Identification of immunogenic surface proteins of *Pasteurella atlantica* genomovar *salmonicida*

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List of abbreviations

Abbreviation	Full name
Ab	Antibody
Ag	Antigen
APC	Antigen Presenting Cell
BA	Blood Agar
BcR	B-cell Receptor
CD4+	Cluster of Differentiation 4 positive
CD8+	Cluster of Differentiation 8 positive
CLR	C-Type Lectin Receptor
DAMP	Damage Associated Molecular Pattern
E	Extracellular
FAb	Fragment Antigen-Binding
Fc	Fragment Crystallizable
FCS	Foetal Calf Serum
g	Gram
x g	Times Gravitational Force
gv	Genomovar
His-tag	Polyhistidine Tag
IFNh	Interferon h
Ig	Immunoglobin
IPTG	Isopropyl β-D-1-Thiogalactopyranoside
IHNV	Infectious Hematopoetic Necrosis Virus
kDa	Kilodalton
L	Liter
LC-MS/MS	Liquid Chromatography and Mass Spectrometry
LRR	Leucine-Rich Repeat
M	Molar
mg	Milligram
MHC	Major Histocompatibility Complex
ml	Millilitre
NBD	Nucleotide Binding Domain

NCBI	National Center for Biotechnology Information
NCC	Non-Specific Cytotoxic Cells
ng	Nanogram
NLR	NOD-Like Receptor
nm	Nanometre
NO	Nitric Oxide
NOD	Nucleotide-Binding Oligomerization Domain
NVI	Norwegian Veterinary Institute
o/n	Overnight
OD	Optical Density
OM	Outer Membrane
OMP	Outer Membrane Protein
P	Periplasmic
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PRR	Pattern Recognition Receptor
RIG-I	Retinoic Acid-Inducible Gene I
RLR	RIG-I Like Receptor
ROS	Reactive Oxygen Species
SAV3	Salmonid Alphavirus 3
SDS-PAGE	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
SOB	Super Optimal Broth
TAP	Transporter Associated with Antigen Processing
TBS	Tris-Buffered Saline
TcR	T-cell Receptor
TLR	Toll-Like Receptor
TSB	Tropic Soy Broth
TTBS	Tween Tris-Buffered Saline
VFDB	Virulence Factor Database
WB	Western Blot
µl	Microlitre

Abstract

Outbreaks of pasteurellosis in Atlantic salmon (*Salmo salar* L.) have progressively increased in the farming industry since 2018, thus intensifying the need of a commercially available vaccine. Presently, our understanding of the bacterias' surface proteins, immunogenic components and virulence factors remains limited.

In this thesis we aim to identify immunogenic proteins of *P. atlantica* genomovar *salmonicida* and express immunogenic proteins recombinantly in *Escherichia coli*. We studied the protein profiles of four *P. atlantica* gv. *salmonicida* isolates through silver stained and Coomassie stained SDS PAGE gels and immunogenic proteins through western blotting (WB). The growth requirements for blood components using Foetal calf serum (FCS) for bacterial growth in broth cultures was also analysed.

Through bacterial growth test in broth cultures supplemented with different concentration of FCS we found that the bacteria are dependent on FCS supplement for growth. Which component of FCS is required, and the optimal amount of supplement is still uncertain. Through WB assays using antisera from fish previously vaccinated with inactivated *P. atlantica* gv. *salmonicida*, we found that the fish produced antibodies binding to proteins with molecular weight roughly around 60- and 90 kDa. Protein bands of this size were analysed using LC-MS/MS: Further, through in silico analysis, a list of proteins that seemed promising vaccine candidates was established. Two proteins, a ExlB-like and a MAM7-like, were selected for the production of recombinant proteins.

We managed to successfully express the recombinant proteins in *Escherichia coli*, but they showed no clear reactivity to sera from vaccinated fish in WB assays. These findings indicate that the recombinant proteins might not be the antigens that the fish immune system primarily responded to following vaccination. The lack of clear reactivity might also be due to varying protein immunogenicity or the recombinant proteins differing from the wildtype protein. Future research may benefit from exploring and selecting additional proteins from the list of potential candidates.

Sammendrag

Utbrudd av pasteurellose hos Atlantisk laks (*Salmo salar* L.) har gradvis økt i den norske oppdrettsindustrien siden 2018, noe som intensiverer behovet for en kommersielt tilgjengelig vaksine. For tiden er kunnskapen om bakteriens overflateproteiner, immunogene komponenter og virulensfaktorer fortsatt begrenset.

I denne avhandlingen forsøker vi å identifisere immunogene proteiner fra «*Pasteurella atlantica* genomovar *salmonicida»* og uttrykke immunogene proteiner rekombinant i *Escherichia coli*. Vi studerte proteinprofiler til fire *P. atlantica* gv. *salmonicida*-isolater gjennom sølvfargede og Coomassie-fargede SDS PAGE-geler, og immunogene proteiner gjennom Western Blott (WB). Vekstkravene for blodkomponenter ved bruk av Fetalt kalveserum (FCS) for å fremme bakteriell vekst i buljongkulturer ble også analysert.

Undersøkelse av bakterievekst i buljongkulturer supplert med forskjellige konsentrasjoner av FCS fant vi at bakteriene er avhengige av FCS-tilskudd for vekst. Hvilken komponent av FCS som kreves, og den optimale mengden av tilskuddet, er fortsatt usikkert. Gjennom WB-tester med bruk av antiserum fra fisk tidligere vaksinert med inaktivert *P. atlantica* gv. *salmonicida*, fant vi at fisken produserte antistoff som binder til proteiner med molekylvekt omtrent rundt 60- og 90 kDa. Proteinbånd av denne størrelsen ble analysert ved hjelp av LC-MS/MS: Videre, gjennom in silico analyse, ble det etablert en liste over proteiner som virket å være lovende vaksinekandidater. Til slutt ble to proteiner, et ExlB-lignende og et MAM7-lignende, valgt for produksjon av rekombinante proteiner.

Vi klarte å uttrykke rekombinante proteiner i *Escherichia coli*, men de viste ingen klar reaktivitet mot serum fra vaksinert fisk i WB. Disse funnene indikerer at de rekombinante proteinene sannsynligvis ikke er antigenet som fiskens immunsystem primært responderte på etter vaksinering. Mangelen på klar reaktivitet kan også skyldes varierende immunogenisitet eller de rekombinante proteinenes forskjell fra villtype-protein. Fremtidig forskning kan dra nytte av å undersøke andre proteiner fra listen over mulige kandidater.

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1 – Introduction

In 2022, there were 52 confirmed cases of pasteurellosis in Norwegian Atlantic salmon (*Salmo salar* L.) fish farms, and the disease was recognized as the 9th largest fish health problem in the Norwegian aquaculture industry according to a survey performed by the Norwegian Veterinary institute (NVI) (Sommerset et al., 2023). The Norwegian salmon farming industry is a significant producer of salmonids, with an annual production of over 1.5 million metric tons. The availability of effective vaccines is crucial to facilitate such large-scale production. Around 455 million salmon were vaccinated in 2022, providing protection against major viral and bacterial diseases. The Norwegian fish farming industry operates with minimal use of antibiotics, with only 605 kg of antibiotics prescribed in 2021 (Directorate of fisheries, 2022; Litleskare, 2022). The absence of commercially available vaccines against the emerging disease, pasteurellosis, in Atlantic salmon is a critical concern both ethically and economically. Therefore, further research is necessary to acquire a better understanding of the causative agent, *Pasteurella atlantica* genomovar *salmonicida*.

1.1 *Pasteurella* sp. infections in Atlantic salmon

The disease caused by bacteria in the genus *Pasteurella* is called pasteurellosis. Pasteurellosis in Norwegian farmed salmon was initially diagnosed after an outbreak in 1989 (Legård & Strøm, 2020) but during the outbreak, the causative pathogen was still unknown. The clinical signs of the disease formed the basis for the proposed name "Varracalbmi," which is a Sami term for "bloody eye" (Valheim et al., 2000). The disease caused panophthalmitis, resulting in haemorrhagic eyes and necrotizing inflammation (Valheim et al., 2000). After the initial outbreak, cases of pasteurellosis in Atlantic salmon occurred sporadically until the spring of 2018, at which point there was a notable rise in the frequency of occurrences (Legård & Strøm, 2020; Sandlund et al., 2021; Sommerset et al., 2023).

Pasteurellosis had thus not been a significant problem in the Norwegian salmon farming industry before 2018, when a new member of the *Pasteurella* species was discovered in diseased salmon (Sommerset et al., 2023). Since then, the occurrences of pasteurellosis caused by this new species have been progressively rising and spreading along the Norwegian coastline (Sommerset et al., 2023). The Norwegian Veterinary Institute (NVI) has proposed a working nomenclature for this new species "*Pasteurella atlantica* genomovar *salmonicida*" until an official name is established. In lumpfish used as cleaner fish in fish farms, pasteurellosis is

associated with another genetic variant, *P. atlantica* genomovar *cyclopteri* (Alarcón et al., 2016). *Pasteruella skyensis*, which is known to cause pasteurellosis in farmed salmon in Scotland, was isolated after an outbreak of disease in farmed Norwegian Atlantic salmon in September 2020. However, this occurrence was confined to just one fish farm and has not resulted in any major issues thus far (Sommerset et al., 2021).

In salmon affected by pasteurellosis resulting from *P. atlantica* gv. *salmonicida* infection, standard clinical macroscopic observations include purulent inflammation of the pericardium, abdominal wall, and pseudobranchia, as well as the presence of abscesses filled with pus in skeletal muscle and at the base of the pectoral fin (Legård & Strøm, 2020; Sommerset et al., 2023). Although protruding, inflamed, and partially bloody eyes are common, they are not present in all fish. Histopathological changes are consistent with the macroscopic observations, revealing signs of acute and chronic inflammation, abundant inflammatory cells, tissue fluid, and the presence of short rod-shaped bacteria in affected organs (Legård & Strøm, 2020; Sommerset et al., 2023).

1.2 Bacterial virulence factors and immunogenic components

Knowledge on a bacterias' virulence factors and immunogenic components are important to understand pathogenesis and for designing new vaccines. The primary interaction between pathogenic bacteria and their host involves the initial attachment of the bacteria to the host's cell surface (Casadevall & Pirofski, 2001). This adherence can lead to the establishment of colonization and the subsequent development of infection and disease. In existing research an adhesin related to adherence, similar to the *Haemophilus influenzae* adhesin (Hia), was identified in *P. atlantica* gv. *cyclopteri* (Ellul et al., 2021). This Hia – like surface protein of *P. atlantica* gv. *cyclopteri* was following the strategies of in silico analysis and reverse vaccinology (Bidmos et al., 2018; Ong et al., 2021) predicted to be the best targeted vaccine candidate.

The cellular components and structures that facilitate the evasion of host defence mechanisms and promote bacterial pathogenicity are commonly referred to as virulence factors. These factors may exist as endotoxins within the bacterial cell, on the surface of the bacterial cell,

such as the bacterial capsule, flagella, fimbriae/pili, or released extracellularly as exotoxins (Casadevall & Pirofski, 2001) as illustrated in Figure 1.

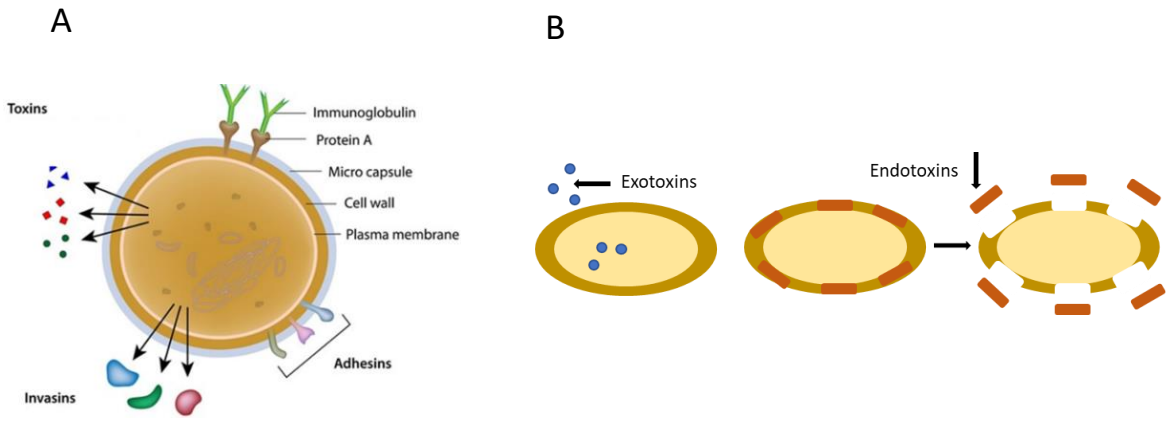

Figure 1: A: *Showing rough structure of a bacterial cell including outer membrane proteins, secretion of toxins and invasins. Modified from Alila Medical Media / Shutterstock.* ***B:*** *Showing the difference between exotoxins, secreted or released from the bacteria into the surroundinggs, and endotoxins that are part of the outer membrane of the cell wall and liberated when the bacteria die.*

Antibody responses that result from the presence of endotoxins (Figure 1), toxic compounds of the outer bacterial cell wall, are usually insufficient in conferring protection against disease when used as vaccine antigens. Lipopolysaccharide (LPS), a prominent endotoxin present in Gram-negative bacteria, may however be a significantly protective antigen (Welch & LaPatra, 2016). In contrast, the active exotoxins are typically immunogenic and known to elicit protective antibody responses. The responses are however subject to several factors, including the size of the toxin (Figure 2). This attribute makes exotoxins possible targets for vaccine development, as they can be utilized to generate a protective immune response in the host (Ellul et al., 2019).

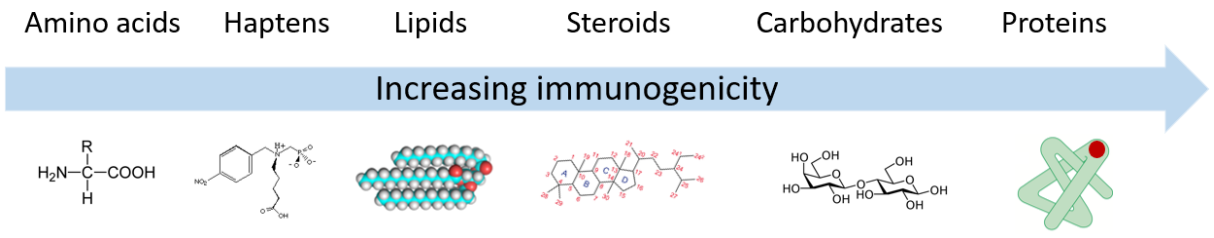

Figure 2: *Molecular size of toxins influence on immunogenicity where large molecules are generally more immunogenic compared to small molecules.*

The process of pathogenic bacteria colonizing and infecting a host is typically facilitated by a group of proteins referred to as adhesins (Casadevall & Pirofski, 2001). They are present on the outer membrane of the bacteria and are regarded as attributes of virulence. The pathogen's aggressiveness can be enhanced through the presence of a protective capsule that surrounds it, providing resistance against phagocytosis or enhancing survival within phagosomes, thereby enabling it to overcome or evade the host's defences (Casadevall & Pirofski, 2001). Additionally, disease can also arise indirectly from bacterial components, as the host's reaction to these components. This can be exemplified by the inflammatory response to polysaccharide components of bacterial capsules, which can cause tissue damage and result in disease (Casadevall & Pirofski, 2001).

Immunogenic components are molecules, usually proteins or polysaccharides, that can stimulate an immune response when recognized by the host's immune system (Casadevall & Pirofski, 1999). Immunogenic components can be derived from pathogens, such as bacteria and viruses, or they can be part of the host's own cells and tissues, as in the case of autoimmunity (Casadevall & Pirofski, 1999). Virulence factors are molecules produced by pathogens that contribute to their ability to cause disease (Diard & Hardt, 2017). They can include adhesins, toxins, and enzymes that help the pathogen to invade and damage host tissues, as well as evade the host's immune response. Some virulence factors can also be immunogenic components, meaning they can trigger an immune response in the host (Diard & Hardt, 2017). However, not all virulence factors are immunogenic components, and not all immunogenic components are virulence factors (Casadevall & Pirofski, 1999). Some virulence factors might not be recognized by the host's immune system, while other molecules that are not directly involved in the pathogen's virulence can still elicit an immune response.

1.3 The teleost immune system

The immune system of teleosts is divided into the innate and adaptive systems. The major lymphoid organs are the thymus, kidney, spleen, and mucosa associated lymphoid tissues (MALTs) (Uribe et al., 2011). The innate immune system applies a generic pathogen recognition mechanism through pathogen recognition receptors (PRRs) recognising conserved motifs of pathogen associated molecular patterns (PAMPs).

There are several types of PRRs that play a crucial role in detecting and responding to invading pathogens. Toll-like receptors (TLRs) are transmembrane proteins expressed on the surface of

membranes of immune cells, such as macrophages and dendritic cells (Magnadóttir, 2006). They recognize a wide variety of PAMPs, including flagellin of the bacterial flagella, viral RNA, and fungal cell wall components. Upon activation, TLRs initiate a signalling cascade that leads to the production of pro-inflammatory cytokines and chemokines, which help recruit other immune cells to the site of infection and initiate the adaptive immune response (Pietretti & Wiegertjes, 2014). Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are cytoplasmic receptors that detect intracellular pathogens or damage-associated molecular patterns (DAMPs) released by damaged cells. They consist of a central nucleotide-binding domain (NBD) and leucine-rich repeat (LRR) domains, which recognize specific PAMPs or DAMPs (Pietretti & Wiegertjes, 2014). Upon activation, NLRs can trigger the formation of multi-protein complexes called inflammasomes, leading to the production of pro-inflammatory cytokines such as IL-1β and IL-18. They can also activate other antimicrobial pathways, such as the production of reactive oxygen species (ROS) and nitric oxide (NO), which help eliminate the invading pathogens (Magnadóttir, 2006). C-type lectin receptors (CLRs) are transmembrane or soluble carbohydrate-binding proteins that recognize specific sugars on the surface of pathogens (Geijtenbeek, T., & Gringhuis, S., 2009). They play a role in mediating phagocytosis and the activation of antimicrobial responses. Some CLRs, such as dectin-1, can also trigger intracellular signalling pathways that lead to the production of pro-inflammatory cytokines and the activation of adaptive immunity. Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are a family of PRRs that play crucial roles in the innate immune response to viral infections. They are involved in the recognition of viral RNA, triggering antiviral signalling pathways leading to the production of type I interferons and proinflammatory cytokines (Chen et al., 2017).

There are several types of bacterial PAMPs and the immunogenicity of a PAMP is determined by various factors, including size, structure, foreignness, and chemical composition. The immunogenicity of PAMPs is mainly determined by their ability to bind to PRRs and trigger signalling pathways that lead to the activation of immune responses (Pietretti & Wiegertjes, 2014). Flagellin, the primary protein component of the bacterial flagella, is recognized by Toll-like receptor 5 in Atlantic salmon (Tsoi et al 2006). Peptidoglycan is a major component of the bacterial cell wall, and it is recognized by NOD-like receptors (NLRs) in the cytoplasm of immune cells, such as NOD1 and NOD2 (Sahoo, 2020). Bacterial lipoproteins are lipid-modified proteins present in the cell envelope of both Gram-positive and Gram-negative

bacteria. They are recognized by TLR2 in mammals, and TLR2 orthologs have been identified in teleost fish (Ribeiro et al., 2010; Zhang et al., 2014)

The innate immune cells do not exhibit an increased efficiency upon subsequent exposure to the same pathogens. On the other hand, the adaptive immune system identifies pathogens via specific antigens and offers targeted immunity and memory against pathogens bearing analogous molecules, thereby granting protection against future infections (Murphy & Weaver, 2017).

Although teleosts possess a well-developed adaptive immune system, the innate immune system play a critical role (Murphy & Weaver, 2017). Its rapid response to new pathogens is essential in providing a window of time for the adaptive immune response to mature sufficiently. Furthermore, the innate immune system is capable of swiftly eliminating some pathogens so quickly there is hardly any need for an adaptive immune response. Hence, both the innate and adaptive immune systems play significant roles in combating diseases, and their activation is necessary through immune stimulation and vaccination (Sommerset et al., 2005).

The innate immune system is the first line of defence against foreign invaders, and it provides protection through physical barriers like skin, scales, and mucus, as well as through humoral and cellular components (Castro & Tafalla, 2015). This system is not known to possess immune memory due to its non-specific nature. Recent studies have however shown that the innate immune system can be "trained" using immune-modulating compounds like β-glucans to confer protection against a secondary infection, independently of T- and B-cells, by relying on macrophages and non-specific cytotoxic cells (NCC) (Petit et al., 2019).

The innate immune response is responsible for initiating the adaptive immune response, which is crucial for long-term protection against pathogens and is the primary goal of vaccines (Sommerset et al., 2005). Adaptive immune responses involve lymphocytes, B-cells and T-cells, playing crucial roles in adaptive immunity. While T-cells are responsible for adaptive cellular immunity, B-cells are responsible for adaptive humoral immunity (Uribe et al., 2011).

Exogenous antigens, recognised by phagocytic receptors and presented by antigen-presenting cells (APCs) are phagocytosed by phagocytic cells like macrophages or dendritic cells (Murphy & Weaver, 2017). Within the cell, through the endocytic pathway, pathogen-derived peptides are bound to major histocompatibility complex class II (MHC class II) molecules and transported to the cell surface where they are introduced to CD4+ T-cells. The CD4+ T-cell recognise MHC class II and the presented peptide through CD4 and the T-cell receptor (TcR)

respectively (Murphy & Weaver, 2017). Recognition and binding leads to activation and differentiation of the CD4+ T-cell into T-cell subsets Th1, Th2, Treg or Th17 cells depending on the cytokines secreted by the APC upon binding (Murphy & Weaver, 2017). For activation of B-cells and development of antibody-secreting cells (plasma cells) in a T-cell dependent-manner, Th2 cells are needed.

B-cells have B-cell receptors (BcR) in the form of cell specific membrane-bound immunoglobulins (Ig). B-cells are responsible for generating specific antibodies against the specific antigens recognized by their BcR (Murphy & Weaver, 2017). Teleost B-cells also possess professional phagocytic abilities, which is a trait normally associated with the innate immune cells (Li et al., 2006).

B-cells can be activated by two different mechanisms, thorough T-cell dependent or T-cell independent pathways (Murphy & Weaver, 2017). In T-cell dependent activation, the BcR binds to the antigen, which is then internalized and degraded. The resulting antigen peptides are displayed on the surface of the B-cell via MHC class II molecules. A helper Th2 cell recognizes the peptide with its specific TcR and binds to the B-cell's CD40 receptor using its CD40L receptor (Murphy & Weaver, 2017). The Th2 cell secretes cytokines, which activates the B-cell and initiates clonal expansion and differentiation to plasma cells and memory cells. In T-cell independent activation, the antigen is often a polymer capable of activating several BcRs and innate membrane receptors simultaneously, leading to clonal expansion without signals from a Th2 cell, this type of activation is however proposed not to induce development of plasma cells and memory cells (Murphy & Weaver, 2017). Plasma cells are responsible for generating antibodies for secretion. Memory B-cells, which are long-lived, can differentiate into plasma cells if they encounter similar antigens during future infections (Murphy & Weaver, 2017). Th1 cells play a more significant role in activating cellular responses, such as effector T-cells (CD8+) and macrophages.

Endogenous antigens are presented on MHC class I molecules, these molecules are present in all nucleated cells (also red blood cells in fish). The antigen is detected within the cell where it is degraded by proteasomes before it is transported to the endoplasmatic reticulum by transporters associated with antigen processing (TAP) (Murphy & Weaver, 2017). Here the antigen derived peptides will bind to MHC class I before it is transported to the surface of the cell and presented to CD8+ T-cells. CD8+ T-cells use T-cell receptors (TcR) to recognize pathogen-derived peptides presented on MCH class I (Murphy & Weaver, 2017). For a full

activation of the CD8+ cytotoxic T-cells, the CD8+ T-cells depend on cytokine signals from derived from Th1 cells.

Immunoglobulins in the form of secreted antibodies are small glycoproteins produced by plasma cells and have a basic Y-shaped structure consisting of four protein chains, each with two identical heavy chains and two identical light chains (Murphy & Weaver, 2017), illustrated in Figure 3. Variations in the amino acid sequence of the heavy chain's constant region determine the immunoglobulin isotype, such as IgM, IgD, and IgT (called IgZ in zebrafish), found in teleost fish (Hordvik, 2015). In contrast to mammals, fish cannot switch isotypes.

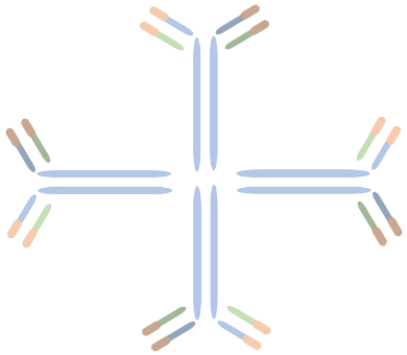

Figure 3: *Schematic drawing of secreted IgM in tetrameric shape. Showing heavy chains (blue), light chains (green) both with antigen binding site, paratope (peach).*

The upper part of the Y-shape is the Fragment antigen-binding (Fab) region, while the lower part is the Fragment crystallizable (Fc) region, with the heavy chain stretching from the Fc region to the Fab region, and the light chain only present in the Fab region (Hordvik, 2015). The N-terminal portion of the heavy and light chains varies greatly between different polypeptides and is therefore called the variable regions (Hordvik, 2015). These variable regions build the antigen binding site, the paratope. The paratope of the antibody binds to an epitope of the antigen. The heterogeneity in the variable regions between antibodies secreted from different plasma cells accounts for the tremendous diversity in antigen specificity between antibody molecules and the ability to mount antibody responses towards several epitopes of an antigen. Thus, the antibodies produced after exposure to an antigen are heterogeneous in specificity and termed polyclonal. Immunoglobulins can create bonds as dimeric, tetrameric, or pentameric structures when secreted as antibodies. IgM of teleosts are tetrameric when secreted and monomeric as membrane anchored BcR (Hordvik, 2015). The secreted tetrameric form can thus crosslink identical epitopes. The secreted antibodies can, in addition to binding to the antigenic determinant, interact with other epitopes with similar structures when there is a partial

or complete similarity in antigen epitopes. This cross reaction of antibodies is usually less stable compared to when binding to the antigen determinant, but it depends on crosslinking (avidity) and the binding energy (affinity) (Mashoof & Criscitiello 2011). Epitopes can be continuous (linear epitope) or discontinuous (Figure 4). This is important considerations when making recombinant proteins. If the folding of the recombinant protein is incorrect there may not be specific binding by protein targeting antibodies or the cross reaction possibly obtained may be weak in nature.

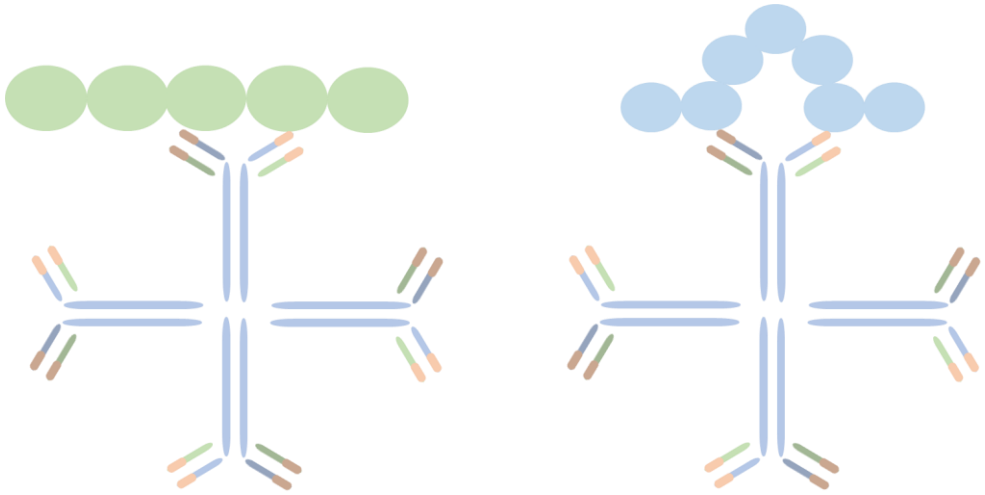

Figure 4: *Antibodies binding to linear epitopes (green circles) and discontinuous epitopes (blue circles).*

The constant region of the antibodies carries out the effector functions. Antibodies can impact pathogens in four ways, by neutralization, agglutination, opsonization, and activation of the complement system (Murphy & Weaver, 2017). Neutralization occurs when antibodies bind to surface proteins of pathogens, blocking their ability to bind to host cells and tissues. Opsonization occurs when phagocytes recognize the Fc-region of antibodies bound to pathogens and phagocytize them (Murphy & Weaver, 2017). This is particularly important in defence against bacterial pathogens. On the other hand, neutralization is more important against viral pathogens. Agglutination is when pathogens and foreign substances are clumped together by antibody binding to several antigens simultaneously, reducing their mobility and infection ability, and increasing the chances of phagocytosis (Murphy & Weaver, 2017). Antibodies can also activate the classical pathway of the complement system, a cascade reaction of over 30 humoral proteins secreted from the liver to the bloodstream. The complement proteins combat pathogens through forming a membrane attack complex, by opsonization followed by phagocytosis and by inducing inflammation (Uribe et al., 2011).

1.4 Vaccines

The fast-paced growth of the worldwide aquaculture industry comes with various obstacles. Disease management and control represent a crucial aspect affecting both the welfare and economic sustainability of the industry. To ensure a sustainable production, it is necessary to improve disease prevention measures and minimize the occurrence of outbreaks that cause substantial fish losses. Vaccination has become a crucial component of intensive aquaculture as it has been shown to be a highly effective method in preventing a wide range of bacterial and viral diseases (Ma et al., 2019). For instance, in Norwegian salmon farming, the reduced use of antibiotics as a result of vaccination programmes has led to a significant increase in production (Directorate of fisheries, 2022; Litleskare, 2022).

A vaccine is a biological substance designed to trigger a safe immune response that provides immunity against infection and/or disease upon future exposure to a pathogen (Pollard & Bijker, 2021). To accomplish this, the vaccine should contain the pathogen itself or antigens that are either naturally derived from the pathogen or artificially produced to mimic components of the pathogen. This exposure prompts B-cells and T-cells to produce effector and memory cells, which allow the adaptive immune system to retain the pathogen's antigens and mount an efficient response to subsequent infections (Murphy & Weaver, 2017).

Fish vaccines traditionally involve inactivated whole microorganisms, although a few live attenuated, subunit protein vaccines and DNA vaccines have been made available commercially (Ma et al., 2019). Inactivated killed vaccines are usually developed by formalin inactivation of a disease-causing microorganism and formulated in an oil adjuvant (Ma et al., 2019). Live vaccines are produced from viruses or bacteria that exhibit attenuated virulence or naturally low virulence towards the specific fish species being targeted. Although attenuated live vaccines have been demonstrated to be safe in most situations and are currently used to combat infections by *Piscirickettsia salmonis* in salmon farming in Chile, there are certain risks that must be addressed to ensure that these products do not revert into a virulent strain, exhibit residual virulence, or cause virulence in immunocompromised vaccinated individuals (Ma et al., 2019; Mondal & Thomas, 2022). The major advantage using these vaccines for intracellular pathogens is their ability to induce both Th1 and Th2 responses, leading to both activation of cytotoxic T-cells and differentiation of plasma cells.

Subunit vaccines rely on immunogenic components, parts of the pathogen, entirely for immunization purposes (Hansson et al., 2000). Since these vaccines lack the ability to replicate

within the host, there is no potential for pathogenicity to the host or unintended species. By utilizing a highly characterized production process, subunit vaccines can specifically target immune responses towards one or a few protective antigens (Hansson et al., 2000). Subunit vaccines can be made by harvesting immunogenic components after bulk production of the target pathogen or through recombinant DNA technology. For recombinant DNA technology the gene encoding the immunogenic protein(s) is identified and inserted into a vector that will express the protein in large quantities before it is harvested and purified. There are several expression systems available having different advantages, including bacterial, mammalian, yeast and insect cells (Nascimento & Leite, 2012). Selection of expression system depends on the requirement of post translational modification such as glycosylation. Although subunit vaccines possess numerous desirable characteristics, such as the possibility to obtain large quantities of the protective protein, their capacity to trigger a robust immune response can often be lower than killed or live whole cell vaccines (Ma et al., 2019). Another important aspect to overcome in expressing protective proteins is the importance of utilizing correctly folded proteins in the vaccine considering the before mentioned issue of linear and conformation epitopes.

The more recently developed DNA vaccines consist of an expression plasmid containing a distinct gene encoding one or a few chosen antigenic proteins (Dalmo, 2018). In fish the plasmid is presented by intramuscular (i.m.) injection. For fish, DNA vaccines with marketing licence are currently available for Atlantic salmon against salmon pancreas disease (salmonid alphavirus 3) (SAV3) and for Infectious Hematopoietic necrosis virus (IHNV) (Dalmo, 2018). The expression of the plasmid encoded protein within the host cells stimulate both innate responses and adaptive cellular and humoral responses (Mondal & Thomas 2022). DNA vaccines can also be administered by Gene Gun delivery, as opposed to i.m. injection which requires micrograms of plasmid DNA, gene gun delivery requires nanogram levels. However recent studies have shown that the two delivery systems induce distinct immune responses, i.m. injection induce predominantly a Th1 response while the gene gun delivery induce a mixed Th1/Th2 response (Nascimento & Leite 2012). Thus, selection of delivery system should be based on if the disease is caused by an intracellular of extracellular infection. When a protective antigen is identified, the development of DNA vaccines can be relatively quick (Mondal & Thomas, 2022).

Vaccine design and identification of protective antigens are traditionally based on understanding pathogenesis and the infectious agent. For the next-generation vaccines

identification of protective antigens and genes encoding them are crucial. Recent advances in gene mining to identify protective antigens in silico has opened the field of reverse vaccinology (Delany et al., 2013). By applying the principles of reverse vaccinology, conducting bioinformatic analyses on pathogen genomes, it is possible to predict immunogenic antigens that can be used as targets for vaccine development in a computer-based approach. This approach is faster than the traditional method of vaccine development and can shorten the development time by approximately two years (He et al., 2010).

Since the establishment and application of reverse vaccinology principles, technology to strengthen the method has advanced significantly. Currently, several programs and databases are accessible that make it easier to predict appropriate targets. These programs utilize criteria based on physical attributes of the bacteria, such as subcellular localization, adhesin probability, and the number of transmembrane domains present in outer membrane proteins (Ong et al., 2021).

1.5 Aims of the study.

Since 2018, there has been an increased prevalence in outbreaks of pasteurellosis in farmed Norwegian salmon. This underscores the need for enhanced knowledge of the disease and the predisposing factors influencing on outbreak of disease. To enable the production of effective vaccines that can be brought to the commercial market, a deeper understanding of the pathogen is needed. There is limited understanding regarding the surface proteins, virulence factors, and immunogenic components of *P. atlantica* gv. *salmonicida*. Additionally, it remains uncertain whether this group is homogenous or whether there exist variations in the expression of surface proteins.

The aims of this study were thus to:

- Analyse growth requirements for FCS for *P. atlantica* gv. *salmonicida* in broth cultures.
- Identify immunogenic proteins of *P. atlantica* gv. *salmonicida*.
- Express immunogenic proteins recombinantly using *E. coli*.

2 – Materials and Methods

2.1 Bacteria

Four isolates of the bacteria *P. atlantica* genomovar *salmonicida*, harvested from Atlantic salmon diagnosed with pasteurellosis were included in this study. The isolates originate from commercial salmon farms in Vestland county, Norway. The isolates will be referred to as PaL-1, PaL-2, PaL-3 and PaL-4 in this thesis.

Table 1 - *Overview of* P. atlantica *isolates included in the thesis, the fish species they were isolated from, which location they were isolated from, and which year they were isolated.*

Isolate name	**Host species**	**Location**	**Year**
PaL-1	Atlantic salmon (*Salmo salar* L.)	Vestland county (Norway)	2019
PaL-2	Atlantic salmon (*Salmo salar* L.)	Vestland county (Norway)	2020
PaL-3	Atlantic salmon (*Salmo salar* L.)	Vestland county (Norway)	2020
PaL-4	Atlantic salmon (*Salmo salar* L.)	Vestland county (Norway)	2021

2.1.1 Culturing bacteria

The bacterial isolates were cultured in Tryptic Soy Broth (TSB) (BD, Lot#1068445) supplemented with 1.5% NaCl and 10% Foetal Calf Sera (FCS) (Gibco – Lot#2233787RP), hereafter called TSB+. Cultivation of bacteria in TSB+ was performed using 50 ml centrifuge tubes (SARSTEDT AG & Co). The bacteria, from freeze stock cultures (1 ml), were suspended in TSB+ (40 ml) and incubated in a shaking incubator (Infors AG, model Minitron) at 200 rpm at 20 °C for 24 hours.

2.1.2 Preparations of glycerol stocks

The four isolates were obtained from the Fish Immunology group at the University of Bergen, as frozen 1 ml bacterial culture stocks. Firstly 40 ml TSB+ (Appendix) was prepared in four separate 50 ml tubes. Once the bacteria stocks had thawed, they were each transferred to the TSB+ and incubated in a shaking incubator (Infors AG, model Minitron) at 200 rpm and 20 °C for 24 hours. Then 16 ml of the bacteria cultured in TSB+ was combined with 4.0 ml of ≥99% glycerol (Sigma-Aldrich) in 50 ml tubes and mixed well. Lastly, 1 ml of the solutions was added to 2.0 ml cryo tubes (VWR), and frozen at -80 °C (Thermo Scientific™, Revco™) to provide stocks for long term storage.

2.1.3 Bacterial growth curves

In this study, all bacteria were collected during their late exponential growth phase. To establish when this phase takes place, growth curves for each isolate were compiled. Bacteria were cultured as described in section 2.1.1, and optical density (OD) was measured over time to portray a growth curve. OD was measured in a spectrophotometer (Spectroquant® Pharo 300, Merck) at 600 nm wavelength. Before measuring, the spectrophotometer was blanked using the growth media used when culturing the bacteria. The OD was then measured by pipetting 1.5 ml bacteria suspension into a cuvette (VWR Cuvettes PS macro, VWR), which was placed in the spectrophotometer. The OD was measured from the time when the culture medium was inoculated with the bacteria, until the curve flattened out (stationary phase).

2.1.4 Bacterial growth at different concentrations of supplemented FCS

Three different concentrations of FCS in TSB (in 50 ml tubes) were included to test the growth rate of the bacteria and their requirement of FCS. The concentrations were 0%, consisting of 40 ml TSB only, 5% consisting of 2.0 ml FCS and 38 ml TSB, and 10% consisting of 4.0 ml FCS and 36 ml TSB (Table 2). The isolates were incubated in a shaking incubator (Infors AG, model Minitron) at 200 rpm at 20 °C for 24 hours. After incubation the OD of the samples were measured using Spectroquant® Pharo 300, Merck. The number of bacterial cells were also counted using a Roche Innovatis AG CASY Model TT cell counter.

Table 2 – *The different concentrations of FCS supplemented in TSB used to test bacterial growth*.

FCS concentration	TSB	FCS	Total
0%	40 ml	-	40 ml
5%	38 ml	2 ml	40 ml
10%	36 ml	4 ml	40 ml

Subcultures of the bacteria with different concentrations of supplemented FCS were prepared. The same growth conditions as described above were followed, but the bacteria came from the already cultured bacteria and not from freeze stocks as before. The subcultures were incubated in shaking incubator (Infors AG, model Minitron) at 200 rpm and 20 °C for 16 hours. The OD and cell count were then measured for the subcultures.

2.2.1 Preparation of *P. atlantica* gv. *salmonicida* antigens for protein gels

Bacteria of the isolates PaL-1, PaL-2, PaL-3, and PaL-4 were used as antigens. Bacterial isolates cultured with different concentrations of FCS as described were harvested after 16h in late exponential growth phase. 1 ml of each of the cultured bacteria suspensions were pipetted into 2.0 ml cryo tubes (VWR) and centrifuged at 1300 x g for 3 min to obtain a pellet of bacteria. The supernatants were discarded, and any excess fluids were gently removed using a pipette. The pellets were resuspended in 50 µl MilliQ.

For the WBs, including associated Coomassie and silver stained gels, bacteria cultured with 10 % FCS supplement were used. To enable comparison across the isolates, the number of cells in each bacterial sample (PaL-1, PaL-2, PaL-3 and PaL-4) were adjusted to the same concentration. The cells were washed by centrifugation at 2500 x g for 10 minutes (Beckman Coulter Inc., Allegra X-15R Centrifuge) and the pellet resuspended in 10 ml PBS. The cells were counted using the CASY TT cell counter. The cell numbers were adjusted by dilution in PBS to a final concentration of $8x10^8$ cells in 200 µl, giving a concentration of $4x10^8$ cells when diluted in sample buffer. When 10 µl of bacterial samples were supplied to the wells this gave a concentration of $1x10^7$ cells per well.

2.2.2 SDS-PAGE gels, Silver and Coomassie staining

To display the protein profiles of the PaL-1, PaL-2, PaL-3 and PaL-4 isolates grown at different concentrations of supplemented FCS, sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining were performed.

Protein gels (12% acrylamide Resolving gel and 4% Stacking gel) were prepared according to the method of Laemmli (Laemmli, 1970). For the electrophoresis a Mini Protean Tetra Cell (Bio-Rad) was used. The bacterial samples were mixed in a ratio of 1:1 in SDS Reducing Buffer (Appendix) supplied with β-mercaptoethanol (Sigma-Aldrich) (1:20) and heated at 98°C for 5 minutes in a heat block (Eppendorf) before 10 µl of the samples were supplied to the wells. 5 µl of unstained low range SDS-PAGE standard (Bio-Rad) diluted 1:20 in the SDS Reducing Buffer, treated equally as the bacterial samples, were used as ladder. The gels were electrophoresed at 190V for 50 minutes in a vertical electrophoresis chamber (Bio-Rad, Mini-PROTEAN Tetra Cell).

After the electrophoresis, the gels for silver staining were fixed in fresh fixative solution (Appendix) for 20 minutes, then washed twice for 10 minutes with Milli-Q (Merck Millipore,

model Milli-Q® Advantage A10® System). The gels were stained using the Silver Stain plus kit (Bio-Rad). Once clear protein bands were visible, the reaction was stopped by adding 5% acetic acid solution to the gels. The gels where then photographed (Bio-Rad, Universal Hood II)

For the Coomassie stained gels the Kaleidoscope™ WB standard (Bio-Rad) was used in one well, combined with the unstained SDS-PAGE standard (Bio-Rad) (5 µL) in another well. Once the electrophoresis was complete, the gel for Coomassie staining were washed with MilliQ, before placed in a staining tray with Coomassie Brilliant Blue (Appendix). The gel was incubated overnight on a flat orbital shaker (IKA, KS 260 basic) at 50 rpm. After staining, the gel was washed using MilliQ to remove excess dye, before adding destaining solution (Appendix). The gel was placed on a flat orbital shaker (IKA, KS 260 basic) at 50 rpm to destain, and once the gel was clear it was washed with MilliQ to remove excess destaining solution.

2.2.3 Western Blot analysis

For Western Blots and the corresponding silver and Coomassie staining, Mini-PROTEAN TGX Stain-Free Precast Gels were used, but otherwise the procedure was the same as described above. The ladders used for the Western Blot gels were a Kaleidoscope™ WB standard (Bio-Rad) (6 µl).

To prepare the Western Blot, a 0.2 µm pore nitrocellulose membrane (Bio-Rad), sponges (Bio-Rad) and filter paper (Ahlstrom Falun) were placed in blotting buffer (Appendix) for 30 minutes. Then the gels were placed in a sandwich of sponges, filter paper, nitrocellulose membrane and gel. The sandwich was mounted in an electrophoresis cell (Bio-Rad, Mini-PROTEAN II), supplied with a cooling block and electroblotting was performed for 60 minutes at 100V to transfer the proteins from the gel to the nitrocellulose membrane.

After the electrophoresis, the proteins from the gel would have transferred on to the nitrocellulose membrane visualised by transfer of the coloured ladder bands. The nitrocellulose membranes were placed in separate glass containers on a flat orbital shaker (IKA, KS 260 basic) at 50 rpm in which they were kept for the rest of the Western Blotting procedure. The membranes were washed in TBS for 1 minute, and then blocked in TBS blocking solution (Appendix) for 60 minutes. The membranes were washed twice with TTBS (Appendix) for 5 minutes, before adding the primary antibody solution. This solution consists of antisera from

fish vaccinated with PaL-1, PaL-2, PaL-3, and sera from non-vaccinated control fish. All sera were diluted 1:100 in antibody buffer (Appendix). The membranes were incubated in this solution overnight.

The membranes were washed twice in TTBS, before 10 ml of the rabbit anti-salmon IgM antisera (diluted 1:1000 in antibody buffer) was added to each membrane to incubate for 120 minutes. Then the membranes were washed twice in TTBS, and the goat anti-rabbit Ig conjugated with HPR antisera (Dako Denmark A/S) diluted 1:1000 was added to the membranes to incubate for 90 minutes. The membranes were then washed twice again and incubated in TBS for 5 minutes before they were supplied with a 1:1 mixed substrate solution of Clarity™ Western ECL (Bio-Rad) and incubated for 5 minutes. The membranes were photographed (Bio-Rad, Universal Hood II).

2.3 Liquid chromatography and mass spectrometry (LC-MS/MS)

Two protein bands of PaL-1 identified by reactivity with antibodies from vaccinated salmon, visualised by WB, were cut from a Coomassie stained protein gel (band 1 and band 2). The proteins present in the bands were analysed at PROBE at Haukeland University Hospital. At PROBE, liquid chromatography and mass spectrometry (LC-MS/MS) were used to identify peptides present in the protein bands based on the annoted genome of PaL-1 (unpublished data). In LC-MS/MS, samples are first separated by liquid chromatography, which separates the components of the sample based on their chemical and physical properties. The separated components are then analysed in a mass spectrometer, which ionizes the molecules and measures their mass-to-charge ratio. The mass spectrometer can be operated in tandem mode, meaning that the ions are fragmented, and the fragment ions are analysed in a second mass spectrometer. This allows for high sensitivity and specificity in the identification and quantification of the proteins in the sample.

2.4 In silico protein selection

The raw data files provided by PROBE was analysed using several different databases in the process of predicating which proteins to select for further analysis. To correlate the raw data to *P. atlantica* gv. *salmonicida* genome encoded proteins the sequenced whole genome of *P. atlantica* gv. *salmonicida* (isolate PaL-1) (unpublished data Fish Immunology group) were annotated by Professor Håkon Dahle (UiB) and the protein sequences aligned to the raw data

files. LC-MS/MS categorized certain proteins as hypothetical proteins because they did not have a similar counterpart in the database. These hypothetical proteins were run through Blastp BLAST® National Center for Biotechnology Information (NCBI), to identify which protein it most likely was. The collection of protein sequences was then analysed with PSORTb v3.0.3., a subcellular localization prediction tool, to identify the localizations of the protein within the cell. Lastly, Virulence Factor Database (VFDB) were used to analyse the protein sequences and compare them to sequences that form proteins which are known to be virulence factors.

2.5 Primer design

The pET21a vector contains multiple cloning sites (MCS) that allow for the insertion of a DNA sequence using specific restriction enzymes. When designing primers, restriction enzymes that were compatible with the MCS of pET21a and that did not cut the gene of the protein were selected. The MCS of pET21a contains sites for the restriction enzymes *Nde*I and *BamH*I and they did not cut the gene of the proteins.

*Nde*I is a type II restriction enzyme that recognizes and cuts DNA at a specific DNA sequence called the restriction site. The restriction site recognized by *Nde*I is 5'-CATATG-3'. *BamH*I is also a type II restriction enzyme, and the restriction site recognized by *BamH*I is 5'-GGATCC-3'. *Nde*I cut site was added in forward primer, and *BamH*I in the reverse primer respectively for both genes.

A polyhistidine tag, also known as a His-tag, was added to the reverse primers to facilitate the detection of the proteins. When designing the primers, a polyhistidine tag consisting of a sequence encoding six histidins (6xHisTag), was added to the 5' end of the reverse primers for both proteins. By adding the His-tag sequence to the primers used in the cloning process, the resulting recombinant protein will contain the His-tag. The His-tag was used to detect the proteins in Western Blot and can also be used to purify recombinant proteins. An antibody that recognizes the His-tag was used as the primary antibody and will specifically bind to the His-tagged protein, allowing for its detection and quantification.

2.6.1 DNA isolation

PaL-1 bacteria from freeze stocks were added to 40 ml TSB+ to incubate for 24 hours on a shaking incubator (Infors AG, model Minitron) at 200 rpm and 20°C. The bacteria cultures were centrifuged at 2500 x g for 10 minutes at 4°C and the supernatant discarded. The pellet was resuspended in RNase/DNase free water in a ratio of 1:1 (w/v). Bacterial genomic DNA (gDNA) were isolated using the GenElute™ Bacterial Genomic DNA Kit Protocol (Sigma-Aldrich) with minor modifications including doubling the amount of Lysis solution T, RNase A solution, Proteinase K and Lysis solution C. The first wash (step 8.) was repeated twice. To increase the efficiency of the elution, the column containing gDNA was incubated for 10 minutes instead of 5 minutes after adding the Elution buffer.

The presence and purity of DNA in the samples were analysed on a 1% agarose gel, where 8 µl of isolated DNA sample were mixed with 2 µl of 5x loading buffer before added to each well. 2,5 µl of Invitrogen 1 Kb Plus DNA Ladder was used as ladder. The samples were electrophorized at 70V for 40 minutes. The amount and purity of the DNA samples were quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies Inc., model NanoDrop™ 1000 Spectrophotometer).

2.6.2 PCR and purification of PCR products

The genes encoding one selected target protein from band one (ExlB-like protein) and one from band two (MAM7-like protein), based on the in silico analysis, were amplified using the primers described above following dilutions shown in Table 3 and incubation programme shown in Table 4.

Table 3: *Mixture for PCR Phusion. Two mixtures (different concentration of template) were prepared for amplification of the gene encoding the ExlB-like protein and two for the MAM7-like protein.*

	ExlB-like protein (P1)	**ExlB-like protein (P1)**	**MAM7-like protein (P2)**	**MAM7-like protein (P2)**
suH_2O	31.5	27.5	31.5	27.5
5xHF buffer	10 µl	10 µl	10 µl	10 µl
dNTP (10mM)	1 µl	1 µl	1 µl	1 µl
Forward primer (10 µM)	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Reverse primer (10 µM)	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Template (ng)	1.5 µl (21,8ng)	5.5 µl (33,5ng)	1.5 µl (27,8ng)	5.5 µl (32,5ng)
Phusion	1 µL	1 µL	1 µL	1 µL
Total	50 µL	50 µL	50 µL	50 µL

Table 4: *Incubation program used for PCR Phusion.*

	Temperature	**Time**	**Cycles**
Initial denaturation	98°C	1.5 min	x1
Denaturation	98°C	15 sec	x30
Annealing	58°C	30 sec	
Extension	72°C	45 sec	
Final extension	72°C	10 min	x1

To confirm successful PCR-reaction, the PCR products were analysed on a 1% agarose gel for one hour at 70V. For each of the four samples 5 µl of PCR product were mixed with 2 µL 5x loading buffer (BlueJuiceTM Gel loading Buffer, Thermo Fisher Scientific) before being added to the well. 2,5 µl of Invitrogen 1 Kb Plus DNA Ladder was used as ladder. The PCR products were purified using a GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) following producers'

descriptions. The purified PCR products were then quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies Inc., model NanoDrop™ 1000 Spectrophotometer). The two samples of each gene (Table 3) were now combined creating two Eppendorf tubes: one with the gene encoding the ExlB-like protein and one with the gene encoding the MAM7-like protein.

2.6.3 Plasmid purification, cutting and ligation.

A culture of 10 ml LB media with ampicillin (100 µg/ml) and 50 µl *Escherichia coli* culture from freeze stock containing pET-21a was incubated overnight at 37 °C in a shaking incubator (Infors AG, model Minitron) at 225 rpm. Plasmid was purified from 3 ml culture using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). The purified plasmids were quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies Inc., model NanoDrop™ 1000 Spectrophotometer).

The PCR products encoding the ExlB-like protein and the MAM7-like protein together with pET-21a plasmid were cut using the restriction enzymes *Nde*I and *BamH*I (Table 5). For ligation, the samples were incubated at 15 °C overnight before being heat inactivated at 65°C for 10 minutes on heat block (Eppendorf).

Table 5: *Mixtures for cutting the PCR products encoding the MAM7-like protein, the ExlB-like protein and pET-21a plasmid with restriction enzymes.*

	pET21a	**ExlB-like**	**MAM7-like**
DNA	41 µl	6.7 µl	5.7 µl
10 x buffer R3.1	5 µl	5 µl	5 µl
*Nde*I	2 µl	2 µl	2 µl
*BamH*I	2 µl	2 µl	2 µl
H_2O	-	34.3 µl	35.3 µl
Total	50 µl	50 µl	50 µl

After cutting, the samples were cleaned using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) and quantified with NanoDrop to determine the amount of DNA insert needed in the ligation. For the ligation, the protocol for T4 DNA Ligase by BioLabs Inc. was followed (Table 6).

Table 6: *Mixture for ligation with T4 DNA ligase of plasmid and gene encoding the ExlB-like protein and plasmid and gene encoding the MAM7-like protein.*

	ExlB-like	**MAM7-like**
Reaction Buffer (10X)	2 µl	2 µl
Plasmid	2.5 µl	2.5 µl
Insert	3.5 µl	2.5 µl
H_2O	11 µl	12 µl
T4 DNA Ligase	1 µl	1 µl
Total	20 µl	20 µl

2.6.4 Transformation and culturing

TOP10 competent cells were obtained from -80°C freezer and kept on ice for slow thawing. 5 µl of each ligation sample was added to the competent cells and incubated for 30 minutes on ice. The bacterial cells in tubes were then heat shocked in water at 42 °C for 30 seconds before being put back on ice. 250 µl of SOC media (Invitrogen, Thermo Fisher Scientific) was then added to each tube before incubating horizontally at 37 °C for one hour in shaking incubator (Infors AG, model Minitron) at 225 rpm. After incubation, 20 µl and 200 µl bacteria suspension from each tube was spread on four separate LB-agar plates containing ampicillin (100 µg/ml) and incubated over night at 37 °C. Eight colonies of TOP10 competent cells containing plasmid with the ExlB-like encoding gene insert and eight colonies of TOP10 competent cells containing plasmid with the MAM7-like encoding gene insert were subcultured onto new LB-agar plates and incubating at 37 °C overnight prior to PCR screening.

2.6.5 PCR screening

To determine whether the transformation was successful or not, a PCR screening was performed. 16 Eppendorf tubes were filled with 20 µl nuclease free water (Sigma-Aldrich). One colony from each of the subcultured colonies were then resuspended by adding them to the separate Eppendorf tubes. PCR tubes (0.2 ml) were prepared for the samples including one tube for control. A mastermix for PCR screening with DreamTaq was mixed (Table 7) and 23 µl was supplied to each PCR tube. 2 µl of bacteria suspension were distributed to each tube and for the control 2 µl of plasmid with insert (pET-21a IFNh) was used. The PCR was run following the program described in Table 8.

Table 7: *Mastermix for PCR Screening with DreamTaq.*

	x 1	**x 18 (Mastermix)**
H_2O	17.4 µl	313.2 µl
10 x buffer	2.5 µl	45 µl
dNTP	0.5 µl	9 µl
pET11a forward primer	1.6 µl	28.8 µl
pET11a reverse primer	0.5 µl	9 µl
DreamTaq	0.5 µl	9 µl
Total	23 µl	414 µl

Table 8: *PCR program for screening with DreamTaq.*

	Temp	**Time**	**Cycles**
Initial denaturation	95 °C	10 min	x 1
Denaturation	95 °C	0.5 min	x 40
Annealing	57 °C	0.5 min	
Extension	72 °C	2 min	
Final extension	72 °C	10 min	x 1

Once the PCR was complete the PCR products were visualized on a 1% agarose gel to confirm a successful PCR reaction and ligation. 5 µl of the PCR product were mixed with 2 µl of 5x loading buffer (BlueJuiceTM Gel loading Buffer, Thermo Fisher Scientific). This mix of 7 µl was then pipetted into each gel chamber. The gel was run at 90V for 40 minutes in 1xTAE buffer (Appendix) before visualizing the bands using UV light (Syngene, G: Box).

2.6.6 Plasmid purification and sanger sequencing

Two colonies of TOP10 competent cells containing plasmid with the ExlB-like encoding gene and two colonies of TOP10 competent cells containing plasmid with the MAM7-like encoding gene was randomly selected for subculturing. The colonies were suspended in 10 ml LB-media supplied with 10 µl ampicillin and incubated at 37 °C in shaking incubator (Infors AG, model Minitron) at 225 rpm overnight. Plasmids from these subcultured colonies were purified using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) as described in section 2.6.3. The purified

plasmids were quantified with NanoDrop and prepared for sanger sequencing by mixing with Big-Dye, Sequencing buffer, water and forward or reverse primer (Table 9) before running the PCR reaction (Table 10). Four 0.2 ml PCR tubes were prepared. Two tubes for each of the two plasmids, one tube containing forward primer and one tube containing reverse primer. Sequencing was performed by the DNA sequencing facility at BIOLOGEN, University of Bergen, Norway. The sanger sequencing results were analysed in Unipro UGENE and base pairs that did not give a clear answer (N) were corrected by analysing the histograms.

Table 9: *Sample preparation for sanger sequencing.*

	Sample	**BigDye**	**Buffer**	**Template (200ng)**	**H_2O**	**Forward primer**	**Reverse primer**
ExlB-like	HS1	1 µl	1 µl	5.5 µl	1.5 µl	1 µl	-
	HS2	1 µl	1 µl	5.5 µl	1.5 µl	-	1 µl
MAM7-like	HS3	1 µl	1 µl	4.5 µl	2.5 µl	1 µl	-
	HS4	1 µl	1 µl	4.5 µl	2.5 µl	-	1 µl

Table 10: *PCR program for sanger sequencing.*

	Temp	**Time**	**Cycles**
1	96 °C	5 min	x 1
2	96 °C	10 sec	x 35
	50 °C	15 sec	
	52 °C	4 min	
3	4°C	-	x 1

2.6.7 Plasmid transformation with electroporation

Purified plasmids containing the gene encoding the ExlB-like protein and purified plasmids containing the MAM7-like protein and two electrocompetent Codon+ *Escherichia coli* in Eppendorf tubes were collected from -80 °C freezer and put on ice for slow thawing. A SOB-like media was mixed containing 9.8 ml LB, 0.1 ml 1M MgCl and 0.1 ml 1ML $MgSO_4$ which gave a total of 10 ml media. 0.2 ml was pipetted out and replaced with 0.2 ml 20% glucose for better growth conditions. Once the thawing was done 2 µl of each purified plasmid sample were

added to the Codon+ cells to incubate on ice for 3 minutes creating a transformation solution. The transformation solutions were then transferred to 0.2 cm electroporation cuvettes. The electroporation was performed with 2.5kV (electric potential), 200 Ω (resistance) and 25 µF (capacitance). After the electroporation, 1 ml of SOB-media was added directly to the cuvettes. The transformation-SOB solutions were then transferred back to the Eppendorf tubes to incubate at 37 °C, horizontally in shaking incubator (Infors AG, model Minitron) at 225 rpm for 30 minutes. After incubation, 20 µl Codon+ and 20 µl LB-media were smeared on to LB plates with ampicillin and chloramphenicol to incubate overnight at 37 °C.

2.6.8 Culturing and preparation of antigens for protein gels

For expression of recombinant proteins, two 15 ml Falcon tubes containing 3 ml LB media supplied with 35 µl chloramphenicol and 35 µl ampicillin were prepared. One for Codon+ *E. coli* containing the plasmid encoding the ExlB-like protein (Codon+/ExlB) and one for Codon+ *E. coli* containing the plasmid encoding the MAM7-like protein (Codon+/MAM7), and one colony were resuspended in the medium. The cultures were incubated at 37 °C in a shaking incubator (Infors AG, model Minitron) at 225 rpm overnight.

After incubating, four 250 ml Erlenmeyer flasks were prepared, two for each of the two Codon+ *E. coli*. Then 100 ml of LB media supplied with 100 µl ampicillin and 100 µl chloramphenicol was supplied to each of the Erlenmeyer flasks before suspending 1 ml of culture in each. The cultures were incubated at 37 °C in shaking incubator (Infors AG, model Minitron) at 225 rpm for 3-4 hours to mid log phase. The culture was split in two and IPTG (isopropylthio-β-galactoside) was added in one of the Codon+/ExlB cultures and in one of the Codon+/ MAM7 cultures. The cultures were then incubated for three more hours.

After incubating, the contents of the Erlenmeyer flasks were transferred to four 50 ml tubes and centrifuged at 2500 x g for 15 minutes (Beckman Coulter Inc., Allegra X-15R Centrifuge). The supernatant was discarded, and the pellets were suspended in RNase free water 10:1 water to wet weight ratio. Four Eppendorf tubes were prepared (one for each sample) with 100 µl bacteria (pellet suspension) and 100 µl sample buffer for SDS-PAGE (Appendix). These samples were then used in protein gels for SDS-PAGE and silver and Coomassie staining (following the procedures described in section 2.2.2) to identify if the target proteins were produced, and in Western Blots to identify if they were targeted by antibodies produced in PaL-1 vaccinated salmon. For the Western Blots the procedures described in section 2.2.3 were

followed with exception of now including an antibody targeting the His-tag as a positive control (6xHisTag Monoclonal Antibody) (HIS.H8) Product# (MA1-21315) (Thermo Fisher – Lot#WJ332937). The 6xHisTag monoclonal antibody was supplied to the membranes in a 1:2000 dilution in antibody buffer and left for incubation overnight. The membranes were washed twice in TTBS, and the goat anti-mouse Ig conjugated with HPR antisera (Dako Denmark A/S) diluted 1:1000 was added to the membranes to incubate for 90 minutes. The following procedure were similar as described in section 2.2.3.

3 – Results

3.1 Growth curves

The growth curves for the bacterial isolates, PaL-1, PaL-2, PaL-3 and PaL-4, are presented in Figure 5. The curves show a similar growth pattern for all four isolates. The curves show that early exponential growth phase was reached for all isolates after 10h and late exponential growth phase was reach after 16h. Accordingly stationary phase was reached after 24h.

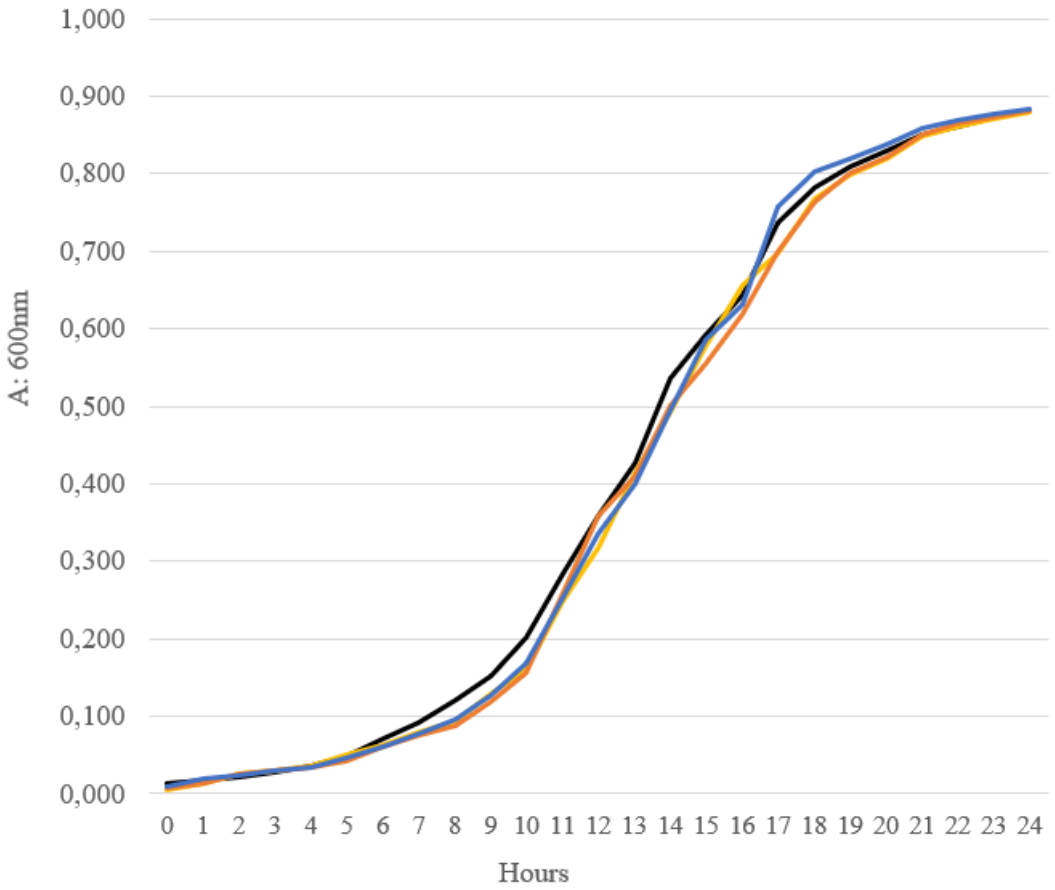

Figure 5: *Growth curves showing optical density over time of broth cultures of PaL-1, PaL-2, PaL-3, and PaL-4. Black line: PaL-1, Yellow line: PaL-2, Orange line: PaL-3, Blue line: PaL-4.*

3.2 Bacterial growth and protein profiles at different concentrations of supplemented FCS in the growth media

Cell counts (bacteria/mL) measured in the CASY cell counter were compiled as graphs showing number of cells and cell sizes (µm) (Figure 6).

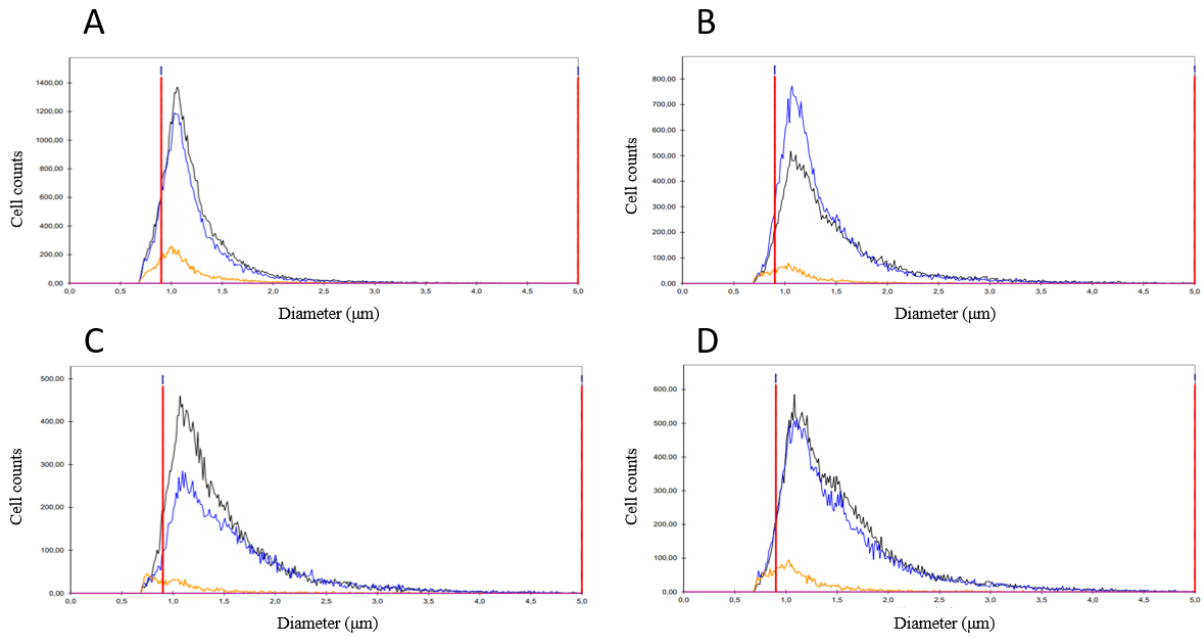

Figure 6: *Graphs from CASY cell counts demonstrating the number and size of cells in the bacteria samples. Black line show bacteria grown in medium containing 10% FCS, blue line 5% FCS and orange line 0% FCS. Note variable cell numbers on y-axis.* ***A:*** *PaL-1,* ***B:*** *PaL-2,* ***C:*** *PaL-3 and* ***D:*** *PaL-4.*

The graphs (Figure 6) show the number and size of cells in the bacteria cultures after 16 hours of incubation in TSB with different concentrations of FCS. Figure 6A, B, C and D represent isolates PaL-1, PaL-2, PaL-3 and PaL-4, respectively, and the colours of the lines represent percentage of FCS supplied to the growth media. In all graphs, black line represents cultures supplied with 10% FCS, blue line represents cultures supplied with 5% FCS and orange line represents cultures without FCS supplement. For all cultures, it is clear that the bacteria are dependent on FCS in the growth media for growth.

The data presented in Table 11 confirms the significance of FCS supplement in the growth media. The number of cells is higher for the 5% and 10% FCS concentration after both the initial incubation with bacteria from freeze stock cultures (1 ml) (Figure 6) and after the subcultivation with bacteria from the initial incubation (1 ml) (Table 11).

Table 11: *Cell counts and aggregation factor for bacterial isolates incubated for 16 hours and subcultured for 16 hours with different concentrations of FCS.*

Isolate	Initial incubation (16h)			Subcultivation (16h)		
	FCS (%)	**Cell Count (cells/mL)**	**Aggregation Factor**	**FCS (%)**	**Cell Count (cells/mL)**	**Aggregation Factor**
PaL-1	10 %	1.913E+09	2.448	10 %	1.943E+09	3.646
	5 %	1.577E+09	2.394	5 %	1.742E+09	4.139
	0 %	4.619E+08	3.656	0 %	8.371E+07	9.578
PaL-2	10 %	1.806E+09	3.962	10 %	1.763E+09	3.882
	5 %	1.659E+09	3.027	5 %	1.727E+09	4.160
	0 %	1.383E+08	3.601	0 %	8.457E+07	4.762
PaL-3	10 %	1.498E+09	3.727	10 %	1.755E+09	4.008
	5 %	1.403E+09	4.638	5 %	5.819E+08	4.168
	0 %	8.726E+07	4.634	0 %	5.808E+07	3.967
PaL-4	10 %	2.451E+09	4.219	10 %	2.024E+09	3.924
	5 %	2.189E+09	4.245	5 %	1.334E+09	4.360
	0 %	1.716E+08	3.611	0 %	7.737E+07	4.770

The significance of FCS supplement was further confirmed with silver stained SDS-PAGE gels (Figure 7). Although 1 ml bacterial sample were harvested from each culture, and the pellets were of approximate similar sizes there are differences in the amounts of proteins in the different bands visualised on the silver-stained gels (Figure 7). The number of bacterial cells was not adjusted to the same amount, therefore there were some differences in the intensity of the bands between the isolates. The different bacteria isolates (Figure 7A-D) all show visual dark coloured protein bands when cultured with 5% and 10% supplemented FCS (lanes 2 and 3 in Figure 7A-D) and only weak protein bands when cultured without FCS supplement (lane 1 in Figure 7A-D). But there is no clear difference in location or presence of protein bands across the isolates or across culture conditions.

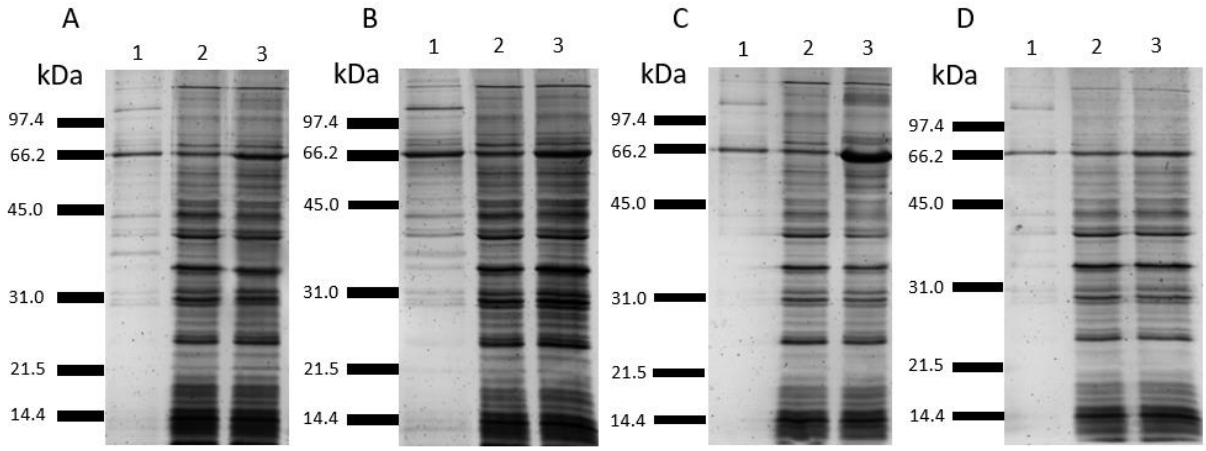

Figure 7: *Silver stained SDS-PAGE gels showing protein profiles of* ***A:*** *PaL-1,* ***B:*** *PaL-2,* ***C:*** *PaL-3, and* ***D:*** *PaL-4 when cultured in TSB supplemented with different concentrations of FCS, lane 1: 0% FCS, lane 2: 5% FCS and lane 3: 10% FCS.*

3.3 Analysis of immunogenic proteins

The Western Blots (Figure 8) were performed using sera from previously vaccinated fish. The fish had been vaccinated with formalin inactivated bacteria, formulated in Freund's incomplete adjuvant, of isolates PaL-1 (Figure 8A), PaL-2 (Figure 8B) or PaL-3 (Figure 8C). Figure 8D show the result from the membrane blotted against sera harvested from non-vaccinated control fish. The WBs in Figure 8, indicate the molecular weights of the proteins that the fish have produced antibodies against following vaccination.

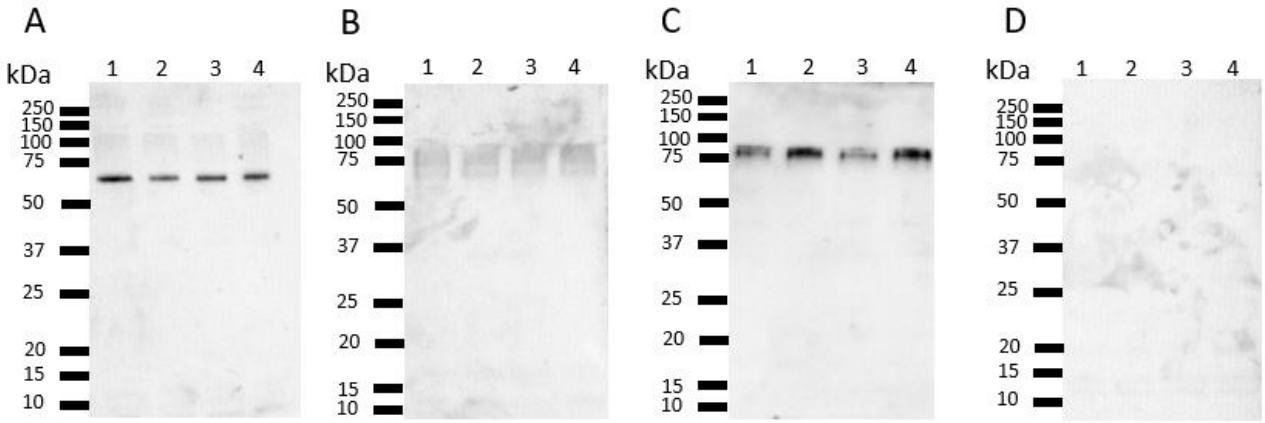

Figure 8*: Western Blot with sera from vaccinated fish.* ***A:*** *vaccinated with PaL-1,* ***B:*** *vaccinated with PaL-2,* ***C:*** *vaccinated with PaL-3 and* ***D:*** *Control (non-vaccinated). Lane 1: PaL-1, lane 2: PaL-2, lane 3: PaL-3, lane 4: PaL-4.*

Sera from fish vaccinated with PaL-1 bound to similar sized proteins of PaL-1, PaL-2, PaL-3 and PaL-4, proteins around roughly 60 kDa of size (Figure 8A). Sera from fish vaccinated with PaL-2, show a weaker staining and more diffuse binding, but show similar results for PaL-1, PaL-2, PaL-3 and PaL-4 as antigens (Figure 8B). Sera from fish vaccinated with PaL-3 bound to similar sized proteins of PaL-1, PaL-2, PaL-3 and PaL-4, around roughly 90 kDa of size (Figure 8C). The WB using sera from non-vaccinated control fish did not indicate presence of cross-reactive proteins targeting the bacterias' expressed proteins (Figure 8D).

Further, protein profiles of PaL-1 – PaL-4 were portrayed in Coomassie stained (Figure 9A) and silver stained (Figure 9B) SDS-PAGE gels. The same bacteria samples (lane 1-4 Figure 9) were used as in the WBs (Figure 8). The location of the proteins where the salmon antibodies bound were estimated as indicated by arrowheads in Figure 9A and B. The protein bands marked with black and white arrowheads were cut out of the Coomassie stained gel for further analysis.

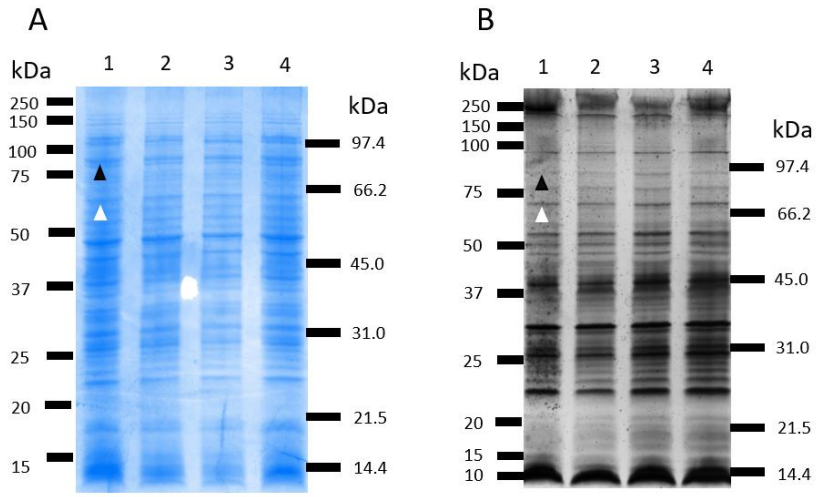

Figure 9: *Coomassie stained gel and silver stained SDS-PAGE gels displaying protein profiles of PaL-1 – PaL-4.* ***A:*** *Coomassie stained gel,* ***B:*** *Silver-stained gel. Lane 1: PaL-1, lane 2: PaL-2, lane 3: PaL-3, lane 4: PaL-4. Black arrowhead: Proteins roughly around 90 kDa. White arrowhead: Proteins roughly around 60 kDa.*

3.4 In silico analysis

The raw data files received after LC-MS/MS analysis of the cut protein bands at PROBE contained information on number of identical peptides, length of amino acid sequences and molecular weight of peptide sequences of proteins within the bands. Certain proteins were categorized as hypothetical proteins by the LC-MS/MS because they did not have a similar counterpart in the database. These hypothetical proteins were analysed with Blastp BLAST®

National Center for Biotechnology Information (NCBI), to identify which protein it most likely was.

Further, in silico analysis was performed to determine the most promising candidates for vaccine development against pasteurellosis, and the results are shown in Table 12 and Table 13. In Table 12 the proteins are narrowed down to molecular weights between 40-70 kDa, and in Table 13 they are narrowed down to 80-100 kDa. This initial selection was performed to match the positions of the proteins having the molecular weight where the salmon antibodies bound (Figure 8). The subcellular localization of the proteins determined by PSORTb software narrowed the proteins down according to location, outer membrane (OM), extracellular (E) and periplasmic (P), with the probability of correct localization (0-100). The Virulence Factor Database (VFDB) further determines if a protein is similar to a known bacterial virulence factor. In addition to this the possible adhesion-related functionality was taken into perspective. The proteins that seemed most promising in the 40-70 kDa selection are highlighted with bold font (number 1 and 7 in Table 12).

Table 12: *Three extracellular, ten outer membrane and eight periplasmic localized proteins are the most promising candidates for vaccine development against* P. atlantica *in the band located at 60kDa after in silico analysis. E: extracellular, OM: outer membrane and P: periplasmic.*

#	Protein	Localization	MW[kDa]	Virulence Factor Database	E-value	Identity
1	**ShlB/FhaC/HecB family hemolysin secretion/activation protein [Pasteurella atlantica]**	**OM(100)**	**68**	**(exlB) hemolysin activator protein [Pseudomonas aeruginosa PA7]**	**1,00E-30**	**25 %**
2	ShlB/FhaC/HecB family hemolysin secretion/activation protein [Pasteurella atlantica].	OM(100)	67,1	(exlB) hemolysin activator protein [Pseudomonas aeruginosa PA7]	3,00E-31	26 %
3	Translocation and assembly module subunit TamA	OM(100)	66,1	n/a		
4	Peptide transport periplasmic protein SapA	P(99)	64,9	n/a		
5	Heme-binding protein A	P(98)	60,3	n/a		
6	Periplasmic oligopeptide-binding protein	P(94)	59,7	n/a		
7	**Hypothetical protein [Pasteurella atlantica]**	**E(95)**	**56,9**	**(sdrE) Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein [Staphylococcus aureus subsp. aureus MW2]**	**8,00E-05**	**23 %**
8	TPR repeat-containing protein	OM(95)	54,9	(ACICU_RS04590) porin family protein [Acinetobacter baumannii ACICU]	7,00E-21	20 %
9	Toxin and drug export protein A	OM(100)	52	(mtrE) multidrug efflux pump channel protein MtrE [Neisseria meningitidis MC58]	3,00E-20	19 %
10	Hypothetical protein [Pasteurella atlantica]	E(96)	51,3	(sdrC) Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein [Staphylococcus aureus subsp. aureus MW2]	1,00E-13	24 %
11	Imelysin [Pasteurella skyensis].	P(97)	50,2	n/a		
12	Periplasmic serine endoprotease DegP	E(98)	50	(mucD) serine protease MucD precursor [Pseudomonas aeruginosa PAO1]	1,00E-72	38 %
13	Patatin-like phospholipase family protein [Pasteurella atlantica]	P(95)	49,2	n/a		
14	Methylamine utilization protein MauG	P(100)	47,5	n/a		
15	Tol-Pal system protein TolB	P(98)	46,5	n/a		
16	47 kDa outer membrane protein	OM(100)	45,4	n/a		
17	Murein hydrolase activator EnvC	P(99)	45,3	(lepB) Dot/Icm type IV secretion system effector LepB, Rab1 GTPase activating protein (GAP) [Legionella pneumophila subsp. pneumophila]	1,00E-08	23 %
18	Maltoporin	OM(100)	44,7	n/a		
19	Hypothetical protein, partial [Pasteurella atlantica]	OM(95)	44,5	(cbpA) collagen-binding adhesin CbpA [Clostridium difficile 630]	0,009	29 %
20	MAG: FIVAR domain-containing protein [Alcaligenaceae bacterium]	OM(95)	43,2	(EF3023) polysaccharide lyase, family 8 [Enterococcus faecalis V583]	6,00E-07	31 %
21	Membrane-bound lytic murein transglycosylase A	OM(99)	41,5	n/a		

The proteins that seemed most promising in the 80-100 kDa selection are highlighted with bold font (number 1 and 4 in Table 13).

Table 13: *Three outer membrane and two periplasmic localized proteins are the most promising candidates for vaccine development against* P. atlantica *from the band at 90kDa after in silico analysis. E: extracellular, OM: outer membrane and P: periplasmic.*

#	Protein	Localization	MW[kDa]	Virulence Factor Database	E-value	Identity
1	**putative protein**	**OM(98)**	**97,6**	**(mam7) multivalent adhesion molecule MAM7 [Vibrio parahaemolyticus RIMD 2210633]**	**e-137**	**32 %**
2	Periplasmic nitrate reductase	P(93)	93,3	n/a		
3	Trimethylamine-N-oxide reductase	P(93)	93	(narG) nitrate reductase subunit alpha [Mycobacterium tuberculosis H37Rv]	5,00E-06	30 %
4	**LPS-assembly protein LptD**	**OM(90)**	**90,3**	**(sca1) cell surface antigen Sca1 [Rickettsia typhi str. Wilmington]**	**0.004**	**24 %**
5	Outer membrane protein assembly factor BamA	OM(90)	89,5	n/a		

Of the most promising proteins, number 1 was chosen for further analysis from each of the Tables 12 and 13. From Table 12 the ExlB-like protein was selected for further analysis, in the following section referred to as Protein 1 (P1). From Table 13 the MAM7-like protein was selected, in the following section referred to as Protein 2 (P2).

3.5 Recombinant protein expression

To express recombinant proteins, genes encoded the proteins were amplified with PCR (Figure 10A). After cloning, PCR screening was performed to select for clones in which the PCR product had been inserted into the vector (Figure 10B, C). Plasmid with pET21a IFNh was used as positive control termed 1 and 2. This confirms that the genes encoding P1 and P2 successfully had been inserted into pET21a. Correct sequences were verified by Sanger sequencing.

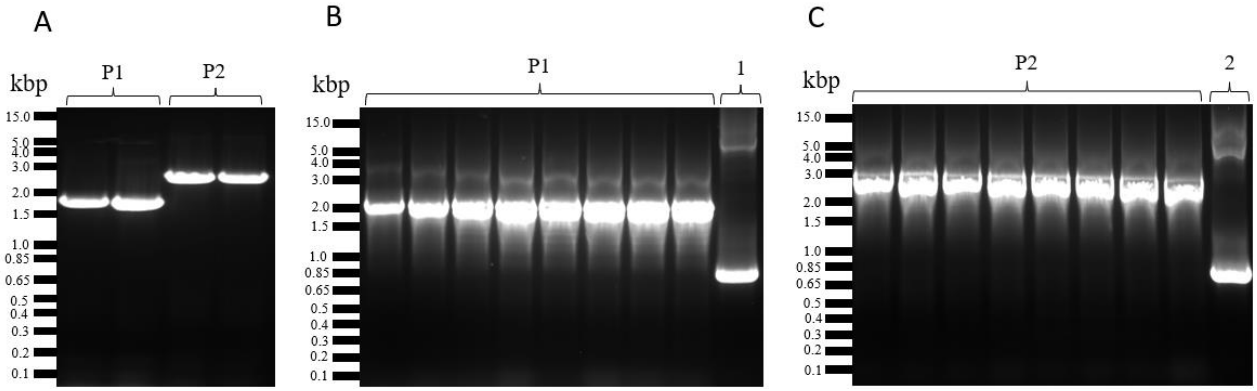

Figure 10: *PCR screening.* ***A:*** *Two samples of Protein 1 (P1) and two samples of Protein 2 (P2) to confirm successful PCR-reaction,* ***B:*** *Eight samples from Protein 1 (P1) and plasmid with insert (pET-21a IFNh) as control (1) to confirm successful PCR-reaction and ligation,* ***C:*** *Eight samples from Protein 2 (P2) and plasmid with insert (pET-21a IFNh) as control (2) to confirm successful PCR-reaction and ligation.*

Thereafter P1 and P2 were transformed into *Codon+ E. coli* for protein expression. They were cultured with and without IPTG. Proteins from bacteria cultured with IPTG is in the following identified with (+) and proteins without IPTG is identified with (-). The proteins were visualized on protein gels and used in WBs (Figure 11). The silver stained SDS-PAGE gel (Figure 11A) show the expressed proteins at approximately 60 kDa and 90 kDa, marked with white and orange arrowheads respectively. WB using sera from vaccinated fish (Figure 11B) show antibody binding for proteins at around 60 kDa for both bacteria cultured with and without IPTG. WB using the monoclonal 6xHisTag antibody (Figure 11C) show antibody binding to the 90 kDa P2+ protein, and successful identification of the MAM7-like recombinant protein.

The P1+ protein was expressed (Figure 11A) but showed no reactivity to the 6xHisTag antibody. Figure 11D is a control with sera from non-vaccinated fish.

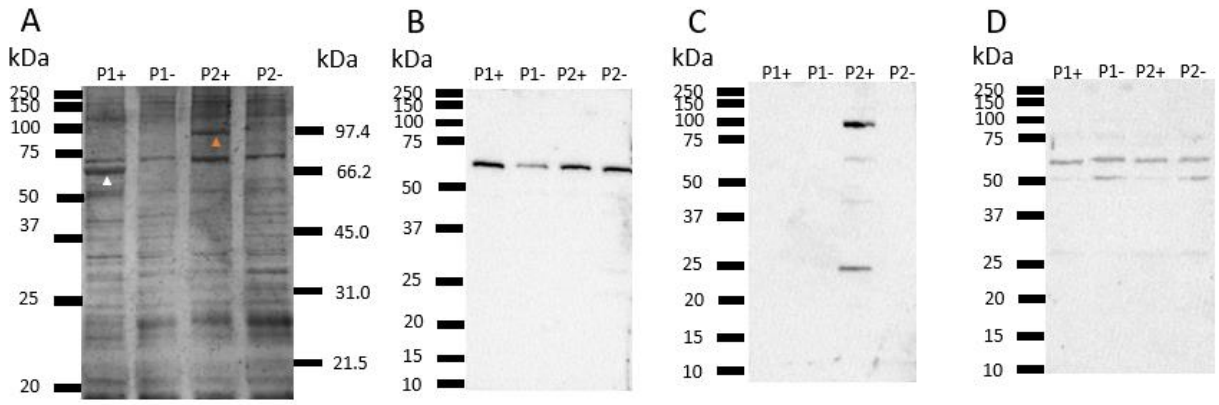

Figure 11: *silver staining and Western Blotting.* ***A:*** *silver stained SDS-PAGE gel of Codon+ E. coli cultured with (P1+) and without (P1-) IPTG,* ***B:*** *WB using sera from vaccinated fish,* ***C:*** *WB using a 6xHisTag antibody,* ***D:*** *WB Control, sera from non-vaccinated fish.* ***A:*** *White arrowhead shows the expressed protein in P1+ and orange arrowhead shows the expressed protein in P2+.*

The results from the trials using new primers for P1 are shown in Figure 12. The protein profiles of bacteria cultured with and without IPTG are shown in Figure 12A and B. In both the silver stained SDS-PAGE gel (Figure 12A) and the Coomassie stained gel (Figure 12B) P1 and P2 are marked with white and orange arrowheads respectively, and clearly show expression of P1 and P2 in cultures incubated with IPTG. The size of the P1+ expressed protein is roughly around 60 and the size of the P2+ expressed protein is roughly around 90 kDa. WB using sera pooled from ten vaccinated fish (Figure 12C) show antibody binding for proteins at around 80 kDa for both bacteria cultured with and without IPTG. Figure 12D show WB using the His-tag antibody and only the P2+ protein is identified. Figure 12E is a control with sera from non-vaccinated fish.

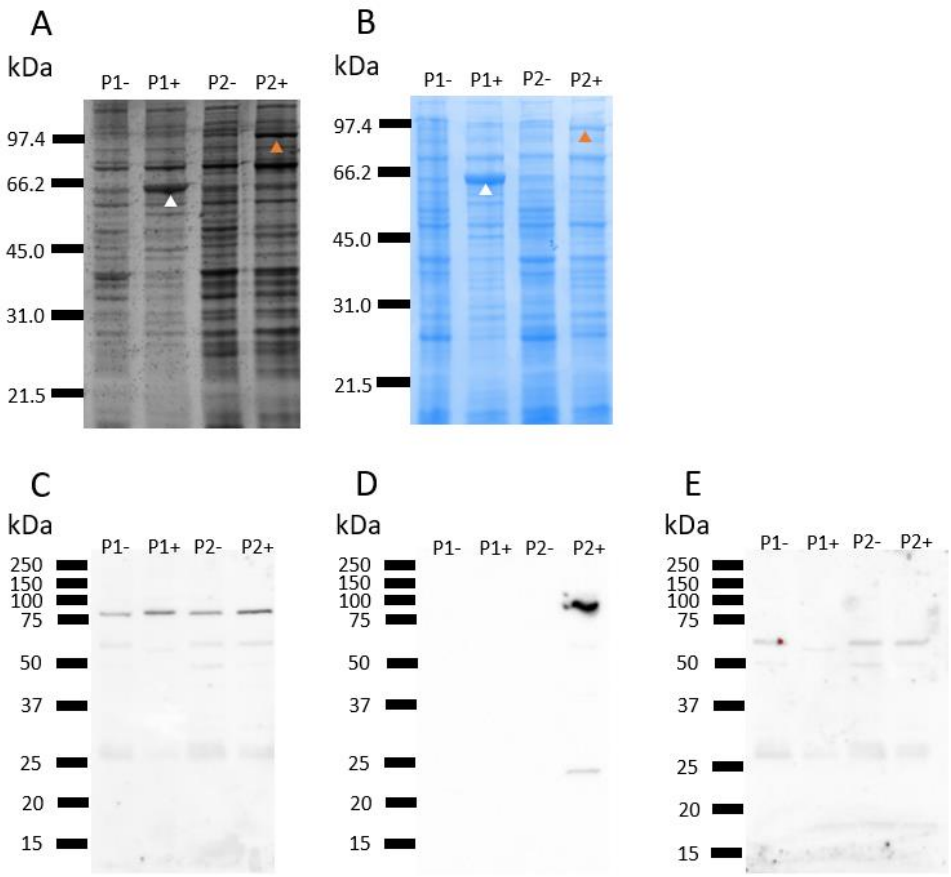

Figure 12: *Silver stained and Coomassie stained SDS-PAGE gels and Western Blotting.* ***A:*** *Silver stained gel,* ***B:*** *Coomassie stained gel,* ***C:*** *WB with sera pooled from ten vaccinated fish* ***D:*** *WB with 6xHisTag antibody, E: WB Control, sera from non-vaccinated fish. P1- is E. coli with plasmid encoding P1 cultured without IPTG, P1+ is E. coli with plasmid encoding P1 cultured with IPTG, P2- is E. coli with plasmid encoding P2 cultured without IPTG and P2+ is E. coli with plasmid encoding P2 cultured2 with IPTG. White arrowhead shows the expressed protein in P1+ and orange arrowhead shows the expressed protein in P2+.*

4 – Discussion

The aims of this study were to identify immunogenic proteins of *P. atlantica* gv. *salmonicida* and to express immunogenic proteins of the bacteria recombinantly in *E. coli*. To better understand the growth requirements of *P. atlantica* gv. *salmonicida* the requirement for blood components were studied by analysing growth at different concentrations of supplemented FCS in broth cultures.

4.1 Growth curves

The four isolates of *P. atlantica* gv. *salmonicida* (PaL-1 – PaL-4) all show great similarity in growth ratio, protein profiles and requirements for FCS in the growth media. This knowledge and establishment of a basic and standardized protocol for culturing is important to ensure consistency and reproducibility across following studies. The compiled growth curves enable harvest of the bacteria at a defined growth stage. This allows better control of experimental conditions which ensures that observed differences are due to experimental variables rather than differences in bacteria cell physiology. In the growth curves the four bacterial isolates show similar growth patterns where they all reach late exponential growth phase after 16 hours of incubation. Harvesting bacteria in the late exponential growth phase has several advantages. The metabolic activity of the bacterial cell is still high, leading to active replication, expression of genes and protein production (Galloway et al., 2003; Khushoo et al., 2004). The cells are also healthier and have a reduced risk of lysis compared to when they are in the stationary phase.

A basic protocol for culturing and knowledge of the bacteria is fundamental in developing functional vaccines. For vaccine production a high yield, high cell number, and optimal expression of immunogenic components are required when harvesting. In the prosses of making a recombinant vaccine the first step is to identify a suitable protein antigen that can induce a protective immune response. This includes bacterial surface proteins, secreted proteins and other components known to interact with the host immune system. The late exponential growth phase represents a stage where the bacteria have optimal protein expression, and potential immunogenic proteins and virulence factors will be present (Galloway et al., 2003; Khushoo et al., 2004).

The silver staining assay presented in Figure 7 demonstrated the protein profiles of all four bacterial isolates which appeared to be identical, both when it comes to sizes of proteins

expressed and the level of proteins at different sizes. Though it should be kept in mind that the proteins visualized in the different bands may be different across the isolates, and also the ratio of proteins within the bands. Even so, the similarities suggests that under the specific growth conditions, the isolates exhibit similar expression of proteins. Consequently, a vaccine developed including one of the isolates would likely offer protection against infection by all four isolates.

4.2 Bacterial growth at different concentrations of supplemented FCS

Through the bacterial growth tests with different concentrations of supplemented FCS in the growth media, it was clear that the bacteria were dependent on FCS in the media for growth. The graphs compiled from cell counts after 16 h incubation (Figure 6) combined with the sub cultivation test (Table 11) clearly showed that the bacteria cultured without supplement of FCS had low growth compared to the ones cultured with supplement of FCS (5% and 10%). The cell counts in Figure 6 show that for PaL-1 and PaL-4 there was almost no difference between 5% and 10% supplement of FCS. In PaL-2 there was higher cell counts for the culture with 5% compared to 10% supplement of FCS. In PaL-3 the opposite was the case, with higher cell counts for the culture with 10% than 5% supplement of FCS. In common for all isolates was that there was lower cell counts in the bacterial cultures without supplement of FCS, and that the cell count decreases in the subcultures without FCS supplement. This was also the case for the silver stained SDS-page gels displayed in Figure 7. There are barely visible bands in the lanes with bacteria cultured without supplement of FCS. Bacteria cultured with 5% and 10% supplement of FCS provide dark and visible protein bands, maybe slightly darker for the ones supplemented with 10% FCS. The individual differences in intensity of the bands between the isolates is because the number of bacterial cells was not adjusted to the same amount. In previous studies on *P. atlantica,* both on salmon and lumpfish, it has been applied 10% of supplemented FCS in the growth media (Ellul et al., 2019; Sandlund et al., 2021). It is not yet known what component(s) in the FCS the bacteria require, but it is undoubtedly important for growth.

4.3 Analysis of immunogenic proteins

In a previous master project (Holstad 2022, unpublished) vaccines based on formalin inactivated whole bacteria (PaL-1 – PaL-3) formulated in Freund's incomplete adjuvant were

made and used for vaccination of salmon. Sera harvested 500 degree days after vaccination showed that antigen specific antibodies were produced in salmon. These sera were in the current study used in WBs to identify immunogenic proteins targeted by the antibodies. The inactivated bacterial vaccine exposes the fish immune system to components of the bacteria, including surface proteins and other molecules such as lipopolysaccharides. The bacterial components serve as PAMPs which are recognized by the fish innate immune cells through PRRs. This recognition initiates an immune response, leading to the activation of APCs, such as macrophages and dendritic cells. APCs process and present bacterial antigens to CD4+ T-cells which differentiate into Th subsets and may activate B-cells. Activated B-cells, which have also recognised the antigen by binding to its BcR, may differentiate into antibody secreting plasma cells secreting antigen specific antibodies binding to the bacterial antigens initially recognized by the BcR.

The WB assay displayed the molecular weight of the proteins that the antibodies bound to. The antibodies seemed to bind to the proteins of the bacterial isolates at the same molecular weight, but there were some differences between the different sera used in the WBs. The sera from fish vaccinated with the PaL-1 vaccine (Figure 8A) had antibodies that bound to proteins with molecular weight roughly around 60 kDa. The sera from fish vaccinated with the PaL-2 vaccine (Figure 8B) did not give a clear result of the molecular weight of the proteins but it seemed to be a response roughly around 90 kDa. The sera from fish vaccinated with the PaL-3 vaccine (Figure 8C) had antibodies that bound to proteins with molecular weight roughly around 90 kDa. However, the identity of these proteins is still unknown, as well as the number of different proteins present in each band. Further analysis was therefore required to identify which proteins were present and which that most likely caused the immune response.

4.4 Reverse vaccinology – In silico analysis

By the methods of reverse vaccinology (RV), it is possible to quickly identify potential vaccine candidates in silico. Some of the many advantages of RV are identification of non-viable vaccine candidates early in the process, enhanced safety by focusing specific antigens instead of live attenuated or inactivated bacteria, and the potential of discovering possible multivalent vaccine candidates in the genome (Delany et al., 2013; Kanampalliwar et al., 2013). Several highly effective recombinant protein subunit vaccines have been developed for the aquaculture industry using RV principles by identifying targets in silico, which ultimately proved to be

protective. A genomic analysis of *P. atlantica* gv. *cyclopteri* (Ellul et al., 2021) highlighted the potential of RV in aquaculture. The recent advancements in in silico analysis tools has thus enabled us to identify and evaluate potential virulence factors and immunogenic components by examining the genome of *P. atlantica* gv. *salmonicida*. These methods form the core principles of RV which streamlines vaccine development by transforming extensive, time-consuming, and broad laboratory work into targeted functional analyses and *in vitro/in vivo* trials. The first standalone RV program, known as NERVE, was published in 2006, and since then, significant progress and comparative analyses have been made in the field of RV (Dalsass et al., 2019; Vivona et al., 2006). A critical aspect of predicting virulence factors is determining the subcellular localization of bacterial proteins. Proteins that are secreted extracellularly or exposed on the bacterial surface have a high likelihood of playing a role in bacterial virulence (Ellul et al., 2021).

The raw data files received after LC-MS/MS analysis performed at PROBE contained information on thousands of peptide sequences encoding proteins. The process of narrowing down the proteins is described in section 2.4. Table 12 and 13 show the proteins in the molecular weight range identified by the antigen specific antibodies, subcellular localization, and if they are similar to proteins known as virulence factors. In addition to this, the raw data files contained information on the length of the amino acid sequences and a sequence score based on the number of identical peptides.

Based on the criteria above the proteins chosen for further analysis was the ExlB-like protein and the MAM7-like protein. The ExlB-like protein had a molecular weight of approximately 68 kDa and was with 100% certainty an outer membrane protein. It consists of a 595 amino acid long sequence and has a sequence score of 107. The MAM7-like protein had a molecular weight of approximately 97,6 kDa and was with 98% certainty an outer membrane protein. It consists of an 881 amino acid long sequence and has a sequence score of 158.

Targeting outer membrane proteins was defined as a criterion based on the recognition and binding of PRRs of APCs in addition to BcR binding and downstream antigen specific antibody production as described above.

The ExlB-like protein was by Blastp recognized as a hemolysin secretion/activation protein in *P. atlantica*. VFDB gave a 26% match with a hemolysin activator protein in *Pseudomonas aeruginosa*, PA7, which is a known virulence factor. ExlB-like proteins are part of the two-partner secretion (TPS) systems found in various Gram-negative bacteria (Filloux, 2022). They

serve as transporters for the secretion of large proteins called exoproteins or TpsA proteins across the outer membrane (Job et al., 2022). These TpsA proteins can have diverse functions, including adhesion, invasion, or other virulence-related activities that contribute to the bacterias' ability to colonize and survive in different environments or hosts (Job et al., 2022). ExlB-like proteins are considered virulence factors because they facilitate the secretion of TpsA proteins, which play a direct role in the pathogenicity of the bacteria. However, not all ExlB-like proteins or TPS systems are associated with virulence. Some TPS systems may have other roles that are unrelated to pathogenicity. The ExlB-ExlA TPS system in *P. aeruginosa*, PA7, is responsible for the secretion of the exoprotein ExlA, which is a member of the TpsA protein family (Bouillot et al., 2020). ExlA has been found to be a potent cytotoxin, capable of causing host cell damage by inducing membrane permeabilization and cytolysis. This cytotoxic activity of ExlA contributes to the virulence of *P. aeruginosa* PA7 and its ability to cause infections.

The MAM7-like protein was by Blastp recognized as a putative protein. VFDB gave a 32% match with a multivalent adhesion molecule (MAM7) in *Vibrio parahaemolyticus*, which is a known virulence factor. This is a bacterial adhesin protein that plays a crucial role in the initial attachment of some Gram-negative bacteria to host cells (Lim et al., 2014). A MAM7-like protein refers to proteins that share structural and functional similarities with MAM7, which is primarily found in *V. parahaemolyticus* (Beltran et al., 2016). MAM7 contains multiple adhesion domains that interact with various host cell receptors, enabling the bacterium to attach to host cells and initiate infection (Lim et al., 2014). In *V. parahaemolyticus* the MAM7 helps the bacteria to hold onto the host cells in early infection. It does so by binding two different molecules on the host, a protein (fibronectin) and phospholipids called phosphatidic acids (Lim et al., 2014). MAM7-like proteins in other bacteria such as *P. atlantica* gv. *salmonicida* may exhibit similar roles in mediating adhesion and potentially contribute to bacterial pathogenicity.

If the recombinant protein is immunogenic there could be several advantages if it also proves to be a virulence factor. Virulence factors are usually highly immunogenic because they interact directly with host tissues during infection (Sharma et al., 2017). If the immune system can recognize and respond to these factors, it can potentially prevent infection or lessen disease severity (Forrellad et al., 2012). Some virulence factors are conserved among different strains or species of pathogens. Thus, a vaccine targeting these factors may offer broad-spectrum protection (Diard & Hardt, 2017). One of the first steps in bacterial infections is attachment to host cells, which is facilitated by adhesins. If antibodies can bind to these adhesins, they can prevent the bacteria from attaching, potentially stopping an infection before it even begins

(Klemm & Schembri, 2000). Adhesins may be prominent virulence factors and suitable candidates for vaccine development (Ellul et al., 2021) and therefore adhesion-related functionality was taken into perspective when conducting in silico analysis.

4.5 Recombinant protein expression

The results from the WBs using the transformed *Codon+ E. coli* cultured with and without IPTG as antigens are shown in Figure 11. The silver stained gel (Figure 11A) clearly show that the recombinant proteins have been expressed in the *E. coli*. The bands visualised in 11B identify proteins having a molecular weight of roughly 60 kDa recognized by antibodies present in PaL-1 vaccinated salmon. This band is however recognised in both *E. coli* expressing the ExlB-like protein and in *E. coli* not expressing the ExlB-like protein. This point to that there are cross reacting antibodies present in the sera binding to epitopes of proteins present in *E. coli.* It is possible that there are ExlB-like protein specific antibodies present, but that their reaction is camouflaged by the reaction of the cross reactive antibodies. To verify the possible presence of ExlB-like specific antibodies it is thus necessary to purify the recombinant proteins and repeat the WBs using the purified proteins as antigens. Arguing against the presence of ExlB-like protein specific antibodies is the presence of bands at similar size, although weaker, in WBs using sera from non-vaccinated salmon (Figure 11D).

As previously mentioned, a His-tag was added to the recombinant proteins to facilitate detection and purification. A monoclonal 6xHisTag antibody was used in the WBs to detect the proteins (Figure 11C and 12D). The MAM7-like recombinant protein (P2) had been successfully expressed with the His-tag and the antibody bound to the His-tag as visualized in Figure 11C. The ExlB-like protein (P1) had also been expressed but the 6xHisTag antibody did not bind to the recombinant protein. This was due to an error in the primer design which had led to failure in attachment of the His-tag. Due to this error, the SDS and WB was repeated (Figure 12) with new primers for P1 and a few adjustments.

The sera from vaccinated fish used in the WBs were now pooled from ten fish vaccinated against PaL-1 (Figure 12C), and the Ag concentration was optimized. From the silver and Coomassie stained SDS-PAGE gels shown in Figure 12A-B it is clear that both recombinant proteins have been expressed in the *E. coli*. In the WB with the 6xHisTag antibody (Figure 12D) the His-tag of the MAM7-like recombinant protein (P2) had successfully bound like earlier, verifying the expression of this protein with the His-tag. In Figure 12C, after incubation with

sera from vaccinated fish, weak bands are present for proteins with a molecular weight of roughly 80 kDa. This does most likely not correspond with the recombinant protein (the MAM7-like protein) and is most likely due to a cross reaction of antibodies in the sera towards *E. coli* proteins. Binding of these proposed cross reactive antibodies (at protein size 80 kDa) were however not identified on WBs using sera from non-vaccinated fish. The ExlB-like protein (P1) had once again not been expressed with the His-tag, and this was due to another error with the primers resulting in that the His-tag was not expressed and therefore the 6xHisTag antibody did not bind to the protein.

Although both recombinant proteins were expressed, as visualized on the protein gels (Figures 11A, 12A-B), there were little to no specific reactivity of the sera from vaccinated fish (Figures 11B, 12C). There could be several reasons for this. The recombinant proteins may not be the antigens that the fish immune system primarily responded to following vaccination. Proteins can also have varying degrees of immunogenicity, and some may provoke a stronger immune response than others. Another reason can be that the recombinant protein might not represent the natural protein as it exists in the fish regarding protein conformation or post-translational modifications. Technical issues in the process cannot be excluded, but this would have to be in the WB procedure, as the proteins were expressed successfully.

4.6 Directions for future research

In future research, it may be beneficial to explore and select additional proteins from the list of potential candidates. Once the immunogenic protein(s) is identified, the process of isolating and purifying the protein commences. This can pose challenges if the protein is insoluble or difficult to work with. However, successfully purified proteins can be employed in various vaccine development strategies. The proteins in the current research have a His-tag added to the gene. The His-tag can be utilized to facilitate protein purification by immobilized metal affinity chromatography (IMAC) (Terpe, 2003). The strong interaction between histidine residues and certain metal ions, such as nickel or cobalt, is taken advantage of in the purification process. The purified protein can be utilized in the creation of a recombinant subunit vaccine, where the isolated protein serves as the primary immunogenic component that elicits an immune response (Nascimento & Leite, 2012). The purified protein can also be used in the development of inactivated vaccines as a control to verify if the bacteria express the protein. By comparing the immune response elicited by the inactivated vaccine to that of the purified protein one gains

insights on the effectiveness of the protein as an immunogenic component. The gene encoding the protein can also be used in development of DNA vaccines by engineering a plasmid to carry the gene.

5 – Conclusions

- *P. atlantica* gv. *salmonicida* is dependent on supplement of serum components, in the current trials FCS, for growth in broth culture.
- Several immunogenic proteins and virulence factors of *P. atlantica* gv. *salmonicida* have been identified.
- Two putatively immunogenic proteins (a MAM7-like protein and an ExlB-like protein) have been expressed recombinantly in *E. coli* and analysed for immunogenicity in WB assays.
- The recombinant proteins (the MAM7-like protein and the ExlB-like protein) are most likely not the most immunogenic antigens of *P. atlantica* gv. *salmonicida* within the protein size range 40-70 kDa and 80-100 kDa.
- Other proteins identified (Table 12 and 13) may prove to be more immunogenic.

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7 – Appendix

Recipes for solutions used in the study.

7.1 – Liquid growth media (TSB+)

Liquid growth media (TSB+) 1150g:

Tropic soy broth (TSB) – BD – catalog# 211825 – lot# 1068445	30 g
NaCl – Honeywell International – catalog# 31434 – lot# L2180	15 g
Fetal calf serum (FCS) – Gibco – catalog# 10099-141 – lot# 2094466RP	105 g
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	1000 g

7.2 – NaOH 10M

NaOH 10M 100 ml:

NaOH – Sigma Aldrich – catalog# S5881 – lot# 106K0004	4.00 g
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	100 ml total volume

7.3 – PBS solution

PBS solution 1000 ml:

Na2HPO4*2H2O – Sigma-Aldrich – catalog# 30435 – lot# SZBE0760V	7.2 g
KH2PO4 – Sigma-Aldrich – catalog# 60220 – lot# SZBD1630V	0.27 g
NaOH 10M – Appendix 7.2	pH = 7.3
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	1000 ml total volume

7.4 – 10x TGS buffer

10x TGS buffer 1000ml:

Tris Base – Sigma-Aldrich – catalog# T1503 – lot# SLBV1855	30.3 g
Glycine – Sigma-Aldrich – catalog# G7126 – lot# BCBJ3736V	144 g
Sodium dodecyl sulfate – Sigma Aldrich – catalog# L4390 – lot# SLBB0911V	10 g
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	1000 ml total volume

7.5 – 1x TGS buffer

1x TGS buffer 1000ml:

10x TGS buffer – Appendix 7.4	100 ml
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	900ml

7.6 – 50x TAE buffer

50x TAE buffer 1000 ml:

Tris Base – Sigma-Aldrich – catalog# T1503 – lot# SLBV1855	242 g
0.5M EDTA – Life Technologies Corporation – catalog# 15575-038 – lot# 1920768	100ml
Acetic acid – Honeywell International – catalog# 33209 – lot# SZBF3510	57.1 ml
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	1000 ml total volume

7.7 – 1x TAE buffer

1x TAE buffer 1000 ml:

50x TAE buffer – Appendix 7.6	20 ml
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	980 ml

7.8 – 0.5M Tris-HCl

0.5M Tris-HCl 100 ml:

Tris Base – Sigma-Aldrich – catalog# T1503 – lot# SLBV1855	6 g
HCl 12M – Sigma-Aldrich – catalog# 258148 – lot# SZBF1100V	pH = 6.8
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	100 ml total volume

7.9 – 1.5M Tris-HCl

1.5M Tris-HCl 150 ml:

Tris Base – Sigma-Aldrich – catalog# T1503 – lot# SLBV1855	27.23 g
HCl 12M – Sigma-Aldrich – catalog# 258148 – lot# SZBF1100V	pH = 8.8
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	150 ml total volume

7.10 – Sample buffer

Sample buffer 9.5 ml:

0.5M Tris-HCl – Appendix 7.8	1.25 ml
Glycerol – Sigma-Aldrich – catalog# G5516-1L – lot# SHBG0744V	2.5 ml
Sodium dodecyl sulfate – Sigma Aldrich – catalog# L4390 – lot# SLBB0911V	200 mg
0.5% Bromophenol blue – Merck – catalog# 8122.0025 – lot# L631122	200 µl
β-mercaptoethanol – Sigma Aldrich – catalog# M3148 – lot# SHBG9616V	500 µl
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	3.55 ml

7.11 – Fixative solution

Fixative solution 160 ml:

Methanol – Honeywell International – catalog# 32213 – lot# SZBG2740H	100 ml
Acetic acid – Honeywell International – catalog# 33209 – lot# SZBF3510	20 ml
Fixative Enhancer Concentrate – Bio-Rad – catalog# 161-0461 – lot# 64075750	20 ml
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	20 ml

7.12 – Silver stain solution

Silver stain solution 65 ml:

Silver Complex Solution – Bio-Rad – catalog# 161-0462 – lot# 64021566	5 ml
Reduction Moderator Solution – Bio-Rad – catalog# 161-0463 – lot# 210012137	5 ml
Image Development Reagent – Bio-Rad – catalog# 161-0464 – lot# P50762	5 ml
Development Accelerator Solution – Bio-Rad – catalog# 161-0449 – lot# 64107923	50 ml

7.13 – Coomassie staining solution

Coomassie staining solution 1000 ml:

Coomassie Brilliant Blue R-250 – catalog# 161-0400	1 g
Methanol – Honeywell International – catalog# 32213 – lot# SZBG2740H	300 ml
Acetic acid – Honeywell International – catalog# 33209 – lot# SZBF3510	50 ml
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	650 ml

7.14 – Coomassie destain solution

Coomassie destain solution 250 ml:

Methanol – Honeywell International – catalog# 32213 – lot# SZBG2740H	125 ml
Acetic acid – Honeywell International – catalog# 33209 – lot# SZBF3510	25 ml
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	100 ml

7.15 – Luria-Bertani (LB) media

Luria-Bertani (LB) media 1000 ml:

Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	1000 ml total volume
Premix formulation (Tryptone, NaCl and Yeast Extract)	25 g
NaOH 10M – Appendix 7.2	7.0 pH

7.16 – Blotting buffer

Blotting buffer 1000 ml:

Tris Base – Sigma-Aldrich – catalog# T1503 – lot# SLBV1855	3.028 g
Glycine – Sigma-Aldrich – catalog# G7126 – lot# BCBJ3736V	14.4g
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	800 ml total volume
Methanol – Honeywell International – catalog# 32213 – lot# SZBG2740H	200 ml

7.17 – TBS solution

TBS solution 500 ml:

NaCl – Honeywell International – catalog# 31434 – lot# L2180	14.61 g
Tris Base – Sigma-Aldrich – catalog# T1503 – lot# SLBV1855	1.21 g
HCl 12M – Sigma-Aldrich – catalog# 258148 – lot# SZBF1100V	pH = 7.5
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	500 ml total volume

7.18 – TBS blocking solution

TBS blocking solution 105 ml:

Skimmed milk powder – Sigma-Aldrich – catalog# 70166 – lot# BCBV5694	5 g
TBS solution – Appendix 7.17	100 ml

7.19 – TTBS

TTBS 700 ml:

TBS solution – Appendix 7.17	700 ml
Tween - Sigma-Aldrich – catalog# P1379 – lot# SZBD2190V	350 µl

7.20 – Antibody buffer

Antibody buffer 202 ml:

Skimmed milk powder – Sigma-Aldrich – catalog# 70166 – lot# BCBV5694	2 g
TTBS – Appendix 7.19	200 ml

7.21 – 5% acetic acid solution

5% acetic acid solution 1000 ml:

Acetic acid – Honeywell International – catalog# 33209 – lot# SZBF3510	5 ml
Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	995 ml

7.22 – SOB-like media

SOB-like media 10 ml:

Luria-Bertani (LB) media – Appendix 7.15	9.8 ml
1M MgCl	0.1 ml
1M $MgSO_4$	0.1 ml