Characterisation of *Vibrio anguillarum* for the development of vaccine in cod (*Gadus morhua*).

A THESIS SUBMITTED TO THE UNIVERSITY OF STIRLING FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

by

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DEC 2008

To Sarah and my parents,

Bénédicte and Benoît, who encouraged me in my love of science.

DECLARATION

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

Remi M. L. Gratacap

ACKNOWLEGEMENTS

I would like to thank my supervisors Dr. Kim Thompson, Professor Alexandra Adams and Professor Ian Bricknell for their assistance and encouragement throughout the course of this study. I would like also to express my gratitude to Professor Patrick Smith and Dr. William Roy, for their insights and practical approach in this project.

I would like to acknowledge Mrs Gillian Dreczkowski, Dr. Margaret Crumlish, Dr. Alison Morgan, Mrs Hilary McEwan, Mrs Karen Snedden, Professor Hugh Ferguson, Dr. Dave Morris, Dr. James Bron, Dr. Charlie McGurk, Dr. Janina Costa, Dr. Saravanane Poobalane, Mr Niall Auchinachie, Mr Robert Aitkin, the staff of the Institute of Aquaculture and of Machrihanish fish farm. I am also thankful to my friends and fellow PhD students for helping me with various aspects of this thesis, most of all Ms Sarah Barker and Dr. Farah Manji.

I am very grateful to the sponsors of this project, Intervet-Schering-Plough and SeaFish for the contribution in making this thesis a reality; I would like to thank the Fishery Society of the British Isles (FSBI), the Scottish International Education Trust (SIET) and the Institute of Aquaculture (Stirling University) for travel grants to present some of this work at international conferences.

I finally thank all the researchers who sent me isolates of *Vibrio anguillarum* and allowed this study to take place: Dr. Dawn Austin (UK), Dr. Jeremy Carson (AUS), Dr. Ysabel Santos (ES), Dr. Duncan Colquhoun (NO), Dr. Nicky Buller (AUS), Dr. Christian Michel (FR), Dr. Philippe Roch (FR), Dr. Amedeo Manfrin (IT), Dr. Harry Birkbeck (UK), Dr. Lene Bay (DK), Dr. Luc Grisez (BE) and Dr. Saturo Nakano (JP).

ABBREVIATIONS

µL: microlitre
µg: microgram
µm: micrometre
A: ampere
AFLP: amplified fragment length polymorphism
BPI: bactericidal/permeability-increasing (protein)
CD: cluster of differentiation
cfu: colony forming units
CRP: C-reactive protein
CSF: colony stimulating factor
Da: Dalton
DGGE: denaturing gradient gel electrophoresis
DIVA: differentiating infected and vaccinated animals
DNA: deoxyribonucleic acid
dph: days post hatch
ECP: extracellular products
e.g.: example
ELISA: enzyme-linked immunosorbant assay
et al.: et alias (and others)
FCS: forward scatter channel
g: gram
X g: multiple of gravity
h: hours
HMW: high molecular weight
HSW: high salt wash-buffer
i.e. id est (that is)
IFN: interferon
Ig: immunoglobulin
IL: interleukin
IP: intraperitoneal
ISH: *in situ* hybridisation
IU: international units
kb: kilo base
L: litre
LBP: lipopolysaccharide binding protein
LD: lethal dose
LMW: low molecular weight
LSW: low salt wash-buffer
LPS: lipopolysaccharides
M: molar
mAb: monoclonal antibody
mg: milligram
MHC: major histocompatibility
min: minutes
mL: millilitre
mm: millimetre
MMW: medium molecular weight
MOMP: major outer membrane protein
MW: molecular weight
nm: nanometre
NMR: nucleic magnetic resonance
OD: optical density
pAb: polyclonal antibody
PAMP: pathogen associated molecular pattern
PBS: phosphate buffer saline
PCR: polymerase chain reaction
PFGE: pulse field gel electrophoresis
PRR: pattern recognition receptors
RAG: recombinant activator gene
RAPD: random amplified polymorphic DNA
RNA: ribonucleic acid
ROS: reactive oxygen species
rpm: rounds per minutes
RPS: relative percentage survival
RT: room temperature (18-22°C)
SAP: serum amyloid-P
SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec: seconds
SH: serum haemolysis
sp.: species
TBS: tris buffer saline
TGF: transforming growth factor
TLR: toll-like receptor
TNF: tumour necrosis factor
TSA: tryptone soy agar
TSB: tryptone soy broth
TTBS: tween tris buffer saline
T: tonne
V: volt
VAR: *Vibrio anguillarum* related (organisms)
v/v: volume/volume
w/v: weight/volume

ABSTRACT

Atlantic cod (*Gadus morhua* L.) is one of the most promising new fish species introduced to cold water aquaculture due to the large established market in Europe and the USA and the decline in wild stock. So far, the production of farmed cod has been relatively low, with the main hindrance due to diseases. *Vibrio anguillarum* has been recognised as the biggest disease problem of farmed cod and has slowed the development of a successful cod aquaculture industry.

When the first incidences of *V. anguillarum* occurred in cod aquaculture, vaccines designed for vibriosis in Atlantic salmon (*Salmo salar* L.) were used in an attempt to combat the disease. However, these vaccines did not provide sufficient protection, possibly because they lacked serotype O2b, which is known to affect cod and to a lesser extent salmonids. Recently, vibriosis vaccines specifically designed to protect Atlantic cod have been formulated, but outbreaks of vibriosis in vaccinated fish are still being reported, suggesting that these formulations are inadequate. The aim of this project was to develop a whole cell inactivated vaccine formulation specifically tailored to protect Atlantic cod against *Vibrio anguillarum*.

The serological classification of *V. anguillarum* was first investigated by producing a set of monoclonal antibodies (mAbs). Using lipopolysaccharides (LPS) extracted with butan-1-ol, 4 mAbs were selected and shown to react specifically with *V. anguillarum* serotypes O1, O2a and O2b. A collection of over 150 *V. anguillarum* isolates were screened using these, which revealed that most of the isolates had been previously correctly classified. A new sub-serotype of *V. anguillarum* O2 was identified from isolates recovered from outbreaks of vibriosis in Norway as well as Scotland. This new sub-serotype was referred to as O2d since the sub-serotype O2c has been recently identified in vibriosis cases from Atlantic cod. However, it was shown that the O2c sub-serotype might not belong to the O2 serotype, but in fact belongs to another serotype. To protect Atlantic cod against all the *V. anguillarum* serotypes (and sub-serotypes) which they are susceptible to, it is recommended that isolates from serotypes O1, O2a, O2b, O2c and O2d should all be included in a bacterin vaccine for cod.

In order to determine which isolates from each of the serotypes to include in the vaccine, a variety of virulence factors of *V. anguillarum* were investigated *in vitro*. The interaction of some

candidate isolates from O1, O2a and O2b serotypes (O2c and O2d were not identified at the time this part of the study took place) with cod phagocytic cells were studied using flow cytometry. Phagocytosis and respiratory burst of cod macrophages and neutrophils as well as cod serum killing of *V. anguillarum* were quantified. It was found that isolates within the same serotype displayed varying degrees of resistance to phagocytosis and the subsequent respiratory burst activity as well as that all the *V. anguillarum* strains tested were resistant to Atlantic cod serum killing. These *in vitro* assays were found to be very useful in assessing the virulence of *V. anguillarum*. The isolate within each serotype eliciting the highest percentage of positive phagocytic cells was selected in order to increase the antigen presentation pathway, thus theoretically enhancing the protection elicited by the vaccine.

A multivalent formalin-inactivated non-adjuvanted vaccine was prepared which included all the serotypes previously described and was injected intraperitoneally into Atlantic cod. A bath challenge was performed on vaccinated and mock-vaccinated fish, 6 weeks post immunisation, using *V. anguillarum* isolates from the serotypes O2b, O2c and O2d that were not included in the vaccine. An excellent level of protection was obtained against O2b and O2d (relative percentage survival 100% and 96.4%, respectively), but the challenge with the sub-serotype O2c isolate did not produce any mortality in the control group and needs to be repeated. The vaccine formulation was very efficient at protecting Atlantic cod against vibriosis but further challenges need to be performed with other serotypes included in the vaccine (O1 and O2a), as well as with more isolates from the O2b, O2c and O2d sub-serotype.

To conclude, Atlantic cod is a species which will certainly have a major influence in marine aquaculture, but many areas have to be improved. The development of an effective and broad range vaccine to protect cod against *Vibrio anguillarum* offers another advance which should help Atlantic cod aquaculture to reach its full potential.

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General Introduction

1.1. Atlantic cod aquaculture

Atlantic cod (*Gadus morhua* L.) farming started in the 1880s with attempts to release millions of larvae to sea in an effort to restock and enhance cod stocks in specific locations. A century later this strategy did not seem to be very efficient (Shelbourne, 1964), especially with Atlantic cod fisheries suffering declining catches since the early 1970s (Figure 1-1, A). Together these have prompted greater efforts in aquaculture research to help substitute for the decreasing supply of cod from fisheries. The production of 70,000 juvenile Atlantic cod in enclosed systems in the mid 1980s, by the Institute of Marine Research in Norway, was considered to be the first success in cod farming (Svasand *et al*., 2004), but more research was needed before the large scale development of the industry could take place. It wasn't until advances in sea bass and sea bream reproduction were made in the late 1990s that the production of farmed cod could take place, using hatchery protocols developed for these species. The ability to delay maturation also represented a major milestone for the industry (Hansen *et al*., 2001). Intensive rearing soon followed with investment in integrated production systems by large companies, often exploiting many small production sites (Rosenlund and Skretting, 2006). The possibility of using equipment developed by the salmon industry for production and processing makes this industry more economically viable.

The main focus of cod production nowadays is in Norway, but the actual level of production is still very low (Figure 1-1, B); with around 12,000 T produced in Norway in 2007 (Hellberg *et al*., 2005-2006-2007). Estimates suggest that worldwide production could reach over 140,000 T by 2010, with Norway as the major producer (50-60%), and Canada and the United States of

America producing the rest (Rosenlund and Skretting, 2006). The United Kingdom is not forecast to be a major player in the cod industry at this time.

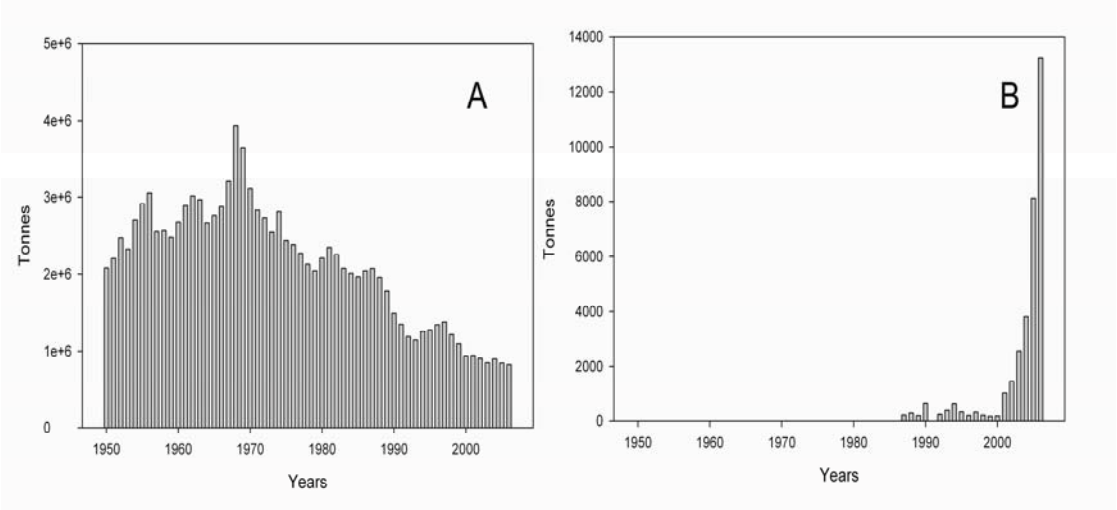

Figure 1-1 (A) Atlantic cod catches and (B) cod aquaculture production from 1950 to 2006. Data source FAO, FishStat software.

Some of the major challenges for successful development of this industry include reduction in production costs, increased quality and an all-year-round supply of juveniles. At the moment around 85 to 95% of mortalities occur in juvenile production (Magnadottir *et al*., 2004). Major efforts have also been made to improve nutrition and rearing conditions of both stock and brood-stock. The increased use of vegetable proteins for cod nutrition is a major advantage over that of salmon, which do not tolerate soybean meal. Recent research has shown that the inclusion of up to 25% of dietary protein derived from Soya could be used in cod diets (Treasurer *et al*., 2008).

Aquaculture production of Atlantic cod is also hindered by several diseases. The main pathogens that can affect cod are reviewed in Bricknell *et al*. (2006) and Samuelsen *et al*. (2006), and in the yearly health reports issued by the Norwegian Veterinary Institute (Hellberg *et al*., 2005-2006-2007). These are summarised in Table 1-1. Further research is needed in

disease management and breeding programmes for disease resistance and should include the development of effective vaccines for Atlantic cod.

Table 1-1 List of the known and potential pathogens of Atlantic cod.

	Bacteria	Virus family (Disease)
Known pathogens	*Vibrio anguillarum* *Aeromonas salmonicida* *Francisella philomiragia* subsp. *noatunensis*	*Birnaviridae* (IPNV) *Nodaviridae* (VNNV) *Rhabdoviridae* (VHSV)
Potential pathogens	*Mycobacterium* spp. *Vibrio ordalii, Vibrio logei*	*Togaviridae* (SPDV)

IPNV: Infectious Pancreatic Disease Virus; **VNNV**: Viral Nervous Necrosis Virus; **VHSV**: Viral Hemorrhagic Septicaemia Virus; **SPDV**: Salmon Pancreas Disease Virus.
The protoctistan and metazoan parasites are not included in this table but can be found in Bricknell *et al*. (2006) and Hemmingsen and MacKenzie (2001).

1.2. Vibriosis in fish

The most economically important disease recorded in farmed cod is vibriosis, caused by *Vibrio anguillarum*, with outbreaks occurring in vaccinated fish every year.

1.2.1. History of the disease

The first report of a suspected vibriosis outbreak in teleost was in 1761 (in eels), and was published by Bonaveri (reported in Drouin de Bouville, 1907), describing this disease as “pestis rubra anguillarum”. Over a hundred years later (1893), the first isolation of the pathogen responsible for vibriosis in eels was reported by Canestrini who referred to the bacterium as *Bacterium anguillarum* (Canestrini, 1907). In 1909 and 1912, Bergman first mentioned the responsible agent of vibriosis as *Vibrio anguillarum* after he described a bacterium isolated from Sweden (Bergman, 1909; Bergman, 1912). In the half century that followed, many related organisms were described and compared to *V. anguillarum* including *Vibrio piscum* in 1927 (David, 1927), *Actinobacter ichthyodermis* later reclassified as *Pseudomonas ichthyodermis* (ZoBell and Upham, 1944) and *Vibrio piscum* var *japonicus* (Hoshina, 1956). In 1971, all these organisms were regrouped under the name proposed by Bergman in 1912, *Vibrio anguillarum* (Hendrie *et al*., 1971). This reclassification was confirmed the following year with 87%

homology by DNA-DNA hybridization for all the strains investigated (Anderson and Ordal, 1972).

The first atypical *Vibrio anguillarum* was described in 1969 (Pacha and Kiehn, 1969), and in the following years additional isolates were described as being closely related to the original strains but not identical (Harrell *et al*., 1976), which prompted the discrimination of *V. anguillarum* into biotype 1 (original strains) and biotype 2 (related). In 1981 the advances in DNA methods highlighted more clearly the differences between biotype 1 and 2, especially regarding plasmid size and its cleavage, which prompted Schiewe and Crosa (1981) to reclassify *Vibrio anguillarum* biotype 2 under a new species, *Vibrio ordalii*. Nowadays, the distinction between these two species is well established (Schiewe *et al*., 1981; Schiewe and Crosa, 1981; Kaper *et al*., 1983; Egidius *et al*., 1986; Grisez *et al*., 1991; Pazos *et al*., 1993; Alsina *et al*., 1994; Alsina and Blanch, 1994a; Alsina and Blanch, 1994b; Tiainen *et al*., 1995).

The classification of *V. anguillarum* and *V. ordalii* has not been straight forward and the results from several different studies have led to confusion. Baumann *et al*. (1978) reclassified both groups (biovar 1 and 2) as *Benecka anguillarum*, but 2 years later retracted this classification (Baumann *et al*., 1980). The most confusing reclassification came in 1985 from an elegant study using ribonucleotide sequences and which led to the total reclassification of *V. anguillarum*, *V. pelagius* and *V. damsela* into the new genus *Listonella* and *V. fischeri* and *V. logei* into *Photobacterium* genus (MacDonell and Colwell, 1985). However Nearhos and Fuerst (1987) were unable to confirm those results and the following year saw additional attempts to reclassify these organisms. Smith *et al*. (1991) put the *Listonella* into *Photobacterium*, Austin *et al*. (1995) considered them better suited as *Vibrio* and finally, Austin *et al*. (1997) could not support the reclassification of *Vibrio* into *Listonella*.

Currently, the biological classification of *V. anguillarum* is very clear and the relatedness of this organism with closely related bacteria is completely characterised but the nomenclature of this pathogen, *Vibrio* or *Listonella*, is still under debate and both appear in the literature. Interestingly, even if no official retraction has been published, Professor Rita Colwell (one of the authors responsible for the creation of the *Listonella* genus) now refers to this organism as *Vibrio anguillarum* (Colwell, 2005). In the present study, the name *Vibrio anguillarum* will be used.

1.2.2. Susceptible species

There are over 85 species of bacteria in the genus *Vibrio* (http://www.bacterio.cict.fr/, updated 2008), of which a dozen are reported pathogenic to fish and shellfish (Toranzo and Barja, 1990) such as *V. anguillarum* and *V. ordalii* (Schiewe *et al*., 1981), *V. tubiashii* (Hada *et al*., 1984), *V. damsela*, now *Photobacterium damselae* (Love *et al*., 1981), *V. vulnificus* II (Tison *et al*., 1982), *V. carchariae* (Grimes *et al*., 1984), *V. salmonicida* (Egidius *et al*., 1986) and *V. cholerae* non-O1 (Muroga *et al*., 1979; Yamanoi *et al*., 1980).

Most of the important economical species cultured in aquaculture are susceptible to *Vibrio* species, such as Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), sea bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*), ayu (*Plecoglossus altivelis*), Atlantic cod (*Gadus morhua*), saithe (*Pollachius virens*) and turbot (*Scophthalmus maximus*) (Larsen *et al*., 1994; Kent and Poppe, 2003).

1.2.3. Clinical signs

Clinical signs of vibriosis were first described in eels in Norway by Bergman (1909) and later in the UK by McCarthy (1976). The disease manifested itself by the appearance of areas of macroscopic haemorrhaging, with swelling and boils. Destroyed epithelium is evident at the top of and around the lesion, while haemorrhaging can be present in the skin. The liver and

intestine may also have haemorrhagic (petechia) tissue and the heart lumen can be filled with fluid. Histopathologically, the muscle fibres are widely separated, with intermuscular connective tissue filling only some of the space, while the rest is filled with fluid (Roberts, 2001). An abundance of red blood cells and few leukocytes can be detected in the intermuscular tissues.

Salmonids are susceptible to vibriosis to varying degrees. The most characteristic clinical signs are splenomegaly, gall bladder distension, petechia of the liver and severe necrotic liquefaction of the kidney (degeneration and necrosis of hepatocytes) with necrosis of the musculature and congestion and prolapse of rectal tissues. Vasodilution of glomerular capillaries, cloudy swelling and hyaline droplet degeneration of the epithelial tubular cells, oedematous gills and desquamation of interstitial mucosa may also be observed (Baumann *et al*., 1984; Ransom *et al*., 1984; Lamas *et al*., 1994).

Atlantic cod was one of the first species of fish to be reported as being susceptible to vibriosis (Bergman, 1912), with the clinical signs reviewed by Egidius (1987). Briefly, the first reports of the gross signs of the disease described infection of the eye, affecting the cornea (Bergman, 1912) and fin erosion and haemorrhages in the head region (Baumann *et al*., 1984; Egidius and Andersen, 1984). Bergman (1912) reported isolating the bacteria from the blood and artificial infecting cod, producing the same symptoms, fulfilling Koch's postulates. Another study reported a slightly different pathology, characterised by ulcers and papules all over the fish (Bagge and Bagge, 1956), and the infection took some time to develop to the ulcerative stage, but vibriosis in this case was thought to be a secondary infection (Egidius, 1987). Nowadays, vibriosis in Atlantic cod, as well as other gadoids such as saithe, presents the typical signs of the disease observed in other species (Egidius *et al*., 1983).

1.2.4. Transmission, route and factors influencing infection

The triggering of an outbreak of vibriosis is the result of host-pathogen-environment interactions (Larsen *et al*., 1994), with the main predisposition being the stress level of the fish. Rise in temperatures has a known effect on the stress level of fish and corresponds well with disease outbreaks (Egidius and Andersen, 1977; Buller, 2004), especially in early to mid summer (Hastein and Holt, 1972), which corresponds also better with the optimal growth temperature of *V. anguillarum*. Fluctuations in salinity, as well as temperature, have been reported as a possible trigger for *V. anguillarum* outbreaks (Rodgers and Burke, 1981). Other factors can also increase the incidence of vibriosis such as transport and transfer from freshwater to seawater (Hastein and Holt, 1972) or pollution (Larsen and Jensen, 1979; Larsen and Willeberg, 1984; Larsen, 1984) or the vaccination of Atlantic cod which is sometimes followed by outbreaks (Dr Colquhoun, personal communication).

The point of entry of *V. anguillarum* has been debated for many years. Several different routes have been proposed, such as directly via the skin (Svendsen and Bogwald, 1997), through the gills (Baudin Laurencin and Germon, 1987) or through the gastrointestinal tracts (Olsson *et al.*, 1996; Grisez *et al.*, 1996). Using bath immersion as the route of infection, the pathogen was first seen in the skin in ayu at 12 h post infection, followed by muscle, spleen and liver at 24 h, and direct contact between the fish led to the highest transmission level (Muroga and Delacruz, 1987; Kanno *et al*., 1989). The rate of infection was increased if the skin was damaged, so the physical and chemical barrier of the mucus and epidermis play an important role in protection against *V. anguillarum* (Kanno *et al*., 1989). Other possible points of entry are the gills (Baudin Laurencin and Germon, 1987) or the digestive tract, but these might be less important than the skin (Funahashi *et al*., 1974; Kawai *et al*., 1981; Evelyn, 1984; Kanno *et al*., 1989), and some studies reported a very low or unsuccessful infection using the gills as a point of infection (Kanno *et al*., 1989; Olsson *et al*., 1996). Most of the different mucus producing tissues (skin,

gills, intestine) in different fish species have been shown to attract *V. anguillarum* (Chen and Hanna, 1992; Bordas *et al*., 1998; Knudsen *et al*., 1999; Larsen *et al*. 2001). *Vibrio anguillarum* was shown to adhere to rainbow trout gut sections (Horne and Baxendale, 1983), and a large number of bacteria could be observed in the peritoneal mesenteric of the gastrointestinal tract of rainbow trout (Nelson *et al*., 1985). It was later shown that the bacterium could use gut mucus as a sole source of carbon in turbot (Olsson *et al*., 1992). The hypothesis of the gastrointestinal tract as a point of entry is fairly well supported, for example, with the recovery of *V. anguillarum* from the spleen after anal and intragastric intubations (Olsson *et al*., 1996). Using transparent zebrafish and a fluorescent-labelled bacteria, the pathogen was observed entering the mouth (probably through drinking water) and then the gastrointestinal tract before producing septicaemia (O'Toole *et al*., 2004).

1.2.5. Antibacterial agents, probiotics and immunostimulants

Antibiotic medication is the only effective way to treat an infectious bacterial disease outbreak. *Vibrio anguillarum* has been shown to be sensitive to most antibiotics, with some differences in sensitivity between serotypes (Table 1-2) (Pedersen *et al*., 1995; Samuelsen and Bergh, 2004; Vik-Mo *et al*., 2005). The most commonly used antibiotic in treating outbreaks of *V. anguillarum* is oxolinic acid (OA) and flumequine (both quinolone), but resistant strains to OA have been identified recently (Colquhoun *et al*., 2007), which highlights the recurrent problems of antibiotic resistance development. This is a well known phenomenon in *Vibrio anguillarum* (Aoki *et al*., 1985; Pedersen *et al*., 1995).

Table 1-2 Sensitivity of *V. anguillarum* serotypes O1 and O2 to selected antibiotics used in aquaculture.

Antibiotic	Serotype O1	Serotype O2
Colistin	Resistant	Sensitive
Ampicilin	Sensitive	Resistant
Cephalothin	Sensitive	Resistant

The introduction of vaccination drastically reduced the levels of antibiotics used in the treatment of vibriosis in salmon in Norway (from 50 T in 1987 to 1 T in 2002). The reduction in their use correlates well with the increased protection offered by vaccination (Hastein *et al*., 2005). Nevertheless certain situations require the use of antibiotics such as the outbreaks of vibriosis occuring just after vaccination (Dr. Colquhoun, personnal communication).

The use of immunostimulants and probiotics are of interest to cod aquaculture since these tend to act on the innate immune system of cod. Cod appear to rely heavily on innate immunity to protect them from pathogens (Magnadottir, 2006). Immunostimulants and probiotics generally improve fish health and together with vaccination, have reduced the used of chemo-therapeutants (Pedersen *et al*., 2004), but many of the mechanisms leading to these benefits are still unknown.

A possible definition of immunostimulant is "a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens" (Bricknell and Dalmo, 2005). The use of these immunostimulants as vaccine adjuvants will be treated more extensively in Section 1.4.1.

Probiotics are defined as live micro-organisms which when consumed in adequate amounts as part of food confer a health benefit to the host (WHO/FAO, Food and Nutrition Paper 85). They work possibly by competitive exclusion, alteration of the microbial metabolism or stimulation of the host immunity (Gatesoupe, 1999; Irianto and Austin, 2002). The use of probiotics have shown minor improvements on survival of Atlantic cod to *V. anguillarum* after feeding on *Carnobacterium dilergens* (Gilberg and Mikkelsen, 1998), and probiotics have also elicited an increased survival in turbot after adding selected bacteria to the water at the same time as challenging with *V. anguillarum* (Hjelm *et al*., 2004; Planas *et al*., 2006). Most of the

mechanisms involved in probiotic benefits are unknown and offer exciting new lines for research.

1.2.6. Characterisation and classification of *Vibrio anguillarum*

1.2.6.1. Culture and biochemical tests

Vibrio anguillarum, as described by Buller (2004) and in the Bergey's manual of Systematic Bacteriology (Baumann *et al*., 1984), is a Gram-negative bacterium with short rods, curved or straight, round ended, occurring singly or in pairs and is pleomorphic. The size of the bacterium is typically 0.5 to 0.8 μm in diameter and 1.4 to 2.6 μm in length. It is a facultative anarobe, capable of both fermentative and respiratory metabolism. It has rapid motility and is β-haemolysin positive. After 2 days of culture on agar, the colonies are 2mm and a glistening cream-colour. The bacterium is catalase and oxidase positive. Optimal growth occurs at pH 7, salinity of 2% and at a temperature of 25°C (Larsen, 1984). The 0/129 Vibriostatic test (2, 4-diamino-6, 7-di-iso-propylpteridine phosphate) is the typical antibiotic test which differentiates *Vibrionaceae* from *Aeromonas* (*Aeromonas* being resistant to both concentrations) but other bacterial species are also sensitive to this test such as *Moritella*, *Photobacterium* and *Flavobacterium* (Buller, 2004). For this reason, the results of these tests have to be considered carefully and are not conclusive, especially since Pedersen *et al*. (1995) showed that some *V. anguillarum* isolates were resistant to this antibiotic. A clearing zone of more than 9 mm on 500 μg discs is considered sensitive for *Vibrio* species (Bernardet and Grimont, 1989).

The first biochemical classification of *V. anguillarum* took place in 1935 (Nybelin, 1935), based on sucrose and mannitol utilisation and indole production, and 2 groups of bacteria were identified. In the next 45 years, 3 additional groups were added (Table 1-3). Only groups A and C were regularly recovered from infected animals (Hastein and Holt, 1972; Egidius and Andersen, 1977), the other three groups represented environmental isolates.

Table 1-3 First biogrouping of *V. anguillarum* with reference to published reports.

Group	Indole	Mannitol	Sucrose	
A	+	+	+	(Nybelin, 1935)
B	-	-	-	(Nybelin, 1935)
C	-	+	+	(Smith, 1961)
D	+	+	-	(Larsen and Jensen, 1979)
E	+	-	-	(Larsen and Jensen, 1979)

With the advances in bacteriology and biochemistry in the early 70's, new phenotypical traits of the bacteria could be analysed and revealed a more complex picture. This was very clearly highlighted by Evelyn (1971), who proposed the first archetype of *V. anguillarum* regarding biochemical reactivity, but recommended that large scale studies should be carried out due to the high heterogeneity observed. Biochemically, the decarboxylase reactions were found to be very useful for differentiating between marine *Vibrio* and other bacteria (Kaper *et al*., 1983) together with arabinose (Egidius and Andersen, 1977) and could be used to distinguish between *V. anguillarum* isolates recovered from wild gadoids and salmonids (Hastein and Smith, 1977).

Several large scale studies were published in the mid-1980s using the increasing power of computers to analyse the biochemical characteristics of bacteria, allowing more complex patterns to be distinguished, usually grouped in phena defined as "a phenotypically reasonably uniform group" (Mayr, 1969). Bryant *et al*. (1986b) reported 38 phenon within the *Vibrio* genera, after analysing over 1000 isolates for 81 parameters. They concluded that 30 of these tests could be used to classify all isolates with a 90% confidence interval, and found all *V. anguillarum* isolates to fall into one group, as in the study by West *et al*. (1986) and Kaper *et al*. (1983). The heterogeneity of the enzymatic capacity of *V. anguillarum* was a characteristic feature found in most studies, with fermentation of carbohydrates and glycosides more

heterogeneous in *V. anguillarum* than in *V. ordalii* (Larsen, 1983). The latter was generally found to be less reactive to many biochemical tests (Colwell and Grimes, 1984).

In 1986, Bryant *et al*. (1986a) compared 5 of the major studies examining numerical classification of the genus *Vibrio* (Lee *et al*., 1981; West *et al*., 1983; Lee and Bryant, 1984; West *et al*., 1986; and Lee and Bryant, unpublished), involving 142 common characteristics and 1091 *Vibrio* strains. They reported 59 clusters with 87.5% similarities and 44 unclustered strains. Almost all the *V. anguillarum* isolates grouped together, with variations in their response to Voges-Proskauer reaction, hydrolysis of elastin and production of acid and growth on L-arabinose.

The API20E system (BioMerieux, France) was the first commercially available kit (Doucet and Paule, 1971) for identifying enterobacteria based on their biochemistry through 20 different biochemical reactions. The use of this kit to identify fish bacterial pathogens was initially disappointing (MacDonell *et al*., 1982), with varying results obtained for *Pasteurella* and *Vibrio* (Kent, 1982), and the possible misidentification of *V. anguillarum* as *Aeromonas hydrophila* (Santos *et al*., 1993). Nowadays an API20E reaction profile is available for *V. anguillarum*, but several tests can vary (Buller, 2004), even within serotypes such as O1 (Pedersen and Larsen, 1995) or O3 (Tiainen *et al*., 1997b). Serotype O2 was found to be even more heterogeneous than O1 (Wiik *et al*., 1989) and therefore classification of *V. anguillarum* based purely on these results is not recommended (Grisez *et al*., 1991).

In 1995, Austin *et al*. (1995) analysed 260 *V. anguillarum* by three different phenotypical methods and found 9 clusters by BIOLOG-GN (including all the strains) , 5 clusters by the API20E (43% of the strains and 117 profiles in total) and 32 clusters based on carbon source, i.e. biotyping (41% of the strains). The authors recommended caution when using commercial kits for identification, and demonstrated once more the biochemical heterogeneity of *V.*

anguillarum. This heterogeneity was also reported by Pedersen *et al*. (1999b) using a PhenePlate system (Kuhn *et al*., 1990), the authors could weakly differentiate between *V. anguillarum* from Nordic countries and those from others countries.

In conclusion, it is clear that no distinct pattern can be seen in biochemical profiles of *V. anguillarum*. This species of *Vibrio* is easy to identify and distinguish from other *Vibrio* species (Alsina and Blanch, 1994a; Alsina and Blanch, 1994b) but the biochemical intra-specific variations do not allow the subdivision of the species in unambiguous groups.

1.2.6.2. Lipopolysaccharides

Lipopolysaccharides (LPS) are integral components of the Gram-negative cell wall of bacteria. Three structural entities are present in LPS, Lipid-A, core-oligosaccharide (attached to the lipid by the 2-keto-3-deoxy-octulosonic acid molecules, KDO) and a polysaccharide known as the O-chain, attached to the core oligosaccharide (Orskov *et al*., 1977). This O-chain consists of a repeating oligosaccharide with a varying degree of polymerisation. Advances in chemistry have led to improvement of LPS analysis such as the use of silver staining for visualising LPS on sodium dodecyl-sulphate-poly-acrylamide gel electrophoresis (SDS-PAGE) (Tsai and Frasch, 1982), or the purification of LPS using proteinase K (Hitchcock and Brown, 1983).

In 1973, Chester *et al*. (1973) analysed the composition of LPS from several *Pseudomonas aeruginosa* isolates and showed that the composition and structure of LPS suggested the existence of serologically common regions in the low molecular weight (LMW) LPS and specific regions in the medium to high molecular weight (MMW and HMW) LPS, schematised in Figure 1-2. Goldman and Leive (1980) and Palva and Makela (1980) suggested that the pattern observed for LPS, characterised by regularly spaced bands, could be due to different numbers of repeating units in the O side chain of the LPS.

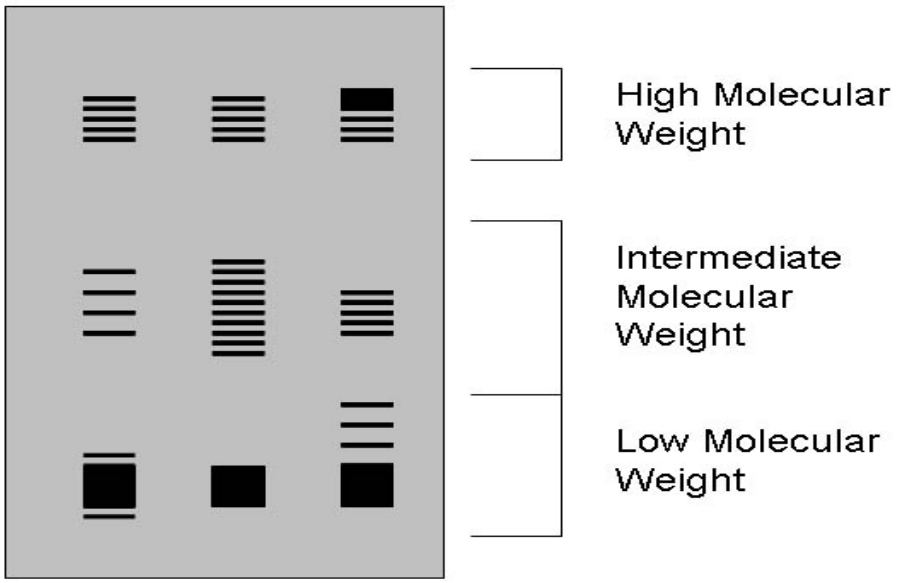

Figure 1-2 Diagrammatic representation of the typical pattern of a Gram-negative bacterial LPS on SDS-PAGE gel.

The segregation of *V. anguillarum* isolates based on their LPS profile was shown to be very consistent and corresponded directly with their serotypes (Nomura and Aoki, 1985). Structural analysis showed that the chemical composition of the LPS was different between different serotypes, as well as between *V. anguillarum* and *V. ordalii*, and may be due to a difference in the relative amount of hydrocarbon structures in the core region for the latter (Salati and Kusuda, 1986). The characteristic LPS patterns seen on SDS-PAGE for different serotypes has been confirmed by many authors (Grisez and Ollevier, 1995; Austin *et al*., 1995; Austin *et al*., 1997; Tiainen *et al*., 1997b; Pedersen *et al*., 1999a), but some reports have described differences in the profiles within serotypes O2 and O3 as well as between membrane bound and extracellular LPS (Santos *et al*., 1995). Using immuno-electrophoretic methods, 2 groups within the serotype O2 of *V. anguillarum* have been reported, i.e. an O2a and an O2b sub-group (Banoub *et al*., 1987; Rasmussen, 1987a; Rasmussen, 1987b). These antigenic differences could not be distinguished on SDS-PAGE (Grisez and Ollevier, 1995; Austin *et al*., 1995). In 1992, the first complete chemical composition of *V. anguillarum* LPS was reported as

well as a molecular structure for the O-polysaccharide (Eguchi *et al*., 1992a; Eguchi *et al*., 1992b).

The antigenicity of the different structures found in the LPS profile of *V. anguillarum* (Figure 1-2) was studied by Mutharia *et al*. (1993) using rabbit antisera. The authors concluded from the resulting profile that O2 and *V. ordalii* had common antigenic epitopes localised in the LMW region of their LPS profile contributing to the extensive cross-reactivity obtained with the rabbit serum, the species-specific antigens seemed to be located in the HMW-LPS.

1.2.6.3. Immunological characterisation

Serological classification is another way of segregating isolates based on the interaction of antibodies with different bacterial antigens. One of the first reports published using polyclonal antibodies (pAbs), segregated 13 isolates of *V. anguillarum* into 3 groups which were geographically and host dependant (Pacha and Kiehn, 1969). Even with the low number of isolates, the classification based on antibody reactions seems to be more homogeneous (less groups) than obtained with biochemical typing. A few years later, Harrell *et al*. (1976) reported an outbreak of vibriosis in fish vaccinated with *V. anguillarum* isolate 775 and showed that the recovered isolate 1669 contained antigenic determinants not present on the vaccine strain.

From 1981 to 1985, advances in immunology and the increasing number of available isolates allowed more robust classification to be carried out. However, a different nomenclature scheme was adopted by each research group (Table 1-4).

Table 1-4 Summary of the different names given to serotypes O1 and O2 from different references until 1985.

	Serotype		Reference
After 1986	O1	O2	(Sorensen and Larsen, 1986)
Before 1986	775		(Harrell *et al*., 1976)
	775A	569	(Strout *et al*., 1978)
	Type I	Type II	(Gould *et al*., 1979)
	C	A	(Kitao *et al*., 1983)
	J-O-3	J-O-1	(Ezura *et al*., 1980)

One of the Japanese classification systems, J-O-1 to J-O-3 (Ezura *et al*., 1980), seemed robust at the time, with 64% of the 195 isolates grouping in the first serogroup, 6% in the second and 30% in J-O-3. They found that isolates within the serotype J-O-1 were almost exclusively recovered from freshwater outbreaks of ayu and J-O-3 was composed almost entirely of isolates from seawater, which was confirmed by Kitao *et al*. (1983). A follow-up study added J-O-4 to J-O-8 to the existing scheme and revealed the presence of a common antigen (heat-labile, k-1) on all the serotype strains and a second one (k-2) only found in some of the J-O-1 isolates and J-O-4 (Tajima *et al*., 1985). Even though the antibody profiles of serotype J-O-1 were homologous in appearance (Harrell *et al*., 1976; Chart and Trust, 1984), it consisted of two distinct biochemical groups; which are now known to be *V. anguillarum* O2 and *V. ordalii*.

In a 5 year study, Sorensen and Larsen (1986) produced a complete classification scheme using thermostable antigens, grouping 90% of the 270 isolates from fish, 30% of the 189 isolates from the environment and 40% of the 36 isolates from invertebrates in 10 serotypes (O1 to O10). Serotype O1 was composed mostly of isolates recovered from farmed salmonids (71% of the isolates recovered from rainbow trout). The predominant group of isolates in the study (O2 serotype) came from marine feral fish but was also found in salmonids. *Vibrio ordalii* isolates grouped with serotype O2. Serotypes O3 to O6 were mostly environmental strains, O7 was isolated from plaice and corresponded to the serogroup J-O-7 (Tajima *et al*., 1985). Serotypes O8, O9 and O10 were isolated from diseased fish, but are rarely recovered from fish. This classification system has now been almost universally adopted and only one subsequent study created a different serotyping system (T-O-I to T-O-VII), where T-O-I, T-O-V and T-O-VI correspond to the serotypes O1, O2 and O3, respectively (Song *et al*., 1988).

In one of the most complete studies on *V. anguillarum*, Austin *et al*. (1995) classified 78% of the 260 isolates in their collection into 15 different serotypes, 10 from Sorensen and Larsen

(1986) and 5 new ones (VaNT1, VaNT2, VaNT4, VaNT5 and VaNT7). They found half of all isolates grouping with O1 and that 60% of the isolates recovered from rotifers, artemia, sediments and water samples were untypable. Pedersen *et al*. (1999a) completed the serotyping scheme using pAbs, by removing two of the VaNT groups and adding 6 serotypes from Japanese isolates (O11 to O16, described in Kitao *et al*., 1983 and Kitao *et al*., 1984, as well as 4 serotypes from the study of Grisez, 1997, O17 to O23). The sub-classification of O2 into O2a and O2b was confirmed by Bolinches *et al*. (1990) using pAbs. Another sub-grouping was revealed on analysis of the extracellular products (ECP) of strains belonging to the O3 serotype, called O3a and O3b (Santos *et al*., 1995). The relationship to pathogenicity was clear, with O3b isolates recovered from the environment and O3a exclusively from disease outbreaks. The latter was very homogenous for both biochemical reactions and ribotype patterns (Tiainen *et al*., 1997b).

Thermostable antigens on *V. anguillarum* are the basis of the serological classification but Mackie and Birkbeck (1987) showed that even after cross-absorption of the pAbs with LPS, they still reacted with two cell envelope proteins (most probably peptidoglycans). The antigenicity of major outer membrane proteins (MOMP) was investigated by Suzuki *et al*. (1996) focusing on Omp35La porin protein (first reported by Murphy, 1994) and found that even if commonly expressed among J-O-1 to J-O-7 serotypes, it did not participate in the antigenicity of this serotyping scheme.

The use of monoclonal antibodies (mAbs) confirmed the results of the serotyping scheme proposed by Sorensen and Larsen (1986) and that LPS was the most antigenic molecule of *V. anguillarum* (Johnsen, 1977; Goerlich, 1987; Bolinches *et al*., 1990). Using mAbs, Tiainen *et al*. (1995) and Toranzo *et al*. (1987) showed clear immunological differences between *V. ordalii* and *V. anguillarum* serotype O2. They found a common heat-labile antigen (protein) between

both species since the pAbs they used (raised against whole bacteria) cross-reacted with both species, as also shown by Mutharia and Amor (1994). Fish serum has been shown to be unable to differentiate between *V. anguillarum* and *V. ordalii*, which prompted Mutharia *et al*. (1993) to conclude that both should be considered as the same species from a fish perspective.

The possible correlation between serotypes of *V. anguillarum*, geographic origin and fish species of the recovered strains has been investigated (Toranzo *et al*., 1987; Larsen *et al*., 1988; Myhr *et al*., 1991; Larsen *et al*., 1994). Salmonids were found to be almost exclusively affected by O1 and O2 serotypes (70 and 20% of the isolates recovered, respectively), the rest consisting of O3, O4, O5 and a few isolates that could not be typed (4%) (Larsen *et al*., 1994). Regarding non-salmonids, the majority of the isolates recovered were serotype O2 (Larsen *et al*., 1994), with O2b isolates almost entirely restricted to this group of fish (Bolinches *et al*., 1990). Kitao *et al*. (1983) found ayu to be infected mostly by O2 isolates, whereas Song *et al*. (1988) reported half of the isolates recovered from ayu were serotype O1. Sea bass and sea bream seemed to be most often infected by O1, while eels are affected with isolates belonging to O2 as well as O3 serotype (Larsen *et al*., 1994).

Atlantic cod are susceptible to most *V. anguillarum* serotypes, i.e. O2, O4, O5, O6, O8, O9 and O10 (Sorensen and Larsen, 1986), as well as VaNT5 (Austin *et al*., 1995). Eleven strains obtained from infected cod in Denmark were also reported to belong to the *Vibrio anguillarum* related organisms (VAR, Santos *et al*., 1996). Juvenile cod seem to be almost exclusively infected by serotype O2 with sub-groups O2a and O2b equally represented (Knappskog *et al*., 1993), but Larsen *et al*. (1994) and Fouz *et al*. (1989) found a bias towards serotype O2a. It seems that the outbreaks documented more recently in farmed cod have seen a shift in the frequency of O2a isolates recovered towards O2b (Santos *et al*., 1995; Pedersen *et al*., 1999a;

Jones *et al*., 2000; Samuelsen *et al*., 2006; Bricknell *et al*., 2006; Hellberg *et al*., 2005-2006-2007). The same phenomenon has been reported for turbot in Spain with a shift from an O1 to an O2a serotype (Toranzo and Barja, 1990). Due to the heterogeneity found within the O2b serotype affecting Atlantic cod, Larsen *et al*. (1994) and Espelid *et al*. (1991) suggested the existence of a third group within the O2 serotype, named O2c by Tiainen *et al*. (1997a), but it was never further characterised. Recently, Mikkelsen *et al*. (2007) presented good evidence to support this in isolates recovered from infected Atlantic cod using pAbs and mAbs, as well as molecular techniques.

Several commercial kits are available for the specific identification of *V. anguillarum* but none are able to discriminate between the different serotypes. The Bionor® agglutination kit was shown to successfully detect O1 to O5 and O7 serotypes but failed to react with the other major serotypes, as well as giving false positives for *V. splendidus* and motile *Aeromonas* (Romalde *et al*., 1995). The AquaRapid-Va® and Aqua-Eia-Va® kits gave good results for O1 and O2 (a and b) but not O3 serotypes and a few non specific reactions were reported (Gonzalez *et al*., 2004).

1.2.6.4. Molecular characterisation

In the late 80s and early 90s, advances in molecular tools allowed new methods to be developed, which allowed a faster and more accurate sequencing of the genetic code. This is very useful for taxonomy since sequencing can differentiate single nucleotide mutations between the genome of two bacterial isolates. Sequence analysis of the 16S small subunit ribosomal RNA led Dorsch *et al*. (1992) to recommend reclassification of *V. anguillarum* from the genus *Listonella* to the *Vibrio* genus. The sequence analysis of the 5S rRNA for 54 different strains of *Vibrio* confirmed this reclassification and helped to establish a clearer picture of the phylogeny of the family *Vibrionaceae* (Ruimy *et al*., 1994). Unfortunately this technique was not discriminatory enough to differentiate between *V. anguillarum* and *V. ordalii*.

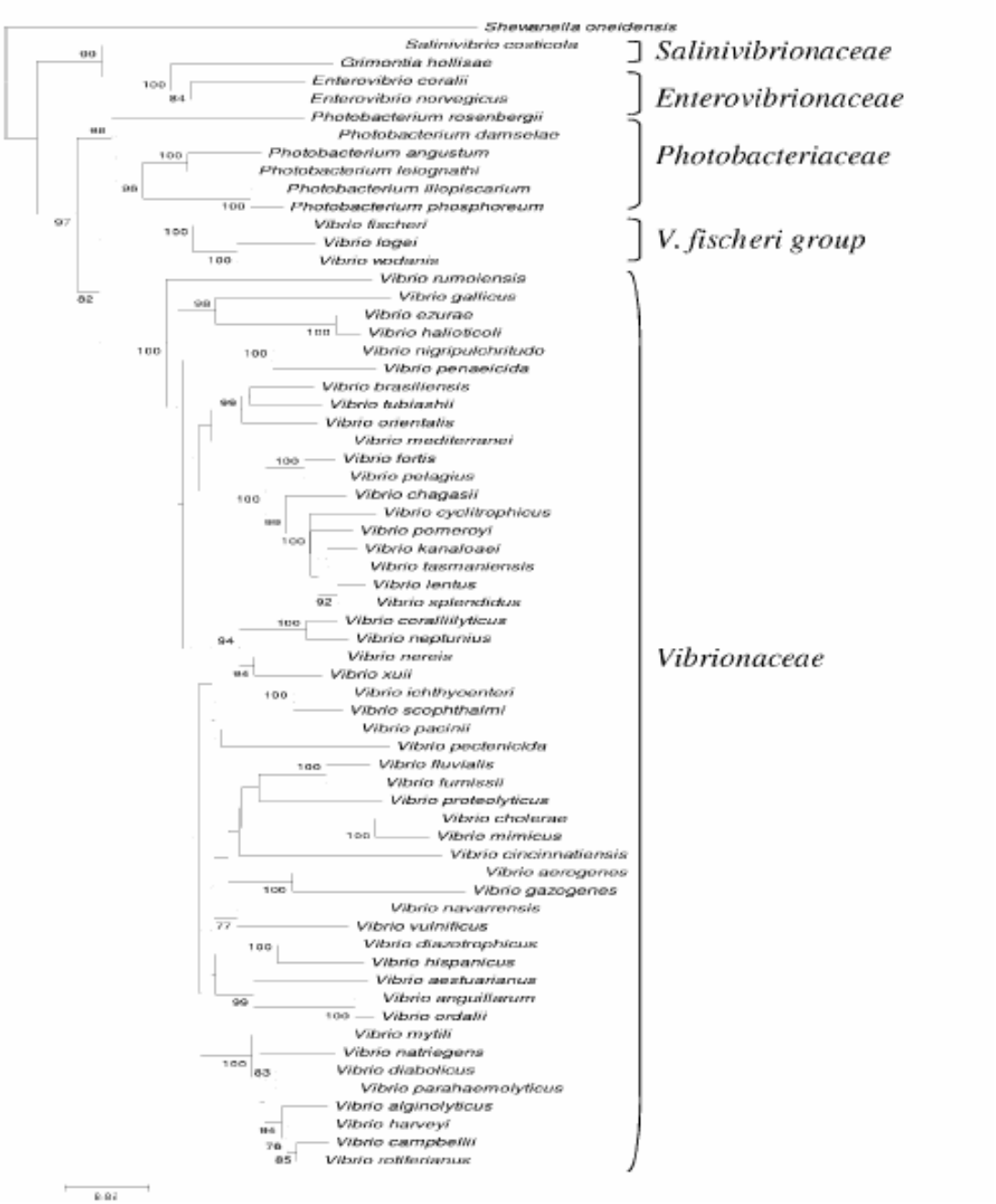

Figure 1-3 Phylogeny of the *Vibrionaceae* family according to Thompson *et al*., 2005 using MLSA of 16S rRNA, *rpo*A, *pyr*H and *rec*A.

The investigation of a 560 nucleotide segment of the 16S rRNA of several *Vibrios* spp. showed that *V. anguillarum* serotypes O1, O2a and O2b grouped closely together, with serotype O9 further away. The sequence of this fragment in *V. ordalii* isolates (DF_1K and DF_3K) was almost identical to *V. anguillarum* isolate NCMB 6 (O2a) and *V. damselae*, while the *V. ordalii* isolate MSC2-75 was distantly related to these (Wiik *et al*., 1995). Recently, the classification of

Vibrionaceae has been investigated using modern molecular techniques such as MultiLocus Sequence Analysis (MLSA), Amplified Fragment Length Polymorphism (AFLP) and rep-PCR (Ben-Haim *et al*., 2003; Gomez-Gil *et a*l., 2004; Thompson *et al*., 2004; Thompson *et al*., 2005; Mikkelsen *et al*., 2007; Urbanczyk *et al*., 2007). These molecular techniques rely on the sequencing of several housekeeping genes (MLSA), the restriction of the genomic DNA followed by amplification of the fragments (AFLP) or the amplification of repetitive extragenic palindromic regions of the genomic DNA (rep-PCR) all followed by the construction of phylogenic trees using similarity matrixes and cluster analysis. These three techniques were found to be more powerfull for the identification of bacteria at the species and strains levels compared to the sequencing of the 16S rRNA alone (Thompson *et al*., 2005). The application of AFLP to *Vibrio anguillarum* lead Mikkelsen *et al*. (2007) to highlight the possibility of the existance of a separate group of bacteria (possibly related to the O2 group). The distinction between O2a and O2b using this technique was not possible. They also found that the NCIMB 6 reference strain (O2a) was grouped away from the O2 cluster, which was also reported by other groups using different techniques (Wiik and Egidius, 1986; Espelid *et al*., 1991; Knappskog *et al*., 1993; Wiik *et al*., 1995).

Ribotyping is another method used for the identification and classification of *V. anguillarum*. It relies on the principle of cleaving ribosomal RNA with specific restriction enzymes, and obtaining a profile after running the fragments on an agarose gel followed by hybridisation with RNA probes. Many restriction enzymes are available and lead to a different restriction profiles. For example, Tsai *et al*. (1990) recommended using restriction enzymes *Cla*I, *Eco*RI, *Hind*III and *Sca*I for *Vibrio* spp. The enzymes *Eco*RI and *Hind*III produced 6 profiles for *V. anguillarum* O1 but no correlation could be found between the geographic origin of the isolates, the fish host or the ribotype profile obtained (Pedersen and Larsen, 1993). The results of ribotyping led Tiainen *et al*. (1997b) to question the grouping of *V. anguillarum* serotype O3, due to closer

similarities between the O3b isolates and *V. aestuarianus* than with *V. anguillarum* O3a. Tiainen *et al*. (1995) showed *V. ordalii* to cluster in a separate group to O2a and O2b sub-serotypes of *V. anguillarum* which shared ribotypes. After cluster analysis, using Dice coefficient (representing the homogeneity of the groups), the authors showed that O1, O3a, O7 and O9 were the most homogenous groups, followed by O2b, O4 and O10; and O2a, O3b, O5 and O8 were the most heterogeneous. This technique was also used by Austin *et al*. (1995), using *Bgl*I to cleave the DNA followed by ribosomal probe labelling. They found a core group of *V. anguillarum* which they considered to represent the "true" species and suggested that the outliers should be questioned as to whether they belonged to this group.

Pulse Field Gel Electrophoresis (PFGE) is another technique which allows classification of isolates into clusters. It relies on the digestion of DNA by restriction enzymes, and the fragments are then separated by three directional gel electrophoresis using variable voltage. This technique generated more profiles for *V. anguillarum* O1 than was found with ribotyping, and therefore has a higher power of discrimination between isolates (Pedersen *et al*., 1999b). For example, PFGE allowed the discrimination of northern and southern (European) clonal lineages of *V. anguillarum* (Skov *et al*., 1995), making this technique a useful tool for epidemiological studies.

Polymerase chain reaction (PCR) has been used very successfully to identify *V. anguillarum*, both accurately and rapidly (Hirono *et al*., 1996; Gonzalez *et al*., 2003). The major advantages of this technique are the specificity of the primers which can be tailored to a particular requirement, the minute amounts of material required (10 pg of template DNA, Hirono *et al*., 1996 or 5 bacteria per reaction, Gonzalez *et al*., 2003), the fact that tissue or blood can be used in the analysis and the possibility of carrying out the tests using non-lethal sampling (e.g. blood).

The random amplified polymorphic DNA (RAPD) was shown to be less powerful for the identification of species or strains than the AFLP, MLSA or rep-PCR (Thompson *et al*., 2004) but leads to the same type of fingerprints, and it allowed Martinez *et al*. (1994) to show clear differences between *V. salmonicida*, *V. anguillarum* and *V. ordalii*. They also showed the possibility to differentiate O1 from other serotypes.

Another application of PCR is the generation of DNA probes which bind specifically to a target region *in situ*. Probes can be used in *in situ* hybridisation (ISH) where they detect the target region in tissue sections. This technique has been extensively used for the localisation of *V. anguillarum* in different tissues (Rehnstam *et al*., 1989; Aoki *et al*., 1989; Martinez-Picado *et al*., 1994; Ito *et al*., 1995; Hirono *et al*., 1996).

Plasmids were initially identified in *V. anguillarum* by Crosa *et al*. (1977), who found a large plasmid, called pJM1,in a very virulent O1 isolate. They showed later that this plasmid was involved in iron sequestering (Crosa, 1979; Crosa, 1980). Serotype O1 appeared to harbour this 67 kbp plasmid (Wiik *et al*., 1989; Larsen and Olsen, 1991; Myhr *et al*., 1991; Pedersen and Larsen, 1995; Pedersen *et al*., 1999b), but some isolates with no plasmids or more than one have also been reported (Olsen and Larsen, 1993). Austin *et al*. (1995) found the same plasmid in the VaNT1 group of isolates, which helped confirm their suspicions that these 2 serotypes were closely related. Serotype O2 was first reported plasmid-less, which prompted the hypothesis that a chromosomal-mediated equivalent to the plasmid mediated iron-sequestering mechanism existed (Toranzo *et al*., 1987; Wiik *et al*., 1989). Some studies later reported evidence that some isolates of the serotype O2 harboured a plasmid, and in some cases more than one (Myhr *et al*., 1991; Tiainen *et al*., 1995; Austin *et al*., 1995; Pedersen *et al*., 1996; Austin *et al*., 1997; Pedersen *et al*., 1999b). The other serotypes (O3, O7, O8, O9, VaNT4 and VaNT5) seem to lack plasmids (Austin *et al*., 1995; Pedersen *et al*., 1996) with the

exception a few isolates belonging to O4, O5 and VaNT7serotypes. Altogether, the power of plasmid analysis seems to be fairly low to segregate intra-serotypes isolates (Austin *et al*., 1995).

1.3. Immune system

1.3.1. Fish immune system

The dichotomic view between the innate and adaptive immune system is being challenged by modern immunologists who consider the whole system as a complex multi-level, highly redundant network of various defence mechanisms (Whyte, 2007), also referred to as combinatorial system (Magnadottir, 2006).

1.3.1.1. Innate immune system

The innate immune system, due to its existence in animals lacking an adaptive immune system, is considered the provider of the "danger signals" (Matzinger, 1994) and the first line of defence until the adaptive response can be mounted. In fish, this system has several components which are more active and show more diversity than their homologues in mammals, such as the high activity of the alternative pathway of the complement system (Sunyer and Tort, 1995). If the physical barrier of the fish is breached, the innate system has a whole range of defences which can protect the host; both humoral (complement, transferrins, anti-proteases, haemolysins, lysozymes, interferon, C-reactive proteins) and cellular (macrophages, neutrophils, lymphocytes and scavenger endothelial cells) systems interact and fight the invading organism. This extremely elaborate structure is called innate because of its non-specificity. This means that Pattern Recognition Receptors (PRR), which can be either soluble (such as mannan-binding lectin or C-reactive proteins) or cellular (Mannose receptor or Toll-like receptors, TLRs), recognise conserved Pathogen-Associated Molecular Patterns (PAMPs) such as LPS, peptidoglycans and β-glucans. This interaction ultimately activates

relevant transcription factors, starting a wide range of responses. In fish, a least 12 different TLRs have been partially sequenced, compared to the 13 found in mammals, all branching from 6 super-families (TLR1, TLR3, TLR4, TLR5, TLR7, TLR11) (Roach *et al*., 2005).

The characteristics of fish complement are similar to the ones known in higher vertebrates but some particular features are unique to poikilotherms (Sunyer and Lambris, 1998). Fish complement tends to be more heat-labile, has stronger antimicrobial action, the optimal temperature seems to be lower than for homeotherms and multiple forms of some of its components have been discovered (Sunyer *et al*., 1997; Nonaka, 2001; Holland and Lambris, 2002; Lange *et al*., 2004). All these features make the fish complement system one of the most effective immune parameters to combat pathogen invasion (Sunyer and Lambris, 1998). Serum of fish consists of many different proteins that interact to either mark the pathogen (opsonisation) for cellular or humoral attack, inhibit its growth or directly destroy it by different biochemical pathways (mainly complement membrane attack complex but also proteases, lysozyme, antibacterial peptides, natural antibodies and cytokines) (Ellis, 1999; Wilson *et al*., 2002; Bergsson *et al*., 2005; Medzhitov, 2007).

Fish non-specific leukocytes, such as macrophages and neutrophils have been studied in many species. Both these types of cells can engulf particles by phagocytosis, the macrophages being more efficient than neutrophils, and kill pathogens primarily by the production of reactive oxygen species (ROS) (Ellis, 1999). Neutrophils have the additional capacity of killing bacteria through a myeloperoxidase driven mechanism involving halide ions (halogenetion) and H_2O_2 (Ellis, 1999). This cell type is the first to respond in a localized infection due to their high mobility, with the macrophages shortly following, driven by chemo-attractants such as chemokines (Secombes *et al*., 2001). The peak response of the two types of cells is reached

within a few days of the initial infection and their numbers and activation thereafter decrease (Secombes, 1996).

1.3.1.2. Adaptive immune system

The kidney is the major lymphoid organ in fish, but the spleen, thymus and mucosa-associated lymphoid tissues are also important in the production of immune cells (Press and Evensen, 1999). No bone marrow is present and the role of the lymph system is still controversial. Lymphoid tissues are generally composed of a framework of cells within which migratory and non-migratory immune cells are present. These migratory populations consist of immunoglobulin positive (B) and negative (T) lymphocytes, monocytes, macrophages, granulocytes and thrombocytes. There are also other types of non specific cells, considered less important in fish but nonetheless necessary, such as mast cells, non-specific cytotoxic cells and dendritic cells (Reite and Evensen, 2006; Bassity and Clark, 2007).

The adaptive immune system is characterised by the emergence of combinatorial antigen specific receptors (Kashahara *et al*., 2004). This system enhances the immune resistance to diseases through the anamnestic response (immunological memory), focussing the innate and adaptive defences towards a particluar target. The fundamental components of this system are immunoglobulins, T-cell receptors, major histocompatibility (MHC) complexes and recombinant activator genes (RAG) (Watts *et al*., 2001). Two types of cells are heavily involved in this response, B and T cells, which are both divided into 2 sub-groups, B1 and B2 as well as T helper (expressing either the cluster of differentiation (CD) 4 and/or CD8 markers) and cytotoxic T-cells (also expressing the CD8 marker). There are two different types of MHC, class I expressed ubiquitously on all nucleated fish cells (interacting with the CD8 molecule) and class II, only expressed on antigen presenting cells (APC), such as B cells, monocytes/macrophages and on activated T cells, interacting with CD4 molecules as shown in

Figure 1-4 (Mannings and Nakanishi, 1996). The latter has not been detected in all studied fish species.

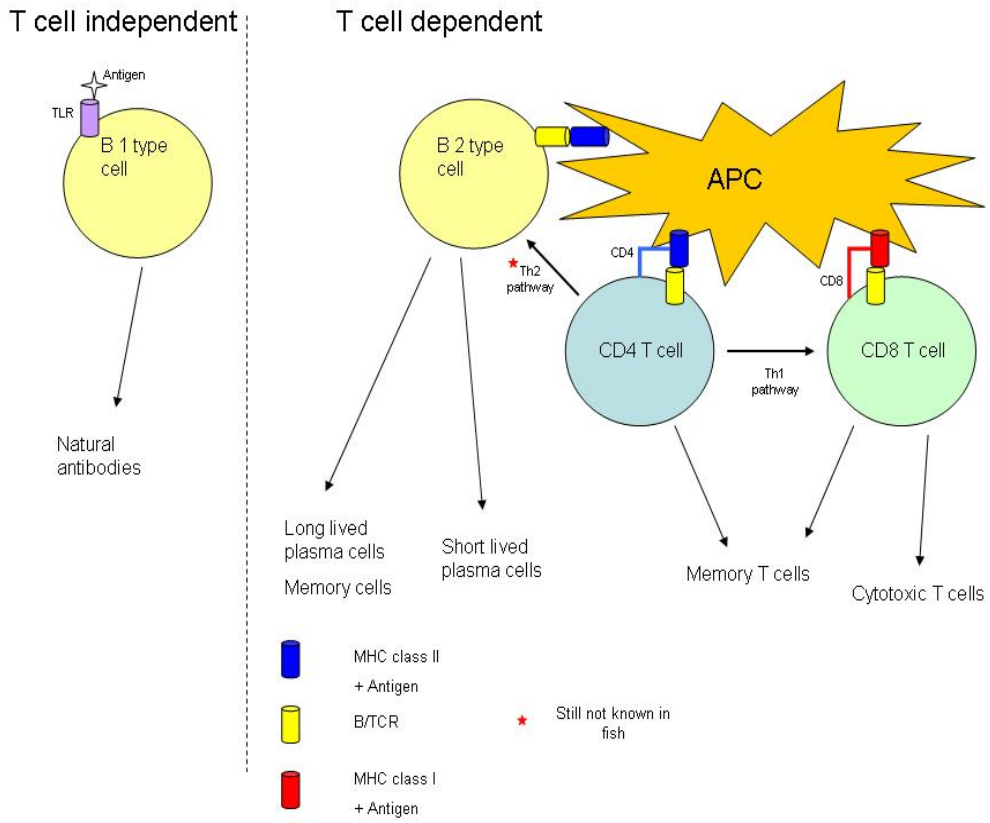

Figure 1-4 Summary of the specific immune system response to a foreign antigen.

Antigen presentation is a crutial part of the specific immune response. In fish, the processing and subsequent presentation of the antigens is believed to be similar to the processes described in mammals (Vallejo *et al*., 1992). In higher vertebrates, the pivotal messenger for antigen presentation promotion may be type I interferons (type I IFNs), through the up-regulation of MHC class I genes (Samuel, 2001) and these genes have been identified in several fish species (reviewed in Robertsen, 2006). The MHC class II (present only on Antigen Presenting Cells) genes have been identified in several fish species and are present on B and T lymphocytes, monocytes/macrophages, epithelial cells and dendritic-like cells (Vallejo *et al*.,

1992; Koppang *et al.*, 2003), as well as on acidophilic granulocytes (neutrophils) (Cuesta *et al*., 2006).

The B cells can also respond to non-specific inducers (Figure 1-4), without the involvement of T-cells, especially since, contrary to what is observed in mammals, the fish B cell is able to phagocytose micro-organisms (Li *et al*., 2006). This T-independent system is based around the interaction of antigens with low affinity poly-binding antibodies (equivalent to natural antibodies in mammals, Avrameas and Ternynck, 1995), which might be produced by a B1 subset equivalent. A specific response requires the interaction of APC, B (2) cells and T helper cells (Figure 1-4). Very recently, a new pathway of T cell differentiation has been elucidated, possibly derived from Th1 and Th2 CD4-positive cells and named Th17, releasing interleukin (IL)-17 and IL-22 (Liang *et al*., 2006); this system appears to exist in fish (Secombes, 2008). This pathway could be involved in the formation of antimicrobial peptides and could act against extracellular bacteria and fungi. The activation of Th cells leads to the specific B cell activation and differentiation, resulting in the production of specific antibodies as well as both B and T memory cells (Figure 1-4). The anamnestic response in fish is considered poor compared to mammals, possibly because immunoglobulin (Ig) M is the main antibody type present, known to have a lower heterogeneity or genetic diversity (Pilstrom and Bengten, 1996). Although a few other Ig molecules and genes have been found, such as IgD (Wilson *et al*., 1997; Stenvik *et al*., 2004), IgT (Hansen *et al*., 2005) and IgZ (Danilova *et al*., 2005), their function is still uncertain. The isotopic diversity of mammals might be compensated in fish by the nature of the IgM secreted which have been shown to be present not only in a tetrameric form but also in mono or dimeric configuration (Magnadottir, 1998). Fish IgM have also a high degree of flexibility which could increase its ability to bind epitopes (Kaattari *et al*., 1998). The environment in which the fish has evolved may have also influenced its specific immune response with sub-optimal conditions, also called non-permissive, such as temperature, affecting the production of

an adequate anamnestic response, especially regarding the T-dependant response (Rijkers *et al*., 1980).

The chemical signals which activate and regulate the specific immune response after an encounter with a recognised antigen are mainly composed of cytokines such as interferons (IFN), ILs, chemokines, colony stimulating factors (CSF) or tumor necrosis factors (TNF) (Secombes *et al*., 1996). After binding of the antigen, a cascade of these polypeptides or glycoproteins is released. Cytokines have been only recently studied in fish and further elucidation of the mechanisms involved still need to be explored. Some of the cytokines involved in mammalian and fish immunity are summarised in Table 1-5.

Table 1-5 Various cytokines involved in different types of immune response in mammals and fish.

Type of response	Cytokines involved	Additional information
Inflammatory	TNF-α, IL-1β, IL-6, IL-8	Mostly produced by macrophages, IL-1 is the precursor of the inflammatory response, TNF can kill cells (with IFN-γ).
Antiviral	Type I IFNs	
Cellular immune response inducers	IL-2, IFN-γ, LT-α, TNF, IL-12, IL-18	IL-2 and IFN-γ are released by Th1 cells and act against intracellular bacteria and some viruses.
Humoral immune response inducers	IL-4, IL-5, IL-6, IL-9,IL-13	Released by Th2 cells.
Regulatory (inhibitory)	IL-6, IL-10, IL-13, TNF-β, IL-23, TGF-β	IL-10 and TGF-β released by regulatory T cells (T reg) but TGF-β also stimulates the differentiation of Th to T reg.
Proliferation	G-CSF, M-CSF, stem cell factor	Induces the proliferation of different non specific immune cells.

TNF: Tumor necrosis factor; **IL**: Interleukin; **IFN**: Interferon; **LT**: Lymphotoxin; **TGF**: Transforming growth factor; **G-CSF**: Granulocyte-colony stimulating factor; **M-CSF**: Macrophage-colony stimulating factor.

The cytokines highlighted have not been identified in fish (Secombes, 2008).

1.3.2. Cod immune system

Atlantic cod has a poor specific immune system, but since this species has continued to thrive in the world's oceans for millions of years, it would appear to have sufficient innate immune defences to allow it to survive infection by many pathogens (Pilstrom *et al*., 2005).

1.3.2.1. Ontogeny

In the early stages of development, the cod immune system, as with most marine species, relies heavily, and for longer, on its innate immune parameters than freshwater species (Magnadottir, 2006).

Post-fertilization, no maternal IgM can be detected in cod eggs (Magnadottir *et al*., 2004), but they nevertheless have an efficient system of defences against pathogens. A small number of molecules associated with innate immune defences are detected before hatching, such as molecules of the complement system (C3) as well as apolipoprotein A-I, cathepsins, and lysosomal enzymes involved in cellular degradation (Magnadottir *et al*., 2004).

At hatching, cod are poorly developed and undergo a long larval period before metamorphosis (Pedersen and Falk-Pedersen, 1992). Post-hatching, many enzymes appear around first feeding (gelatinase, caseinase and collagenase, 6 days post hatch, dph), followed by haemoglobin a few days later (Magnadottir *et al*., 2004). Before metamorphosis, no immunoglobulin producing cells are detected and immunological organs are immature; the first plasma cells are detected around 58 dph (Schroder *et al*., 1998).

The onset of feeding, with the intake of bacteria from food and water, has been correlated with the start of the development of the specific immune cells in Atlantic cod (Pedersen and Falk-Pedersen, 1992).

1.3.2.2. Immune physiology

In adult fish, the head kidney is bilobular and dominated by haemopoietic tissues (Sorensen *et al*., 1997). In cod, this organ has been shown to contain many immune cells, with most identified as granulocytes resembling neutrophils, macrophages, and lymphocytes and plasma cells (Heumann and Roger, 2002). The head kidney and the spleen are major organs for trapping and clearance of foreign particulate material, with the accumulation of melano-

macrophages (especially in the head kidney) allowing a long retention of the antigens at these sites (Ronneseth *et al*., 2007). The spleen plays a more minor role in antibody production (Ronneseth *et al*., 2007), although some degree of organisation of antigen uptake and plasma cells has been observed in the spleen of cod (Arnesen *et al*., 2002). However, no co-localisation of antigen and plasma cells could be observed in the head kidney or spleen, or any clustering of B cells in the head kidney (Stenvik *et al*., 2001). Sorensen *et al*. (1997) found the leukocyte population of the head kidney of cod to consist of 50% granulocytes (in accordance with Ronneseth *et al*. (2007)), 33% macrophages and 7% lymphocytes. There was also a high proportion of neutrophils in the blood, twice that of salmon blood (Fausa Pettersen *et al*., 2000); important in protecting the cod against bacterial infections (Sorensen *et al*., 1997).

1.3.2.3. Cellular innate immunity

Leukocytes involved in innate immunity in cod, i.e. macrophages and neutrophils, have been isolated and cultured from head kidney (Sorensen *et al*., 1997; Steiro *et al*., 1998) as well as from blood (Nikoskelainen *et al*., 2006). Granulocytes, resembling neutrophils, from cod appear to vary in size, and are predominantly granular and mono-nucleated with a cytoplasm containing electron-dense granules; macrophages were roughly the same size as the granulocytes but with a more irregular shape and the absence of electron-dense granules (except for a few lysosomes) (Sorensen *et al*., 1997). The majority of macrophages, enriched by adhesion, are able to survive and remain phagocytic for a week in cell culture and up to 3 weeks with regular changes of media (Sorensen *et al*., 1997; Steiro *et al*., 1998). Unstimulated cells have been shown, by the same two studies to have a poor level of respiratory burst, and a fairly high level of variation in this response has been reported between individuals. A significant increase in respiratory burst (O_2^- and H_2O_2) was obtained with cod macrophages after incubation with LPS as well as β glucans, tuftsin, chitosan and, to a lesser extent, acid peptides (Sorensen *et al*., 1997). Acid phosphatase activity in macrophages was also

enhanced by culture in LPS-supplemented media (Sorensen *et al*., 1997). Generally, cod are considered to have a low response for those non-specific immune parameters (Steiro *et al*., 1998). This contradicted the findings of Nikoskelainen *et al*. (2006), who showed the respiratory burst of cod blood leukocytes to be at least double that reported in trout, although this may be due to the relative numbers of phagocytic cells in the blood of the two species.

1.3.2.4. Humoral innate immunity

Magnadottir (2000) described some very peculiar aspects of cod serum, such as the optimal temperature for serum haemolysis (SH), using sheep red blood cells, found to be 37°C. The addition of EDTA, a traditional inhibitor of both the classical and alternative complement pathways, as well as PMSF, was found to increase the SH capacity. Nevertheless, some common features were found with other teleosts, such as the inhibition of the SH by high levels of LPS and zymosans (partial in this case) and serum of other fish, goat or ascites (Magnadottir, 2000). The last point is the fact that cod SH activity seems to be driven by heat stable molecules since heating it at 56°C for 120 min only reduced the SH activity by 45%. The author concluded that haemolytic factors other than complement may be involved, either together or independently. In recent years, the haemolytic activity of cod reared in Iceland was reported to be very low (or not detectable) and a shift from the data presented previously (Magnadottir, 2000) seems to have occurred (Magnadottir, 2006). This activity seemed to be more “normal”, heat sensitive and inhibited by EDTA. One of the explanations given for this was the possible association of apolipoprotein A-I with complement in the low SH activity cod, which was not found in the unusually high activity group (Magnadottir and Lange, 2004). In most studies the level of variation of the haemolytic activity in cod was found to be very high (Magnadottir *et al*., 2001; Magnadottir *et al*., 2002). The variability in SH was hypothesised to be due to either genetic traits or to its sensitivity to undefined external or inherent factors and that there might be a seasonal effect contributing to this phenomenon.

Lysozymes are not thought to be involved in this high haemolytic activity of cod since very low levels are detected in the serum and no activity has been recorded in mucus (Bergsson *et al*., 2005), but a high chitinase activity was found in the plasma and various organs (Fletcher and White, 1973; Lindsay and Gooday, 1985). Other studies reported levels of lysozyme approximately half of that found in Atlantic salmon (Fagan *et al*., 2003; King V *et al*., 2006) as well as a high expression of a g-type lysozyme gene after vaccination (Caipang *et al*., 2008). The anti-protease activity of serum is generally high and seemed to be unaffected by immunisation or infection, but the seasonal effect could explain the slight variations observed (Magnadottir *et al*., 2001). Agglutinins and precipitins (lectins) are found in cod which produce C-reactive protein (CRP) pentraxin, but not serum Amyloid-P component (SAP). The resting level of CRP seems to be high (over 300 $\mu g.mL^{-1}$) and showed a strong individual variability in its reduced form (1 to 4 monomeric units of the 125 kDa molecule) (Magnadottir, 2006).

Antibodies present in the serum have been shown to very important in the early life defence system of fish through antiviral or antibacterial activity (Gonzalez *et al*., 1988; Sinyakov *et al*., 2002). The total immunoglobulin concentration in the serum of cod is somewhat 5 to 10 times higher than that measured in Atlantic salmon or other marine fish species (Israelsson *et al*., 1991; Magnadottir *et al*., 2001), and a positive correlation between IgM concentration and size/age, as well as rearing temperature of cod has been reported (Magnadattir *et al*., 1998). The total IgM can represent as much as 80% of the total protein in the serum of some cod, but generally, it constitutes 20% of the total protein (Stenvik *et al*., 2001). These results are reported to vary greatly between individuals (Magnadattir *et al*., 1998). Atlantic cod serum is often referred to as “sticky” and the reasons for this high level of immunoglobulins are not known. Samuelsen *et al*. (2006) hypothesised that this might be due to the uptake of bacterial antigens through the gut or the autonomous development of B cells to plasma cells without the involvement of particular antigens. The fact that different clusters of plasma cells, with separate

V-H families and no co-localisation, were found in non immunised cod, led Stenvik *et al*. (2001) to the hypothesis that immunoglobulin-producing B cells might have developed autonomously into plasma cells, or with the involvement of a "natural antigen" even if no evidence in any higher vertebrates exists for this phenomenon. This could also explain the increase in total IgM with the size of the fish. A similar idea is also mentioned in Solem and Stenvik (2006) regarding a possible weak clonal selection or an ongoing clonal expansion and differentiation of B cells in the particular clustered distribution of plasma cells. High levels of natural antibodies have been shown to possibly inhibit the production of specific antibodies in goldfish (Sinyakov *et al*., 2002) and due to the very high levels of natural antibodies in cod; this could contribute to the lack of detectable specific antibody response. Nevertheless, wolfish although having a high level of natural antibodies in the serum can still produce a high specific immune response to immunisation with different bacteria. In this case, the non-immunised fish natural antibodies showed very low cross-reaction with the different bacteria tested (Espelid *et al*., 2001).

1.3.2.5. Atlantic cod adaptive immunity

The development of the lymphoid organs in cod larvae seem to follow the "normal" ontogeny of these organs (head kidney, spleen and thymus) observed in other marine teleosts (Schroder *et al*., 1998), even if they seem to develop more slowly than in freshwater species (Razquin *et al*., 1990). In Atlantic cod, the head kidney and the spleen are the first organs to develop and are both present at the time of hatching, and the thymus is first detected 30 dph (Schroder *et al*., 1998). These immune organs seem to be fully developed by 50 dph but the first plasma cells only appeared a week later, apparently leaving the cod larvae entirely dependant on innate immunity to defend itself against pathogens for almost 2 months.

Gadoids have been described as poor or non-responders to hapten-carriers (Magnadottir *et al*., 2001) or bacteria (Espelid *et al*., 1991; Schroder *et al*., 1992; Lund *et al*., 2006). Cod seem to have all the components for a normal immune response regarding gene numbers, structure,

organization, diversity or expression of IgM and IgD chains (Solem and Stenvik, 2006), although the structure of cod IgD was shown to be different from that of Atlantic salmon (Stenvik and Jorgensen, 2000), this cannot fully explain the very low specific immunity in Atlantic cod (Samuelsen *et al*., 2006). Pilstrom *et al*. (2005) reviewed the results of a decade of research and summarised that cod do have B cells and that these cells produce immunoglobulin. They found cod B cells to have the machinery to express membrane receptors for antigens, and described that the Ig heavy chain structure, organisation and expression (VH, D, JH segments) cannot explain the lack of specific antibody response, even if the number of V-gene families is lower in cod than rainbow trout, with 4 and 11 families respectively (Roman *et al*., 1996; Stenvik *et al*., 2001). Pilstrom *et al*. (2005) also reviewed that the Ig light chain structure, organisation, transcription and expression cannot explain the lack of specific antibody response, while the sequences for TCR and MHC class I imply that the cod is immuno-competent against intracellular pathogens.

The poor adaptive immune response seen in cod is also found in other gadoids, such as haddock (Bentley, 2002) and pollock (Magnadottir *et al*., 1999b), which suggests that a possible impairment in specific immune response to certain pathogens (i.e. *V. anguillarum*) is common to some, if not all, gadoids.

The inflammatory response seen in Atlantic cod after vaccination is no different to that observed in other fish (Mutoloki *et al*., 2008). Cod also seem to have a normal proportion of plasma cells, approximately 1% of the total cell population in the head kidney, spleen and thymus (Schroder *et al*., 1998). The B cell proportion was found to be higher in the spleen than in the head kidney or the blood and their distribution in the spleen scattered (Ronneseth *et al*., 2007). These Ig (heavy chain) mRNA positive cells (most probably blast or plasma cells) were found to be mostly clustered close to the connective tissues surrounding the arterioles in head

kidney and spleen but did not seem to be co-localised with centres of pigmented cells in the head kidney (Stenvik *et al*., 2001), as described in other fish species (Press *et al*., 1994). Positive cells were also observed in the gut and the gills but their distribution seemed to be more scattered (Schroder *et al*., 1998).

Results from vaccination seem to suggest that cod are able to mount an immune response and obtained good protection against *V. anguillarum* and *V. salmonicida* (Schroder *et al*., 1992; Stromsheim *et al*., 1994; Lund *et al*., 2006). The low levels of specific antibodies detected in the serum of immunised cod seems to be directed towards LPS (T cell-independent response), which could explain the apparent successes of vaccination against pathogenic bacteria (Lund *et al*., 2006). Levels of specific antibodies to bacteria, haptened and non-haptened molecules, were very poor (Magnadottir *et al*., 2001). The same was observed against *V. salmonicida* (Schroder *et al*., 1992) and *V. anguillarum*, but *A. salmonicida* was reported to elicit a stronger immune response, even though still poor (Lund *et al*., 2006).

1.4. Vaccination

1.4.1. Vaccine design in fish

In 1796, Edward Jenner demonstrated that humans could be protected against smallpox virus by injecting a nine-year old boy with a live cowpox virus (Vaccinia); the term vaccine was then coined (Jenners, 1798). Almost 150 years later, Snieszko *et al*. (1938) showed that fish too were also able to mount an anamnestic response after injection of carp with heat killed *Aeromonas punctata*. A few years later, oral immunisation was successfully used to protect trout against *Bacterium salmonicida* (now known as *Aeromonas salmonicida*) (Duff, 1942). In the following two decades, the development of efficient sulfa drugs and the discovery of potent antibiotics diminished the interest in vaccine development for fish. However, in the late 1960's and early 1970's, the emergence of antibiotic resistant bacteria and the realisation of the

potential dangers of these treatments in fish for human health (Van Muiswinkel, 2008) refocused disease research towards fish vaccinology. Nowadays, vaccination is considered the most effective method for preventing infectious diseases in fish (Sakai, 1999).

The definition of vaccination (or immunisation) is the administration of a substance to produce immunity to a disease. The modern design of a successful vaccine requires two parts, first the identification of an antigen on the surface of the pathogen which could be recognised by the fish immune system, and the induction of an optimal immune response, so that the animal develops a strong protective immunity (Gudding *et al*., 1999). There are advantages working in fish for testing and development of vaccines, as the possibility to obtain licences to investigate the effect of very diverse formulations is easier than if using mammals, the number of animals that can be included in the studies (often several thousands of fish vaccinated and subsequently challenged) or the fact that fish are now regarded as very good animal models to understand the mechanisms investigated in higher vertebrates (van der Sar *et al*., 2004; Novoa *et al*., 2006). Three main types of vaccines have been used in aquaculture: (I) whole pathogens (live or dead), (II) subunit and (III) DNA vaccines (which can technically be classed as a subunit vaccine, (Clark and Cassidy-Hanley, 2005)).

Whole pathogen vaccines were the only type available until recently and present the immune system with a wide diversity of antigens present on the pathogen. This can sometimes mean that cross protection is acquired against closely related organisms, as between cowpox and smallpox viruses. However, this is not always the case, for example, different serotypes of *V. anguillarum* offer limited protection against other serotypes (Mikkelsen *et al*., 2007). The organism to be used in a vaccine can either be killed or attenuated. Inactivated vaccines are very safe since they are non-replicating, and are therefore non-infectious, but the majority of those vaccines lack the ability to induce a robust immunological response and are generally

considered inferior to live attenuated vaccines (Rogan and Babiuk, 2005). The killed pathogen may not contain important *de novo* proteins induced during infection, such as secreted toxins which could potentially be protective, for this reason, the media in which the bacteria is cultured is often added to the vaccine. The vast majority of bacterial vaccines used in aquaculture nowadays consist of inactivated pathogens (mostly multivalent formulations) and these have been proven extremely efficient for the prevention of many diseases (Hoel *et al*., 1998; Hastein *et al*., 2005). Nevertheless, the inclusion of several pathogens in a vaccine can lead to the inhibition of the specific response expected even if the individual pathogen induced protection, as seen with the combination of *Tenacibaculum maritimum* and *Photobacterium damselae* subsp. *piscicida* bacterins (Romalde *et al*., 2005). Several vaccines formulated with inactivated pathogens have failed to protect fish against bacterial and viral diseases, such as BKD (Piganelli *et al*, 1999), nevertheless, this approach should be studied first and if the results are not satisfactory after testing different formulations and delivery methods, other types of vaccines should be tried.

Advances in genetic engineering now allow live pathogens to be attenuated for use in vaccines. Live attenuated vaccines induce infection but have a reduced or a complete lack of ability to produce the disease (Rogan and Babiuk, 2005). These offer great advantages regarding the cost of production, since they are injected live and little processing is necessary, but the safety of such products is still debated and the efforts to commercialise them has been slow (Clark and Cassidy-Hanley, 2005). The potential risk of reversion of the pathogen to its original form is a very real danger, especially since the pathogen could be potentially shed from the infected animal into the aquatic environment. Attenuated vaccines are still rare in aquaculture although a lot of research is taking place with some promising results for many pathogens such as *Streptococcus iniae* (Locke *et al*., 2008), *Aeromonas hydrophila* (Vivas *et al*., 2004), *Yersinia*

ruckeri (Temprano *et al*., 2005), *Flavobacterium psychrophilum* (LaFrentz *et al*., 2008) and *Edwardsiella ictaluri* and *Flavobacterium columnare* (Klesius *et al*., 2005).

Subunit vaccines are based on the production of a particular antigen either purified directly from culture of the pathogen, expressed by a prokaryotic or eukaryotic system (Euzeby, 1997), or chemically synthesised, e.g. peptide vaccines. Their main advantage is that they are free of pathogen-derived toxins or immunosuppressive components. Any antigen can be included in the vaccine as long as it is accessible on the surface of the pathogen for the immune system of the host to recognise it (Grandi, 2001). The ease from a licensing and regulatory standpoint compared to attenuated or DNA vaccines makes this type of vaccine very attractive since they have no ability to replicate or invade the host's genome. The main hurdle of the development of a subunit vaccine is first identifying and then expressing the target protein in its correct three-dimensional structure. Bacteria and yeasts have been shown to often produce structurally deficient molecules from a structural point of view and the use of mammalian cells, which can overcome this problem, is extremely costly. Nevertheless, many new organisms are being investigated for protein expression, and ciliates (e.g. *Tetrahymena thermophila*) show great potential both from a cost and an expression point of view (Gaertig *et al*., 1999). The novel adjuvants and delivery method (mentioned later in this section) will also help to make subunit vaccines a realistic option for the future. Many of the subunit vaccines have been shown to be very efficient against some fish viruses and bacteria but one of the future potential uses of subunit vaccines is against parasites (reviewed in Clark and Cassidy-Hanley, 2005). This technology has already been used to produce successful vaccines against viral and bacterial diseases such as infectious pancreatic necrosis virus (IPNV) using the VP2 antigen (Christie, 1997), *Piscirickettsia salmonis* with the OspA protein (Kuzyk *et al*., 2001) or the T2 fragment against infection by striped jack nervous necrosis virus, (SJNNV, *Nodaviridae*) (Husgag *et al*., 2001).

DNA vaccines are an exciting new field in vaccinology. The principle is based on the injection of a plasmid coding a protective antigen, letting the host machinery transcribe and translate the selected gene, thus basically producing the vaccine *in situ* (Liu, 2003). DNA vaccines are for this reason, considered safe; they are also very stable and several protective antigens (as genes) can be theoretically injected in different plasmids (Gurunathan *et al*., 2000). Other advantages of DNA vaccines are the price of production once the design is proven protective and the possibility to tailor the type of response produced (Liu, 2003). The first DNA vaccine was approved in the US was for horse West Nile virus in July 2005 (CDC press release, http://www.cdc.gov), but the second was for fish (Infectious Haematopoietic Necrosis Virus DNA vaccine, Novartis) and the hurdle of licensing this vaccine was greater since the fish were farmed for human consumption. DNA vaccines, also called "vector vaccines" present similar problems to attenuated vaccines, e.g. the plasmid could integrate into the host's genome, be released into the environment, be integrated into other organisms, including humans or mutate (Rogan and Babiuk, 2005). Nevertheless, this possibility has been reported to be extremely unlikely (Lorenzen and LaPatra, 2005). Some of the DNA vaccine constructs designed have been shown to be very efficient at protecting fish against diseases which have failed using other methods. The plasmid encoding the G protein of rhabdoviruses (VHSV and IHNV) in salmonids (Lorenzen *et al*., 2000) is the biggest success to date and has been licensed for vaccine use in Canada (Norvartis). But many attempts using DNA technology to prevent diseases caused by viruses in fish have failed so far (reviewed by Lorenzen and LaPatra, 2005).

Most commercial vaccines contain adjuvants, a substance used to help boost the immune response so that less vaccine is needed or the overall protection is increased. They are generally considered to (I) increase the antigen uptake and cell presentation, (II) induce a "danger signal" through pattern recognition receptor signalling, (III) provide a secondary signal

(co-stimulatory) for lymphocyte activation (Secombes, 2008), and (IV) allow prolonged delivery of antigens (Evensen *et al*., 2005). Several substances, called immunostimulants, are used to initiate activation of innate immune mechanisms that may result, for example, in production of antimicrobial peptides and hence protect the fish more efficiently against pathogens (Bricknell and Dalmo, 2005). Beta-glucans, bacterial products such as lipopolysaccharides (LPS), brown and red algae and terrestrial fungi can be used as immunostimulants (Bricknell and Dalmo, 2005). Hormones and cytokines have also been considered as immunostimulants and can be used to enhance the defence system of fish (Secombes *et al*., 2001). As with most molecules having an effect on the immune system, the timing and doses of the compound have to be very carefully judged not to produce immuno-suppression or other non-beneficial effects (Sakai, 1999). Many adjuvants are used in fish vaccines with the main substances comprising oil (which seem to produce the best results), glucans and aluminium salts (Midtlyng, 1996). Some vaccines against common fish diseases, such as furunculosis, require the addition of an adjuvant to produce satisfactory levels of protection (Hamid, 2003). In Atlantic cod, several compounds have been tested, immunostimulants or adjuvants, such as cod milt (histone antimicrobial effect), lipopolysaccharides, polymannuronic acid, levimasol, chitosan and others, but only the first two were shown to have a beneficial effect on cod survival (Pedersen *et al*., 2004; Magnadottir *et al*., 2006). The LPS can have a significant effect on different cell populations, such as the possible maturation of monocytes into macrophages (Iliev *et al*., 2005a; Belosevic *et al*., 2006) and to prime macrophages and consequently increase the total respiratory burst level of macrophages and neutrophils in Atlantic salmon (Solem *et al*., 1995), goldfish (Wang *et al*., 1995; Neumann *et al*., 1995; Neumann and Belosevic, 1996), rainbow trout (Novoa *et al*., 1996) and dab (Tahir and Secombes, 1996). Phagocytosis of glucan particles by Atlantic salmon macrophages was shown to increase after incubation with LPS (Solem *et al*., 1995), in trout macrophage (MacKenzie *et al*., 2003) as well as Mx production

(Acosta *et al*., 2004). Gene expression of many cytokines was also found to increase after incubation of rainbow trout monocytes and macrophages with LPS, such as TNFα, TNF2, Interleukin (IL)-1β1 and COX-2 (MacKenzie *et al*., 2003; Iliev *et al*., 2005a). Nevertheless, fish have been reported to acquire tolerance to the long term use of immunostimulants (Matsuo and Miyazano, 1993; Zapata *et al*., 1997) and more research is needed to assess the risks of these to fish health.

Fish injected with oil-adjuvanted vaccines have been reported to present side effects, ranging from none to quite severe (Midtlyng *et al*., 1998). The extent of the lesions vary between fish species and the formulation of the oil adjuvants used, but reduced growth rate (fairly common), local reactions with the appearance of granulomas, and organ adhesion in the peritoneal cavity have been reported (Poppe and Breck, 1997; Midtlyng and Lillehaug, 1998). However, these draw-backs are considered to be offset by their protective effect.

Numerous vaccine delivery methods are available to the fish farmer such as injection, immersion, oral, hyperosmotic immersion and spray vaccination (Smith, 1988). The method offering highest protection seems to be intraperitoneal (IP) injection of the vaccine, followed by immersion and finally oral vaccination (Palm *et al*., 1998). The trend in protection is reversed when focussing on the practicality of vaccination, oral vaccines are easier to deliver than immersion treatments and injection is the most time consuming and results in the highest level of stress for the fish (Vandenberg, 2004; Gravningen and Berntsen, 2007). New delivery methods for vaccinating fish have been developed in recent years, and show promising results such as the multipuncture/immersion, eliciting better protection than achieved by immersion alone and similar to the results seen with IP injection (Nakanishi *et al*., 2002). Renewed interest is also been shown in hyperosmotic immersion method of vaccination (Huising *et al*., 2003), which was developed in the 1970s (Amend and Fender, 1976).

1.4.2. Vaccination in Atlantic cod

Vaccines against *V. anguillarum* specifically tailored for Atlantic cod have been developed in the last 10 years because the typical formulation used to protect Atlantic salmon against this pathogen did not include any isolates from the O2b sub-serotype, which is often recovered from outbreaks of vibriosis in farmed Atlantic cod. Homologous type vaccination-challenge experiments have been shown to be efficient against *V. anguillarum* (Espelid *et al*., 1991; Mikkelsen *et al*., 2004). Even in the case of heterogeneous type experiments (a different strain used for the challenge and the vaccine), juveniles and adult fish have shown very encouraging results for protection against the most common bacteria such as *A. salmonicida*, *V. salmonicida* and *V. anguillarum* (Schroder *et al*., 1992; Jones *et al*., 2000; Mikkelsen *et al*., 2004).

In order to protect cod against pathogens that tend to produce very high mortality during the larval and juvenile stages, it is very important to determine the earliest age at which the vaccination can be administered efficiently (Schroder *et al*., 1998). So, early vaccination is critical for the protection of juvenile cod and since plasma cells were first detected at 0.5 g in Atlantic cod (Schroder *et al*., 1998), the onset of immuno-competence was investigated regarding vaccine efficiency around this size. Schroder *et al*. (2006a) found that the minimal weight for vaccinating Atlantic cod juveniles was 2 g (also shown by Gravningen and Berntsen, 2007), but better results were obtained with 10 g fish dip-vaccinated at 28 weeks post hatch.

So far, vaccines seem to be efficient at protecting Atlantic cod against *V. anguillarum*, but specific antibody response cannot be detected in some studies (Espelid *et al*., 1991; Gravningen *et al*., 2005a; Gravningen *et al*., 2005b), or is extremely variable between individuals (Lund *et al*., 2006), with conventional methods such as ELISA unable to detect an antibody response to vaccination (Bricknell *et al*., 2006). At this point in time, the only reliable

method for investigating vaccine efficacy in cod is based on relative percentage survival (RPS) data post-challenge (Amend, 1981).

Other factors than the direct response to vaccination can be measured to give indications as to the efficacy of vaccines such as the antigen uptake or the cellular and humoral factors expressed and activated after vaccination. Vaccination of Atlantic cod with heat or formalin-inactivated bacteria produces a typical inflammatory reaction around the site of injection with both oil and water based vaccines as well as liposome encapsulation (Mutoloki *et al*., 2008). They authors showed a normal recruitment of mononuclear cells, more pronounced in groups injected by oil-based vaccines. Inflammation is prolonged for longer when oil adjuvanted vaccines are used compared to liposome and water-based vaccines (Gravningen *et al*., 2005a; Mutoloki *et al*., 2008). Caipang *et al*. (2008) showed that a water-based vaccine (heat inactivated *V. anguillarum*) elicited an up-regulation of the cytokines IL-8 and IL-1β in cod. These cytokines are involved in the inflammation process and the priming of leukocytes for respiratory burst and phagocytosis. The response of IL-8 was maintained for 10 days post-vaccination, possibly by the infiltration of neutrophils to sustain the elevated level of the cytokines. Some of the adjuvanted vaccines have also been shown to cross-protect against other pathogens and adjuvant-only formulations can protect Atlantic cod and other species against pathogens (Olivier *et al*., 1985; Mikkelsen *et al*., 2004; Gravningen *et al*., 2005b), which could be due to the recruitment of non-specific immune cells. This possibility was also raised by Caipang *et al*. (2008) after showing an up-regulation of NCCRP-1 which is a signal molecule to the non-specific cytotoxic cells. A possible down-fall of multivalent vaccines could be more severe reaction at the site of injection (Mutoloki *et al*., 2008), possibly due to the higher numbers of PAMPs presented to the immune system.

The antibacterial activity of cod serum seems to increase following vaccination. Caipang *et al*. (2008) identified a higher antibacterial activity against *V. anguillarum* after injection of an inactivated *V. anguillarum*, suggesting that innate immunity is involved in early defence against bacteria, and that this system can be enhanced by vaccination. Gene expression in some of the systems involved in antimicrobial activity were up-regulated as a result of vaccination and this offers an insight into the mechanisms that cod use to combat infections. Up-regulation of the bactericidal/permeability-increased protein and lipopolysaccharide-binding protein (BPI/LBP, Solstad *et al*., 2007; Caipang *et al*., 2008), goose-type lysozyme (Caipang *et al*., 2008; Larsen *et al*., In Press), transferrin and apolipoprotein A-I genes (Caipang *et al*., 2008) were all reported as early as one day post-vaccination, which seems to suggest that cod rely on innate immunity immediately after infection. Transferrin up-regulation could imply an important reliance on the iron regulation and sequestration when bacterial infection occurs, however, Arnesen *et al*. (2002), as well as Caipang *et al*. (2008), were not able to detect any increases in transferrin after water based vaccine injection.

Unfortunately, cell markers and molecular probes to detect mRNA of important proteins involved in the immune response of Atlantic cod are still not available, and the mechanisms involved in protecting this species for long periods after vaccination are still a mystery. Massive amounts of antigens of *V. anguillarum* were shown to be taken up in the spleen of cod 24 h post-vaccination, and first appeared in the spleen 1 hour after vaccination (Arnesen *et al*., 2002). The head kidney was also heavily involved in the clearance of these bacteria, with the atrium of the heart containing fewer antigens. Both these organs showed antigen-positive cells for 2 weeks post vaccination, but no antigens could be detected after this time. No co-localisation of IgM producing cells and antigen presenting cells could be detected.

In general, vaccines for Atlantic cod against *V. anguillarum* seemed to be very efficient in the first instance, although the mechanisms involved are still not understood. In recent years it has been apparent that commercial formulations used in the field do not protect this commercially important species against all the strains present in the environment. Several outbreaks in vaccinated fish have been reported (Hellberg *et al*., 2005-2006-2007) and more work is needed to investigate the variation of pathogenic strains, the immune response of cod against these strains and the formulation of vaccines used to provide broad protection against various serotypes of *V. anguillarum*.

1.5. Aim and objectives

The aim of this study was to investigate the possibility of designing a broad-range vaccine to protect Atlantic cod from *Vibrio anguillarum*.

The specific aims of this study were addressed through the following objectives:

To develop a set of monoclonal antibodies reacting specifically with the different pathogenic serotypes of *V. anguillarum*, and establish if any subgrouping previously unknown exists. This would allow inclusion of all the relevant serotypes of *V. anguillarum* affecting Atlantic cod in the vaccine formulation.

To develop a set of *in vitro* assays to investigate the immune response of cod phagocytic cells and serum to different virulence factors of different serotypes of *V. anguillarum*. This would assist in determining which isolates should be included in the vaccine.

To design and test a vaccine for Atlantic cod against the different *V. anguillarum* serotypes they are known to be susceptible to.

Chapter 2.

General material and methods

2.1. Bacteriology

2.1.1. *Vibrio* collection

A collection of over 150 isolates of *Vibrio anguillarum* was made with the help and generosity of a number of researchers worldwide, who very kindly provided the isolates listed in Table 2-1 and represent isolates from different fish species collected in different geographic regions.

Table 2-1 *Vibrio anguillarum* isolates used in the present study.

Species	Designation	Origin of the strains	Origin of isolation
Vibrio anguillarum	VA 16-RT 32	IZSV, IT	*Scophthalmus maximus*, 1985, ES
Vibrio anguillarum	VA 12-3402/D	IZSV, IT	*Oncorhynchus mykiss*, Kidney/29/09/99
Vibrio anguillarum	VA 15-408/ittio da UI	IZSV, IT	*Dentex dentex*, 12/10/00
Vibrio anguillarum	VA 14-325/ittio ceppo	IZSV, IT	*Oncorhynchus mykiss*, 17/08/01
Vibrio anguillarum	VA 13-138/I02 ceppo	IZSV, IT	*Solea solea*, Kidney/17/04/02
Vibrio anguillarum	VA 10-76/I03	IZSV, IT	*Dicentrarchus labrax*, Kidney/12/03/03
Vibrio anguillarum	VA 9-O2-43 F	IZSV, IT	*Scophthalmus maximus*, 14/06/99, ES
Vibrio anguillarum	VA 7-34/I04/1	IZSV, IT	*Dicentrarchus labrax*, Kidney/13/02/04
Vibrio anguillarum	VA 8-11008	IZSV, IT	*Scophthalmus maximus*, 14/06/99, ES
Vibrio anguillarum	VA 11-48/I02	IZSV, IT	*Dicentrarchus labrax*, Kidney/04/03/02
Vibrio anguillarum	VA 20-07-84(1)	INRA, FR	*Oncorhynchus mykiss*, IT
Vibrio anguillarum	VA 21-NATA 98-23	INRA, FR	*Scophthalmus maximus*, Noirmoutier, FR
Vibrio anguillarum	VA 17-15-99	INRA, FR	*Dicentrarchus labrax*, Mediterrenea, FR
Vibrio anguillarum	VA 18-49-00	INRA, FR	*Dicentrarchus labrax*, Antibes, FR
Vibrio anguillarum	VA 22-08-02	INRA, FR	*Dicentrarchus labrax*, Corsica, FR
Vibrio anguillarum	VA 19-09-02	INRA, FR	*Dicentrarchus labrax*, Corsica, FR
Vibrio anguillarum	VA 23-10-02	INRA, FR	*Dicentrarchus labrax*, Corsica, FR
Vibrio anguillarum	VIB 64-UB A023	HWU, UK	*Scophthalmus maximus*, ES
Vibrio anguillarum	VIB 65-UB A024	HWU, UK	*Scophthalmus maximus*, ES
Vibrio anguillarum	VIB 67-UB A055	HWU, UK	*Scophthalmus maximus*, ES
Vibrio anguillarum	VIB 68-UB A056	HWU, UK	*Scophthalmus maximus*, ES
Vibrio anguillarum	VIB 87-NCMB 1873	HWU, UK	*Salmo salar*, UK
Vibrio anguillarum	VIB 126-RVAU 840606-2/5	HWU, UK	*Oncorhynchus mykiss*, DK
Vibrio anguillarum	VIB 134-RVAU 91-8-178	HWU, UK	*Scophthalmus maximus*, NO
Vibrio anguillarum	VIB 135-RVAU 91-11-244	HWU, UK	*Scophthalmus maximus*, NO
Vibrio anguillarum	VIB 195-LMG 13222	HWU, UK	*Artemia spp*, ES
Vibrio anguillarum	VIB 227-CIP P03.82	HWU, UK	*Scophthalmus maximus*, FR
Vibrio anguillarum	VIB 236-CIP 10283	HWU, UK	*Scophthalmus maximus*, FR

Table 2-1 *Vibrio anguillarum* isolates used in the present study (cont.).

Species	Designation	Origin of the strains	Origin of isolation
Vibrio anguillarum	VIB 238-CIP 10466	HWU, UK	*Scophthalmus maximus*, FR
Vibrio anguillarum	VIB 253-Carson 85/3954-4	HWU, UK	*Oncorhynchus mykiss*, Tasmania, AU
Vibrio anguillarum	VIB 266-Carson 89/3748-1	HWU, UK	Rotifer, Tasmania, AU
Vibrio anguillarum	VIB 269-Evelyn, C1	HWU, UK	*Oncorhynchus keta*, CA
Vibrio anguillarum	VIB 546-UB A0236	HWU, UK	*Scophthalmus maximus*, ES
Vibrio anguillarum	VIB 549-UB A054	HWU, UK	*Scophthalmus maximus*, ES
Vibrio anguillarum	VIB 605-PT 213	HWU, UK	*Plecoglossus altivelis*, JP
Vibrio anguillarum	VIB 72T-LMG 4437-NCIMB6	HWU, UK	*Gadus morhua*, NO
Vibrio anguillarum	VIB 98-RVAU 860908-3	HWU, UK	*Scophthalmus maximus*, NO
Vibrio anguillarum	VIB 99-RVAU 89-2-62	HWU, UK	*Scophthalmus maximus*, NO
Vibrio anguillarum	VIB 100-RVAU 91-7-175	HWU, UK	*Scophthalmus maximus*, DK
Vibrio anguillarum	VIB 140-RVAU 910614-1/1	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VIB 242-CIP 11164	HWU, UK	*Scophthalmus maximus*, ES
Vibrio anguillarum	VIB 102-RVAU 1076/2	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VIB 103-RVAU 1089/2	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VIB 104-RVAU 1474/1	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VIB 105-RVAU 6828c	HWU, UK	*Scophthalmus maximus*, DK
Vibrio anguillarum	VIB 77-LMG 12099	HWU, UK	*Plecoglossus altivelis*, JP
Vibrio anguillarum	VIB 604-PT 493	HWU, UK	*Plecoglossus altivelis*, JP
Vibrio anguillarum	VIB 111-RVAU 1321/1	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VIB 114-RVAU 1712/1	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VIB 118-RVAU 1629	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VIB 122-RVAU 6828g	HWU, UK	*Scophthalmus maximus*, DK
Vibrio anguillarum	VIB 606-PB 15	HWU, UK	*Plecoglossus altivelis*, JP
Vibrio anguillarum	VIB 608-ET 1	HWU, UK	*Anguilla sp*, JP
Vibrio anguillarum	VIB 127-RVAU 3737/1	HWU, UK	*Oncorhynchus mykiss*, IT
Vibrio anguillarum	VIB 216-LMG 13243	HWU, UK	*Dicentrarchus labrax*, ES
Vibrio anguillarum	VIB 129-RVAU 850617-1/3	HWU, UK	*Gadus morhua*, FI
Vibrio anguillarum	VIB 215-LMG 13242	HWU, UK	*Dicentrarchus labrax*, ES
Vibrio anguillarum	VIB 197-LMG 13224	HWU, UK	Rotifer, GR
Vibrio anguillarum	VIB 198-LMG 13225	HWU, UK	Rotifer, GR
Vibrio anguillarum	VIB 199-LMG 13226	HWU, UK	Water, GR
Vibrio anguillarum	VIB 200-LMG 13227	HWU, UK	*Sparus aurata*, GR
Vibrio anguillarum	VIB 201-LMG 13228	HWU, UK	*Sparus aurata*, GR
Vibrio anguillarum	VIB 202-LMG 13229	HWU, UK	*Dicentrarchus labrax*, GR
Vibrio anguillarum	VIB 82-LMG 13186	HWU, UK	*Dicentrarchus labrax*, GR
Vibrio anguillarum	VIB 128-RVAU 850610-1/8b	HWU, UK	*Oncorhynchus mykiss*, DK
Vibrio anguillarum	VIB 141-RVAU 840523-2/4b	HWU, UK	*Oncorhynchus mykiss*, DK
Vibrio anguillarum	VIB 142-RVAU 840808-3/4a	HWU, UK	*Oncorhynchus mykiss*, DK
Vibrio anguillarum	VIB 149-RVAU V2 5/3	HWU, UK	Water, DK
Vibrio anguillarum	VIB 808	HWU, UK	*Scophthalmus maximus*, ES
Vibrio anguillarum	VIB 555	HWU, UK	*Scophthalmus maximus*, ES
Vibrio anguillarum	VIB 558	HWU, UK	*Scophthalmus maximus*, ES
Vibrio anguillarum	VIB 547	HWU, UK	*Scophthalmus maximus*, ES
Vibrio anguillarum	VIB 130-RVAU 91-7-152	HWU, UK	*Scophthalmus maximus*, DK
Vibrio anguillarum	VIB 133	HWU, UK	*Scophthalmus maximus*, DK
Vibrio anguillarum	VIB 66-UB A044	HWU, UK	*Scophthalmus maximus*, Sp
Vibrio anguillarum	VIB 29-HWU VA73	HWU, UK	*Dicentrarchus labrax*, GR
Vibrio anguillarum	VIB 30-HWU VA75	HWU, UK	*Sparus aurata*, GR

Table 2-1 *Vibrio anguillarum* isolates used in the present study (cont.).

Species	Designation	Origin of the strains	Origin of isolation
Vibrio anguillarum	VIB 2-1173/1	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VIB 4-1356/1	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VIB 5-1384/1	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VIB 6-1406/1	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VIB 8-1733/2	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VIB 9-1247/1	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VIB 10-1347/1	HWU, UK	*Gadus morhua*, DK
Vibrio anguillarum	VA 33-O2α	NVI, NO	*Gadus morhua*, NO
Vibrio anguillarum	VA 34-O2β	NVI, NO	*Gadus morhua*, NO
Vibrio anguillarum	VA68-02.40.5355	NVI, NO	*Gadus morhua*, NO
Vibrio anguillarum	VA-69-04.09.428	NVI, NO	*Gadus morhua*, NO
Vibrio anguillarum	VA70-04.09.325	NVI, NO	*Gadus morhua*, NO
Vibrio anguillarum	VA71-01.09.824	NVI, NO	*Gadus morhua*, NO
Vibrio anguillarum	VA72-03.09.449	NVI, NO	*Gadus morhua*, NO
Vibrio anguillarum	VA73-07.09.297	NVI, NO	*Gadus morhua*, NO
Vibrio anguillarum	VA74-01.50.1853	NVI, NO	*Gadus morhua*, NO
Vibrio anguillarum	VA75-04.09.412	NVI, NO	*Gadus morhua*, NO
Vibrio anguillarum	VA76-06.09.28	NVI, NO	*Gadus morhua*, NO
Vibrio anguillarum	VA 5-91079	UGDM, UK	*Scophthalmus maximus*, Scotland, UK
Vibrio anguillarum	VA 6-17/99	UGDM, UK	*Gadus morhua*, Scotland, UK
Vibrio anguillarum	VA 4-06/00	UGDM, UK	*Gadus morhua*, Scotland, UK
Vibrio anguillarum	VIB 1-6018/1	HWU, UK	*Oncorhynchus mykiss*, DK
Vibrio anguillarum	VIB 3-6062/A	HWU, UK	*Oncorhynchus mykiss*, DK
Vibrio anguillarum	VA 41-00/2552-1 (850)	DPIWE, AU	*Salmo salar*, Tasmania, AU
Vibrio anguillarum	VA 42-99/2107-1-AS (844)	DPIWE, AU	*Salmo salar*, Tasmania, AU
Vibrio anguillarum	VA 43-00/1147-6k (792)	DPIWE, AU	*Oncorhynchus mykiss*, Tasmania, AU
Vibrio anguillarum	VA 44-00/1676-1 (824)	DPIWE, AU	*Oncorhynchus mykiss*, Tasmania, AU
Vibrio anguillarum	VA 49-89/3748-2	DPIWE, AU	Rotifers, Tasmania, AU
Vibrio anguillarum	VA 45-00/2354-1k (849)	DPIWE, AU	*Oncorhynchus mykiss*, Tasmania, AU
Vibrio anguillarum	VA 46-85/3526 (778)	DPIWE, AU	*Oncorhynchus mykiss*, Tasmania, AU
Vibrio anguillarum	VA 47-85/3954-2	DPIWE, AU	*Oncorhynchus mykiss*, Tasmania, AU
Vibrio anguillarum	VA 48-89/1838	DPIWE, AU	*Oncorhynchus mykiss*, Tasmania, AU
Vibrio anguillarum	VA 50-98/2963-4 (753)	DPIWE, AU	Rotifers, Tasmania, AU
Vibrio anguillarum	VA 51-98/2963-1 (755)	DPIWE, AU	*Latris forsteri*, Tasmania, AU
Vibrio ordalii	V9-85/1039 (1367)	DPIWE, AU	*Oncorhynchus tshawytscha*, Marlborough Sounds NZ
Vibrio ordalii	V10-85/0679 (1366)	DPIWE, AU	*Oncorhynchus tshawytscha*, Stewart Island, NZ
Vibrio ordalii	V11-85/10739 (1365)	DPIWE, AU	*Oncorhynchus nerka*, Marlborough Sounds NZ
Vibrio anguillarum	VA 37-VIB 166 (PB 15)	KVL, DK	*Plecoglossus altivelis*, JP
Vibrio anguillarum	VA 38-VIB 167 (PB 28)	KVL, DK	*Plecoglossus altivelis*, JP
Vibrio anguillarum	VA 39-VIB 168 (ET 1)	KVL, DK	*Anguilla anguilla*, JP
Vibrio anguillarum	VA 52-AS-02-1977-1	AHL, AU	Brackish water fish, AU
Vibrio anguillarum	VA 53-AS-03-2336-3	AHL, AU	*Cheerax destructor* (freshwater crayfish), AU
Vibrio anguillarum	VIB102A (passaged)	Own	*Gadus morhua*, / passaged 17.11.06
Vibrio anguillarum	VIB2A (passaged)	Own	*Gadus morhua* / passaged 22.11.06
Vibrio harveyi	V 12	DRIM, FR	Not available
Vibrio splendidus	V 13-LGP32	DRIM, FR	Not available
Vibrio vulnificus	V 14	DRIM, FR	IT (gulf of Taranto)

Table 2-1 *Vibrio anguillarum* isolates used in the present study (cont.).

Species	Designation	Origin of the strains	Origin of isolation
Vibrio tubiashii	V 15-CRL10	DRIM, FR	Not available
Vibrio anguillarum	VA56	DRIM, FR	Not available
Vibrio anguillarum	VA 31	LEA, BE	*Dicentrarchus labrax* larvae, ES
Vibrio anguillarum	VA 29	LEA, BE	*Sparus aurata* larvae, GR
Vibrio anguillarum	VA 28	LEA, BE	*Sparus aurata* larvae, GR
Vibrio anguillarum	VA 30	LEA, BE	*Sparus aurata* larvae, GR
Vibrio anguillarum	VA 32	LEA, BE	*Sparus aurata* larvae, GR
Vibrio anguillarum	VA 58-V06-01	S-P, JP	JP
Vibrio anguillarum	VA 60-V06-08	S-P, JP	JP
Vibrio anguillarum	VA 62-V06-22	S-P, JP	JP
Vibrio anguillarum	VA 59-V06-03	S-P, JP	JP
Vibrio anguillarum	VA 61-V06-19	S-P, JP	JP
Vibrio anguillarum	VA 40-O1 AVL (78 skid)	S-P, UK	*Salmon salar*, UK
Vibrio anguillarum	VA40(2)-V. ang 1 (O1 AVL)	S-P, UK	UK
Vibrio anguillarum	VA 35-220 Strain B	S-P, UK	UK
Vibrio anguillarum	VA 36-259 Strain A	S-P, UK	UK
Vibrio ordalii	V6-	S-P, UK	CL
Vibrio ordalii	V7-	S-P, UK	CL
Vibrio ordalii	V8-	S-P, UK	CL
Vibrio anguillarum	VA 57-NB10	VDL, UK	SE
Vibrio anguillarum	VA 1-NCIMB6	VDL, UK	*Gadus morhua*, 1956, NO
Vibrio anguillarum	VA 66-Serotype O1	VDL, UK	Tasmania, AU
Vibrio anguillarum	VA 64-Serv 2b	VDL, UK	Machrihanish, Scotland, UK
Vibrio anguillarum	VA 67-Vib O1	VDL, UK	Machrihanish, Scotland, UK
Vibrio ordalii	VA 65-MSC-275	S-P, UK	Not available
Vibrio ordalii	V4-Vib O2	VDL, UK	Machrihanish, Scotland, UK
Vibrio carchariae	V 1-Flounder	VDL, UK	
Vibrio vulnificus I	V3-ATCC27562	VDL, UK	Human blood
Vibrio vulnificus II	V2-ATCC33148	VDL, UK	*Anguilla anguilla*
Vibrio ordalii	V5 (2)-Dom F3 Kid DF3K	VDL, UK	Kidney, *Oncorhynchus kisutch*
Vibrio anguillarum	VIB 7-6192/3	DMP, ES	*Anguilla anguilla*, DK
Vibrio anguillarum	VA 24-TM14	DMP, ES	*Oncorhynchus mykiss*, sea water, ES
Vibrio anguillarum	VA 27-RC71	DMP, ES	*Scophthalmus maximus*, ES
Vibrio anguillarum	VA 25-643/04	DMP, ES	*Solea senegalensis*, ES

IZSV: Istituto Zooprofilattico Sperimentale delle Venezie, Dr A. Manfrin, Padova, Italy; INRA: Institut National de la Recherche Agronomique,Dr C. Michel, Jouy-en-Josas, France; HWU: Heriot Watt University, Dr D. Austin, Edinburgh, United Kingdom; NVI: National Veterinary Institute, Section for Fish Health, Dr D. Colquhoun, Oslo, Norway; UG DM: University of Glasgow, Department of Microbiology , Dr H. Birkbeck, Glasgow, United Kingdom; DPIWE: Department of Primary Industry, Water and Environment, J. Carson, Tasmania, Australia; KVL: Royal Veterinary and Agricultural University, Dr L. Bay, Copenhagen, Denmark; AHL: Animal Health Laboratories, Department of Agriculture, Western Austrlai, Dr N. B. Buller, Perth, Australia; DRIM: Defense et Resistance chez les Invertebres Marins (IFREMER-CNRS), Dr P. Roch, Montpellier, France; LEA: Laboratory voor Ecologie en Aquacultuur, Zoologish Instituut, Dr. L. Grisez, Leuven, Belgium; S-P JP: Intervet-Schering-Plough, Dr S. Nakano, Hialeah, Japan; S-P UK: Intervet-Schering-Plough, Prof P. Smith, saffron Walden, United Kingdom; VDL: Veterinary Diagnostic Limited, University of Stirling, Prof H. Ferguson, Stirling, United Kingdom; DMP: Departamento de Microbiología y Parasitología, Universidad de Santiago de Compostela, Dr Y. Santos, Santiago de Compostela, Spain.

2.1.2. Growth and preparation of bacteria

Tryptone Soya Broth+NaCl (Appendix 1.2) was aliquoted aseptically into either 15 mL or 50 mL centrifuge tubes (Greiner Bio-one, Stonehouse, UK) depending on the final volume required. A single colony of the bacteria was collected from a plate or a loopful from a slope and inoculated into the broth overnight at 22°C with shaking (Adolf Kuhner AG, Basel, CH, 140 rev.min^{-1}). *Vibrio anguillarum* is a fast growing bacterium (growing overnight) while *Vibrio ordalii* required 36 h incubation to produce sufficient bacteria for use. Once the culture was sufficiently dense (using MacFarland Nº 4 as a reference, BioMerieux, Paris, FR), the tube was centrifuged at 3500 x g for 15 min (MSE, Mistral 2000R, 4°C), the supernatant was discarded and the bacterial pellet resuspended in the same volume with sterile phosphate buffered saline (PBS, 0.02M phosphate, Appendix 2.1). After vigorous vortexing, the tube was centrifuged, as previously described, and the supernatant discarded. The bacteria were finally resuspended in a small volume of sterile PBS, and after vortexing vigorously, the absorbance of the suspension was measured at 610 nm using a spectrophotometer (CECIL, CE 2041), using PBS as a reference. The optical density (OD) of the suspension was adjusted to 1.00 (±0.05) with sterile PBS.

2.1.3. Storage of bacteria

2.1.3.1. Long term storage

Vibrio anguillarum strains were streaked onto plates of TSA+NaCl (2%, Appendix 1.1) with a flamed metal loop and grown overnight in a 22°C incubator. If the colonies which grew showed a typical *Vibrio*-like morphology (Section 1.2.6.1), the bacteria were then further characterised by performing a Gram stain and 0/129 test (Section 2.1.4.2 and 2.1.4.3). If those tests were as expected, the strain was stabbed into 50x12 mm glass vials (Scientific Laboratory Supplies Ltd, Nottingham, UK) filled with 2 mL of TSA+NaCl solidified at a 45° angle (slope) and kept at RT.

Every 12 months each slope was checked for viability by sub-culturing the bacteria on TSA+NaCl plates and if identified as *Vibrio anguillarum*, the bacteria were stabbed in a new slope. Bacterial strains were also stored on cryo-beads (Technical Services Consultants Ltd, Heywood, UK) by inoculating a loopful of the strain (collected from a plate) into a cryo-vial, followed by shaking and removing the preserving media. The vial was stored at -70°C. To revive the strain from both storage media, a bead or loopful of bacteria was collected from the cryo-vial or slope and grown overnight in broth (Section 2.1.2) as well as streaking it onto TSA+NaCl.

2.1.3.2. Short term storage at -70°C in glycerol

To investigate the effect of short term storage at -70°C, in different concentrations of glycerol (Sigma-Aldrich, between 5 and 50% in PBS) and the subsequent recovery of bacteria, viability cell count (2.1.5) was measured. The highest recovery of live bacteria was found when using a 15% solution of glycerol.

Bacteria were grown overnight in TSB+NaCl (Section 2.1.2) and 1 mL of the solution was aliquoted into centrifuge tubes, centrifuged at 3500 x g for 15 min and resuspended in 1 mL of 15% glycerol in PBS. The tubes were then centrifuged as previously described and the supernatant discarded, leaving only 40 µL to cover the pellet. The different bacteria were stored at -70°C until used. To revive the bacteria, 960 µL of PBS were added to the bacteria of interest, and after 5 min at RT the bacteria were vortexed vigorously before centrifugation at 3500 x g for 15 min. The pellet was then resuspended in 1 mL of PBS and kept on ice until used. To estimate the percentage survival of the bacteria, viable cell counts (Section 2.1.5) were performed with an average recovery superior to 80% (data not shown).

2.1.4. Identification of *Vibrio anguillarum*

Vibrio anguillarum is a Gram negative bacterium, described as short and curved rods under the microscope, forming creamy colour colonies on non-selective media with a requirement for salt (Section 1.2.6.1).

2.1.4.1. API20E

API20E is a 20 reactions system test used typically for the identification of enterobacteria including amino acid decarboxylation and carbohydrate fermentation. Following the manufacturer's instructions (BioMerieux, FR), different profiles were found for *Vibrio anguillarum*, but the most common reaction profile is shown in Figure 2-1.

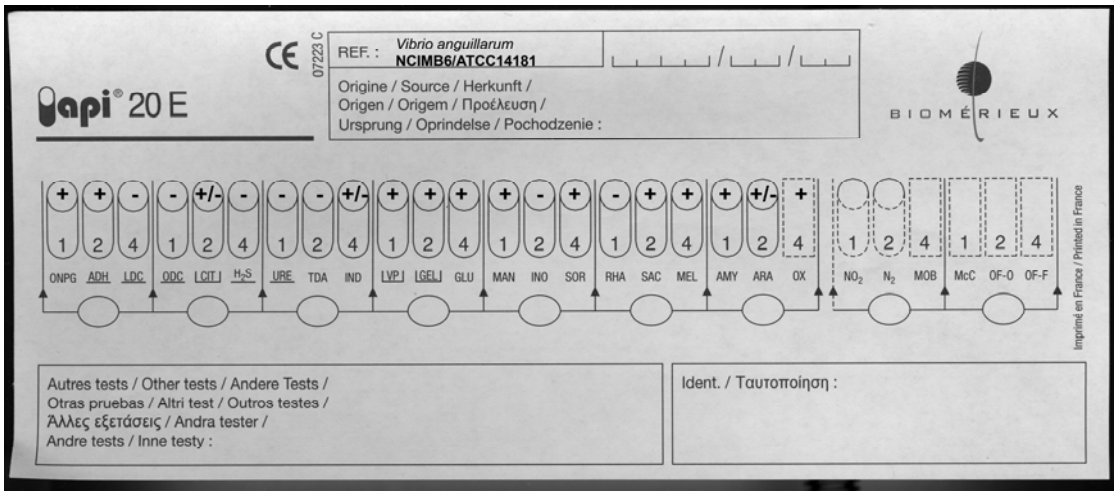

Figure 2-1 Typical API20E profile for *V. anguillarum* NCIMB6 type strain.

2.1.4.2. Gram staining

The technique used during this study was adapted from Gram (1884). Briefly, part of a colony of the bacterium of interest was mixed with one drop of sterile 2% NaCl solution (w/v, Sigma-Aldrich, Poole, UK) onto a glass slide (Surgipath, Bretton, UK) using a sterile metal loop. The slide was left to air dry at room temperature (RT, 20-22°C) and was then passed 3 times though the flame of a Bunsen-burner to fix the bacteria. A few drops of Crystal violet solution (Appendix 1.3.1) were placed onto the fixed bacteria and left for 1 min at RT. The stain was washed off under running tap water and replaced with Iodine solution (Appendix 1.3.2) and

incubated for 1 min. The slide was rinsed in acetone (VWR, Lutterworth, UK) and then under running water. Safranin solution (Appendix 1.3.3) was placed onto the slide and incubated for 2 min at RT. Finally the slide was rinsed under running tap water and left to air dry at RT. The morphology of the colony was then observed using an Olympus BH2 microscope at 100x magnification under oil immersion.

2.1.4.3. 0/129 Vibriostatic test

One colony of the bacterium under investigation was mixed in 2 mL of sterile 2% NaCl solution and vortexed vigorously. Five drops of the solution were spread onto a Tryptone Soya Agar plates containing 2% NaCl (TSA+NaCl, Appendix 1.1) using a sterilised glass rod. The plate was left to dry at RT for a few minutes and discs of 10 µg and 150 µg of 2,4-diamino-6,7-diisopropylpteridine (Oxoid, Thermo fisher Scientific Ltd., Cambridge, UK) were placed on opposite sides of the plate using flamed forceps. The plate was incubated overnight at 22°C and the size of the growth-inhibition zone was measured. A clearing zone of 9 mm or greater around the 150 µg disc was considered as a sensitive result (Bernardet and Grimont, 1989), while any inhibition zone around the 10 µg disc was considered sensitive.

2.1.5. Bacterial concentration using viability counts

Viable cell counts were carried out using the technique of Miles *et al*. (1938). First, a standard curve for *Vibrio anguillarum* was constructed. Briefly, 8 different strains of *V. anguillarum* were grown overnight (Section 2.1.2) and after measuring the OD (absorbance at 610 nm), six ten-fold serial dilutions of the bacteria suspension were made by adding 500 µL of the bacterial suspension to 4.5 mL of 2% NaCl (final dilution 10^{-6}). A TSA+NaCl plate was marked in 6 equal sectors and 20 µL of the suspension was added to each sector (6 replicates). After incubation overnight at 22°C, the number of colony forming units (cfu) was counted in each sector on the 10^{-6} dilution plate and multiplied by 5 x10^{7} to obtain the number of cfu.mL^{-1} of solution. A

standard curve (concentration vs. OD) was generated for 8 *V. anguillarum* isolates after estimating the concentration of bacteria using the viability count method previously described for different bacterial dilutions, and is represented on Figure 2-2, using SigmaPlot 10.0 software (Systat software Inc, Hounslow, UK).

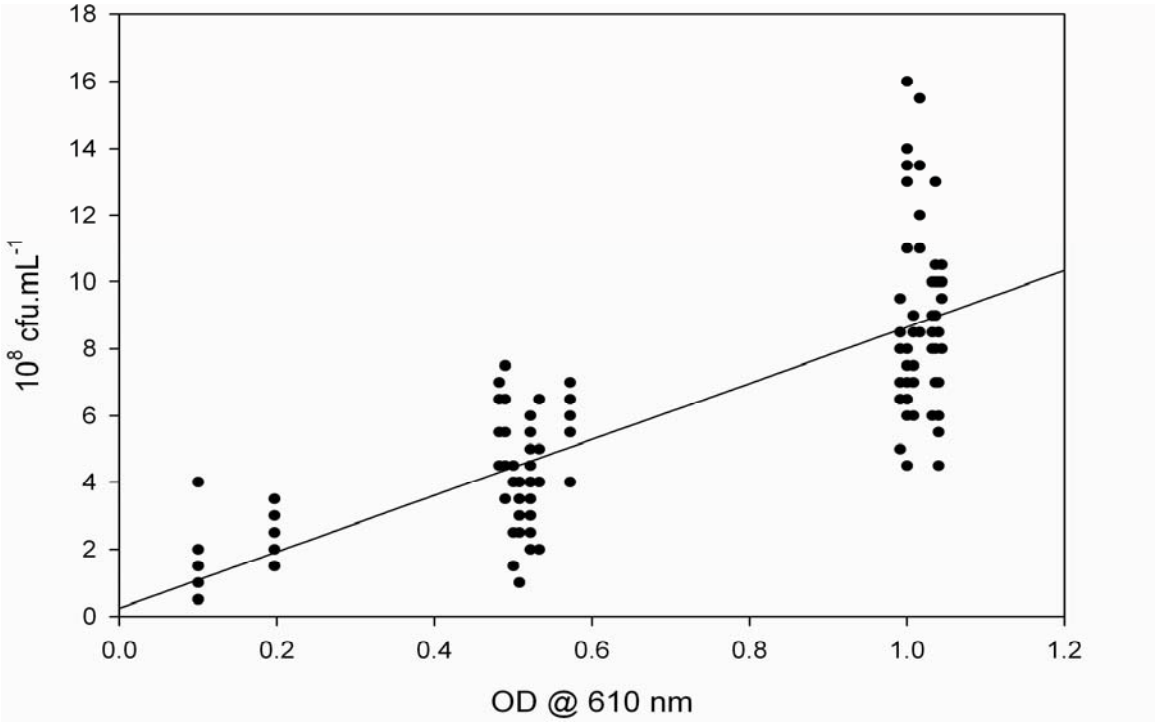

Figure 2-2 Concentration (cfu.mL^{-1}) of different *V. anguillarum* isolates calculated after determining the viable cell counts and absorbance at 610 nm of different dilutions of bacteria.

Using the regression curve on the Figure 2-2, the concentration of *Vibrio anguillarum* can then be estimated at any given OD using Equation 2-1.

$$Y = (8.41\ X + 0.24)\ x10^8$$

Equation 2-1 Relationship between optical density of *Vibrio anguillarum* at 610 nm (X) in PBS and concentration in colony forming units per mL of solution (Y).

Equation 2-1 gives a concentration of *V. anguillarum* between 8.23 and 9.07 x10^8 cfu.mL^{-1} for an optical density of 1.00 ± 0.05 at 610 nm.

In order to estimate the number of bacteria in any sample, either the standard curve was used after measuring the OD of the bacteria at 610 nm or a viable cell count was carried out following the method described earlier.

2.1.6. Formalin killing of bacteria

Bacteria were grown overnight in TSB+NaCl (Section 2.1.2) and formalin (Sigma-Aldrich) was added at a final concentration of 0.5% (v/v). After incubation overnight at 4°C, the bacteria were streaked onto TSA+NaCl plates to ensure that they were dead (no growth recorded after 24 h at 22°C). The formalin-killed bacteria were then centrifuged at 3500 x g for 15 min and resuspended in the same volume of 15% sodium meta-bisulfite (w/v, Sigma-Aldrich) and incubated overnight in PBS to neutralise the formalin. The bacteria were then washed three times with PBS. The optical density was adjusted to 1.0 in PBS and the bacteria used immediately or stored at -20°C.

2.2. SDS PAGE

2.2.1. SDS PAGE protocol

The gel caster (Biorad Labs Ltd, Hemel Hampstead, UK) was assembled according to the manufacturers' instructions. The separating gel (12% polyacrylamide) was made by mixing 5 mL of SDS separating buffer (Appendix 2.3) with 7 mL of distilled water and 8 mL of 30% acrylamide/Bis acrylamide solution (Severn Biotech Ltd, Kidderminster, UK). The solution was degassed for 30 min and 15 µL of TEMED (Sigma-Aldrich) and 70 µL of ammonium persulphate (Sigma-Aldrich, 100 $mg.mL^{-1}$ in distilled water) were added. After mixing by gentle inversion, the separating gel was poured into the gel caster and 200 µL of iso-propanol (VWR, UK) layered on top. The gel was left to polymerise at RT for 60 min. The stacking gel (4% polyacrylamide) was prepared by mixing 2.5 mL of SDS stacking buffer (Appendix 2.4) with 6.1 mL of distilled water and 1.34 mL of 30% polyacrylamide solution. The solution was degassed

for 30 min and 10 µL of TEMED and 50 µL of APS were added. The solution was added on top of the separating gel after washing off the iso-propanol several times with distilled water, and was left to polymerise for 60 min. After polymerisation, the gel was assembled in the electrophoresis system (BioRad, Labs Ltd) and SDS running buffer (Appendix 2.5) was added to the reservoir tank.

After growth of the bacteria of interest (Section 2.1.2), 500 µL of each bacterial suspension was mixed with 500 µL of 2x SDS sample buffer (Appendix 2.2). The samples were boiled for 10 min and vigorously vortexed before aliquoting in 100 µL and stored at -20°C.

Ten µL of bacterial sample in SDS sample buffer was added to the wells of the gel, as well as 7 µL of protein marker (New England Biolabs, Hitchin, UK) in the last well. When the gel was destined for Western Blotting (Section 2.3), 7 µL of Rainbow marker (Bio-Rad, UK) was used instead. The gel was subjected to electrophoresis at 150 V for 20 min (or until the proteins reached the 12% gel) followed by 200 V for 40 min (or until the samples reached the bottom of the gel). The protein bands were stained with either Coomassie blue staining (Section 2.2.2) or Silver staining (Section 2.2.3) or the gel used for Western blotting (Section 2.3). After staining, the gel was scanned (EPSON expression 1680 Pro at 600 dpi, using Pagemanager, Presto software 4.80.15, Newsoft Inc., Milpitas, US) and dried in a cellophane casting (Section 2.2.4).

2.2.2. Protein Coomassie Brilliant Blue staining

The gel was left overnight in 50 mL of Coomassie blue solution diluted following the manufacturers' instructions (Brilliant blue G solution, Sigma-Aldrich). The gel was destained with several 10 min washes with destain solution (40% methanol (Thermo fisher scientific Ltd, UK), 10% acetic acid (VWR) in distilled water) until the bands clearly appeared. The gel was then soaked in distilled water until scanned and dried (Section 2.2.4).

2.2.3. Silver Staining

This staining technique was adapted from Swain and Ross (1995). All the solutions used were 100 mL unless otherwise stated. Briefly, the gel was fixed overnight in a solution of 10% acetic acid (VWR) and 40% ethanol (Thermo fisher scientific Ltd) in distilled water (fixative solution). The gel was then rinsed for 10 min in distilled water and incubated for 5 min in the fixative solution plus 0.05% gluteraldehyde (v/v, Sigma-Aldrich) and 0.01% formaldehyde (v/v, Sigma-Aldrich). The gel was rinsed in fixative solution for 20 min and then in distilled water for another 20 min. The sensitiser solution was added for 1 min (Sodium thiosulphate, VWR, 0.49 $mg.mL^{-1}$ in distilled water) and the gel rinsed twice for 1 min in distilled water. The gel was stained with silver nitrate (Sigma-Aldrich, 1 $mg.mL^{-1}$ in distilled water) for 15 min before 2 rinses of 1 min in distilled water. The gel was developed in sodium carbonate solution (Sigma-Aldrich, 2.5 $mg.mL^{-1}$ in distilled water) with 0.04% formaldehyde. The reaction was stopped by rinsing several times in 5% acetic acid (VWR) in distilled water. The gel was then soaked in distilled water until scanned and dried (Section 2.2.4).

2.2.4. Gel Drying

The gel was dried by placing it between two sheets of hydrated cellophane (VWR) in the middle of a drying frame clamped together and left at RT overnight. The dried membrane was then cut around the gel leaving a 1 cm margin.

2.3. Western blotting

The protocol used was adapted from Wiens *et al*. (1990). The SDS gel was soaked for 30 min in blotting buffer (Appendix 3.1) together with 6 pre-cut pieces of filter paper (Whatman n°1, GE Healthcare, Maidstone, UK) and nitrocellulose paper (Amersham Biosciences, Little Chalfont, UK), all cut to the size of the gel. The wet-blotter tank (Thermo fisher Scientific Ltd) was filled with the blotting buffer and the blot prepared by placing 3 layers of filter paper on each side of

the gel and the nitrocellulose membrane (Figure 2-3). Air bubbles were removed by rolling a plastic pipette on top of the stack.

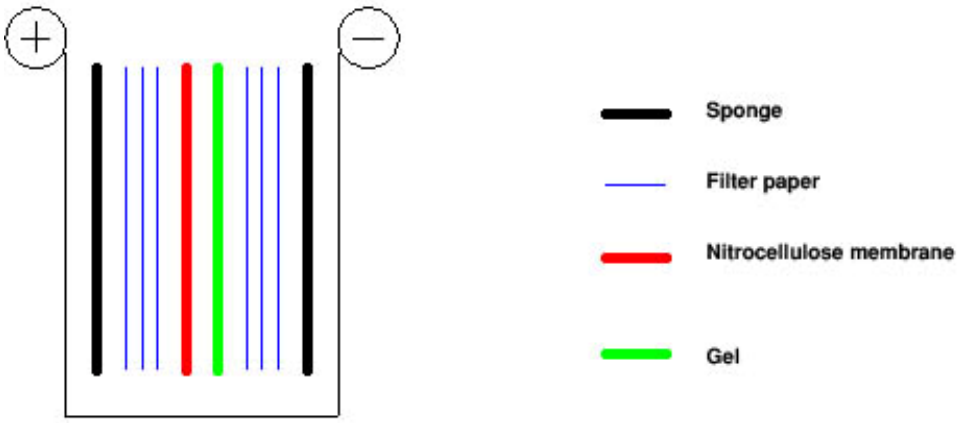

Figure 2-3 Layout of gel/nitrocellulose membrane used for transferring proteins from SDS gel to nitrocellulose membrane.

The proteins were transferred from the gel to the nitrocellulose membrane by applying a current of 60 V for 60 min. The SDS gel and the filter paper were discarded after ensuring that the proteins had transferred to the nitrocellulose membrane (visualised through the transfer of the Rainbow marker). The membrane was then blocked overnight in 100 mL of 1% Bovine Serum Albumin (w/v, BSA, Sigma-Aldrich) in Tris Buffered Saline (TBS, Appendix 3.2) at 4°C. The paper was rinsed 3 times for 5 min in Tween-TBS (TTBS, Appendix 3.3) on a rocker at 40 $rev.min^{-1}$. The membrane was incubated in 10 mL of neat monoclonal antibody (mAb) supernatant (Section 3.3.2.1) for 60 min at RT on a rocker and rinsed 3 times as previously described. The membrane was then incubated in 10 mL of goat anti-mouse IgG conjugated to Horse Radish Peroxidase (Sigma-Aldrich, 1:400 in TBS) for 60 min at RT on a rocker at 40 $rev.min^{-1}$. After rinsing 3 times for 5 min in TTBS followed by one 1 min rinse in TBS, the bands recognised by the antibodies were stained by adding the developing solution (Appendix 3.4) at RT for 7 to 10 min. The reaction was stopped with several rinses in distilled water. The membrane was then scanned and dried as for the SDS gel.

2.4. Enzyme Linked Immuno-Sorbant Assay (ELISA)

Indirect ELISA was used throughout the study; all the reactions were carried out at RT unless otherwise stated.

The technique used for this assay was adapted from Adams and Thompson (1990). A 96-well ELISA plate (Immunlon, Thermo Ficher Scientific) was coated with 50 µL.well^{-1} of 0.05% poly-L-lysine (w/v, Sigma-Aldrich) diluted in coating buffer (Appendix 4.3) and incubated for 1 h. The plate was then washed twice in Low Salt Wash buffer (LSW, Appendix 4.1) and tapped dry. One hundred µL of bacteria (Section 2.1.2) or butan-1-ol extracted LPS (Section 3.2.1.2, at 2.5 mg.mL^{-1} in PBS) was added to each well (in duplicates) and incubated at 4°C overnight. Fifty µL of 0.05% gluteraldehyde (v/v, Sigma-Aldrich) in PBS was added to each well and incubated for 20 min to fix the bacteria. The plate was then washed three times in LSW buffer and 250 µL of 1% bovine serum albumin (w/v, Sigma-Aldrich) or 3% casein (w/v, Marvel, UK) in distilled water was added and incubated for a minimum of 2 h at 37°C. The plate was washed again three times in LSW buffer and 100 µL of neat mAbs supernatant (Section 3.3.2.1) was added to the appropriate wells for 1 h, 2 wells per bacteria isolate were incubated with PBS instead of the mAbs to calculate the cut off point of the reaction (negative background). The plate was washed five times in High Salt Wash buffer (HSW, Appendix 4.2) with the last wash left for 5 min before tapping the plate dry. The plate was incubated with 100 µL of secondary antibody (Goat-anti-mouse HRP conjugated, Sigma-Aldrich, 1:4000 in 1% BSA (w/v) in LSW buffer) for 1 h. The plate was washed again 5 times as previously described with HSW buffer and 100 µL of developing solution (Appendix 4.4) was added to all the wells and incubated until a blue colour appeared for the positive control (1 to 10 min). The reaction was stopped by adding 50 µL of 2M H_2SO_4 (VWR). The absorbance was read on a spectrophotometer (DYNEX technologies, Worthing, UK) at 450 nm.

Chapter 3.

Development of monoclonal antibodies for serotyping *Vibrio anguillarum*

3.1. Introduction

The principle behind serotyping is the grouping of organisms (bacteria or viruses) based on their cell surface antigens. Serotyping establishes groups within a species called serotypes (or serovars), but those groups are only defined by their reaction to a particular antibody, so the detection of different antigens (or epitopes) on the surface of a bacterium by different antibodies can potentially lead to different serotyping schemes, sometimes called epigroups or epitypes.

Complete understanding of serological classification of strains within a bacterial species facilitates the development of vaccines, as well as epidemiological studies of the different groups of bacteria involved in disease outbreaks.

Serotyping relies on the interaction of antigens with antibodies. These immunoglobulin molecules are produced by plasma cells (fully differentiated antibody producing B lymphocytes) through the expression of genes (V-D-J segments of the light anf heavy chain) which have a very high number of possible combinations, thus allowing an almost infinite number of epitope binding domains to be produced (Ollila and Vihinen, 2005). Each B cell produces a single antibody type with a unique specificity, and this is the basis of specific antibody production for specific recognition and serotyping.

3.1.1. Monoclonal and polyclonal antibodies

Two types of antibodies are used for serotyping i.e. monoclonal antibodies (mAb), containing antibodies able to recognise only one epitope and polyclonal antibodies (pAb), containing a mixture of antibodies with different specificities.

Polyclonal antibody serum is produced by injecting an animal (usually rabbit) with a selected antigen (virus, whole bacteria or molecules extracted from the pathogens), this results in the production of antibodies reacting against the different epitopes present of the antigens injected. The advantage of using pAbs lies in the ease of production and the relatively large quantities of serum with high antibody concentrations that can be obtained. One problem with using pAb for serotyping arises from the multitude of antibodies present in the serum with different specificities. These can react with closely related serotypes because of the presence of common epitopes, and thus the serum may need to be cross-absorbed to obtain a specific reaction. Cross-absorption is the removal of antibodies from the serum which cross-react with other serotypes being investigated, leaving only antibodies which react with the serotype against which the serum was made. To study sub-serotypes using pAbs, a large number of isolates need to be screened with different cross-absorbed sera, making the task very laborious. Another problem is that the supply of the serum is limited and when finished, another animal has to be immunised and the serum collected can vary from the original.

Monoclonal antibodies, compared with pAbs, are produced by a single B-cell clone. This results in the production of antibodies with identical specificity to a particular epitope. The innovative method for producing mAbs was developed by Kohler and Milstein (1975), who received the Nobel Prize for Physiology or Medicine in 1984 for this discovery. They produced B-cell descendants after fusing together healthy plasma cells and neoplastic myeloma cells to produce so called hybridoma cells which have the combined ability of the plasma cell (to

secrete the same specificity immunoglobulins) and the characteristics of a tumour cell (to divide in an unlimited fashion, making them virtually immortal). Another advantage of working with mAbs is that they are easily produced in culture and are relatively easy to purify (Luttmann *et al*., 2006). The disadvantages are easily compensated by the advantages but the selection of a suitable hybridoma cell for a specific epitope can be labour intensive. The cells can also lose their ability to produce mAbs (Frame and Hu, 1990), and finally, the production of an epitope specific antibody can be a problem for some applications which require a large or broad binding capacity.

Almost any type of molecule can be used as an antigen for antibody production, as long as it elicits an immune response. One of the most antigenic molecules of Gram negative bacteria is the lipopolysaccharide (LPS) complex (Johnsen, 1977). This molecule, found on the surface of bacteria, is a complex of sugars and lipids and is heat-stable (Section 1.2.6.2). For these reasons, LPS is often chosen as the antigen for serotyping Gram negative bacteria.

3.1.2. LPS immunogen

LPS molecules are very immunogenic and may constitute the most potent and multivalent molecule of bacterial origin for mammals (Jacques, 1996). This might be explained by the fact that the innate immune system seems to have evolved towards an increased recognition of structurally conserved molecules such as fungal cell walls, bacterial LPS and peptidoglycans or viral double stranded RNA (Robertsen, 1999). The LPS molecules are present on the majority of Gram negative bacteria membranes, this structure is then easily recognised by a number of specific binding molecules, such as LPS binding protein (LBP, Tobias *et al*., 1986) and a closely related protein, bactericidal/permeability-increasing protein (BPI, Elsbach and Weiss, 1993) as well as antibodies. The LBP-LPS complex has been shown to activate some branches of the innate immune system of mammals and BPI can exert strong antibacterial

activity, acting as an opsonin and having neutralising activity against LPS. In fish, only one gene was identified in carp (Thompson *et al*., 2004), catfish (Xu *et al*., 2005) and cod (Stenvik *et al*., 2004), showing more homology to the BPI sequence than to the LBP; however in rainbow trout two homologous genes where found (Inagawa *et al*., 2002), which may be the precursors of LBP and BPI in mammals. Since it is mainly the activation of the LBP that leads to endotoxic shock, this could explain why fish have a higher tolerance to LPS (discussed later). There are also questions about the existence of TLR4-mediated cellular responses to LPS in fish (Iliev *et al*., 2005b), which in mammals is involved in the specific recognition of LPS motifs. This needs to be clarified but could add weight to the reasons for low LPS toxicity in fish.

Enterobacteria have LPS consisting of three distinct regions (Section 1.2.6.2), the lipid A, the core oligosaccharide and the O side chain (or O antigen) as seen on Figure 3-1. The O antigen is extensively variable in its composition and conformation (repeating units of oligosaccharides) and provides the basis for classification of enterobacteria through sometimes hundreds of different thermostable O serogroups, for example *Escherichia coli* has over 170 different serotypes (Caugant *et al*., 1985).

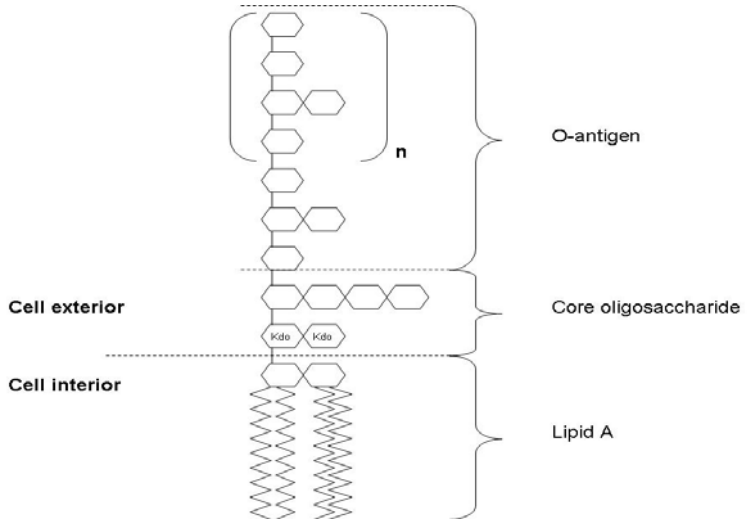

Figure 3-1 Typical LPS molecule on the surface of Gram-negative bacteria.

Lipid A is the toxic component of LPS and with KDO represents the most conserved region throughout the Gram negative bacteria (Jacques, 1996). The lipid A has been shown to be a good adjuvant and mitogen (Chiller *et al*., 1973), and responsible for the immuno-modulatory effect of this molecule (Robertsen, 1999) in mammals and fish.

Fish respond differently to LPS than mammals, probably due to the lower toxicity observed in fish to LPS (Harbell *et al*., 1979; Yongjuan *et al*., 2002). Fish cells survive doses 100 times higher than the lethal concentrations found with mammalian cells (Iliev *et al*., 2005a). When interacting with the immune system of fish, LPS basically acts as an immunostimulant (Section 1.4.1; Sakai, 1999). It has several stimulatory effects on the immune system by acting on both the cellular and humoral parts of the defences. For example, incubation of Atlantic cod macrophages with LPS enhanced the production of superoxide anions acid phosphatase activity, and increased respiratory burst activity (Sorensen *et al*., 1997; Steiro *et al*., 1998). The LPS has been shown to activate the complement system of fish with an increase in C5a and C3a anaphylatoxins, which act as chemotactic molecules for many immune cells (Kato *et al*., 2003; Li *et al*., 2007).

The LPS molecules of certain pathogenic bacteria participate in the inhibition of the complement system, offering them a great advantage over the immune system (Reeves, 1995). The lipid A component seems to be sensitive to the lytic activity of complement, but the O-antigen chain, present on most pathogenic strains, can protect this structure by binding the C3 molecules (Reeves, 1995). The length of the O-antigen chain has been correlated with serum resistance, e.g. an increase in the length of the LPS molecule in *V. anguillarum* (after growth in fish blood supplemented media), correlated with an increase in resistance to serum killing (Mutharia and Amor, 1994). The reverse is also true, isolates with fewer high molecular weight

O-antigen (after growth in glucose rich medium), showed a reduced resistance to serum killing (Boesen *et al*., 1999).

The structure of the *V. anguillarum* O side chain (O-antigen) has been elucidated by Eguchi *et al*. (1992b) and Sadovskaya *et al*. (1996) for serotype O2 and O3 respectively. The O-antigen structure is easily distinguished between those 2 serotypes on SDS-PAGE, but acid hydrolysis of these molecules showed similarities based on the repeat units of tetrasaccharides. The KDO molecule has been reported in *V. anguillarum* (Iguchi *et al*., 1989) but Salati and Kusuda (1986) could not find any in *V. ordalii*, showing the existence of structural differences between these two bacterial species.

Lipopolysaccharides are found in almost all Gram-negative bacteria, are highly immunogenic and a good correlation between LPS profiles and serogroups is often found (i.e. for *V. anguillarum*, Austin *et al*., 1995), which makes this molecule a very good candidate for the production of mAbs for serotyping *V. anguillarum*.

3.1.3. *Vibrio anguillarum* serotypes

Vibrio anguillarum has 23 known serotypes, as mentioned in Section 1.2.6.3, with two sub-serotypes identified for both O2 (O2 a and b, Rasmussen, 1987b, or α and β, Bolinches *et al*., 1990) and O3 (O3A and O3B, Muino *et al*., 2001). Espelid *et al*. (1991) reported the existence of additional sub-serotypes within the O2 group, with 4 groups identified using mAbs. The mAbs were made to 1 isolate (probably O2b serotype) and if discounting the non reactive strain (serotype D), they found 3 serotypes. After correlation of the results with other serotyping schemes, the group C seems to be represented by the O2a serotype and the serotype O2b was divided into 2 groups (O2b group A and B). Knappskog *et al*. (1993) also reported the existence of a subgroup within the sub-serotype O2b after cross-absorption with a rabbit polyclonal serum against O2b. Tiainen *et al*. (1997a) suggested the existence of additional sub-

serotypes on the basis of the results obtained with rabbit polyclonal antisera to O2a and O2b using slide agglutination as four isolates of *V. anguillarum* reacted with unabsorbed sera from O2a, but not with the O2a or O2b serum (cross-absorbed) and thus proposed the possibility of the existance of a new serogroup, which they temporarily named O2c. The loss of reactivity of polyclonal serum after cross-absorption with O2a or O2b has also been reported by Larsen *et al*. (1994), for a few isolates. Mikkelsen *et al*. (2007) suggested a new sub-group within isolates classified as O2 serotypes in *V. anguillarum* strains recovered from diseased cod. They used two of the mAbs produced by Espelid *et al*. (1991), one which reacted with all the O2 serotype isolates (6E10) and one with only O2b subgroup strains (1C12). They found that all the isolates reacted with 6E10 by ELISA, except for 5 isolates which did not react with either of the mAbs. As these isolates reacted with a rabbit polyclonal against O2, they concluded that this group belonged to the O2 serotype. It has to be mentioned that the sub-group they found in this study is not the same as the sub-groups A and B from Espelid *et al*. (1991), since those 2 sub-groups both reacted with 1C12 and so belonged to the O2b sub-group. For ease of nomenclature and discussion, the name O2c will be used for the subgroup identified by Mikkelsen *et al*. (2007) throughout this study.

3.1.4. Aims

The aim of this chapter was to investigate the serotyping classification of *Vibrio anguillarum* in order to determine the serological relationship of the different pathogens to different fish species. From the results of this study, it is anticipated that a more accurate decision may be taken regarding which serotypes and sub-serotypes of *V. anguillarum* should be included in a vaccine to protect Atlantic cod against vibriosis. For this reason a panel of monoclonal antibodies was developed, these were selected on the basis of their cross-reaction and specificity with the different serotypes and sub-serotypes responsible for cod disease outbreaks (i.e. O1, O2a, O2b and O3). Lipopolysaccharide was used in order to screen and select the

antibodies, since this molecule is the basis of the existing serotyping schemes for *V. anguillarum*.

3.2. Materials and methods

3.2.1. Lipopolysaccharide extraction

3.2.1.1. Culture of bacteria on cellophane

The method for collecting large quantities of bacteria using cellophane sheets was adapted from Liu (1957). Briefly, circles of cellophane (Medicell International Ltd, London, UK) were cut using the lid of a 13 cm Petri dish (Sterilin Ltd, Caerphilly, UK) as a template and stacked with a sheet of filter paper (Whatman N°1, GE Healthcare, Maidstone, UK) in between each layers. The sheets were hydrated and autoclaved in foil (123°C for 20 min). Each cellophane sheet was placed onto a 13 cm TSA+NaCl plate (Appendix 1.1) and the filter paper removed. Different isolates of *V. anguillarum* were grown overnight in TSB+NaCl (Section 2.1.2) and 1 mL of the suspension (OD 1, corresponding to 10^9 cfu.mL^{-1}) was spread with a flamed glass rod onto the plate. Five plates were inoculated per isolate.

The plates were incubated for approximately one week in a 22°C incubator. Once satisfactory growth was obtained, the bacteria were collected, using a flame-sterilised metal scraper and placed into a pre-weighed sterile glass universal.

3.2.1.2. Butan-1-ol extraction method

Butan-1-ol was used to extract the LPS from bacteria using a modification of the procedure described by Morrison and Leive (1975). The bacteria collected were resuspended in 20 mL of PBS and equally divided between 2 sterile glass universals (10 mL each) which were boiled for 10 min to kill and disrupt the membrane of the bacteria. An equal volume of butan-1-ol (VWR, Lutterworth, UK) was added to the bacteria after transfer into two 35 mL PPCO centrifuge tubes (Nalgene, US), and the solution incubated on ice with shaking for 15 min. The solution

was then centrifuged at 35000 x g for 20 min at 4°C (Sorvall, Thermo Fisher Scientific Inc., Loughborough, UK). The aqueous phase (lower layer) was collected and filtered through a 0.20 µm Vivaspin concentrator tube (Sartorius, Epsom, UK) by centrifugation at 3000 x g for 30 min at 4°C. The high molecular weight particles were removed using a 300,000 Da Vivaspin concentrator tube (Sartorius, UK) centrifuged for 60 min at 3000 x g. The filtrate was then concentrated using a 10,000 Da Vivaspin concentrator tube (Sartorius) at 3000 x g until around 1 mL was left. The concentrate was resuspended twice in PBS and centrifuged as previously described to remove all traces of butanol. The flow-through was discarded each time and the concentrated solution collected. Proteinase K (Sigma-Aldrich, Poole, UK, final concentration 0.5 mg.mL^{-1} in distilled water) was added to the solution and incubated overnight at 60°C in a water-bath. Nuclease-mix (Amersham Biosciences, GE Healthcare, UK, final concentration 80 units.mL^{-1} of DNAse and 1.2 units.mL^{-1} of RNAse) was added and incubated at RT for 60 min. The samples were then boiled for 10 min to inactivate the enzymes and resuspended in 10 mL of distilled water. An equal volume of butan-1-ol was added and after incubation on ice for 15 min, the tubes were centrifuged at 35000 x g for 20 min at 4°C. The aqueous phase was collected and filtered through a 10,000 Da concentrator tube for 30 min at 3000 x g, resuspended in 5 mL of distilled water in the same tube and re-centrifuged twice. The product was aliquoted into freeze-drying vials (Scientific Laboratory Supplies Ltd, Nottingham, UK) and frozen overnight at -70°C. The samples were then lyophilised over-night in a freeze-dryer (Martin Christ, Osterode am Harz, DE). The final product was collected into sterile 5 mL glass vials and kept at -20°C until required.

3.2.1.3. Quantification of LPS using the Purpald assay

The quantity of LPS in the samples was measured using the Purpald method described by Lee and Tsai (1999). Fifty µL of the LPS standards, ranging from 0.5 to 5 µg.well^{-1} of butanol extracted LPS in distilled water, as well as bacterial samples grown in TSB+NaCl overnight

(Section 2.1.2) were placed into a flat bottomed micro-titre plate (Immunlon 4 HBX, Thermo Fisher Scientific Inc., UK). Fifty µL of sodium peroxide (Sigma-Aldrich, 32 mM in distilled water) was added to each well and incubated for 20 min at RT. Fifty µL of Purpald solution (Sigma-Aldrich, $NaIO_4$ at 136 mM in 2M NaOH) was added and incubated at RT for 20 min. Another 50 µL of 64 mM $NaIO_4$ was added followed by 20 µL of propan-2-ol (Sigma-Aldrich) for 20 min at RT to stop the solution from foaming. The absorbance was then read on a spectrophotometer (Dynex Technologies Ltd, Worthing, UK) at 550 nm.

3.2.1.4. Analysis of extracted LPS

Purification of LPS using proteinase K

In order to compare the LPS profiles produced by butan-1-ol extraction and proteinase K treatment, samples were treated with proteinase K according to Hitchcock and Brown (1983). Briefly, the bacteria were grown in TSB+NaCl (Section 2.1.2) and 1.5 mL of a bacterial suspension was centrifuged at 3500 x g for 15 min. The pellet was resuspended in 50 µL of 2x SDS sample buffer (Appendix 2.2) and boiled for 10 min. Once cooled, 10 µL of Proteinase K (Sigma-Aldrich, 2.5 $mg.mL^{-1}$ in Urea buffer) was added to the solution and incubated overnight at 60°C in a water bath. The solution was then boiled for 5 min to inactivate the enzyme and frozen at -20°C.

Protein concentration after LPS extraction

In order to verify the elimination of the proteins from the purified LPS, the protein content of the batun-1-ol extracted LPS (2.5 $mg.mL^{-1}$ of LPS in distilled water) was assessed for VIB2 and VIB3 samples using a BCA kit (Pierce, Thermo Fisher Scientific Inc) following the manufacturer's instructions.

SDS-PAGE protocol

The LPS profiles of the butanol extracted LPS were compared to the profile of LPS extracted using only proteinase K. The SDS-PAGE gels (12%) were prepared (Section 2.2.1) and 10 µL of the proteinase K treated bacteria or pure butanol extracted LPS at different concentrations (1:1 in 1x SDS sample buffer) were loaded into the wells of the gel. Once the gel had run, the resulting bands were stained with silver staining specific for LPS (Tsai and Frasch, 1982). Briefly, the gels were fixed overnight at RT in 100 mL of Solution A (Appendix 5.1) in a glass staining trough placed on a rocker (RT, 40 rpm). The solution was replaced with 100 mL of Solution B (Appendix 5.2) for 5 min and the gels were washed 8 times for 3 min in distilled water. The LPS were stained with Solution C (Appendix 5.3) for 10 min, followed by 3 washes of 10 min each in distilled water. The bands were then developed in 100 mL of Solution D (Appendix 5.4) until clearly visible. The reaction was stopped by 3 quick rinses in 0.35% acetic acid (VWR, UK) in water and left for at least 1 h in distilled water before scanning and drying the gel (Section 2.2.4).

3.2.2. Monoclonal antibody production

3.2.2.1. Immunisation of mice

Five Balb/C mice (3-4 weeks old) were obtained from Charles River Laboratories (Edinburgh, UK) and left to acclimatise for 3 weeks in the Animal Facility at the University of Stirling. The antigen was prepared by growing *V. anguillarum* VIB1 (O1 serotype), VIB72T (O2a serotype), VIB102 (O2b serotype) and VIB3 (O3 serotype) in TSB+NaCl overnight (Section 2.1.2). Bacteria were formalin-killed (Section 2.1.6) and the OD adjusted to 2.0 at 610 nm. *Vibrio anguillarum* VIB102 LPS only (extracted with butanol, Section 3.2.1.2) was also prepared as an antigen (280 µg.mL^{-1} of LPS in PBS) and injected in one mouse. Each solution (formalin killed bacteria and LPS) was emulsified (1:1) with Titermax (Interchim S. A. Montlucon, Fr) and 100

µL of the different solutions were injected subcutaneously into separate mice. The ears of the mice were clipped for differentiation between animals and a pre-injection bleed collected. The mice were re-injected after 3 weeks with the same quantity of antigen. A test sample of blood was collected a week after each injection in sterile 1.5 mL centrifuge tubes to determine the antibody response elicited by each immunisation. The mice were finally boosted after a further 3 weeks, intravenously into the tail with no adjuvant. After four days, the mice were culled by CO_2 asphyxiation and bled out by cardiac puncture. The animals were then transported from the Animal Facilities to the Aquatic Vaccine Unit laboratory.

3.2.2.2. Myeloma cell culture

Myeloma cells (SP2, Sigma-Aldrich) were expanded a week before performing the fusion in DMEM* (Appendix 9.1) with 20% FCS (v/v, Sigma-Aldrich) at 37°C in 5% CO_2. The media was changed every 2 to 3 days to maintain cells in the log phase.

3.2.2.3. Fusion

The fusion of myeloma cells with spleen cells was performed according to the method of Campbell (1984) with modifications. All work took place in a laminar flow cabinet and the instruments were kept in 70% ethanol to insure sterility during the procedure. The spleen was dissected from each individual mice and the cells collected by several passages of DMEM through it using a syringe with a 27 gauge needle. The medium containing the spleen cells was transferred into a 50 mL sterile centrifuge tube and placed at 37°C with 5% CO_2.

Meanwhile SP2 cells were collected by centrifugation at 150 x g for 7 min and the cells resuspended in 5 mL of DMEM* without FCS, this step was repeated to remove all traces of FCS.

Both the myeloma and the spleen cell suspensions were mixed together (around 10 myeloma cells per spleen cell). The cells were centrifuged at 100 x g for 10 min, the supernatant

discarded, and the pellet gently resuspended by the addition of 1 mL of 50% polyethylene glycol (w/v, Sigma-Aldrich, in DMEM) warmed to 37°C over 1 min. The cells were allowed to stand for 60 sec before adding one mL of DMEM over 30 sec, then 3 mL over 30 sec and finally 16 mL over 60 sec. The cells were allowed to stand for 5 min before centrifugation at 400 x g for 5 min. The supernatant was gently discarded, and the pellet was carefully resuspended in 4 mL of DMEM* plus 0.3% mouse blood (v/v) and HAT (100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine, Sigma-Aldrich). One hundred and eighty µL of the solution was added to each well of 14 96-well plates. The plates were incubated at 37°C with 5% CO_2 taking care not to disturb the cells for at least 48 h.

3.2.2.4. Screening, cloning and expansion

After a week, 100 µL of the 180 µL original media was removed and replaced with 100 µL of DMEM*+10% FCS and HT (100 µM hypoxanthine, 16 µM thymidine, Sigma-Aldrich). The media was then changed every three days by removing 150 µL of the media, without disturbing the clones, and adding 150 µL of fresh media (DMEM*+10% FCS+HT).

Screening was performed to establish positive hybridoma wells by ELISA (Section 2.4) using either butanol extracted LPS (Section 3.2.1.2) or whole bacteria (Section 2.1.2) to coat the ELISA plate.

The first screening took place two weeks after the fusion, and out of the 14 96-well plates approximately 100 wells were selected on the basis of the highest reaction with LPS in the ELISA. The media from these selected wells was changed and the cells were left to rest for two days. The clones were then resuspended and 150 µL of the suspension transferred to a 24-well plate containing 1.5 mL of DMEM*+10% FCS and mouse red blood cells (0.3%) in each well. The strains used to immunize the mice were also used to coat the ELISA plates for the second screening. Between 15 and 20 hybridoma wells were selected based on highest optical

density by ELISA. These wells were cloned by adding 100 µL of resuspended cells to the top left well of a 96-plate, which contained 150 µL of DMEM*+10% FCS + mouse red blood cells (0.3%) in each well with an extra 150 µL in the first column. This was then diluted 1:2 down the 8 wells of the first column before diluting across the plate (also 1:2) up to column 6. The same was done for the other half of the plate with another selected clone.

After 4 days incubating at 37°C with 5% CO_2, wells with a single clone were selected (ideally 6 for each cloning). The third screening was carried out to select clones not only positive for the homologous strain used but also against different *V. anguillarum* serotypes. At this stage the positive and non cross-reacting clones, respectively, were re-cloned as described previously. Clones cross reacting with O2a and O2b isolates were also selected. The clones with the highest reaction in the ELISA were selected and stored in liquid nitrogen (Section 3.2.2.5).

3.2.2.5. Freeze/Thawing of hybridomas

Hybridomas with mid-log phase growth were harvested in 15 mL centrifuge tubes and centrifuged at 150 x g for 7 min. The cells were resuspended in DMEM*+20% FCS+10% DMSO to obtain a concentration of cells around 2 $x10^6$ cells.mL^{-1} and the media aliquoted into 1.5 mL cryo-vials (Nunc, Thermo-Fisher), wrapped in bubble-wrap and placed at -70°C overnight. The following day the bubble-wrap was removed and half the cells were transferred to liquid nitrogen, the other half were kept at -70°C.

In order to recover the hybridomas after storage at -70°C, the cryo-vial containing the hybridoma of interest was thawed in a 37°C water bath and immediately layered onto 1 mL of FCS and 9 mL of DMEM* (layered above the FCS) in a 15 mL tube. The tubes were centrifuged at 150 x g for 7 min and resuspended in 4 mL of DMEM*+20% FCS. The cells were then incubated in duplicate wells of a 24-well plate in 2 mL of medium.

3.2.2.6. Expansion and harvesting of mAb

After 24h, the medium of the cells was changed, and after 4 days the cells were transferred to a 50 mL flask and 8 mL of DMEM*+10% FCS was added. The cells were checked after 4 days and if the growth was satisfactory the medium was replaced by 30 mL of DMEM*+10% FCS. After a few days the cells were rechecked and transferred to a 200 mL flask with 100 mL of DMEM*+10% FCS. From there, the cells could be grown continuously by transferring the cells to a new flask each week and refilling the “mother” flask with fresh medium. The medium was changed once on the “daughter” flask before leaving it for 10 days in the incubator to let the cells produce mAbs before dying.

The mAbs were collected by centrifuging the contents of the flasks at 500 x g for 10 min in 50 mL centrifuge tubes and the supernatant pooled together, aliquoted it into 50 mL centrifuge tubes and frozen at -20°C. When required, the mAbs were thawed and 0.01% thimerosal (w/v, Sigma-Aldrich) was added. The monoclonals were then kept at 4°C until used.

3.2.2.7. ELISA and Western blot

The entire bacterial collection (Section 2.1.1) was screened with the selected mAbs using the indirect ELISA method described in Section 2.4. The ELISA results are presented for this section as percent cross-reactivity (Table 3-1). These values were calculated for each mAb by subtracting the average background from the absorbance recorded for each strain and dividing the resulting value by the absorbance of the strain used to produce the mAbs in question, and then multiplying by 100. Reactions with over 75% cross reactivity were considered strongly positive and values between 30 and 75% cross reactivity considered weakly positive.

In order to visualise the binding properties of the mAbs selected, as well as verifying that the LPS extraction method did not modify the banding patterns of the recognised bands, SDS-PAGE (Section 2.2.1) and Western blotting (Section 2.3) were carried out using the selected

mAbs on selected bacteria, purified butan-1-ol extracted LPS and proteinase K treated whole bacteria (Hitchcock and Brown, 1983).

3.3. Results

3.3.1. Lipopolysaccharides extraction using butan-1-ol

3.3.1.1. Yield of LPS extraction

An average of 5 g of bacteria was collected for each of the 10 different bacterial isolates grown on TSA+NaCl covered with cellophane,. The average weight of LPS after extraction with butan-1-ol from these isolates was 7.8 mg, making the average yield of LPS obtained by butan-1-ol extraction 1.56 mg of LPS per g of bacteria.

The concentration of protein in the LPS preparations of VIB2 and VIB3 (after butan-1-ol extraction) was found to be 14.3 and 62.0 $\mu g.mL^{-1}$, respectively, which corresponds to 0.6 and 2.0% protein content in these samples.

3.3.1.2. SDS-PAGE and Western blot profiles of proteinase K treated whole cell bacteria and butanol extracted LPS

After treatment of whole bacteria with proteinase K, different LPS banding pattern was observed for each serotype as represented in Figure 3-2. Differences were evident between serotypes in their high molecular weight (HMW) and low molecular weight (LMW) LPS, which appeared as 2 distinct regions on the gels, separated approximately around 35 kDa. Each serotype can be easily differentiated on the basis of their LPS profile either on the HMW LPS region or the LMW LPS bands, but it has to be noted that there seems to be no differences between the LPS profiles of serotype O2a and O2b (Figure 3-4) and that the pattern of banding can vary slightly between strains of the same serotype (data not shown). Serotype O1 appears to have only one broad band in both the HMW and LMW LPS regions which is different from the laddering pattern typically observed in most serotype. The LPS of serotype O2 appears to

have the typically repetitive pattern in both the LMW and HMW LPS regions, also seen for serotype O3. The difference between these two serotypes seems to lie in the distribution of bands between the two different regions; both the HMW and the LMW LPS regions in serotype O3 profile less dispersed than that of the O2 LPS profile. Nevertheless the same number of bands, 6 to 7, is present for both these serotypes in the LMW LPS region.

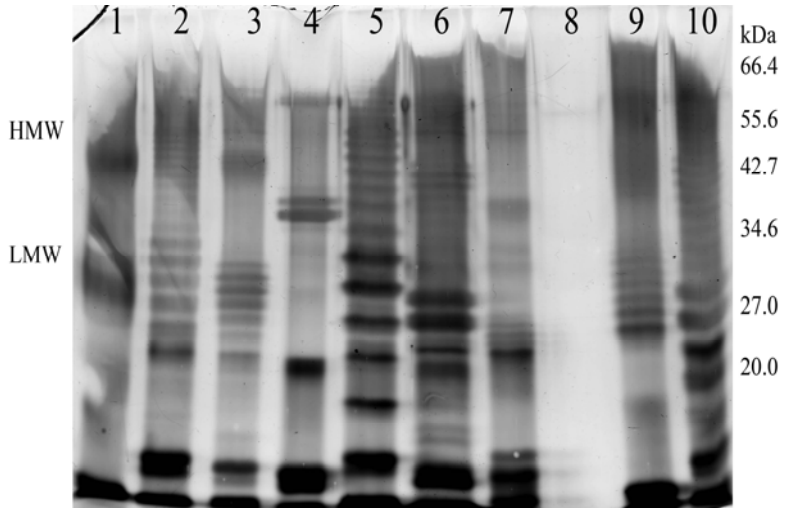

Figure 3-2 SDS-PAGE profile of proteinase K treated *V. anguillarum* O1 to O10 serotypes (O8 missing) Silver stained (Section 3.2.1.4). Lanes: (1): VIB1 (O1); (2): VIB2 (O2a); (3): VIB3 (O3); (4): VIB4 (O4); (5): VIB5 (O5); (6): VIB6 (O6); (7): VIB7 (O7); (9): VIB9 (O9); (10): VIB10 (O10).

The profiles of the LPS extracted using butanol on SDS-PAGE are very similar to those obtained with proteinase K digestion (Figure 3-3). The bands present, for both treatments, in the HMW and the LMW LPS regions appear at the same location on the gel for serotype O2a (VIB2), i.e. the HMW LPS region lays between 37 and 55 kDa for both O2a samples and the LMW LPS region between 20 and 32 kDa. Both the regions for O3 LPS appear around the same molecular mass but show a narrower range. The spacing of each band within the HMW and LMW LPS also seem to correspond between the two preparation methods. The butanol extracted preparation appears cleaner than the proteinase K treated samples, with better definition of the bands, especially for the O2 samples.

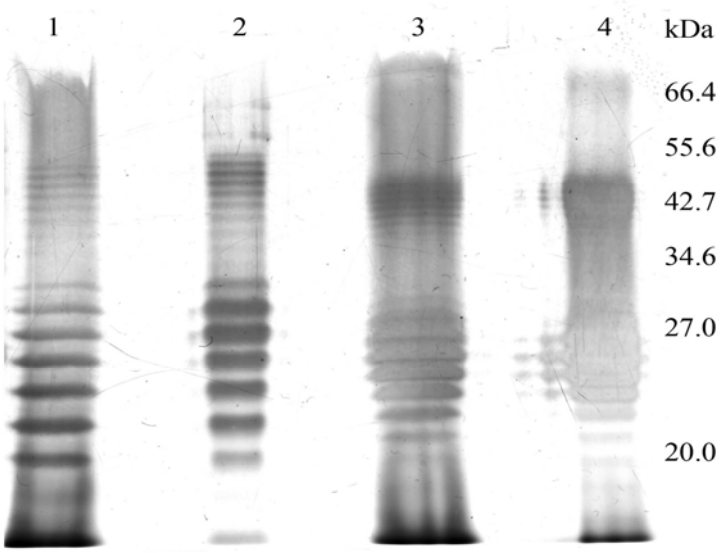

Figure 3-3 SDS-PAGE profile of proteinase K treated and butanol extracted *V. anguillarum* Silver stained (Section 3.2.1.4). Lanes: (1) VIB2 protK (O2a); (2) VIB2 But (O2a); (3) VIB3 protK (O3); (4) VIB3 But (O3).

On Figure 3-4, some bands in the profile are more easily distinguished in the butanol extracted LPS than in the proteinase K treated samples and some bands seem to be present only in the butanol extracted LPS as for the VIB7 (lane 3&4). It is impossible to distinguish between O2a (VIB2 or VIB72T) and O2b (VIB102) serotypes (Figure 3-4 lanes 1&2 or 5&6 and 7&8, respectively). Some protein or carbohydrate still appears to remain in the proteinase K treated samples (Figure 3-4, bottom of the gel, lanes 1, 3 and 7) resulting in poor resolution of the low molecular weight bands in this region. This intensely stained region at the bottom of the gel (<20 kDa) is also present in the VIB7 and VIB102 butanol extracted samples (lane 4 and 8) but was not evident in the butanol extracted LPS profiles of the VIB2 and VIB72T samples (lane 2 and 6).

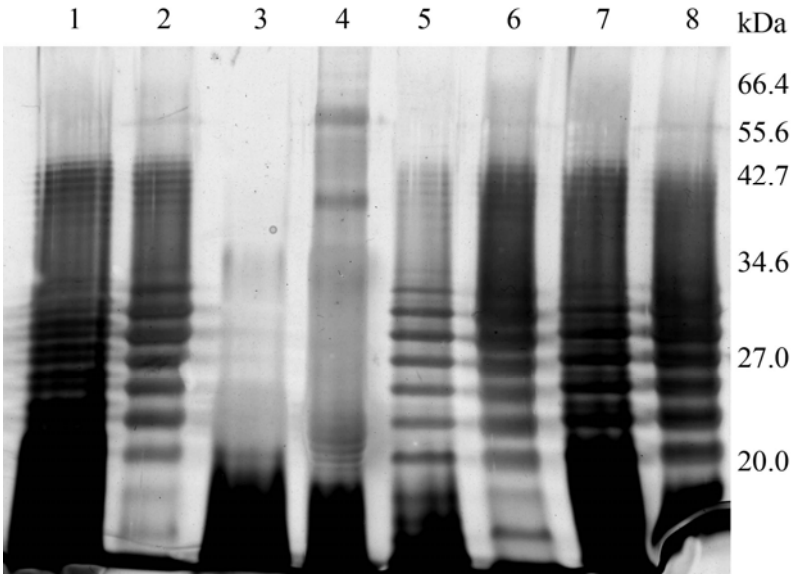

Figure 3-4 SDS-PAGE profile of proteinase K treated and butanol extracted *V. anguillarum* Silver stained (Section 3.2.1.4). Lanes: (1) VIB2 protK (O2a); (2) VIB2 But (O2a); (3) VIB7 protK (O7); (4) VIB7 But (O7); (5) VIB72T protK (O2a); (6) VIB72T But (O2a); (7) VIB102 protK (O2b); (8) VIB102 But (O2b).

3.3.1.3. Western blot profile of extracted LPS

The banding pattern observed for both the O2a and O2b serotypes in SDS-PAGE analysis using silver staining is clearly visible for both the proteinase K treated and the butanol extracted samples (Figure 3-5 A). The comparison between the SDS-PAGE (Figure 3-5, A) and the Western blot immunostained with O2a specific mAb (Figure 3-5, B) reveals a similar ladder pattern with the exception of the O2a isolate VIB2 butanol extracted LPS, where the mAb used bound to a HMW LPS higher than that visualised with the silver staining (red box).

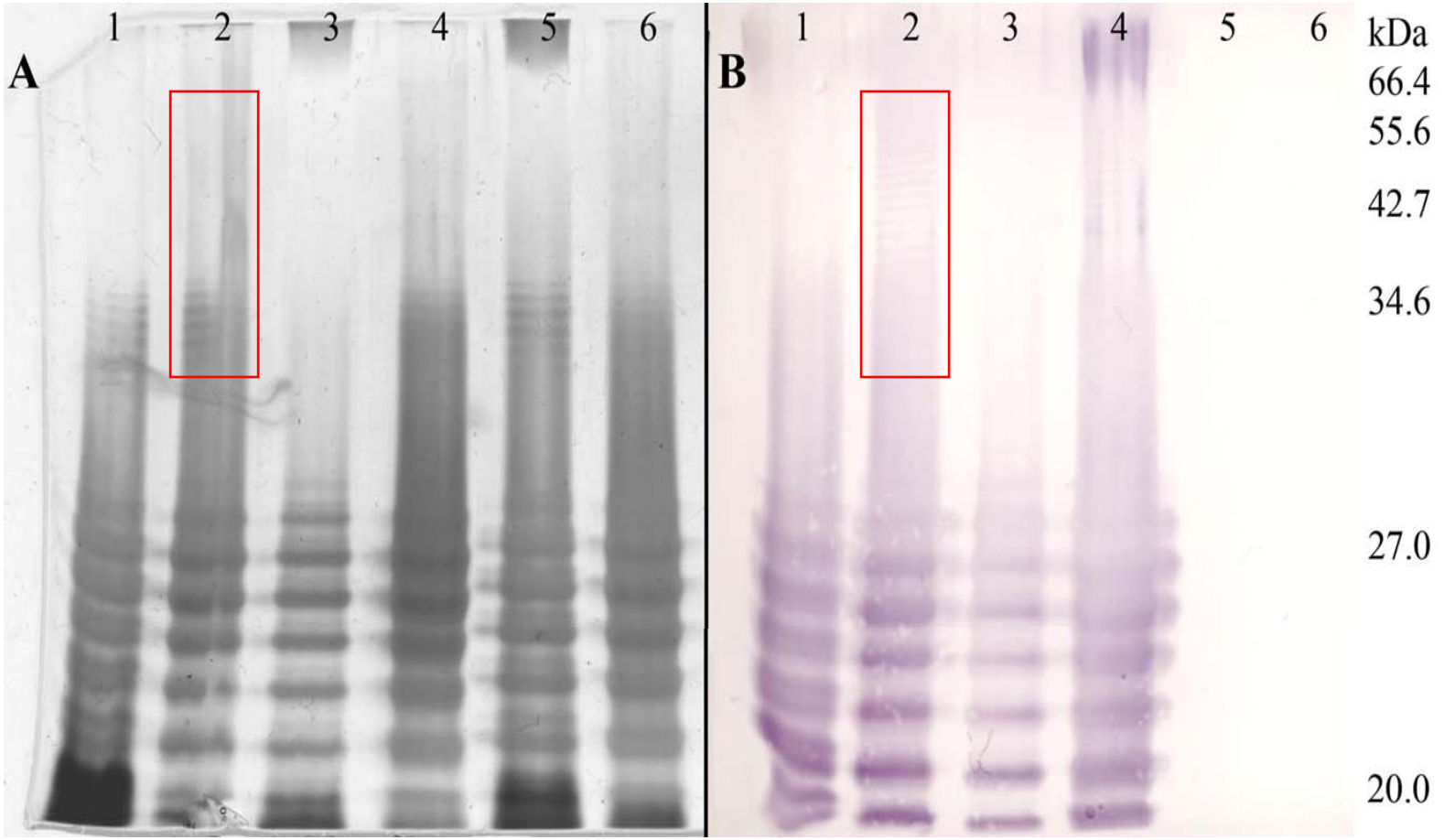

Figure 3-5 (A): SDS-PAGE gel Silver stained (Section 3.2.1.4) and (B) Western blot profile using O2a specific mAb (V.angO2a mab3)on proteinase K treated (protK) and butanol extracted (But) *V. anguillarum*. Lanes: (1) VIB2 protK (O2a); (2) VIB2 But (O2a); (3) VIB72T protK (O2a); (4) VIB72T But (O2a); (5) VIB102 protK (O2b); (6) VIB102 But (O2b).

3.3.1.4. Semi-quantification of LPS by SDS-PAGE

The extracted LPS were used to estimate the total amount of LPS in a given sample by running different concentrations of the purified LPS (of known concentration) by SDS PAGE, and comparing band profile intensity with these of a whole bacterial cell sample after silver staining.

Comparison of the intensity of the LPS standards with the bands obtained with whole cell bacteria revealed that the whole cell sample (Figure 3-6, Lane 7) contained between 0.1 and 0.2 $mg.mL^{-1}$ of purified LPS (Figure 3-6, lane 6 and 5, respectively). The estimated amount of LPS in the sample was 0.15 $mg.mL^{-1}$ and since it was diluted 1:5 after mixing it 1:1 in SDS sample buffer, this represents approximately 1.5 $mg.mL^{-1}$ of LPS for the sample of VIB2 bacteria at an OD of 1.0 (10^9 $cfu.mL^{-1}$).

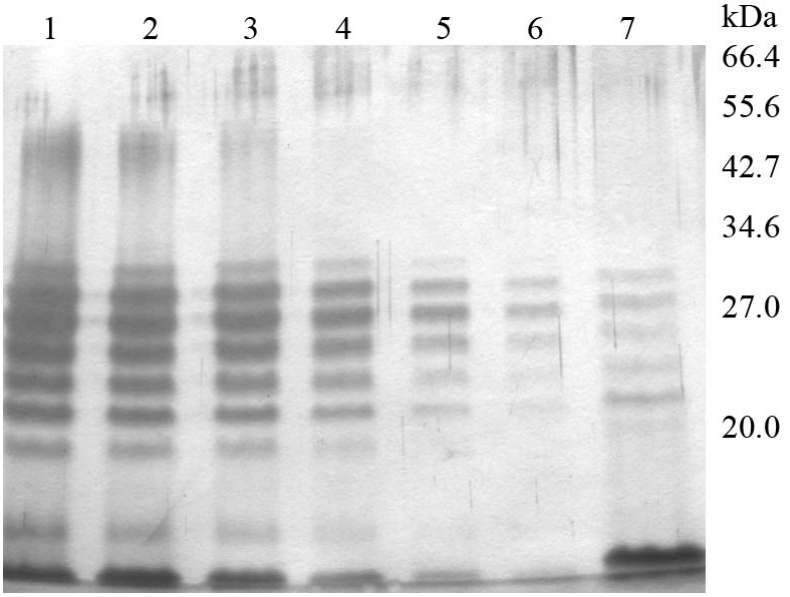

Figure 3-6 SDS-PAGE profiles of butanol extracted LPS at different concentrations ($mg.mL^{-1}$) and whole cell *V. anguillarum* VIB2 (O2a). Silver stained (Section 3.2.1.4). Lanes: (1): 2 $mg.mL^{-1}$; (2): 1.5; (3): 1.0; (4): 0.5; (5): 0.2; (6): 0.1; (7): VIB2 whole cell sample at OD 1.0 (diluted 1:1 in SDS sample buffer and 1:5 in distilled water).

3.3.1.5. Quantification of LPS in whole bacteria using purpald assay

The purpald assay standard curve (Figure 3-7) using butanol extracted LPS gave a good linear correlation between the amount of LPS and the optical density measured (i.e. R^2=0.99 for

VA33 isolate, O2a), confirming that the purpald assay can be used accurately for estimating the amount of KDO (and so LPS) in bacterial samples.

The estimation of the amount of LPS in a given sample cannot be extrapolated from one isolate to the next, even if they are from the same serotype, since variations in the standard curve occurred between isolates (data not shown). Thus a standard curve needs to be constructed for each individual isolate investigated.

Samples digested with proteinase K could not be analysed by purpald assay since this enzyme interfered with the reaction and the absorbance was too high to quantify.

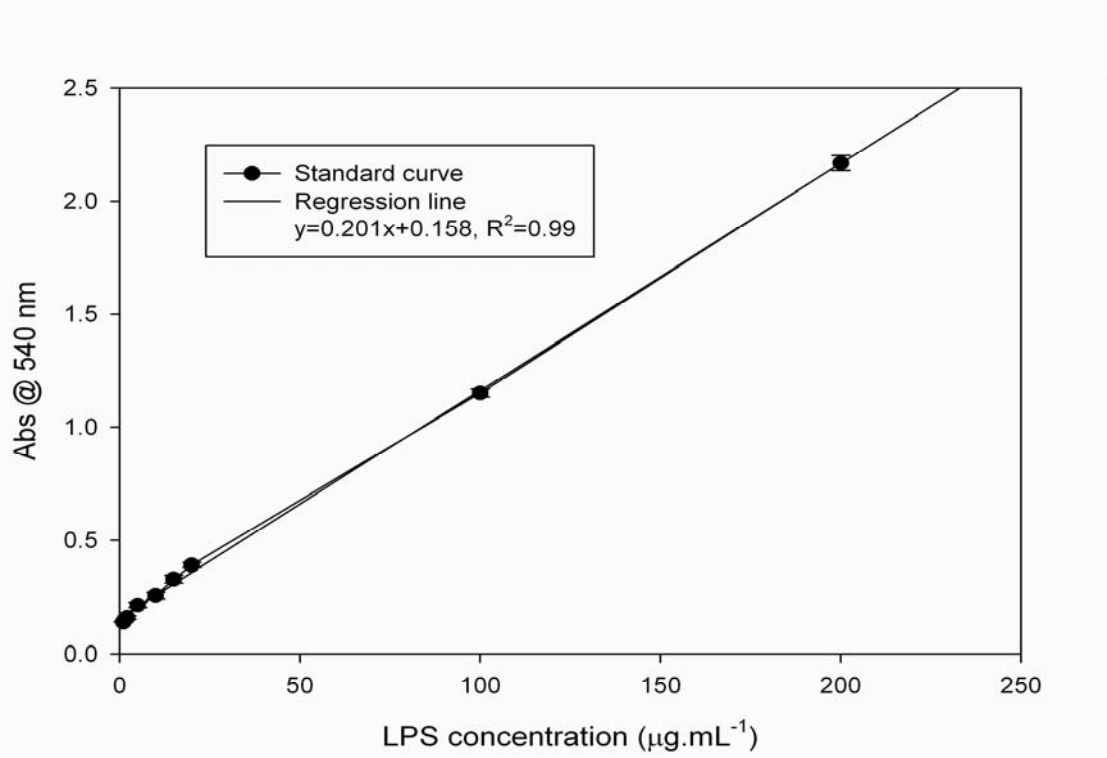

Figure 3-7 Standard curve of butanol extracted LPS in purpald assay using *V. anguillarum* O2a strain (VA33).

The amount of LPS in any given sample can be calculated from the regression equation of the standard curve. For example, VA33 strain (O2a serotype) grown in TSB+NaCl overnight (Section 2.1.2), diluted to an OD of 1.0, contained 18 mg of LPS per mL of bacteria when compared to the corresponding butanol extracted LPS standard.

3.3.2. mAbs Results

3.3.2.1. Monoclonal antibodies

After the third cloning, 4 monoclonal antibodies were selected for the O1 antigen, labelled V.angO1 1.2, V.angO1 2.3, V.angO1 6.3 and V.angO1 7.2. After screening these four mAbs and checking their cross reaction against *V. anguillarum* serotype type strains O2 to O10, V.angO1 6.3 and V.angO1 7.2 were found to cross-react with most of the serotypes and were therefore not used for any further work (data not shown). The V.angO1 1.2 and V.angO1 2.3 mAbs were found to react only with O1 type isolates and on the basis of the highest binding (data not shown), V.angO1 2.3 was selected as the anti O1 specific mAb.

Ten mAbs selected from the O2a injected mice were found to cross-react (all of them) between the O2a and the O2b strains and no further selection was done. A mAb produced by Fuad Matori at the Institute of Aquaculture in 2001 against VIB72T (V.angO2a mab3) was used in this study since it was found to be specific to the O2a sub-serotype.

The third screening for the O2b antigen led to 6 mAbs selected, V.angO2b 3.2, V.angO2b 5.5, V.angO2 6.3, V.angO2 7.1, V.angO2b 9.2 and V.angO2b 14.1. The mAbs V.angO2b 5.5, V.angO2b 6.3 and V.angO2b 14.1 were found to react only with the O2b type strain (data not shown). On the basis of the intensity of the reaction, V.angO2b 6.3 was selected as the mAb specific for O2b identification (data not shown). Both V.angO2b 3.2 and V.angO2b 9.2 cross reacted with both the O2a and the O2b type strains, but not with any of the O1 or O3 to O10 type strains (data not shown). V.angO2b 3.2 gave a slight reaction with the O1 strains so taking this as a selection factor and on the basis of the intensity of the reaction; V.angO2b 9.2 was selected and renamed V.angO2a-b 9.2 since it cross-reacted with both sub-serotypes (data not shown).

The mAbs produced from the mouse injected with the O3 serotype type strain (VIB3) only reacted with this particular isolate and no other O3 isolates, so no further work was carried out with these mAbs.

3.3.2.2. ELISA

Generally, the results of the classification using the selected mAbs (Table 3-1) agreed with the serotypes of previously typed isolates obtained from the different sources (Section 2.1.1).

There appeared to be some discrepancies regarding the classification of some isolates when first serotyped with the mAbs in the ELISA. Some isolates did not appear to belong to the serotype under which they had been classified by other researchers and were therefore re-grown from frozen stock (Section 2.1.3.1) to confirm their classification. The results obtained after this second screening agreed with the initial results found with the mAbs.

Out of the 38 isolates previously classified as O1, 4 were found to be misclassified using this set of mAbs (10.5% of the total of O1 strains) and 15 unclassified isolates were found to belong to the O1 serotype. The anti-O1 mAb (V.angO1 2.3) generally had a strong reaction in the ELISA, the majority of the cross reactivity values were greater than 75% (true positive), weak positive values were obtained for isolates VA14, VIB269, VIB64, VA21, VA35 and VA61. Only one isolate was just under the cut off value for weak positive, VA59 (25.6% cross reactivity), it was recovered from Japan in 2006 and might be a related organism. VIB269 was a Canadian isolate collected in 1968 (Evelyn, 1971) and could have changed over the numerous years of sub-culturing.

Table 3-1 Percentage cross-reactivity of the anti-*V. anguillarum* monoclonal antibodies (Anti O1: V.angO1 2.3; Anti O2: V.angO2a-b 9.2; Anti O2a: V.angO2a mab3; AntiO2b: V.angO2b 6.3) with the *V. anguillarum* collection by ELISA.

Isolate	Serotype	Anti O1	Anti O2	Anti O2a	Anti O2b
VIB1	**O1**	**100.0**	6.8	6.9	4.9
78 skid	O1	88.3	2.6	1.6	1.2
VA10	O1	96.8	3.1	2.0	1.4
VA12	O1	75.7	4.0	2.1	0.7
VA13	O1	83.5	0.3	0.8	0
VA14	O1	63.0	3.7	2.2	0.4
VA15	O1	83.2	3.0	2.2	0.5
VA16	O1	96.5	6.0	2.1	3.1
VA24	O1	123.3	10.0	2.1	8.1
VA40	O1	109.7	3.4	5.0	0.9
VA41	O1	95.6	3.3	1.0	0
VA42	O1	121.5	4.6	2.3	0.9
VA43	O1	91.4	3.4	2.0	1.4
VA44	O1	106.4	4.8	4.9	1.9
VA46	O1	102.6	3.9	3.2	0.6
VA48	O1	97.1	5.0	1.2	1.8
VA57	O1	103.9	4.8	5.5	0.1
VIB134	O1	91.3	2.8	1.8	1.6
VIB135	O1	103.4	2.0	0.1	0
VIB226	O1	92.0	3.5	1.7	1.3
VIB227	O1	88.6	3.1	1.5	0.9
VIB236	O1	96.8	3.6	3.2	1.1
VIB238	O1	91.1	3.1	2.5	0.8
VIB253	O1	93.9	3.3	0.8	0.9
VIB269	O1	34.9	1.4	0.9	0.3
VIB546	O1	75.4	4.3	3.3	2.6
VIB549	O1	100.9	0	0	0
VIB605	O1	124.8	9.6	2.3	2.3
VIB64	O1	66.5	1.7	0.8	0.9
VIB65	O1	83.2	4.7	4.1	0.4
VIB67	O1	91.9	2.2	1.3	0.5
VIB68	O1	105.1	3.9	2.6	1.3
VA45	O1	100.6	4.7	1.8	1.1
VA47	O1	85.0	0.4	0	0
VA17	Untyped	88.8	0	3.4	0
VA18	Untyped	82.9	4.2	5.5	0.8
VA20	Untyped	89.4	3.8	2.4	1.4
VA21	Untyped	68.6	4.3	5.5	1.2
VA22	Untyped	98.6	7.5	3.2	1.0
VA23	Untyped	107.8	4.9	3.0	1.9
VA35	Untyped	71.8	2.5	1.4	1.4
VA58	Untyped	91.9	2.8	1.6	1.0
VA59	Untyped	25.6	2.7	1.1	0.7
VA60	Untyped	88.1	5.9	1.2	0.4
VA61	Untyped	45.9	3.9	3.3	1.1
VA62	Untyped	80.2	2.7	2.0	0
VA64	Untyped	101.0	4.9	4.5	0.9
VA66	Untyped	102.4	3.7	2.9	1.2
VA67	Untyped	93.0	3.1	1.3	1.8
VIB126	O1	1.2	0.4	0.5	1.3
VIB195	O1	1.8	2.5	1.5	0.6
VIB217	O1	0	0	0	2.1
VIB266	O1	0.8	1.6	2.3	0.8
VIB2	O2a	0	77.8	61.2	3.9
VA25	O2	7.5	85.4	67.3	0
VA27	O2a	3.6	82.5	72.8	1.9
VA33	O2a	1.1	79.1	74.7	31.0
VA36	Untyped	8.2	84.9	66.8	6.2
VA56	O2	2.4	101.2	66.5	1.4
VIB242	O2a	4.3	98.6	64.2	0.2
VIB2A	O2a	11.3	88.7	66.8	2.9

Table 3-1 Percentage cross-reactivity of the anti-*V. anguillarum* monoclonal antibodies (Anti O1: V.angO1 2.3; Anti O2: V.angO2a-b 9.2; Anti O2a: V.angO2a mab3; AntiO2b: V.angO2b 6.3) with the *V. anguillarum* collection by ELISA (Cont.)

Isolate	Serotype	Anti O1	Anti O2	Anti O2a	Anti O2b
VIB2B	O2a	16.0	111.3	88.5	11.9
VIB72T	**O2a**	9.4	84.7	**100.0**	8.9
VIB77	O2	5.4	107.3	63.6	0
VIB98	O2a	10.8	92.4	83.9	3.9
VIB99	O2a	8.6	99.1	80.5	2.4
VA68	O2a	0	72.92	57.54	1.83
VA69	O2a	0	54.64	49.87	0.74
V4	V. ordalii	0	91.7	70.0	5.6
VIB102	**O2b**	4.8	**100.0**	1.3	**100.0**
VIB102A	O2b	0	85.9	0	84.2
VIB102B	O2b	2.6	104.0	2.0	96.4
VIB103	O2b	0	77.8	0	121.3
VIB103A	O2b	5.5	99.4	1.9	102.6
VIB104	O2b	0.5	87.3	1.5	86.4
VIB105	O2b	1.2	80.4	2.2	80.0
VA34	O2b	5.1	105.4	6.3	69.1
VA64	Untyped	3.6	82.4	3.5	83.8
B02061	Untyped	7.2	104.4	3.9	103.3
B03064 K1	Untyped	7.2	91.1	2.4	91.8
B03064 Lesion	Untyped	8.2	95.2	2.3	84.5
B03068	Untyped	3.3	92.8	2.0	97.9
VA71	O2b	0.61	83.17	1.84	77.78
VA72	O2b	6.68	82.35	3.56	86.42
B01103 2(1)	Untyped	3.2	99.0	4.3	2.1
B01103 5-4	Untyped	3.6	95.7	2.4	2.0
B02006 SLC1	Untyped	6.6	92.9	0.9	1.4
VA73	O2b	0.34	67.43	0.82	0.96
VA5	O2a	0	0	0	0
VIB100	O2a	0	1.0	1.8	0.6
VIB140	O2a	0.5	2.6	1.3	1.1
VA7	O2	1.1	2.8	2.0	0.7

Isolate	Serotype	Anti O1	Anti O2	Anti O2a	Anti O2b
VA9	O2	1.6	1.1	0.2	0.6
VA70	O2a	0.08	0	0	0.08
VA74	O2c	0	2.16	0.43	0.11
VA75	O2c	0	0.64	0.25	0.66
VA76	O2c	3.32	0.66	0.45	0.05
VIB3	O3	0.5	3.5	1.9	2.2
VA11	O3	2.6	1.9	1.0	1.4
VA8	O3	0.8	3.1	1.2	0.9
VIB111	O3	2.0	1.8	0.3	0
VIB604	O3	0.8	3.2	1.9	1.0
VIB4	O4	2.4	3.5	1.3	0.8
VA49	O4	3.5	4.0	1.7	1.0
VIB114	O4	4.6	4.8	3.9	1.5
VIB5	O5	3.0	4.4	2.5	1.0
VIB118	O5	3.6	4.7	1.6	1.8
VIB6	O6	6.2	2.1	0.9	3.1
VIB7	O7	2.9	3.1	1.4	1.4
VIB122	O7	2.9	3.3	2.0	1.2
VIB8	O8	5.5	3.0	1.6	1.0
VIB9	O9	6.0	2.4	0.9	1.3
VIB10	O10	2.3	4.3	0.9	1.4
VA37	O11	4.8	2.6	0.8	1.2

Table 3-1 Percentage cross-reactivity of the anti-*V. anguillarum* monoclonal antibodies (Anti O1: V.angO1 2.3; Anti O2: V.angO2a-b 9.2; Anti O2a: V.angO2a mab3; AntiO2b: V.angO2b 6.3) with the *V. anguillarum* collection by ELISA (Cont.).

Isolate	Serotype	Anti O1	Anti O2	Anti O2a	Anti O2b
VIB606	O11	5.5	2.6	1.3	0.9
VA38	O12	6.2	2.9	2.3	1.2
VA39	O13	4.0	5.0	0.6	1.1
VIB608	O13	4.5	1.6	1.1	1.1
VA31	O17	5.9	2.4	17.5	3.0
VIB127	O18	13.2	2.2	1.0	0.8
VIB216	O18	3.1	5.0	4.2	1.6
VIB129	O19	5.6	2.5	0.6	0.4
VIB215	O19	5.6	1.6	0.4	1.5
VIB197	O20	6.5	3.5	2.0	1.8
VIB198	O20	3.8	3.2	3.2	2.0
VIB199	O20	5.3	2.5	0.9	1.2
VIB200	O20	3.2	4.8	2.2	1.1
VIB201	O20	3.0	3.2	1.3	0.8
VIB202	O20	3.9	3.2	4.9	0.6
VA29	O21	2.4	5.3	4.2	1.5
VA30	O21	3.4	2.7	0.8	1.0
VA32	O22	3.6	2.4	0.8	0.2
VIB128	VaNT2	2.3	4.0	1.0	0.8
VIB141	VaNT2	3.4	4.0	1.8	1.0
VIB142	VaNT2	2.1	5.3	4.5	0.5
VIB149	VaNT2	4.9	5.6	7.2	1.3
VIB82	VaNT2	5.2	2.9	1.6	0.8
VA4	Untyped	4.4	3.1	0.9	2.0
VA50	Untyped	3.2	2.8	1.3	1.8
VA51	Untyped	3.2	2.9	1.0	1.6
VA52	Untyped	1.6	2.4	0.9	0.3
VA53	Untyped	3.2	3.7	2.5	1.8
VA6	Untyped	7.6	2.4	0.6	0.8
VIB130	Untyped	9.7	2.6	0.6	1.2
VIB547	Untyped	11.6	2.2	1.1	2.2
VIB555	Untyped	2.2	2.1	1.4	0.6
VIB558	Untyped	0.8	0.7	0.0	0
VIB66	Untyped	6.7	1.4	0.4	0.6
VIB808	Untyped	8.4	2.4	2.3	1.2
V10	*V. ordalii*	5.3	2.6	1.3	1.0
V11	*V. ordalii*	7.4	3.8	1.6	1.0
V5	*V. ordalii*	4.2	15.0	19.0	2.0
V6	*V. ordalii*	10.9	1.9	1.3	1.6
V7	*V. ordalii*	1.2	5.9	7.5	2.4
V8	*V. ordalii*	0.8	11.7	16.2	1.8
V9	*V. ordalii*	5.2	2.4	1.6	2.6
V1	*V. carchariae*	3.1	3.4	2.5	1.6
V3	*V. vulnificus I*	2.8	2.9	1.6	0.4
V2	*V. vulnificus II*	2.9	2.7	0.9	0.8
V12	*V. harveii*	3.3	1.3	0.0	1.0

Dark grey cells: true positives, light grey cells: weak positives (Section 3.2.2.7). Isolates in bold are strains used for the preparation of the mAbs, **VIB1, VIB72T** and **VIB102**.
Average absorbance at OD of 450, **VIB1** Anti O1: 1.24; **VIB72T** Anti O2: 1.18, Anti O2a: 1.77; **VIB102** Anti O2: 1.37, Anti O2b: 1.31

Regarding the classification of O2 serotype isolates, 21 isolates previously classified as O2 grouped with this serotype; 7 were added (5 O2b, 1 O2a and one isolate previously classified as *Vibrio ordalii* re-classified as O2a), 3 were sub-typed (previously known as O2, 2 were O2a and 1 O2b) and 6 were found to have been misclassified in the O2 sero-group, which represents 13.6% (6/44) of the isolates from this group. None of the 6 misclassified isolates reacted with any mAbs used. The three isolates classed as O2c did not react either with any of the monoclonal antibodies used in this study. The majority of the isolates which reacted with the O2 specific mAb (V.angO2a-b 9.2) reacted with either the O2a or the O2b specific mAb, confirming that this antibody reacted with both sub-serotypes. There were 4 isolates, B01103 2(1), B01103 5-4, B02006 SLC1 (recovered from Atlantic cod, Machrihanish fish farm, Argyll, Scotland) and VA73 (recovered from Atlantic cod, Norway), which reacted with the O2 specific antibody, but with neither the O2a nor the O2b mAbs.

The overall reactivity of V.angO2b 6.3 and V.angO2a-b 9.2 was very good, with both of them reacting strongly with cross-reactivity percent over 75; reactions against V.angO2a mab3 were weaker but this was due to the very high absorbance of the VIB72T isolate used for calculating the cross-reactivity (1.77). The only weak positive values recorded for the anti-O2b mAb were for VA33, and VA34, both recovered from Atlantic cod in Norway. The isolate VA33 gave a higher result with the O2a mAb (74.7%) than with the O2b (31.0%) and was considered to belong to the O2a serotype, whereas the isolate VA34 was considered to belong to the O2b group.

All the other isolates (non-O1 and non-O2), 18 groups and a group of untyped isolates, did not react significantly with any mAbs used in this study.

All the mAbs selected were tested against two non-*Vibrio* type strains *Aeromonas hydrophila* (V16-NCIMB 9240) and *Edwardsiella tarda* (V17-NCIMB 2034) and did not cross-react by ELISA with any of the selected mAbs (data not shown).

3.3.2.3. Western blotting using the selected mAbs

The anti-O1 mAb (V.angO1 2.3) did not react with any bacteria in Western blot analysis even with the strains known to be of an O1 serotype (data not shown).

The anti-O2 mAb (V.angO2a-b 9.2) reacted with all the O2a, O2b and O2 strains tested, but not with the O1 strains (Figure 3-8, Lane 1 and 2). This mAb reacted the strongest with O2a isolates (lane 3 and 4) than with the O2b or the O2 non-O2a non-O2b strains (lane 5 to 8) as seen with the HMW LPS, between 75 and 200 kDa. The LMW LPS, between 20 and 35 kDa, was very similar for all the O2a, O2b and O2 strains, with around 7 distinct bands stained. A very low molecular weight non-protein molecule was also detected by V.angO2a-b 9.2 with a molecular weight below 15 kDa and was present in all O2a, O2b or O2 strains.

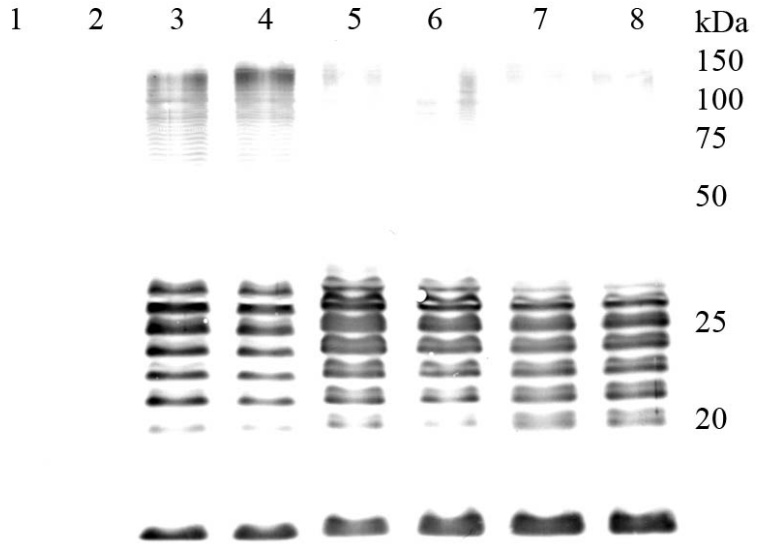

Figure 3-8 Western blot of proteinase K treated *V. anguillarum* isolates with O2 specific mAb (V.angO2a-b 9.2). Lane1&2: O1, VIB1 & VIB227; 3&4: O2a, VIB2 & VIB72T; 5&6: O2b, VIB102 & VIB103; 7&8: O2, B01103 2(1)&B02006 SLC1.

Only O2a isolates were detected using the mAb specific for O2a serotype (V.angO2a mab3, Figure 3-9 A, lane 3 and 4) and no staining was detected against the O1, the O2b or the O2 (non-O2a non-O2b)isolates. The HMW LPS were stained more strongly than on Figure 3-8, and the banding pattern extended lower, down to 55 kDa. The LMW LPS presented same staining pattern as with the V.angO2a-b 9.2 mAb (Figure 3-8), with 7 distinct bands

The O2b specific mAb (V.angO2b 6.3) bound to HMW LPS on both of the O2b isolates (Figure 3-9 B, lane 5&6) which was not the case when the anti-O2 mAb was used (V.angO2a-b 9.2, Figure 3-8, lane 5&6). The staining of the O2b antibody was especially strong for the HMW LPS of the isolate VIB103 (lane 6). The number of LMW LPS bands stained seems to be lower than that detected using the V.angO2a-b 9.2 mAb, only 5 bands were detected by mAb V.angO2b 6.3.

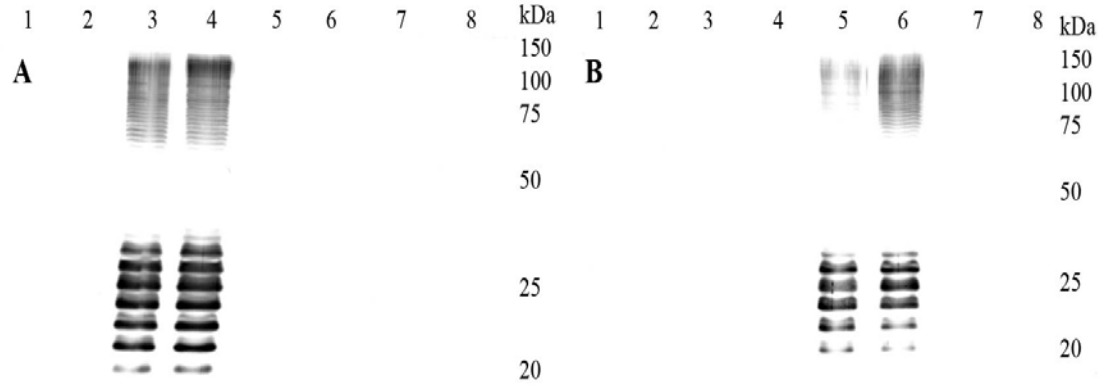

Figure 3-9 Western blot of proteinase K treated *V. anguillarum* isolates with A: O2a specific mAb (V.angO2a mab3); B: O2b specific mAb (V. angO2b 6.3). Lane1&2: O1, VIB1 & VIB227; 3&4: O2a, VIB2 & VIB72T; 5&6: O2b, VIB102 & VIB103; 7&8: O2, B01103 2(1)&B02006 SLC1.

None of the O2 strains (B01103 2(1) and B02006 SLC1) reacted with either the V.angO2a mab3 or the V.angO2b 6.3.

3.4. Discussion

3.4.1. Lipopolysaccharides

The most common method used for the extraction of LPS from Gram-negative bacteria is based on the procedure developed by Westphal and Jann (1965). It relies on the solubilisation of LPS in phenol by heating the samples to 60°C in a phenol solution, after which the solution is centrifuged to obtain a two-phase solution; the aqueous phase is then collected and concentrated. Phenol is a very dangerous chemical, which produces toxic fumes, burns the skin and has anaesthetic properties, thus an individual does not immediately feel the burns and severe injuries can occur.

An alternative method to this dangerous procedure is the use of butan-1-ol to replace the phenol (Morrison and Leive, 1975). Butan-1-ol has the advantage of being much safer than hot phenol, and does not affect the biological activity of the LPS.

The butanol extraction procedure used here was simple, rapid and gave satisfactory yields of LPS. Phenol extraction tends to give higher yields of LPS compared with butanol extraction (Morrison and Leive, 1975; Salmani *et al*., 2008). Bogwald and Hoffman (2006) extracted LPS using the phenol-chloroform method from *V. salmonicida* and obtained a yield of 1.5% (w/w) which is ten times higher than found here (0.16%) but they used dried bacteria compared to semi dry in our study, which might explain the high difference in the yields obtained. In addition, the amount of LPS on the surface of bacteria varies from species to species and serotype to serotype, such as for *V. anguillarum* serotype O2 and O3 (J-O-2) where 2% and 0.77% (w/w) of LPS were extracted by the phenol method (Eguchi *et al*., 1992a; Sadovskaya *et al*., 1996). The yield of LPS obtained with the butanol extraction method was low compared to phenol extraction, but could possibly be increased by permeabilising the membrane of the bacteria in order to release LPS molecules with enzyme treatment such as lysozymes (Salati and Kusuda,

1986) or proteinase K, prior to the extraction. Due to the amount of enzyme required, these pre-treatments are not feasible for the production of large quantities of LPS, such as in this study. The amount of bacteria used for the extraction could be increased by growing the bacteria in large volumes of broth instead of on cellophane sheets. Optimisation of LPS extraction of *Brucella abortus* using either phenol or butanol has recently been published (Salmani *et al*., 2008), with extraction yields improved by 70% and 17%, respectively, compared to the original methods (Westphal and Jann, 1965; Morrison and Leive, 1975). This optimised butanol extraction method was modified by the alternative use of ethanol precipitation followed by tri-chloro-acetic acid (TCA) treatment. The amount of LPS on the surface of the bacteria could also be increased by growing the bacteria in different media, for example supplemented with fish blood (Mutharia and Amor, 1994). The possibility that the two extraction methods could yield structurally different products could also explain the different yields recorded. However the products from both these extraction methods seem to be fairly similar (Morrison and Leive, 1975), with corresponding properties (chemical as well as immunological); the authors highlighted that butanol extraction is a milder procedure to phenol extraction with less denaturation of the LPS.

Quantification of the LPS using the purpald assay, (Lee and Tsai, 1999) relies on the measurement of the amount of KDO. This method was found to be very reliable for the quantification of LPS in this study since the standard regression curve was very good (R^2=0.99). The improved extraction protocol using butanol presented by Salmani *et al*. (2008) resulted in an increase of KDO molecule far higher than the increase in LPS extraction yield, 13 times more KDO was observed for an increase in LPS yield of 17% only. This could imply that free KDO molecules, not covalently linked in LPS molecules, were present in the final product. Since purpald reacts with KDO molecules, it is possible if free molecules are present, that the total amount of LPS could be over-estimated. However, KDO are small molecules (estimated

molecular mass, 238 Da) and the use of a 10 kDa filter in the extraction procedure of this study should have removed free KDO molecules.

The quantification of LPS using the purpald assay is very simple, rapid (only taking an hour), accurate and could be used to investigate the total amount of LPS present after culturing the bacteria in different growth or biological media.

Butan-1-ol extracted LPS were used to screen the mAbs for *V. anguillarum* specificity, because this screening requires large amounts of antigen to coat the ELISA plates, and the use of purified LPS (at very low concentrations, 2.5 $\mu g.mL^{-1}$) allowed a large number of 96-well plates to be coated without having to grow large quantities of bacteria for this purpose.

The quality of the extracted LPS was satisfactory for our requirements. The profile of the butanol extracted LPS on SDS-PAGE was very similar to the whole cell LPS after proteinase K treatment and the use of mAbs specific to *V. anguillarum* LPS showed that the antigenicity of the butanol extracted LPS was conserved as shown by ELISA and Western blotting.

Extraction of lipopolysaccharides using butan-1-ol is simple, fast and safe even if not leading to the highest yields. It would also be interesting to investigate the molecular structure of the LPS extracted by this method in comparison with the phenol extracted products by nuclear magnetic resonance (NMR) spectroscopy (Bogwald and Hoffman, 2006) to make sure that no structural modifications occur when using butanol. The use of LPS extracted with this method could give insights into the interaction of the bacterial LPS from different serotypes of any isolate of *V. anguillarum* with the cod immune system, such as the serum killing inhibition, the complement activation and cellular activation (respiratory burst priming). Bacterin vaccines for *V. anguillarum* elicit high protection in salmonids, but the specific immune system in cod does not seem to respond to these vaccines; injection of purified LPS into cod could prove very valuable from a protection point of view (Salati *et al*., 1989). It is also possible that the lower antibody

response in ayu associated with low rearing temperature shown in this study could be linked with the metabolisation of LPS at low temperature. The possibility of injecting LPS extracted from different serotypes of *V. anguillarum*, in the form of a polyvalent vaccine, may also overcome antigenic competition, which sometimes results when a multivalent vaccine is used (Nikoskelainen *et al*., 2007).

3.4.2. Monoclonal antibody characterisation

The mAbs were produced by injecting different serotypes of *V. anguillarum* (O1, O2a, O2b and O3) as formalin inactivated whole cells into four mice. In order to cut the number of hybridomas to be cloned and to only select hybridomas producing antibodies which would be useful for the serotyping scheme, the mAb clones were screened with butan-1-ol extracted LPS. This allowed a selection of clones reacting only with the molecule of the serotypes of interest to be made.

All the mAbs selected (anti O1-V.angO1 2.3; anti O2a-V.angO2a mab3; anti O2b-V.angO2b 6.3; anti O2-V.angO2a-b 9.2) reacted with the LPS in ELISA, but the anti-O1 mAb did not react with any of the strains used by Western blot. This problem of loss of reactivity between ELISA and Western blot has been previously reported. Mutharia and Amor (1994) observed this with a rabbit pAb (cross reacting with O2a and O2b on ELISA) which lost reactivity with O2b on Western blot. Tiainen *et al*. (1997a) also reported that strains of an O2 serotype, reacting positively by slide agglutination with rabbit polyclonal serum, lost their reactivity when analysed by Western blotting using the same serum. The most likely explanation for this loss of reactivity is that the epitope recognised by ELISA had lost its conformation after treatment with SDS and so no longer reacts in Western blot. Since this mAb was selected against LPS, it is possible that an LPS-LPS or protein-LPS complex was lost during the Western blotting process (Mutharia and Amor, 1994). Another hypothesis is that the LPS from the O1 serotype bound

less efficiently (or did not transfer) on the nitrocellulose membrane as described for *V. ordalii* (Mutharia *et al*., 1993).

The reaction of the different mAbs raised against the O2 sub-serotypes (V.angO2a mab3, v.angO2b 6.3 and V.angO2a-b 9.2) allowed a visualisation of specific regions of the different sub-serotypes. The fact that the anti-O2a-b mAb reacted with the LMW region of the LPS indicates that this was a common region between all of the O2 isolates. This mAb also reacted with a band lower than the repetitive pattern found at the very bottom of the gel (around 10-15 kDa), which could be the lipid A-core oligosaccharide. This is supported by Chart and Trust (1984) who showed that the shared epitopes between *V. ordalii* and *V. anguillarum* O2 are mostly localised in the very low molecular region on SDS PAGE with a molecular weight of 15 kDa (also described by Mutharia *et al*., 1993).The anti-O2a-b mAb also reacted with some of the HMW LPS of the O2a strains which means that the binding epitope is also expressed in this region. This reaction was unexpected since this mAb was prepared using an O2b strain and so it would be more logical that it would react with the HMW LPS from only this sub-serotype. Both the anti-O2a (V.angO2a mab3) and the anti-O2b (V.angO2b 6.3) mAbs recognised both the HMW and the LMW LPS regions on each respective sub-group. Thus, from these results, the sub-grouping specificity appears to lie in the HMW part of the LPS, and the LMW region of the LPS is identical in these strains, as described by Mutharia *et al*. (1993). This was also observed by Espelid *et al*. (1991) who found that 2 out of 3 mAbs they produced were specific for a sub-group of O2 reacting in the HMW LPS region of the profile, and the one cross-reacting between those sub-groups reacted mostly with the LMW LPS region.

3.4.3. Serological classification of *V. anguillarum*

The analysis of 154 different bacteria, 143 belonging to *V. anguillarum* and 11 to other *Vibrio* species, gave a good correlation between the anticipated results and the reactions actually obtained using the selected mAbs described previously.

As described in Section 3.1.3, *V. anguillarum* O2 serotype has been reported to possibly have a third sub-group, but on analysis of the studies by Espelid *et al*. (1991) and Mikkelsen *et al*. (2007), it seems that actually 4 groups could be discerned in the O2 serotype, the O2a sub-group, the two sub-groups of O2b described by Espelid *et al*. (1991) and another different group described by Mikkelsen *et al*. (2007).

Regarding the results obtained during this study, a third group of *Vibrio anguillarum* belonging to the O2 serotype was found, which reacted positively to the mAb V.angO2a-b 9.2 but neither with the O2a or the O2b specific antibodies. These isolates (B01103 2(1); B01103 5-4, B02006 SLC1 and VA73) were all isolated from vaccinated cod that died of suspected vibriosis. The comparison of these isolates with the O2c strain (non-O2a non-O2b strains from Norway, kindly provided by Dr Duncan Colquhoun), revealed that these 4 isolates did not belong to the O2c sub-group since none of these reacted with any of the mAbs used in this study. For the ease of analysis, this group (O2 but non-O2a non-O2b highlighted in this study) was labelled O2d. The O2d group identified in this study has not been previously reported, and could very well be related to the third group mentioned by Espelid *et al*. (1991), however, further investigation is required to determine this. It should be noted that in the study by Mikkelsen *et al*. (2007), this group of strains were not given a name but rather described as non-O2a non-O2b and that we used in this study the name O2c for facilitating the discussion. Furthermore, the O2c sub-group found was grouping quite far apart from the O2a and O2b serotype strains when analysed by AFLP. From the results of our study, we have doubts whether the O2c sub-serotype should be

grouped within the O2 serotype; it could represent another serotype (maybe O24). It would be very interesting to investigate the AFLP dendrogram of the O2d sub-group to see if these isolates are grouping close to the O2a and O2b strains. It is also imperative to screen the strains used from these different studies (Espelid *et al*., 1991; Knappskog *et al*., 1993; Tiainen *et al*., 1997a; Mikkelsen *et al*., 2007), using the mAbs developed here, to ensure that these groups are well characterised.

It is important to have a fuller understanding of the classification of the O2 isolates of *V. anguillarum* involved in cod disease. To do this, it is necessary to screen as many isolates from this group as possible, especially from Scandinavian countries, where cod aquaculture is an important economical activity. This study revealed some unsuspected differences between the isolates which compose the O2 serotype of *V. anguillarum*. Since the isolates grouping within this possibly new O2d sub-serotype were recovered from diseased fish, this finding could be of significant importance for vaccine formulation as well as epidemiological studies.

The lipopolysaccharide extraction method presented here was successful at producing LPS for the purpose of screening mAbs. The quality of these extracted products was also satisfactory for its application to other areas of fish immunology. The mAbs developed during this study will be useful for examining the prevalence of different serotypes of *V. anguillarum* involved in the vibriosis outbreaks recorded in vaccinated cod in Scandinavia, as well as more basic diagnostic tools for the rapid screening of isolates.

Chapter 4.

Study of the pathogenicity of *Vibrio anguillarum in vitro* in Atlantic cod

4.1. Introduction

The *Vibrio* inactivated whole cell vaccine is considered the most successful vaccine in marine aquaculture (Sakai, 1999), however, protection of Atlantic cod against *V. anguillarum* has not been as straight-forward as for salmonids, and after a decade of research, outbreaks are still occurring in vaccinated fish (Hellberg *et al*., 2005-2006-2007; Mikkelsen *et al*., 2007). The formulation of vaccines to protect cod more efficiently against *V. anguillarum* will require a better understanding of the virulence mechanisms employed by the bacterium and the elucidation of how the cod immune system responds to *Vibrio* infections.

Fish rely on their innate immune system more than their mammalian counterparts, possibly because of their more ancient origin (Magnadottir, 2006) or due to different evolutionary pathways. The development of acquired immunity in cod following infection with *V. anguillarum* has still not been fully elucidated (Section 1.3.2.5) and due to the importance of innate immunity in this species for pathogen clearance (Magnadottir, 2006), it is clear that further investigations are needed into the innate immune response of cod to *V. anguillarum*.

4.1.1. Innate immune response

Destruction of bacteria by phagocytic killing is a major mechanism in the prevention of colonisation of pathogenic bacteria (Stuart and Ezekowitz, 2005). Phagocytosis involves the ingestion and intracellular destruction of microbial pathogens, as well as apoptotic host cells and debris (Ernst, 2000; Djaldetti *et al*., 2002; Heale and Speert, 2002; Stuart and Ezekowitz,

2005). As previously mentioned in Section 1.3.1.1 and 1.3.2.3, the non-specific nature of phagocytosis is a very important feature of cod defence mechanisms. The ingestion of bacteria involves the attachment of the bacterium by the phagocytic cell and its intake within a phagosome (Secombes, 1996). The recognition of common antigens on the surface of bacteria, i.e. PAMPs by PRRs, activates the engulfment processes, involving actin polymerization (Heale and Speert, 2002). Once internalised, phagocytic and lysosomic granules migrate and fuse together to form a phago-lysosome (Ross and Auger, 2002). Bacterial killing in the phago-lysosome can occur through two separate processes: oxygen dependant or independent mechanisms which take place within the first hour of internalisation for most animal species. The lysosome fuses with the phagosome (acidification) within 15 minutes of ingestion in mammals (Ross and Auger, 2002). Reactive oxygen species (ROS, oxygen dependant) are produced in the phago-lysosome by the reduction of oxygen to superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) catalysed by NADPH (Babior, 1984). The oxygen free radicals are very toxic to bacteria through damage to their cell membranes and DNA (Hampton *et al*., 1998). The resting level of those ROS can be greatly increased through the activation effect of several chemicals (Section 1.3.2.3). Another type of oxygen dependant molecule used by the cells for bacterial killing is nitric oxide species (NOS) and its intermediates. It has been shown that, as for ROS, the basal level of NOS can be increased after cell activation by LPS and IFN-γ (Nathan and Hibbs Jr, 1991). Nitric oxide, which itself is not particularly toxic, can lead to the production of more toxic substances such as peroxidising (OH^-), nitrating (NO_2) or nitrosating (N_2O_3) species. These are toxic to bacteria by inactivating DNA and proteins and have the ability to damage membrane integrity (Kaplan *et al*., 1996).

Oxygen-independent processes of the cellular innate immunity are also involved in the killing of bacteria within the phago-lysosome, such as the acidification of the vesicle, sequestration of iron or other nutritional requirements of the bacteria (Heale and Speert, 2002). In addition,

antimicrobial peptides (elastases, collagenases, lipases, sulfatases, phosphatases and defensins) present in the lysosome are able to form pores in the bacterial membrane (Heale and Speert, 2002) and cationic proteins and various enzymes such as lysozymes and hydrolytic molecules are also involved (Wilson *et al*., 2002).

The humoral innate immunity is also heavily involved in the defences against pathogens. Complement is probably the main component in the serum to interact with bacteria, and is a much more ancient system than the adaptive immune response (Nonaka, 2001). The function of this system in mammals is to provide antimicrobial substances to (i) bind to particles and enhance their phagocytosis through opsonisation, (ii) enhance inflammatory events through production of chemo-attractants (anaphylotoxins) and antibody formation and (iii) directly kill the bacteria by the lytic complex (Wilson *et al*., 2002). Briefly, complement acts in several ways but the C3 molecule seems to have a central role; when activated, it splits into C3a (binding to leukocytes and initiating an inflammatory response) and C3b (opsonising the pathogen recognised); the membrane attack complex (MAC), a combination of C5 to C9 molecules can also directly lyse the micro-organism (Boshra *et al*., 2006). The complement of Atlantic cod does not seem to present multiple forms of the C3 molecules as found in other fish species (Olivier *et al*., 1992), although this study is not conclusive and more research is needed to confirm it. Complement is however not the only component in serum that has antimicrobial properties (Section 1.3.1.1). Cod serum has been reported to have some peculiar characteristics compared to other bony fish (Section 1.3.2.4) such as its haemolytic activity or the lack of lysozyme activity. The opsonisation of normal cod serum has been shown to be poorer, compared to rainbow trout serum, regarding the increase in respiratory burst levels against *Aeromonas salmonicida* (Nikoskelainen *et al*., 2006), but this could be due to the specific immune system differences in these two fish rather than due to complement. This bacterium was also reported to be resistant to cod serum (Magnadottir, 2000), which could be

due to a specific avoidance of C3 binding (Rautemaa and Meri, 1999). Nevertheless, cod serum has some strong antimicrobial properties that have been shown to be drastically increased by vaccination with *V. anguillarum* (Section 1.4.2).

4.1.2. Evasion of the immune system by *Vibrio anguillarum*, virulence factors

The definition of pathogenesis according to the online Oxford English dictionary is: "The production or development of disease; the events occurring during the development of a particular disease; the mechanisms whereby a disease is produced" (http://www.oed.com).

The evolution of pathogenic bacteria and the counter-measures developed by the immune system of fish is a two-way process, the goal of which is ultimately the survival of the host or the pathogen (Wilson *et al*., 2002). There are a few requirements in order for a pathogenic bacteria to create a disease in its host,: (I) once in contact with its host, the pathogen needs to establish itself in the host; (II) it must then avoid being destroyed or expelled by the host's immune system and at the same time obtain essential nutrients for its survival; (III) it must escape from its host for transmission to another individual (Wilson *et al*., 2002).

There are hundreds of different mechanisms that bacteria use to survive attack by the immune system, maybe as many as there are ways of the immune system defending itself against pathogens, as shown in Figure 4-1.

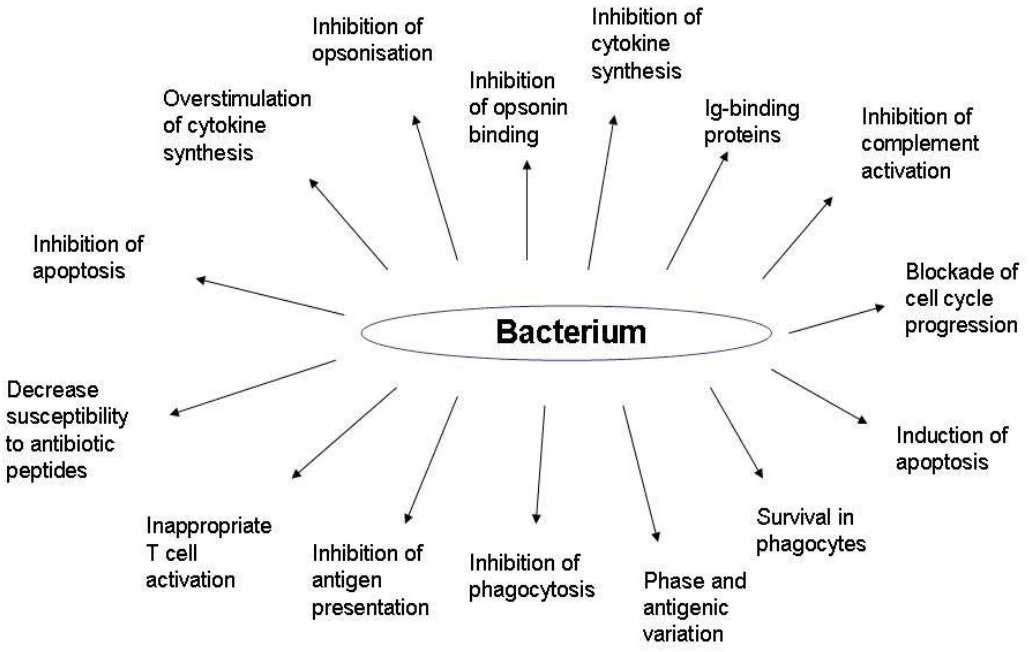

Figure 4-1 Different strategies of pathogenic bacteria to avoid host immune defences (Wilson *et al*., 2002).

Pathogenic bacteria manipulate the cells and humoral components of the host's immune system in order to survive. Most of the mechanisms used rely on secreted or membrane-bound chemicals and target mainly the cytoskeleton or the intracellular signalling pathway of the cell (Bhavsar *et al*., 2007). Manipulation of the host cell sometimes involves the injection of specialised effector molecules to subvert the machinery of the cell such as YopH (potent phagocytosis blocking agent) through Type III Secretion System (T3SS) (Cornelis and Wolf-Watz, 1997; Frihtz-Lindsten *et al*., 1997), or the disruption of the nuclear factor (NF) -κB regulatory pathway by T3SS molecules (Bhavsar *et al*., 2007).

Bacteria can inhibit phagocytosis (reviewed in Wilson *et al*., 2002) by either direct interaction with the cell, blocking internalisation (Ernst, 2000) or the oxidative killing once it is internalised (Buchmeier and Heffron, 1991; Bliska and Black, 1995). Processing pathways resulting in the

presentation of antigens on the surface of the phagocytic cells can also be blocked (Noss *et al*., 2001) and sometimes the phagocytic cells can be destroyed through apoptosis (Bhavsar *et al*., 2007). Inhibition of the binding of complement molecules is also a common mechanism of many bacteria to avoid opsonisation and phagocytosis. Avoidance of complement binding is therefore another very important virulence factor encountered in many pathogens and as shown in Figure 4-1, bacteria have evolved many systems to inhibit (active) or avoid (passive) the killing mechanisms in serum (reviewed in Rooijakkers and Strijp, 2007). These mechanisms are intimately linked to surface molecules that help protect the integrity of the bacterium, such as sialic acid, and LPS molecules (reviewed in Boshra *et al*., 2006).

Vibrio anguillarum is highly pathogenic to many fish species (Section 1.2.2) and is easily cultured *in vitro* (Section 2.1.1), making it a very good model for host-pathogen interactions. However, most of the studies of this pathogen have concentrated on the immune response of the fish to the pathogen using dead bacteria, so little is known about the defence mechanisms of the live pathogen. Phagocytic cells from many different fish species have been shown to be capable of phagocytosing *V. anguillarum* e.g. sea bream (*Sparus aurata* L.) (Esteban *et al*., 1998), Coho salmon (*Oncorhynchus kisutch*) (Balfry *et al*., 2001), rainbow trout (Lamas *et al*., 1994) and sea bass (*Dicentrarchus labrax* L.) (Sepulcre *et al*., 2007). Very few studies have compared the pathogenic mechanisms between live isolates of the same species (between or within serotypes). Boesen *et al*. (2001) studied the interaction of different live isolates of *V. anguillarum* (O2a) with trout leukocytes, while a few other studies can be found using other fish pathogens such as *Edwardsiella tarda* (Ishibe *et al*., 2008), *Aeromonas salmonicida* ((Karczewski *et al*., 1991; Olivier *et al*., 1992; Lamas and Ellis, 1994), *Photobacterium damselae* subsp. *piscicida* (Skarmeta *et al*., 1995).

Some strains of *V. anguillarum* are able to resist trout serum killing (Boesen *et al*, 1999) and some of the growth inhibitors present in serum, such as the mechanisms resulting in iron sequestration through the presence of the plasmid pJM1 (Crosa *et al*., 1977) or its chromosomal equivalent (Toranzo *et al*., 1983). However, other mechanisms are involved in serum resistance as shown by the work of Trust *et al*. (1981) with *V. anguillarum* mutant isolates. Differences in the resistance of different serotypes of *V. anguillarum* to serum killing has also been shown, with O1 being sensitive to killing via the classical complement pathway (antibody mediated) and O2a seemingly resistant (Boesen *et al*., 1999). A good correlation was found between the resistance of *V. anguillarum* to serum killing and pathogenicity (Austin *et al*., 1995; Boesen *et al*., 1999), showing that resistance to serum is one of the main mechanisms involved in the progress of the disease. The influence of LPS in resistance to serum killing has been clearly shown, through the use of LPS-deficient mutants (Welch and Crosa, 2005) as well as the use of different growth media (Section 3.1.2).

Protease activity of *V. anguillarum* has also been investigated (Zhang *et al*., 2006) and it was reported that strains grown in gastrointestinal mucus of Atlantic salmon produced five *de novo* proteins of which four form part of the membrane fraction of *V. anguillarum* (Garcia *et al*., 1997). Using the same type of experiment, a nine-fold increase in the extracellular protease activity was reported compared to bacteria grown in bacteriological media (Denkin and Nelson, 1999). It is still unclear if protease secretion by the bacterium is directly linked to virulence since contradictory results have been published using protease deficient mutants (Norqvist *et al*., 1990; Milton *et al*., 1992).

4.1.3. Assessment of virulence and pathogenicity

The use of *in vitro* assays is becoming more common for studying the pathogenesis of bacteria due to advances in cellular and molecular techniques, as well as the pressure on scientists to

reduce, replace and refine the use of animals in experimental work (UK Home Office, Animals, Scientific Procedures Act, 1986). Moreover, conventional assays to study the pathogenesis of bacteria *in vivo* are costly. Although *in vitro* assays are unlikely to completely replace testing *in vivo*, they can offer a valuable alternative.

In the last 30 years, the techniques used to investigate bacterial pathogenicity have come a long way from toxicity studies examining cell survival to nowadays more elaborate experiments where thousands of genes expressed in response to infection are measured using micro-arrays. The use of knockout animals has also greatly increased the understanding of the pathways and interactions taking place in healthy and diseased animals.

Flow cytometry is a fairly recent technology (dating from the early 80s) which allows the properties of individual particles to be examined (in most cases cells, but bacteria can also be analysed) (Sklar, 2005). It is based on the principle of acquiring information on single particles in a sample regarding their ability to scatter light emitted from a laser source. Light scatter is recorded by two detectors, a Forward Scatter Channel (FSC) where the intensity of the light detected provides information about the size of the cell, and a Side Scatter Channel (SSC) which provides information about the granular content of the particles (Shapiro, 2003). A number of other channels detect fluorescence at specific wavelengths. The specific binding of fluorochrome-associated molecules onto cells allows a very wide range of applications such as the identification and quantification of distinct populations of cells, detection of particular markers within or on the cell surface, quantification of biological processes, and studies on nucleic acids or apoptosis.

The advantages of flow cytometry are numerous, but the speed of measurements (up to 50,000 cells per sec, potentially 100,000 samples a day) and the possibility of multiplexing the assay (4

different lasers recording simultaneously different fluorescent markers) can allow a huge number of highly statistically relevant parameters to be measured (Sklar, 2005).

Many areas of biology can find an application for flow cytometry (e.g. diagnostics, therapeutics, agriculture, reproductive biology, protein engineering, etc…) and immunology and bacterial pathogenesis research have benefited immensely from the data acquired by flow cytometry (Valdivia and Falkow, 1998). Investigations of phagocytic activity by labelling bacteria with fluorescent dyes (commonly FITC but other dyes are available, Drevets and Elliott, 1995) and discriminating between internalised (phagocytosed) and adhered bacteria (through the use of quenching (Van Amersfoot and Van Strijp, 1994) or inhibiting agents (Steinkamp *et al*., 1982; Mansfield *et al*., 2000), has generated data that has contributed to a better understanding of the interaction between pathogenic bacteria and the immune system (Lehmann *et al*., 2000). Assessment of the effect of opsonisation with specific antibodies (Bassoe and Bjerknes, 1984; Drevets and Elliott, 1995; Lehmann *et al*., 2000; Rodriguez *et al*., 2001), and respiratory burst which results after phagocytosis can actually be measured together through a combined staining protocol (Perticarari *et al*., 1991; Smits *et al*., 1997; Busque *et al*., 1998).

Recently, aquaculture and fish immunology researchers have been applying the use of flow cytometry in their analysis. One of the most simple application is the discrimination of different cell populations in fish, based on either granularity/size (FSC/SSC) (Stafford *et al*., 2001; Belosevic *et al*., 2006) or by staining cells with a fluorescent label (Inoue *et al*., 2002). The use of mAbs that recognise different cell types also allow the differentiation and quantification of populations such as leukocyte populations in healthy cod (Ronneseth *et al*., 2007), or after treatment with immunostimulants (Huttenhuis *et al*., 2006), but specific antibodies to many fish markers are still missing.

Studies of fish phagocytic and respiratory burst activity have been applied to many species such as rainbow trout (Chilmonczyk and Monge, 1999; Boshra *et al*., 2006), sea bream (Esteban *et al*., 1998; Ortuno *et al*., 2000; Chaves-Pozo *et al*., 2004), ayu (*Plecoglossus altivelis*) (Moritomo *et al*., 2003), Brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*) (MacKenzie *et al*., 2003). Most of these studies used latex beads as a target for the phagocytic cells and only a few used labelled inactivated bacteria (Esteban *et al*., 1998; Coteur *et al*., 2002; Chaves-Pozo *et al*., 2004).

The status of bacteria, i.e. whether live or dead, can also be determined by flow cytometry. It is possible to discriminate (different emmision wavelength) between stained live plus dead bacteria with Thiazole Orange, which stains all cells, and damaged plus dead bacteria incorporating Propidium Iodide (G. Nebe-von-Caron *et al*., 2000). This technique is very useful since bacteria do not need to be cultured and cfu counts performed for enumeration. The procedure has many applications such as determining the bactericidal activity of individual sera (Virta *et al*., 1998), the testing of disinfectants (Wozniak-kosek and Kawiak, 2005) or antimicrobial agents like antibiotics (Durodie *et al*., 1995; Alvarez-Barrientos *et al*., 2000).

4.1.4. Aim of the study

The aim of this study was to investigate the mechanisms behind the ability of *V. anguillarum* to avoid being killed by the innate immune system of Atlantic cod. A set of *in vitro* assays were developed to examine interactions between different isolates of the same serotype of *V. anguillarum* and the main innate defences (i.e. phagocytosis, respiratory burst and serum killing) of cod. These assays were developed as an alternative to *in vivo* strain selection. Ultimately, this work would provide a better understanding of the virulence mechanisms of *V. anguillarum* and in turn would enable more appropriate strains to be included in the vaccine formulation and thus provide a better *V. anguillarum* vaccine product for cod (Chapter 5).

4.2. Materials and methods

4.2.1. Fish and cell collection

Forty healthy unvaccinated Atlantic cod were obtained from Machrihanish fish farm (West coast of Scotland) and transported to the University of Stirling in oxygenated bags. The fish (approximately 80 g in weight) were housed in 4 circular tanks (1.5 m diameter and 1.5 m deep) independent from the main recirculation system, with individual pump and oxygenation systems. The temperature was maintained at 12°C.

The cod were left to acclimatise for a minimum of 3 weeks and fed on commercial cod sinking pellets (BioMarine Pearl, BioMar) at 1% of their body weight per day.

The fish (106.1 ± 8.9 g and 22.75 ± 0.61 cm) were then euthanised with an overdose of 2-phenoxyethanol (Sigma-Aldrich, Poole, UK, 1 $mL.L^{-1}$ in sea water) and the brain immediately destroyed by dissection. The fish were bled using a Vacuette (Greiner Bio-One) and the blood left to clot overnight at 4°C. The head kidney was aseptically dissected and kept on ice in 5 mL of Leibovtz media (L15*, Appendix 9.2) plus 15 $IU.mL^{-1}$ of heparin (Sigma-Aldrich). The serum was collected by centrifuging the clotted blood at 3000 x g for 7 min. It was aliquoted in 1.5 mL centrifuged tube and kept at -20°C until used.

Leukocytes were extracted from cod head kidney following the method of Braun-Nesje *et al*. (1981) with slight modifications. All cell work took place in a laminar flow cabinet. The head kidney was immediately transported to the laboratory and passed through a cell strainer (100 μm mesh, BD Falcon, Oxford, UK) in a sterile Petri dish. The cells were collected by adding 5 mL of L15* and centrifuged for 10 min at 200 x g. The supernatant was discarded and the cells resuspended in 10 mL of L15* (without FCS) and layered on top of a discontinuous Percoll gradient (Appendix 8). The tubes were centrifuged at 400 x g for 45 min at 7°C and the

leukocyte band located at the interface of the 34%/51% layers collected using a disposable sterile Pasteur pipette. The medium containing the cells was made up to a volume of 15 mL with L15* and the cell suspension centrifuged for 10 min at 200 x g. The supernatant was discarded and the cells resuspended in 10 mL of L15*. The cell concentration was estimated using a Haemacytometer (improved Neubauer, 0.0025 mm^2) following the manufacturer's instructions and kept overnight in the incubator set at 12°C.

4.2.2. Bacteria

The bacteria used in this study (Table 4-1) were cultured in TSB+NaCl (Section 2.1.2) from stocks maintained on cryo-beads. The bacteria were also cultured on TSA+NaCl to check the purity and biochemical characteristics of the isolates (Section 2.1.4). Bacteria were either stained with FITC on an orbital rotator (Stuart Scientifics) for the phagocytosis assay according to Weingart *et al*. (1999) with modifications, where different concentrations of FITC and incubation times were tested on one isolate (VIB2), or left unstained for the respiratory burst assay. The bacteria were kept thereafter as 1 mL aliquots in a 15% glycerol solution at -70°C until used (Section 2.1.3.2).

Table 4-1 *Vibrio anguillarum* isolates used in the phagocytosis, the respiratory burst and the serum killing assays.

Vibrio strain	Serotype	Origin	Vibrio strain	Serotype	Origin
VIB1	O1	Trout, DK	VIB102	O2b	Cod, DK
VIB227	O1	Turbot, FR	VIB103	O2b	Cod, DK
VA40-78skid	O1	Salmon, UK	VIB104	O2b	Cod, DK
VA65	O1	Turbot, ES	VIB105	O2b	Turbot, DK
VIB2	O2a	Cod, DK	VA64	O2b	Cod, UK
VIB72T	O2a	Cod, NO	VIB3	O3	Trout, DK
VA25	O2a	Sole, ES	VIB149	Untyped	Water, DK
VIB98	O2a	Turbot, NO	V5	V. ordalii	Salmon, US
VA34	O2b	Cod, NO			

Isolate VA65 was not used for the respiratory burst or serum killing assays.

To revive the frozen stocks of prepared bacteria, 1 mL aliquots of stained or unstained bacteria were removed from the -70°C, centrifuged at 3500 x g for 10 min and resuspended in 1 mL of

sterile PBS. The effect of the freezing in 15% glycerol (Section 2.1.3.2) was investigated by flow cytometer to ensure that it did not affect the staining of the FITC label.

4.2.3. Phagocytosis and respiratory burst assay

Different reaction conditions were tested to optimise the assay before performing the analysis. The phagocytosis assay was carried out according to Esteban *et al*. (1998) and the respiratory burst according to Richardson *et al*. (1998). Briefly, to optimise the assays, different bacteria:leukocytes ratios were used (from 1:1 to 1000:1), the time for maximum ingestion of the bacteria by the phagocytes was established (5 min to 6 h), and different blocking agents (for the phagocytosis assay) were examined. The inhibition of phagocytosis was necessary in order to discriminate between phagocytosed bacteria and bacteria attached onto the surface of the phagocytes, which could lead to an over-estimation of the total positive cells. The agents tested included different concentrations and incubation times of paraformaldehyde (Sigma-Aldrich) or sodium azide (Sigma-Aldrich) to kill the leukocytes and Colchicine (Sigma-Aldrich) and 2,3 butanedione monoxime (BDM) to block the phagocytosis in these cells. Cells for the negative control in the phagocytosis assay were supplemented with 20 μL of BDM (Sigma-Aldrich, 420 mM), according to Mansfield *et al*. (2000) and incubated for 1 h at 12°C.

Cod leukocytes prepared in Section 4.2.1 were centrifuged at 200 x g for 10 min and diluted to 5 $x10^6$ cells.mL^{-1} in L15*. The leukocyte suspension of each fish was aliquoted into sterile FACS tubes (BD Falcon) at 200 μL.$tube^{-1}$. Twenty μL of each selected bacterial suspension prepared in Section 4.2.2 was added to one of the tubes at a bacteria:leukocyte ratio of 20:1, gently vortexed and centrifuged at 150 x g for 3 min. Additional tubes without bacteria or cells treated with BDM were used as negative controls. The tubes were then returned to the incubator (12°C) for 2 h to allow phagocytosis or respiratory burst to occur.

Phagocytosis assay:

After incubation of the cells with bacteria (negative controls containing no bacteria), for 2 h, 500 μL of ice cold PBS +1% BSA (w/v, Sigma-Aldrich) was added to each tube which was then gently vortexed and centrifuged for 5 min at 200 x g. The supernatant was discarded and the cells resuspended in 1 mL of ice cold PBS +1% BSA. The tubes were again centrifuged as described above, and the cells resuspended in 500 μL of PBS +0.02% (w/v) EDTA (Sigma-Aldrich) and immediately acquired on the flow cytometer.

A 100 μL sample was collected from a few samples just before analysis on the flow cytometer. Cytospin preparations were made of these by adding 100 μL of 10% FCS to the sample and centrifugation for 5 min at 117 x g in a Cytospin3 centrifuge (Thermo-Shandon, Runcorn, UK). The smears were then stained with Rapid Romanowski stain (Raymond A Lamd, Eastbourne, UK) after optimisation of the staining for cod leukocytes, briefly 30s in solution A, 2 x 20 sec in solution B and 2 x 15 sec in solution C. This was done to visualise if the pre-treatment of cells with BDM and the centrifugation were efficient in inhibiting phagocytosis and removing unphagocytosed bacteria from the surface of the phagocytes, respectively.

Respiratory burst assay:

After incubation of the cells and bacteria for 2h, 100 μL of Dihydrorhodamine 123 (DHR, Appendix 6.1) was added to all the tubes and incubated for 5 min at RT followed by 30 min at 12°C. Five hundred μL of ice cold L15* was then added to each tube and the level of respiratory burst activity immediately measured on the flow cytometer. Positive controls consisted of leukocytes incubated with DHR and stimulated by adding 100 μL of PMA (Appendix 6.2) after the 5 min incubation at RT, and the negative controls comprised leukocytes with 100 μL of PMA but no DHR. The auto-respiratory burst of cod leukocytes were also measured by incubating cod leukocytes with DHR only.

4.2.4. Serum killing assay

Sera collected from individual cod (the same fish as used in the phagocytosis and respiratory burst assays) were defrosted and half the serum heat inactivated by heating it to 56°C for 30 min. The sera were then diluted 1:10 in sterile PBS and filtered through a 0.20 μm syringe filter (Sartorius Minisart, Epsom, UK) before use. Twenty fish sera were used to analyse VIB1 and VIB227 while only 10 sera (Fish1 to Fish10) were used for the remaining isolates tested (Table 4.1).

The serum killing assay was performed on the cod sera based on the protocol of Virta *et al*. (1998) but several parameters needed to be optimised. Naïve serum from either rainbow trout or Atlantic salmon was used to validate the assay using *V. anguillarum* isolate VIB87 (serotype O1), known to be sensitive to the serum of these fish species. Briefly, the sera (normal and heat inactivated by heating at 56°C for 30 min) from 2 fish per species was diluted 1:10 in PBS. The assay was performed using the same protocol as outlined for the cod serum except that the temperature was set at 25°C.

Bacteria (Table 4-1, except VA65) were grown overnight and diluted to an OD of 1 at 610 nm (Section 2.1.2). The bacteria were diluted 1:10 in sterile PBS and 10 μL of bacteria was added to 90 μL of serum (normal or heat inactivated) plus 100 μL of serum buffer (Appendix 7.1) in FACS tubes. The tubes were gently vortexed and incubated for 2 h at 12°C. After incubation, 500 μL of ice cold PBS+EDTA (1 mM EDTA) was added to each tube, vortexed quickly and centrifuged at 3000 x g for 10 min. This wash was repeated and the bacteria resuspended in 500 μL of PBS+EDTA. The staining procedure was based on the BD cell viability kit protocol (Alsharif and Godfrey, 2002). Three μL of Thiazole Orange (Appendix 7.2) and 3 μL of Propidium Iodide (Sigma-Aldrich, 4.3 mM in PBS) were added to each tube, incubated for 5

min at RT and placed on ice until read. Negative controls comprised bacteria without serum to make sure that the protocol did not affect the survival of the different isolates.

4.2.5. Flow cytometry settings and Statistics

4.2.5.1. Phagocytosis and respiratory burst assay settings

The settings of the flow cytometer (FACScalibur, Becton Dickinson, Oxford, UK) were set separately for the phagocytosis and the respiratory burst to obtain the best repartition of the cell population on the FSC/SSC dot plot; for both experiments, the Threshold (set on SSC-H primary parameter) and the Compensation levels were not modified (Figure 4-2).

Regarding the respiratory burst assay, the following modifications were applied to the settings: FSC: 2.02; SSC: 2.76; FL1: 258 and FL2: 258. All the other settings were left as for the phagocytosis assay.

The data were collected from the flow cytometer for both assays using CellQuest (Version 3.3, Becton Dickinson, UK). Five thousands events were recorded after gating the population of phagocytic cells (red polygon on Figure 4-2). The data were expressed as the percent of positive cells (i.e. fluorescent) over the background level for the relevant assays, after transformation according to Equation 4-1 in order to normalise the data.

$$x_i = \text{Log}\,(x)$$

Equation 4-1 Transformation formula applied to the percentage data of the phagocytosis and respiratory burst data prior to statistical analysis.

The phagocytosis data were calculated by subtracting the results of the phagocytosis blocked with BDM from the data acquired without the blocking agent.

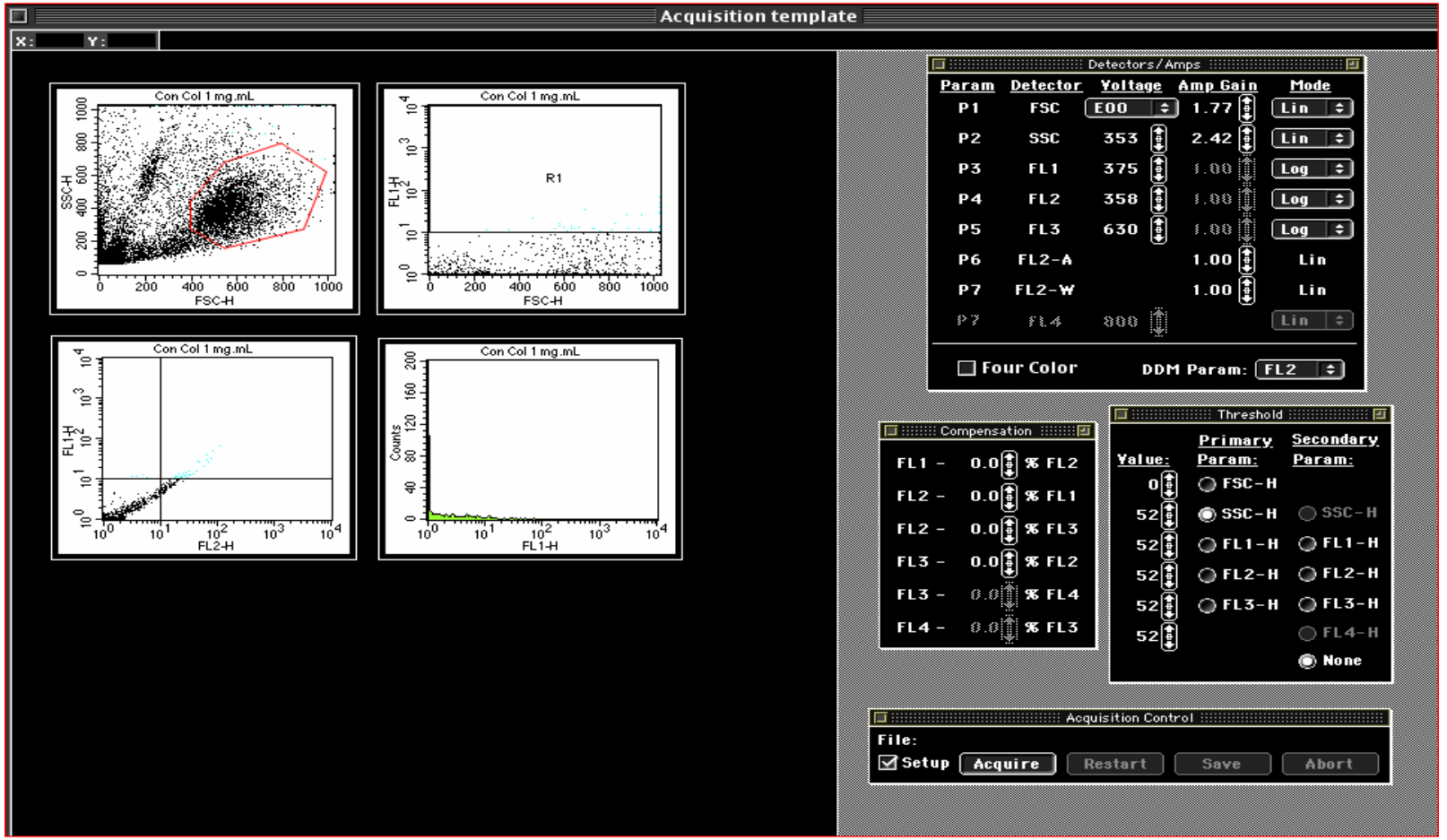

Figure 4-2 Settings of the flow cytometer for phagocytosis assay; red polygon represents the gating of the phagocytic leukocyte population (Software: CellQuest version 3.3).

The normality of the distribution, as well as the equal variance test (Anderson-Darling and Bartlett's tests, respectively), were run using Minitab 15.1.0 (Minitab Inc., Coventry, UK). All data are normally distributed unless otherwise stated. The test chosen to establish the significance of the differences was a General Linear Model (GLM) with the output: log of the percentage of positive events (Equation 4-1) and the factors: bacterial strain, serotype and fish. The fish parameter was set as a random factor due to the high variation known to exist between individual cod. The test was followed by a pair-wise comparison procedure (Holm-Sidak method).

The different graphs presented in this chapter were produced using SigmaPlot 10.0 software (Systat software Inc, Hounslow, UK). Figures 4-8, 4-9, 4-10 and 4-12 were plotted using the median of data sets with the inter-quartile range represented by vertical bars. This was carried out to simplify the interpretation of the values due to the non-normal distribution of the data or the very large standard deviation encountered. It is much more useful to express the data in this way than to use standard deviations as it is less affected by outliers (Dytham, 2003). Figure 4-5 was plotted using the mean of the data set and the vertical bars represent one standard deviation.

4.2.5.2. Serum killing assay settings

Instructions to set the flow cytometer for the bacteria live/dead assay from Alsharif and Godfrey (2002) were followed and the settings used are shown in Figure 4-3 A.

The plot of fluorescence channels FL1/FL3 (Figure 4-3 B) was used to gate the bacteria falling in one of the three possible categories: Live (Figure 4-3 B lower right quadrant), Dead (Figure 4-3 B upper left quadrant) or Injured (Figure 4-3 B upper right quadrant). The data were collected from the flow cytometer using CellQuest (Version 3.3, Becton Dickinson) and 10,000 events were acquired in the gate encompassing these 3 populations.

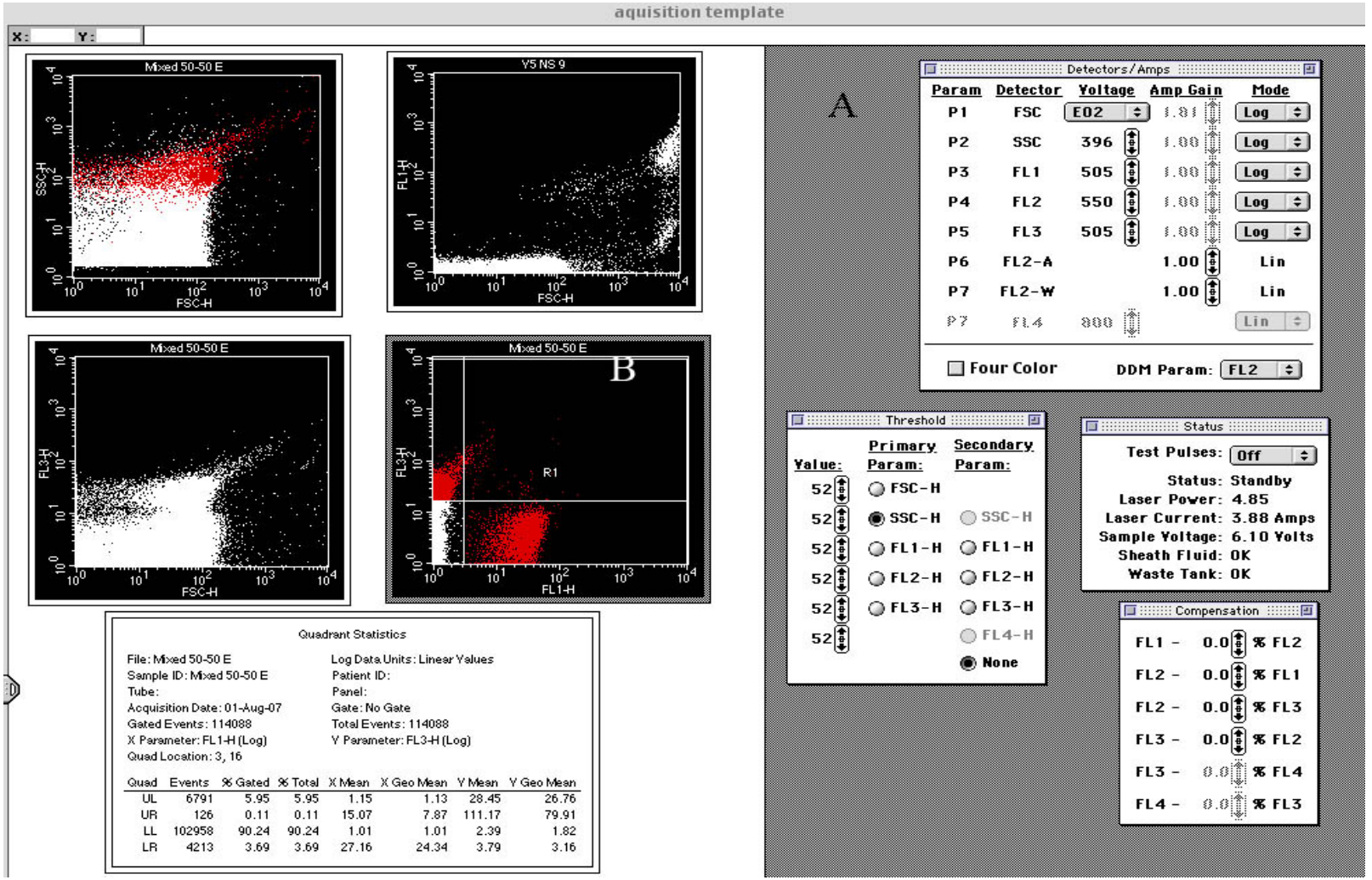

Figure 4-3 Settings of the flow cytometer for the serum killing assay; A: laser settings; B: dot plot used to calculate the bacteria state (live, injured or dead). Software: CellQuest version 3.3.

The statistics were run on the ratio of dead + injured to live bacteria of different isolates using Minitab software. An example of these data can be found on Figure 4-3 in the "Quadrant Statistics" box, where the lower left (LL) quadrant encompass the debris, upper left (UL) the dead bacteria, lower right (LR) the live ones and upper right (UR) the injured bacteria.

4.3. Results

4.3.1. Phagocytosis assay

4.3.1.1. FITC staining

The FITC staining of *V. anguillarum* was investigated using VIB2 strain (O2a) by adding different concentrations of the FITC for 1h or over-night. The data were analysed on the flow cytometer as shown in Figure 4-4. The highest fluorescence intensity recorded on the FL-1 channel (A-2 to G-2) was observed with 0.05 (F-2) and 0.1 $mg.mL^{-1}$ (E-2) of FITC for 1 h. Visually, the distribution of the bacteria plots recorded on the FSC/SSC (Figure 4-4, A-1 to G-1) revealed that the FSC/SSC graphs with 0.05 (F-1) and 0.1 $mg.mL^{-1}$ of FITC (E-1) as well as the over-night incubation with 0.05 $mg.mL^{-1}$ of FITC (A-1) were visually the most similar to the control graph (B-1). The intensity of the staining with 1 $mg.mL^{-1}$ for 1 h (C-2) seems to have two peaks of fluorescence intensity, a very high peak of fluorescence and a lower one.

The survival of the bacteria after staining with different concentrations of FITC was investigated to ensure staining did not affect the viability of the bacteria (Figure 4-5). The bacteria were incubated for 1 h with the different concentrations of FITC and cfu numbers were determined to assess the viability of the bacteria (Section 2.1.5).

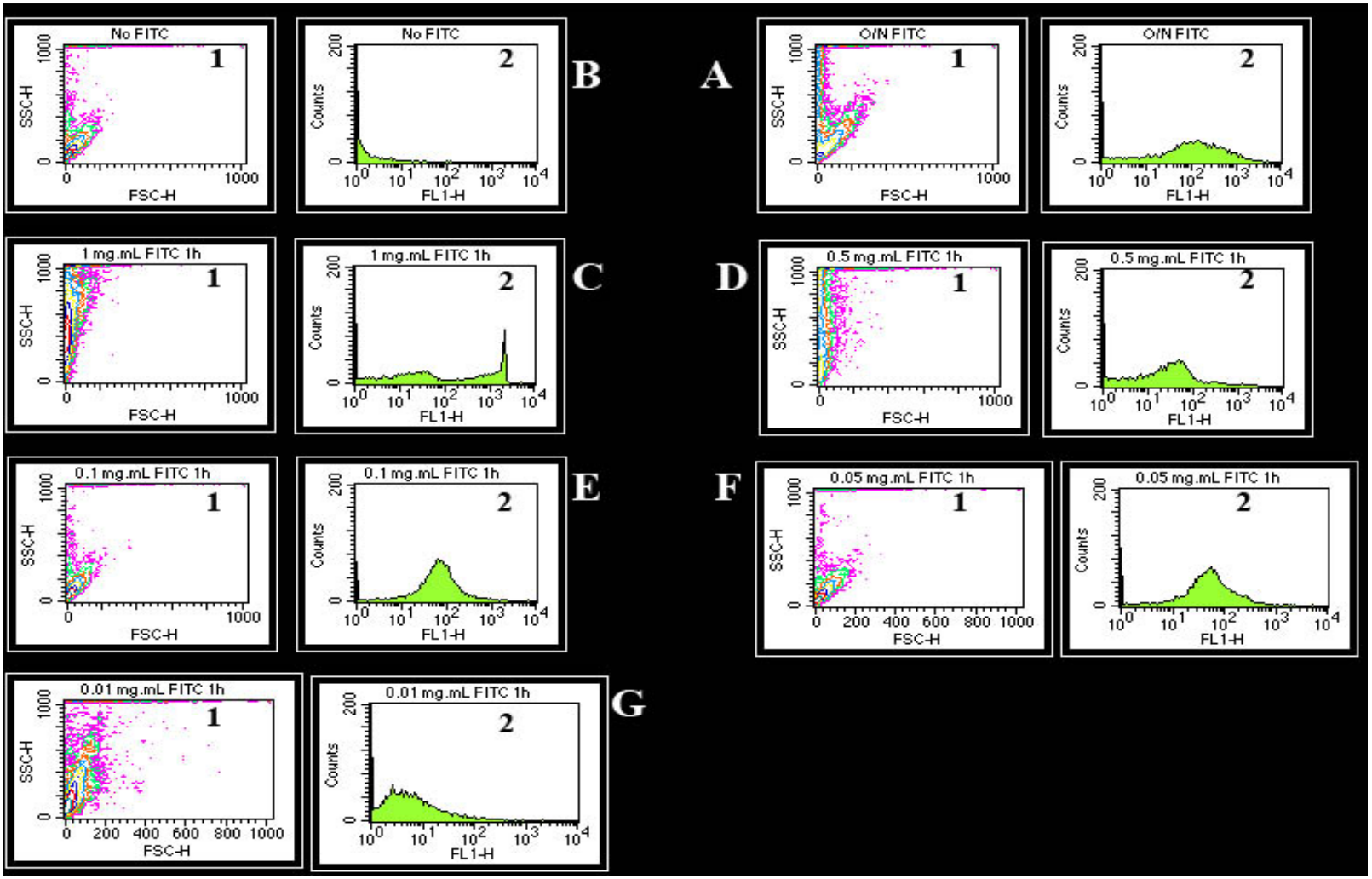

Figure 4-4 Flow cytometry data after staining *V. anguillarum* with FITC. (A): 12h incubation with 0.05 mg.mL^{-1} FITC; (B to G): 1 h incubation with 0.01 to 1 mg.mL^{-1} FITC. (1): FSC/SSC plots of the bacteria; (2) FL1 histogram of the fluorescence intensity.

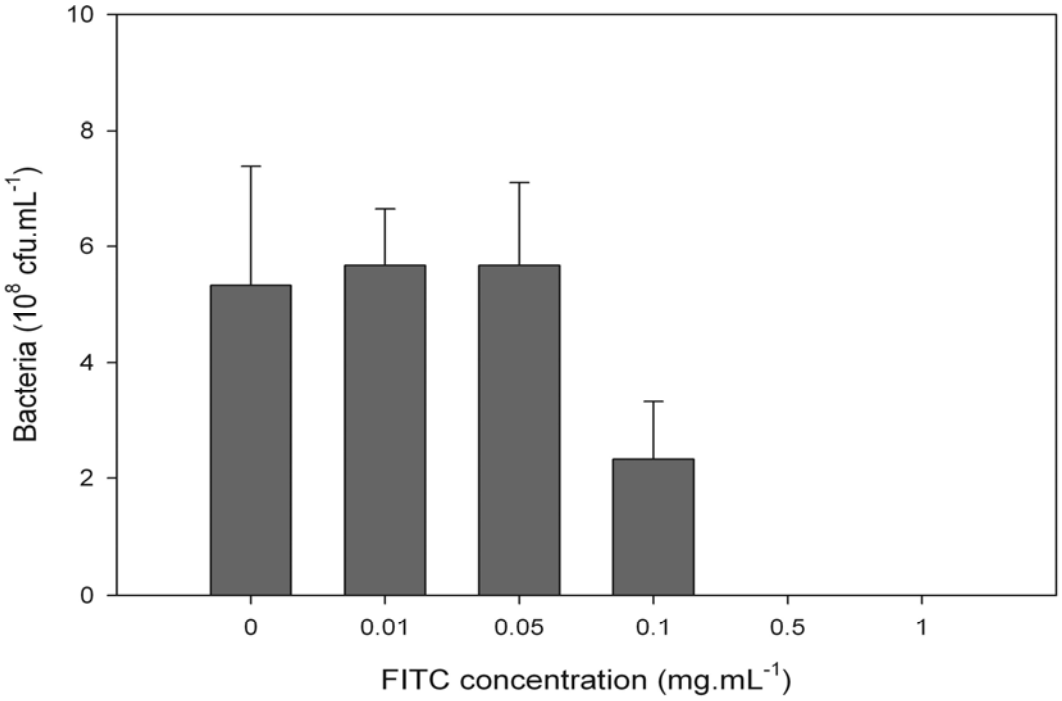

Figure 4-5 Survival of *V. anguillarum* after incubation in different concentrations of FITC for 1 h.

Concentrations of FITC over 0.05 mg.mL^{-1} for 1 h were shown to have a detrimental effect on the survival of the bacteria; below this concentration, no survival differences were seen between the control (no FITC) and the stained bacteria.

With respect to the intensity of the staining (Figure 4-4, A-2 to G-2), the distribution of the bacteria (Figure 4-4, A-1 to G-1) and survival of the bacteria (Figure 4-5), the optimum concentration of FITC for staining *V. anguillarum* was found to be 0.05 mg.mL^{-1} for 1 h on an orbital shaker.

The effect of storing the bacteria at -70°C, prior to use, was investigated regarding the retention of FITC stain. From the results shown in Figure 4-6, the intensity of the fluorescence appeared to be higher and the peak narrower in the thawed frozen samples (A to C), which could be due to a lower uptake of the stain by the isolates which were not frozen (D to F).

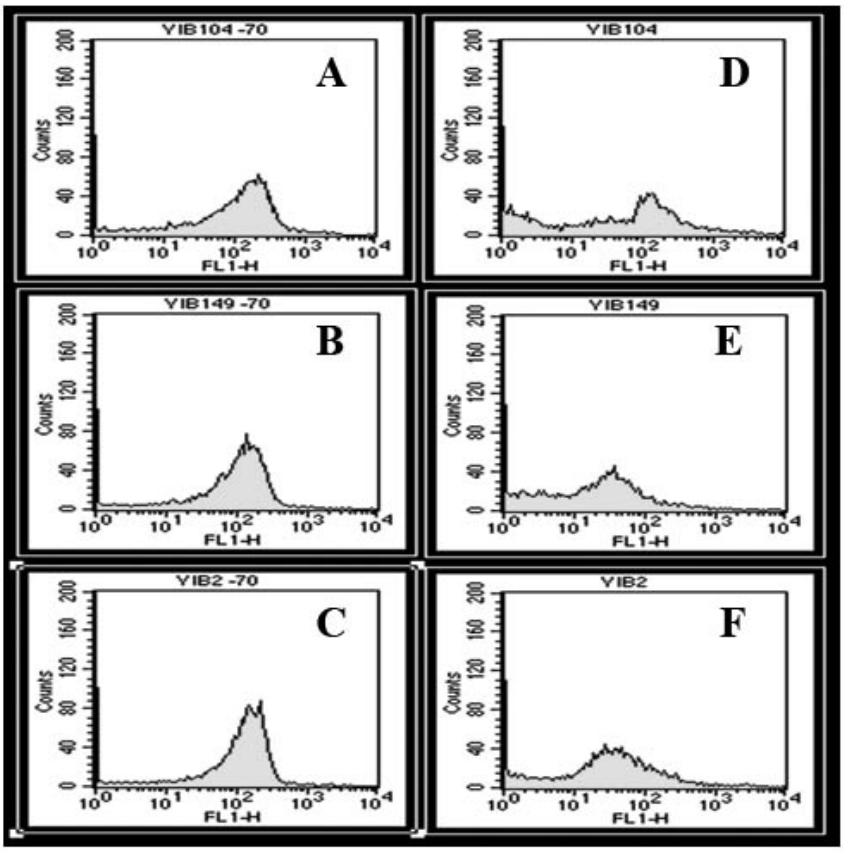

Figure 4-6 Flow cytometry data (FL1 histogram of the fluorescence intensity) of FITC stained *V. anguillarum* at 0.05 mg.mL^{-1} for 1 h. Stored bacteria overnight at -70°C (A to C); same bacteria stained on the day (D to F).

All together, freezing the stained bacteria at -70°C did not seem to interfere with the intensity of the FITC stain on the bacteria.

4.3.1.2. Phagocytosis inhibition

Sodium azide and paraformaldehyde were used to kill the leukocytes and were found to be efficient at killing the phagocytes but paraformaldehyde caused the cells to auto-fluoresce and could therefore not be used. Sodium azide changed the morphology of the leukocytes, making it harder to gate them and compare the results with live cells.

Colchicine and BDM were both found to block phagocytosis without changing cell morphology, as observed from the FSC/SSC dot plot and they did not cause any auto-fluorescence of cells (data not shown). BDM used at a concentration of 38.3 mM for a minimum of 60 min at 12°C

was found to be more efficient at blocking phagocytosis than colchicine (data not shown) and was chosen as the blocking agent.

4.3.1.3. Phagocytosis assay

Examination of cod leukocytes under light microscopy after 2 h phagocytosis revealed that a small proportion of the macrophage and neutrophil populations had internalised bacteria (data not shown). Within each positive cell, an average of 4 to 5 bacteria, with up to 10 bacteria, could be clearly seen within the cytoplasm (Figure 4-7).

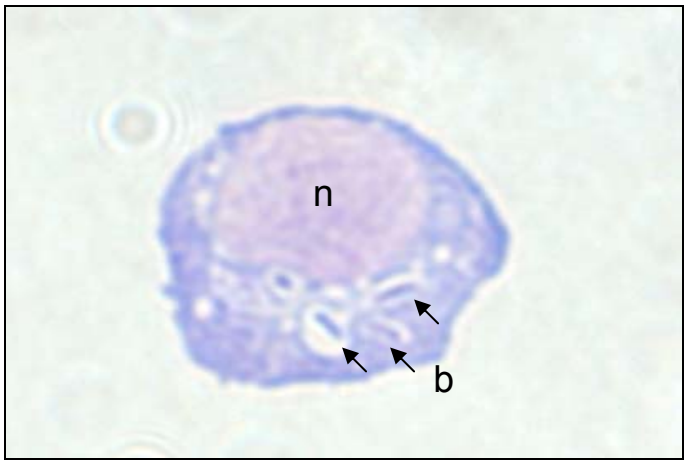

Figure 4-7 Light microscopy of *V. anguillarum* (VIB149) bacteria engulfed in the cytoplasm of a cod macrophage (magnification x1000). n: nucleus; b: bacteria (arrows).

The phagocytosis values obtained by FACS analysis for each isolate were significantly higher than that obtained for the leukocytes incubated without bacteria, with the exception of VIB1. It can be seen from the inter-quartile range in Figure 4-8, that the variation within the data set for each isolate was very high. This was also the case with the standard deviation (sd); for example, for VIB2, the phagocytosis level was 7.34 % ± 8.46 (mean ± 2x sd).

The lowest level of phagocytosis was obtained with an O1 serotype isolate, VIB1, with 0.1% of the cells having phagocytosed bacteria. The highest level of phagocytosis was found with an O2a serotype isolate, VIB2, with 7.3% of the cells having phagocytosed bacteria after 2 h incubation.

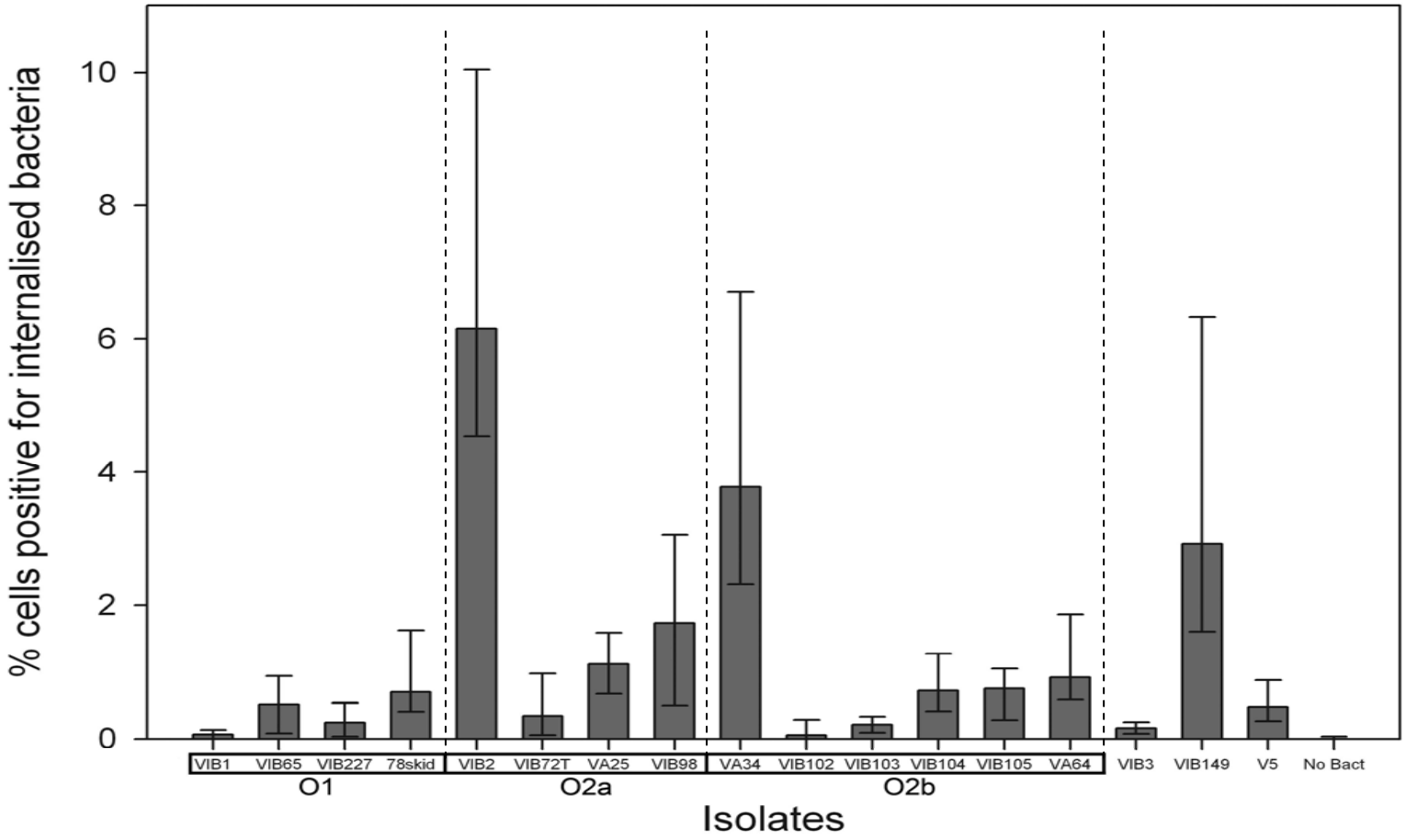

Figure 4-8 Phagocytosis of different strains of *V. anguillarum* and *V. ordalii* (V5) by cod phagocytic leukocytes after 2h incubation at 12°C.

Each serotype was analysed separately since the purpose of the assay was to investigate the ability of the different isolates within a serotype to be phagocytosed by cod leukocytes.

A significant difference was found ($p<0.001$) between the phagocytosis of the strains of the O1 serotype and the pair-wise comparison showed that the level of phagocytosis of VIB1 was significantly different from that of VIB65 and 78skid but not VIB227, and that the phagocytosis of 78skid was different from that of VIB1 and VIB227 but not VIB65.

The difference between the phagocytosis of the O2a serotype strains was also significant, and the pair-wise analysis revealed that only the phagocytosis level of cod leukocytes towards VIB98 and VA25 was not significantly different from each other. The average phagocytosis of VIB2 was 3 times greater than that seen with VIB98, and approximately 15 fold greater than phagocytosis observed with VIB72T.

A significant difference was found in the level of phagocytosis within serotype O2b, isolate VA34 level of phagocytosis was significantly higher than all the other O2b strains (mean phagocytosis of 5.3%). The mean value of phagocytosis obtained with the other isolates in this group ranged from 0.3 % with VIB103 and 1.2% with VA64, with the phagocytosis of isolates VIB102 and VIB103 not significantly different to each other, and a third group composed of isolates VIB104, VIB105 and VA64, had an intermediate level of phagocytosis, not significantly different from each others.

When statistical analysis was carried out between each of the serotypes as groups, significant differences were found (normality test passed) between the groups represented in Figure 4-9. The fact that O3, non-pathogenic *V. anguillarum* (Non-Patho) and *V. ordalii* groups are only represented by 1 isolate diminishes largely the relevance of the statistical analysis. The non pathogenic isolate to rainbow trout, VIB149 (Non-Patho group) was the highest phagocytosed

bacteria, with 4.8% of the cells showing phagocytosis, followed by O2a with 2.9%; the minimum was found for the VIB3 (O3) isolate with only 0.2% of the cells containing phagocytosed bacteria. The Holm-Sidak test performed following GLM model analysis of this data revealed that the phagocytosis of the Non-Patho group and the sub-serotype O2a were significantly higher than that of all the other groups.

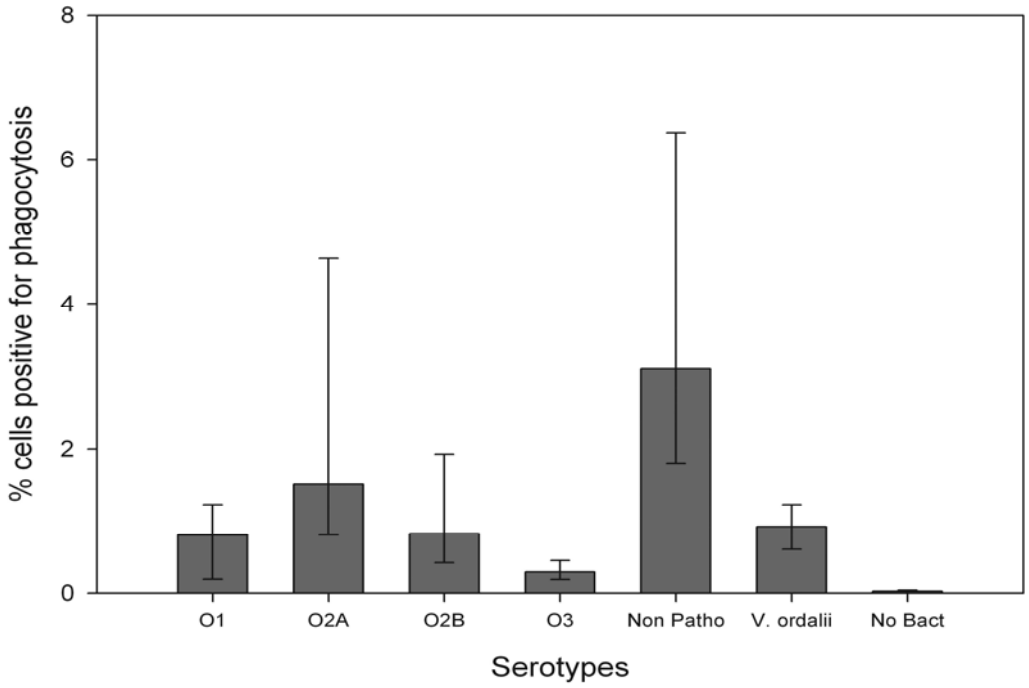

Figure 4-9 Phagocytosis of different serotypes of *V. anguillarum* and *V. ordalii* by cod phagocytic leukocytes after 2h incubation at 12°C.

4.3.2. Respiratory burst assay

The data collected for the respiratory burst assay were transformed using Equation 4-1 and analysed in the same manner as for the phagocytosis assay. Figure 4-10 represents the median of the respiratory burst activity of cod leukocytes with the inter-quartile intervals presented.

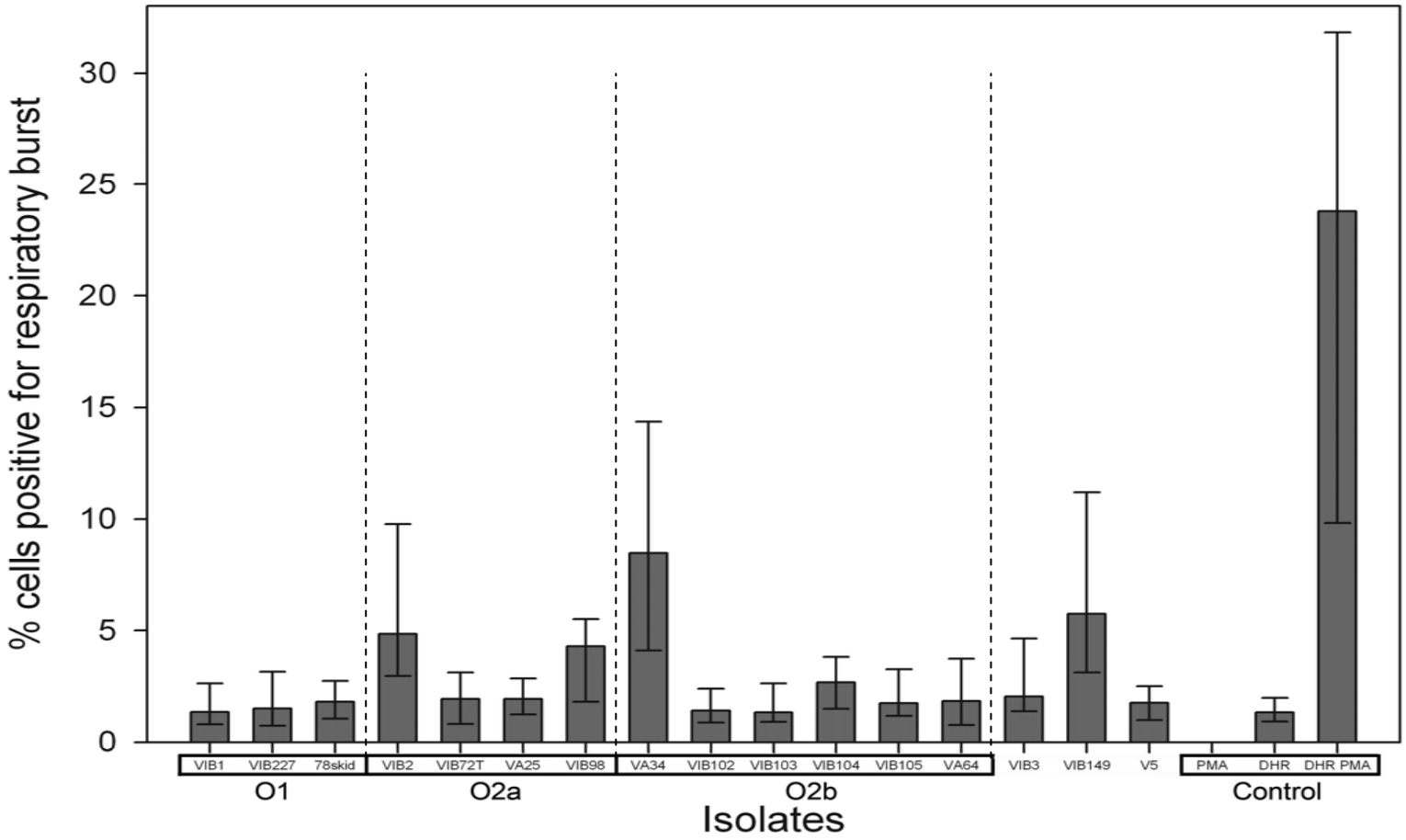

Figure 4-10. Respiratory burst of cod leukocytes after 2h incubation at 12°C with different isolates of *V. anguillarum* and *V. ordalii* (V5).

Only a few strains of *V. anguillarum* had a significantly higher level of respiratory burst activity compared with the background level of auto-respiratory burst, measured in a tube containing leukocytes with DHR but no bacteria. These isolates are VIB2 (O2a), VIB98 (O2a), VA34 (O2b), VIB149 (Non-Patho) and the positive control (no bacteria +DHR +PMA). The cod leukocytes incubated with isolate VA34 had the highest number of cells undergoing respiratory burst with 10.7% of the gated phagocytic cells showing a positive activity. Both isolates VIB2 (O2a) and VIB149 (Non-Patho) activated approximately 7.5% of the total phagocytic cell population. The lowest percentage of cells which were still significantly different from the negative control (DHR only) was with isolate VIB98 (O2a) where only 5.6% of the leukocytes were undergoing respiratory burst. The positive control cells stimulated with PMA contained a mean value of 23.4% of the phagocytic cells undergoing respiratory burst.

There appeared to be two distinct phagocytic populations undergoing respiratory burst (contributing 44 and 56% of the phagocytic population), observed in the positive control for each fish, determined by their difference in fluorescence intensity (Figure 4-11), but the size/granularity dot plot did not allow clear differentiation between these two populations (data not shown).

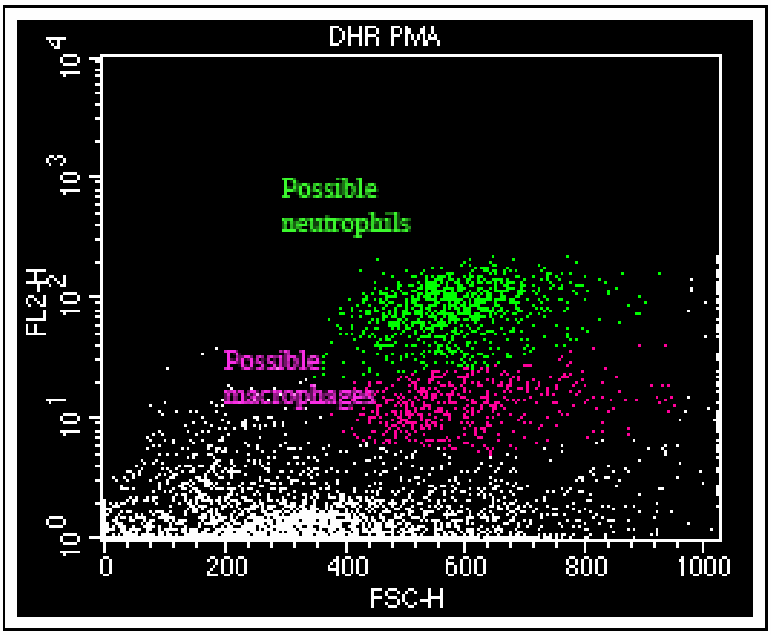

Figure 4-11 Fluorescence intensity (FL2) of cod leukocytes incubated with DHR and PMA against size of the cells (FSC).

4.3.3. Serum killing assay

As shown in Figure 4-12, all the isolates of *V. anguillarum* seem to be resistant to the killing activity of Atlantic cod serum, with both normal or heat-inactivated serum. The highest rate of killing was found for isolate VIB105 (O2b) with only 0.1% of the bacteria dead or injured after 2 h incubation in normal serum diluted 1:10.

Incubation of *Vibrio ordalii*, isolate V5, with normal or heat inactivated serum resulted in 4.9 and 4.5% death, respectively. This result has to be considered carefully and it may have been due to an artefact when gating the different populations, since *V. ordalii* appear to have a different size/granularity to *V. anguillarum* (data not shown).

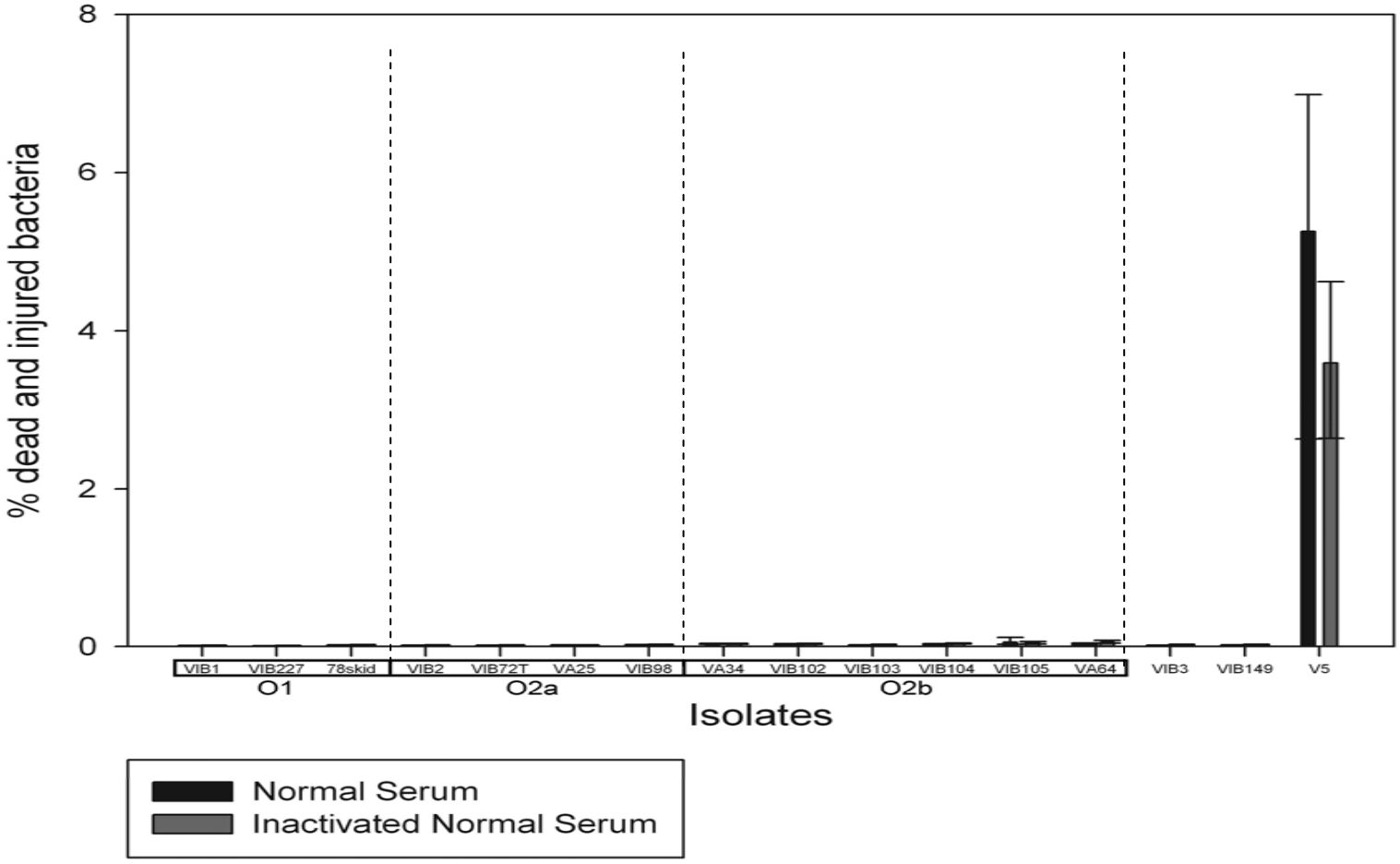

Figure 4-12 Serum killing activity of cod serum (normal and heat-inactivated) after incubation of different strains of *V. anguillarum* and *V. ordalii* (V5) for 2 h at 12°C.

4.4. Discussion

4.4.1. General considerations on the Atlantic cod leukocytes, serum and *V. anguillarum* bacteria

Three assays were developed to examine the interactions between *V. anguillarum* and the immune response of cod *in vitro*. The assays included phagocytosis and respiratory burst by head kidney leukocytes, and a serum killing assay, which were optimised by modifying methods reported in the literature.

Extraction of leukocytes from cod head kidney was performed following the methods of Braun-Nesje *et al*. (1981), Steiro *et al*. (1998) and Sorensen *et al*. (1997). The cod leukocytes could be incubated at 12°C for 24 h using the conditions described in Section 4.2.1 without any major loss of cell viability. Steiro *et al*. (1998) showed that macrophage viability and function, i.e. phagocytosis, respiratory burst or cell attachment, was optimal at 4-6°C and performed less efficiently at 12°C, while Magnadottir (2000) observed the optimal temperature for serum killing with cod serum to be 37°C. In order to perform all the assays at the same temperature, 12°C was chosen specifically as it was the temperature the fish were reared at.

Atlantic cod serum was kept at -20°C for 3 weeks prior to examination of the serum killing capacity on *V. anguillarum*. The haemolytic activity, usually attributed to the alternative complement pathway, has been shown to be unaffected by storage at this temperature (Magnadottir *et al*., 1999a; Magnadottir *et al*., 1999b).

Due to the dynamic relationship between host and pathogen (Drevets and Elliott, 1995), live bacteria should be used in pathogenicity experiments. Therefore, live bacteria were used in this study, however, it was not possible to prepare bacteria and perform the assays on the day of the experiment, bacteria (stained or not) were stored at -70°C prior to use (Trust *et al*., 1981)

and a survival recovery from the frozen stocks of approximately 75% (data not shown) was considered satisfactory.

The state of the bacteria could have affected the outcome of the experiments performed. Storage of the pathogen (e.g. on slopes, subcultures, lyophilisation) can lead to a reduction in the virulence or pathogenicity of the bacterium as well as differential expression of regulatory genes or the loss of plasmids, all of which has been previously documented Yamamoto *et al*., 1993; Pedersen, 1997; Lesne *et al*., 2000; Somerville *et al*., 2002). The isolates used in this study were all laboratory isolates, subcultured many times on TSA+NaCl and this may have lowered the virulence of these isolates compared to when they were first recovered. The use of isolates freshly recovered from outbreaks or passaged several times through fish would have been more relevant but due to the number of isolates tested in this study and the fact that no field sampling was scheduled, this could not be done.

Due to the fact that each bacterial isolate was treated in the same manner within each experiment, the results obtained are considered to be a true reflection of the differences between the various isolates examined at the time of investigation, regarding both their interaction with and evasion mechanisms towards cod leukocytes. This does not mean that the *in vitro* model used here reflects exactly what is happening *in vivo* since the interaction of the various components of the immune system (humoral and cellular) were not assessed together (e.g. leukocytes without serum for the phagocytosis assay and respiratory burst assay, and serum without leukocytes for the bacterial killing assay).

4.4.2. *In vitro* flow cytometry assays

Flow cytometry is extremely useful for processing a large number of samples, but it required a certain amount of expertise to become familiar with its settings, and published protocols had to be adapted for use with Atlantic cod leukocytes.

The bacteria used in the phagocytosis assay needed to be labelled with a fluorochrome (FITC, (Boesen *et al*., 2001) for analysis on the flow cytometer. The FITC staining proved to be a simple method for labelling bacteria, although some researchers have used the insertion of a green fluorescent protein (GFP) gene into the genome of bacteria. In hindsight, GFP labelling of *V. anguillarum* may have been a better alternative than using FITC labelling due to the potential problems associated with this fluorochrome. The FITC covalently binds to amines on the amino acids present on the N terminus of proteins and on lysine residues (Grunwaldt *et al*., 2002). It was shown that this binding reduced the adenylate cyclase toxin activity of *Bordetella pertussis*, and in turn increases the number of bacteria phagocytosed by human neutrophils compared with GFP labelled bacteria (Weingart *et al*., 1999). The authors concluded that since FITC labelling affected the activity of the adenylate cyclase toxin, interference of FITC with activity of other immune proteins could not be ruled out. GFP labelling has been shown to conserve all pathogenic traits of the bacteria (Weingart *et al*., 1999; Chu and Lu, 2008) and also to give a more stable fluorescent signal. Many studies have however successfully used FITC staining of bacteria to investigate host-pathogen interactions. Drevets and Elliott (1995) did not report any differences in the phagocytosis of *Listeria monocytogenes* labelled with carboxy-X-rhodamine (CR), hydrazine derivate of fluorescein (FH) or Lucifer Yellow (LY) by mice macrophages compared to unlabeled bacteria.

The detection of the intensity of fluorescence of a gated population (thought to be phagocytes) was used to quantify the proportion of cod leukocytes which had engulfed fluorescent bacteria. These cells can be recorded positive (intensity above a threshold) either because they have internalised FITC labelled bacteria or the labelled bacteria have attached non-specifically to the surface of the cell. Different methods are available for discriminating between attached and internalised bacteria. Quenching fluorescent bacteria attached to the surface of the macrophage or neutrophils is a very efficient way to overcome this problem, but the bacteria

have to be dead to allow penetration of the quenching agent into the organism (Van Amersfoot and Van Strijp, 1994). The quenching of live bacteria is more problematic, and attempts to do so using Crystal Violet or Trypan Blue gave unsatisfactory results (data not shown), even after permeabilising the membrane of the bacteria with different concentrations of lysozyme. The other method used in this study to overcome this problem was to inhibit phagocytosis with a chemical agent and from this measure the number of cells with only attached bacteria. The level of cells with surface bound bacteria after blocking with BDM was found to be different for each isolate, and so blocking phagocytosis was necessary for each bacterial isolate analysed. It might also have been possible to mark the bacteria attached to the surface of the cells using specific antibodies (Section 3.3.2.1) labelled with a different fluorochrome instead of calculating a negative background. This would have avoided the use of any chemicals on the cells such as BDM which can modify the adherence of the bacteria to the cell surface. Peak levels of phagocytosis were reached within 2h of incubation (data not shown), as found by Esteban *et al*. (1998), with as much as 10 bacteria per macrophage (Figure 4-7).

4.4.2.1. *Vibrio anguillarum* and cellular innate immunity studies

Vibrio anguillarum is a very heterogeneous species of bacteria with differences in biochemical profiles within each serotype (Section 1.2.6.1). The interaction of this bacterium with cod phagocytic cells has been reported, with less phagocytosis occurring with this species compared to other bacteria species as seen with a pathogenic and a mutant strain of *A. salmonicida* (Nikoskelainen *et al*., 2006), but no studies have been published on the different mechanisms used by *V. anguillarum* to resist cod innate immunity.

The phagocytic capacity of cod leukocytes was found to be between 0.1% for isolate VIB1 (serotype O1) to over 7% for VIB2 (O2a serotype). Within serotypes O1, O2a and O2b, cod leukocytes phagocytosed to a higher extent isolates 78skid, VIB2 and VA34 respectively, with the serotype O3 the most resistant to phagocytosis when compared by serotypes (Figure 4-9).

These results are much lower than the levels of phagocytosis found with sea bream leukocytes using *V. anguillarum* serotype O1, where Esteban *et al*. (1998) reported approximately 80% of the leukocyte population had phagocytosed bacteria using flow cytometry. The main differences between these two experiments were the fish species used and Esteban *et al*. (1998) used dead bacteria while live bacteria were used in the present study. The latter could have a large impact on the level of phagocytosis of bacteria because dead bacteria could loose their ability to actively resist this mechanism. However, in pre-experiments using dead bacteria, no significant differences could be seen between the level of phagocytosis by cod leukocytes of live and dead bacteria (data not shown). The isolate VIB2 from O2a serotype was phagocytosed by 7% of the cells gated in Figure 4-9; this represents 28% of the total macrophage and neutrophil population which could undergo respiratory burst (Section 4.4.2.2) having phagocytosed at least one bacteria. This level of phagocytosis by cod macrophages is similar to the results found by Boesen *et al*. (2001), i.e. 25%, using the same serotype of *V. anguillarum* with trout phagocytes after 2 h incubation at a ratio of 5:1 (bacteria:leukocytes). In the present study, a ratio of 20:1 was found optimal (data not shown). If the same calculation is applied to the most phagocytosed bacteria, VIB2, serotype O2a, 7.3% of the total phagocytic population equates to 64% of the macrophages (able to undergo respiratory burst) having phagocytosed a least one bacteria.

The phagocytic ability of cod leukocytes was found to vary between individual fish, individual isolates of *V. anguillarum* and between serotypes. The range of different phagocytic values obtained with individual isolates reflects the wide heterogeneity found among *V. anguillarum* isolates; a hundred times more cells had phagocytosed VIB2 (7.2%) than VIB1 (0.08%).

Only a low percentage of the gated cells underwent respiratory burst after incubation with bacteria (i.e. higher than the auto-respiratory burst level). This level (DHR only in Figure 4-10)

was found here to be approximately 2% of the cells and this has been reported by others (Nikoskelainen *et al*., 2006). This, coupled with the fact that a very low level of respiratory burst was observed for most bacterial strains reduced the power of the analysis of the data. The highest respiratory burst was found when incubating the non pathogenic strain (VIB149) with cod leukocytes (approximately 6% underwent a respiratory burst), which corresponds to 24% of the total macrophage and neutrophil population capable of undergoing respiratory burst (Section 4.4.2.2). The serotype which produced the lowest percentage of cells positive for a respiratory burst was O1, giving a level similar to the negative control (data not shown). Respiratory burst by cod leukocytes has been reported to be very variable between individuals (Sorensen *et al*., 1997), as found here. Some authors have observed the level produced by the addition of PMA to be twice as high in cod leukocytes as in rainbow trout (Steiro *et al*., 1998), but others have shown that this level is generally lower than in other fish species (Nikoskelainen *et al*., 2006). Since the respiratory burst is linked to phagocytosis, it is interesting to compare these results in order to investigate the possible inhibition of the respiratory burst once *V. anguillarum* has been phagocytosed. Boesen *et al*. (2001) showed that *V. anguillarum* was able to block the respiratory burst of rainbow trout leukocytes, also shown for sea bass (Sepulcre *et al*., 2007), which might be the case for cod too. The relationship between the respiratory burst and the phagocytic activity of cod leukocytes is fairly good, with the same isolates of *V. anguillarum* highly phagocytosed also producing a high level of respiratory burst. This implies that a possible inhibition of the respiratory burst by *V. anguillarum* is unlikely since a lower level of respiratory burst would be expected compared to phagocytosis. There was actually a higher respiratory burst level for almost all strains than phagocytosis (i.e. VA34 with 5.2% of the cells positive for phagocytosis and 10.7% of the cells undergoing a respiratory burst). The fact that more cells underwent respiratory burst after incubation with the same number of bacteria than for phagocytosis assay implies either an

under-estimation of phagocytosis level in this assay or that these isolates actively trigger the macrophage and neutrophils to undergo a respiratory burst without prior phagocytosis. The discrepancy noted here could also be due to the preparation of the samples since the bacteria used for the respiratory burst were not stained with FITC and this may have influenced the overall phagocytic capacity of cod leukocytes. The possibility of double staining for measuring the proportion of the population positive for phagocytosis and respiratory burst at the same time (mentioned in Section 4.1.3) could have given more information on the interaction of the pathogen with the cod leukocytes; it would also have reduced the time and increased the accuracy of the assay.

To verify if the respiratory burst was inhibited by live *V. anguillarum*, it would have been useful to incubate the cod leukocytes with both live and heat-inactivated bacteria to establish if any differences in the level of respiratory burst could be detected. Alternatively, triggering respiratory burst with PMA post-phagocytosis would have shown if the phagocytic cells had a reduced ability to undergo respiratory burst (Boesen *et al*., 2001). It would be also very important to assess the killing of the bacteria after phagocytosis and respiratory burst since the bacteria can survive within the phagocytic cell such as seen with *Edwarsiella tarda* (intracellular pathogen, Rao *et al*., 2001).

4.4.2.2. General considerations of the different cell populations

In order to increase the number of cells recorded by the flow cytometry assay using cod leukocytes, a gate was drawn around the cells thought to be phagocytic (Figure 4-2). It is possible to obtain information about the different cell populations undergoing a respiratory burst by flow cytometry. Since only macrophages and neutrophils have the ability to produce reactive oxygen species after triggering this mechanism using PMA, the total proportion of reactive phagocytes within the gated population can be estimated. In this study around 25% of the gated cell population was able to produce a respiratory burst higher than the threshold limit set

(Figure 4-10). Since the population gated was fairly well defined on the SSC/FSC dot plot, it is believed that these cells were only macrophages and neutrophils but an investigation by light microscopy after cell sorting would elucidate if there was any "contamination" of other cell types. Two populations were distinguishable on the flow cytometer after triggering respiratory burst in samples containing only cod leukocytes (Figure 4-11), identified by the difference in respiratory burst intensity. The neutrophil population is known to produce a higher level of free radical species than macrophages (Secombes, 1996). If Atlantic cod neutrophils and macrophages behave the same way, it seems possible to estimate the relative percentage of these two populations using this method. In this study, the neutrophil:macrophage ratio was 56:44, where 62:38 was found by light microscopy observation of head kidney cells (Sorensen *et al*., 1997). If the values found for the ratio of neutrophils and macrophages are related to the total population gated, then these represent respectively 14 and 11% of the gated population (total phagocytes 25%). Sorting the gated cell populations by FACS and then identifying them morphologically under light microscopy would help establish if this assumption is correct.

4.4.2.3. *Vibrio anguillarum* and humoral innate immunity study

Atlantic cod serum bactericidal activity has not been extensively studied despite the importance of this system in fish and the fact that the specific immune response in Atlantic cod is still open to debate (Section 1.3.2.4). Specific antibodies to *V. anguillarum* are not found (or in very low quantities) in cod after infection or vaccination (Section 1.3.2.5), this seems to suggest that the non-specific or non-antibody based defence system is playing a central role in Atlantic cod. The opsonisation capacity of *V. anguillarum* by cod serum, measured by respiratory burst activity, has also been shown to be poorer than with trout serum (Nikoskelainen *et al*., 2006). Magnadottir (2000) reported that *Aeromonas salmonicida* seems to be completely resistant to cod bactericidal activity. This fish pathogen is notoriously resistant to serum killing mostly due to the A layer protein on its surface (Munn *et al*., 1982; Merino *et al*., 1994).

After 2 hours incubation in normal cod serum diluted 1:10, all strains of *V. anguillarum* seemed able to resist membrane lysis by cod serum. This result was surprising in respect to the known heterogeneity of resistance to complement killing within serotypes (Boesen *et al*. 1999). Heat inactivation of serum tends to inhibit bacterial killing, but in this study, inactivation of the serum had no marked effect on its ability to kill the bacterium. This was not unexpected since Magnadottir (2000) showed a very moderate loss of haemolytic activity in cod serum heated at 63°C for 30 min, concluding that the haemolytic activity was partially heat-stable and so might be only relying partially on complement activity. Regarding the dilution, Boesen *et al*. (1999) showed that immunised serum from trout could be diluted up to 1:8192 and still kill 30% of *V. anguillarum* O1, unfortunately data regarding the normal levels of serum killing were not presented in this study. Neat serum from normal rainbow trout was shown to kill some *V. anguillarum* strains (mostly from serotype O1) after 48 h incubation at 20°C (Boesen *et al*., 1999), and maybe in our study cod serum needed to be used neat and incubation with the bacteria for more than 2 h to obtain any bactericidal activity. Complement-mediated killing via the classical pathway (antibody dependant) can be faster acting than the alternative pathway in lysing bacteria (Traub and Kleber, 1976), and since cod might rely only partially (if at all) on specific antibodies, the killing of *V. anguillarum* by cod serum may need longer incubation times to be effective. Nevertheless, Caipang *et al*. (2008) showed that Atlantic cod normal serum (PBS injected fish) did not have an effect on *V. anguillarum* mortality after incubation for 48 h at 15°C in 1:1 bacteria solution:serum. This reinforces the results found here that *V. anguillarum* is resistant to killing by cod serum.

The investigation of serum killing activity of serum using flow cytometry allowed rapid measurement of the bactericidal activity of cod serum towards *V. anguillarum*. This type of assay has the advantage over the classical protocol (cfu enumeration after growth on agar

plates) to be extremely quick, very precise and able to take into account the potential non-culturable bacteria.

Several factors could have influenced the serum assay, firstly the temperature of the assay was set at 12°C, but Magnadottir (2000) reported higher serum haemolysis activity at 37°C. In preliminary experiments conducted in this study, the cod bactericidal activity with normal serum of *V. anguillarum* VIB87 (serotype O1) incubated at 37°C was found to be around 4%, with no differences between normal and heat inactivated serum (data not shown), which is a lot higher than the results found at 12°C presented here. In the same experiment rainbow trout serum was found to kill 70% of the same bacterial isolate at 25°C, with the inactivated trout serum value under 1% (data not shown).

LPS is a very important outer membrane molecule on the surface of *V. anguillarum* and is responsible for triggering a whole cascade of events e.g. cytokine production (Caipang *et al*., 2008), and activation of the complement system (Boshra *et al*., 2006), but they may also play a part in avoidance of phagocytosis (Rittig *et al*., 2003) or serum inhibition (Magnadottir, 2000). It would be very interesting to do the same type of assays as presented here with a reduced amount of LPS on some strains (Boesen *et al*., 1999) or using some LPS deficient mutants to establish the role of this molecule in relation to the avoidance of phagocytosis as well as the respiratory burst or serum killing.

4.4.3. Pathogenicity of *Vibrio anguillarum*

Vibrio anguillarum is a very heterogeneous group of bacteria, and even within each serotype differences are found in their biochemical profiles (Austin *et al*., 1995), genetic make-up (Pedersen and Larsen, 1993) or pathogenicity (Boesen *et al*., 1997). The high heterogeneity of the results in the different assays was expected, with the majority of the strains highly resistant to the different defence mechanisms tested and a few presenting a lower resistance to the cod

cellular innate immune system. This high resistance trend was expected since most of the isolates were from *V. anguillarum* outbreaks in fish and so these strains were expected to be highly virulent (Table 4-1). Regarding the results of the phagocytosis assay, considering the average for each serotype (Figure 4-9), it seems that serotype O3, O2b and O1 would be more susceptible to produce an infection in Atlantic cod since these isolates, on average, were less phagocytosed than O2a. In reality, O2a and O2b subseroytpes are the most pathogenic. Altogether the assays described here allowed an insight into the individual virulence mechanisms between isolates of the same bacteria species, with VA40-78skid (O1), VIB2 (O2a) and VA34 (O2b) the most phagocytosed and VIB1 (O1), VIB72T (O2a) and VIB103 (O2b) the most resistant to phagocytosis by Atlantic cod leukocytes.

The problems encountered in Norway of outbreaks in vaccinated fish (Hellberg *et al*., 2005-2006-2007) and of the different vaccines giving variable protection require a better understanding of what makes an isolate pathogenic and which strains are more prone to produce a disease state in Atlantic cod. *Vibrio anguillarum* is obviously able to bypass the cod immune system defences since O1, O2 and O3 serotypes are able to produce the disease in healthy fish. These evasion mechanisms are not fully understood and the evasion of the phagocytosis as well as the serum resistance (shown in this study) are not the only virulence factors that the pathogenic strain possess but seem to be part of the most important resistance mechanisms.

When presented with a catalogue of sometimes hundreds of possible candidates for use in the development of a vaccine, we believe that the study of some of the factors involved in the pathogenesis *in vitro* through the use of flow cytometry, coupled with a reliable serotyping scheme, provides valuable information for making an educated and scientific decision towards which isolate, within each serotype, should be included in the vaccine.

Chapter 5.

Efficacy of whole cell vaccine in cod against *Vibrio anguillarum*

5.1. Introduction

Vibrio anguillarum was one of the first bacterial diseases of fish to be investigated by bacteriologists, immunologists and vaccinologists, because of its significant impact on salmonids when first cultured. From the late 60's to the end of the 70's, most of the studies related to vibriosis vaccines were published by researchers in the USA (Antipa, 1976; Amend and Fender, 1976; Antipa and Amend, 1977; Croy and Amend, 1977; Gould *et al*., 1979) and Japan (Hayashi *et al*., 1964; Kusuda *et al*., 1978; Nakajima and Chikahata, 1979). The results of these studies showed that heat or formalin-killed preparations of *V. anguillarum* were very efficient in protecting salmonids and ayu from vibriosis.

Commercial vaccines for vibriosis in salmonids generally consist of a mixture of 2 or 3 different serotypes of *V. anguillarum* (O1, O2a and sometimes *V. ordalii*). These serotypes are usually combined with other inactivated fish pathogens in a polyvalent vaccine formulation, and tend to cover the main pathogens associated with heavy losses of fish. Intervet-Schering-Plough, for example, produce a vaccine, Norvax® Minova 6, which protects against 5 different diseases (furunculosis caused by *Aeromonas salmonicida* subsp. *salmonicida*, vibriosis caused by *Vibrio anguillarum*, coldwater vibriosis caused by *Vibrio salmonicida*, cold water ulcer disease caused by *Moritella viscosa* and Infectious Pancreatic Necrosis caused by IPN virus). Many commercially devastating diseases are mostly covered by vaccines, but vaccines protecting against viral diseases are also being produced. Many of the viral vaccines are still at an experimental stage, as shown in Table 5-1 (Biering *et al*., 2005; Hastein *et al*., 2005).

Table 5-1 Selection of bacterial and viral diseases controlled by vaccination.

Bacterial disease	Bacteria
Yersiniosis	*Yersinia ruckeri*
Pasteurellosis	*Photobacterium damselae* subsp *piscicida*
Edwardsiellosis	*Edwardsiella ictaluri*
Streptococcosis	*Streptococcus iniae*
Lactococcosis	*Lactococcus garviae*
Warm water vibriosis	*V. alginoliticus, V. vulnificus, V. parahaemolyticus*
Flavobacteriosis	*Flavobacterium columnare*
Flexibacteriosis	*Tenacibaculum maritimum*
Vibriosis	*V. anguillarum; V. ordalii; V. salmonicida*
Piscirickettsiosis	*Piscirickettsia salmonis*
Furunculosis	*Aeromonas salmonicida* subsp. *salmonicida*
MAS	*Aeromonas hydrophila*
Bacterial kidney diseases (BKD)	*Renibacterium salmoninarum*

Virus	Family
IPNV*	*Birnaviridae*
IHNV*	*Rhabdoviridae*
VHSV	*Rhabdoviridae*
SVCV	*Rhabdoviridae*
SPDV*	*Togaviridae*
SDV	*Togaviridae*
SJNNV	*Nodaviridae*
ISAV*	*Orthomyxoviridae*
KHV	*Herpesviridae*
LCDV	*Iridoviridae*

MAS: Motile Aeromonas Septicaemia; **IPNV**: Infectious Pancreatic Necrotic Virus; **IHNV**: Infectious Haematopoietic Necrosis Virus; **VHSV**: Viral Hemorrhagic Septicaemia Virus; **SVCV**: Spring Viraemia Carp Virus; **SPDV**: Salmon Pancreas Disease Virus; **SDV**: Sleeping Disease Virus; **SJNNV**: Stripped Jack Nervous Necrosis Virus; **ISAV**: Infectious Salmon Anaemia Virus; **KHV**: Koi Herpes Virus; **LCDV**: Lymphocystis Disease Virus. * indicates commercial viral vaccine available.

Vibrio vaccines are available commercially for many fish species such as rainbow trout, sea bass, sea bream, barramundi (*Lates calcarifer*), tilapia (*Tilapia spp*), Japanese amberjack (*Seriola quinqueradiata*), turbot, yellowtail amberjack (*Seriola dumerili* and *S. lalandei*), white trevally (*Pseudocaranx dentex*) and channel catfish (*Ictalurus punctatus*) (Hastein *et al*., 2005).

Atlantic cod can be infected by *V. anguillarum*, principally O2a and O2b serotypes, the vaccines developed to protect salmonids and used for Atlantic cod lacked the second sub-serotype, leaving Atlantic cod vulnerable to infection by this group of bacteria (Section 1.4.2). A very important difference between Atlantic cod and salmon in their vaccination strategy is that the salmonid first life cycle occurs in fresh water, where they are vaccinated against *V. anguillarum* and where infection very rarely occurs. For Atlantic cod, from the time they are only a few grams, they can encounter *V. anguillarum* and the vaccination has to happen at a lot earlier stage. Nowadays, both Pharmaq and Intervet-Schering-Plough, arguably the two leading fish vaccine manufacturers, have developed a *Vibrio* vaccine formulation specifically for this species, under the names Alpha Marine® and Norvax Vibriose Marine®, respectively. These formulations include *Vibrio* isolates belonging to serotypes O1 and O2a, but also O2b.

Norway is the largest aquaculture producer of cod in the world (FAO stats, 2006), as well as the largest supplier of injectable oil-based fish vaccines (Gravningen and Berntsen, 2007). Numerous outbreaks of vibriosis in vaccinated Atlantic cod have been reported from this country (Hellberg *et al*., 2005-2006-2007; Samuelsen *et al*., 2006), despite the systematic vaccination strategy implemented at 1 and 5 g (dip vaccination) and again at 30 g (i.p.vaccination). A possibly new serotype of *V. anguillarum*, isolated from cod in Norway, (referred to as O2c in this study) has been shown to be genetically and immunologically different to O2a and O2b isolates and, as yet, has not been included in any commercial vaccines (Mikkelsen *et al*., 2007). The authors showed that the protection of the Pharmaq commercial vaccine was fairly poor against this (O2c) serotype isolate (RPS 32%), and thus could explain why outbreaks of vibriosis are still occurring in commercially-reared vaccinated cod, even if the number of outbreaks due to this particular serotype is low.

Numerous studies comparing the different serotypes of *V. anguillarum* suggested that representative isolates from all the relevant sub-groups should be included in future *Vibrio* vaccines (Airdrie *et al*., 1989; Tiainen *et al*., 1997a; Mikkelsen *et al*., 2004), to protect cod against as broad a range of relevant *V. anguillarum* serotypes as possible. It should be kept in mind that the grouping of isolates into different serotypes is based on the use of mammalian antibodies (mouse mAbs and rabbit pAbs), and there is no indication that fish antibodies are able to differentiate between the different serotypes in the same way as mammalian antibodies, i.e. mAbs might reveal differences that may not be recognised by fish (Espelid *et al*., 1991; Mutharia *et al*., 1993; Jakobsen *et al*., 1999).

As mentioned in Section 1.4.1, several different types of vaccines are under development for aquaculture, and the “new technology” vaccines, such as DNA or subunits vaccines, offer the greatest choice of formulation since single molecules (or combinations of these) can be

injected, fine-tuning the immune response for a particular type of response (Rogan and Babiuk, 2005). These have been developed as an alternative to the "traditional" approach of vaccine development (i.e. inactivated pathogens) where the latter had been ineffective. Nonetheless, the simplest and most cost effective way of making a commercial vaccine, is to incorporate (after inactivation) all the representative and relevant isolates of a pathogen family belonging to different serotypes. Since inactivated whole cell vaccines have been shown to offer good protection against *V. anguillarum* in many species of fish (Sakai, 1999), this approach was used in the formulation of a vaccine to protect Atlantic cod from the bacterium.

Autologous vaccines, also called "emergency vaccines" or autogenous vaccines are tailored for use at a particular farm by including some (or all) of the isolates recovered from this farm in the vaccine. The licensing regulations for this type of vaccine are fairly minimal (The Veterinary Medicines Regulations 2007, Nº 2539); and they have been found to be extremely effective but protection against other isolates of the same species can be "hit or miss" (Gudmundsdottir and Gudmunsdottir, 1997). This type of vaccine has been used for Atlantic cod since the commercial vaccines have been awarding incomplete protection, but a product offering a broad spectrum of protection is a commercial necessity for the cod industry.

The aim of this chapter was to investigate the protection elicited by a multivalent whole cell inactivated vaccine against *V. anguillarum* in Atlantic cod. From the analysis performed in Chapter 3, four groups of *V. anguillarum* O2 serotype were identified and one isolate from each of these was included in the vaccine; an isolate from serotype O1 was also included, see Section 5.4.1. As mentioned previously, the immune system of Atlantic cod appears different from other fish species, mostly in its inability to produce measurable specific antibodies to *V. anguillarum*. Keeping this point in mind, we took the approach of trying to identify specific isolates from serotypes O1, O2a and O2b which were phagocytosed by cod leukocytes at a

higher level than other isolates within the same serotype. Since only a very small proportion of an injected antigen is involved in the immune response (Nossal, 1995), the presentation of this antigen is considered to be a limiting factor for the immune response (Paglia and Colombo, 2002). Maximising antigen uptake and resulting antigen presentation has been shown to increase the immune response (antibody production), with this type of approach well characterised when adjuvants are used in the vaccine formulation (Dupuis *et al*., 1998). In order to increase the antigen uptake of the different components of the vaccine, the *V. anguillarum* isolates resulting in the highest number of positive phagocytic cod leukocytes and therefore also resulted in higher proportion of phagocytic cells undergoing respiratory burst, were selected. The capacity of cod serum to kill the different selected isolates of *V. anguillarum* could not be used since all isolates were found resistant. These assays were carried out in order to include isolates which theoretically offered the highest level of antigen presentation and subsequently protection within each serotype. The choice of which isolate to include from sub-serotypes O2c and O2d was made randomly due to the fact that their existence was unknown prior to performing the pathogenesis studies carried out in Chapter 4.

5.2. Materials and methods

All the infection experiments took place at the Marine Environmental Research Laboratory (MERL), Machrihanish, Argyll, Scotland. This facility was supplied with natural seawater, the temperature of which was maintained at 10°C. The average salinity of the water was 33 ppt and the light regime used was 12:12 (L:D).

5.2.1. Determination of challenge-dose

The challenge-dose experiment was carried out in order to find the optimal dose of *V. anguillarum* necessary to achieve a level of mortality in unvaccinated fish of approximately 60%.

5.2.1.1. *Vibrio anguillarum* challenge isolates

The *Vibrio anguillarum* isolates used in the challenge-dose experiment were chosen from serotype O2 strains recovered from Atlantic cod *Vibrio* outbreaks (Table 2-1) which included the sub-serotypes O2b (isolate VIB102b), O2c (isolate 04.09.412) and O2d (isolate B01103 5-4). These isolates were different to the isolates used in the vaccine (heterologous design).

5.2.1.2. Pre-challenge protocol

The 3 isolates of *V. anguillarum* selected as challenge strains were grown in two sterile 1 L bottles containing 750 mL each of TSB+NaCl and incubated for 48h with agitation (Section 2.1.2). Two concentrations of bacteria (Table 5-2) were used to estimate, after measuring the OD of the solution, the optimal lethal dose with 60% mortality (LD_{60}), using a waterborne exposure protocol established at MERL (10^4 cfu.mL^{-1}) as a guideline.

Six groups of 6 unvaccinated Atlantic cod, average weight 270 g, were placed in 6 tanks (1.36 m diameter, 450 L) housed in the Isolation Unit (IU) of MERL. The volume in each tank was lowered to 100 L and the water flow stopped. Two doses were tested for each challenge isolate by pouring 500 mL and 1 L of each of the 3 bacterial suspensions in the respective tanks (Table 5-2) and the fish exposed to the bacteria for an hour. Oxygenation was increased and the O_2 concentration monitored every 10 min during this time. The water flow was then restored and the mortality recorded twice daily until 2 consecutive days without any mortality was recorded.

Table 5-2 Concentration of *V. anguillarum* isolates used in the challenge-dose experiment.

Serotype	Isolate	Bacterial load (cfu.mL^{-1})
O2b	VIB102b	Dose 1: 3.60 x10^6 Dose 2: 1.80 x10^6
O2c	04.09.412	Dose 1: 7.40 x10^6 Dose 2: 3.70 x10^6
O2d	B01103 5-4	Dose 1: 8.90 x10^6 Dose 2: 4.45 x10^6

A swab of the head kidney of two moribund fish per tank was streaked onto TSA+NaCl plates (Appendix 1.1) and incubated at 22°C to confirm the presence of *V. anguillarum*. After 48 h, colonies resembling those of *V. anguillarum* (Section 1.2.6.1) were subcultured and identified by Gram staining, Vibriocidal test 0/129 and API20E (Section 2.1.4), as well as polyclonal antibody agglutination test (Bionor MonoVa, following the manufacturers' instructions) and ELISA (Section 2.4) using the mAbs described in Section 3.3.2.1. Head kidney and spleen were also collected from the same fish and stored in 4% buffered formalin.

5.2.2. Vaccination

5.2.2.1. Vaccination isolates

One isolate from each of the five serotypes deemed as important constituents to the vaccine (O1, O2a, O2b, O2c and O2d) were selected from the results obtained in Chapter 4. The isolates used for the vaccination as well as their origin are summarised in Table 5-3.

Table 5-3 *Vibrio anguillarum* isolates used in the vaccine preparation.

Serotype	Isolate name	Origin of the isolate	Concentration (cfu.mL^{-1})
O1	VA40-78skid	*Salmo salar*, S-P UK	4.36 x10^{9}
O2a	VIB2-1173/1	*Gadus morhua*, HWU	4.23 x10^{9}
O2b	VA34	*Gadus morhua*, NVI	4.60 x10^{9}
O2c	06.09.28	*Gadus morhua*, NVI	4.29 x10^{9}
O2d	07.09.297	*Gadus morhua*, NVI	4.46 x10^{9}

NVI: National Veterinary Institute, Section for Fish Health, Dr D. Colquhoun, Oslo, Norway; **HWU**: Heriot Watt University, Dr Dawn Austin, Edinburgh, United Kingdom; **S-P UK**: Intervet-Schering-Plough, Prof P. Smith, Saffron Walden, United Kingdom.

5.2.2.2. Vaccination protocol

Vaccine preparation

The bacteria were grown in 2 x 50 mL of TSB+NaCl (Section 2.1.2) from the original bacterial slopes. After 24 h, the bacteria were collected, the supernatant kept and the bacteria formalin-killed as described in Section 2.1.6. Both tubes were combined and the optical density of each isolate adjusted to a final concentration in the vaccine of approximately 4.5 x10^{9} cfu.mL^{-1}. The isolates were centrifuged at 5525 x g for 30 min and the pellet resuspended in 5 mL of the

previously collected supernatant, after sterilising it using 0.22 μm filters. The bacteria were then mixed all together (1:1:1:1:1), making 25 mL of vaccine. The concentration of each isolate used in the vaccine can be found in Table 5-3. No adjuvant was used for this study and the vaccine was stored at 4°C for 2 weeks.

Vaccination of Atlantic cod

Three hundred cod (average weight 269.9 g) were injected intraperitoneally with 100 μL of either the vaccine (200 fish) or sterile PBS (100 fish). The fish were anaesthetized in 50 ppm solution of MS222 (Sigma-Aldrich) in sea water until the fish were observed unresponsive, vaccinated IP and weighed. The vaccinated fish were then separated into 2 tanks labelled V1 and V2 (100 fish per tank, 2 m diameter, 1500 L) and the PBS vaccinated fish were placed in a third tank, labelled C (100 fish).

5.2.3. Challenge

Seven weeks post-vaccination, the three groups of fish (2 vaccinated and 1 control) were experimentally challenged with the bacteria. The fish were transferred before infection from their on-growing tanks to the IU and divided randomly and equally in 3 tanks per group (9 x 450 L, 30 fish per tank, 1.36 m diameter).

The bacteria recovered from the pre-challenge were grown in three 2 L bottles containing 1.5 L each of TSB+NaCl and incubated for 72h with agitation (Section 2.1.2). The optical density of the bacterial suspension was measured just prior to the challenge.

The bath-challenge procedure of vaccinated fish was the same as that carried out in the pre-challenge experiment (Section 5.2.1.2), with the only difference being the number of fish used per tank and the number of tanks used. The challenge dose used was chosen from the results of the challenge-dose experiment to reach an LD_{60} in the control group. Briefly, the water in

each tank was lowered to 100 L, the water flow stopped and the oxygen monitored. Five hundred mL of the bacterial solution prepared from the different isolates was poured into the corresponding tanks (Table 5-4) and after 1 h, the water flow was restored.

Table 5-4 Experimental design for the bath-challenge of vaccinated cod with *V. anguillarum* O2b, O2c and O2d isolates.

Challenge strain	Bacterial load (cfu.mL^{-1})	Group	Fish number	Tank N°
O2b	1.25 x10^6	V1	30	2
		V2	31	9
		C	28	7
O2c	3.45 x10^6	V1	30	1
		V2	30	5
		C	28	3
O2d	1.8 x10^6	V1	29	4
		V2	30	6
		C	27	8

The fish mortality was recorded for 2 weeks, at which point the challenge was terminated.

Kidney swabs were taken from fish that died during the challenge. They were plated on TSA+NaCl and kept at 22°C until colonies had grown. The colonies were then analysed by agglutination test (BioNor) and ELISA (Section 2.4) using the mAbs developed in Chapter 3.

At the end of the challenge period, 10 fish were collected from each of the tanks and their infection status examined by taking a swab from the spleen of each fish and inoculating on one half of a TSA+NaCl plate (i.e. 90 samples in total). The plates were incubated at 22°C for 48 h. An average of 3 different bacterial colony types could be observed on each plate (around 250 different colonies). Those resembling *V. anguillarum* colonies (Section 1.2.6.1) were screened by Gram staining (Section 2.1.4.2) and the bacteria similar to *V. anguillarum* were grown in the presence of Vibriocidal 0/129 discs (Section 2.1.4.3). The isolates, which were selected after this test, were grown overnight in TSB+NaCl (Section 2.1.2) and analysed by ELISA (Section 2.4), as described previously.

5.2.4. Analysis of the mortality data

The Relative Percent Survival (RPS, Amend, 1981) was calculated according to Equation 5-1.

Equation 5-1 Formula of the Relative Percent Survival

$$RPS = \left(1 - \frac{\%\ \text{mortality in vaccinated fish}}{\%\ \text{mortality in control fish}}\right) X\ 100$$

The statistical differences in mortality between vaccinated and control groups were determined running a one-tailed Fisher's Exact Test. The test was considered significant if the p value was less than 0.05.

5.3. Results

5.3.1. Determination of challenge-dose (pre-challenge)

The mortality of cod recorded following a bath challenge with *V. anguillarum* after 2 weeks is summarised in Table 5-5.

Table 5-5 Percentage mortality recorded after pre-challenge using different doses of three different *V. anguillarum* isolates.

Serotype	Isolate	Bacterial load (cfu.mL^{-1})	Mortality %
O2b	VIB102b	3.6 x10^6	83% (5/6)
		1.8 x10^6	66% (4/6)
O2c	04.09.412	7.4 x10^6	33% (2/6)
		3.7 x10^6	33% (2/6)
O2d	B01103 5-4	8.9 x10^6	83% (5/6)
		4.45 x10^6	66% (4/6)

All the isolates recovered from the dead fish after pre-challenge were identified as *V. anguillarum*, except for one from Tank 15 (O2c, low dose), which did not agglutinate using the BioNor anti-*Vibrio anguillarum* polyclonal antibody. The reaction of the mAbs in ELISA revealed that the isolates recovered from both challenge dose from the O2b and O2d groups belonged to the correct subserotype.

One identified isolate from each of the serotypes tested (O2b, O2c and O2d), recovered from the pre-challenge experiment was selected to be used in the challenge post-vaccination.

The lower dose of bacterium for serotype O2b and O2d (VIB102b at 1.8 $x10^6$ $cfu.mL^{-1}$ and B01103 5-4 at 4.45 $x10^6$ $cfu.mL^{-1}$) gave satisfactory levels of mortality (i.e. 4 out of 6 fish died, 66%) in the pre-challenge experiment and these concentrations were therefore selected as the challenge dose for future challenges. Only 33% of fish died with the highest dose of the serotype O2c isolate 04.09.412 when used at 7.4 $x10^6$ $cfu.mL^{-1}$, and therefore a higher dose was deemed necessary to produce a higher level of mortality.

5.3.2. Vaccinated Atlantic cod bath challenged with *V. anguillarum*

The levels of mortality obtained in vaccinated fish 2 weeks after challenging them with *V. anguillarum* are shown in Table 5-6.

Table 5-6 Levels of mortality in vaccinated Atlantic cod after bath-challenge with *V. anguillarum*.

Serotype	Group	Mortality %	RPS
O2b	Vaccinated Control	0% (0/61) 14.3% (4/28)	100%
O2c	Vaccinated Control	0% (0/60) 0% (0/28)	0%
O2d	Vaccinated Control	1.7% (1/59) 48.1% (13/27)	96.4%

The mortality data from both vaccinated tanks were combined.
Mortality (number of dead fish/total number of vaccinated fish), 2 fish died in the O2c vaccinated group but their death could not be attributed to vibriosis and so do not appear in this table.

Isolates recovered from the dead or moribund fish sampled during the challenge period (5 fish from Tank 8 (O2d control), 3 from Tank 7 (O2b control) and 1 fish from Tank 5 (O2c vaccinated)) were all identified as *V. anguillarum* (by the BioNor agglutination test) except for the isolate obtained from Tank 5 (O2c) which showed no agglutination with the BioNor test. All the other isolates (5 recovered from Tank 8 (O2d) and 3 from Tank 7 (O2b)) were identified as the expected serotypes of either O2b or O2d. The dynamics of the challenge mortality in the

control tanks can be seen in Figure 5-1. No mortalities occurred in control fish infected with the O2c isolate (Tank 3). The mortalities in the control fish infected with serotype O2b (Tank 7) started after 5 days and did not seem to plateau by the end of the experiment (2 fish dying on Day 14). The mortality in the control fish infected with O2d (Tank 8) was regularly increasing until the experiment was stopped on Day 15.

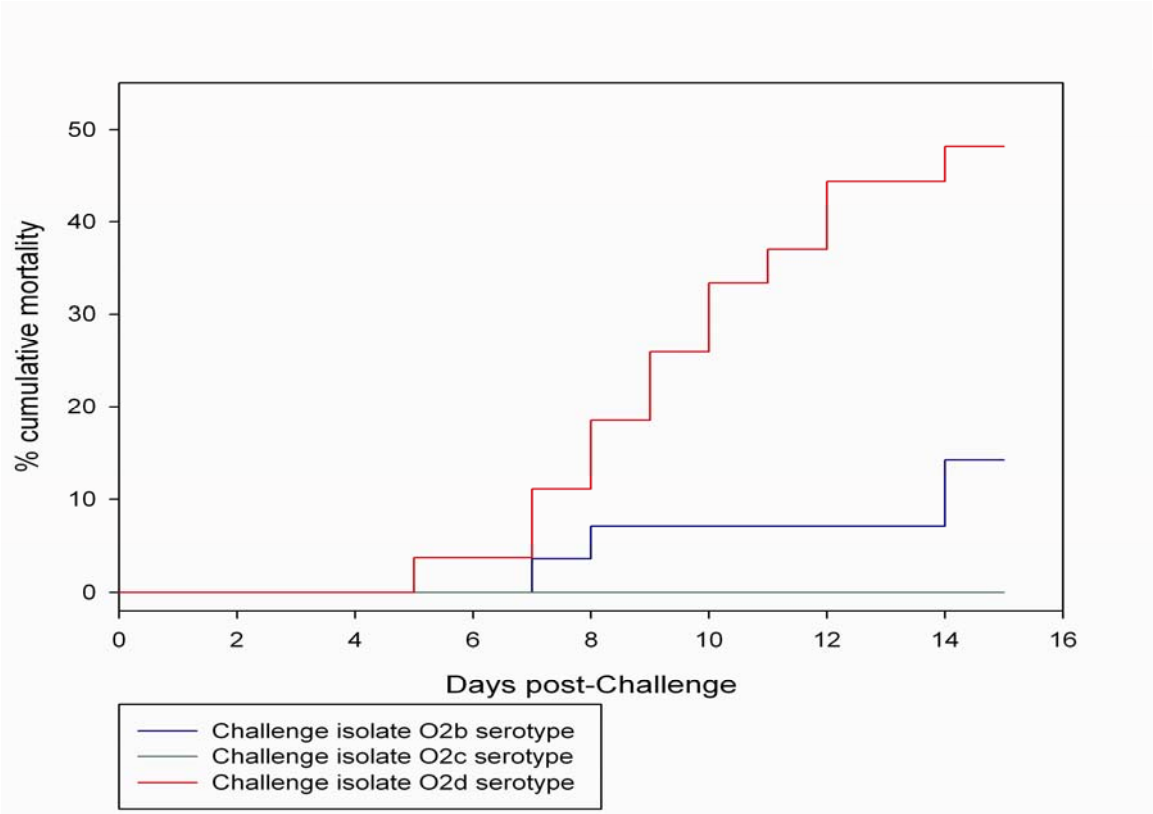

Figure 5-1 Percent cumulative mortality in control tanks of fish challenged with O2b, O2c and O2d *V. anguillarum* sub-serotypes.

Ten fish per tank were sampled at the end of the experiment to establish if they were infected with the bacterium. Of the 250 bacterial isolates recovered from the spleen samples, only 5 bacteria were found to react positively with the mAbs used. Two isolates recovered from Tank 7 (challenged with serotype O2b) were grouped with the sub-serotype O2b (as expected) and 3 isolates recovered from Tank 8 (challenged with serotype O2d) presented an O2d reaction profile. Both these tanks were control tanks with mortality still occurring when the challenge had

to be terminated (Figure 5-1). No *V. anguillarum* were recovered from any of the vaccinated fish.

The level of infected fish at the end of the experiment was extrapolated to the total population of fish in the tanks and since no mortality in the vaccinated groups occurred as a consequence of an infection by *V. anguillarum*, the Figure 5-2 only represents the control groups. The prevalence of infection established in Tank 7 was 20% and 33% in Tank 8, this means that 4 fish were infected but not dead at the end of the experiment in Tank 7 (20% of 24 fish live) and 4 fish were infected in Tank 8 (33% of 14 fish live).

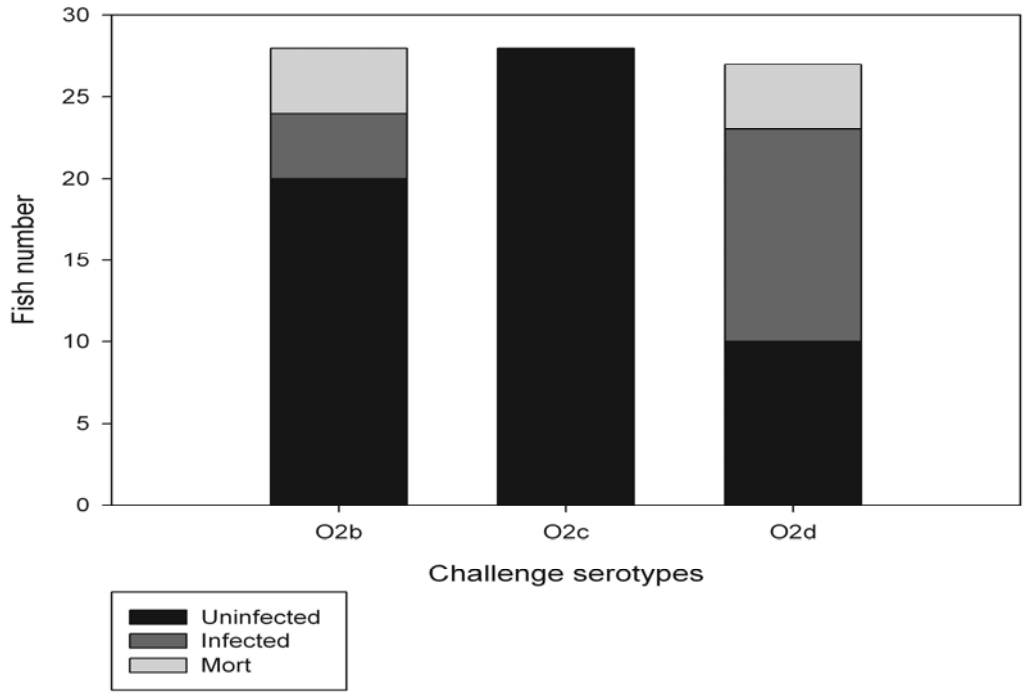

Figure 5-2 Cumulative mort, infected and uninfected control fish after challenge with different serotypes of *V. anguillarum*.

There was a significant difference between the number of mort in the vaccinated and control tanks for the O2b ($p=0.0084$) and the O2d ($p<0.0001$) serotypes (Table 5-6).

5.4. Discussion

5.4.1. Experimental design and vaccine formulation

The formalin-inactivated *Vibrio anguillarum* vaccine used in this study was designed to include 4 sub-serotype isolates from O2 (O2a, O2b, O2c and O2d) as well as an O1 isolate. Serotype O1 is shown to be pathogenic to some marine species, but outbreaks in Atlantic cod are rare (Larsen *et al*., 1994). Toranzo *et al*. (1997) suggested that it was not necessary to include this strain in the vaccine; nevertheless the O1 serotype was included in the vaccine because it was designed to be potentially used in other fish species. The possibility of a shift in the prevalence of a particular serotype to another one in disease outbreaks is also a possibility (Section 6.2.3).

It was decided to use a water-based vaccine preparation, not containing any adjuvant. It has been shown that the short term protection of Atlantic cod after bath-vaccination was not improved by the addition of an adjuvant (Mikkelsen *et al*., 2004). Oil adjuvants have been used in cod and were shown to be well tolerated, although adverse reactions to adjuvants have been observed in other bony fish, such as the development of adhesion (Samuelsen *et al*., 2006; Schroder *et al*., 2006a). These side effects are considered acceptable when the benefits of vaccination are taken into consideration (Gravningen *et al*., 2005a).

In the vaccine preparation, the supernatant from the bacterial preparation following centrifugation was used to resuspend the bacteria. This was done so as to also include the ECP secreted by the bacteria during its growth, as this may contain protective antigens.

It has been shown that vaccinating and challenging fish by the same route (IP) can temporarily protect them against infection, through development of local immunity at the site of injection (Nordmo and Ramstad, 1997), possibly masking the effect of any antigen-specific protection. It is recommended that the experimental infection should be performed using a natural route of infection (Griffin, 2002). The fish were vaccinated via IP injection and challenged through bath

exposure. The pathogen is therefore exposed to the full repertoire of immune mechanisms (gut, skin and mucus), which might be bypassed by injecting the bacteria straight into the peritoneal cavity of the fish. Nevertheless, the European Pharmacopoeia regulation requires that experimental challenge be administered by injection for vaccine licensing tests (Midtlyng, 2005).

There was a difference in the virulence between the different isolates of *V. anguillarum* used to challenge the fish. Serotype O2b and O2d were found to be highly virulent, while the isolate used from serotype O2c was found to be less virulent since 4 times more bacteria was required to achieve only half the number of mortalities in the pre-challenge, compared with the other two serotypes (i.e. 33% Vs 66%) and no mortality occurred in the actual challenge of the vaccinated fish. This finding reflects the results of Mikkelsen *et al*. (2007), where isolates of the O2c serotype appeared to be less virulent to cod than the classical O2a and O2b, with only 50 to 75% mortality occurring after bath-challenge at a high dose (10^6 cfu.mL^{-1}).

The concentrations of bacteria used to infect adult Atlantic cod in this study were fairly similar (1.8 to 8.9 x10^6 cfu.mL^{-1}) compared to those generally used by other researchers. Concentrations of bacteria added to the tank during the bath-challenge in other studies usually ranged from 5 x10^5 to 5 x10^7 cfu.mL^{-1} (Gilberg and Mikkelsen, 1998; Pedersen *et al*., 2004; Samuelsen and Bergh, 2004; Vik-Mo *et al*., 2005; Schroder *et al*., 2006a), with fairly variable mortality rates reported, especially since different *V. anguillarum* isolates were used. Generally, the mortality levels in the control tanks were over 75%.

In the current study, the size of the fish could also have had an influence on the outcome of the challenge since in most studies, the fish vaccinated are around 20 g. The temperature at which the challenge took place was fairly low (10°C) and since outbreaks in the wild tend to happen

during summer, when the sea temperature is rising (1.2.4), this could have participated to the low mortalities observed.

The doses used to challenge the vaccinated fish in this study did not achieve the target level of mortality in the control groups of 66%, as obtained in the pre-challenge response experiment. The final mortality levels obtained in the control fish in the vaccine trial were 14.3, 0 and 48.1% after infection with O2b, O2c and O2d, respectively. The recommended levels of mortality for vaccine studies is over 50% (Griffin, 2002). The lower levels of mortality obtained compared to the pre-challenge response trial were probably due to the lower level of bacteria used (i.e. O2c, 3.45×10^6 vs. 7.4×10^6, respectively). There were practical reasons for the difference in bacterial dose used for the two experiments. Firstly the assumption was made that different volumes of broth would sustain the same growth rate of *V. anguillarum* (750 mL for the pre-challenge and 1.5 L for the vaccination challenge). The absorbance of the bacterial suspension was made at Machrihanish, just prior to the challenge. The MERL is a 4 h drive away from the Institute of Aquaculture and time constraints did not allow postponing the challenge once it was realised that the level of bacteria was lower than the pre-challenge dose. The scaling up of the fish numbers from the pre-challenge to the vaccination-challenge experiments (from 6 to 30 fish per tank), could also explain the low mortality in the control groups since the number of bacteria available per fish was reduced by a factor of 5. Maybe the challenge could have been carried out with the exact same protocol as the challenge-dose experiment, bathing fish in groups of 6 and then transferring them into another tank.

Variability in the level of infection has also been reported by other researchers when bath-challenge has been used. This method of infection, although artificial, mimics a more natural route; however the IP route of infection is generally far more reproducible. Schroder *et al*. (2006a) reported a 77% level of mortality in their pre-challenged fish by bath administration with

an O2b isolate (5 x10^5 cfu.mL^{-1}), but levels of mortalities of 18 and 40% in duplicate tanks and up to 89% in another experiment, all using the same challenge protocol. Mikkelsen *et al*. (2007) found inconsistencies in their challenge experiments, with mortalities of 75% obtained with a dose of 9.3 x10^6 cfu.mL^{-1}, while 83% mortalities were obtained with a dose 10 times lower. Corripio-Miyar *et al*. (2007) reported a similar problem when challenging haddock (*Melanogrammus aeglefinus* L.) with *V. anguillarum* O2 serotype by bath. The mortality recorded reached 60% in the pre-challenge and only 28% in the experimental challenge.

The isolates used to challenge the vaccination fish (O2b, O2c and O2d) were re-isolated from fish that died during the pre-challenge experiment. On reflection, these isolates should have been the same bacteria that were used in the pre-challenge experiment and not the ones recovered from the pre-challenge experiment. An error could have been made during the recovery of the isolates, especially regarding the O2c isolate since it does not react with any of the mAbs used in this study and could only be confirmed as *V. anguillarum* by using the BioNor agglutination test.

The number of bacteria recovered post vaccination (at the end of the experiment) was fairly high and it is possible that another disease occurred during this experiment. No identification of these other bacteria was carried out but the fish had to be transported from the challenge facilities (Machrihanish) to the laboratory (Stirling university) on ice and this transport took 10 hours, which could explain why the number of bacteria isolated was high.

The two fish that died in the vaccinated group challenged with serotype O2c (Tank 5) were not thought to be infected with *V. anguillarum*. The first of the two mortalities occurred the day of the challenge and could be stress related. No *V. anguillarum* could be recovered from the second dead fish in this tank (BioNor negative) and so it seems that the challenge of this group of fish failed. No vaccine safety testing (toxicity of the vaccine formulation), by injecting twice

the dose of the vaccine in 10 fish, was done and it would be very important to do so in order to prove that the death of these two fish was not due to the vaccine itself. Nevertheless, this possibility seems remote since no fish died for the 6 weeks post vaccination.

5.4.2. Vaccine protection

Excellent protection was achieved in two of the three groups challenged with *V. anguillarum* at 12 weeks post vaccination. The vaccine was very efficient in protecting against heterologous isolates of serotypes O2b and the O2d, with RPS values of 100% and 96.4%, respectively. The proportion of fish dying in the O2d serotype control group, at almost 50%, was statistically different to the mortality in fish vaccinated, while the low mortality in the O2b group (only 4 fish out of 28) was also shown to be statistically different from the vaccinated group. These results suggest that the vaccine formulation may protect Atlantic cod against isolates of *V. anguillarum* not included in the vaccine formulation. The fact that no *V. anguillarum* were recovered from the vaccinated fish indicates that the vaccine formulated allowed the fish to clear the pathogen. However, the vaccination study should be repeated on a larger scale to confirm this. It was shown that some fish (i.e. 20 and 33% of the control fish challenged with serotypes O2b and O2d, respectively) were infected with the bacteria but did not die or develop signs of the disease. The protection elicited by the vaccine against O2c is still uncertain and the experiment would need to be repeated. A more thorough investigation of its virulence (challenge-dose) in cod should also be performed. In addition, as well as the development of characterisation tools (mAbs) to help screen or detect this serotype, it is important to evaluate the protection of the vaccine against more isolates from each serotype (O2b, O2c and O2d) and also against O2a and maybe O1, to prove that the vaccine elicits broad-spectrum protection, as suggested from the results of this study.

Experiments in our laboratory have shown that the specific antibody response to vaccination or infection could not be detected by ELISA (data not shown) and thus no antibody levels were measured post-vaccination.

5.4.3. Measuring the protection elicited in Atlantic cod

In the search for an effective vaccine, achieving protection against all of the different strains of a pathogen, the vaccinologist is faced with a number of questions: (I) how to make an antigen immunogenic, or select the most antigenic strain, (II) how to induce the right type of response in the fish, eliciting good protection for an extended period of time and (III) how to determine the effectiveness of a pilot vaccine (Secombes, 2008).

Regarding the design of *V. anguillarum* vaccines, the lipopolysaccharide layer of Gram negative bacteria has been shown to elicit a strong immunological response and is specific for the different *V. anguillarum* serotypes of the species (Grisez, 1997). So, as long as all the representative strains of the pathogen are included in a polyvalent vaccine, the fish should be protected to the infection. Nevertheless, the injection of purified LPS as antigen would increase drastically the production cost of vaccines and currently immunisation of fish with whole cells is generally very effective (5.4.2). The question of how to specifically tailor a vaccine to stimulate a particular immune response in the fish, such as a strong and long-lasting memory T cell response (Salerno-Goncalves and Sztein, 2006), is not easy to answer since cellular immune responses in fish are still being unravelled. One way to increase the potential effectiveness of vaccines is related to measuring the fish response to vaccination. Experimental infections of hundreds of vaccinated fish (if not thousands) is the common method for testing whether a vaccine is protective, but such trials are becoming increasingly expensive, generate limited information (Secombes, 2008), as well as challenge the replacement, reduction and refinement of animal use in scientific experiments (Home Office Act 1986). Another common way of

measuring the relative effect of a vaccine is to measure the antibody response of the fish after vaccination, which can be done fairly easily and in a non-lethal sampling but the relationship between antibody levels and protection has to be verified (Reitan and Secombes, 1997). Unfortunately, direct detection of anti-*V. anguillarum* IgM by ELISA in vaccinated or infected fish is not a reliable method for cod (Bricknell *et al*., 2006).

In order to investigate the effect of vaccination in Atlantic cod, other components of the immune system can be measured which may give an indication of the reaction of the cod immune system to vaccination. Since cod seems to be protected against *V. anguillarum* by vaccination for almost a year (44 weeks, Lund *et al*., 2007), a particular type of specific (and long-lasting) immune response has to be present in the vaccinated animals. A possible explanation is that Atlantic cod do in fact produce specific antibodies which are not detectable by ELISA (Dr Tony Ellis, personal communication). Opsonophagocytosis, the increase in phagocytosis after incubation with immune sera, of a particular pathogen has been shown to increase post-vaccination (Verho *et al*., 2005). This indirect measurement of antibodies in cod could help determine the effectiveness of a vaccine formulation, even if preliminary experiments in our lab did not shown any increase in the phagocytic activity of cod leukocytes after incubation with immune sera. The expression of the signalling molecules such as cytokines could also give indications of the effectiveness of the vaccination (Secombes, 2008). Other components of the immune system of cod could also explain the increased resistance to challenge such as acute phase proteins or antibacterial activity (G-type lysozyme, BPI/LBP and Transferin genes and serum killing of *V. anguillarum*). Both these factors, as well as several cytokines (IL-1β and IL-8), apolipoprotein and natural cytokine cell receptor protein genes have been shown to increase shortly after vaccinating fish (Caipang *et al*., 2008). Nevertheless, most of the genes previously mentioned are part of the acute phase response and are thought to be involved in early and not long term protection. More work should be conducted to determine why Atlantic

cod remain protected against *V. anguillarum* weeks or months after vaccination, when most of the acute phase proteins should have reduced back to pre-vaccination levels and so should offer no more protection than before the vaccination.

Research on Atlantic cod B and T cells is under way by several laboratories and it seems that, at the moment, there is no plausible explanation for the lack of specific immune response after vaccination or infection. Hopefully, through the advancement of cell characterisation and the understanding of the molecular mechanisms involved in the specific immunity of Atlantic cod, a working hypothesis can be developed. This would give great insights into the general understanding of the immune system of cod and possibly help design more effective vaccines.

Chapter 6.

General Discussion

6.1. Atlantic cod Aquaculture

Norway was the first country in the world to farm Atlantic cod and, at the moment, is the only country where cod aquaculture is well established. In 2006, there were 213 active cod farming concessions (sites) in Norway, with a total of 500 licenced sites and production of 100,000 T authorised by the government. The actual production was an estimated 12,000 T in 2007 (Hellberg *et al*., 2005-2006-2007), which makes an average of a little over 50 T per farm per year.

Cod aquaculture in Norway seems to be flourishing under the governmental motto "Go for Cod". At the moment, the vast majority of the licences are exploited by a few companies with the 18 largest owning over 200 licences, and 120 companies having a single licence. The three major companies are Spon Fish (capacity of 22,000 T), Marine Harvest (20,000 T) and Codfarmers (15,000 T) (www.Worldfishing.net, Norway, 2007). The estimated production of the cod aquaculture industry is estimated at around 100,000 T by 2010-2015 (Rosenlund and Skretting, 2006), with potential to quadruple this amount.

The total aquaculture industry in Scotland was estimated to be worth £400 million in 2006. This comprised £382 million for farmed salmon, approximately £10 million for rainbow, brown and sea trout, £6 million for shellfish and approximately £2 million for halibut and cod (www.scotland.gov.uk). The cod industry produced 543 T (and 233 T of halibut), accounting for 0.3% of the total aquatic farming value.

The bankruptcy of the Shetland based Johnson Seafood, No Catch operation, in February 2008 saw the end of industrial production of Atlantic cod in the UK, after only 4 years of production. No Catch, producing "organic" cod, was selling their fish in 13 different countries through major supermarket retailers and was planning to supply 10% of the British market by 2010 with an estimated production of 30,000 T. Thus, today, aquaculture production of Atlantic cod in the UK is zero, but this does not mean that the Norwegian model cannot be successfully implemented, especially in Scotland since the environment, infrastructure (based on salmon farms) and aquaculture companies such as Marine Harvest are all present. At the moment, the UK is importing over 90% of the cod consumed, approximately 300,000 T (www.seafish.org), which leaves scope for future development.

6.2. Atlantic cod vaccines and *Vibrio anguillarum* serotypes

6.2.1. Vaccines and Atlantic cod vibriosis

Disease is the major hindrance to the cod industry, and *Vibrio anguillarum* is still considered the most important bacterial disease affecting cod (Section 1.1).

Virtually all Atlantic cod produced in Norway are vaccinated with the most up-to-date vaccine available (composed of *V. anguillarum* O2a and O2b isolates), but as mentioned in Section 5.1, outbreaks of vibriosis are still occurring on farms, illustrating that the current commercial vaccines used, need to be improved.

The general trend in *V. anguillarum* outbreaks in vaccinated cod from 2003 to 2007 has shown the number of infections due to *V. anguillarum* O2a sub-serotype to have decreased in proportion to outbreaks caused by O2b (Section 1.2.6.3 and Table 6-1). *Vibrio anguillarum* has been responsible for 30 reported cases of disease outbreaks from 19 farm sites in Norway in 2006. The identification of a new variant of the O2a sub-serotype was also reported (Schroder

et al., 2006b) and a comprehensive study published the following year (Mikkelsen *et al*., 2007). Retrospectively, it was found that this new variant (called O2c in this work) was identified in samples dating from 2001 and may have been responsible for some of the outbreaks recorded since then. The proportion of O2c outbreaks seems to be increasing rapidly, but since this sub-serotype has only been identified very recently, this might not represent a true trend.

Table 6-1 Summary of the number of *V. anguillarum* outbreaks identified in Norway (Hellberg *et al*., 2005-2006-2007).

	Year				
Serotype	2003	2004	2005	2006	2007
O1	Not found	Not found	Not found	Not found	Not found
O2a	6	9	1	5	5
O2b	11	18	17	15	15
O2c				3	6
Total farms investigated	19	27	18	19	19

Some isolates have not been serotyped and some had multiple serotypes present.

Thus, the number of outbreaks reported in Norway by the National Veterinary Institute (Hellberg *et al*., 2005-2006-2007), shows very clearly that vaccines still need to be improved. Reformulation of the vaccine used appears to be the most appropriate way to address this issue. Therefore, the first part of this study investigated the classification of *V. anguillarum* with respect to identifying the different *V. anguillarum* serotypes responsible for disease outbreaks in the cod industry.

6.2.2. Monoclonal antibodies

A set of monoclonal antibodies were developed during this study to identify the different serotypes of *V. anguillarum* responsible for disease outbreaks in Atlantic cod as well as in other fish species (Chapter 3). After selection of O1 (V.angO1 2.3), O2a (V.angO2a mab3), O2b (V.angO2b 6.3) and O2a-b (V.angO2a-b 9.2) specific mAbs, the entire *V. anguillarum* collection, shown in Table 2-1, was screened using these antibodies. A new sub-serotype belonging to the O2 group was identified and referred to as O2d, which reacted with the O2

specific mAb but not with the O2a or O2b sub-serotype specific mAbs. Further analysis of *V. anguillarum* isolates from Norway (courtesy of Dr D. Colquhoun), revealed that (I) the O2c sub-serotype did not appear to react with any of the mAbs developed in this study, suggesting it was a completely different serotype (II) the serotype of some of the isolates previously identified did not match with the results of this study (i.e. the serotypes of some isolates had been misclassified), and (III) the O2d sub-serotype was not restricted to Scottish waters, but was also present in Norway.

These findings show that the set of *V. anguillarum* specific mAbs developed in this study are useful tools for identifying isolates recovered from outbreaks to a sub-serotype level, or for use in studies of virulence or pathogenicity. After presumptive identification of *V. anguillarum* by Gram staining and the 0/129 test, isolates could be screened with commercially available polyclonal antibodies specific for *V. anguillarum* (Mono-Va, BioNor) and the set of mAbs developed here to confirm the identity and the serotype of the *V. anguillarum* associated with a disease outbreak. The results expected for the corresponding serotypes are summarised in Table 6-2.

Table 6-2 Presumptive results of diagnostic test for serotyping *V. anguillarum* recovered from Atlantic cod.

	Isolate serotype				
Antibody	O1	O2a	O2b	O2c	O2d
BioNor (Mono-Va)	+	+	+	+	+
O1 specific mAb	+	-	-	-	-
O2a specific mAb	-	+	-	-	-
O2b specific mAb	-	-	+	-	-
O2a-b specific mAb	-	+	+	-	+

6.2.3. Evolution of the *V. anguillarum* serotypes affecting cod

The use of vaccination has profound effects on the pathogen population being targeted. For example, it might mean that these *V. anguillarum* strains (included in the vaccine), may disappear from the *V. anguillarum* population or at least decrease in infection due to a

decrease in number. This may lead to accelerated pathogen evolution (Martcheva *et al*., 2008), sometimes referred to as immune escape, with the result that more pathogenic strains may evolve in the environment, as was shown for Marek's disease in poultry (Gimeno, 2008). The increase of other pathogenic species may also occur following vaccination. Such a phenomenon has been reported when *Staphylococcus aureus* infection was found to be increasing in human ear otitis cases after vaccination against *Streptococcus pneumoniae* (Bogaert *et al*., 2004). The possible adaptation of the pathogen, to avoid detection by the immune system, is also possible, for example antigenic drift observed with the influenza virus (Boni, 2008).

The evolutionary pressure of vaccination (Day *et al*., 2008) can also lead to changes not only within the pathogen, as described above, but within the pathogen population, with a rise of new serotypes not included in the vaccine. This phenomenon, reported in mathematical and epidemiological models as well as field observations, is described as "vaccine-induced pathogen strain replacement" (Martcheva *et al*., 2008). It explains the emergence and subsequent dominance of a once-rare pathogen strain induced by vaccination (or chemotherapy) which has a differential effectiveness towards different pathogenic strains. This theory is relatively new and has been applied mostly to humans pathogens, primarily for pneumococcal diseases such as *Haemophilus influenza* and *Streptococcus pneumoniae* (Carrat and Flahault, 2007; Martcheva *et al*., 2008). The reasons for the rise of the non-vaccine strains are numerous and complex, but can be mainly attributed to competition for the niche; the dominant strain previously dominating this niche, is removed from the environment through vaccination, enabling the sub-dominant strain to emerge.

This phenomenon does not appear to have been described for fish pathogens or aquaculture systems, but logically it can be assumed that it occurs. Some examples from the infection of

Atlantic cod with *V. anguillarum* seem to "fit" fairly well with this theory. As mentioned previously, isolates recovered from outbreaks of vibriosis in the early 2000's in farmed cod belonged mostly to the O2a serotype, but with the successful vaccination of Atlantic cod using vaccines designed for salmonids (containing O2a but not O2b), a rise in the number of outbreaks of serotype O2b was observed and is now considered to be the main pathogenic sub-serotype in this fish species. Recently, serotype O2b was included in the vaccine for cod but it now appears that two "new" sub-serotypes (O2c and O2d) have been recovered in Norway and Scotland.

Vaccine-induced pathogen strain replacement should be kept in mind when designing vaccines and the possibility of making the situation worse could be an outcome by allowing a more virulent (but less infectious) strain to establish itself in the host, a phenomenon described as scrambling competition in ecology (De Jong and Bouma, 2001). The vaccinologist has to be aware that this is a possibility and that even the removal of a particular pathogen could allow another species to establish itself. This may be what has been happening with *Francisella philomiragia* subsp. *noatunensis* and *Vibrio ordalii* in Norway in recent years, which are increasing in prevalence (Hellberg *et al*., 2005-2006-2007). Nevertheless several other factors are most probably also involved in the emergence of these pathogens.

6.3. Vaccine formulation

For the reasons described above, it is important to include all the serotypes (if feasible) responsible for outbreaks in a vaccine, even the ones that are not as significant regarding the number of cases, thereby increasing the breadth of the vaccine. Including antigens shared between these serotypes, targeting vulnerable features common to all strains (Martcheva *et al*., 2008), is also important. This is why a recommendation was made here to leave the O1 serotype in the vaccine, as it is known to be pathogenic to Atlantic cod, and maybe also include

the O3 and O5 serotypes which have been isolated in sporadic outbreaks (Larsen *et al*., 1994). This would provide the cod aquaculture industry with a vaccine that is not only able to respond to the threats experienced at the moment, but hopefully protect the cod population in aquaculture for extended periods without the need to update the vaccine after serotype replacement occurs.

The first part of the vaccine design was achieved by understanding which serotypes to include in the vaccine (Chapter 3), in order to elicit protection in Atlantic cod against a wide range of strains, with representative isolates from each of the serotypes.

In the present study, the inactivated whole cell approach was taken because bacterin vaccines against Gram-negative bacteria generally give very good protection (Section 1.4.1).

As described in Section 1.4.1, the identification of protective antigens in bacteria is not an easy task. Selection of a particular strain from hundreds of isolates is equally difficult. As reviewed in Curtis (2002), Grandi (2001) and Bramwell and Perrie (2005), the search for virulence factors is currently normally accomplished using genetic techniques, rather than LD_{50}-type experiments. The latter leads to limited information, even if the data from surviving animals are extremely useful to get insights on protective antigens. In the present study (Chapter 4), the same approach was taken by trying to identify the level of virulence of *V. anguillarum in vitro*; but with the difference that the physiological response to the pathogenic bacteria was observed in Atlantic cod leukocytes, instead of molecular markers. This was achieved by measuring the resistance of different isolates against components of the immune response of Atlantic cod *in vitro* using flow cytometry.

The selection of vaccine candidates from several bacterial isolates within each serotype was performed by determining which strains were more virulent than others. For this, the relative degree of resistance of the killing by complement, phagocytosis and the respiratory burst

activity was investigated. The presence of such virulence mechanisms would enable the pathogen to have a higher chance of surviving in the fish (Wilson *et al*., 2002). Thus, if strains could be identified that were better at resisting the immune defences, the chances of these having virulence factors that could be protective, as well as immunogenic, would be higher than in less immunogenic/virulent strains (Curtis, 2002).

All the isolates tested in Chapter 4 were found to be resistant to complement-mediated serum killing; therefore it was not possible to select isolates on the basis of this characteristic. Regarding phagocytosis activity and respiratory burst of the cod leukocytes, a contradiction was encountered while taking the decision of which isolates to include in the vaccine; a strain resisting phagocytosis would have an advantage and so could be considered more virulent. In this instance, this strain should be selected on this basis to be included in the vaccine. On the other hand, from a vaccine efficacy point of view, a bacterium that would be more easily phagocytosed appears to be a better option since the antigens would be more easily expressed by APC, and so the chances of stimulating a higher immune response would follow (Section 5.1). Thus, the most highly phagocytosed isolates from each selected serotype were included in the vaccine, in order to elicit the highest possible response in Atlantic cod, since this animal is known to display a poor (if existing) specific antibody response to vaccination with *V. anguillarum*.

The vaccine formulation used in Chapter 5 was not supplemented with adjuvant but it is important to remember that since cod has a poor specific immune response to vaccination with *V. anguillarum*, it might be possible to increase this response, or direct the immune system towards a more appropriate pathway in order to increase protection with the use of an adjuvant (Section 1.4.1).

The vaccine produced was efficient at protecting Atlantic cod against heterologous strains of *V. anguillarum* O2b and O2d with very encouraging values for the RPS (100 and 96%, respectively). The challenge procedure used (low dose) did not allow the investigation of the protection against *V. anguillarum* sub-serotype O2c and should be repeated. In addition, the formulation should be tested against other strains of all the serotypes included in the vaccine to determine the "broadness" of protection elicited. Nevertheless, the vaccine formulation appears to be a good combination of bacterial candidates for protecting Atlantic cod against *Vibrio anguillarum*, which allowed the vaccinated fish to clear the pathogen.

It would have been interesting to test two vaccines to estimate the relationship between the choice of the strains and the protection elicited. Together with the vaccine designed in this study, the immunisation of Atlantic cod with a vaccine containing the strains from Chapter 4 which had the highest resistance to phagocytosis (VIB1 (O1), VIB72T (O2a) and VIB102 (O2b)), might have shown if this choice was clearly affecting the elicited protection. But due to the number of fish available for this study, this double vaccination experiment could not be designed.

No evaluation of the antibody levels elicited has been carried out in this experiment and the immunological mechanisms which allow Atlantic cod to stop infection by *V. anguillarum* after vaccination have still not been elucidated. Maybe the protection of the fish is due to a "normal" anamnestic response with the activation of B cells to long-lived plasma cells and the secretion of specific antibodies but these have not been clearly shown to be detectable at this point. The possibility of a T-dependant type response is also to be considered, with the production of memory T cells and the subsequent activation of the cellular mediated immunity with the activation of phagocytic and cytotoxic cells. An interesting experiment which could give insights into the mechanisms of the cod vaccine protection would be the collection of B and T cells after

vaccination and passively immunise naïve fish with these. A subsequent challenge with *V. anguillarum* could give indications as to whether the antigen is producing an immune response and if either the B or the T cells are involved. Unfortunately, the advances in fish cellular immunology have not reached the point where many antibodies are available as markers, especially for fish species such as Atlantic cod. This type of experiment, relying on the use of B and T cell specific antibodies is not possible at the moment.

In conclusion, Atlantic cod will certainly become a major fish species for cold-water marine aquaculture. *Vibrio anguillarum* is the main pathogen affecting cod and even though all fish produced in captivity are vaccinated, outbreaks of vibriosis are still reported. The problems regarding the specific immune response of Atlantic cod to pathogens, especially *V. anguillarum*, still evades researchers and this has hampered vaccine development for this species. Nevertheless, the tools developed in this study produced valuable information for the development of a suitable vaccine for cod. The identification of the different serotypes affecting this species (using the mAbs) as well as the *in vitro* assays developed for analysis by flow cytometry to examine the interaction of cod leukocytes with different serotypes of *V. anguillarum* enabled the investigation of some pathogenic mechanisms of isolates belonging to different serotypes. This helped in the decision of which isolates to include in the vaccine, so as to provide a broad range protection against different *V. anguillarum* serotypes. The vaccine specifically tailored to protect Atlantic cod against *V. anguillarum* in this study showed very promising results, eliciting high protection and therefore would be a very valuable product for the cod farming industry.

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Appendix 1. Bacteriology Buffers and Solutions

All solutions and buffers were stored at 4°C unless otherwise stated.

Appendix 1.1. Tryptone Soya Agar + 2% NaCl (TSA+NaCl):

Tryptone Soya Agar (Oxoid)	20 g
NaCl (Sigma-Aldrich)	7.5 g
Distilled water	500 mL

The solution was shaken and autoclaved. After cooling to 50°C in a water bath, 25 mL was poured into 90 mm Agar plates (Sterilin) and left to solidify overnight at RT.

Appendix 1.2. Tryptone Soya Broth + 2% NaCl (TSB+NaCl):

Tryptone Soya Broth (Oxoid)	15 g
NaCl	7.5 g
Distilled water	500 mL

The solution was mixed and autoclaved.

Appendix 1.3. Gram Staining Solutions:

Appendix 1.3.1. Crystal Violet Solution:

Methyl violet (Fissons)	2 g
Ethanol (95%, VWR)	20 mL
Ammonium oxalate (Sigma-Aldrich)	0.5 g
Distilled water	80 mL

The solution was left overnight at RT and filtered through a filter paper N°1 (Whatman).

Appendix 1.3.2. Iodine Solution:

Iodine (VWR)	1 g
Potassium Iodine (VWR)	2 g
Distilled water	300 mL

The solution was left overnight at RT and filtered through a filter paper N°1 (Whatman).

Appendix 1.3.3. Safranin Solution:

Safranin (Sigma-Aldrich)	0.25 g
Ethanol (95%, VWR)	10 mL
Distilled water	90 mL

The solution was left overnight at RT and filtered through a filter paper N°1 (Whatman).

Appendix 2. SDS Gel Electrophoresis Buffers and Solutions

Appendix 2.1. Phosphate Buffered Saline (PBS, 10x):

NaH2PO4 (VWR)	4.38 g
Na2HPO4 (VWR)	12.8 g
NaCl	43.85 g
Distilled water	500 mL

For use, the solution was diluted 1:10 in distilled water, the pH adjusted to 7.2 and autoclaved.

Appendix 2.2. SDS Sample Buffer (2x):

Trisma HCl (Sigma-Aldrich, 0.5 M, pH 6.8)	2.5 mL
Glycerol (Sigma-Aldrich)	2 mL
SDS (10% w/v, Sigma-Aldrich)	4 mL
DTT (Sigma-Aldrich)	310 mg
Bromophenol Blue (Sigma-Aldrich)	a few grains
Distilled water	adjusted to 10 mL

The solution was vortexed vigorously, aliquoted in 500 µL and frozen at -20°C.

Appendix 2.3. SDS Separating Buffer:

Trisma Base (Sigma-Aldrich)	91 g
SDS (Sigma-Aldrich)	2g
Distilled water	500 mL

The pH of the solution was adjusted to 8.7 and kept at 4°C for 1 month.

Appendix 2.4. SDS Stacking Buffer:

Trisma Base	6.05 g
SDS	0.4 g
Distilled water	100 mL

The pH was adjusted to 6.8 and the solution kept at 4°C for 6 months.

Appendix 2.5. SDS Running Buffer (5x):

Trisma Base	7.5 g
Glycine (Sigma-Aldrich)	36 g
SDS	2.5 g
Distilled water	500 mL

The solution was kept at 4°C for 6 months. For use, 60 mL of running buffer was diluted with 240 mL of distilled water and the pH adjusted to 8.3.

Appendix 3. Western blot Buffers and Solutions

Appendix 3.1. Blotting Buffer:

Glycine	14.4 g
Trisma base	3.03 g
Methanol (Thermo Fisher Scientific)	200 mL
Distilled water	800 mL

The pH was adjusted to 8.3, if required.

Appendix 3.2. Trisma Buffered Saline (TBS, 10x):

Trisma base	12.1 g
NaCl	146.2 g
Distilled water	500 mL

For use, the solution was diluted 1:10 in distilled water and the pH adjusted to 7.5.

Appendix 3.3. Tween Trisma Buffered Saline (TTBS):

TBS solution (1x)	500 mL
Tween 20 (Sigma-Aldrich)	250 µL

The pH was adjusted to 7.5.

Appendix 3.4. Substrate Buffer and Developing Solution:

Appendix 3.4.1. Substrate Buffer:

4-chloro-1-naphtol (Sigma-Aldrich)	0.15 g
Methanol	50 mL

The solution was aliquoted in 10 mL and kept in the dark at -20°C.

Appendix 3.4.2. Developing Solution:

Substrate buffer	2 mL
TBS (1x)	10 mL
H_2O_2 (Sigma-Aldrich)	10 µL

The H_2O_2 was added just before use.

Appendix 4. ELISA Buffers and Solutions

Appendix 4.1. Low Salt Wash Buffer (x10):

Trisma base	12.1 g
NaCl	111.1 g
Tween 20	2.5 mL
Distilled water	500 mL

For use, the solution was diluted 1:10 in distilled water and the pH adjusted to 7.3.

Appendix 4.2. High Salt Wash Buffer (x10):

Trisma base	12.1 g
NaCl	146.1 g
Tween 20	5 mL
Distilled water	500 mL

For use, the solution was diluted 1:10 in distilled water and the pH adjusted to 7.7.

Appendix 4.3. Coating Buffer:

Carbonate-bicarbonate buffer (Sigma-Aldrich)	1 tablet
Distilled water	100 mL

Appendix 4.4. ELISA Developing Solutions

Appendix 4.4.1. Substrate Buffer:

Citric acid (VWR)	2.1 g
Sodium acetate (Sigma-Aldrich)	0.82 g
Distilled water	100 mL

The pH was adjusted to 5.4.

Appendix 4.4.2. TMB Solution:

3'3'5'5'-Tetramethylbenidine dihydrochloride (TMB, Sigma-Aldrich)	79 mg
Acetic acid (VWR)	2 mL
Distilled water	4 mL

The solution was vortexed and kept in the dark at 4°C.

Appendix 4.4.3. Developing Solution:

TMB solution	150 µL
Substrate buffer	15 mL
H_2O_2	5 µL

Appendix 5. LPS Silver Staining

Appendix 5.1. Solution A:

Ethanol	45 mL
Acetic acid	5 mL
Distilled water	50 mL

Appendix 5.2. Solution B:

Acetic acid	7 mL
Iso-propanol (Sigma-Aldrich)	1 mL
Periodic acid (Sigma-Aldrich)	1.05 g
Distilled water	92 mL

Appendix 5.3. Solution C:

Sodium Hydroxyde (VWR, 0.1M)	28 mL
Ammonium hydroxide (Concentrated, Sigma-Aldrich)	1.5 m
Silver nitrate solution (20% w/v, Sigma-Aldrich)	5 mL
Distilled water	115 mL

The solution was prepared in the fume hood.

Appendix 5.4. Solution D:

Citric acid (Sigma-Aldrich)	25 mg
Formaldehyde (Sigma-Aldrich)	250 µL
Distilled water	100 mL

Appendix 6. Respiratory Burst Solutions

Appendix 6.1. DHR:

Dihydrorhodamine 123 (Sigma-Aldrich)	10 mg
Dimethyl sulfoxide (DMSO, Sigma-Aldrich)	1 mL

The solution was aliquoted in 20 µL and kept at -20°C until use. For use, 20 µL of the solution was thawed and diluted with 1.980 mL of sterile PBS (1x).

Appendix 6.2. PMA:

Phorbol 12-myristate 13-acetate (Sigma-Aldrich)	100 µg
Dimethyl sulfoxide	1 mL

The solution was aliquoted in 20 µL and kept at -20°C until use. For use, 20 µL of the solution was thawed and diluted with 1.980 mL of sterile PBS (1x).

Appendix 7. Serum Killing Buffers and Solutions

Appendix 7.1. Serum Buffer:

Tris HCl	630 mg
NaCl	877 mg
Distilled water	100 mL

Appendix 7.2. Live Stain Solution:

Thiazole Orange (Sigma-Aldrich)	200 mg
Dimethyl sulfoxide	1 mL

The solution was aliquoted in 20 µL and kept at -20°C until use. For use, 20 µL of the solution was thawed and diluted with 1.980 mL of sterile PBS (1x).

Appendix 8. Cell Separation Solutions

Appendix 8.1. 34% Percoll:

Percoll (Sigma-Aldrich)	3.4 mL
PBS (10x)	1 mL
Distilled water	5.6 mL

Appendix 8.2. 51% Percoll:

Percoll	5.1 mL
PBS (10x)	1 mL
Distilled water	3.9 mL

Appendix 8.3. Preparation of the discontinuous gradient:

To prepare the discontinuous gradient, 5 mL of 34% Percoll solution was poured into a 20 mL universal tube (Sterilin) and 10 mL of 51% Percoll solution was layered below the 34% layer by placing a 10 mL pipette filled with 51% Percoll solution and very slowly expelled. The tube was then kept at 4°C with care not to mix the layers while moving it.

Appendix 9. Cell culture media

Appendix 9.1. DMEM*:

DMEM (Sigma-Aldrich)	500 mL
Sodium pyruvate (Sigma-Aldrich, 100mM)	5 mL
L-glutamine (Sigma-Aldrich, 200mM)	5 mL
Penicillin-Streptomycin (Sigma-Aldrich, 200 $IU.mL^{-1}$/200 $\mu g.mL^{-1}$)	2.5 mL

Appendix 9.2. L15*:

L15 (Sigma-Aldrich; without glutamine)	500 mL
L-glutamine (Sigma-Aldrich, 200mM)	5 mL
Fetal Calf Serum (Sigma-Aldrich)	10 mL

Companies:
Sterilin Ltd, Caerphilly, UK
Whatman, GE Healthcare, Maidstone, UK
Oxoid, Thermo Fisher Scientific Inc., Loughborough, UK
Fissons, Thermo Fisher Scientific, UK
VWR, Lutterworth, UK
Sigma-Aldrich, Poole, UK