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Identification and cloning of immunogenic *Aliivibrio salmonicida* Pal-like protein present in profiled outer membrane and secreted subproteome

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ABSTRACT: *Aliivibrio salmonicida* is the aetiological agent of cold water vibriosis affecting farmed fish species, a disease that today is fully controlled by vaccination. However, the molecular mechanisms behind the successful vaccine are largely unknown. In order to gain insight into the possible mechanisms of *A. salmonicida* vaccines, we report here the profiles of both the outer membrane and secreted subproteomes of *A. salmonicida* LFI315. The 2 subproteomes were resolved by 2-dimensional electrophoresis that identified a total of 82 protein entries. Monoclonal antibodies specific to an unidentified protein antigen were utilized in the immunoproteomic analysis of both outer membrane proteins and extracellular proteins. The immunogenic protein was located in both subproteomes and identified as a 20 kDa peptidoglycan-associated lipoprotein (Pal). The identity of the antigen was verified by heterologous expression of the cloned *A. salmonicida pal* gene (VSAL_I1899). It is likely that the immunogenic Pal-like protein is among the constituents that act as a protective antigen in the successful vaccine used today. In view of this, it may be considered a potentially useful component in future vaccine development and pathogenicity studies.

KEY WORDS: *Aliivibrio salmonicida* · Outer membrane protein · OMP · Extracellular product · ECP · Immunogenic

INTRODUCTION

Aliivibrio salmonicida (formerly *Vibrio salmonicida* Urbanczyk et al. 2007) is the aetiological agent of cold water vibriosis in sea-farmed Atlantic salmon *Salmo salar*, rainbow trout *Oncorhynchus mykiss* and Atlantic cod *Gadus morhua* (Egidius et al. 1981, 1986, Jørgensen et al. 1989). All farmed Atlantic salmon are today vaccinated with oil-adjuvanted multicomponent vaccines, and no outbreaks were detected in Norwegian farms in 2009 (Bornø et al. 2010). However, the detailed molecular mechanisms underlying the protective nature of the vaccine are unknown. It may result from the *A. salmonicida* constituent, cross-protection from one of the other vaccine components, the oil adjuvant, or a combination of 2 or more of these factors. Disease outbreaks are associated with low temperatures (<10°C), but no pathogenicity mechanisms have been verified in *A. salmonicida*. Also, little is known about the protective immune responses against the pathogen in Atlantic salmon.

Genes and systems that might play central roles in the virulence of *Aliivibrio salmonicida* include several protein secretion systems (3 T1SS, 1 T2SS, 2 T6SS and 1 Flp-type pilus system; Hjerde et al. 2008) and 2 putative haemolysins. Intriguingly, in contrast to the majority of pathogenic bacteria, *A. salmonicida* has not been shown to induce any extracellular protease activity or cytotoxicity in cell models although numerous experiments have been performed (Hjelmeland et al. 1988,

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present study data not presented). Other candidate genes and systems are, for example, iron acquisition systems linked to virulence mechanisms in several vibrios (Stork et al. 2004). Iron acquisition in *A. salmonicida* is proposed to constitute a temperature-sensitive virulence factor, as siderophore production is only detected at ≤10°C. An alternative non-siderophore-based iron assimilation system was also detected. In addition, 3 high-molecular-weight outer membrane proteins (OMPs) were found expressed under iron-restricted growth at 6 and 10°C, but suppressed at 15°C (Colquhoun & Sørum 2001). Genomic analysis of *A. salmonicida* revealed 3 *tonB* systems and 1 heme uptake system that may be involved in iron acquisition (Hjerde et al. 2008). The 76 kDa outer membrane protein VS-P2 that is produced only in unshaken cultures at 10°C or less does not have the ability to stimulate a specific humoral response (Colquhoun et al. 2002). An outer membrane proteomic study demonstrated that flagellin production and motility were regulated in response to salt concentration, as seawater-like conditions elevate flagellin production and motility compared to physiological-like salt conditions (Karlsen et al. 2008). Furthermore, fish skin mucus has been shown to stimulate expression of flagellar proteins and proteins involved in oxidative stress responses (Uttakleic Raeder et al. 2007). Flagella and motility in several species within the *Vibrio* and *Aliivibrio* groups are linked to host colonization and virulence (Richardson 1991, Graf et al. 1994, Ormonde et al. 2000, Lee et al. 2004). It has also been hypothesized that the production of hydrogen peroxide may act as a possible virulence factor in *A. salmonicida* (Fidopiastis et al. 1999).

Similar to other rough-type Gram-negative bacteria, *Aliivibrio salmonicida* carries a rough-type lipopolysaccharide (LPS) surface structure composed of Lipid A linked to low-molecular-weight oligosaccharides (Bøgwald et al. 1991, Edebrink et al. 1996). The immunogenicity of *A. salmonicida* resides mainly in 2 molecules associated with the outer membrane fraction of the bacterium (Espelid et al. 1987, 1988). The dominant immuno-component is the surface layer complex VS-P1, secreted from living cells into growth medium, fish tissue, or fish serum (Hjelmeland et al. 1988). Thirteen out of 17 monoclonal antibodies (MAbs) against *A. salmonicida* LFI83001 demonstrated specificity for VS-P1, comprising both the antigenic low molecular LPS molecules and a 40 kDa OMP. The 4 remaining MAbs showed specificity to a membrane-associated 24 kDa protein (Espelid 1986, Espelid et al. 1987, 1988, Bøgwald et al. 1990, 1991). The molecule was later detected in the culture supernatant when grown in minimal media, but not in marine broth (Knudsen 1991, Stuge 1992). The importance of VS-P1 in *A. salmonicida* pathogenesis is well documented, and it has been hypothesized that, as the bacterium releases VS-P1, specific antibodies will bind to the complex and thus save the bacterium from complement-mediated killing and phagocytosis (Hjelmeland et al. 1988). The outer membrane of Gram-negative bacteria encompasses various antigenic structures that are recognized by antibodies and, therefore, initiate the protective immune response. By administrating such antigens through vaccinations, acquired immunity could be induced in the recipients, which would develop an increased protection against the disease. A variety of OMPs of pathogens have been repeatedly detected by immunoproteomics (McKevitt et al. 2005, Ying et al. 2005); among them is the peptidoglycan-associated lipoprotein (Pal), which is also found circulating in Gram-negative sepsis. The protein is anchored in the outer membrane, where it constitutes a part of a multiprotein membrane-spanning Tol-Pal complex involved in maintaining the outer membrane integrity of Gram-negative bacteria (Godlewska et al. 2009). Pal proteins are highly conserved, and homologs to the *pal* sequences have been widely reported (Parsons et al. 2006). Gene clusters of Tol-Pal systems are detected in most sequenced Gram-negative bacteria, but not in Gram-positive bacteria. In order to reveal the immunogenic surface structures of *A. salmonicida*, the present study deals with proteomics in combination with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to identify the native and heterologously expressed locus VSAL_I1899 of *A. salmonicida* LFI1238.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions. *Aliivibrio salmonicida* Strain LFI315 (NOFIMA culture collection; Tromsø, Norway), originally isolated from the head kidney of an Atlantic salmon diagnosed with cold water vibriosis (Norwegian fish farm, 1996) was plated on blood agar (agar base No. 2, Oxoid CM271, 7% human whole blood, 2% [w/v] NaCl). A single colony was expanded overnight at 200 rpm in 5 ml Luria-Bertani (LB) containing 1.0% NaCl at 7.5°C. The pre-culture was diluted and further expanded in batches of 250 ml. Cultures were harvested in mid-log phase (OD = 0.65) by centrifugation (4000 × *g*, 10 min at 4°C). The *A. salmonicida* gene encoding Pal (VSAL_I1899) was amplified from total DNA (Forward: 5′-AAA AAG CAG GCT TCA TGC AAC TAA ATA AAC TTC TTA AG-3′ and Reverse: 5′-AGA AAG CTG GGT CTT AGT ATA CTA ATA CTG CAC GAC G-3′), cloned and heterologously expressed utilizing the Gateway system (Invitrogen). Two *Escherichia coli* strains, BL21 (DE3) (Invitrogen) and BL21 CodonPlus (DE3) (Stratagene), were transformed with the

pDEST17 vector containing the *A. salmonicida pal* gene and induced with 1 mM isopropyl β-D-1-thio-galactopyranoside at 20°C overnight. The constructs were verified by DNA sequencing.

Preparation of outer membrane and extracellular proteins. OMPs were isolated as previously described (Karlsen et al. 2008). Extracellular products (ECPs) were extracted from the culture supernatant by supplementing it with phenylmethylsulfonyl fluoride to a final concentration of 5 µM before filtering it through a 0.2 µm membrane (MF75, Nalgene). Proteins were precipitated on ice over night in 6% (w/v) trichloroacetic acid (Fluka) with 0.2 µg ml^{-1} of Na-deoxycholate (Sigma). The precipitate was centrifuged (6000 × *g*, 1 h, 4°C) and washed 3 times with ice-cold acetone to remove traces of the trichloroacetic acid. Protein concentration was measured (Bradford 1976) before it was resolubilized in rehydration solution: 7 M urea, 2 M thiourea, 4% (w/v) CHAPS and 10% (v/v) glycerol with freshly made 0.2% (w/v) dithiotreitol (DTT) and 0.5% (v/v) pharmalyte (pH 3 to 11 non-linear, NL).

Separation and visualization of protein fractions. Two-dimensional PAGE was performed as previously described (Karlsen et al. 2008). Briefly, protein samples (150 mg) were passively rehydrated for 1 h on a nonlinear immobilized pH gradient strip (3.0 to 11.0 NL IPG 13 cm, GE Healthcare). Isoelectric focusing was completed at 45 kVh, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation was performed with 12% acrylamide gels run in a Protean II xi 2-D cell system (Bio-Rad) with a Laemmli buffer system (Laemmli 1970). Gels applied 150 V and 10 mA gel^{-1} overnight were stained with GelCode Blue Stain Reagent (Pierce Biotechnology) and imaged with Fluor-S MultiImager (Bio-Rad). Samples separated by SDS-PAGE electrophoresis utilized the Nupage Pre-Cast gel system (Invitrogen) in accordance with the manufacturer's protocol. Protein samples (30 mg) were mixed with 2× sample buffer (100 mM Tris-HCl pH 6.5, 4% [w/v] SDS, 20% [v/v] glycerol 200 mM DTT and 0.05% [w/v] bromphenol blue) and heated to 80°C for 10 min before being applied on 4 to 12% Bis-Tris gels run in 1× MES buffer (Invitrogen) at 200 V/120 mA for 45 min. Gels were stained with Simply Blue SafeStain (Invitrogen) according to the microwave protocol and imaged by a GEL DOC 2000 (Bio-Rad).

Monoclonal antibodies. Four different MAbs (5E10, 7G1, 4H4 and 1G11) developed against *Aliivibrio salmonicida* Strain LFI 83001 (Espelid 1986), with specificity to an uncharacterized 24 kDa protein, were utilized. In the present study, the pooled MAbs, which were to be identified by mass spectrometry (MS), showed specificity towards a cell surface antigen with a molecular weight corresponding to 20 kDa.

Western blot analysis. Proteins separated by SDS-PAGE were transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences) by semi-dry electrophoretic blotting with a 2117-250 Novablot electrophoretic transfer kit (LKB). Protein transfer was performed using the Towbin buffer system, with the transfer carried out at room temperature applying 20 V and 150 mA for 90 min. Non-specific binding was blocked by incubating the membrane in blocking buffer of 5% (w/v) skim milk (Nestle) in TBS-T (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.1% Tween-20), for 1 h with gentle agitation at 20°C. MAbs diluted in TBS-T were added and incubated overnight at 4°C with gentle agitation. Horseradish peroxidase conjugated goat anti-mouse IgG (BD-Pharmingen) identified the positive antibody reactions developed using the luminol reagent solution (Santa Cruz Biotechnology) in combination with a Lumi-ImagerF1 (Mannheim Boehringer).

Protein spot preparation and collection of MS data. Excision, reduction and alkylation of protein spots and extraction of trypsin-generated peptides are identical to previous descriptions (Karlsen et al. 2008). Protein mass fingerprint (PMF) mass spectra were obtained on a MALDI micro MX (Waters Corporation). MS/MS data were obtained on a Q-TOF UltimaGlobalTM MS equipped with an electro-spray ion source interface online with a CapLC autosampler (nanoACQUITY Ultra Performance LC). Mass spectral data were recorded by means of automated data-dependent switching between MS and MS/MS mode based upon ion intensity, mass and charge state. Fragmentation spectra were collected from mass to charge ratios (m/z) 50 to 2000 and 800 to 3200 for MS/MS and PMF, respectively. PMF spectra were collected as a summation of up to 100 laser shots. Both MS instruments were operated in the positive ion mode under the MassLynx 4.1 software on a Windows XP workstation. For both MS analyses, peptides were concentrated and desalted on C-18 pipette tips (Varian). Peptide ionization for MALDI was achieved by mixing the peptide solution with 1,1-cyano-4-hydroxy-trans-cinnamic acid (10 mg ml^{-1} in 0.1% trifluoroacetic acid: acetonitrile, 1:1, v/v) directly on the target. Electrospray ionization peptides were eluted in 0.1% formic acid. Instruments and software were from Waters Corporation.

Protein identification. MS data were searched against the NCBInr database and the recently sequenced and completed genome of *Aliivibrio salmonicida* (Hjerde et al. 2008) on an in-house Mascot server (Ver. 2.2; Matrix Science). We assume that peptide ions were monoisotopic (MALDI) or contained up to 3 charged residues (MS/MS), oxidized at methionine residues and carbamidomethylated at cysteine resi-

dues with up to 1 missed trypsin cleavage. Mass accuracy of 100 ppm was the window of error allowed for matching the peptide mass values, while tolerances were set to 100 ppm and 1 Da for the peptide precursors and the fragment ions, respectively. For unmatched peptides, however, good quality MS/MS spectra were subjected to automated de novo sequencing using the Peaks Studio software Ver. 3.0 (Bioinformatics Solutions) (Ma et al. 2003).

RESULTS

Identification of an immunogenic 20 kDa OMP

The OMP fraction of *Aliivibrio salmonicida* was utilized to determine the immunogenic protein recognized by the pooled MAbs. As revealed by Western blot, 1 single band reacted with the MAbs (Fig. 1, Lane III). To pinpoint the exact immunoreactive protein on the SDS-PAGE gel (Fig. 1, Lane II), 4 bands in the same molecular weight area were isolated from the gel and transferred separately before being blotted. The immunogenic protein band was allocated (Fig. 1, Lane IV), and analysis of the collected MS data identified the antigenic OMP as a Pal-like protein.

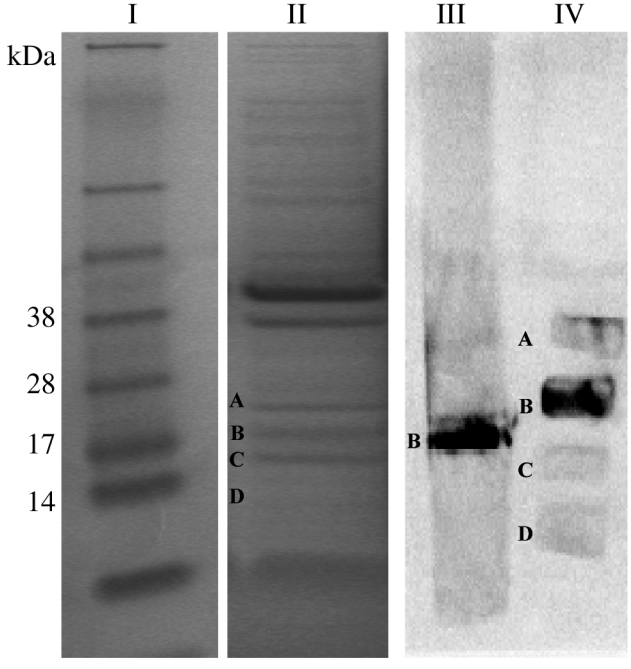

Fig. 1. *Aliivibrio salmonicida*. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot of *A. salmonicida* outer membrane proteins. Lane II: SDS-PAGE of membrane proteins. Lane III: Western blot of the protein band containing the immunoreactive protein, corresponding to Protein Band B in Lanes II and IV. Protein bands indicated by A, B, C and D in Lane II were all blotted individually to pinpoint the immunoreactive protein. Lane IV: Western blot of Protein Bands A, B, C and D from Lane II. Lane I represents the protein standard SeeBlue Plus2 Standard (Invitrogen)

2-D analysis of the outer membrane and secreted proteome

OMPs and ECPs of *Aliivibrio salmonicida* resolved in the pH range of 3 to 11 and separated in a 12% SDS-PAGE are presented in Fig. 2. The spots represent proteins expressed at low temperature at low osmotic pressure. The numbers of identified spots were quantified to 120 and 180 for the OMPs and ECPs, respectively. Spots marked by arrowheads (82) are identified protein entries, annotated in accordance with expected cell compartments or function (Table 1). Several of the identified proteins are also annotated as putative membrane or exported proteins in the genome of *A. salmonicida* (Hjerde et al. 2008). Some highly expressed proteins, such as chaperone DnaK and GroEL, are identified in both the outer membrane and extracellular fraction of *A. salmonicida* (Spot 234/142 and 233/143, respectively). The presence of the immunogenic Pal (Spot 227) was also confirmed in the OMP fraction by MS analysis and by Western blot of both fractions (Fig. 2). Membrane association of homologs to many of the predicted *A. salmonicida* OMPs and ECPs were confirmed by proteomic studies in other bacteria (Table 2). Nonetheless, several of the metabolic proteins like Eno, Fba, AccC/D are well characterized cytoplasmic proteins and are likely to represent cytoplasmic contaminants. Other metabolic proteins, such as GapA and GlyA, have been extracted from OMPs and ECPs in other bacteria (Ying et al. 2005, Voigt et al. 2006). Although they may be cytoplasmic contaminants, they cannot entirely be excluded as legitimate components of the *A. salmonicida* outer membrane.

Cloning and sequence analysis

To further verify the identity of the putative Pal protein, the corresponding gene was cloned into the Gateway pDEST17 vector and expressed in *Escherichia coli* of CodonPlus cells. Both the insoluble and soluble fractions of the resulting cell lysate contained the heterologously expressed Pal protein (Fig. 3). Pooled MAbs demonstrated specificity to both fractions and the *Aliivibrio salmonicida* OMP extract in a molecular weight area of 20 kDa. The estimated sizes correspond to the theoretical molecular weight of the *A. salmonicida* Pal protein, which is 19755 Da. Expression of the Pal pro-

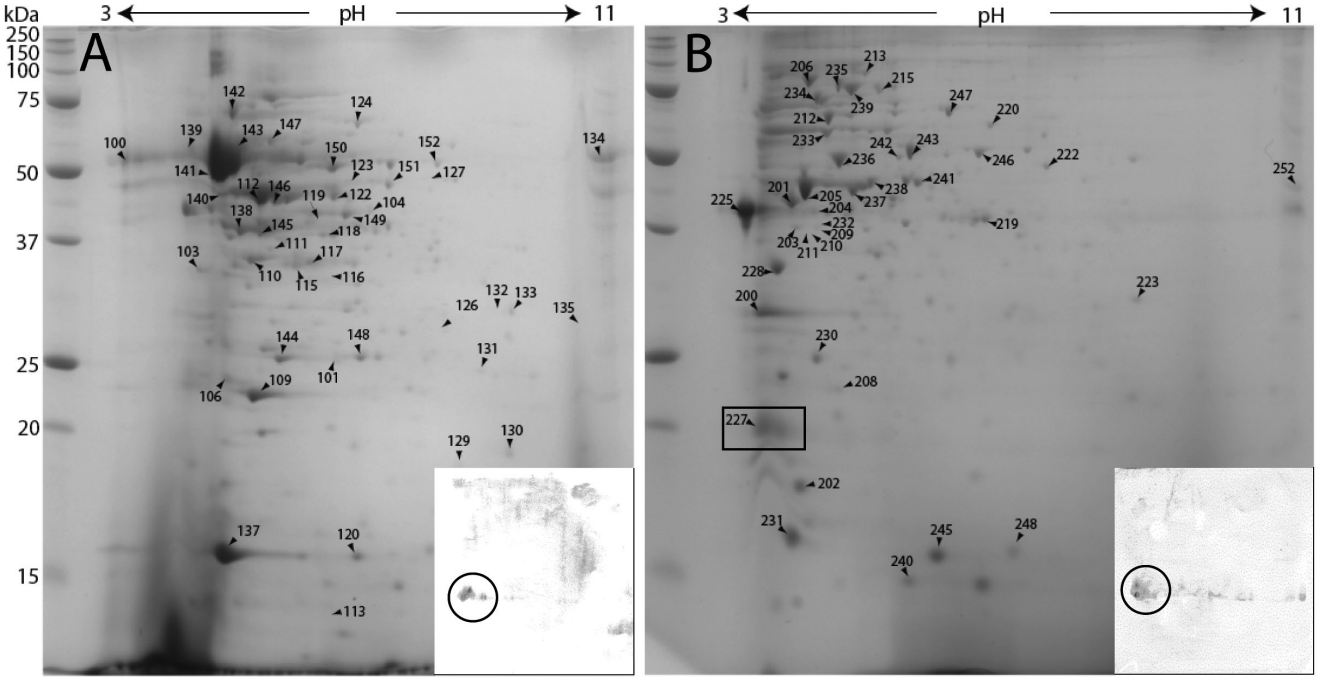

Fig. 2. *Aliivibrio salmonicida* Strain LFI315. Proteome reference map of (A) *A. salmonicida* extracellular products (ECPs) and (B) outer membrane proteins (OMPs). Proteins (150 µg) isolated from mid-log phase cells were focused on 13 cm NL pH 3 to 11 immobilized pH gradient strips and by 12% SDS-PAGE. Arrowheads represent spots with protein entries. See Table 1 for protein annotation. The marked rectangular area enclosing Spot 227 in the OMP reference map indicates the immunogenic Pal. The Pal protein was not present to a detectable level by Coomassie in the ECP fraction. Western blot of *A. salmonicida* ECPs (A, inset) and OMPs (B, inset) identified the immunoreactive protein (circled) in both fractions. The antigenic protein was recognized by use of peritoneal fluid (ascites) from immunized mice

Table 1. (Continued on next page) *Aliivibrio salmonicida*. Identification and predicted function of *A. salmonicida* outer membrane proteins (OMPs) and extracellular products (ECPs) grown. Cells grown at 7.5°C in Lubria-Bertina containing 1.0% NaCl, and harvested in mid-log phase (OD = 0.65). Spot numbers match those marked in Fig. 2 (ECPs = 100 numbers, OMPs = 200 numbers). Proteins/peptides with no identity score are not listed. Gi: GenInfo identifier

Protein Gi no.	Protein	Spot ID
Cell motility and secretion		
209695754	Flagellin subunit A, FlaA	232
209695749	Flagellin subunit C, FlaC	209/145
209695748	Flagellin subunit D, FlaD	211
209695747	Flagellin subunit E, FlaE	204/210/138
209695935	Flagellin subunit F, FlaF	203
209695756	Putative flagellar hook-associated protein, FlgK	124
Folding/repair/assembly		
209695865	Putative peroxidase/antioxidant, AhpC	109
209808931	Peptide methionine sulfoxide reductase	111
209693674	Thiol:disulfide interchange protein, DsbA	130
209693911	FKBP-type peptidyl-prolyl cis-trans isomerase, FkpA	200
209695843	Outer membrane protein assembly factor, YaeT	235
209695891	Chaperone, DnaK	234/142
209693650	Chaperone, GroEL	233/143
Transport		
209695418	Lysine-, arginine-, ornithine-binding protein, ArgT	148
209693642	Putative cystine ATP-binding cassette transporter	144
209695701	Outer membrane fatty acid transport protein precursor, FadL	201
209696083	Outer membrane channel protein, TolC	205/140
209696285	Putative OMP-associated TonB-dependent receptor	212
209694180	Major outer membrane protein, OmpU	228
209696443	ATP synthase beta chain, AtpD	236

Table 1 (continued)

Protein Gi no.	Protein	Spot ID
209808860	Outer membrane efflux protein	237
209696445	ATP synthase alpha subunit, AtpA	242/243
Outer membrane integrity		
209693974	Organic solvent tolerance protein precursor, Imp	206
209694546	Rare lipoprotein A	219
209695366	Peptidoglycan-associated lipoprotein precusor, Pal	227
209694545	Penicillin-binding protein 5 precursor, DacA	255
Transcription/translation		
209694252	Ribosome-associated inhibitor protein A, RaiA	125
209694218	Elongation factor G	239
209696169	50s ribosomal subunit protein L9	240
209695311	Seryl-tRNA synthetase, SerS	242
209809781	Helix-turn-helix-type transcriptional regulator, AsnC family	248/106
Extracellular proteins		
208009581	Putative exported protein	101
208009252	Putative exported protein	104
208010625	Putative exported protein	112
209693976	Exported protein	131
209695629	Putative exported protein	134
209809063	Putative exported protein	146
Metabolism/biosynthesis		
209693789	Polysaccharide biosynthesis protein	104
209694150	Fructose-bisphosphate aldolase, Fba	110
209695316	Glyceraldehyde-3-phosphate dehydrogenase, GapA	115
208007913	Malate dehydrogenase, Mdh	117
209694652	Iron-containing alcohol dehydrogenase	118
209695632	Adenylosuccinate lyase, PurB	123
209694483	Formyltetrahydrofolate deformylase, PurU	126
209808904	Succinylarginine dihydrolase, AstB	127
209696337	Glutamine synthetase, GlnA	139
209694149	Phosphoglycerate kinase	145
209695306	Alanine dehydrogenase, Ald	149
209809185	Glycerol kinase, GlpK	150
209696119	Dihydrolipoamide dehydrogenase	150
209693743	Glutathione reductase	151
209694326	Inosine-5′-monophosphate dehydrogenase, GuaB	152
209695933	CTP synthase, PyrG	220
209696240	Biotin carboxylase, AccC	222
209694635	Acetyl-CoA carboxylase subunit beta, AccD	223
209695932	Enolase, Eno	238
209694441	Serine hydroxymethyltransferase, GlyA	241/122
209696033	Dihydrolipoamide dehydrogenase, LpdA	246
209694423	Succinate dehydrogenase flavoprotein subunit, SdhA	247
209693783	Putative pseudaminic acid biosynthesis protein	254
Attachment and colonization		
209808958	N-acetylglucosamine-binding protein A, GbpA	100/141
DNA transposition		
209809238	Transposase	129
Hypothetical proteins		
209693960	Hypothetical protein	133
209694793	Hypothetical protein	135
209809718	Unidentified product	137
Membrane proteins with unknown function		
209694987	Outer membrane protein	202/253
209694766	Outer membrane protein	213
209694253	Outer membrane protein	230
209694320	Putative outer membrane protein	208
209694682	Putative outer membrane protein	225/252
209693880	Putative outer membrane protein	234
209695331	Putative outer membrane protein	245
209696425	Putative outer membrane protein	245

Table 2. *Aliivibrio salmonicida*. Identified homologs of membrane-associated protein confirmed by proteomic studies in other bacteria. Cells grown at 7.5°C in Lubria-Bertina containing 1.0% NaCl, and harvested in mid-log phase (OD = 0.65). Gi: GenInfo identifier. Spot numbers match those marked in Fig. 2 (ECPs = 100 numbers, OMPs = 200 numbers)

Protein Gi no.	Protein	Species	Function	Spot ID
81728747	General secretion pathway protein E	*Vibrio parahaemolyticus*	Component of T2SS	101
78033454	Hypothetical protein	*Magnetospirillum gryphis-waldense*	Unknown	113
58254493	DNA alkylation repair enzyme	*Lactobacillus acidophilus*	DNA repair	120
262192021	ISSod13 transposase	*Vibrio cholerae*	Genetic rearrangement	132
59713085	Phosphoenolpyruvate carboxykinase, PckA	*Aliivibrio fischeri*	Energy metabolism carbohydrates	147
61212678	Exodeoxyribonuclease 7 large subunit	*Vibrio vulnificus*	DNA excision	151
22653130	Outer membrane protein, PorB	*Neisseria meningitidis*	Membrane transport	231
28897600	Asparagine synthetase B, AsnB	*Vibrio parahaemolyticus*	Metabolism	252
83716747	Flagellum-specific ATP synthase, FliI	*Burkholderia thailandensis*	Cell motility and secretion	252

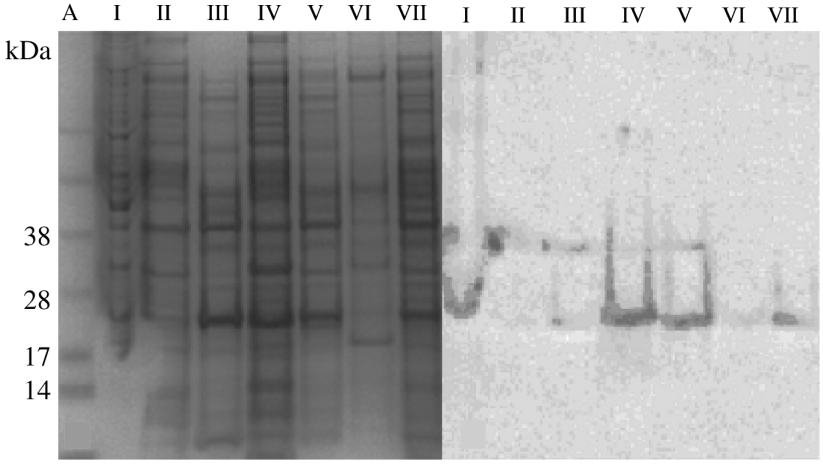

Fig. 3. *Aliivibrio salmonicida*. SDS-PAGE and Western blot of *A. salmonicida* outer membrane proteins (OMPs) and the heterologously expressed Pal protein. Lane I: *A. salmonicida* OMP extract. Lanes II/III: negative controls; insoluble and soluble cell fractions of BL21 CodonPlus (DE3) expressing an irrelevant protein (VP4 from infectious pancreatic necrosis virus). Lanes IV/V: insoluble and soluble cell fractions of *Escherichia coli* Strain BL21 CodonPlus (DE3) expressing the *A. salmonicida* Pal protein. Lanes VI/VII: insoluble and soluble cell fractions of *E. coli* Strain BL21 (DE3) expressing the Pal protein. Lane A: SeeBlue Plus2 Standard (Invitrogen)

tein or the folding to its native state appeared to be temperature dependent, as the detection level on the immunoblot was best retained when expressed at 20°C compared to 30°C (data not shown). The transformed *E. coli* Strain BL21 (DE3) did not express the recombinant protein to any detectable level.

DISCUSSION

Aliivibrio salmonicida is the aetiological agent of cold water vibriosis in farmed fish species, a disease that today is fully controlled by vaccination. The molecular mechanisms behind the protective nature of the vaccine are unknown, and no specific antigen has been found. It is therefore in our interest to search for immunogenic OMPs that induce responses in fish, as well as in mouse and rabbit (Espelid et al. 1987). In the present study we found a Pal that was present in both the OMP extract as well as in the ECPs from *A. salmonicida* grown in LB medium. The mature expressed protein has an apparent molecular mass of 20 kDa, similar to the theoretical molecular weight of 19755 Da, predicted from the 185 amino acid sequence. Also, the first 28 amino acids on the N-terminal part of the Pal protein sequence are predicted to constitute a hydrophobic lipoprotein signal sequence of 2873 Da. Tol-Pal systems appear ubiquitous in Gram-negative bacteria, and the gene cluster present in *A. salmonicida* is identical to, for example, *Escherichia coli* and *Vibrio cholerae* (*ybgC-tolQ-tolR-tolA-tolB-pal-ybgF*). No designed experiment has demonstrated that the protein is in fact peptidoglycan-associated in *A. salmonicida*. However, its highly conserved homology to other Pals within the Vibrionaceae and in *E. coli* and its location in the outer membrane suggest that the protein belongs to the Pal family. Pal proteins are shed from the bacterial surface into the circulation of animals and induce a strong antibody response in many different species (Liang et al. 2005, Godlewska et al. 2009). Although the Pal protein is highly immunogenic in Atlantic salmon, it is assumed that VS-P1 has a stronger effect in mediating the humoral immune response. This assumption is based on the fact that >90% of the antigen response in the serum of Atlantic

salmon immunized with *A. salmonicida* is targeting the VS-P1 complex (Espelid et al. 1987). Additional experiments are required to elucidate whether *A. salmonicida* Pal plays a role in the pathogenesis of cold water vibriosis.

The heterologously expressed *Aliivibrio salmonicida* Pal-like product was found to be immunogenic by Western blot analysis, which further supports the correct annotation and antigenic nature of the protein. The immunogenic property of the recombinantly expressed protein excludes any possible cross immunogenicity
owing to *A. salmonicida* LPS, as in the VS-P1 complex.

Little is known about the constituents of the *Aliivibrio salmonicida* surface layer that circulates in sepsis. The LPS layer or OMP/LPS complexes such as VS-P1 were suggested to act as the immunodominant antigens that triggered the immune responses in Atlantic salmon. However, immunization of Atlantic salmon with LPS provided little protection, whereas an improved immunity was observed with the VS-P1 complex. Still, whole inactivated bacterial cells improve the efficacy (Bøgwald et al. 1992), which suggests that antigens other than LPS or the OMP/LPS complex are involved in the stimulation of the fish immune response, such as the Pal protein. The LPS of Gram-negative bacteria may, in sepsis, act as toxins that elicit inflammation activity. Recent studies indicate that lipoproteins and outer surface proteins can also modulate the host immune response, as the lipoproteins are recognized by Class II Toll-like receptors (TLR2) of antigen presenting cells (Aliprantis et al. 1999). Some lipoproteins, such as Pal, can also be secreted into the bloodstream during infection and contribute to the development of septic shock (Hellman et al. 2002, Liang et al. 2005). Although the role of Pal in pathogenesis is not clearly defined, mutations in the *pal* gene result in decreased virulence (Godlewska et al. 2009).

The fact that we have cloned and expressed the immunogene VSAL_I1899 from *Aliivibrio salmonicida* LFI1238 makes it possible to test the protective properties of the protein. Whether it can be regarded as a suitable candidate in developing a subunit vaccine for cold water vibriosis in Atlantic salmon remains to be elucidated. The application of Pal in immunoprophylaxis is supported by the fact that all Pal proteins studied so far are highly immunogenic. By construction of a gene-specific knockout the importance of the Pal protein in cold water vibriosis can be tested in an infection model.

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