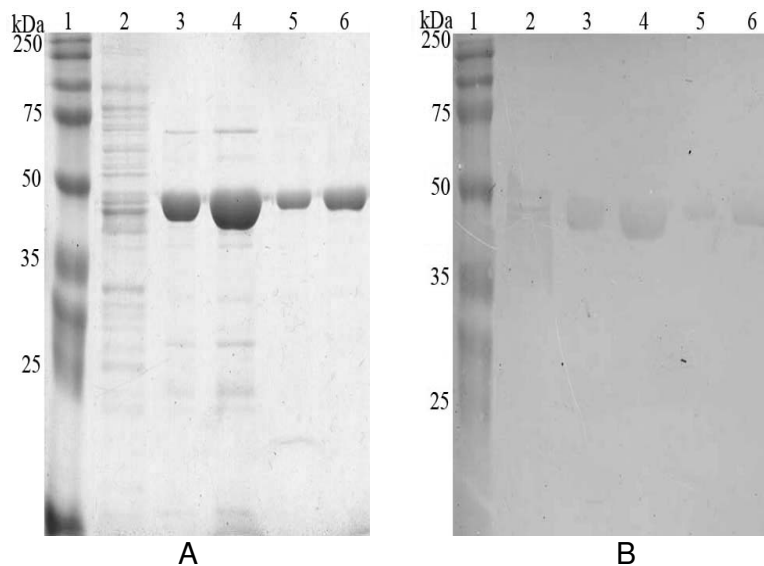


Figure 4.7: Recombinant S-layer protein of *A. hydrophila* purified from *E. coli*

(A) 12 % SDS-PAGE stained with Coomassie blue (B) Western blot against anti-*A. hydrophila* T4 isolate antibody from carp. Lanes: (1) Standard protein marker; (2) WC protein of *A. hydrophila*; (3 & 4) protein separated from insoluble fractions of recombinant *E. coli*; (5 & 6) protein separated from soluble fractions of recombinant *E. coli*.

4.3.3. Efficacy of recombinant S-layer protein against *A. hydrophila* in common carp

4.3.3.1. Standardisation of the challenge of common carp with *A. hydrophila*

All six strains T4, 98140, 98141, Hh, B2/12 and Vds were passaged two times through common carp and the bacteria were successfully recovered from both passages. During the first passage, no mortalities occurred in any of the groups of fish, while most fish died upon repassaging the bacterium a second time with all strains except T4. The values obtained in the preliminary challenge experiment in which the LD₅₀ dose for each strain was determined are given in Table 4.1. The highest LD₅₀ value of 1×10^8 bacteria ml⁻¹ was obtained with isolate T4, while the lowest dose (7.5×10^6 bacteria ml⁻¹) was found with isolate B2/12. An LD₅₀ value of 2×10^7 bacteria ml⁻¹ was found with isolates 98140, 98141 and Vds.

Table 4.1: The LD₅₀ values of *A. hydrophila* strains for common carp

| Strains of <i>A. hydrophila</i> | LD ₅₀ Value (bacteria ml ⁻¹) |
|---------------------------------|---|
| T4 | 1×10^8 |
| Hh | 5×10^7 |
| 98140 | 2×10^7 |
| 98141 | 2×10^7 |
| Vds | 2×10^7 |
| B2/12 | 7.5×10^6 |

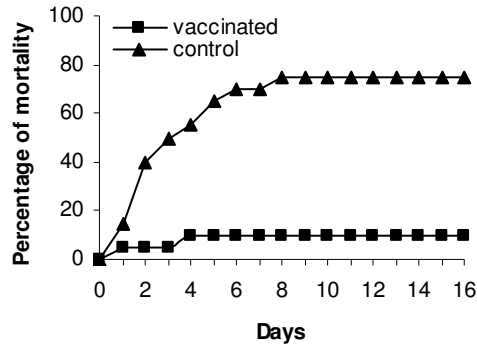
4.3.3.2. Vaccination of common carp with recombinant S-layer protein of *A. hydrophila*

Some mortalities (10 %) occurred in both the vaccinated and control groups of fish following injection, which then stabilised the next day and no more mortalities occurred prior to challenging the fish with *A. hydrophila*. In fish challenged with isolate T4, 75 % of control and 10 % of vaccinated fish died. A high percentage of mortalities were recorded in control fish challenged with isolate T4 compared with fish challenged with the other isolates of *A. hydrophila*. Fifteen percent of the control group died by the first day post-challenge and 25 % had died by Day 2 post-challenge. The levels of mortality decreased to 10 % by Day 3 post-challenge and thereafter it varied between 5 and 10 % until the mortalities stopped by Day 8 post-challenge. The mortality in the vaccinated group was 5 % on Day 1 post-challenge and another 5 % had died by Day 5 (Figure 4.8a). A relatively high relative percentage survival (RPS) value (87 %) was found with isolate T4 compared with other isolates (Table 4.2).

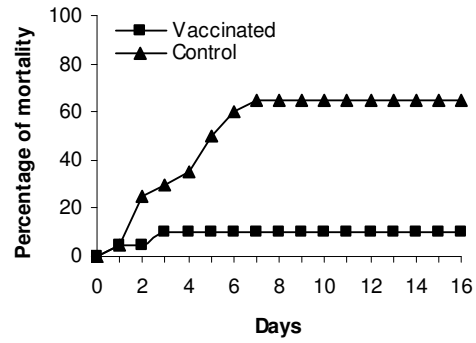
Mortality of 5 % was noted in the control group challenged with isolate Hh on Day 1 post-challenge. However, the mortality increased to 20 % by Day 2, 15 % occurred on Day 5 and 10 % on Day 6. The remainder of mortalities (*i.e.* 15 %) were distributed over the period after Day 7. In the vaccinated group, 5 % of mortalities were recorded on the first and third day post-challenge (Figure 4.8b). The second highest RPS value (85 %) in the trial was observed with this isolate.

Fifty percentage mortality was seen with the control group challenged with isolate 98140. Thirty percent died in the control group during the first two days post-challenge and remainder died over the course of the experiment (16 days post-

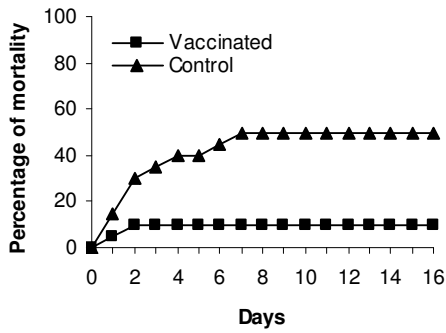
A



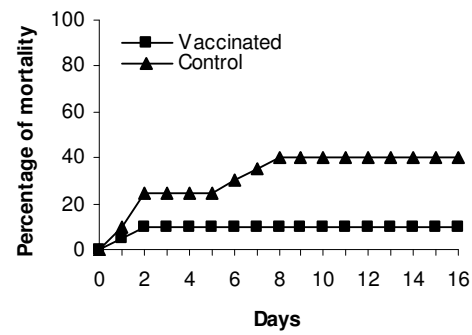
B



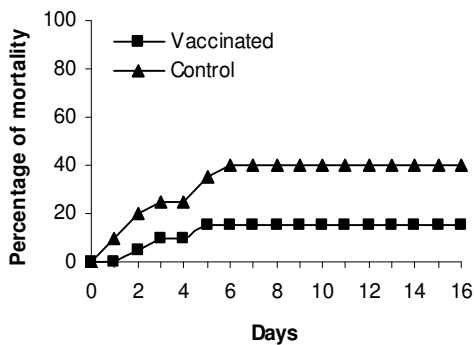
C



D



E



F

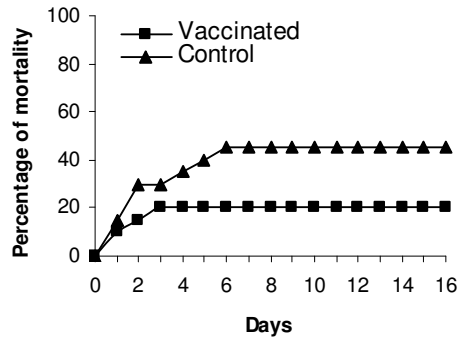


Figure 4.8: Cumulative percentage mortality of carp vaccinated with recombinant S-layer protein and challenged with *A. hydrophila* isolates (A) T4, (B) Hh, (C) 98140, (D) 98141, (E) Vds, (F) B2/12.

challenge). Five percent mortality was recorded in the vaccinated group during the first two days post-challenge, and no further mortalities occurred in this group leading to an 80 % RPS value for this isolate (Figure 4.8c).

In the control group challenged with isolate 98141, 25 % of mortality occurred over the first two days of the experiment and thereafter 15 % mortalities occurred. The mortality with the vaccinated group was similar to that of the mortality recorded with vaccinated group challenged with isolate 98140 (Figure 4.8d). An RPS value of 75 % was recorded with this isolate.

The control group challenged with isolate Vds experienced a 10 % mortality on Day 1, Day 2 and Day 5 post-challenge, while 5 % mortalities occurred on the third and sixth day post-challenge (Figure 4.8e). A total of 15 % mortality occurred in the vaccinated group distributed over Day 2, 3 and 5 post-challenge. The RPS value with this isolate was 62.5 %.

Percentage mortality in the control group rose to 30 % during the first two days after challenging the fish with isolate B2/12. Another 15 % mortality occurred in this group over the remainder of the experiment. The highest percentage mortality amongst vaccinated fish was recorded in the group challenged with B2/12. Ten percent mortality was observed in this group on the next day post-challenge and 5 % of mortality occurred on the second day and the third day post-challenge (Figure 4.8f). The RPS value was low (56 %) with this isolate compared to other isolates.

Table 4.2: Percentage mortality and relative percentage survival of carp vaccinated with recombinant S-layer protein of *A. hydrophila* and then challenged against the bacteria 35 days post-vaccination

| Strain of <i>A. hydrophila</i> | Total mortality (%) | | Relative percentage survival (%) | P-value (Chi-square test) |
|-----------------------------------|---------------------|-----------------|--|---------------------------------|
| | Vaccinated fish | Control fish | | |
| T4 | 10 | 75 | 87 | 0.000 |
| Hh | 10 | 65 | 85 | 0.000 |
| 98140 | 10 | 50 | 80 | 0.006 |
| 98141 | 10 | 40 | 75 | 0.028 |
| Vds | 15 | 40 | 62.5 | 0.077 |
| B2/12 | 20 | 45 | 56 | 0.091 |

All the fish that died during the experiment showed the presence of *A. hydrophila* in their kidneys, determined from swabs plated onto TSA. In contrast, *A. hydrophila* was not cultured from kidney swabs taken from the surviving fish except very few colonies from one fish in the vaccinated group challenged with isolate 98140 and one fish in the control group challenged with isolate 98141, when the experiment was completed. Statistical analysis revealed that survival against isolates T4, 98140, 98141 and Hh were significant in vaccinated fish compared to control fish, while levels of survival were not statistically significant for isolates B2/12 and Vds (Table 4.2).

4.4. Discussion

Different preparations of vaccines against *A. hydrophila* have been used including sonicated preparations (Thune and Plumb, 1982), heat or formalin-inactivated bacterial extracts (Koval, 1988), live cells (Loghothetis and Austin, 1994) and heat-killed cells (Tafalla *et al.*, 1999). For example, Rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) carp immunised with pooled WC of three *A. hydrophila* isolates had RPS values of around 53.5 % and 35 % respectively, when challenged against three *A. hydrophila* isolates together (Chandran *et al.*, 2002a). They found RPS values of 48 % and 38 % with rohu and mrigal respectively, when fish were challenged with three pooled *A. hydrophila* isolates after immunisation with pooled ECP from those isolates. Azad *et al.*, (2000b) observed RPS values of 82-100 % in common carp immunised with an *A. hydrophila* biofilm (heat inactivated) vaccine, while an RPS value of 76-81 % was seen in fish immunised with heat inactivated free-cell suspension of *A. hydrophila*. An RPS value of 90.8-100 % was obtained with three batches of catfish, *Clarias batrachus* vaccinated with heat inactivated *A. hydrophila* biofilm and challenged with same *A. hydrophila* isolate compared with RPS values of 28.8-42.1 % for fish immunised with heat inactivated free cell suspension of *A. hydrophila* (Nayak *et al.*, 2004b). Although these attempts seem promising, the level of protection has only been investigated against one or a few isolates, and the vaccines do not appear to have been evaluated against a range of isolates to establish if they cross-protect against a range of *A. hydrophila* isolates.

Recently, attention has focused on recombinant protein vaccine production. This technique has a number of advantages over using the WC or separate components from a pathogen as vaccines. These protein vaccines are inexpensive, safe and mostly efficacious compared with the traditional vaccines (Clark and Cassidy-Hanley, 2005). One of the other major advantages is that this method of preparation avoids unwanted antigens from the pathogen in the vaccine compared to traditional vaccines (WC) where inclusion of all antigens may lead to suppression of the host immune system by some antigen. For example, some of the surface proteins (p22 and p57) of *Renibacterium salmoninarum* have been found to suppress the immune system of fish, and therefore, the WC preparations of this bacterium may not be used as vaccines (Fredriksen *et al.*, 1997). Recombinant protein vaccines, on the other hand, can induce a specific immunity against the particular antigen used, which could protect the host efficiently during infection (Potter and Babiuk, 2001). These vaccines have been developed for a variety of human and animal pathogens, for example, mice immunised with a recombinant *Salmonella typhimurium aroA* vaccine that secretes the naturally secreted 30 kDa protein of *Mycobacterium bovis* conferred protection against *M. tuberculosis* (Hess *et al.*, 2000). Protective immune response against piscine nodavirus was observed with sevenband grouper, *Epinephelus septemfasciatus* vaccinated by recombinant 42 kDa protein of the virus (Tanaka *et al.*, 2001). A mixture of recombinant proteins containing two heat shock proteins and one flagella protein of *Piscirickettsia salmonis* protected Atlantic salmon and exhibited a RPS value of 95 % when fish were challenged with the bacterium (Wilhelm *et al.*, 2006).

In this study, a recombinant S-layer protein of *A. hydrophila* was produced to confirm the protection efficacy of this protein in common carp against different isolates of *A. hydrophila*. The low quantity of protein eluted from SDS-PAGE gels was a major constraint for efficacy testing, and preliminary efficacy trials had to be conducted on very small numbers of fish as discussed in Chapter 3. A larger number of fish was vaccinated with recombinant S-layer protein of *A. hydrophila* in this Chapter to establish if this protein conferred protection. Vaccinated fish were then challenged with a range of different *A. hydrophila* isolates to confirm if the protein could cross-protect between the isolates.

The protein (S-layer) identified by MALDI-TOF MS is the outermost component of the cell envelope of bacteria and such structures are widely distributed throughout the prokaryotic kingdom. The S-layer proteins are composed of regularly arranged protein or glycoprotein monomers, which self-assemble into a precise supramolecular structure enclosing the bacterial cell (Koval, 1988; Thomas and Trust, 1995a). The unit cell dimension of the S-layer is approximately 12 nm in *A. hydrophila* (Murray *et al.*, 1988). The S-layer often tends to be lost during prolonged cultivation of the bacterium in the laboratory (Sleytr and Messner, 1983 and 1988).

Six bases were found to be different in the gene encoding S-layer protein from *A. hydrophila* T4 isolate compared with the S-layer genome sequence of isolate TF7 reported by Thomas and Trust (1995a). This in turn could result with changes in 4 amino acids in the S-layer protein of *A. hydrophila* isolate T4 compared with S-layer amino acid sequence reported for isolate TF7. However, this needs to be further characterised by nucleotide sequencing of the amplified gene encoding S-

layer protein from *A. hydrophila* T4 isolate and peptide mass fingerprinting of the overexpressed protein. These characterisation might resolve whether the sequence substitutions are the result of isolate variation or due to mis-incorporation of nucleotides by Taq polymerase during amplification. Such changes of amino acids in a particular gene are reported to be involved in modifications of some functional properties of the gene (Tran *et al.*, 2000). For example, an amino-acid substitution (from Gly in the wild type to Asp in the mutant) in *pufl* gene of purple bacteria, *Rubrivivax gelatinosus* coding for the L subunit of the reaction centre conferred resistant to an herbicide, terbutryn (Ouchane *et al.*, 1995). Similarly, an increased sensitivity to oxidative stress was found with a spirochetal bacterium, *Borrelia burgdorferi* when a single nucleotide substitution, converting an arginine to a lysine was observed with a mutated strain compared with a parent strain (Seshu *et al.*, 2004). Alterations in the amino acid sequence of a secreted protein (dense granule antigens) between strains of a parasite, *Toxoplasma gondii* plays an important role in both pathogenicity and antigenicity (Fazaeli *et al.*, 2000).

As with the natural S-layer protein isolated from *A. hydrophila*, the recombinant S-layer protein proved reactive in Western blot analysis against anti-*A. hydrophila* T4 common carp serum. Although it took some time to establish a recombinant S-layer protein, especially due to the standardisation of primers, PCR conditions, ligation and transformation, this method is more convenient and effective than the electro-elution of the S-layer protein from SDS-PAGE gels as a source of protein for vaccination studies. The recombinant protein was uncontaminated and the expression was consistently reproduced. Problems were encountered, however, during the development process, mainly in primer design with the selective

restriction enzymes used and vector transformation into competent cells. Initially, various sets of primers with different restriction enzymes; Eco RI and Nde I were investigated. These primers were lengthy with six histidine tag, stop codon, restriction enzyme (Nde I) and 4 'T' bases in the reverse primer (tgtggtacttgcgtaaggtagtagtagtagtaattgatactttt), while restriction enzyme and another 4 'T' bases were present in the forward primer (tttcatatgggtaactctggacactggtgc). Initial attempts to clone the 1353 bp fragment encoding the S-layer protein from *A. hydrophila* T4 isolate was unsuccessful due to the ineffective ligation of the amplified S-layer gene using these primers and 'T' vectors. Finally, these problems were resolved as the effective ligation of the amplified gene and the pQE 60 vector was attained when the primers with restriction enzymes mentioned in the Figure 4.1 were used for the amplification of S-layer gene. Transformation of the pQE 60 vector carrying the amplified S-layer gene into competent cells was also successful after using these primers for the S-layer gene amplification.

Variations in the degree of virulence was observed between different isolates of *A. hydrophila* during determination of the LD₅₀ values in the virulence study carried out in Chapter 2. Causes for variation in virulence of different *A. hydrophila* isolates has been previously explained by Santos *et al.* (1988), Zhang *et al.* (2000) and Sirirat *et al.* (1999). The authors found a wide variation in the gene expression of different *A. hydrophila* isolates, which in turn lead to different expression of virulent factors, such as extracellular products (ECP) and surface proteins. In this study, the lowest virulence was seen with isolate T4 and the highest virulence with isolate B2/12. High mortality rate was observed both in vaccinated and control

group within two days post-challenge compared with the mortality from Day 3 post-challenge as described in Figure 4.8. This result suggests that artificial challenge with *A. hydrophila* could result in sudden mortality of fish due to toxic shock (Allan and Stevenson, 1981; Chakraborty *et al.*, 1984; Khalil and Mansour, 1997; Perez *et al.*, 2002). Such a pattern of mortalities may not occur during a natural infection, and efficacy testing of the recombinant S-layer protein vaccine produced in this Chapter may therefore perform better against natural infection. The protection elicited by the S-layer protein in vaccinated fish indicates a potential role for this protein in the virulence of *A. hydrophila*.

The pathogenicity of *A. hydrophila* is mostly due to surface properties and extracellular enzymes (Nord *et al.*, 1975; Larsen and Jensen, 1977; Dooley and Trust, 1988; Santos *et al.*, 1988; Favre *et al.*, 1993; Thomas and Trust, 1995a and b). The virulence of the S-layer protein of *A. hydrophila* has previously been reported by Dooley *et al.* (1986), Sakata and Shimojo (1991) and Thomas and Trust (1995a and b). As a major surface protein, the S-layer (with a molecular weight of 52 kDa) has been associated with isolates of *A. hydrophila* with high levels of virulence (Dooley *et al.*, 1985). It is a predominant cell surface protein seen in the SDS-PAGE profiles of WC lysates and outer membrane fractions of *A. hydrophila* (Murray *et al.*, 1988). The S-layer of *A. hydrophila* has the ability to bind to host proteins such as fibronectin, laminin and vitronectin, as well as providing resistance to serum killing and protease digestion (Noonan and Trust, 1997).

This S-layer protein has been studied in various bacteria because of its role as one of the major virulence factors in bacteria, and helps the bacterium to evade

the host's immune system (Thompson, 2002). Moreover, being on the outermost layer of the bacterium, the S-layer protein has more possibility of interacting readily with the host than any other protein components of the bacterium (Koval, 1988; Thomas and Trust, 1995a). This property could be one reason why the S-layer protein appears to be more immunogenic than other proteins in the bacterium. A number of authors have studied the S-layer protein in *Aeromonas*, mainly examining its virulence and its evasion of the host's immune system. For example, Kokka *et al.* (1992) found that *A. hydrophila* strains belonging to the serogroup O:11 had an S-layer protein, and suggested that the S-layers may provide protection for bacteria in their natural environment or may provide a selective advantage in the ability of bacterium to cause infection. The presence of S-layer protein among highly virulent strains of *A. hydrophila* had also been confirmed by Thomas and Trust (1995a and b). Diseases in humans and animals (particularly fish) caused by *A. hydrophila*, possessing S-layers are often associated with invasive systemic infection (Janda *et al.*, 1994a).

Virulence characteristics of other *Aeromonas* species have also been reported as being associated with surface layers of the bacterium, for example those playing a role in binding with iron-containing proteins of the host (Ascencio *et al.*, 1992). The S-layer of *A. salmonicida* had been shown to be a prerequisite for virulence, by increasing hydrophobicity and enhancing macrophage association to efficiently adhere, enter, and survive within macrophages (Garduno *et al.*, 2000). The S-layer of *A. salmonicida* physically protects the cell against bacteriophages, proteases, as well as immune and non-immune complement and is required for macrophage infiltration and resistance (Kay and Trust, 1991). Chang *et al.* (1992)

showed that resistance of *A. salmonicida* to complement-mediated lysis was acquired by having an S-layer in conjunction with lipopolysaccharide (LPS). Kay and Trust (1991) confirmed that mutants containing a disorganized S-layer are avirulent and are able to confer significant protection to salmonids when applied by immersion exposure. Our studies indicate that the S-layer protein antigen of *A. hydrophila* appears to have conferred protection against the different isolates of *A. hydrophila* tested, although the RPS values of carp did vary between the different challenge isolates.

No mortalities occurred in any of the groups of fish after Day 11 post-challenge in the vaccination trial described in this Chapter. Moreover, no colonies of *A. hydrophila* grew from the kidney swabs taken from surviving fish at the end of experiment except for two fish. This suggests that most of the surviving fish in the control group had cleared the bacterium through their own immune response, as fish can produce an antibody response against different components of bacterium and clear the bacteria in blood circulatory system within seven days post-infection (Leung and Stevenson, 1988b; Chandran *et al.*, 2002b).

Recombinant outer membrane proteins (OMPs) have been tested as possible vaccine antigens for *A. hydrophila*. Fang *et al.* (2004) showed significant protection against two isolates of *A. hydrophila* in blue gourami, *Trichogaster trichopterus* (75 and 87.5 % RPS) immunised with a recombinant 43 kDa OMP. More recently, a recombinant OMP (37 kDa) of *A. hydrophila* was produced and was shown to be immunogenic in rohu carp (Khushiramani *et al.*, 2007a). Fish vaccinated with this recombinant OMP showed a RPS value of 57 after

challenging the fish with a virulent *A. hydrophila* isolate (Khushiramani *et al.*, 2007b). Cross-protection against a range of *A. hydrophila* isolates was not reported. The protection obtained in the present study, by the recombinant S-layer protein antigen against 6 different isolates of *A. hydrophila*, suggests that it may be a suitable vaccine against a range of *A. hydrophila* isolates.

In summary, the results from this Chapter suggest that the S-layer protein of *A. hydrophila* may be important in inducing specific antibodies that confer protection in common carp against a variety of different virulent isolates of *A. hydrophila*. The S-layer protein was also shown to protect against an *A. hydrophila* challenge in goldfish in Chapter 3. The protection obtained in goldfish and common carp against *A. hydrophila* suggests that the recombinant S-layer protein of *A. hydrophila* might protect different species of fish from infection by this pathogen in aquaculture systems.

Chapter 5. Discussion and final conclusions

Aeromonas hydrophila is an important fish pathogen (Merino *et al.*, 1995; Rahman and Kawai, 2000; Vivas *et al.*, 2005) that has been reported to cause a wide range of diseases in fish and shellfish (Tanasomwang and Saitanu, 1979; Karunasagar *et al.*, 1989; Borrel *et al.*, 1998; Nielsen *et al.*, 2001) in the aquatic environment. Farmers have found it difficult to treat *A. hydrophila* infections in fish due to the resistance of this pathogen to a number of antibiotics (Shariff, 1998; Dixon *et al.*, 1990; Vivekanandhan *et al.*, 2002). Thus, researchers have been trying to develop a vaccine to protect fish against diseases caused by *A. hydrophila*, but this has proven difficult mainly because of the heterogeneity of the pathogen (Lamers *et al.*, 1985; Baba *et al.*, 1988b; Leung *et al.*, 1997; Rahman and Kawai, 2000).

A variety of approaches have been used to try to develop an effective vaccine against *A. hydrophila*. Various preparations of *A. hydrophila*, such as whole cell (WC) (killed, live and live attenuated), extracellular products (ECP), outer membrane protein (OMP) and lipopolysaccharide (LPS) have been found to be protective. In most studies the efficacy of the vaccine was performed using only one isolate (homologous strains) and it is not known if these vaccines will cross-protect against other isolates (Baba *et al.*, 1988b; Leung *et al.*, 1997; Hernanz *et al.*, 1998; Azad *et al.*, 1999; Rahman and Kawai, 2000; Nayak *et al.*, 2004). Among the vaccines evaluated, the highest relative percentage survival (RPS) of 100 % was found with an inactivated biofilm vaccine for the bacterium (Azad *et al.*, 1999; Nayak *et al.*, 2004). Only a few studies have addressed the issue of cross-protection. For example, protection against two isolates of *A. hydrophila* was reported in fish immunised with a plasmid-free *A. hydrophila* mutant (Majumdar *et al.*, 2006), while Fang *et al.* (2000) found protection against two heterologous

isolates in gourami (*Trichogaster trichopterus*) vaccinated with OMP (43 kDa) of *A. hydrophila*. Generally, the levels of protection against a range of isolates have not been evaluated despite the antigenic diversity of *A. hydrophila* being highlighted as a major problem for developing a successful vaccine (Karunasagar *et al.*, 1991; Loghothesis and Austin, 1996a; Rahman and Kawai, 2000). In addition, from the work conducted by Baba *et al.* (1988b) it appears that antigenic components may be denatured in *A. hydrophila* when vaccines are used as inactivated WC preparations. They found increased and long lasting protection in fish immunised with LPS of *A. hydrophila* in comparison to protection achieved in fish immunised with formalin inactivated WC vaccine. Most of the vaccines (discussed earlier in this paragraph) have been developed on an experimental scale and have not been field-tested for commercialisation, possibly due to the fact that licensing vaccines is a long and complicated process, also requiring field trials or it may not be cost effective to produce a commercial vaccine. However, Chandran *et al.* (2000a) investigated the protection efficacy of a polyvalent vaccine containing inactivated WC and ECP of *A. hydrophila* in the field using two species of Indian major carp (rohu, *Labeo rohita* and Mrigal, *Cirrhinus mrigala*) but the vaccine failed to protect the fish upon artificially challenging them with the bacterium. Therefore, in the present study the approach of using immunoproteomic techniques was used to identify a common protective antigen between *A. hydrophila* isolates (cultured *in vitro* and *in vivo*) in order to develop an effective vaccine against the pathogen. Recombinant protein production technology was then utilised to produce sufficient quantities of the immunogenic protein to carry out vaccine efficacy trials, using a variety of *A. hydrophila* isolates to challenge the fish.

Electrophoresis (1 and 2 dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS-PAGE) was used in the current study to determine WC, OMP and ECP profiles of different isolates of *A. hydrophila* grown *in vitro* and *in vivo*. Generating reproducible 2D gels was difficult initially and it took considerable time to attain gel consistency before accurate analysis of the protein profiles could be made. Moreover, the presence of streaking in the 2D gels affected the position of spots and clarity of the gels. The 2D gels were repeated several times using a variety of modifications for sample preparation to eliminate this streaking problem. This was eventually eliminated by incubating the bacterial samples with DNase and RNase while preparing the samples for 2D SDS-PAGE (Berkelman and Stenstedt, 2002). The use of silver staining was very effective in staining the 2D SDS-PAGE gels, and made visualising the smaller spots easier. Similarly, the software for 1 and 2D gel analysis was extremely effective for differentiating the location of the bands and spots expressed. The 2D SDS-PAGE was more informative than the 1D SDS-PAGE with reference to up and down-regulation of proteins from bacteria cultured *in vitro* and *in vivo*, as this method separated the proteins with respect to their pI and MW, while 1D analysis is based on MW only.

Up and down-regulation or expression of unique proteins *in vivo* has been reported for a variety of bacterial pathogens affecting fish, such as *A. salmonicida*, *Vibrio salmonicida* and *Photobacterium damsela* subsp. *piscicida*, when these were cultured in dialysis bags implanted within the peritoneal cavity of different fish (Jung, 1999; Colquhoun and Sorum, 1998; Dacanay *et al.*, 2003; Wang *et al.*, 2004; Ebanks *et al.*, 2004; Bakopoulos *et al.*, 2004). Such up-regulated or unique

proteins expressed by the pathogen *in vivo* (in the host) have been reported to be associated with virulence (e.g. *A. salmonicida*) (Ebanks *et al.*, 2004). In some cases, the growth of bacteria *in vivo* has been imitated *in vitro* by using iron-restricted culture medium (obtained by adding the iron-chelator (e.g. 2,2'-dipyridyl) to the medium), glucose rich medium, as well as autolysate of fish viscera medium. Such modified *in vitro* systems have been used to investigate changes in bacterial expression (e.g. *A. hydrophila*, *A. salmonicida* and *Edwardsiella tarda*) to try to identify molecules that may be useful for vaccine development (Aoki and Holland, 1985; Hirst and Ellis, 1994; Martinez *et al.*, 1995; Aguilar *et al.*, 1999). For example, the antigens expressed by *A. salmonicida* grown under iron-restricted culture conditions have been shown to be immunogenic and protective (Ellis *et al.*, 1997). In the current study, a selection of virulent and avirulent isolates of *A. hydrophila* were cultured *in vitro* and *in vivo* to investigate the up and down-regulation of proteins for the identification of potential common antigens for vaccine development. Virulent isolates of *A. hydrophila* cultured *in vivo* expressed bands of increased intensity at 70, 55, 50 and 25 kDa in the 1D SDS-PAGE with WC preparations, while unique bands at 58 and 55 kDa for WC and OMP preparations, respectively, were observed for all the isolates cultured *in vivo*. Analysis of WC preparations of *A. hydrophila* by 2D SDS-PAGE showed a number of unique and up-regulated spots in the bacteria grown *in vivo*, mostly between 30 and 80 kDa with pI values ranging from 5.0-6.0 (Table 2.5 and 2.6).

Differences in the formation of bacterial capsules have also been reported between bacteria cultured *in vitro* and *in vivo*. Bacterial capsules may promote the adherence of the bacterium to different surfaces to facilitate colonisation (Roberts,

1996). It was reported that such capsule formation in *A. hydrophila* can confer serum resistance, and thus it can protect the bacterium from the defence mechanisms of the host (Aguilar *et al.*, 1999). Capsular expression has been seen in *A. hydrophila* recovered from artificially infected trout (Mateos and Paniagua, 1995). In addition, culture environments mimicking growth conditions *in vivo* (*i.e.* yeast extract peptone glucose mineral salts medium and autolysate of fish viscera medium) have also been shown to induce the formation of such capsules in *A. hydrophila* and *A. salmonicida* (Merino *et al.*, 1996b; Aguilar *et al.*, 1999). Capsule formation was also seen in the present study with *A. hydrophila* grown *in vivo* compared with bacteria grown *in vitro*.

Antibodies raised against bacteria are useful tools to assist in the identification of potential vaccine antigens. Researchers have explored a wide range of approaches using antibodies to determine vaccine candidates for *A. hydrophila*. For example, agglutination, immunodiffusion and immunoelectrophoresis assays were used to screen different components of *A. hydrophila* (lipopolysaccharide (LPS), exopolysaccharide, flagella, S-layer protein and haemolytic components of ECP) using antibodies raised against inactivated and live bacteria in rabbits and trout, respectively, to identify immunogenic components (Loghothetis and Austin, 1994 and 1996b). Antibodies have also been raised against inactivated *A. hydrophila* in rabbits and used to screen LPS and OMP from *A. hydrophila* by Western blot (Dooley *et al.*, 1986; Sendra *et al.*, 1997). In another approach, different OMP fractions were screened using enzyme linked immunosorbent assay (ELISA) with antibodies raised against crude OMP of *A. hydrophila* in rabbits in search of immunogenic antigens (Maji *et al.*, 2006). Jung (1999) suggested raising

antibodies against live bacteria in the host (fish) and using these to screen bacteria cultured *in vivo* to accurately identify potential protective antigens. This was in contrast to using inactivated bacteria for raising the antibodies and then using these to screen bacteria grown *in vitro*. The author also highlighted that potential immunogenic antigens may not be recognised by the antibodies raised against live bacteria in fish if the bacteria used for screening had been grown *in vitro* (i.e. the appropriate antigen may not be expressed *in vitro*). Therefore, in the present study, antibodies were raised against different live isolates of *A. hydrophila* in common carp (*Cyprinus carpio*), in order to acquire host antibodies against the proteins which are expressed *in vivo*. These were then utilised to identify potential protective antigens of *A. hydrophila* using various preparations (WC, OMP and ECP) of different *A. hydrophila* isolates grown *in vitro* and *in vivo*. A common protein of molecular mass 50 kDa was found to be expressed in the bacteria grown under two growth conditions when the preparations were screened with the antibodies raised against the corresponding isolates in common carp (Chapter 3). Similarly, a common protein of molecular mass 50 kDa was also found in most WC preparations of virulent and avirulent isolates (14 isolates) cultured *in vitro* when screened with pooled sera raised against six different isolates (4 virulent and 2 avirulent). This 50 kDa protein was identified as the S-layer protein of *A. hydrophila*. The 2D Western blot performed with a WC preparation of isolate T4 provided additional information on the expression of the antigens recognised by the fish sera. There appears to be post-translational modification (addition of other biochemical functional groups such as acetate, phosphate, various lipids and carbohydrates with proteins) of the S-layer protein, since three spots were found very close together at 50 kDa but with slightly

different pI values (Figure 3.4). Although such modifications can change the chemical nature of proteins all three spots did react with the fish anti-sera suggesting that the immunogenic region of the S-layer protein was conserved. Thomas and Trust (1995a) showed post-translational modification of the *A. hydrophila* S-layer protein due to phosphorylation (addition of a phosphate (PO₄) group to S-layer protein), and reported that this process can result in a change in pI values as well as the MW of the S-layer protein. It has been reported that post-translational modification of expressed bacterial proteins might alter function of the protein in relation to its virulence (Cozzone *et al.*, 2004). For example, a *Helicobacter pylori* protein (Cag A), causing gastric carcinoma in humans is reported to be more virulent following post-translational modification (by phosphorylation) (Higashi *et al.*, 2002).

Direct and passive immunisation have been used by a number of researchers to determine the protection elicited by different bacterial vaccines in fish (Kusuda *et al.*, 1987; Yin *et al.*, 1997; Irie *et al.*, 2005; Akhlaghi, 1999; Shelby *et al.*, 2002). For direct immunisation, various types of antigens were used such as killed, attenuated and live *A. hydrophila*, and different components (OMP and LPS) (Baba *et al.*, 1988b; Leung *et al.*, 1997; Rahman and Kawai, 2000; Chandran *et al.*, 2002a; Vivas *et al.*, 2004b; Nayak *et al.* 2004b). In passive immunisation, anti-sera against a variety of pathogens, raised in different animals such as fish, sheep and rabbits have been used (Akhlaghi, 1999; Shelby *et al.*, 2002; Pasnik *et al.*, 2006). In the present study, goldfish were vaccinated (preliminary trials) with the 50 kDa protein from *A. hydrophila* (electro-eluted from the SDS-PAGE gel) and then challenged with one of the most virulent isolate (T4) to assess protection prior

to up-scaling investigations with this protein (Chapter 3). Similarly, fish were also passively immunised with goldfish serum raised against 50 kDa protein of *A. hydrophila* and then challenged to test protection efficacy. The 50 kDa protein appeared to confer protection in goldfish in these small-scale studies. The RPS value was 67 % in the vaccination trial, while the results of the passive immunisation trial were inconclusive due to the small number of fish used and the fact that only 33 % mortality occurred in the control group, however no mortalities occurred in the passively immunised fish.

The antigen used for vaccination and for raising the serum for passive immunisation trial (*i.e.* 50 kDa protein) was prepared by electro-elution from the WC SDS-PAGE of *A. hydrophila* (T4 isolate). Although it was possible to elute sufficient amounts of protein from the gel for a small trial, it was a very laborious process as a number of gels had to be run to harvest enough protein (100 µg). The protection achieved in the goldfish immunisation experiment suggests that the antigen identified in the present study (50 kDa) elicits a protective antibody response. The Western blot results indicate that using live bacteria for raising the antibodies in fish might be a more valuable approach rather than using killed or inactivated bacteria, when identification of protective vaccine candidates are sought. This is in agreement with Jung (1999).

It has been shown that matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is a powerful tool to identify proteins within a short time compared with the traditional approaches such as nucleic acid sequencing (Chen *et al.*, 2004; Ying *et al.*, 2005). This technology was used in the

current study to confirm that 50 kDa protein was the S-layer protein of *A. hydrophila*. The S-layer protein has been reported to be a major virulence factor of *A. hydrophila* as discussed in Chapter 1, while it is also found to be an important virulence factor for many other bacteria such as *Clostridium botulinum*, *A. salmonicida*, *Campylobacter fetus* subsp. *fetus*, *C. rectus* and *Mycobacterium bovis* (Boulanger *et al.*, 1977; Messner and Sleytr, 1992). It assists pathogens in evading host immune responses and provides resistance to serum killing and protease digestion in *Aeromonas* and *Campylobacter* species (Noonan and Trust, 1997; Thompson, 2002).

Localisation of the S-layer protein on the surface of the bacterium and its contribution in infection could be one reason why this molecule elicited protection against *A. hydrophila* in goldfish. The surface components of bacteria can be recognised by the fish immune system soon after infection occurs and thus the fish could elicit antibodies to encounter the pathogen. Although, it has been reported that bacterial S-layer proteins are lost during culture *in vitro*, work done with *Wolinella recta* (a human Gram-negative bacterial pathogen) suggests that it may not be completely lost (Borinski and Holt, 1990). They found that the S-layer proteins were missing or faintly stained in both 1 and 2D SDS-PAGE with the isolate passaged *in vitro* a number of times compared with the isolate passaged several times *in vitro*. In the current study, the S-layer proteins appeared to be up-regulated in bacteria grown *in vivo* compared with bacteria cultured *in vitro* (Figures 2.4, 2.5 and 2.6b). The down-regulation of the S-layer protein of *A. hydrophila* cultured *in vitro* and post-translational modification of the S-layer protein may be important factors involved in both heterogeneity and pathogenicity.

Recombinant DNA technology is considered to be a useful tool in speeding up the development of vaccines and the drug discovery process for animals and fish (Munn, 1994; Chakravarti *et al.* 2000; Potter and Babiuk, 2001). It has several advantages compared with the production of traditional vaccines, as discussed in Chapter 4, including large yields of a consistent protein in a short period of time, low production cost, safety and efficacy. However, incorrect protein folding (denatured protein), low expression of proteins due to problems with efficiency of promoters in plasmids, and the translational efficiency of *E. coli* mRNAs are some times encountered during recombinant protein production (De Bernardez Clark, 1998; Baneyx, 1999). In the current study, the gene encoding S-layer protein was cloned and overexpressed and was found to react with the antibodies raised against live *A. hydrophila* in the Western blot (Figure 4.7). However, there were difficulties with the primer design for DNA amplification, the restriction enzymes used for ligating vectors with the S-layer protein genes and transforming the vectors into competent cells. These factors were overcome after designing suitable primers and using different restriction enzymes; the modifications adapted during the process were discussed in Chapter 4.

Recombinant protein vaccines have been shown to protect humans and other animals (including fish) from a variety of pathogens, including bacteria, *Mycobacterium tuberculosis* (Skeiky, *et al.*, 2004), parasites (*Plasmodium falciparum*) (Saul *et al.*, 2005) and virus e.g. infectious pancreatic necrosis virus (IPNV) (*Aquabirnavirus* sp.) (Biering *et al.*, 2005). Recombinant protein vaccines have also been developed for different other virus of the *Rhabdoviridae* family in fish, such as infectious hematopoietic necrosis virus (IHNV), viral haemorrhagic

septicaemia virus (VHSV) and spring viraemia of carp virus (SVCV) but these have not yet been commercialised due to limited protection (Noonan *et al.*, 1995; Cain *et al.*, 1999). The recombinant protein vaccine for IPNV is commercially available in Norway and Chile (Biering *et al.*, 2005). Licensing vaccines for commercial use, on the other hand, is a problem globally due to the number of regulations to be followed in aspects of safety, purity, potency, efficacy, handling, storing and administration of the vaccines (Sethi *et al.*, 1997; Midtlyng, 2005). Important control measures such as limitation on the use raw materials/chemicals for culturing bacteria and methods for production of recombinant proteins, including removal of antibiotic resistance genes in the expression vector, and bacteria are also a constraint for licensing recombinant protein vaccines (Personal communication, Prof. Patrick Smith, Schering-Plough Aquaculture, UK). In the current study, the recombinant S-layer protein conferred protection in common carp against all six virulent isolates examined with the RPS values ranging from 56 to 87 % between the different isolates of *A. hydrophila*. Protection was considered significant only for isolates T4, 98140, 98141 and Hh compared with Vds and B2/12. Isolate B2/12 was the most virulent strain used although the levels of virulence of other isolates are not consistent compared to each other between virulence and LD₅₀ determination trials (Tables 4.1 and 4.2 and Figure 2.3). The variation in protection obtained suggests that there might be a need for a second antigen to be added to the vaccine for increasing the vaccine's efficacy and improve cross-protection against isolates such as Vds and B2/12. For example, OMPs (43 and 37 kDa) and LPS of *A. hydrophila* could possibly be selected as additional antigens as these components have been shown to be immunogenic and protective in various investigations reported in the literature (Baba *et al.*,

1988b; Loghothetis and Austin 1996b; Fang *et al.*, 2004; Khushiramani *et al.*, 2007a and 2007b). Despite the variation in protection between challenge isolates, the recombinant S-layer protein vaccine may be a useful tool to help manage the disease problem in the aquaculture industry caused by *A. hydrophila* as the natural infection could be less severe compared to artificial challenge. However, further trials in the field using this recombinant S-layer protein vaccine are required to examine the accurate performance of the vaccine. Challenge against a larger number of isolates is essential to investigate cross-protection of the vaccine prior to commercialising the vaccine. In addition, the antibiotic resistance gene from the plasmid used in the current study needs to be eliminated before expressing the proteins for commercialisation.

Problems with the development of successful traditional WC vaccines against *A. hydrophila* could be due to the expression of inappropriate antigens on bacteria grown *in vitro*. These antigens may not be represented on the bacteria, *in vivo*, during infection and thus protection is poor or not effective against a wide range of isolates. Although the S-layer protein appears to be present in *A. hydrophila* grown *in vitro* and *in vivo* it does appear to be up-regulated *in vivo*. The conformation of the S-layer protein may also be important for attaining protection. However, further work is required to fully evaluate the role of the S-layer protein in conferring protection.

In conclusion, the results from the present study suggest that it is potentially achievable to develop a commercial vaccine against *A. hydrophila* which will overcome the issues of the heterogeneity of the bacterium. It may be possible to

improve the vaccine by adding additional antigens to the formulation. The aquaculture industry desperately needs such a product to manage this devastating disease problem.

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Appendix I. Buffers and reagents

Phosphate buffered saline (pH 7.2)

| | |
|---|---------|
| NaH ₂ PO ₄ ·2H ₂ O (5.6 mM) | 0.876 g |
| Na ₂ HPO ₄ ·2H ₂ O (14.4 mM) | 2.56 g |
| NaCl (0.15 M) | 8.77 g |
| Distilled water | 1000 ml |

Reagents used for Gram's staining

Crystal violet solution

| | |
|------------------|-------|
| Crystal violet | 2 g |
| 95 % ethanol | 20 ml |
| Ammonium oxalate | 0.5 g |
| Distilled water | 80 ml |

Iodine Solution

| | |
|------------------|--------|
| Iodine | 1 g |
| Potassium iodide | 2 g |
| Distilled water | 300 ml |

Safranin Solution

| | |
|-----------------|--------|
| Safranin | 0.25 g |
| 95 % ethanol | 10 ml |
| Distilled water | 90 ml |

Alcohol / Acetone mixture

| | |
|---------|--------|
| Ethanol | 950 ml |
| Acetone | 50 ml |

Buffers used for Electrophoresis

Sample buffer (2 X)

| | |
|--------------------------|----------|
| Tris-Hcl (0.5 M, pH 6.8) | 2.5 ml |
| Glycerol (1 M) | 2.0 ml |
| SDS (10 % w/v = 0.35 M) | 4.0 ml |
| Dithiothreitol (0.2 M) | 0.31 g |
| Bromophenol blue | 2.0 mg |
| Distilled water | to 10 ml |
| Stored at -20°C | |

Reservoir buffer for 1D SDS-PAGE (5 X, pH 8.3)

| | |
|--------------------|--------|
| Tris base (123 mM) | 7.5 g |
| Glycine (0.96 M) | 36 g |
| SDS (17 mM) | 2.5 g |
| Distilled water | 500 ml |

Reservoir buffer for 2D SDS-PAGE (5 X, pH 8.3)

| | |
|--|-----------|
| Tris base(125 mM) | 7.575 g |
| Glycine (1 M) | 37.5 g |
| SDS (17 mM) | 2.5 g |
| Distilled water | to 500 ml |
| Filter the buffer through 0.45 µm filter | |

Separating gel buffer (pH 8.7)

| | |
|---------------------|--------|
| Tris base (1.5 M) | 91 g |
| SDS (0.4 %,13.8 mM) | 2 g |
| Distilled water | 500 ml |
| Stored at -4°C | |

Stacking gel buffer (pH 6.8)

| | |
|----------------------|--------|
| Tris base (0.5 M) | 6.05 g |
| SDS (0.4 %, 13.8 mM) | 0.4 g |
| Distilled water | 100 ml |
| Stored at -4°C | |

Separating gel (12 %)

| | |
|---|---------|
| Separating gel buffer | 24.90 % |
| Distilled water | 34.85 % |
| Acrylamide (30 % w/v) (29 % acrylamide + 1 % bis acrylamide) | 39.83 % |
| N,N,N,N-tetramethylethylenediamine TEMED (1 M) | 0.075 % |
| Ammonium persulphate (10 % = 438 mM) | 0.35 % |

Stacking gel (4 %)

| | |
|---|--------|
| Stacking gel buffer | 25 % |
| Distilled water | 61 % |
| Acrylamide (30 % w/v) (29 % acrylamide + 1 % bis acrylamide) | 13.4 % |
| TEMED (1 M) | 0.10 % |
| Ammonium persulphate (10 % = 438 mM) | 0.05 % |

Buffers used for ELISA

Coating buffer (pH 9.6)

| | |
|---|--------|
| Na ₂ CO ₃ (15 mM) | 1.59 g |
| NaHCO ₃ (38.9 mM) | 2.93 g |

Low salt wash buffer (10 X, pH 7.3)

| | |
|---------------------|---------|
| Trisma base (0.2 M) | 24.2 g |
| Nacl (3.8 M) | 222.2 g |
| Tween20 | 5 ml |
| Distilled water | 1000 ml |

High salt wash buffer (10 X, pH 7.7)

| | |
|---------------------|---------|
| Trisma base (0.2 M) | 24.2 g |
| Nacl (5 M) | 292.2 g |
| Tween20 | 10 ml |
| Distilled water | 1000 ml |

Antibody buffer

| | |
|-----|--------|
| BSA | 1 g |
| PBS | 100 ml |

Conjugate buffer

| | |
|----------------------------|--------|
| BSA | 1 g |
| Low salt wash buffer (1 X) | 100 ml |

Substrate buffer (pH 5.4)

| | |
|----------------------|---------|
| 0.1 M Citric acid | 21 g |
| 0.1 M Sodium acetate | 8.2 g |
| Distilled water | 1000 ml |
| Stored at -4 °C | |

Chromogen solution

| | |
|---|-----------|
| 3'3'5'5'-Tetramethylbenidine dihydrochloride (42 mM) | 0.07896 g |
| Acetic acid:distilled water (1:2) | 6 ml |
| Stored at -4 °C | |

Chromogen in substrate buffer

| | |
|-------------------------------|--------|
| Substrate buffer | 15 ml |
| Chromogen solution | 150 µl |
| H ₂ O ₂ | 5 µl |

Buffers used for Western blot

Transblot buffer (pH 8.3)

| | |
|-------------------|--------|
| Glycine (192 mM) | 14.4 g |
| Tris base (25 mM) | 3.03 g |
| Methanol | 200 ml |
| Distilled water | 800 ml |

Tris buffered saline (pH 7.5)

| | |
|--------------------|---------|
| Tris base (0.02 M) | 2.42 g |
| NaCl (0.5 M) | 29.24 g |
| Distilled water | 1000 ml |

Tris buffered saline with Tween

| | |
|----------------------|---------|
| Tween 20 | 1 ml |
| Tris buffered saline | 1000 ml |

Substrate solution (10 X)

| | |
|-----------------------------|--------|
| 4-chloro-naphthol | 0.15 g |
| Methanol | 50 ml |
| Stored in the dark at -20°C | |

Appendix II. Mixtures and vector used for recombinant protein production

PCR mixture (40 μ l volume with 32 cycles)

| | |
|------------------------|--------------|
| Genomic DNA | 3 μ l |
| Primer forward | 1.0 μ l |
| Primer reverse | 1.0 μ l |
| dNTP | 5 μ l |
| 10 x buffer | 4 μ l |
| Taq DNA polymerase | 0.5 μ l |
| Double distilled water | 24.5 μ l |

Colony PCR mixture (10.5 μ l volume with 32 cycles)

| | |
|------------------------|-------------|
| Small pinch of colony | |
| Primer forward | 0.2 μ l |
| Primer reverse | 0.2 μ l |
| dNTP | 1 μ l |
| 10 x buffer | 1 μ l |
| Taq DNA polymerase | 0.1 μ l |
| Double distilled water | 8 μ l |

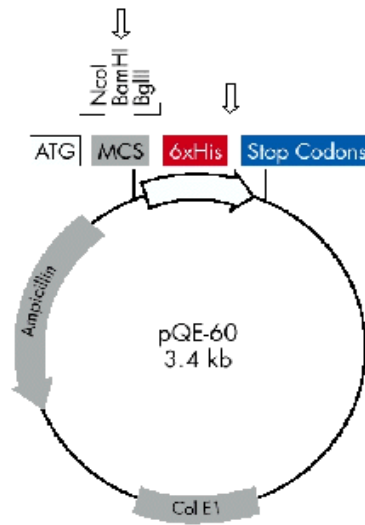
PCR products digestion mixture

| | |
|------------------------|-------------|
| Purified PCR products | 10 μ l |
| 10 x buffer (H) | 1 μ l |
| Nco I enzyme | 0.5 μ l |
| Bgl II enzyme | 0.5 μ l |
| Double distilled water | 8 μ l |

Vector digestion mixture

| | |
|------------------------|-------------|
| pQE 60 vector | 2 μ l |
| 10 x buffer (H) | 1 μ l |
| Nco I enzyme | 0.5 μ l |
| Bgl II enzyme | 0.5 μ l |
| Double distilled water | 8 μ l |

Map of the expression vector pQE 60



Appendix III. Publications and presentations from the project

Poobalane S., Thompson K.D., Diab A., Ardo L., Jeney G., Smith P. and Adams A. Protein expression by *Aeromonas hydrophila* during growth *in vitro* and *in vivo*. *Microbial pathogenesis* (Submitted).

Poobalane S. *Aeromonas hydrophila* vaccine development using immunoproteomics. Oral presentation, Departmental seminar, 29.11.2006, Institute of Aquaculture, University of Stirling, Scotland, UK.

Poobalane S., Thompson K. D., Jeney G., Jeney Z. and Adams A. Antigenic analysis of different strains of *Aeromonas hydrophila* grown *in vitro* and *in vivo*. Oral presentation, European Association of Fish Pathologist 12th International conference, 11-16 September 2005, Copenhagen, Denmark. Book of Abstracts p. 88.

Poobalane S., Thompson K. D. and Adams A. Proteomic and immunological analysis of *A. hydrophila* for vaccine production. Orals presentation, Scottish fish immunology research centre seminar day, 14.06.2005, Institute of Aquaculture, University of Stirling, Scotland, UK.