Characterization of immunogenic surface proteins of *Pasteurella atlantica* genomvar *salmonicida*

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List of abbreviations

APC	Antigen-presenting cells
BcR	B-cell receptor
CD4+	Cluster of Differentiation 4 positive
CD8+	Cluster of Differentiation 8 positive
CMI	Cell mediated immune responses
CV	Column volumes
DAMP	Danger associated molecular patterns
DC	Dendritic cell
dd	Degree days
DTT	Dithiothreitol
FCS	Foetal Calf Serum
gDNA	Genomic DNA
GSH	Reduced glutathione
GSSG	Oxidized glutathione
gv.	Genomvar
Hia	*Haemophilus influenzae* adhesin
HisTag	Polyhistidine tag
Ig	Immunoglobulin
i.m.	Intramuscular
i.p.	Intraperitoneal
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex

NLR	NOD-like receptors
Nm	Nanometre
NOD	Nucleotide-Binding Oligomerization Domain
NVI	Norwegian Veterinary Institute
OD	Optical density
OM	Outer membrane
OMP	Outer membrane proteins
PAMP	Pathogen associated molecular patterns
PBS	Phosphate Buffered Saline
PRR	Pathogen recognition receptors
RAS	Recirculatory aquaculture systems
RE	Restriction enzyme
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I Like receptor
Rpm	Revolutions per minute
RV	Reverse vaccinology
SAV3	Salmonid alphavirus 3
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TcR	T-cell receptor
Th	T-helper cells
TLR	Toll like receptors
Tm	Melting temperature
Treg	Regulatory T-cell
TSB	Tryptic Soy Broth

UoB	University of Bergen
VFDB	Virulence Factor Database
WB	Western blot
x g	Times gravity
µl	Microliter

Abstract

Pasteurellosis caused by *Pasteurella atlantica* genomvar *salmonicida* has been an emerging disease in Norway since 2018, causing poor fish welfare and mortalities in the fish farming industry. The development of a commercial vaccine is challenging, and knowledge of the bacteria's pathogenesis, virulence factors, and immunogenic components is still restricted.

The aim of this study was to predict immunogenic proteins of *P. atlantica* gv. *salmonicida* (PaL-1 isolate) by *in silico* analysis, express two proteins recombinantly using *E. coli* and test the antigenicity of the proteins by western blot analysis (WB). Furthermore, the growth of PaL-1 and a *Pasteurella atlantica* gv. *cyclopteri* isolate in TSB supplied with 1.5% NaCl and 10% FCS (TSB+) at different temperatures (10°C, 20°C and 30°C) was investigated. Lastly, the growth of PaL-1 in TSB+ was compared to growth in TSB (2% NaCl) supplemented with reduced and oxidized glutathione (GSH and GSSG, respectively), and the ligand binding of one of the recombinant proteins with GSH and GSSG was assessed.

PaL-1 grew well in TSB+ at 20°C, and the exponential growth phase started after approximately 8 hours. The *P. atlantica* isolates incubated at 20 °C and 30 °C gave a higher yield in cell number compared to when cultured at 10 °C. Several immunogenic proteins were identified by *in silico* analysis of the PaL-1 proteome. The most prominent were a Heme binding protein A-like protein (HbpA) and a BamA-like protein, which were chosen for recombinant protein expression. The HbpA-like protein was successfully expressed recombinantly but showed no reactivity when blotted against sera from PaL-1 vaccinated fish.

The growth of PaL-1 in TSB supplemented with GSH/GSSG gave a higher yield than the growth in TSB+. By further testing, this may have the potential to be a serum-free growth medium for *P. atlantica*. The ligand binding assessment of the recombinant HbpA-like protein and GSH/GSSG did not show any reactivity. However, this may be due to methodological issues.

Sammendrag

Pasteurellose forårsaket av *Pasteurella atlantica* gv. *salmonicida* har vært et økende problem i norsk fiskeoppdrett siden 2018, og fører til nedsatt fiskevelferd og dødelighet. Utvikling av en kommersiell vaksine er utfordrende, da kunnskapen om bakteriens patogenese, virulens faktorer og immunogene komponenter fortsatt er begrenset.

Målet med denne studien var å identifisere immunogene proteiner for *P. atlantica* gv. *salmonicida* (PaL-1-isolatet) ved *in silico* analyser, uttrykke to proteiner rekombinant ved bruk av *E. coli* , og teste antigenisiteten til proteinene ved western blot analyser (WB). Videre ble veksten av PaL-1 og et *P. atlantica* gv. *cyclopteri* isolat i TSB tilsatt 1.5 % NaCl og 10 % FCS (TSB+) ved forskjellige temperaturer (10°C, 20°C and 30°C) analysert. Til slutt ble veksten av PaL-1 i TSB+ sammenlignet med vekst i TSB (2 % NaCl) supplert med redusert og oksidert glutation (henholdsvis GSH og GSSG), og ligandbindingen til ett av de rekombinante proteinene med GSH og GSSG ble analysert.

PaL-1 isolatet vokste godt i TSB+ ved 20°C, og nådde den eksponentielle vekstfasen etter ca. 8 timer. *P. atlantica* isolatene inkubert ved 20 °C og 30 °C vokste bedre og viste høyere celletall, enn isolatene som ble inkubert på 10 °C. Flere immunogene proteiner ble identifisert ved *in silico* analyse av PaL-1 proteomet. De mest spennende var et Heme bindende protein A-lignende protein (HbpA) og et BamA-lignende protein, som begge ble valgt for rekombinant proteinuttrykk. Det HbpA-lignende proteinet ble vellykket uttrykt rekombinant, men det viste ingen reaktivitet når det ble blottet mot sera fra PaL-1 vaksinert fisk.

Veksten av PaL-1 i TSB supplert med GSH/GSSG ga et høyere utbytte enn veksten i TSB+. Ved ytterligere testing kan dette ha potensial for et nytt serum-fritt vekstmedium for *P. atlantica.* Ligandbinding mellom det rekombinante HbpA-lignende proteinet og GSH/GSSG viste ingen reaktivitet, noe som muligens kan skyldes feil metode.

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1. Introduction

Intensive production with high stocking densities in the Norwegian fish farming industry years has led to significant issues related to mortality and welfare. In Norway in 2023, approximately 62.8 million sea cage farmed salmon died, corresponding to a mortality rate of 16.7% (Sommerset et al., 2024). This high mortality can be attributed to various factors, including infectious disease caused by viral, bacterial, and parasitic agents, and physical injuries caused by handling and delousing. The most frequently reported bacterial diseases in 2023 were winter ulcers, yersiniosis, and pasteurellosis. Among the mentioned diseases, there were 27 cases of pasteurellosis reported from farmed Atlantic salmon in 2023 (Sommerset et al., 2024).

Challenges caused by the salmon lice (*Lepeophtheirus salmonis*) are a major issue for the salmon farming industry, causing poor fish welfare and mortalities primarily caused by delousing procedures (Overton et al., 2019). The intensive production in fish farms with high stock densities facilitates favourable conditions for horizontal transmission and faster reproduction of the salmon lice (Bowers et al., 2000; Overton et al., 2019). To prevent transmission of lice from the farmed salmon to wild fish, legislation with strict limits of 0.2 to 0.5 (season dependent) adult female lice per fish is being practised in Norway (Vormedal, 2023). Anti-parasitic chemotherapeutics have traditionally been the preferred method for delousing. However, due to the limited availability of commercially approved chemotherapeutics and increasing resistance in lice populations, the aquaculture industry has implemented non-medicinal de-lousing methods such as mechanical (brushing and flushing), thermal (hot water treatment), and biological (cleaner fish) delousing (Fjørtoft et al., 2020; Østevik et al., 2022). The use of mechanical and thermal methods can result in high stress levels and suppression of the immune system of the fish, in addition to injuries, which subsequently can function as a portal of entry for opportunistic bacteria such as *Pasteurella* sp. (Østevik et al., 2022). Biological delousing includes cleaner fish, and the most popular species are lumpsuckers (*Cyclopterus lumpus* L.), as they are easy to produce and are reported to have better delousing activity in low water temperatures (Imsland and Reynolds, 2022). However, there have been several bacterial diseases in farmed lumpsuckers, which highlights the potential risk of horizontal transmission between Atlantic salmon and lumpsuckers (Haugland et al., 2020).

To address challenges related to fish welfare, mortality, and the environment, the industry needs to focus on new methods, innovative production regimes, and effective vaccines to increase

control of the pathogen burdens and reduce fish handling (Afewerki et al., 2023). Significant attention is being directed toward the development of new farming technology, including semi-enclosed cages, offshore farming, and recirculatory aquaculture systems (RAS) on land. Such farming technologies can provide better control of factors such as temperature and salinity, salmon lice manifestation, reduce stress caused by handling of fish, and better control of transmission from pathogenic agents. The traditional smolt production strategy is to raise smolt up to approximately 80-100 grams in land-based flow-through- and RAS systems before transfer to seawater. New smolt strategies involve the production of post-smolt up to 1 kg to reduce the production time after transfer to open cages in the sea (Afewerki et al., 2023; Mota et al., 2022). In addition, the development of effective vaccines as a prophylactic measure to prevent infection/disease is considered a key factor for the intensive production of Atlantic salmon in today's industry (Sommerset et al., 2005). However, the absence of commercially licenced vaccines for some emerging diseases, such as pasteurellosis in Atlantic salmon, is critical (Sommerset et al., 2024). Further research thus needs to focus on the bacteria, including pathogenesis and immunogenic components of the emerging pathogen *Pasteurella atlantica* genomvar *salmonicida,* to gain better insight into host-pathogen interactions, which is crucial for developing efficient vaccines providing long-lasting immunity.

1.1 Pasteurellosis in Norwegian aquaculture

Pasteurellosis is a disease that results from an infection by a bacterium belonging to the genus *Pasteurella* spp. (Legård and Strøm, 2020). In Norwegian aquaculture, the disease has been detected in Atlantic salmon and lumpfish (Alarcón et al., 2016; Ellul et al., 2019; Legård and Strøm, 2020). The first detection of pasteurellosis in Atlantic salmon in Norway was in a fish farm in Northern Norway, back in 1989 (Legård and Strøm, 2020; Valheim et al., 2000). Diseased fish showed skin ulceration, necrosis, and haemorrhagic granulomas in the pseudobranch, and the characteristic infection of the eyes caused panophthalmitis (Valheim et al., 2000). The disease was given the name *Varracalbmi,* a term in Sami for blood eye. There were sporadic reports of the disease in fish farms at low sea temperatures in the winter until 1992. The causative agent of the disease remained unknown. However, through isolation and cultivation of bacteria from diseased fish, they were identified and characterized as gram-negative, non-motile short rods (Valheim et al., 2000). Following the cases from 1989-1992, the occurrence of pasteurellosis in Norwegian fish farms became more sporadic (Legård and Strøm, 2020).

In 2018, seven cases of farmed Atlantic salmon from different fish farms were diagnosed with pasteurellosis along the western coast of Norway (Sommerset et al., 2024). The number of reports rose in the following years, with 14 cases in 2019, 57 cases in 2020, 45 cases in 2021, and 52 cases in 2022 (Sommerset et al., 2024). In 2023, there was a decrease with 27 reported cases, but the number is still high. There can be many and combined reasons for the decrease: an increased focus on biosecurity measures, development and use of autogenous vaccines, or underreporting (Sommerset et al., 2024). Different from the detection of *Varracalbmi* that occurred at low temperatures, the outbreaks after 2018 are reported all year and at different water temperatures (Legård and Strøm, 2020). The bacteria isolated from cases of diseased farmed Atlantic salmon in Norway after 2018 has not yet been officially named, as there is still insufficient knowledge about its physiological and genetic characteristics. In an article published by the Norwegian Veterinary Institute (NVI) in 2020, a temporary nomenclature was established, "*Pasteurella atlantica* genomvar *salmonicida*" (Gulla et al., 2020). Based on reports, the disease typically manifests in large salmon, typically 3-4 weeks after lice treatment or other types of handling, causing unnecessary stress to the fish (Legård and Strøm, 2020). There are variations in the mortality rates and clinical findings (Legård and Strøm, 2020; Sommerset et al., 2024). A common clinical feature is inflammation in the pericardium, the abdominal wall, and the pseudobranch (Legård and Strøm, 2020; Sommerset et al., 2024). Reports also include abscesses in the skeleton musculature, ulcer formation at the base of the pectoral fins, and bloody and infected eyes, like the descriptions from 1989 (Gulla et al., 2020; Legård and Strøm, 2020; Sommerset et al., 2024). By histopathological examination, bacterial aggregates and necrotizing inflammation are observed in affected tissues, along with epicarditis in the heart (Gulla et al., 2020; Legård and Strøm, 2020). In addition to infections caused by *P. atlantica* gv. *salmonicida* in Atlantic salmon, there has been one report in Norway of pasteurellosis caused by *Pasteurella skyensis* (known to cause pasteurellosis in Scottish Atlantic salmon) (Gulla et al., 2020).

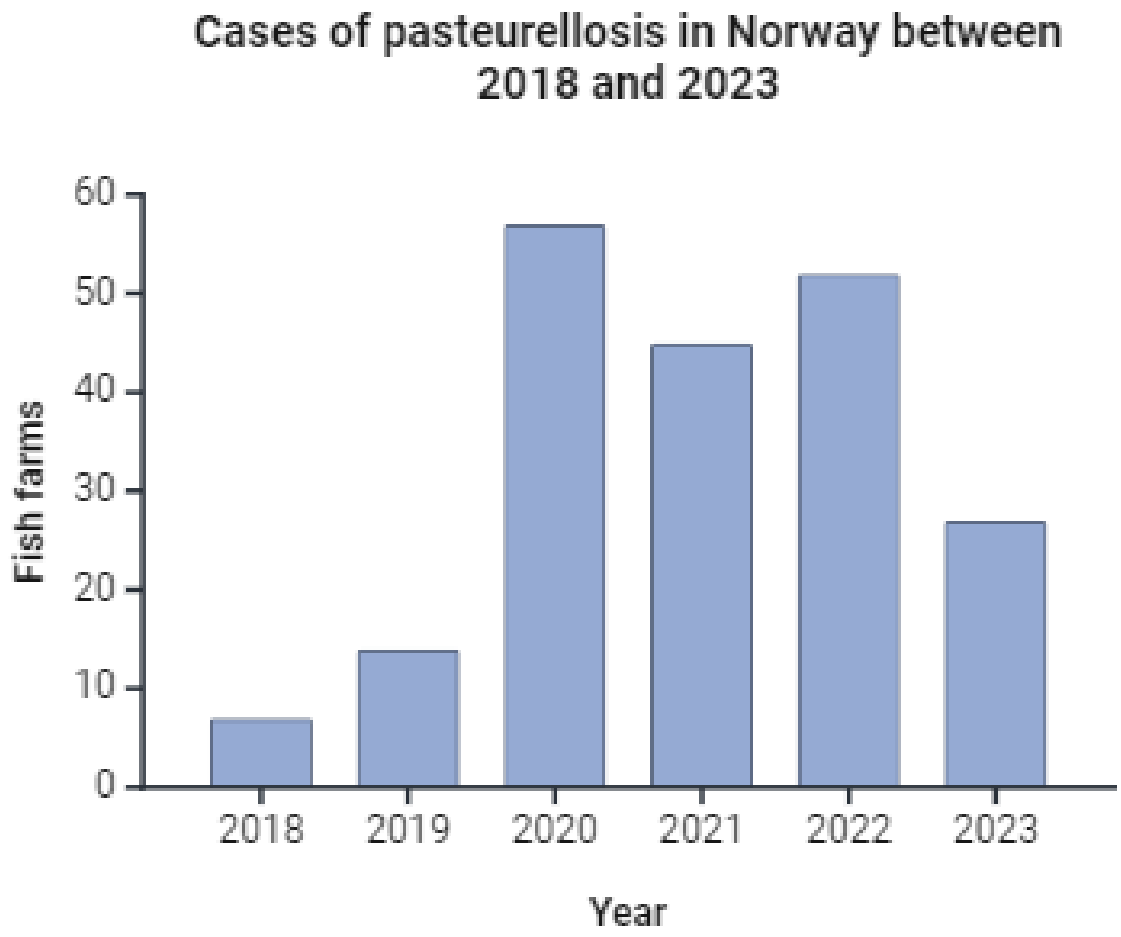

Figure 1: *Number of fish farms in Norway with detection of* P. atlantica *gv.* salmonicida *from 2018 to 2023. The numbers from 2018 and 2019 are based on samples submitted to the Norwegian Veterinary Institute (NVI) and may be inaccurate, while the statistics from 2020 – 2023 also include detections from private diagnostic laboratories. The statistics are gathered from the Fish Health Report from NVI (2023). Created with BioRender.com.*

Pasteurellosis in farmed lumpfish in Norway is caused by a bacterium going under the working nomenclature *P. atlantica* gv. *cyclopteri* (Alarcón et al., 2016; Gulla et al., 2020). The first detection of the disease was in 2012, and the occurrence of the disease has been increasing (Alarcón et al., 2016; Sandlund et al., 2021). Unlike the variable mortality rates observed in disease outbreaks of farmed Atlantic salmon, high mortality levels are frequent in cases with diseased lumpfish (Alarcón et al., 2016; Ellul et al., 2021; Sandlund et al., 2021). The possibility of horizontal transmission between lumpfish and Atlantic salmon was tested by Sandlund et al. in 2021. The results showed that the lumpfish are susceptible to both *P. atlantica* gv. *cyclopteri* and *P. atlantica* gv. *salmonicida* isolates, while the Atlantic salmon did not show any clinical signs of disease during the challenge experiment (Sandlund et al., 2021).

The current taxonomic placement of the *P. skyensis* isolates from Scotland and *P. atlantica* isolated from Atlantic salmon and lumpfish in Norway are in the genus *Pasteurella* within the

family *Pasteurellaceae* (Alarcón et al., 2016; Ellul et al., 2021; Gulla et al., 2023). Analysis shows that the *Pasteurella* isolates have phenotypic similarities but are genomic and serologically distinct from each other (Alarcón et al., 2016; Ellul et al., 2021; Gulla et al., 2023). A recent study from 2023 performed by Gulla et al. shows that *P. skyensis* and *P. atlantica* share high nucleotide identity with *Phocoenobacter uteri*, and the authors suggest that the isolates should be moved to the genus *Phocoenobacter* (Gulla et al., 2023).

1.2 Immunogenic components and virulence factors

To develop prophylactic measures such as vaccines, it is important to have a general understanding of the components involved in the host-pathogen interaction, such as the bacteria's ability to stimulate the host immune responses (immunogenicity), its immunogenic components (the specific bacterial components that elicit an immune response) and the virulence factors that mediate bacterial pathogenicity, which includes starting and maintaining an infection. The bacterial virulence factors are often referred to as cellular molecular components produced by the bacteria that promote the bacterium's pathogenicity (Sharma et al., 2017; Ellul, 2021). Immunogenic components are specific structural parts of the pathogenic bacterium that can be recognized by the host's immune system (Goldsby et al., 2002).

Some virulence factors are toxins that can exist either as exotoxins that are secreted into the host cells or tissue, or as membrane-bound endotoxins, such as flagella, fimbria, lipopolysaccharide (LPS), and the bacterial capsule (Ellul, 2021; Goldsby et al., 2002). The secreted toxins have different functions, such as causing cell lysis in host cells (Sharma et al., 2017). Membrane-bound virulence factors are responsible for adhesion to host cells and tissues and some for evasion of the host immune responses. The first interaction between a bacterial cell and a host is the attachment of host cells and tissue through adhesins on the outer membrane of the bacterial cell (Ben Hamed et al., 2018; Maiti et al., 2020). This adherence between the bacteria and the host is necessary and lays the foundation for further pathogenicity by, for example, regulating the delivery of toxins. Furthermore, the application of vaccines targeting adhesins has shown the ability to confer protection against the pathogen by inducing neutralizing- and opsonizing antibodies that prevent the first stage of infection (Raynes et al., 2018). By *in silico* analysis of the proteome of *P. atlantica* gv. *cyclopteri* following the procedures of reverse vaccinology, Ellul et al. (2021) predicted a potential adhesin that was

closely related to an earlier described adhesin found in *Haemophilus influenzae* (Hia adhesin) to be the most promising vaccine candidate.

The most prominent immunogenic components known to trigger the cellular and humoral immune responses are proteins and polysaccharides (Goldsby et al., 2002). In addition, lipids and nucleic acids can show high immunogenicity when connected with proteins and polysaccharides. The level of immunogenicity is based on factors such as antigenicity, size, structural complexity, and the ability to be processed and presented to adaptive immune cells. The antigenicity describes the ability of a molecule to be recognized by membrane anchored immunoglobulins on B-cells (the B-cell receptor, BcR) or the T-cell receptor (TcR), while the immunogenicity of a compound also includes the ability to stimulate the proliferation of effector cells and plasma cells after binding (Goldsby et al., 2002). In vaccinology, outer membrane proteins (OMPs) and membrane-bound components are prominent vaccine candidates that can be both immunogenic and antigenic and confer the host a long-lasting protection against pathogens (Maiti et al., 2020). These structures often play a crucial role in bacterial cell integrity and permeability, as well as they may also function as virulence factors. Lipopolysaccharides on the bacterial outer membrane play a key role in bacterial survival by inhibiting the host's complement proteins and lysozymes, and have shown the ability to confer protective immunity when purified and used in a vaccine against *Yersinia ruckeri* (Maiti et al., 2020; Welch and LaPatra, 2016).

1.3 The teleost immune system

The marine environment, promote survival of pathogenic microorganisms and the ability for horizontal transmission, and fish are constantly exposed to these microorganisms (Ben Hamed et al., 2014). This means that the fish immune system is constantly exposed to pathogens. The immune system is divided into innate- and adaptive immune responses, and includes physical, humoral, and cellular responses for protection against potentially pathogenic microorganisms. The main difference is that the innate system is inherited, it gives the fish a nonspecific protection and reacts quickly, while the adaptive immune system is more slowly activated, but provides a more specific, powerful, and long-lasting protection against pathogens by forming memory cells.

1.3.1 The innate immune system

The innate immune system includes physical barriers, cellular and humoral components (Uribe et al., 2011). Skin and mucosa constitute the physical barriers that prevent pathogens from entering the fish and causing infections (Esteban, 2012; Magnadóttir, 2006; Uribe et al., 2011). The humoral part of the innate immune system consists of molecules, mainly antibodies, that can lead to killing, opsonizing, and agglutination of pathogens, in addition to signalling substances (cytokines) to alert and activate other cells. Other important compounds are antibacterial peptides, transferrin, lytic enzymes and complement proteins found in the body fluids such as blood and mucosa (Magnadóttir, 2006). The immune cells that take part in the innate cellular response include the phagocytes and cells with cytotoxic functions. Some of the phagocytes, dendritic cells (DC), macrophages and B cells, can serve as antigen-presenting cells (APC) by presenting antigen derived peptides on the Major Histocompatibility Complex (MHC) class II.

The recognition mechanisms in the innate immune system are composed of phagocytic, secreted, and signalling pathogen recognition receptors (PRRs) that recognizes pathogen- or danger-associated molecular patterns (PAMPs or DAMPs) (Magnadóttir, 2006). Bacterial PAMPs that can be recognized by the phagocytic receptors are for example OMPs that when recognized and facilitate phagocytosis. The signalling PRRs, which include the Toll like receptors (TLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and the NOD-like receptors (RLRs), ensure tailored immune responses. The TLRs are found on outer membranes and on membranes of endosomes, exemplified by TLR-5 recognising flagellin which is a building block of the bacteria flagella. By binding to signalling PRRs a cascade of cellular and molecular mechanisms is activated leading to production cytokines and chemokines, which induce inflammatory responses and recruitment of other immune cells (Jørgensen, 2014). The RLRs and NLRs are cytoplasmic receptors present in the cytosol of the cell and will recognise pathogen derived PAMAs, mainly nucleic acids. Binding of these receptors will lead to similar responses as binding to TLRs. The secreted PRRs comprise the complement proteins and pentraxins and have important functions in opsonization and lysis of pathogens as well as inducing inflammation.

1.3.2 The adaptive immune system

The APCs of the innate immune system have important roles in activating the adaptive immune responses (Mutoloki et al., 2014). The main type of cells that mediate the adaptive immune responses are B-cells and T-cells. B- and T-cells are equipped with specific antigen recognition receptors, BcR and TcR, respectively. The adaptive immune system can be divided into a cellular and humoral part, where T-cells are responsible for the cell mediated immune responses (CMI), and the B-cells are responsible for the humoral part, producing antibodies (Mutoloki et al., 2014; Uribe et al., 2011).

Activation of effector B- and T-cells occur through presentation of antigen fragments on MHC class I or II on the membrane of cells (Mutoloki et al., 2014; Uribe et al., 2011). Exogenous antigens are recognized by phagocytic receptors of APCs. After binding antigens are engulfed, processed, and degraded into antigen fragments through phagocytosis, and pathogen derived peptides are bound to MHC class II molecules and presented on the surface of the cells. Molecules presented on MHC class II are introduced to CD4+ T-helper cells, which can further differentiate into Th1 cells that stimulate CMI responses (through activation of CD8+ cytotoxic T-cells), Th2 cells that stimulate humoral responses (through activation of B-cells), Th17 cells that improve inflammatory responses or Treg cells that have function in regulating the immune responses (Tang et al., 2021).

B-cells are responsible for the humoral part of the adaptive immune system. The BcR consist of membrane-bound immunoglobulins (Ig), and so far, IgM, IgD, and IgT have been identified in salmonid species (Hordvik, 2015; Mutoloki et al., 2014). B-cells in teleost have shown the ability of phagocytosis and antigen presentation (Li et al., 2006). The activation of B-cells can be direct (independent of Th2 cells) after recognition of antigens by several BcRs or by binding of both the BcR and other innate receptors, or dependent on Th2-cells that can recognize fragments of antigens presented on MHC II on the surface of the B-cells. The Th2 cells secrets stimulating cytokines that start the clonal expansion of B-cells. Subsequently, the proliferation of the B-cells leads to maturation of antibody secreting plasma cells and memory cells. The production of long-lived memory B-cells happens through the process where B-cells are dependent on stimuli from Th2 cells to proliferate (Ellul, 2021).

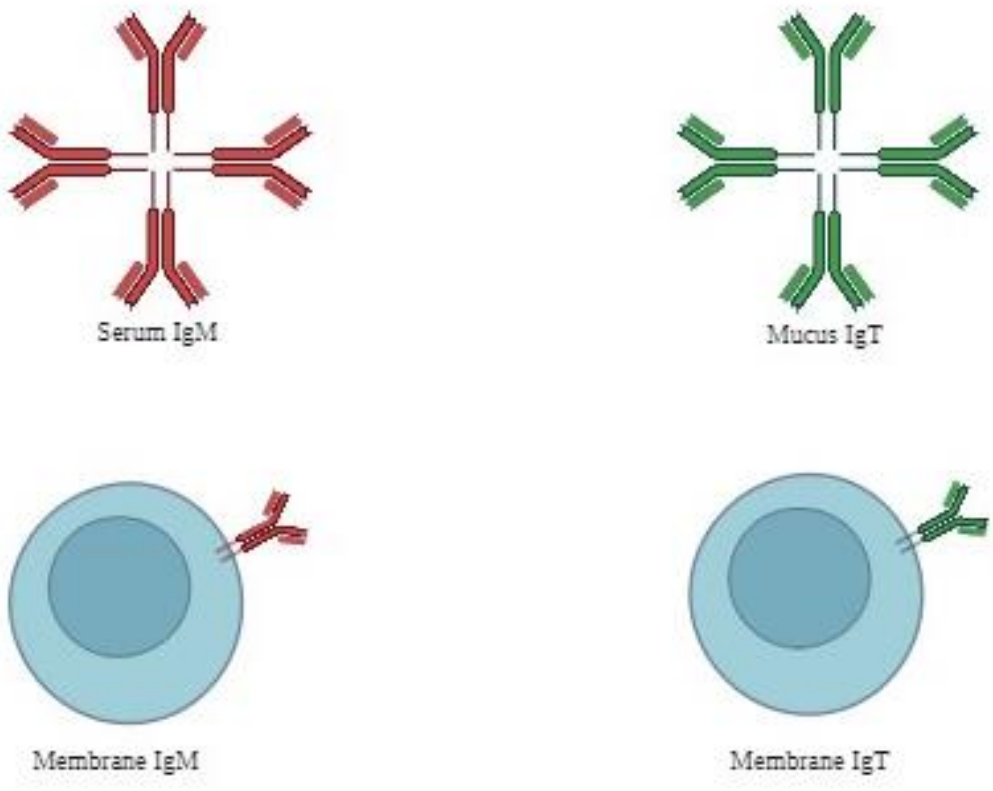

Figure 2: *Schematic illustration of membrane bound and secreted IgM and IgT. Membrane bound IgM and IgT are monomers on the surface of B-cells. Secreted IgM are in serum as tetramers, and IgT are mucosa associated tetramers. Created with BioRender.com.*

T-cells are responsible for the cellular response in the adaptive immune system. Endogenous antigens from intracellular pathogens are presented on MHC I molecules, which are present in all nucleated cells (Ellul, 2021; Mutoloki et al., 2014). Infected cells process the antigens with proteasomes, and fragments of the antigen are transported for binding to MHC class I molecules before being presented on the cell surface (Mutoloki et al., 2014). The TcR of CD8+ T-cells recognize the antigen fragments on MHC class I molecules, and activation into an effector cytotoxic T-cell is facilitated through stimuli from cytokines produced by Th1 cells.

1.4 Vaccines for farmed fish

One of the main factors contributing to the high production of salmon today is the development of successful vaccines to prevent diseases (Sommerset et al., 2024). A vaccine is a biological product such as whole pathogens or components of a specific pathogen that are processed to induce a protective immune response, through activation of memory B- and T-cells (Mondal and Thomas, 2022). Vaccines can be administered either orally through the feed, by immersion

(dip or bath), and by intraperitoneal (i.p.) or intramuscular (i.m.) injections (Mondal and Thomas, 2022).

Vaccines available for fish are divided into the categories: inactivated vaccines, live attenuated vaccines, subunit vaccines and DNA vaccines (Mondal and Thomas, 2022; Pollard and Bijker, 2021). The most applied vaccines in aquaculture are based on whole inactivated/killed pathogens (Maiti et al., 2020).

Whole cell inactivated vaccines are considered safe vaccines as they can induce protective immunity against a disease without causing infection. Inactivated vaccines are often whole bacterial cells replicated in broth culture, that have been inactivated with formalin (Mondal and Thomas, 2022). However, whole cell inactivated vaccines generally confer low protection against intracellular pathogens as they do not stimulate the CMI responses (Maiti et al., 2020).

Live attenuated vaccines are based on bacteria or viruses that are genetically or chemically weakened so that they have reduced virulence, or on non-pathogenic isolates with similar immunogenic epitopes. These vaccines stimulate the immune system through infection but without giving disease. The immune response post vaccination is thus very similar to a natural infection and can confer protection also against intracellular pathogens depending on the vaccine strain, as they may stimulate both humoral and CMI responses (Maiti et al., 2020). The disadvantage of attenuated vaccines is the risk of the pathogen reverting into virulent form and causing infection/disease in vaccinated fish and the risk of spread in the aquatic environment.

Subunit vaccines and DNA vaccines are the most common vaccines for delivery of immunogenic components derived from the pathogen (Maiti et al., 2020). Subunit vaccines are based on specific immunogenic components such as outer membrane proteins (OMPs) instead of using the whole pathogen in the vaccine (Ellul, 2021; Maiti et al., 2020). The immunogenic components are either purified directly from the pathogen or produced by recombinantly protein expression through vectors and competent *E. coli* cell systems into big quantities before purification (Ma et al., 2019). These vaccines are considered as safer than the inactivated and attenuated vaccines and can be more specific with antigens that are highly immunogenic.

DNA vaccines stimulate both the CMI and humoral responses of the adaptive immune system (Mondal and Thomas, 2022; Pollard and Bijker, 2021). The development of DNA vaccines includes *in silico* analysis, cloning of the gene of interest, and insert of the gene of interest into plasmids which are produced into big quantities in bacterial cells (Kurath, 2008; Ma et al., 2019). The bacterial plasmids that contain both the gene coding for the antigen and a promotor

that initiates the expression of the antigen in the host cells are provided to the fish through i.m. injection, which leads to both CMI response and production of antibodies targeting the produced antigen (Kurath, 2008). In Norwegian aquaculture, there are one commercial licenced DNA vaccine available targeting pancreas disease caused by the salmonid alphavirus 3 (SAV3) (Thorarinsson et al., 2024).

1.4.1 Reverse vaccinology

The procedures of vaccinology are evolving rapidly, making the timeframe for the development of effective vaccines against emerging pathogens more rapid. Following the principles of reverse vaccinology (RV) (Rappuoli, 2000), the identification and characterization of key antigens can be performed by analysis of the bacterial genome/proteome in different databases *in silico* (Ellul et al., 2021; Maiti et al., 2020). There are several bioinformatic tools that enhance the process of selecting the most prominent immunogenic components. These tools include databases that help predict the localization of the proteins in the bacteria and analysis of genes encoding proteins known as virulence factors and immunogenic components (Maiti et al., 2020).

1.5 Aims of the study

Identification of immunogenic proteins is essential in the development of vaccines. For *P. atlantica* gv. *salmonicida* little knowledge is available so far on immunogenicity and immunogenic surface proteins. The study builds on a previous master thesis (Skaar, 2023) where immunoproteomics were used to investigate the immunogenicity of two protein candidates. In the current study *in silico* analysis is performed on the proteome of the isolate PaL-1, to predict vaccine candidates, and to produce two of them as recombinant proteins.

Specifically, the aims of the study were:

- To identify immunogenic proteins of *P. atlantica* gv. *salmonicida* (PaL-1 isolate) by *in silico* analysis.
- To clone genes encoding two vaccine candidates into expression vector.
- To express and purify immunogenic proteins recombinantly using *E. coli.*
- To analyse antigenicity of the recombinant proteins.
- To do functionality analysis of the recombinant proteins.
- To analyse temperature range for growth and protein expression for the PaL-1 isolate when cultured in TSB+.

2. Materials and Methods

2.1 Bacterial isolates

One isolate of *P. atlantica* gv. *salmonicida* was included in this study. The isolate was harvested from diseased salmon during an outbreak of pasteurellosis at a commercial fish farm in Vestland county in Norway in 2019. For comparison, in some of the analyses, on isolate of each of the bacteria, *P. atlantica* gv. *cyclopteri, Vibrio anguillarum* and atypical *Aeromonas salmonicida* isolated from lumpfish were also included.

2.1.1 Cultivation of bacteria

The *P. atlantica* gv. *salmonicida* isolate, hereafter termed PaL-1, was cultivated in Tryptic Soy Broth (TSB) supplemented with 1.5% NaCl and 10% Foetal Calf Serum (FCS) (TSB+) (appendix). The bacteria stock was collected from a -80°C storage freezer in 1 ml cryotubes and suspended into 50 ml tubes (SARSTEDT AG & Co. KG) with 40 ml TSB+ (4 ml FCS). The 50 ml tubes with bacterial suspension were incubated in a shaking incubator (Infors AG, model Minitron) at 200 rpm and 20°C for 12 hours.

2.1.2 Glycerol stocks

After 12 hours in the incubator, the bacterial suspension was harvested to make glycerol stocks for long-term storage at -80°C to ensure that all following analysis were based on the same bacterial stock. Firstly, the optical density (OD) was measured in a spectrophotometer (Spectroquant® Pharo 300, Merck), and the number of cells per millilitre was determined using a CASY cell counter (CASY® Cell Counter). The procedure for the CASY cell counter involved pipetting 10 µl of bacterial suspension into 10 ml of CASYton, a defined buffer solution, before running the test in the CASY cell counter using pre-set analysis parameters. The CASY cell counter was cleaned using CASYton before and after each test. The OD was measured at 600nm wavelength. The spectrophotometer was blanked using a cuvette (VWR Cuvettes PS macro, VWR) containing 1,5 ml TSB+. Next, 1,5 ml bacterial suspension was pipetted into cuvettes and placed in the spectrophotometer for the OD measurements.

After the measurements, glycerol stocks were made to ensure that the bacteria were well preserved for later use. A solution containing 2.3 ml 86% glycerol (Sigma-Aldrich) and 7.7 ml

bacterial suspension in 50 ml tubes, with a final concentration of 20 % glycerol, was mixed. Subsequently, 1 ml was pipetted into cryotubes (VWR) and stored in the -80 °C freezer.

2.1.3 Growth curves

A growth curve for PaL-1 was compiled to visualize the lag and exponential growth phase. The bacteria were cultivated in TSB+ in the same manner as described in section 2.1.1. The growth curve was constructed by measuring OD over time following the procedure described in section 2.1.2. OD was measured every hour and plotted in Microsoft Excel to illustrate the growth phases, OD as a function of time.

In addition, measurements of cells per milliliter were conducted using a CASY cell counter, as described in section 2.1.2.

2.2.1 Analysis of temperature tolerance of *P. atlantica*

In this study, three novel bacterial isolates were included for comparative purposes. One isolate of *P. atlantica* genomvar *cyclopteri* isolated from lumpfish was included for comparison.

Table 1: *Cultivation of PaL-1 and* P. atlantica *gv.* cyclopteri *at 10 °C, 20 °C and 30 °C.*

Bacteria	Fish species	Growth media	Growth condition
PaL-1	Salmon	40 ml TSB +	200 rpm and 20°C
P. atlantica gv *cyclopteri*	Lumpfish	40 ml TSB +	200 rpm and 20°C
Subculture PaL-1	Salmon	40 ml TSB +	200 rpm and 10°C
	Salmon	40 ml TSB +	200 rpm and 30°C
Subculture *P. atlantica* gv. *cyclopteri*	Lumpfish	40 ml TSB +	200 rpm and 10°C
	Lumpfish	40 ml TSB +	200 rpm and 30°C

The bacteria were cultured overnight at 20 °C at 200 rpm in a shaking incubator according to the conditions described in Table 1. The *P. atlantica* isolates were cultured following the procedure described in section 2.1.1.

The bacteria were harvested after 24 hours. Cell counts of all cultures were measured using the CASY cell counter, following the procedure outlined in section 2.1.2. Subsequently, two

subcultures were prepared from the *P. atlantica* isolates following the cultivation procedure in TBS+ for further incubation for 24 hours at 10 °C and 30 °C.

Bacterial pellets from the cultures were prepared by centrifugation, for analysis on Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Two samples from each isolate containing 1 ml of bacterial culture were centrifuged at 13000 x g for 3 minutes. Following centrifugation, the supernatant was carefully discarded by pipetting, and one pellet from each isolate was resuspended in 50 µl of PBS and one in 100 µl PBS. Additionally, bacterial pellets were isolated similarly for the subcultures, with 100 µl of PBS added to each pellet. All bacterial pellets were carefully resuspended and stored at -20 °C until further analysis by SDS-PAGE. Lastly, glycerol stocks of the *P. atlantica* gv *cyclopteri* isolate was prepared, following the method described in section 2.1.2.

2.2.2 SDS-PAGE and coomassie staining.

SDS-PAGE and Coomassie staining were performed to observe differences in the protein profiles of the different bacteria and to assess whether the incubation temperature had an impact on these protein profiles.

A Mini-PROTEAN® Tetra Cell and precast gels (Mini-PROTEAN® precast gels) were employed. Two gels were arranged within the electrophoresis chamber, which was subsequently filled with 1x TGS electrophoresis buffer (appendix).

The bacterial pellets resuspended in PBS were collected from the -20 °C freezer. Then, 10 µl of each sample was mixed in a 1:1 ratio with SDS-PAGE sample buffer (appendix) containing dithiothreitol (DTT) within Eppendorf tubes. The samples were subjected to heat treatment at 98°C for 5 minutes. Additionally, a molecular weight marker (Silver Stain SDS-PAGE Standards Low Range, BioRad) was diluted 1:20 in SDS-PAGE sample buffer with DTT and heat treated in the same manner as the bacterial samples. For electrophoreses, 10 µl of the bacterial samples and 5 µl of the molecular weight marker were supplied to the gel wells. The gels were electrophoresed at 190V for 45 minutes. Following electrophoresis, the gels were prepared for Coomassie staining. For thorough staining of the protein bands, the gels were covered with Coomassie staining solution (appendix) and incubated overnight on a flat orbital shaker (IKA® KS 260 basic) at 50 rpm. The gels were washed in Milli-Q water to remove excess dye and incubated in a Destaining solution (appendix) at 50 rpm. Once the protein bands

on the gels appeared visible and clear, the gels were washed in Milli-Q water and photographed in a GelDoc Go Imaging System (BioRad).

2.3 *In silico* analyses of the PaL-1 proteome

The entire proteome of the PAL-1 isolates (data sourced from the Fish Immunology group at UoB) was analysed *in silico* to get an insight into potential outer membrane proteins (OMP) and virulence factors with antigenic properties, including their cellular localization and potential functions, to select relevant proteins for further examination. The whole proteome was analysed in PSORTB version 3.0.3 (https://www.psort.org/psortb/), which predicts the cellular localization of proteins. Some sequences within the proteome were designated as "hypothetical proteins". These sequences were examined through blastp, a function in the database BLAST® National Centre for Biotechnology Information, to compare and decide what proteins they could be. All relevant proteins were checked and investigated in the Virulence Factor Database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm) to compare them to other proteins with known virulence properties.

2.4 Primer design

Primers were designed to specifically amplify the target proteins chosen by the *in silico* analysis, an Outer membrane protein assembly factor BamA-like protein (BamA-like protein) and a Heme binding protein A-like protein (HbpA-like protein). It was decided to make primer sequences at a minimum length of 20 nucleotides and melting temperature (Tm) at approximately 60 °C (calculated using a TM calculator by ThermoFisher ().

Restriction sites recognized by the two type II restriction enzymes, NdeI and XhoI, were incorporated into the primer sequences. As shown in Table 2, one forward and one reverse primer was designed for both genes. The sequence 5`…CATATG…3` was added to the forward primers to be recognized by NdeI. At the reverse primers, the sequence 5`…CTCGAG…3` was added to be recognized by XhoI. A polyhistidine tag (HisTag) was also added to the reverse primers, which will play a central role in the purification of recombinant proteins and in detecting proteins by western blot (WB) at later stages. The tag was added from 5`…GTGGTGGTGGTGGTGGTG…3`, encoding six histidins.

For sequencing, two primers which bind in the vector was used. In addition, a primer was designed to bind in the middle for the BamA-like sequence, to ensure the whole sequence was covered in Sanger sequencing. One more overlapping primer was therefore designed to cover the middle of the sequence, as shown in Table 2.

Table 2: *Forward and reverse primers for BamA- and HbpA-like proteins.*

Gene	Primer	Application	Primer sequence (5`… 3`)
BamA	Forward	Cloning / sequencing	CCCGACATATGTCACCTTTTGTTGTAAAAGATAT ACGTAT
BamA	Reverse	Cloning / sequencing	CCGCTCGAGTCATCAGTGGTGGTGGTGGTGGTGG A ACGAACCACCAATACTAAATTG
BamA	Middle	Cloning / sequencing	AGCAAAAGATATTGATGCACTAAAAT
HbpA	Forward	Cloning / sequencing	CCCGACATATGTCTGGTAAAACTTTTTATTATTG TACAGATA
HbpA	Reverse	Cloning / sequencing	CCGCTCGAGTCATCAGTGGTGGTGGTGGTGGTGT TTATCTTTTAATTTTGTTCCATATAAATAAA
pET-11a	Forward	PCR-screening	CGACTCACTATAGGGGAATTGTG
pET-11a	Reverse	PCR-screening	CAAGGGGTTATGCTAGTTATTGC

2.5 Plasmid purification and agarose gel

The PaL-1 and *P. atlantica* gv. *cyclopteri* were cultivated in a shaking incubator, according to Table 1. In addition, one atypical *A. salmonicida* isolate was cultivated in TSB, and one the *V. anguillarum* was cultivated in a TSB supplemented with 2% NaCl. Following the incubation period, cell counts and glycerol stocks of the atypical *A. salmonicida* and *V. anguillarum* were measured. Further, 10 ml of each bacterial isolate were pipetted to 15 ml tubes for pelletizing in an Allegra® X-15R Benchtop Centrifuge (Beckman Coulter®) at 2500 x g, and a temperature of 4 °C, for 15 minutes. The remaining bacterial culture was pelletized and stored at -20 °C for genomic DNA (gDNA) isolation.

The pellets from all bacteria were plasmid-purified following the procedure outlined in the GenElute™ plasmid miniprep kit (Sigma-Aldrich), with minor adjustments. All centrifugation steps in the protocol were performed at 16,000 x g, and 50 µl milli-Q water was used for elution.

For analysis, a 1% agarose gel was prepared. Firstly, 0.4 g agarose powder (SeaKem® LE Agarose, Lonza) and 40 ml 1x TAE (appendix) in 250 ml Erlen Meyer flasks. Next, the solution was heated to the boiling point in a microwave, and 4 µl GelRed was mixed into the agarose solution. The solution was loaded in a Mini-Sub Cell GT Cell (BioRad), and a comb was inserted. After approximately 30 minutes, the gel was ready. The plasmid samples were diluted in Milli-Q water and 5x Loading buffer (BlueJuiceTM Gel loading Buffer, Thermo Fisher Scientific), and a Supercoil DNA ladder (NEW ENGLAND LABS) was diluted in loading buffer and milli-Q. For the analysis, 10 µl diluted sample, and 4 µl Supercoil DNA ladder were added to the wells for electroporation at 80V for 40 minutes and photographed in GelDoc Go Imaging System (BioRad).

2.6.1 Genomic DNA-isolation

The remaining bacterial pellets from section 2.2.2 were collected from the freezer. Bacterial genomic DNA was isolated using the GenElute™ Bacterial Genomic DNA Kits (Sigma-Aldrich) with some modifications. The amount of Lysis solution T, RNase A, Proteinase K, and Lysis solution C was doubled. The first wash in step 8 was performed twice, and the incubation was at room temperature. The time of elution in step 9 was doubled to 10 minutes, and the "optional" elution in step 10 was performed to test if there was any difference.

The samples of genomic DNA were analysed on both nanodrop (Thermo Scientific NanoDrop™ 1000 Spectrophotometer) and on a 1% agarose gel to check if the g-DNA isolation was successful. A 1% agarose gel was prepared according to the procedure in section 2.5, and samples were diluted in a 5x loading buffer. Next, 10 µl of samples and 2.5 µl of Invitrogen 1 Kb+ DNA ladder (ThermoFisher Scientific) was added to the gel and electrophorized at 80 V for 40 minutes and photographed with GelDoc Go Imaging System (BioRad).

2.6.2 Amplification with PCR

Two genes, selected *in silico*, encoding HbpA-like and BamA-like proteins, were amplified using PCR. The PCR reactions contained buffer, dNTPs, forward and reversed primers from Table 2, template, and polymerase as described in Table 3. Sample 1, 2, 4 and 5 was run in the PCR machine using the program shown in Table 4 and sample 3 was following the program in Table 5. Some modifications to the amount of template and temperature had to be made to achieve a successful PCR reaction of the gene encoding BamA-like protein (Table 3).

Table 3: *Mixture for PCR reactions for HbpA and BamA.*

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
	HbpA	**BamA**	**BamA**	**BamA**	**BamA**
suH_2O	28 µl	28 µl	28 µl	30.5 µl	8 µl
5xHF buffer	10 µl	10 µl	10 µl	10 µl	10 µl
dNTP (10mM)	1 µl	1 µl	1 µl	1 µl	1 µl
Forward Primer (10µM)	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Reverse primer (10µM)	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Template (18.3 ng/µl)	5 µl	5 µl	5 µl	2.5 µl	25 µl
Phusion polymerase	1 µl	1 µl	1 µl	1 µl	1 µl
Total	**50 µl**	**50 µl**	**50 µl**	**50 µl**	**50 µl**
Tm	58.8 °C	61.3 °C	58.8 °C	58.8 °C	61.3 °C

Table 4: *PCR program for samples 1, 2, 4, and 5.*

	Temperature	**Time**	**Cycles**
Initial Denaturation	98 °C	1,5 min	x1
Denaturation	98 °C	15 sec	x30
Annealing	59 °C	30 sec	
Extension	72 °C	1,5 min	
Final extension	72 °C	10 min	x1

Table 5: *PCR program for sample 3.*

	Temperature	**Time**	**Cycles**
Initial Denaturation	98 °C	1,5 min	x1
Denaturation	98 °C	15 sec	x30
Annealing	61 °C	30 sec	
Extension	72 °C	2 min	
Final extension	72 °C	10 min	x1

The PCR products were analysed on a 1% agarose gel at 80V for 40 minutes. Before adding the samples to the agarose gel, 5 µl PCR product was mixed with 2 µl of 5x loading buffer. To control the size of the product, 2,5 µl of 1kb+ DNA ladder was supplied to the agarose gel.

Selected PCR products of HbpA-like and BamA-like were purified using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich).

2.6.3 Cutting and ligation in pET-21a

pET-21a plasmid was used for cutting and ligation with the PCR products coding for the BamA- and HbpA-like proteins. Both pET-21a and the PCR products were mixed with restriction enzymes (RE), as shown in Table 6 for cutting, and incubated overnight at 37 °C.

Table 6: *Mixture for cutting the pET-21a and PCR products with restriction enzymes NdeI and XhoI.*

	pET-21a	**HbpA**	**BamA**
DNA	25 µl	28 µl	28 µl
10 x buffer R3.1	5 µl	5 µl	5 µl
BSA 1:10	5 µl	5 µl	5 µl
NdeI	1 µl	1 µl	1 µl
XhoI	1 µl	1 µl	1 µl
H_2O	13 µl	10 µl	10 µl
Total	**50 µl**	**50 µl**	**50 µl**

The RE digestion reaction were heat-inactivated at 65 °C for 20 minutes in a heat block and purified using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich), before quantification on NanoDrop to determine the amount of DNA insert needed. The pET-21a samples were run on a 1% agarose gel, where the bands were excised for purification using the E.Z.N.A.® Gel Extraction kit and quantified on NanoDrop. For ligation, the recipe shown in Table 7 was calculated with purified pET-21a and PCR product in a ratio of 1:3, following the protocol for T4 DNA Ligase (NEW ENGLAND BioLabs Inc.).

Table 7: *Ligation mixture to insert BamA-like and HbpA-like genes into pET-21a.*

	HbpA	**BamA**
H_2O	3.0 µl	2.5 µl
pET-21a (50ng)	12.5 µl	12.5 µl
PCR product	1.5 µl	2.0 µl
Buffer	2.0 µl	2.0 µl
T4 DNA ligase	1.0 µl	1.0 µl
Total	20 µl	20 µl

The mixtures (Table 7) were mixed in 2 ml tubes and held at room temperature for 20 minutes before being incubated overnight at 16 °C.

2.6.4 Transformation in TOP10 cells

Eppendorf tubes containing 50 µl TOP10 chemically competent cells were collected from the -80 °C freezer and thawed on ice. Next, 5 µl from the ligation mixtures containing pET-21a plasmids with the BamA- and HbpA-like encoding genes as inserts was pipetted into the vials with competent cells and incubated for 10 minutes on ice. After 10 minutes on ice, the samples were heat-shock treated in a 42 °C warm water bath for 30 seconds and placed back on ice. Subsequently, 250 µl SOC medium (appendix) was added to the mixture with TOP10 competent cells and ligation plasmid and incubated horizontally in a shaking incubator (Infors AG, model Minitron) at 225 rpm and 37°C. After 40 minutes, the TOP10 suspensions were collected and spread on two separate LB-agar plates containing ampicillin (100 ug/ml), one with 20 µl- and 200 µl TOP10 suspension, and incubated overnight at 37 °C.

2.6.5 PCR screening

A PCR screen was performed to confirm that the genes were successfully inserted into pET21a. The bacterial colonies formed on LB-agar plates were harvested, and four 1.5 Eppendorf tubes for each protein were prepared with one randomly selected colony from each suspended in 20 µl LB + ampicillin (100 ug/ml). Then, 2 µl from each Eppendorf tube containing bacterial colony was mixed with 23 µl master mix (Table 8) in 0.2 PCR tubes. The PCR program is shown in Table 9.

Table 8: *Mastermix for PCR Screening with Dream Taq.*

	x1	x7
H_2O	17.4 µl	121.8 µl
10 x buffer	2.5 µl	17.5 µl
dNTP	0.5 µl	3.5 µl
pET11a forward primer	1.6 µl	7 µl
pET11a reverse primer	0.5 µl	7 µl
Dream Taq DNA polymerase	0.5 µl	3.5 µl
Total volume	23 µl	161 µl

Table 9: *PCR program for screening with Dream Taq.*

	Temperature	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	1.5 min	
Final extension	72 °C	10 min	x 1

After the PCR, the samples underwent analysis using a 1% agarose gel. To prepare for the gel electrophoresis, 5 µl of each PCR sample was mixed with 2 µl 5x loading buffer. Additionally, 2 µl of a 1KB+ standard was loaded onto the gel for reference.

Following confirmation of a successful transformation, the remaining 18 µl TOP10 competent cells in the Eppendorf tubes were cultivated overnight in 15 ml falcon tubes with 3 ml LB-medium + ampicillin (100 ug/ml) in a shaking incubator (Infors AG, model Minitron) at 225 rpm and 37°C, for plasmid purification.

2.6.6 Plasmid purification and sanger sequencing

Plasmids from the overnight culture were purified following the protocol for QIAprep® Spin Miniprep Kit (QIAGEN), with minor modifications. Firstly, 1.5 ml of the bacterial isolation was pelletized at 13000 x g in 3 minutes in a benchtop centrifuge (Allegra® X-15R Benchtop Centrifuge, Beckman Coulter®). The kit was used following the instructions for microcentrifuge. After elution, the plasmid concentrations were determined following nanodrop (Thermo Scientific NanoDrop™ 1000 Spectrophotometer).

The purified plasmids were submitted for Sanger sequencing at Azenta Life Science to confirm the DNA sequence of the genes transformed into the TOP-10 competent cells corresponding to the sequence of the genes encoding BamA- and HbpA-like proteins. Two samples for each gene were prepared in 1.5 Eppendorf tubes containing 5 µl purified plasmid mixed with 5 µl of either forward- or reversed primers. Before being added to the Eppendorf tubes, the primers were diluted 1:1 with RNase-free water. An additional sample was submitted for the sequence encoding for BamA-like protein, where a specific primer (Table 2) was added to cover the middle part of the sequence.

2.7.1 Transformation with BL21-Codon+ competent cells

The purified plasmids encoding the BamA- and HbpA-like proteins were transformed into competent Codon+ cells using the same procedure described in section 2.6.4 with some modifications, such as 2 µl purified plasmid was added to the Eppendorf tubes containing Codon+ *E. coli* cells. Next, 25 µl of ampicillin (100 ug/ml) and 25 µl of chloramphenicol (25 mg/ml) were spread on LB-agar plates to prevent the growth of other bacteria, and 25 µl from each transformation was smeared on the LB-agar plates for incubation overnight at 37°C. Next, one randomly selected colony from each LB-agar plate was collected and suspended in 5 ml LB media supplemented with 44 µl of chloramphenicol (25 mg/ml) and 44 µl of ampicillin (100 ug/ml) in 50 ml falcon tubes. The culture was incubated in a shaking incubator (Infors AG, model Minitron) at 225 rpm and 37°C overnight.

2.7.2 Expression of recombinant protein induced by IPTG

The culture with competent Codon+ *E. coli* cells containing plasmids encoding the BamA- and HbpA-like proteins, hereafter called "Codon+/HbpA start culture" and "Codon+/BamA start culture", was harvested for subcultivation with Isopropyl β-D-1-thiogalactopyranoside (IPTG). In two 250 ml Erlenmeyer flasks, one for each gene, 1 ml of Codon+ cells were suspended in 100 ml LB-media (appendix) supplied with 100 µl of chloramphenicol (25 mg/ml) and 100 µl of ampicillin (100 ug/ml) and incubated in a shaking incubator (Infors AG, model Minitron) at 200 rpm and 37°C for 2 hours. After 2 hours, OD was measured to confirm optimal growth. The cultures were split in half, and 50 ml from each culture was transferred into two new 250 ml Erlenmeyer flasks supplemented with 50 µl IPTG. At this point, there were two 250 ml Erlenmeyer flasks containing 50 ml of each culture, one supplemented with 50 µl IPTG and one without for each culture. The four Erlenmeyer flasks were incubated in a shaking incubator (Infors AG, model Minitron) at 225 rpm and 37°C for 3 hours and 30 minutes. After incubation, the cultures were pelleted in a benchtop centrifuge (Allegra® X-15R Benchtop Centrifuge, Beckman Coulter®) to prepare for SDS-PAGE electroporation. The weights of four empty 50 ml falcon tubes were measured to ensure accurate measurement of the pellet weight. Subsequently, the cultures with and without IPTG were transferred into the 50 ml falcon tubes and centrifuged at 4000 x g for 10 minutes. Following centrifugation, the supernatant was carefully discarded, and the weights of each tube were measured again. The pellets were resuspended in PBS buffer in a ratio of 1:100 based on their weight, for further analysis on

SDS-PAGE gel electrophoresis to determine the production of targeted proteins, and Western Blot (WB) to confirm their binding to antibodies.

For SDS-PAGE analysis and Western Blot, three precast gels (Mini-PROTEAN® precast gels) were arranged with samples of BamA- and HbpA-like proteins. The difference between the gels is that a molecular weight marker (Silver Stain SDS-PAGE Standards Low Range, BioRad) was used as a ladder for SDS-PAGE analysis, while a Kaleidoscope™ WB standard (Bio-Rad) was used for WB analysis. The samples for SDS-PAGE analysis were electrophoresed and Coomassie stained in the same manner as in section 2.2.2.

2.7.3 Western Blot

The protein bands on the precast gels (Mini-PROTEAN® precast gels) were transferred to 0.2 µm nitrocellulose membranes (Bio-Rad) with electroblotting. For preparations, filter papers, sponges, and nitrocellulose membranes were cut in the same size as the SDS-PAGE gels and immersed in 1 x blotting buffer (appendix) for 30 minutes. To transfer the protein bands from the gels to the membrane, a sandwich was arranged in a gel holder as follows: one sponge at the black side, three filter papers, SDS-PAGE gels, nitrocellulose membrane, three filter papers, and one sponge on top. The sandwich was mounted according to the manufacturer's instructions in an electrophoresis cell (Bio-Rad, Mini-PROTEAN II), supplied with a cooling block. The electrophoresis cell was filled with 1x blotting buffer (appendix), and the electroblotting was performed at 100 V for 60 minutes. During electrophoresis, the electrophoresis cell was placed on a magnetic stirrer.

All steps following the electroblotting were carried out at room temperature with gentle shaking on a flat orbital shaker (IKA® KS 260 basic). After electroblotting, the protein bands of the molecular weight standard show as coloured bands on the nitrocellulose membrane. One of the membranes, hereafter called membrane 1, was subjected to blotting with antibodies, and the other, membrane 2, served as a control. Initially, both membranes were incubated in a blocking solution (appendix) for 1 hour to block unbound sites at the membrane. Next, the membranes were washed with TTBS (appendix) for 5 minutes. Membrane 1 was incubated overnight in a solution with primary antibody, 10 µl His-Tag (6xHisTag Monoclonal Antibody) (Thermo Fisher Scientific) diluted 1:1000 in antibody buffer (appendix). Similarly, membrane 2 underwent overnight incubation in an antibody buffer (without primary antibody). Following this, the membranes were washed twice in TTBS (appendix) for 5 minutes. Membrane 1 and 2

was then incubated for 90 minutes in secondary antibody solution, 5 µl of goat anti-mouse (Polyclonal Goat Anti-Mouse Immunoglobulins/HRP from Dako Denmark A/S), diluted at 1:2000 with antibody buffer. Membranes 1 and 2 underwent an additional two washes in TTBS for 5 minutes before both membranes were incubated for 5 minutes in TBS. The membranes were prepared for imaging following the protocol outlined for the Clarity™ Western ECL kit (BIO-RAD) and photographed in ChemiDoc™ XRS+ System with Image Lab™ (BIO-RAD).

2.7.4 Expression of recombinant HbpA-like protein in ZYP media

After the Western blot, it was decided to proceed with expressing the HbpA–like protein recombinantly. Firstly, 10 µl of Codon+/HbpA startculture from section 2.7.2 was inoculated in 50 ml PA – 0.5 G medium (appendix) in 250 ml Erlen Meyer flasks, and incubated in a shaking incubator (Infors AG, model Minitron) at 225 rpm and 37°C overnight. To mix the ZYP 5052 – medium (appendix), the ZY – medium (appendix) was mixed and autoclaved to ensure no other bacteria grew in the culture. The next day, 10 ml from the 50 PA – 0.5 G medium culture was inoculated in 1 liter ZYP 5052 media (appendix) in 2.5 L Erlen Meyer flasks and incubated in a shaking incubator (Infors AG, model Minitron) at 225 rpm and 37°C for 24 hours.

After incubation, the ZYP culture was transferred to 1 L centrifuge tubes and centrifuged in an Avanti® J-26S centrifuge (Beckman Coulter). The weight of the pellet was determined after it was transferred to 50 ml falcon tubes and stored in the –80 °C freezer for purification with Chelating Sepharose.

2.7.5 Purification of HbpA-like protein with Chelating Sepharose

The pellet from the ZYP cultures was collected from the -80°C freezer for purification with Chelating Sepharose. To prepare for protein purification, buffer A (lysis buffer) (appendix), buffer B (elution buffer) (appendix), buffer C (Ni-buffer 8x) (appendix), and buffer D (stripping buffer) (appendix) were made, and 3 ml of resin was loaded into the column. The ZYP culture was resuspended in 10 mL Buffer A until the pellet was dissolved entirely, followed by sonication using the “630-0422” microtip (for volumes between 0.5 ml – 10 ml). The sonication lasted 3 minutes with the amplitude set to 30% and the pulse set to 30 – 30. After sonication, 100 µl cell extract was pipetted into a 1.5 ml Eppendorf tube for testing on SDS-PAGE at a

later stage (kept on ice). The culture was centrifuged in a benchtop centrifuge (Allegra® X-15R Benchtop Centrifuge, Beckman Coulter®) at 4000 x g for 20 minutes. During centrifugation, the column with resin was washed by adding 4 column volumes (CV) of milliQ, 3 CV of 1x Ni (appendix), 3 CV of milliQ, and 2 CV of buffer A. Following centrifugation, the supernatant was transferred to a new 50 ml falcon tube, 100 µl was pipetted to a 1.5 ml Eppendorf tube for SDS-PAGE test, and the pellet was stored at -80 °C freezer. The resin was transferred to the supernatant and incubated with shaking at 4 °C on a Polymax 1040 (Heidolph Instruments GmbH & Co. KG) for 90 minutes to ensure binding between the resin and HisTag proteins. After incubation, the culture was centrifuged three times in a benchtop centrifuge (Allegra® X-15R Benchtop Centrifuge, Beckman Coulter®) at 500 x g for 1 minute each. The supernatant was discarded between each centrifugation, and the pellet was carefully resuspended in 25 ml of buffer A. After the final centrifugation, the pellet was resuspended in 10 ml buffer A and loaded onto the column. The column was washed with 2 CV of buffer A before elution with fractions ranging from 50-300 mM imidazole. The elution was performed twice per fraction (Table 10) and collected in 15 ml falcon tubes. After elution, the column was rinsed with 1 CV buffer D, 1 CV milliQ, and 1 CV 20% EtOH and stored at 4 °C.

Table 10: *Dilution of imidazole used to eluate.*

Imidazole (mM)	Buffer B (500 mM imidazole)	Buffer A (0 mM imidazole)
50	1µl	9µl
100	2µl	8µl
150	3µl	7µl
200	4µl	6µl
250	5µl	5µl
300	6µl	4µl

The samples from the cell extract, supernatant, and elution's from 50 – 300 mM imidazole (Table 10) were tested by SDS-PAGE and Coomassie staining to confirm if the proteins were present in the supernatant. The samples were diluted in milliQ and SDS-sample buffer before being loaded onto the precast gels (Mini-PROTEAN® precast gels).

As the recombinant protein was insoluble, the bacterial pellet was collected from the -80°C freezer for purification. The procedure described for the supernatant was performed with some modifications. Urea was added to Buffer A to a final concentration of 8M, and all steps were

performed at room temperature because of urea's low freezing point. Following the elution, all samples (cell extract, supernatant, and elution fractions) were tested on SDS-PAGE gels in the same manner as the purified supernatant.

2.7.6 Renaturation of proteins with dialyze cassettes.

After purification of proteins using urea, denatured proteins need to be renatured using dialysis cassettes. The SDS-PAGE gels were analysed to identify the eluate fraction with purest and highest concentration of recombinant proteins. Dialysis cassettes were filled with 3 ml of eluate using a 3 ml syringe (FINE-JECT®). The cassettes were incubated in 1 L dialysis buffer with 4M urea (appendix) for 2 hours on a magnet stirrer. After 2 hours, the cassettes were transferred to 1 L of dialysis buffer (appendix) (without urea) and incubated overnight. The next day, the solution containing renatured proteins was transferred from the dialysis cassettes to 15 ml Falcon tubes using a 3 ml syringe, ready to measure protein concentration in the sample.

2.7.7 Measurement of protein concentration of HbpA-like protein

The protocol outlined in the Bio-Rad Protein Assay was applied to assess the total protein concentration in the protein samples with HbpA-like protein. Four 1 ml cuvettes to calculate the standard curve and two additional 1 ml cuvettes to measure the protein samples were prepared.

To create the standard curves, 900 µl of Diluted Dye concentrate (appendix) was pipetted into the cuvettes. BSA-solution (appendix) and H_2O were pipetted directly to the cuvettes according to the recipe in Table 11. The cuvettes were then inverted three times with parafilm to blend the samples.

Following this, two cuvettes were prepared to measure the protein concentration in 1 µl and 5 µl protein samples. 0.2 ml of Bio-Rad Protein Assay Dye Reagent Concentrate, 0.8 ml H_2O, and the protein sample were pipetted directly into the cuvettes. The cuvettes were inverted three times with parafilm to blend the samples.

The absorbance of the samples was measured at 595 nm using a spectrophotometer. Initially, the spectrophotometer was blanked with a sample containing 0 µg BSA.

Table 11: *Mixture with BSA for standard curve.*

Amount BSA solution (µl)	H_2O	Total amount BSA (ug)
0	10	0
10	90	2
25	75	5
50	50	10

2.8 Analysis of antigenicity of the HbpA-like protein

The HbpA-like protein was analysed by WB using sera collected from salmon vaccinated against PaL-1 and sera from unvaccinated salmon. Initially, three samples were prepared in reducing buffer for SDS-PAGE electrophoresis following the procedure described in section 2.2.2. Sample 1 contained the full protein profile of PAL-1, sample 2 contained the recombinant HbpA-like protein diluted 1:10 in PBS, and sample 3 contained recombinant HbpA-like protein diluted 1:50 in PBS. The samples were arranged on three precast gels (Mini-PROTEAN® precast gels) and electrophorized at 190V for 45 minutes. After the electroporation gel 1 was coomassie stained and photographed following the procedure for coomassie staining described in section 2.2.2, whereas gels 2 and 3 were subjected to WB against sera from vaccinated and unvaccinated salmon.

For the WB of gel 2 and 3 the procedure described in section 2.7.3 was followed, but with different antibodies. For the step with incubation overnight in primary antibody, gel 2 was incubated in sera from PaL-1 vaccinated salmon diluted 1:100 in antibody buffer, and gel 3 was incubated in sera from unvaccinated salmon diluted 1:100 in antibody buffer. Next, both gels were incubated in rabbit anti-salmon IgM diluted 1:1000 with antibody buffer for 2 hours, before one last incubation in a third antibody, goat anti-rabbit Ig conjugated with HPR antisera (Dako Denmark A/S), diluted 1:1000 in antibody buffer for 90 minutes.

Following incubation, the samples were prepared for analysis following the protocol described in the Clarity™ Western ECL kit (BIO-RAD) and photographed using the ChemiDoc™ XRS+ System with Image Lab™ (BIO-RAD), similarly as in section 2.7.3.

2.9.1 Cultivation of PaL-1 in TSB supplemented with GSH and GSSH

To investigate the impact of glutathione on PaL-1 growth, the bacteria was incubated in four different media; TSB supplemented with 1.5% NaCl, TSB+, and TSB supplemented with 1.5% NaCl and either reduced glutathione (GSH) or oxidized glutathione (GSSH). Initially, a starter culture of PaL-1 was incubated overnight following the procedure described for cultivation in section 2.1.1. After incubation the starter culture of PAL-1 was centrifuged in an Allegra® X-15R Benchtop Centrifuge (Beckman Coulter®) at 2500 x g for 10 minutes, the supernatant was discarded, and the pellet was resuspended in TSB (1.5% NaCl).

Subsequently, four 15 ml falcon tubes were prepared with 10 ml of TSB (1.5% NaCl), TSB+, TSB (1.5% NaCl) supplemented with 5 µg/ml GSH, and TSB (1.5% NaCl) supplemented with 5 µg/ml GSSH. Then, 250 µl of the resuspended pellet was supplied to each growth media, and cell counts of each culture was estimated following the procedure of CASY cell counter (CASY® Cell Counter) outlined in section 2.1.3, before the cultures was incubated in a shaking incubator at 200 rpm and 20 °C. Cell counts was then measured after 5 hours, 24 hours and 28 hours to assess growth over time.

2.9.2 Assessment of ligand binding interactions between HbpA-like protein and GSH/GSSH

Cryo tubes with recombinant HbpA-like protein (1 g/ml), were gathered from the -80 °C freezer, and 10 mM stocks with GSH and GSSH were prepared. The molecular weight of GSH is 307.32 g/mol, and GSSH is 612.32 g/mol. To gain 10 mM in the stock solution, 6 mg GSH was diluted in 2000 µl milliQ water, and 12 mg GSSH was diluted in 2000 µl milliQ. The HbpA-like protein (20 µl/tube) was mixed with 40 µl of GSH or GSSH stock dilutions resulting in 0, 1, 2, 3, 4, 5, and 10 mM of GSH or GSSH in the different tubes. The samples were incubated for 45 minutes at room temperature. The first sample (0 mM) contained HbpA-like protein without GSH or GSSG.

Two SDS-PAGE gels were made for the analysis. The gels were prepared within glass plates with resolving gel (10%) (appendix) at the bottom and stacking gel (4%) (appendix) at the top. For analysis on two SDS-PAGE gels (one for GSH and one for GSSH), seven 1.5 Eppendorf tubes for each gel were prepared. After 45 minutes, the samples were diluted 1:1 in SDS-PAGE sample buffer without DTT (appendix), and 8 µl of the sample and 5 µl protein standard

(Precision Plus Protein™ Kaleidoscope™) were included for the analysis. The electroporation, Coomassie staining, and photographs of the gels were performed as outlined in section 2.2.2.

3. Results

3.1 Growth curve PaL-1

The growth curve of PaL-1 cultivated in TSB+ is illustrated in Figure 3. The figure shows that the PaL-1 isolate grows slowly in the lag phase until growth increases after approximately 8 hours of incubation, when exponential growth starts. The exponential growth phase continues until the stationary phase is reached after 20 hours of incubation.

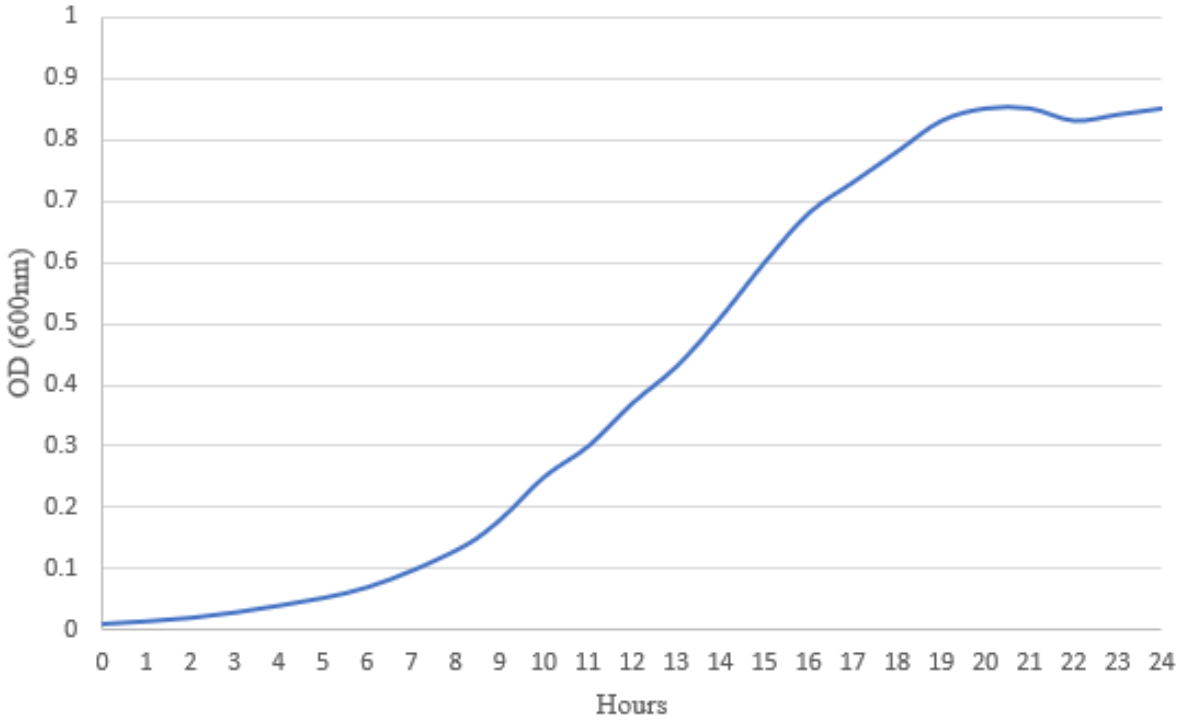

Figure 3: *Growth curve of the PAL-1 isolate cultivated in TSB+ at 200 rpm and 20 °C. The x-axis shows hours of incubation, and the y-axis shows optical density measured at 600 nm.*

Table 12 shows the OD_{600} measurements forming the growth curve presented in Figure 3 and the correlation between measured OD_{600} and bacterial cells per milliliter measured in the CASY cell counter. The table shows that during the exponential growth phase, after 8 hours, the number of bacterial cells in the culture increases more than during the lag phase. The table also shows that when the bacterial growth reaches the stationary phase based on the OD measurements, after 20 hours the increase in cells per milliliter slows down and stabilizes.

Table 12: *Optical density (OD) measured at 600 nm and cells per millilitre of the PAL-1 isolate when cultured in TSB+ over time.*

Cell counts and optical density		
Hours	**OD_{600}**	**Cell counts (cells/ml)**
0	0.01	4.00E+07
2	0.02	9.94E+07
4	0.04	4.05E+07
6	0.04	1.44E+08
8	0.13	2.35E+08
9	0.18	3.40E+08
10	0.25	4.02E+08
11	0.30	5.55E+08
12	0.37	7.08E+08
13	0.43	9.00E+08
14	0.51	1.20E+09
15	0.6	1.60E+09
16	0.68	1.84E+09
17	0.73	1.93E+09
18	0.78	2.50E+09
19	0.83	2.70E+09
20	0.85	3.10E+09
21	0.85	2.90E+09
22	0.83	2.10E+09
23	0.84	2.80E+09
24	0.85	3.00E+09

3.2.1 Analysis of temperature tolerance of *P. atlantica* isolates

The cell counts of PaL-1, *P. atlantica* gv. *cyclopteri*, *V. anguillarum*, and atypical *A. salmonicida* after 24 hours of cultivation at 20 °C, and two subcultures for each of the *P. atlantica* isolates, one cultured at 10 °C and one cultured at 30 °C are present in Table 13. The results indicate that the number of cells/ml is higher for the PaL-1 isolate when incubated at 20

°C compared to the subcultures at 10 °C and 30 °C. For the lumpfish isolate, *P. atlantica* gv. *cyclopteri,* the subculture incubated at 30 °C shows the highest number of cells/ml.

Table 13: *Cell counts of* P. atlantica *cultivated for 24h at 10 °C, 20 °C, and 30 °C.*

Bacteria	Growth condition	Cells per ml
PaL-1	200 rpm and 20°C	2.30E+09
Subculture PaL-1	200 rpm and 10°C	4.60E+08
	200 rpm and 30°C	1.30E+09
P. atlantica gv. *cyclopteri*	200 rpm and 20°C	1.40E+09
Subculture *P. atlantica* gv. *cyclopteri*	200 rpm and 10°C	1.30E+08
	200 rpm and 30°C	1.50E+09

The protein profiles of the bacteria cultured at 20 °C, and the subcultures at 10 °C and 30 °C (Table 13) were illustrated on coomassie-stained SDS-PAGE gels (Fig. 4). The protein profiles of the PaL-1 isolate subcultured at 10 °C and 30 °C (lane 1 and 3) and the starter culture at 20 °C (lane 2) are shown in Figure 4A. The protein profiles of the *P. atlantica* gv. *cyclopteri* isolate subcultured at 10 °C and 30 °C (lane 1 and 3 respectively) and the starter culture at 20 °C (lane 2) are shown in Figure 4B. Figure 4A demonstrates darker protein bands for the PaL-1 isolate when cultured at 20 °C and 30 °C compared to when cultured at 10 °C. In particular for proteins at approximately 38 kDa size (indicated by white arrows in Fig 4A). Similarly, the *P. atlantica* gv. *cyclopteri* isolate (Fig. 4B) shows more intensified protein bands of approximately 66 kDa size (indicated by white arrows in Fig. 4B) when cultured at 20 °C and 30 °C compared to when cultured at 10 °C. The protein profiles do not indicate any absence or differences in the localization of protein bands in the different cultures.

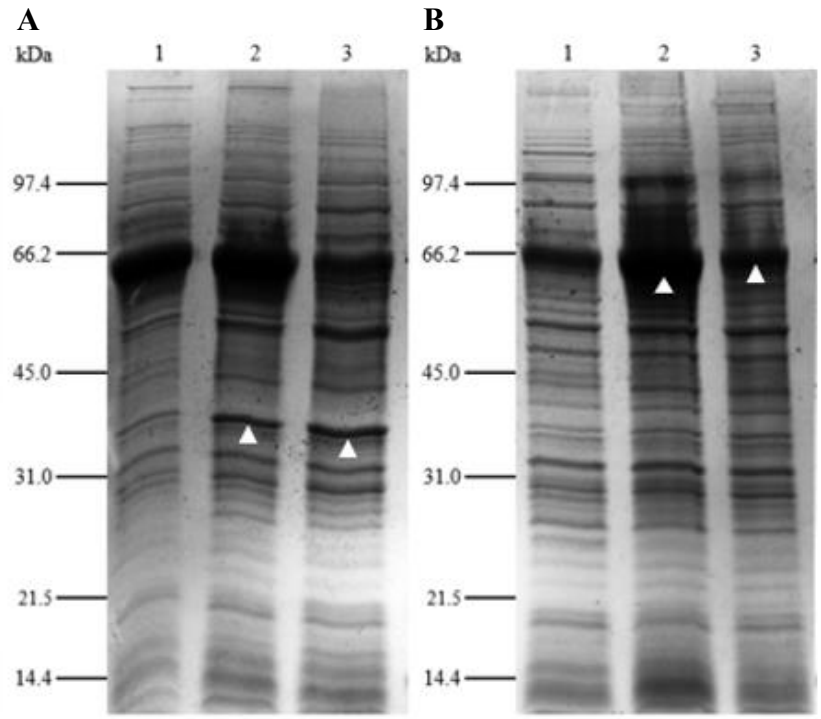

Figure 4: *Coomassie-stained SDS-PAGE gels showing protein profiles.* ***A:*** *Protein profiles of PaL-1 starter culture and both subcultures, lane 1: 10 °C, lane 2: 20 °C, and lane 3: 30 °C.* ***B:*** *Protein profiles of* P. atlantica *gv.* cyclopteri *starter culture and both subcultures, lane 1: 10 °C, lane 2: 20 °C, and lane 3: 30 °C.*

3.2.2 Analysis of the presence of plasmids in PaL-1

The protein profiles of PaL-1, *P. atlantica* gv. *cyclopteri, V. anguillarum,* and atypical *A. salmonicida* cultivated at 20 °C are presented in Figure 5A lane 1-4 respectively. In addition, the presence of plasmids for all the isolates was analysed in Figure 5B. The isolates of *P. atlantica* gv. *salmonicida* and *V. anguillarum* had previously been analysed and found not to contain plasmids, the atypical *A. salmonicida* isolate had previously been found to contain plasmids, these isolates were therefore included as controls to demonstrate presence or absence of plasmids in the PaL-1 isolate. The results are illustrated in Figure 5 and demonstrate that only the isolate of atypical *A. salmonicida* contains plasmids (lane 2). The isolate atypical *A. salmonicida* in lane 2 displays three bands on the gel, which represent three separate plasmids. No plasmids were identified in the PaL-1 isolate.

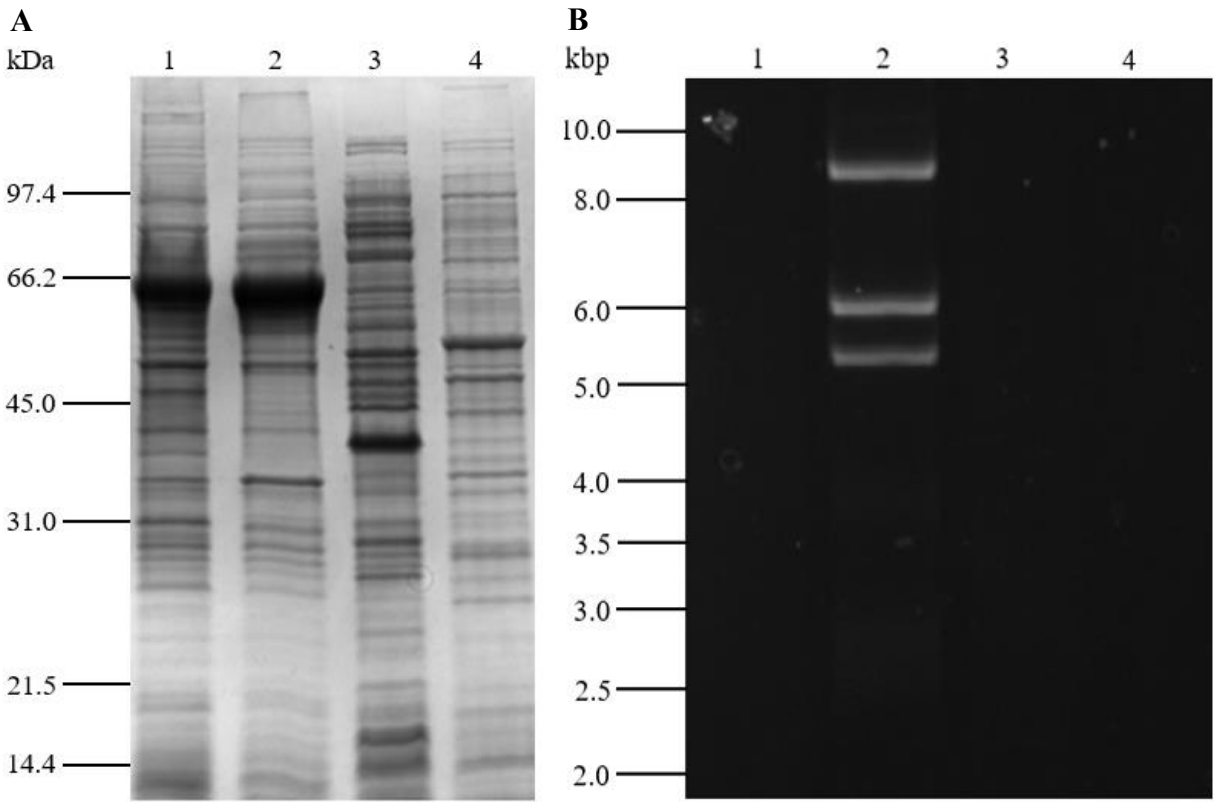

Figure 5: A: *Coomassie stained SDS-PAGE gel showing protein profiles of PaL-1 (1.2 x 10^9 cells/ml) (lane 1),* P. atlantica *gv.* cyclopteri *(0.7 x 10^8 cells/ml) (lane 2),* V. anguillarum *(0.7 x 10^8 cells/ml) (lane 3), and atypical* A. salmonicida *(1.3 x 10^9 cells/ml) (lane 4).* ***B:*** *1% agarose gel showing purified plasmids.* V. anguillarum *(lane 1),* P. atlantica *gv.* Cyclopteri *(lane 3), and PaL-1 (lane 4) do not show plasmids on the gel. The atypical* A. salmonicida *isolate shows three plasmids in lane 2.*

3.3 *In Silico* analysis

The results from the in-silico analysis of the PaL-1 proteome are shown in Tables 14 and 15. The most promising proteins for a vaccine against pasteurellosis in Atlantic salmon were narrowed down to lists of proteins sized between 40 - 70 kDa (Table 14) and 80 – 100 kDa (Table 15), localized either extracellular, periplasmic or in the outer membrane of the bacteria. The selected proteins were further narrowed down to two proteins, one Heme binding protein A (table 1) (in bold typing), hereafter called HbpA-like protein, and one Outer membrane protein assembly factor BamA (Table 15) (in bold typing), hereafter called BamA-like protein.

Table 14: *Proteins in the range of 40-70 kDa, localized either extracellular (E), periplasmic (P), and in the outer membrane (OM), their access number in the proteome, and similar proteins with known bacterial virulence factors from VFDB. VFDB show protein name, protein function and bacterial species from where it has been described.*

#	Protein	MW	Localization	VFDB	E-value	Id	Blastp	Access
1	Hypothetical protein	68.0	OM (100)	(hrpB) two-partner secretion system transporter HrpB. Effector delivery system. Neisseria meningitidis MC58	1,00E-101	37 %	ShlB/FhaC/HecB family hemolysin secretion/activation protein	WP_306347399.1
2	Hypothetical protein	67.1	OM (100)	(hrpB) two-partner secretion system transporter HrpB. Effector delivery system. Neisseria meningitidis MC58	1,00E-104	36 %	ShlB/FhaC/HecB family hemolysin secretion/activation protein	WP_306353493.1
3	Translocation and assembly module subunit TamA	66.1	OM (100)	(omp89) outer membrane protein assembly factor BamA. Adherence. Bartonella quintana str. Toulouse	6,00E-08	32 %	autotransporter assembly complex family protein	WP_306346823.1
4	Peptide transport periplasmic protein SapA	64.9	P (100)	(oppA) peptide ABC transporter substrate-binding protein. Nutritional/Metabolic factor. Listeria ivanovii subsp. ivanovii PAM 55	1,00E-07	19 %	ABC transporter substrate-binding protein	WP_306347629.1
5	**Heme-binding protein A**	**60.3**	**P (98)**	**(oppA) peptide ABC transporter substrate-binding protein. Nutritional/Metabolic factor. Listeria seeligeri serovar 1/2b str. SLCC3954**	**4,00E-18**	**24 %**	**ABC transporter substrate-binding protein**	**WP_306346933.1**
6	Periplasmic oligopeptide-binding protein	59.7	P (94)	(oppA) peptide ABC transporter substrate-binding protein. Nutritional/Metabolic factor. Listeria seeligeri serovar 1/2b str. SLCC3954	4,00E-27	25 %	peptide ABC transporter substrate-binding protein	WP_322631725.1
7	hypothetical protein	56.9	E (95)	(rhsP2) ADP-ribosyltransferase toxin. Effector delivery system. Pseudomonas fluorescens Pf0-1	7,00E-05	27 %	hypothetical protein	WP_306371852.1
8	TPR repeat-containing protein	54.98	OM (95)	(BJAB0715_RS05250) porin family protein. Nutritional/Metabolic factor. Acinetobacter baumannii BJAB0715	2,00E-20	20 %	surface lipoprotein assembly modifier	WP_306346273.1
9	Toxin and drug export protein A	52.0	OM (100)	(mtrE) multidrug efflux pump channel protein MtrE Antimicrobial activity/Competitive advantage.Neisseria gonorrhoeae NCCP11945	3,00E-20	19 %	TolC family protein	WP_306347092.1
10	hypothetical protein	51.3	E (96)	(sdrD) Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein. Adherence. Staphylococcus aureus subsp. aureus N315	5,00E-14	22 %	hypothetical protein	WP_306355447.1
11	hypothetical protein	50.2	P (97)	No hit			imelysin family protein	WP_306347725.1
12	Periplasmic serine endoprotease DegP	50.0	P (98)	(mucD) serine protease MucD precursor. Biofilm Pseudomonas aeruginosa PA7	3,00E-72	38 %	DegQ family serine endoprotease	WP_306346961.1
13	Methylamine utilization protein MauG	47.5	P (100)	No hit			cytochrome c peroxidase	WP_306347729.1
14	Tol-Pal system protein TolB	46.5	P (98)	No hit			Tol-Pal system beta propeller repeat protein TolB	WP_306346604.1
15	hypothetical protein	45.3	OM (100)	(lepB) Dot/Icm type IV secretion system effector LepB, Rab1 GTPase activating protein (GAP). Effector delivery system. Legionella pneumophila str. Paris	1,00E-09	23 %	murein hydrolase activator EnvC	WP_306347595.1
16	Maltoporin	44.7	OM (100)	No hit			maltoporin [Pasteurella atlantica]	WP_306346224.1
17	hypothetical protein	44.5	OM (95)	(SPR_RS06970) G5 domain-containing protein. Adherence. Streptococcus pneumoniae R6	5,00E-05	23 %	hypothetical protein [Pasteurella atlantica]	WP_306373349.1
18	hypothetical protein	43.2	OM (95)	(EF3023) polysaccharide lyase, family 8. Exoenzyme. Enterococcus faecalis D32	4,00E-06	32 %	hypothetical protein [Pasteurella atlantica]	WP_322631698.1
19	Membrane-bound lytic murein transglycosylase A	41.5	OM (99)	No hit			murein transglycosylase A [Pasteurella atlantica]	WP_211596950.1

Table 15: *Proteins within the range of 80-100 kDa, localized either extracellular (E), periplasmic (P), and in the outer membrane (OM), their access number in the proteome, and similar proteins with known bacterial virulence factors from VFDB. VFDB show protein name, protein function and bacterial species from where it has been described.*

#	Protein	MW	Localization	VFDB	E-value	Id	Blastp	Access
1	putative protein	97.7	OM-(95)	(mam7) multivalent adhesion molecule MAM7. Adherence. Vibrio parahaemolyticus RIMD 2210633	1,00E-137	32 %	MlaD family protein	WP_306347287.1
2	Periplasmic nitrate reductase	93.3	P-(99)	(nuoG) NADH-quinone oxidoreductase subunit G. Immune modulation. Mycobacterium gilvum PYR-GCK	1,00E-14	28 %	nitrate reductase catalytic subunit NapA	WP_306346495.1
3	Trimethylamine-N-oxide reductase	93.0	P-(100)	(narG) nitrate reductase subunit alpha. Nutritional/Metabolic factor. Mycobacterium gilvum PYR-GCK	3,00E-06	25 %	trimethylamine-N-oxide reductase TorA	WP_306346876.1
4	LPS-assembly protein LptD	90.3	OM-(100)	No hit			LPS assembly protein LptD	WP_306346585.1
5	**Outer membrane protein assembly factor BamA**	**89.5**	**OM-(100)**	**(omp89) outer membrane protein assembly factor BamA. Adherence. Bartonella quintana str. Toulouse**	**9,00E-76**	**26 %**	**Outer membrane protein assembly factor BamA**	**WP_306347077.1**

3.4.1 Expression of recombinant protein

Genomic DNA was successfully isolated from the *V. anguillarum,* atypical *A. salmonicida, P. atlantica* gv. *salmonicida, and* PaL-1 isolates, and presented in Figure 6A lane 1-4 respectively. Further, the genes encoding the BamA-like- and HbpA-like proteins of the PaL-1 isolate were amplified with PCR (Fig. 6B). PCR screening after cloning in pET-21a vectors, transformed in TOP10 competent cells, verified that the PCR product had successfully been inserted into the vector (Fig. 6C-D). In Figure 6A, clear and distinct bands indicate that the samples are pure. Analysis of the PCR reaction with amplificated genes coding for the BamA-like and HbpA-like proteins (Figure 6B) shows protein bands at approximately 2500 base pairs and 1500 base pairs respectively, which correspond to the theoretical size of the genes. Figure 6C-D displays PCR products of four samples with TOP10 cells on a 1% agarose gel and confirms that the genes encoding the BamA-like and the HbpA-like proteins were correctly inserted into pET-21a vectors.

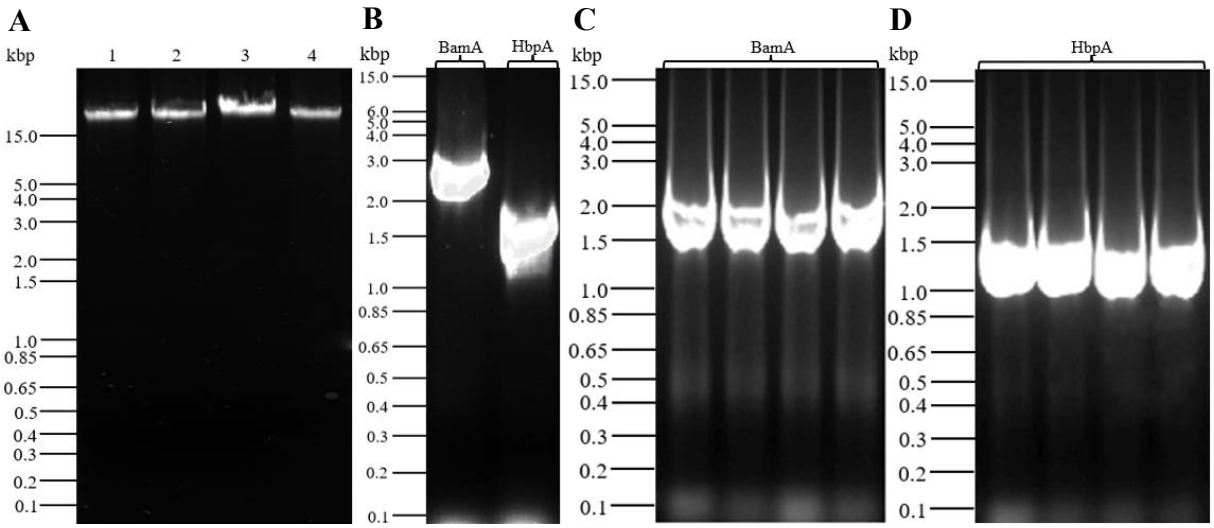

Figure 6: *1% agarose gels illustrating:* ***A:*** *Genomic DNA of* V. anguillarum *(lane 1), atypical* A. salmonicida *(lane 2),* P. atlantica *gv.* cyclopteri *(lane 3), and PaL-1 (lane 4).* ***B:*** *PCR products of the genes encoding the BamA-like protein and HbpA-like protein.* ***C:*** *PCR-screen showing four samples of the pET-21a plasmids with the BamA-like protein as insert to control successful ligation.* ***D:*** *PCR-screen showing four samples of the pET-21a plasmids with the HbpA-like protein as insert to control successful ligation.*

The sequences of the inserts were verified by Sanger sequencing.

With confirmed Sanger sequencing, the plasmids containing genes encoding the HbpA-like and the BamA-like protein inserts were transformed into Codon+ *E. coli* cells. Following transformation, the Codon+ cells were incubated, and one culture was supplied with IPTG to induce the production of the target proteins, and the other was incubated without IPTG. For the coomassie stained gel (Fig. 7A), the cultures supplied with IPTG show darker protein bands at approximately 60 kDa for the HbpA-like protein (lane 2) and at approximately 90 kDa for the BamA-like protein (lane 4). The WB analysis (Fig. 7B) performed to test antibody binding to the 6xHisTag showed that the HbpA-like protein was successfully expressed (Fig. 7B, lanes 1 and 2). For the BamA-like protein, no visual bands were identified by the WB (Fig. 7B, lanes 3 and 4), indicating no reactivity to the 6xHisTag antibody and that the protein has not been produced. The same effect of IPTG, as shown on the coomassie stained gel (Fig. 7A), is demonstrated by the WB analysis, with more prominent bands for the HbpA-like protein in lane 2.

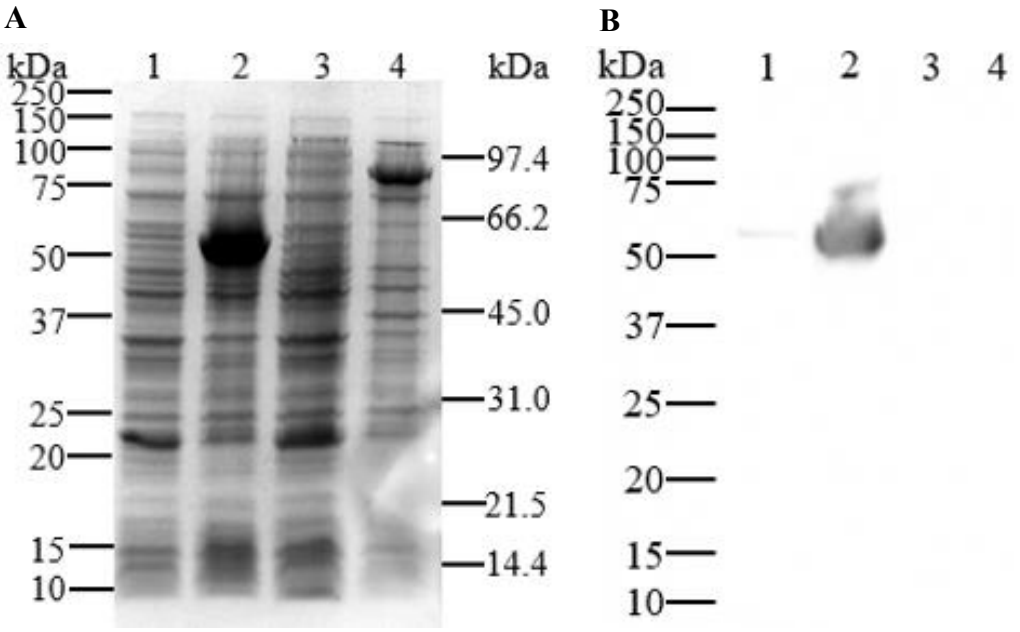

Figure 7: *Coomassie-stained SDS-PAGE gel and WB of Codon+ cells containing plasmids with BamA- and HbpA-like proteins induced by IPTG.* ***A:*** *Coomassie-stained SDS-PAGE with HbpA without IPTG (lane1), HbpA with IPTG (lane 2), BamA without IPTG (lane 3), and BamA with IPTG (lane 4).* ***B:*** *WB of proteins of SDS-PAGE, blotted against 6xHisTag antibody.*

3.5 Purification with chelating Sepharose

HbpA-like proteins were successfully purified from the pellet of the Codon+ culture with Chelating Sepharose (Fig. 8). Following sonication of the Codon+ culture the cell extract (Fig. 8A, lane 1) and supernatant (Fig. 8A, lane 2), were gathered for analysis along the purified proteins. Further, recombinant HbpA-like proteins were purified with different Imidazole concentrations to obtain the purest sample with the highest concentration of recombinant protein. Two elution's, with each concentration of Imidazole (Table 10), were applied to the lanes in Figure 8A lanes 3-8 and Figure 8B lanes 1-6, respectively. The gel analysis (Fig. 8A-B) shows a big dark protein band at approximately 60 kDa in all lanes, indicating a successful purification. The purest sample with the most distinct protein bands, indicating the highest protein concentration, was observed in the samples purified with 100 mM and 150 mM Imidazole (Fig. 8A, lanes 5, 6, and 7). In contrast to the purified samples, the lanes containing cell extract and supernatant (Fig. 8A, lanes 1 and 2) displayed multiple bands, including the distinct band at 60 kDa. Additionally, it was observed that the protein bands became weaker in the samples eluted with higher concentration of Imidazole (Fig. 8B), indicating a decrease in proteins with higher concentrations of Imidazole.

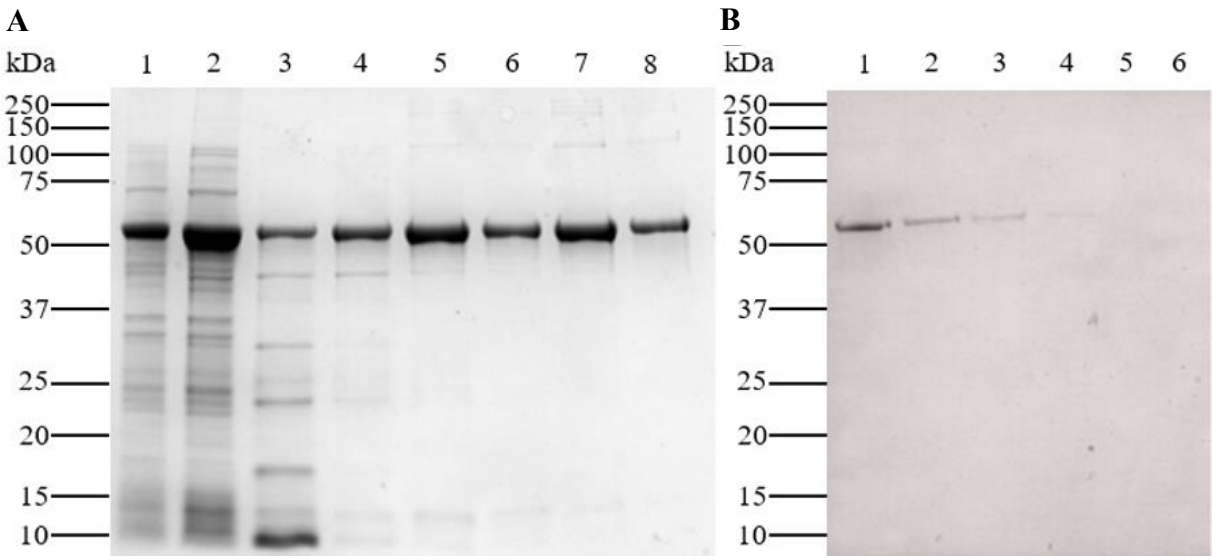

Figure 8: *Coomassie-stained SDS-PAGE of cell extract, supernatant and eluates at different concentrations containing purified proteins :* ***A:*** *cell extract (lane 1), supernatant (lane 2), E-50 (1) (lane 3), E-50 (2) (lane 4), E-100 (1) (lane 5), E-100 (2) (lane 6), E-150 (1) (lane 7), E-150 (2) (lane 8), and* ***B:*** *E-200 (1) (lane 1), E-200 (2) (lane 2), E-250 (1) (lane 3), E-250 (2) (lane 4), E-300 (1) (lane 5), and E-300 (2) (lane 6).*

The recombinant protein in the sample purified with a concentration of 100 mM imidazole (E-100 (1)) (Fig. 8A, lane 5) was renatured in dialyze cassettes, before the protein concentration in the sample was calculated to be 0.9338 µg/µl using BioRad Protein Assay.

3.6 Antigenicity of the HbpA-like protein

A coomassie stained SDS-PAGE gel illustrating the protein profile of PaL-1 and the recombinant HbpA-like protein is illustrated in Figure 9A. WB analysis of PaL-1 and the recombinant HbpA-like protein using salmon sera collected 500 degree days (dd) post vaccination against PaL-1 is shown in Figure 9B. Sera from non-vaccinated salmon were included as controls (Fig. 9C). On the coomassie-stained SDS-PAGE gel (Figure 9A), the protein profile of PaL-1 shows protein bands of PaL-1 expressed proteins (Fig. 9A, lane 1). The recombinant HbpA-like protein (Fig. 9A, lanes 2 and 3) shows darker bands for the sample diluted 1:10 (lane 2) compared to the sample diluted 1:50 (lane 3). The WB analysis against sera from vaccinated salmon shows antibody binding as visual bands on proteins of the PaL-1 isolate (Fig. 9B, lane 1) for proteins at approximately 50 kDa in size. In contrast, the samples of recombinant protein (Fig. 9B, lanes 2 and 3) were not identified by the antibodies produced

in vaccinated fish. The WB analysis presented in Figure 9C does not show bands, indicating that no cross-reactive antibodies to PaL-1 were present in the non-vaccinated fish.

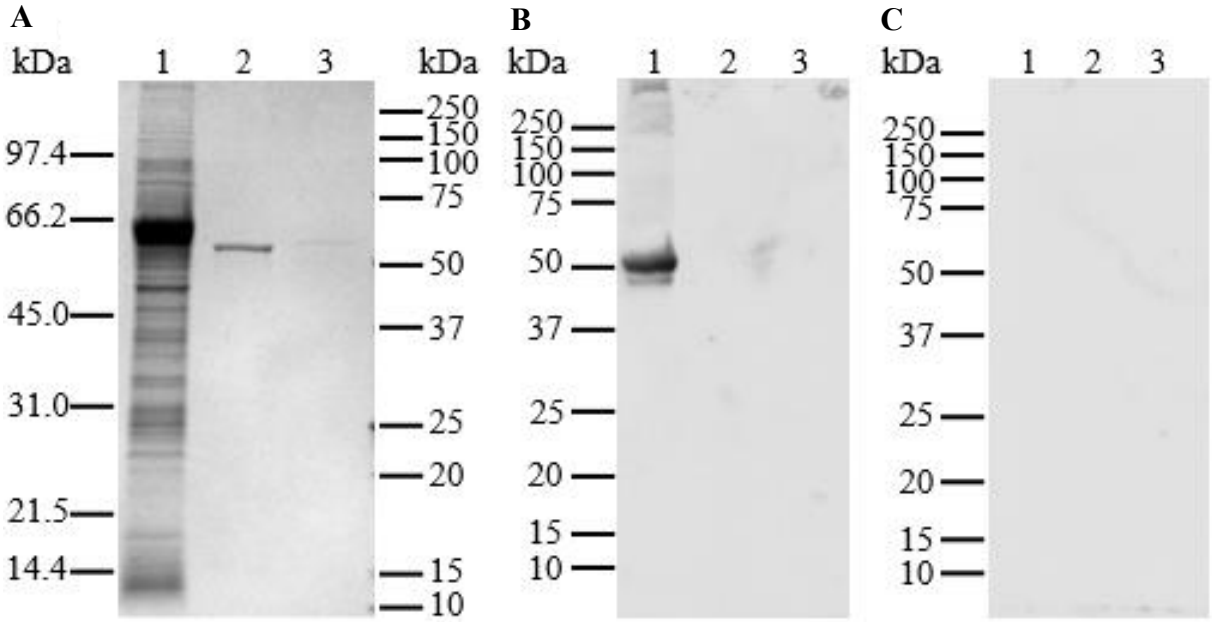

Figure 9: A: *Coomassie-stained SDS-PAGE gel; B: WB against sera from PaL-1 vaccinated salmon; and C: WB against sera from unvaccinated salmon. Lane 1 contains the PaL-1 protein profile, lane 2 contains recombinant HbpA-like protein diluted 1:10, and lane 3 contains recombinant HbpA-like protein diluted 1:50.*

3.7 Cultivation of PaL-1 with GSH and GSSG supplement

Table 16 demonstrates measurements of cells/ml over time of the PaL-1 isolate cultivated in either TSB supplemented with 1.5% NaCl, TSB+, or TSB supplemented with 1.5% NaCl and either reduced glutathione (GSH) or oxidized glutathione (GSSG). The isolate cultivated in TSB shows the least increase among the cultures, with a slight increase after the cell count at 24 hours. The isolate cultivated in TSB+ showed a good increase in cells/ml over time, as expected. Further, the isolates cultivated in TSB supplemented with GSH shows growth quite like the isolate cultured with TSB+. Lastly, the culture cultivated with TSB supplemented with GSSG showed a significant increase in growth after 5 hours, and the highest yield measured by cells/ml after 28 hours.

Table 16: *Cell counts of a PaL-1 starter culture subcultured in four different growth media, measured before incubation and after 5-, 24-, and 28 hours past incubation.*

Viable cells per milliliter				
	Growth media			
Hours	TSB	TSB+	TSB (GSH)	TSB (GSSG)
0	4.02E+07	3.78E+07	3.84E+07	4.32E+07
5	8.65E+07	1.19E+08	1.02E+08	7.71E+07
24	2.51E+08	9.05E+08	5.91E+08	1.06E+09
28	3.18E+08	1.14E+09	1.90E+09	3.32E+09

3.8 Binding of the HbpA-like protein to GSH/GSSG

A demonstration of the binding of the recombinant HbpA-like protein to GSH and GSSG was attempted (Fig. 10). The analysis was carried out by testing the binding of the HbpA-like protein to GSH and GSSG at concentrations 0 mM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, and 10 mM. A protein band at approximately 60 kDa (size of the HbpA-like protein) are visualised on the coomassie stained gels of protein GSH/GSSG combinations for all the concentrations. Further, there are no visible changes in the intensity of the bands by comparing the samples with different concentrations of GSH/GSSG. This analysis was thus not sufficient to demonstrate binding.

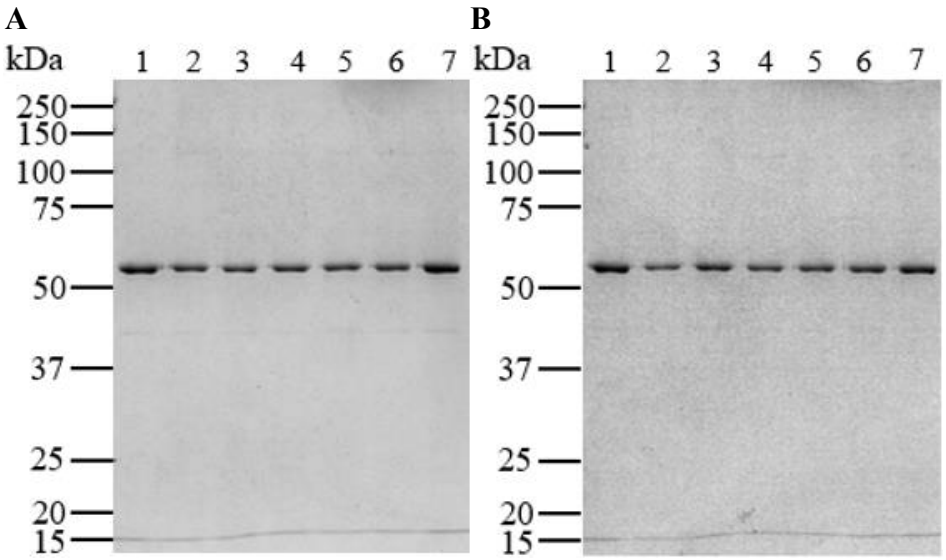

Figure 10: *Coomassie-stained SDS-PAGE gels.* ***A:*** *Analysis of ligand binding between HbpA-like protein and GSH, lane 1: 0 mM ligand, lane 2: 1 mM ligand, lane 3: 2 mM ligand, lane 4: 3m M ligand, lane 5: 4 mM ligand, lane 6: 5 mM ligand and lane 7: 10 mM ligand.* ***B:*** *analysis of ligand binding between HbpA-like protein and GSSG, lane 1: 0 mM ligand, lane 2: 1 mM ligand, lane 3: 2 mM ligand, lane 4: 3m M ligand, lane 5: 4 mM ligand, lane 6: 5 mM ligand and lane 7: 10 mM ligand.*

4. Discussion

4.1 Bacterial growth at different temperatures

In this study, the growth conditions of two *P. atlantica* isolates, PaL-1 isolated from diseased Atlantic salmon and a *P. atlantica* gv. *cyclopteri* isolated from diseased lumpfish, were analysed. Initially, the growth curve of the PaL-1 isolate cultivated in TSB+ growth media was analysed to determine the exponential phase. The growth curve (Fig. 3) show that the exponential phase takes place approximately between 8 and 20 hours of incubation. The growth curve shows similar patterns as demonstrated in a previous study (Skaar 2023, unpublished). The exponential growth phase is when there is sufficient nutrition, and the bacterial cells are most active and multiply rapidly. In laboratory methods, such as for recombinant protein expression, it is advantageous to harvest bacteria in the late exponential phase when there are high amounts of viable cells in the culture, and minimal waste and dead bacterial cells.

Further, the growth of the PaL-1 and *P. atlantica* gv. *cyclopteri* isolates at different temperatures were compared. A starter culture from each isolate cultivated at 20 °C and two subcultures at 10 °C and 30°C for each isolate subcultured at 10 °C and 30 °C were analysed. The results (Table 13) show that the number of cells/ml in the bacterial isolates subcultured at 10 °C was lower compared to the starter culture. This indicates that the low temperature had a negative impact on the bacterial growth. However, the results from the subcultures at 30 °C showed quite similar numbers of cells/ml compared to the starter culture. This shows that the bacterial growth is better at 30 °C compared to at 10 °C, and that 30 °C might be closer to the optimal growth temperature for the *P. atlantica* isolates. By comparing the results for the two *P. atlantica* isolates (Table 13), there are little difference in the amount of cells/ml at the same temperature. The coomassie stained protein profiles (Fig. 4) shows a similar pattern as the cell counts, with more prominent bands for the protein profiles illustrated in lanes 2 and 3 for both bacterial isolates, which may indicate that the bacteria are expressing more proteins at 20 °C and 30 °C. However, the cell numbers have not been adjusted so the results also indicate higher protein load in these lanes. These findings indicates that there are similarities between the *P. atlantica* isolates from Atlantic salmon and lumpfish. These findings also indicate that the bacteria grow more slowly and thus express less proteins at lower temperatures, which can influence the bacterial yield and virulence upon harvest.

These findings also indicates that the *P. atlantica* isolates may be adapted to warmer environments. In a newer taxonomic study from Snorre Gulla et al. (2023) the taxonomic

placement of the *P. atlantica* isolates were questioned. By analysing the genome of *P. atlantica* isolates from both Atlantic salmon and lumpfish it was found that they shared a nucleotide identity at approximately 81-83 % with the bacteria *Phocoenobacter uteri* within the genus *Phocoenobacter.* The study suggests marine mammals as a potential reservoir, as the *Phocoenobacter uteri* are isolated from harbor porpoise (*Phocoena phocoena).* The temperature on the external mucosa of marine mammals is known to be around 30 °C (Melero et al., 2015), which correlates well with the bacterial growth showed in this study.

4.2 Recombinant protein expression

The principles of reverse vaccinology (RV) have made the development of effective vaccines against novel emerging diseases more efficient. The method has several advantages, such as reducing the timeline of development and requiring fewer animals for experimental testing of the vaccines. RV targets immunogenic components such as OMPs through bioinformatic tools that can predict surface-associated proteins, and subsequently express the most prominent targets recombinantly (Bidmos et al., 2018). Through *in silico* analysis of the *P. atlantica* gv. *cyclopteri* proteome, Ellul et al. (2021) discovered a Hia-like protein, previously found in *H. influenzae*, that are predicted to function as an adhesin. However, the Hia-like protein found in *P. atlantica* gv. *cyclopteri* was not found in analysis (unpublished) of the bacterial proteome of *P. atlantica* gv. *salmonicida.*

In previous studies, vaccines based on formalin inactivated whole bacteria, each with one of three different *P. atlantica* gv. *salmonicida* isolates (PaL-1, PaL-2, and PaL-3) was made, and sera from vaccinated salmon was collected 500 dd post vaccination (Holstad, 2022). Furthermore, Skaar (2023, unpublished) analysed immunogenic components by WB, and predicted that the molecular weight of the immunogenic components to be around 60- and 90 kDa. In the same study, Skaar analysed the protein bands at this size by LC-MS/MS, to analyse the proteome of *P. atlantica* gv. *salmonicida* (unpublished) *in silico* following the principles of reverse vaccinology, to predict the most prominent proteins as immunogenic components. Two proteins, one ExlB-like protein and one MAM7-like protein was successfully expressed recombinantly but neither of them showed any reactivity against sera from the vaccinated salmon in WB analysis.

In the current study the proteome of *P. atlantica* gv. *salmonicida* was analysed *in silico* as outlined in section 2.3, and two tables (Table 14 and 15) with the most prominent proteins as

potential immunogenic components for a vaccine was compiled. It was decided to process and express a HbpA-like protein and a BamA-like protein recombinantly in Codon+ *E. coli* cells.

The HbpA-like protein has a molecular weight of 60.3 kDa and are predicted to be a periplasmic protein. In Blastp analysis, it was identified as an ABC transporter substrate-binding protein in *P. atlantica.* Further, VFDB analysis gave a 24 % match with a (oppA) peptide ABC transporter substrate-binding protein from *Listeria seeligeri.* Heme binding proteins are substrate-binding lipoproteins proteins in the ABC-transporter complex, which bind to substrates with high affinity (Delepelaire, 2019). HbpA proteins are known as periplasmic lipoproteins that have a specific function in heme utilization for the bacteria by transporting heme from the extracellular space into the cytosol (Morton et al., 2005).

The BamA-like protein has a molecular weight of 89.5 kDa and is predicted to be an outer membrane protein. In Blastp analysis, it was identified as an outer membrane protein assembly factor BamA in *P. atlantica*. Additionally, VFDB analysis gave a 26% match with an outer membrane protein assembly factor BamA (omp89) which is associated with potential adhesion functions, form *Bartonella quintana.* BamA proteins are part of the β-barrel assembly machinery (BAM) which provides the structural basis of outer membranes by of folding outer membrane proteins (Gessmann et al., 2014; Singh et al., 2017). BamA is considered a protein that is highly conserved among Gram-negative bacteria, and in a study by Sing et al. (2017), recombinant BamA elicited a high IgG antibody response in mice.

In this study, the BamA-like and HbpA-like proteins were expressed recombinantly in Codon+ *E. coli* cells. The coomassie stained SDS-PAGE gel in Table 7A shows more prominent protein bands for the cultures with IPTG at approximately 60kDa (correlates with the size of HbpA) in lane 2 and 90kDa (correlates with the size of BamA) in lane 4. IPTG is a synthetic molecule that functions as an inductor of gene expression on the lac-operon in *E. coli* bacteria (Simas et al., 2023). Subsequently, the proteins were blotted against a 6xHisTag monoclonal antibody to detect the HisTag added to the proteins. The BamA-like protein (Fig. 7B lanes 3 and 4) did not show any reactivity to the antibody, indicating that there was an error in the primers. The culture with the HbpA-like protein did show reactivity to the 6xHisTag antibody with a protein band at approximately 60 kDa (Fig. 7B, lanes 1 and 2). The effect of IPTG was also detectable in the WB analysis with a more intense protein band in lane 2.

Furthermore, the HbpA-like protein that had successful binding to the 6xHisTag antibody was purified with Chelating Sepharose for further testing of antigenicity, ligand binding and supplement for bacterial growth.

4.3 Antigenicity of immunogenic proteins

Antigenicity refers to molecules, such as recombinant proteins, which have epitopes that can be recognized by antibodies. As mentioned earlier, Skaar (2023, unpublished) predicted that the molecular weight of the immunogenic components is around 60- and 90 kDa. The antigenicity of the HbpA-like protein was tested against sera from vaccinated salmon. No binding of antibodies was detected by WB analysis, indicating that the antibodies produced in PaL-1 vaccinated salmon did not show reactivity to recombinant protein. In the coomassie stained SDS-PAGE gel (Fig. 9A) there are two visible bands at approximately 60 kDa in lanes 2 and 3, corresponding to the size of HbpA-like protein. These findings show that the recombinant HbpA-like protein was successfully purified but was not recognized by antibodies present in sera from vaccinated salmon.

The lack of binding between the antibodies and the recombinant HbpA-like protein may result from incomplete folding of the recombinant protein during expression in Codon+ E. coli cells or renaturation in dialyse cassettes. In recombinant protein expression, it is important that the proteins are correctly folded to maintain their natural structure (Beygmoradi et al., 2023). When a cell such as Codon+ *E. coli* cells are induced to express a specific protein, there are several problems that can influence the natural structure and functions of the produced protein such as incomplete folding, proteolysis, and aggregation. In *E. coli* cells the proteins are going through post-translational modifications that are critical for the structure and function (Beygmoradi et al., 2023).

4.4 Bacterial growth supplemented with GSH and GSSG

P. atlantica has proven difficult to grow in high numbers. When the first detections of pasteurellosis in lumpfish occurred, a suitable growth medium was lacking and the bacteria was only possible to grow on blood agar supplemented with blood and sodium chloride (Alarcón et al., 2016). A study presented by Ellul et al. in 2018 found that the *P. atlantica* gv. *salmonicida* was possible to cultivate in TSB enriched with 1.5% NaCl and 10 % FCS (TSB+). Skaar (2023, unpublished) tested the bacterial growth of *P. atlantica* isolated from salmon in TSB with

different concentrations of FCS and found out that the bacteria's growth was best with 10% FCS. It is crucial to get a high yield of bacteria for laboratory analysis and for the production of vaccines. Which component from FCS that promotes bacterial growth is still unknown.

The vaccine developing industry aims to avoid the use of products derived from animals such as FCS due to ethical, welfare and economic reasons. In this study, *P. atlantica* gv. *salmonicida* which is known to grow in TSB+, was tested with supplements of GSH and GSSG instead of FCS. It has been shown that FCS contains glutathione (Bump and Reed, 1977), which is an antioxidant that plays a key role in cellular functions such as regulating redox reactions in the bacteria and protecting bacterial cells against oxidative stress (Smirnova and Oktyabrsky, 2005; Vergauwen et al., 2010).

The results presented in Table 16 show that the bacteria cultivated with GSH and GSSG have higher yields after 28 hours than the standard protocol with FCS as a supplement. This may open the possibility of cultivating *P. atlantica* in serum-free cell culture media. Further testing is important to understand the functions of glutathione's impact on bacterial growth and how glutathione is transported from the extracellular space. Subsequently, this method can also be tested as an option for bacterial growth in other bacteria with fastidious growth requirements.

4.5 Ligand binding between HbpA-like protein and GSH/GSSG

As previously mentioned, HbpA proteins are known for their role in heme acquisition in some gram-negative bacteria such as *Haemophilus influenzae* (Morton et al., 2005). This was tested in a study from Vergauwen et al. (2010), where they showed that HbpA proteins binds heme with low affinity, and in the same study showed that HbpA proteins binds both GSH and GSSG with high affinity.

In this current study, the ligand binding interactions between the recombinant HbpA-like protein and GSH/GSSG was tested and analysed on a coomassie stained SDS-PAGE gel. The results (Fig. 10) show protein bands at approximately 60 kDa for both the test with GSH (Fig. 10A) and the test with GSSG (Fig. 10B), which correlates with the size of the HbpA-like protein. As mentioned earlier, the HbpA protein is associated with the ABC transporter system in bacteria, providing specific transport of molecules over the bacterial membrane. The results from this study cannot exclude the ligand binding interactions between the HbpA protein and GSH/GSSG. In the study that shows high affinity between the HbpA-like protein and GSH and GSSG the ligand binding was quantified with Isothermal titration calorimetry (ITC)

(Vergauwen et al., 2010). In the study it was proposed to change the name of HbpA to glutathione-binding protein A, based on the results. This method can be applied to confirm the binding, and further testing is needed to understand the interactions between HbpA and glutathione.

4.6 Directions for Future Research

Further research on characterizing the immunogenic components for a future vaccine is important. Further testing needs to be done on the HbpA-like protein and the BamA-like protein. The crystal structure of HbpA-like protein can be established to analyse epitopes and further testing to express the BamA-like protein recombinant and test its antigenicity against serum from PaL-1 vaccinated fish. In addition, new immunogenic proteins from the lists with prominent targets can be expressed and tested for antigenicity against sera from vaccinated fish.

5. Conclusion

- The most promising immunogenic components with potential virulence function of the PaL-1 isolate were identified using bioinformatic tools *in silico*.

- Two proteins, one HbpA-like protein and one BamA-like protein, were chosen for recombinant protein expression. The BamA-like protein was not detected in the WB analysis when blotted against 6xHisTag antibody, and we failed to express this protein recombinantly. The HbpA-like protein was successfully expressed recombinantly but did not show antigenicity when blotted against sera from PaL-1 vaccinated fish.

- The bacterial growth of PaL-1 showed better growth when cultivated with GSH and GSSG than FCS, which may show the potential of a serum-free growth media.

- Ine the current experiment we could not show ligand binding of the recombinant HbpA-like protein to GSH/GSSG.

- *P. atlantica* grew best when cultivated at 20°C and 30°C.

6. References

Afewerki, S., Asche, F., Misund, B., Thorvaldsen, T., Tveteras, R., 2023. Innovation in the Norwegian aquaculture industry. Reviews in Aquaculture 15, 759–771. https://doi.org/10.1111/raq.12755

Alarcón, M., Gulla, S., Røsaeg, M.V., Rønneseth, A., Wergeland, H., Poppe, T.T., Nilsen, H., Colquhoun, D.J., 2016. Pasteurellosis in lumpsucker *Cyclopterus lumpus*, farmed in Norway. J Fish Dis 39, 489–495. https://doi.org/10.1111/jfd.12366

Ben Hamed, S., Tavares Ranzani-Paiva, M.J., Tachibana, L., de Carla Dias, D., Ishikawa, C.M., Esteban, M.A., 2018. Fish pathogen bacteria: Adhesion, parameters influencing virulence and interaction with host cells. Fish Shellfish Immunol 80, 550–562. https://doi.org/10.1016/j.fsi.2018.06.053

Ben Hamed, S., Guardiola, F.A., Mars, M., Esteban, M.Á., 2014. Pathogen bacteria adhesion to skin mucus of fishes. Veterinary Microbiology 171, 1–12. https://doi.org/10.1016/j.vetmic.2014.03.008

Beygmoradi, A., Homaei, A., Hemmati, R., Fernandes, P., 2023. Recombinant protein expression: Challenges in production and folding related matters. International Journal of Biological Macromolecules 233, 123407. https://doi.org/10.1016/j.ijbiomac.2023.123407

Bidmos, F.A., Siris, S., Gladstone, C.A., Langford, P.R., 2018. Bacterial Vaccine Antigen Discovery in the Reverse Vaccinology 2.0 Era: Progress and Challenges. Front. Immunol. 9. https://doi.org/10.3389/fimmu.2018.02315

Bowers, J.M., Mustafa, A., Speare, D.J., Conboy, G.A., Brimacombe, M., Sims, D.E., Burka, J.F., 2000. The physiological response of Atlantic salmon, *Salmo salar* L., to a single experimental challenge with sea lice, *Lepeophtheirus salmonis*. Journal of Fish Diseases 23, 165–172. https://doi.org/10.1046/j.1365-2761.2000.00225.x

Bump, E.A., Reed, D.J., 1977. A unique property of fetal bovine serum: high levels of protein-glutathione mixed disulfides. In Vitro 13, 115–118. https://doi.org/10.1007/BF02615075

Delepelaire, P., 2019. Bacterial ABC transporters of iron containing compounds. Research in Microbiology, ABC systems in microorganisms 170, 345–357. https://doi.org/10.1016/j.resmic.2019.10.008

Ellul, R.M., 2021. Pasteurella atlantica in Norwegian lumpsuckers : Characterisation, pathogenicity, vaccine development and immune responses (Doctoral thesis). The University of Bergen.

Ellul, R.M., Kalatzis, P.G., Frantzen, C., Haugland, G.T., Gulla, S., Colquhoun, D.J., Middelboe, M., Wergeland, H.I., Rønneseth, A., 2021. Genomic Analysis of *Pasteurella atlantica* Provides Insight on Its Virulence Factors and Phylogeny and Highlights the Potential of Reverse Vaccinology in Aquaculture. Microorganisms 9. https://doi.org/10.3390/microorganisms9061215

Ellul, R.M., Walde, C., Haugland, G.T., Wergeland, H., Rønneseth, A., 2019. Pathogenicity of *Pasteurella sp*. in lumpsuckers (*Cyclopterus lumpus* L.). Journal of Fish Diseases 42, 35–46. https://doi.org/10.1111/jfd.12905

Esteban, M., 2012. An Overview of the Immunological Defenses in Fish Skin. ISRN Immunology 2012. https://doi.org/10.5402/2012/853470

Fjørtoft, H.B., Nilsen, F., Besnier, F., Espedal, P.G., Stene, A., Tveten, A.-K., Bjørn, P.A., Aspehaug, V.T., Glover, K.A., 2020. Aquaculture-driven evolution: distribution of pyrethroid resistance in the salmon louse throughout the North Atlantic in the years 2000–2017. ICES Journal of Marine Science 77, 1806–1815. https://doi.org/10.1093/icesjms/fsaa062

Gessmann, D., Chung, Y.H., Danoff, E.J., Plummer, A.M., Sandlin, C.W., Zaccai, N.R., Fleming, K.G., 2014. Outer membrane β-barrel protein folding is physically controlled by periplasmic lipid head groups and BamA. Proc Natl Acad Sci U S A 111, 5878–5883. https://doi.org/10.1073/pnas.1322473111

Goldsby, R.A., Kindt, T.J., Osborne, B.A., Kuby, J., 2002. Immunology, 5th ed. ed. Freeman, New York.

Gulla, S., Colquhoun, D.J., Olsen, A.B., Spilsberg, B., Lagesen, K., Åkesson, C.P., Strøm, S., Manji, F., Birkbeck, T.H., Nilsen, H.K., 2023. Phylogeography and host specificity of

Pasteurellaceae pathogenic to sea-farmed fish in the north-east Atlantic. Front. Microbiol. 14. https://doi.org/10.3389/fmicb.2023.1236290

Gulla, S., Nilsen, H., Olsen, A.B., Colquhoun, D., 2020. Fiskepatogene Pasteurella i Norge. URL https://www.vetinst.no/nyheter/fiskepatogene-pasteurella-i-norge (accessed 3.20.24).

Haugland, G.T., K. Dagbjartarson Imsland, A., Reynolds, P., Treasurer, J., 2020. 10 - Application of biological control: use of cleaner fish, in: Kibenge, F.S.B., Powell, M.D. (Eds.), Aquaculture Health Management. Academic Press, pp. 319–369. https://doi.org/10.1016/B978-0-12-813359-0.00010-5

Holstad, V.M., 2022. Adaptive immune responses in Atlantic salmon after vaccination with bacterins of Pasteurella atlantica (Master thesis). The University of Bergen.

Hordvik, I., 2015. Immunoglobulin Isotypes in Atlantic Salmon, Salmo Salar. Biomolecules 5, 166–177. https://doi.org/10.3390/biom5010166

Imsland, A.K.D., Reynolds, P., 2022. In lumpfish We Trust? The Efficacy of Lumpfish *Cyclopterus lumpus* to Control *Lepeophtheirus salmonis* Infestations on Farmed Atlantic Salmon: A Review. Fishes 7, 220. https://doi.org/10.3390/fishes7050220

Jørgensen, J.B., 2014. The Innate Immune Response in Fish, in: Gudding, R., Lillehaug, A., Evensen, Ø. (Eds.), Fish Vaccination. Wiley-Blackwell.

Kurath, G., 2008. Biotechnology and DNA vaccines for aquatic animals. Rev Sci Tech 27, 175–196. http://dx.doi.org/10.20506/rst.27.1.1793.

Legård, B.K., Strøm, S., 2020. Pasteurellosis in Atlantic salmon (*Salmo salar*) in Western Norway. Bulletin of the European Association of Fish Pathologists 40, 148–155. Accessible at: https://eafp.org/bulletin-archive/2020-volume40/issue_4/#wpfb-cat-267.

Li, J., Barreda, D.R., Zhang, Y.-A., Boshra, H., Gelman, A.E., LaPatra, S., Tort, L., Sunyer, J.O., 2006. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. Nat Immunol 7, 1116–1124. https://doi.org/10.1038/ni1389

Ma, J., Bruce, T.J., Jones, E.M., Cain, K.D., 2019. A Review of Fish Vaccine Development Strategies: Conventional Methods and Modern Biotechnological Approaches. Microorganisms 7, 569. https://doi.org/10.3390/microorganisms7110569

Magnadóttir, B., 2006. Innate immunity of fish (overview). Fish & Shellfish Immunology, Reviews in Fish Immunology 20, 137–151. https://doi.org/10.1016/j.fsi.2004.09.006

Maiti, B., Dubey, S., Munang'andu, H.M., Karunasagar, Iddya, Karunasagar, Indrani, Evensen, Ø., 2020. Application of Outer Membrane Protein-Based Vaccines Against Major Bacterial Fish Pathogens in India. Front Immunol 11, 1362. https://doi.org/10.3389/fimmu.2020.01362

Melero, M., Rodríguez-Prieto, V., Rubio-García, A., García-Párraga, D., Sánchez-Vizcaíno, J.M., 2015. Thermal reference points as an index for monitoring body temperature in marine mammals. BMC Res Notes 8, 411. https://doi.org/10.1186/s13104-015-1383-6

Mondal, H., Thomas, J., 2022. A review on the recent advances and application of vaccines against fish pathogens in aquaculture. Aquac Int 30, 1971–2000. https://doi.org/10.1007/s10499-022-00884-w

Morton, D.J., Madore, L.L., Smith, A., VanWagoner, T.M., Seale, T.W., Whitby, P.W., Stull, T.L., 2005. The heme-binding lipoprotein (HbpA) of *Haemophilus influenzae*: Role in heme utilization. FEMS Microbiology Letters 253, 193–199. https://doi.org/10.1016/j.femsle.2005.09.016

Mota, V.C., Striberny, A., Verstege, G.C., Difford, G.F., Lazado, C.C., 2022. Evaluation of a Recirculating Aquaculture System Research Facility Designed to Address Current Knowledge Needs in Atlantic Salmon Production. Front. Anim. Sci. 3. https://doi.org/10.3389/fanim.2022.876504

Mutoloki, S., Jørgensen, J.B., Evensen, Ø., 2014. The Adaptive Immune Response in Fish, in: Fish Vaccination. Wiley-Blackwell.

Overton, K., Dempster, T., Oppedal, F., Kristiansen, T.S., Gismervik, K., Stien, L.H., 2019. Salmon lice treatments and salmon mortality in Norwegian aquaculture: a review. Reviews in Aquaculture 11, 1398–1417. https://doi.org/10.1111/raq.12299

Pollard, A.J., Bijker, E.M., 2021. A guide to vaccinology: from basic principles to new developments. Nat Rev Immunol 21, 83–100. https://doi.org/10.1038/s41577-020-00479-7

Rappuoli, R., 2000. Reverse vaccinology. Curr Opin Microbiol 3, 445–450. https://doi.org/10.1016/s1369-5274(00)00119-3

Raynes, J.M., Young, P.G., Proft, T., Williamson, D.A., Baker, E.N., Moreland, N.J., 2018. Protein adhesins as vaccine antigens for Group A Streptococcus. Pathogens and Disease 76, fty016. https://doi.org/10.1093/femspd/fty016

Sandlund, N., Rønneseth, A., Ellul, R.M., Nylund, S., Sandlund, L., 2021. *Pasteurella spp*. Infections in Atlantic salmon and lumpsucker. Journal of Fish Diseases 44, 1201–1214. https://doi.org/10.1111/jfd.13381

Sharma, A.K., Dhasmana, N., Dubey, N., Kumar, N., Gangwal, A., Gupta, M., Singh, Y., 2017. Bacterial Virulence Factors: Secreted for Survival. Indian J Microbiol 57, 1–10. https://doi.org/10.1007/s12088-016-0625-1

Simas, R.G., Junior, A.P., Long, P.F., 2023. Mechanistic aspects of IPTG (isopropylthio-β-galactoside) transport across the cytoplasmic membrane of *Escherichia coli*—a rate limiting step in the induction of recombinant protein expression. Journal of Industrial Microbiology & Biotechnology 50. https://doi.org/10.1093/jimb/kuad034

Singh, R., Capalash, N., Sharma, P., 2017. Immunoprotective potential of BamA, the outer membrane protein assembly factor, against MDR *Acinetobacter baumannii*. Sci Rep 7, 12411. https://doi.org/10.1038/s41598-017-12789-3

Smirnova, G.V., Oktyabrsky, O.N., 2005. Glutathione in Bacteria. Biochemistry 70, 1199–1211. https://doi.org/10.1007/s10541-005-0248-3

Sommerset, I., Krossøy, B., Biering, E., Frost, P., 2005. Vaccines for fish in aquaculture. Expert Review of Vaccines 4, 89–101. https://doi.org/10.1586/14760584.4.1.89

Sommerset, I., Wiik-Nielsen, J., Moldal, T., Oliveira, V.H.S. de, Svendsen, J.C., Haukaas, A., Brun, E., 2024. Fiskehelserapporten 2023. URL https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2024/fiskehelserapporten-2023 (accessed 3.13.24).

Tang, H., Jiang, X., Zhang, J., Pei, C., Zhao, X., Li, L., Kong, X., 2021. Teleost CD4+ helper T cells: Molecular characteristics and functions and comparison with mammalian counterparts. Veterinary Immunology and Immunopathology 240, 110316. https://doi.org/10.1016/j.vetimm.2021.110316

Thorarinsson, R., Ramstad, A., Wolf, J.C., Sindre, H., Skjerve, E., Rimstad, E., Evensen, Ø., Rodriguez, J.F., 2024. Effect of pancreas disease vaccines on infection levels and virus transmission in Atlantic salmon (*Salmo salar*) challenged with salmonid alphavirus, genotype 2. Front. Immunol. 15. https://doi.org/10.3389/fimmu.2024.1342816

Uribe, C., Folch, H., Enriquez, R., Moran, G., 2011. Innate and adaptive immunity in teleost fish: a review. Vet. Med. 56, 486–503. https://doi.org/10.17221/3294-VETMED

Valheim, M., Håstein, T., Myhr, E., Speilberg, L., Ferguson, H.W., 2000. Varracalbmi: a new bacterial panophthalmitis in farmed Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 23, 61–70. https://doi.org/10.1046/j.1365-2761.2000.00209.x

Vergauwen, B., Elegheert, J., Dansercoer, A., Devreese, B., Savvides, S.N., 2010. Glutathione import in Haemophilus influenzae Rd is primed by the periplasmic heme-binding protein HbpA. Proceedings of the National Academy of Sciences 107, 13270–13275. https://doi.org/10.1073/pnas.1005198107

Vormedal, I., 2023. Sea-lice regulation in salmon-farming countries: how science shape policies for protecting wild salmon. Aquacult Int. https://doi.org/10.1007/s10499-023-01270-w

Welch, T.J., LaPatra, S., 2016. *Yersinia ruckeri* lipopolysaccharide is necessary and sufficient for eliciting a protective immune response in rainbow trout (*Oncorhynchus mykiss*, Walbaum). Fish & Shellfish Immunology 49, 420–426. https://doi.org/10.1016/j.fsi.2015.12.037

Østevik, L., Stormoen, M., Evensen, Ø., Xu, C., Lie, K.-I., Nødtvedt, A., Rodger, H., Skagøy, A., Manji, F., Alarcón, M., 2022. Effects of thermal and mechanical delousing on gill health of farmed Atlantic salmon (*Salmo salar* L.). Aquaculture 552, 738019. https://doi.org/10.1016/j.aquaculture.2022.738019

7. Appendix

7.1 Solutions

7.1.1 Growth media solutions

TSB: 1000 ml

1. Tryptic soy broth (TSB) – BD – catalog# 211825	30 g
2. Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	1000 ml

TSB+: 1000 ml

1. Tryptic soy broth (TSB) – BD – catalog# 211825	30g
2. NaCl – Honeywell International – catalog# 31434	15g
3. Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	1000 ml
4. Foetal calf serum (FCS) – Gibco – catalog# 10099-141	10 %

SOB-medium: 950 ml

1. Bacto™ Tryptone – Thermo Fisher – catalog# 211705	20 g
2. Bacto™ Yeast Extract – Thermo Fisher – catalog# 212750	5 g
3. NaCl – Honeywell International – catalog# 31434	0.5 g
4. Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	950 ml

SOC-medium: 100 ml

1. SOB-medium (Appendix)	98 ml
2. 20% Glucose – Thermo Fisher - catalog#CA2494001	2 ml

Luria-Bertani (LB) media: 1000 ml

1. Bacto™ Tryptone – Thermo Fisher – catalog# 211705	20 g
2. Bacto™ Yeast Extract – Thermo Fisher – catalog# 212750	5 g
3. NaCl – Honeywell International – catalog# 31434	0.5 g
4. NaOH 10 M - (Appendix)	7.0 pH
5. Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	1000 ml total volume

NaOH 10 M: 100 ml

1. NaOH – Sigma Aldrich – catalog# S5881	4.0 g
2. Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	100 ml total volume

50 x 5052: 200 ml

1.	Glycerol – Sigma-Aldrich – catalog# G5516	50 g
2.	20 % glucose – Thermo Fisher -catalog#CA2494001	5 g
3.	α – Lactose – Sigma-Aldrich – catalog #5989-81-1	20 g
4.	Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	146 ml

20 x NPS: 1000 ml

1.	$(NH_4)_2SO_4$ – Biowhittaker – catalog#A4418	66 g
2.	KH_2Po_4 – Fluka – catalog# 7079054307003	136 g
3.	Na_2HPO_4 – Sigma Aldrich – catalog#10028-24-7	142 g
4.	Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	900 ml

PA – 0.5 g medium: 50 ml

1.	1M $MgSO_4$ – Sigma Aldrich – catalog#BCBB1371	50 µl
2.	40 % glucose – Sigma Aldrich – catalog#G8270	0.625 ml
3.	20 x NPS (appendix)	2.5 ml
4.	Ampicillin (100 µg/ml)	25 µl
5.	Chloramphenicol (25 mg/ml) 50 µl	
6.	suH_2O – Thermo Fisher – catalog#J60610	46.1 ml

ZY-media: 1000 ml

1.	N-Z CASE PLUS – Sigma Aldrich – catalog#N4642	20 g
2.	Bacto™ Yeast Extract – Thermo Fisher – catalog# 212750	10 g
3.	Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	925 ml

ZYP – 5052 medium: 1000 ml

1.	ZY-medium (appendix)	928 ml
2.	1 M $MgSO_4$ – Sigma Aldrich – BCBB1371	1 ml
3.	50 x 5052 buffer (appendix)	20 ml
4.	20 x NPS buffer (appendix)	50 ml
5.	Ampicillin (100 µg/ml)	500 µl
6.	Chloramphenicol (25 mg/ml) 1000 µl	

7.1.2 SDS-PAGE solutions

10x TGS (electrode buffer): 1000 ml

1.	Tris Base – Sigma-Aldrich – catalog# T1503	30.3 g
2.	Glycine – Sigma-Aldrich – catalog# G7126	144 g
3.	Sodium dodecyl sulfate – Sigma Aldrich – catalog# L4390	10 g
4.	Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	1000 ml total volume

1x TGS buffer (electrode buffer): 1000 ml

1.	10x TGS buffer (appendix)	100 ml
2.	Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	900 ml

0.5M Tris-HCl: 100 ml

1.	Tris Base – Sigma-Aldrich – catalog# T1503	6 g
2.	HCl 12M – Sigma-Aldrich – catalog# 258148	pH 6.8
3.	Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	100 ml total

4x SDS-PAGE sample buffer: 38 ml

1.	Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	14.2 ml
2.	0.5M Tris-HCl (appendix)	5 ml
3.	Glycerol – Sigma-Aldrich – catalog# G5516	10 ml
4.	10% SDS – Sigma Aldrich – catalog# L4390	8 ml
5.	0.5% Bromophenol blue – Merck – catalog# 8122.0025	0.8 m

SDS-sample buffer with DTT: 250 µl

1.	4x SDS-PAGE sample buffer (appendix)	245 µl
2.	DTT – Sigma-Aldrich – catlog#3483-12-3	5 µl

Coomassie staining solution: 1000 ml

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

Coomassie destain solution: 250ml

1.	Methanol – Honeywell International – catalog# 32213	125 ml
2.	Acetic acid – Honeywell International – catalog# 33209	25ml
3.	Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	100 ml

7.1.3 Agarose solutions

50x TAE buffer: 1000 ml

1.	Tris Base – Sigma-Aldrich – catalog# T1503	242 g
2.	0.5M EDTA – Life Technologies Corporation – catalog# 15575-038	100 ml
3.	Acetic acid – Honeywell International – catalog# 33209	57,1 ml
4.	Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	1000 ml total volume

1x TAE buffer: 1000 ml

1.	50 x TAE buffer (appendix)	20 ml
2.	Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	980 ml

1% Agarose gel: 40 ml

1.	1x TAE (appendix)	40 ml
2.	SeaKem® LE Agarose – Lonza – catalog# 50004	0,4 g
3.	GelRed – Biotium – catalog#19G1205	4 µl

5x loading buffer:

1.	Bromophenol Blue sodium salt – Merck – catalog#B5525	125 g
2.	0.5M EDTA – Life Technologies Corporation – catalog# 15575-038	10 ml
3.	Glycerol – Sigma-Aldrich – catalog# G5516	17.24 ml
4.	Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	22.75 ml

7.1.4 WB solutions

10 x Blotting buffer: 250 ml

1.	Tris Base – Sigma-Aldrich – catalog# T1503	7.57 g
2.	Glycine – Sigma-Aldrich – catalog# G7126	14.4 g
3.	Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	250 ml total volume

1 x Blotting buffer: 1000 ml

1. 10 x Blotting buffer (appendix)	100 ml
2. Methanol – Honeywell International – catalog# 32213	200 ml
3. Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	700 ml

10 x TBS: 500 ml

1. Tris Base – Sigma-Aldrich – catalog# T1503	12.11 g
2. NaCl – Honeywell International – catalog# 31434	146.1 g
3. HCl 12M – Sigma-Aldrich – catalog# 258148	pH = 7.5
4. Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	500 ml total

1 x TBS: 1000 ml

1. 10 x TBS (appendix)	100 ml
2. Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	900 ml

TTBS: 700 ml

1. Tween - Sigma-Aldrich – catalog# P1379	350 µl
2. 1 x TBS (appendix)	700 ml

Blocking solution: 105 ml

1. Skimmed milk powder – Sigma-Aldrich – catalog# 70166	5 g
2. 1 x TBS (appendix)	100 ml

Antibody buffer: 200 ml

1. Skimmed milk powder – Sigma-Aldrich – catalog# 70166	2 g
2. TTBS (appendix)	200 ml

7.1.5 Solutions for purification and renaturation

Buffer A (lysis buffer): 200 ml

1. Tris Base – Sigma-Aldrich – catalog# T1503	50 mM
2. NaCl – Honeywell International – catalog# 31434	0.5 mM
3. Glycerol – Sigma-Aldrich – catalog# G5516	20 %

Buffer B (elution buffer): 100 ml

1. Tris Base – Sigma-Aldrich – catalog# T1503	50 mM
2. NaCl – Honeywell International – catalog# 31434	0.5 mM
3. Glycerol – Sigma-Aldrich – catalog# G5516	20 %
4. Imidazole – Sigma Aldrich – I202	0.5 M

Buffer C (8x Ni buffer):

1. $NiSO_4x6H_2O$ – Sigma Aldrich – catalog#N4882	3.2 M

Buffer D (stripping buffer): 10 ml

1. 0.5 M EDTA	2 ml
2. NaCl – Honeywell International – catalog# 31434	1 ml
3. Tris Base – Sigma-Aldrich – catalog# T1503	0.2 ml
4. Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	6.8 ml

1x Ni: 20 ml

1. Buffer C (appendix)	2.5 ml
2. suH_2O - Thermo Fisher – catalog#J60610	17.5 ml

Dialyse buffer: 1000 ml

1. Tris Base – Sigma-Aldrich – catalog# T1503	20 ml
2. Glycerol – Sigma-Aldrich – catalog# G5516	100 ml
3. NaCl – Honeywell International – catalog# 31434	5.85 g
4. Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	875 m

Dialyse buffer with 4M UREA:

1. Tris Base – Sigma-Aldrich – catalog# T1503	20 ml
2. Glycerol – Sigma-Aldrich – catalog# G5516	100 ml
3. NaCl – Honeywell International – catalog# 31434	5.85 g
4. Urea – Merck – catalog#K31980287	240 g
5. Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	635 ml

BSA-solution: 300 µl

1. BSA – BIO-RAD – catalog#5000206	3 µl
2. Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	297 µl

Diluted dye concentrate: 0.9 ml

1. Protein Assay Dye Concentrate – BIO-RAD - catalog#5000006	0.2 ml
2. Milli-Q® – Merck Millipore, model Milli-Q® Advantage A10® System	0.7 ml