Analysis of the *Flavobacterium psychrophilum* outer-membrane subproteome and identification of new antigenic targets for vaccine by immunomics

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Flavobacterium psychrophilum is an important infectious Gram-negative bacterium causing cold-water disease (CWD) and rainbow trout fry syndrome. Outer-membrane proteins (OMPs) are key molecules with regard to the interface between the cell and its environment. Therefore, we sought to define the outer-membrane (OM) subproteome of *F. psychrophilum* in order to gain insight into the biology and pathogenesis of this bacterium and to identify the dominant antigens targeted by the rainbow trout (*Oncorhynchus mykiss*) immune system during infection. First, OMs were prepared from a cell-envelope suspension by differential Sarkosyl (sodium lauryl sarcosinate) solubility. We then isolated the OMPs and identified 36 proteins from 34 spots resolved by two-dimensional electrophoresis and LC-MS/MS. An immunoproteomic approach using antibodies from CWD-convalescent rainbow trout was then used to identify 25 immunoreactive *F. psychrophilum* antigens that may be relevant in pathogenesis and diagnosis. These included the previously characterized surface-exposed OMPs OmpA, OmpH/P18 and FspA, as well as newly described antigenic proteins. This study provides a number of novel candidate proteins for developing vaccine(s) against flavobacteriosis infection in aquaculture.

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INTRODUCTION

Flavobacterium psychrophilum is a yellow-pigmented, Gram-negative, gliding bacterium that predominantly affects salmonid fish (Borg, 1960), such as coho salmon (*Oncorhynchus kisutch*) or rainbow trout (*Oncorhynchus mykiss*), and occasionally other fish species, such as ayu (*Plecoglossus altivelis*) (Iida & Mizokami, 1996). This bacterium is therefore responsible for considerable economic losses in fish aquaculture. Infections with *F. psychrophilum* have several clinical manifestations, the most significant of which include mortality associated with haemorrhagic septicaemia and spleen hypertrophy in juvenile fish, referred to as rainbow trout fry syndrome, and in adults, septicaemia preceded by extensive necrotic

lesions, called cold-water disease (CWD) (Bernardet & Bowman, 2006). However, the actual mechanism of pathogenesis is not well understood, although virulence has been suspected to be related to the ability of *F. psychrophilum* to produce exotoxins (Dalsgaard, 1993), extracellular metalloproteases (Fpp1-2; Secades *et al.*, 2001, 2003) or enzymes involved in the degradation of products such as chondroitin sulfate, collagen and fibrinogen (Bertolini *et al.*, 1994). Clearly, the flavobacterial outer membrane (OM) is important when we consider interactions of bacteria with host cells and tissues in the context of pathogenesis and immunity to infection. Several surface components of *F. psychrophilum* have been implicated in flavobacterial pathogenesis and identified as possible vaccine and diagnostic candidate macromolecules; they include lipopolysaccharide O antigen (MacLean *et al.*, 2001) and surface-exposed antigens [e.g. 20 kDa antigen (Crump *et al.*, 2001) and OmpA (Merle *et al.*, 2003; Dumetz *et al.*, 2007)], some of which may be good candidates for an *F. psychrophilum* subunit vaccine, such as the surface-localized *Flavobacterium*-specific protein (FspA; Crump *et al.*, 2005). Indeed, a protective immune

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Abbreviations: ACN, acetonitrile; ASB-14, 3-[*N,N*-dimethyl(3-myristoyl-aminopropyl)ammonio]propanesulfonate; CWD, cold-water disease; GRAVY, grand average of hydropathy; OM, outer membrane; OMP, outer-membrane protein; Sarkosyl, sodium lauryl sarcosinate.

response has been shown experimentally in rainbow trout by P18 (Massias *et al.*, 2004), a surface-exposed protein belonging to the OmpH family (Dumetz *et al.*, 2006).

Elucidation of the *F. psychrophilum* genome sequence has provided information about the structure and function of flavobacterial proteins (Duchaud *et al.*, 2007). Proteomic analysis is a useful approach for examination of global protein synthesis and is particularly good as an indicator of gene expression in bacteria. Such analyses have allowed identification of virulence determinants, antigens and vaccine candidates for many bacterial pathogens (e.g. *Pasteurella multocida*; Boyce *et al.*, 2006), since changes in the proteome depend on developmental stage, disease state and environmental conditions. Recently, an immunoproteomic analysis of a virulent and a non-virulent strain of *F. psychrophilum* has been done to identify possible factors associated with virulence and to identify specific proteins recognized by the host immune response (Sudheesh *et al.*, 2007). Only minor differences were seen between the two strains when total cell proteins were analysed and four immunogenic proteins were conclusively identified: the heat-shock proteins HSP60 and HSP70, ATP synthase and thermolysin. The ribosomal L10-like protein also seems to elicit a protective response in fish (Crump *et al.*, 2007). Are such antigens promising candidates for vaccine development? Indeed, *F. psychrophilum* OM preparations (Rahman *et al.*, 2002) and a highly enriched fraction of the surface-exposed antigen OmpH/P18 (Dumetz *et al.*, 2006) have been shown previously to be involved in fish protection against infection. Moreover, it has been demonstrated that OM proteins (OMPs) are depleted in two-dimensional (2-D) electrophoresis displaying whole-cell extracts (Santoni *et al.*, 2000). The common underrepresentation of OMPs is not only due to their poor solubility, but also sometimes to low expression levels, which renders detection and thus identification difficult. As they are located on the bacterial surface, OMPs are likely to interact with the environment and, particularly, with the host immune system. Therefore, the aims of our study were (i) to perform subcellular fractionation and subsequent enrichment of OMPs with the ionic detergent sodium lauryl sarcosinate (Sarkosyl), (ii) to systematically define the protein content of the OM from the virulent *F. psychrophilum* strain JIP02/86 and (iii) to identify a number of candidate proteins as possible vaccine targets probed with antibodies collected from naturally infected fish.

METHODS

***F. psychrophilum* cell culture.** *F. psychrophilum* strain JIP02/86 (INRA, Jouy-en-Josas, France) was used in this study. Cells were cultured in a modified Anacker & Ordal's (AOAE) liquid medium supplemented with 5% bovine serum under standard growth conditions (Dumetz *et al.*, 2006). Purity was checked at the end of growth ($OD_{600}=0.8$) by examination of Gram-stained smears. Cells were collected by centrifugation at 6000 g for 10 min at 4 °C. Following removal of supernatant and two washing steps with PBS

(50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4), the pellet was dispersed into cold water supplemented with a protease inhibitor cocktail (Roche). Flavobacteria were disrupted by 10 repeated ultrasonication steps (24 W, 10 s, 0 °C). Unbroken cells and cellular debris were removed by centrifugation at 10 000 g for 10 min at 4 °C. Cell envelopes were recovered by an ultracentrifugation step (260 000 g, 15 min, 4 °C) in a Beckman Coulter MicroUltracentrifuge Optima TLX in TLA-110 rotor. Then they were washed twice with PBS.

Sucrose density-gradient centrifugation. Whole-cell envelope suspension (200 µl per tube) was applied to the discontinuous sucrose density gradient (20–60%, w/w, sucrose in 5 mM EDTA) reported previously for the separation of inner- and outer-membrane vesicles from *Escherichia coli* (Osborn & Munson, 1974). The gradients were ultracentrifuged (215 000 g, 24 h, 4 °C) and fractions were collected by piercing the bottom of each tube with an 18-gauge syringe needle.

OM preparation by differential solubility in Sarkosyl. The OM fraction was isolated from the *F. psychrophilum* cell envelope with the ionic detergent Sarkosyl (Sigma) as described for the bacterial pathogen *Bartonella henselae* (Rhomberg *et al.*, 2004). Briefly, whole-cell envelopes were dispersed in 1 vol. 10 mM HEPES buffer (pH 7.4) and this envelope suspension was treated with 1 vol. 2% Sarkosyl in the same buffer at room temperature for 20 min. The insoluble material (i.e. the OM fraction) was collected by ultracentrifugation and washed twice with HEPES buffer to remove residual traces of detergent contaminants.

Protein and enzyme assays. The OMP content was estimated using our standard procedures (Merle *et al.*, 2003). The OM preparation was finally adjusted to 10 mg protein ml⁻¹ and stored at -80 °C. NADH dehydrogenase activity was measured as an indicator for inner-membrane contamination of the OM preparation (Kasahara & Anraku, 1974).

Electrophoresis. SDS-PAGE analyses were performed in polyacrylamide gel slabs (10 × 8 × 0.075 cm; 10% acrylamide, 0.26% bisacrylamide; 200 V, 1 h) (Laemmli, 1970). 2-D electrophoresis was performed following the ZOOM IPG runner system procedure described by Invitrogen with the following modifications. To generate an electrophoretic map of OMPs, about 200 µg protein extract was mixed in 160 µl IPG rehydration buffer [7 M urea, 2 M thiourea, 2% 3-[N,N-dimethyl(3-myristoylamino)propyl]ammonio]propanesulfonate (ASB-14), 25 mM dithiothreitol, 0.01% bromophenol blue, 1%, v/v, carrier ampholytes 3-10 (Invitrogen)]. After sonication, samples were clarified by centrifugation (5000 g, 5 min, 20 °C), loaded on 3–10 NL IPG ZOOM strips (Invitrogen) and allowed to swell overnight. The following IEF parameters were applied: (i) 200 V for 20 min, (ii) 450 V for 15 min, (iii) 750 V for 15 min, and (iv) 2000 V for 30 min. After the focusing was complete, IPG ZOOM strips were sequentially equilibrated in two equilibration buffers: buffer I (6 M urea; 50 mM Tris; 2% SDS; 30% glycerol; 50 mM dithiothreitol) and buffer II (6 M urea; 50 mM Tris; 2% SDS; 30% glycerol; 125 mM iodoacetamide). The separation in the second dimension was performed on SDS-PAGE (12% polyacrylamide) for 2 h under a constant voltage of 120 V. Following the electrophoretic runs, gels containing proteins were stained with Coomassie brilliant blue G-250.

Protein detection by immunoblotting. Proteins separated by SDS-PAGE or by 2-D IEF/SDS-PAGE were electroblotted onto a nitrocellulose filter (Bio-Rad) using a Bio-Rad TransBlot electrophoretic transfer cell as specified by the manufacturer. The blots were blocked with 5% BSA in PBS for 1 h at room temperature and then incubated for 1 h with anti-*F. psychrophilum* OmpA rabbit serum (Dumetz *et al.*, 2007), with a pool of antisera (dilution 1:2000 in

0.05 % Tween 20 in PBS) collected from 20 healthy trout (generously given by Scott E. LaPatra, Clear Springs Foods, Inc., Buhl, Idaho, USA) or with a pool of antisera (dilution 1 : 1000 in 0.05 % Tween 20 in PBS) collected from 20 CWD-convalescent trout taken from an infected French farm. The washing steps were performed three times with PBS-Tween 20. An additional incubation was made with a rabbit antiserum anti-trout immunoglobulin (dilution 1 : 2000); the primary antibodies were from trout antiserum. Bound antibodies were visualized on immunoblots using an incubation step with alkaline phosphatase-conjugated anti-rabbit IgG antibodies (dilution 1 : 2000; DakoCytomation), followed by a colour reaction with an NBT/BCIP cocktail (4-nitroblue tetrazolium chloride; 5-bromo-4-chloro-3-indolyl phosphate).

Protein identification by LC-MS/MS, data interpretation and database searching. Protein spots selected for analysis were manually excised from the 2-D gels and destained in H₂O/acetonitrile (ACN) (50 : 50). Spots were subsequently rinsed twice in ultrapure water and shrunk in ACN for 10 min. After removal of ACN, gel pieces were dried at room temperature, covered with trypsin solution (10 ng ml⁻¹ in 50 mM NH₄HCO₃), rehydrated at 4 °C for 10 min and finally incubated overnight at 37 °C. Spots were then incubated for 15 min in 50 mM NH₄HCO₃ at room temperature with rotary shaking. The supernatant was collected and an H₂O/ACN/HCOOH (47.5 : 47.5 : 5) extraction solution was added onto the gel slices and left for 15 min. The extraction step was repeated twice. Supernatants were pooled and concentrated in a vacuum centrifuge to a final volume of 25 µl. Digests were finally acidified by addition of 1.5 µl 5 % acetic acid and stored at -20 °C. The peptide mixture was analysed by online capillary HPLC (300 µm × 5 mm C18 PepMap trap column; LC Packings) coupled to a nanospray LCQ IT mass spectrometer (Thermo Finnigan). The samples used were 10 µl of the peptide digests. The flow rate was set at 200 nl min⁻¹. Solvent A was used for column equilibration (0.1 % formic acid in 5 % ACN) and solvent B was 0.1 % formic acid in 80 % ACN. Peptides were eluted with a 5–50 % linear gradient of solvent B for 30 min. The mass spectrometer operated in positive ion mode at a 2 kV needle voltage and a 46 V capillary voltage. Data were acquired in data-dependent mode alternating an MS scan survey over the range *m/z* 300–2000 and three MS/MS scans in an exclusion dynamic mode. MS/MS spectra were acquired using a 2 *m/z* units ion isolation window, a 35 % relative collision energy and a 0.5 min dynamic exclusion duration. Data were searched by SEQUEST through the Bioworks 3.2 interface (Thermo Finnigan) against the *F. psychrophilum* complete proteome database (Duchaud *et al.*, 2007). Data files were generated for MS/MS spectra that reached both a minimal intensity (5 × 10⁴) and a sufficient number of ions (Fraser *et al.*, 1999). Generation of the data file allowed the averaging of several MS/MS spectra corresponding to the same precursor ion with a tolerance of 1.4 Da. Spectra from precursor ions higher than 3500 Da or lower than 500 Da were rejected. The search parameters were as follows: mass accuracy of the peptide precursor and peptide fragments was set to 1.5 Da and 0.5 Da, respectively. Only b- and y-ions were considered for mass calculation. Oxidation of methionines (+16) and carbamidomethylation of cysteines (+57) were considered as differential modifications. Two missed trypsin cleavages were allowed. Only peptides with Xcorr values higher than 1.5 (single charge), 2 (double charge) and 2.5 (triple charge) were retained. In all cases, ΔC_n had to be greater than 0.1. All protein identifications were based on a minimum of two peptide assignments. To be considered, the protein spot had to show a reproducible pattern in three gels which had been run separately.

In silico analysis. Protein homology searches were carried out with the SWISS-PROT database with the BLAST (Altschul *et al.*, 1990; Schaffer *et al.*, 2001) and FASTA (Pearson, 1990) algorithms. Identification of peculiar domains in proteins was conducted using the HMMPFAM program in the HMMER package with the Pfam HMM

library (www.sanger.ac.uk/Software/Pfam/) (Finn *et al.*, 2006). Prediction of membrane-spanning regions and their orientation was performed using the TMpred program (Hofmann & Stoffel, 1993). Membrane anchor and signal peptides were analysed with LipoP 1.0 and SignalP 3.0 software (www.cbs.dtu.dk/services/) (Nielsen *et al.*, 1997; Juncker *et al.*, 2003; Bendtsen *et al.*, 2004). The subcellular locations of OM candidates were predicted using the program PSORTb v. 2.0 (www.psort.org/psortb/) (Gardy *et al.*, 2005). The hydrophobicity of proteins was estimated using the grand average of hydropathy (GRAVY) value (ProtParam program; ExPASy proteomics tools, www.expasy.org/tools/protparam.html) as the sum of the hydropathy value of all amino acids divided by the length of the amino acid sequence (Kyte & Doolittle, 1982).

RESULTS AND DISCUSSION

OMPs preparation

High-quality subcellular fractionation was a prerequisite for a proteomic analysis of the OM subproteome. First, we tried to isolate OMs from the whole-cell envelope of *F. psychrophilum* JIP02/86 using discontinuous sucrose gradient ultracentrifugation. This method was not fruitful, since only one layer containing the envelope material was seen in the gradient, even when the gradient curves were modified (data not shown). Consequently, the ionic detergent Sarkosyl was used on an *F. psychrophilum* cell-envelope suspension (Fig. 1) because of its capability to selectively release proteins from the inner membrane of ruptured Gram-negative bacteria while conserving the integrity of the OM. The purity of the OM preparation (Fig. 1a; lane 2) was shown by immunoblot analysis using antibodies directed against an OM-specific marker (OmpA; Dumetz *et al.*, 2007) and by an enzymic assay using NADH dehydrogenase activity as an inner-membrane marker (Fig. 1b). The signal for OmpA could only be seen in the OM preparation, whereas NADH dehydrogenase activity could only be detected in the Sarkosyl-soluble fraction. This isolation method results in a sufficiently pure OM fraction since no NADH dehydrogenase activity was recorded, underlining the absence of cytoplasmic membrane contaminants.

2-D electrophoresis of *F. psychrophilum* OMPs and protein assignment

OMs prepared by Sarkosyl insolubility were used for concise 2-D mapping of *F. psychrophilum* JIP02/86 OMPs (Fig. 2a). Using the non-ionic detergent ASB-14, proteins were solubilized and resolved within a non-linear pH range of 3–10. A total of 34 OMP spots were selected from the 2-D gel. LC-MS/MS analysis of these spots allowed the identification of 36 ORFs comprising 1.5 % of the 2432 predicted protein-encoding genes in *F. psychrophilum* (Table 1). All of the detected spots were resolved within a molecular mass range of <120 to about 18 kDa.

Identified proteins were analysed for hydrophobicity by determining their GRAVY scores. A calculated GRAVY score of up to -0.4 indicates a hydrophobic protein,

Fig. 1. SDS-PAGE analysis of *F. psychrophilum* JIP02/86 proteins. Lanes: 1 (a, b), cell-envelope fraction (20 µg protein); 2 (a, b), Sarkosyl-insoluble OM fraction (10 µg protein); 3 (b), Sarkosyl-soluble inner-membrane fraction (10 µg protein). All samples were solubilized with 1% SDS. Proteins were silver-stained (a) or probed with a rabbit antiserum directed against OmpA (b). NADH dehydrogenase activity in each fraction is indicated by + or – under each in (b).

suggesting a membrane association. Here, such a criterion revealed that 75 % of the proteins identified in the course of the 2-D analysis should be considered to be hydrophobic (Table 1). It should be stressed that even with a score of <-0.4 , a protein might still be membrane-associated as has been shown for some lipoproteins; such hydrophilic components that carry out functions on the membrane surface are tethered to the plasma membrane via acyl chains (e.g. OmpA from *Borrelia burgdorferi*, Bouchon *et al.*, 1997; spiralin from *Spiroplasma melliferum*, Le Hénaff & Fontenelle, 2000). Sixteen of the identified proteins are predicted to be OMs, but the subcellular location could not be determined for the 20 other identified spots. However, the identity of some spots suggests that the OM fraction might have been weakly contaminated by cytoplasmic and/or periplasmic components: a probable cytoplasmic cysteine aminopeptidase (spot 16, FP2369; PepC, the

bacterial homologue of the eukaryotic bleomycin hydrolase; Mistou & Gripon, 1998) and a protein of unknown function (spot 24, FP1439; FkpA/FKBP-type, a possible homologue of an *Escherichia coli* periplasmic chaperone; Saul *et al.*, 2004).

Our earlier observations (Duchaud *et al.*, 2007) suggest that the Sec system is the major route contributing to the export of proteins across the plasma membrane. All identified proteins were predicted to contain typical Sec signal N-termini lacking sequence similarity. Twenty-one out of the 36 identified proteins seem to be synthesized as a proprotein containing a typical signal peptide (about 20 aa) with a cleavage specific for signal peptidase I (SPase I). The 15 other spots are proteins predicted to be processed in the same way as Braun's lipoprotein, the prototype for bacterial lipoproteins (Sankaran *et al.*, 1995), since they contain a putative cleavage site for the prolipoprotein-specific signal peptidase II (globomycin-sensitive SPase II). A few proteins were resolved as multiple spots, as shown in Fig. 2(a) (e.g. spot 13, OmpA, FP0156). This might be due to post-translational modifications. Indeed, such modifications can modify the pI while the apparent molecular masses of the proteins are not affected, since the modifications are mainly on the side chains of the amino acids. Heterogeneous sugar modifications do explain the large multiple spots seen for OmpA since it is a glycoprotein (Merle *et al.*, 2003). Spots assembled into a single circle were also identified as OmpA, although their molecular masses were less than 30 kDa, suggesting possible degradation in the course of sample preparation. Interestingly, a probable lipoprotein precursor of an uncharacterized iron-regulated protein (spot 20, FP1478, IrpA) was found as two spots with a difference in their apparent molecular mass of less than 3 kDa, suggesting its presence in the OM in both unprocessed and processed forms. However, the Sec-independent system must be functional since we have previously identified one possible component of the type 1 secretion system from Sarkosyl-insoluble membrane proteins resolved by 1-D electrophoresis (probable multidrug resistance protein, AcrB/AcrD/AcrF family protein, FP0880; Dumetz, 2006) and the gene encoding a probable OM efflux protein TolC (FP0296) is present in the *F. psychrophilum* genome (Duchaud *et al.*, 2007). In Gram-negative bacteria, TolC efflux has been described as a tripartite pump that consists of the TolC OMP and a specific plasma membrane channel (such as AcrB) connected with a periplasmic protein known as the adaptor (e.g. AcrA) (Fernandez-Recio *et al.*, 2004).

Iron import is often the limiting factor in bacterial growth, largely because of the insolubility of Fe(III), but also because mammals reduce the availability of iron to potential pathogens by the use of very high affinity iron-chelating molecules (lactoferrin, transferrin and haemoglobin) (for a review see Bullen *et al.*, 2005). As a consequence, host-adapted pathogens have evolved means of using these iron-bearing molecules as an iron source, as well as in some cases synthesizing their own chelators

Fig. 2. 2-D gel map of *F. psychrophilum* JIP02/86. Proteins were prepared from Sarkosyl-insoluble OM material (see Methods) and resolved using non-linear pH 3–10 IPG and 12% SDS-PAGE. Proteins were stained with Coomassie brilliant blue G-250 (a) or probed with a pool of antisera collected from CWD-convalescent trout (b). Protein spots are annotated according to FPORF (*F. psychrophilum* gene annotation; Duchaud *et al.*, 2007).

(siderophores), which are secreted, trap iron and are then transported back into the cell. Interestingly, we found seven proteins in the OM of *F. psychrophilum* which may be involved in iron acquisition/metabolism. They include a probable OMP belonging to the Omp121 family (FP1199), five probable TonB-dependent OM receptors (FP0144, FP1500, FP2456, FP1922 and FP0521), and iron-regulated protein A (IrpA, FP1478). Genes encoding such components have been identified in bacteria belonging to the *Cytophaga-Flavobacterium-Bacteroides* phylum (e.g. *omp200*, a porin gene complex from *Bacteroides fragilis*, *omp121* and *omp71*; Wexler *et al.*, 2002). Because of the importance of iron to the survival of bacterial pathogens within the host environment, the TonB system has been shown to be essential for virulence in diverse organisms (Beddek *et al.*, 2004; Wyckoff *et al.*, 2006) and components are the main targets of the humoral immune response

(e.g. the siderophore receptor FhuA in *Haemophilus parasuis*; del Río *et al.*, 2006). However, functional studies are needed to assign possible roles of these components to iron acquisition and flavobacterial virulence.

Analysis of the OM proteome indicated four spots that were identified as probable cell-surface proteins containing leucine-rich repeats (Lrr proteins: FP0169, FP0171, FP0172 and FP0175). Leucine-rich repeats consist of 2–45 motifs of 20–30 aa in length (Enkhbayar *et al.*, 2004) and appear to provide a structural framework for the formation of protein–protein interactions (Kobe & Kajava, 2001). Proteins containing Lrr include cell-adhesion molecules, virulence factors and extracellular matrix-binding glycoproteins, involved in a variety of biological processes (signal transduction, cell adhesion, disease resistance, immune response, etc.). The four Lrr proteins identified in *F. psychrophilum* are encoded by four genes belonging to a family of 15 genes (Duchaud *et al.*, 2007). They show similarities with BspA and LrrA from the periodontopathogenic bacteria *Tannerella forsythensis* (Inagaki *et al.*, 2005, 2006) and *Treponema denticola* (Ikegami *et al.*, 2004), respectively. These proteins have been reported to be immunogenic cell-surface determinants that bind strongly to extracellular matrix components and play a role in the attachment to human oral tissue, a property that may be important in the virulence of these organisms.

Several known proteins have been retrieved from the OM preparation. They are OmpH/P18 (FP2098; Massias *et al.*, 2004; Dumetz *et al.*, 2006), OmpA (FP0156; Merle *et al.*, 2003; Dumetz *et al.*, 2007) and flavobacterial-specific protein antigen (FspA) (FP2019; Crump *et al.*, 2005). All of them are probably involved in bacteria–host interactions since they are included in a set of antigenic flavobacterial proteins recognized predominantly by antisera from convalescent fish that were naturally infected. Several other proteins and lipoproteins with unknown functions were found (see Table 1). They include a flavomodulin (FP0097) that could be a member of the thiol-activated cytolysin family of pore-forming toxins (TACyS). Such toxins are involved in the pathogenesis mechanism of a number of Gram-positive species of bacteria, such as suliyisin from *Streptococcus suis* (Lun *et al.*, 2003). They may interfere with immune cell function and induce cytokine production (Billington *et al.*, 2000). In *F. psychrophilum*, flavomodulin may be present because of a possible lateral gene transfer event from a Gram-positive bacterium, and it may be related to flavobacterial virulence as has been shown for suliyisin (Takamatsu *et al.*, 2002).

Immunogenic OMPs of *F. psychrophilum*

The OM fraction prepared by Sarkosyl insolubility was used for identification of immunoreactive proteins using a pool of antisera collected from CWD-convalescent trout. A total of 25 immunolabelled protein spots were observed from the immunoblot (Fig. 2b) and they matched the protein spots seen in the preparative 2-D gel (Fig. 2a). No immunoreactive

Table 1. Protein identification in the OM of *F. psychrophilum* JIP02/86

Assignment of single spots to the locus tag of the complete *F. psychrophilum* genome. Putative cleavage sites for signal peptides, subcellular location and possible transmembrane helices were predicted using SignalP, PSORTb or ExPASy proteomics tools, respectively.

Spot no	NCBI acc. no	Protein description	Peptide matching	Sequence coverage	pI/molecular mass (kDa)	GRAVY score	Putative cleavage site	Subcellular location	Transmembrane helix
1	FP1199	Probable OMP (Omp121 family)	136/22	25.8	8.94/115.1	-0.285	SpI 22-23	OM	0
2	FP0144	Probable TonB-dependent OM receptor	64/36	51.6	9.04/104.9	-0.431	SpI 18-19	OM	1
3	FP2096	Probable OMP	35/25	35.1	9.13/99.9	-0.437	SpI 21-22	OM	0
4	FP1500	Probable TonB-dependent OM receptor	27/22	37.1	9.21/92.2	-0.556	SpI 19-20	OM	0
5	FP2456	Probable TonB-dependent OM ferrichrome-iron receptor	13/13	21.1	8.98/86.5	-0.290	SpII 19-20	OM	0
–	FP0690	Putative OMP involved in nutrient binding	17/17	29.7	9.22/89.5	-0.368	SpI 21-22	OM	1
6	FP1922	Probable TonB-dependent OM ferrichrome-iron receptor FhuA	19/18	35.7	9.19/83.2	-0.300	SpI 20-21	OM	0
7	FP0521	Probable TonB-dependent OM receptor	22/17	40.4	9.18/66.3	-0.324	SpI 18-19	OM	0
8	FP0110	Probable lipoprotein of unknown function	105/21	46.2	6.35/60.7	-0.225	SpII 21-22	Unknown	0
9	FP1198	Probable lipoprotein of unknown function	105/15	37.8	8.18/58.3	-0.066	SpII 19-20	Unknown	0
10	FP1402	Protein of unknown function	36/16	42.4	9.03/56.4	-0.462	SpI 19-20	OM	0
11	FP1346	Probable lipoprotein of unknown function	166/23	57.9	5.49/54.5	-0.346	SpII 20-21	Unknown	1
12	FP2239	Probable lipoprotein of unknown function	93/10	27.6	5.61/50.4	-0.155	SpII 18-19	Unknown	1
13	FP0156	OMP P60 (OmpA family)	364/32	68.6	4.57/49.4	-0.324	SpI 21-22	OM	0
14	FP0073	Protein of unknown function	15/13	40.2	6.68/46.3	-0.248	SpI 22-23	Unknown	0
15	FP1486	Probable lipoprotein of unknown function	215/19	56.1	5.61/44.7	-0.168	SpII 18-19	Unknown	0
16	FP2369	Bleomycin hydrolase minopeptidase C	68/22	63.8	5.97/44.2	-0.454	SpI 21-22	Unknown	0
17	FP0175	Probable cell-surface protein (Lrr protein)	6/4	14.7	5.26/44.1	+0.321	SpI 16-17	Unknown	1
18	FP2433	Protein of unknown function, putative OMP	17/11	27.0	9.58/43.6	-0.067	SpI 19-20	OM	1
19	FP2425	Probable lipoprotein of unknown function	20/12	42.0	5.70/43.4	-0.480	SpII-17-18	Unknown	0
20	FP1478	Probable lipoprotein IrpA	137/15	49.2	4.88/42.4	-0.132	SpII 18-19	OM	0
21	FP0097	Flavomodulin	27/11	32.2	8.26/42.0	-0.185	SpII 18-19	OM	0
22	FP1456	Probable lipoprotein ('S-layer')	106/10	32.6	5.33/37.8	+0.074	SpII 19-20	Unknown	0
23*	FP0171	Probable cell surface protein (Lrr protein)	17/3	13.8	8.88/30.3	+0.239	SpI 18-19	Unknown	1
–	FP0172	Probable cell-surface protein (Lrr protein)	17/3	11.1	8.04/37.2	+0.305	SpI 18-19	Unknown	1
24	FP1439	Protein of unknown function (FKBP-type)	60/9	28.4	4.73/37.2	-0.506	SpII 19-20	Unknown	0
25	FP0169	Probable cell-surface protein (Lrr protein)	12/3	11.8	7.55/35.3	+0.284	SpI 16-17	Unknown	0
26	FP0261	Protein of unknown function	136/15	54.5	7.82/34.0	-0.323	SpI 19-20	OM	1
27	FP0578	Protein of unknown function	57/9	34.7	6.10/29.4	-0.010	SpII 20-21	Unknown	1
28	FP0451	Protein of unknown function	38/10	28.7	8.69/26.0	-0.593	SpII 18-19	Unknown	0
29	FP2138	Protein of unknown function	6/6	31.6	5.73/26.2	-0.333	SpI 16-17	Unknown	1
30	FP1507	Probable lipoprotein YiaD precursor	12/5	29.4	9.06/23.9	-0.217	SpII 21-22	OM	3
31	FP1493	Protein of unknown function	111/9	65.9	8.61/22.7	-0.015	SpII 19-20	Unknown	0
32	FP2019	Flavobacterial-specific protein antigen FspA	176/12	83.3	9.35/21.3	+0.017	SpI 19-20	Unknown	1
33	FP0139	Protein of unknown function	164/7	32.7	7.87/18.4	+0.027	SpI 18-19	OM	0
34	FP2098	OMP P18 (OmpH family)	35/7	48.8	8.58/18.7	-0.456	SpI 23-24	Unknown	1

*The peptides identified here did not allow us to differentiate the probable cell-surface proteins FP0171 and FP0172.

spot was observed when a pool of antisera obtained from healthy trout was used to probe the blot (data not shown).

The first group of proteins identified in the study included strong immunogens which have been previously well-characterized in *F. psychrophilum*: (i) FspA, (ii) the OmpA family, and (iii) OmpH/P18 (see above). As expected, these proteins comprise some of the major, abundant protein spots on the 2-D immunoblot. It should be stressed that the protein spot corresponding to OmpH/P18 was very poorly stained with Coomassie brilliant blue R-250 (see circle with dashed line, Fig. 2a), whereas it was visible clearly on the immunoblot (Fig. 2b). As a surface-adsorbed protein (Dumetz *et al.*, 2006), OmpH/P18 should be lost during the Sarkosyl treatment of cell envelopes during preparation of the OM fraction. In Gram-negative bacteria, OmpA is highly abundant in the cell wall and is involved in several functions, including the ability of bacteria to invade host cells (adhesin, invasins), to participate in biofilm formation and to act as both an immune target and an evasin. Many of these properties are related to four short protein loops that emanate from the protein to the outside of the cell (for a review see Smith *et al.*, 2007). Although the N-terminal amino acid sequence of *F. psychrophilum* OmpA shows no similarity with any other OMPs of Gram-negative bacteria outside of the family *Flavobacteriaceae*, we have recently shown that OmpA contains five possible thrombospondin type 3 repeat: OmpA/MotB domains, suggesting that it might be involved in the interaction/adherence of the pathogen with the host cells, as for other members of the OmpA family (Dumetz *et al.*, 2007). In addition, OmpA is one of the immunodominant antigens, and antibodies raised against it are capable of killing flavobacterial cells in the presence of complement. Preliminary data suggest also that vaccine cocktails containing OmpA protect rainbow trout against flavobacteriosis. The surface-localized protein FspA was first identified since it reacts strongly with serum from CWD-convalescent fish (Crump *et al.*, 2005). As for OmpA, FspA was shown to be unique and only homologous to unspecified sequences reported from bacteria belonging to the family *Flavobacteriaceae*. However, weak sequence homologies were found with a 17 kDa OMP from *Yersinia pseudotuberculosis* that mediates adhesion to mammalian cells and contributes to serum resistance (Yang *et al.*, 1996). Taking these data as a whole, proteins from this group appear to be the main immunogens, widely exposed at the cell surface of *F. psychrophilum* and, consequently, are the most promising subunit candidates for vaccine design. However, functional studies are needed to clarify their functions, particularly their interactions with host cells and their capabilities to stimulate the immune system.

The second group consisted mainly of a large set of probable proteins or lipoproteins of unknown function, for which there is no homologous equivalent in micro-organisms outside the *Cytophaga-Flavobacterium-Bacteroides* phylum. However, it contained the four probable cell-surface proteins with leucine-rich repeats

identified above that show similarities to BspA and LrrA proteins from *Tannerella forsythensis* and *Treponema denticola*, respectively. Like BspA and LrrA proteins, we have reported here that Lrr proteins in *F. psychrophilum* are immunogenic cell-surface determinants. They might play an important role in attachment to fish tissues, as do their homologues from periodontopathogens which bind strongly to extracellular matrix components (Sharma *et al.*, 1998; Ikegami *et al.*, 2004). The identification of such components belonging to the second group of immunoreactive proteins provides a set of novel antigens that could be unique to *F. psychrophilum* and a useful reservoir for the design of new vaccines. Biochemical characterization is required to establish the function of FP0139 (corresponding to spot 33; Fig. 2) which seems to be a good antigenic candidate. Indeed, the CWD-convalescent trout antisera contain a high titre of antibodies directed against this protein, as suggested by the intensity of the immunolabelled spot. The question concerning the protective activity of such antibodies remains open.

In conclusion, an objective of proteomic investigation concerning the study of bacterial pathogens is the location of proteins within or near the cell surface. Such proteins are known to have pivotal functions related to adhesion and invasion of host tissues, weakening of the host immune response and acquisition of host metabolites necessary for bacterial survival. Consequently, OMPs are good vaccine candidates, since they are usually abundant proteins and are in direct contact with the host immune system. In this study, we have mapped the OM proteome and identified 25 proteins that can elicit a humoral immune response by rainbow trout. Several of them have been identified previously and used as subunit vaccines for experimental challenge/vaccine assays (e.g. OmpH/P18; Dumetz *et al.*, 2006). More interestingly, this study draws up an inventory of new *F. psychrophilum* immunoreactive proteins that may be used either as single antigens or in combinations of several surface-exposed antigens. Further work is needed to evaluate the capability of these components to induce high titres of protective antibodies and their protection efficacy upon challenge with *F. psychrophilum* in fish.

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