Analysis of the \textit{Flavobacterium psychrophilum} outer-membrane subproteome and identification of new antigenic targets for vaccine by immunomics

Fabien Dumetz,\textsuperscript{1} Eric Duchaud,\textsuperscript{2} Stéphane Claverol,\textsuperscript{3} Nicolas Orieux,\textsuperscript{1} Sandrine Papillon,\textsuperscript{1} Delphine Lapaillerie\textsuperscript{3} and Michel Le Hénaff\textsuperscript{1}

Correspondence
Michel Le Hénaff
m-lehenaff@enitab.fr

\textsuperscript{1}Université Bordeaux 1, CNRS UMR 5805 EPOC, Place du Dr Peyneau, F-33120 Arcachon, France
\textsuperscript{2}Unité de Virologie et Immunologie Moléculaires, INRA, Domaine de Vilvert, F-78352 Jouy-en-Josas Cedex, France
\textsuperscript{3}Plateforme Génomique Fonctionnelle, Université Victor Segalen Bordeaux 2, 146 Rue Léo Saignat, F-33076 Bordeaux Cedex, France

Received 10 January 2008
Revised 18 February 2008
Accepted 6 March 2008

\textit{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 \textit{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 (\textit{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 \textit{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.

INTRODUCTION

\textit{Flavobacterium psychrophilum} is a yellow-pigmented, Gram-negative, gliding bacterium that predominantly affects salmonid fish (Borg, 1960), such as coho salmon (\textit{Oncorhynchus kisutch}) or rainbow trout (\textit{Oncorhynchus mykiss}), and occasionally other fish species, such as ayu (\textit{Plecoglossus altivelis}) (Iida & Mizokami, 1996). This bacterium is therefore responsible for considerable economic losses in fish aquaculture. Infections with \textit{F. psychrophilum} have several clinical manifestations, the most significant of which include mortality associated with haemorrhagic septicemia and spleen hypertrophy in juvenile fish, referred to as rainbow trout fry syndrome, and in adults, septicemia 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 \textit{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 \textit{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 \textit{F. psychrophilum} subunit vaccine, such as the surface-localized \textit{Flavobacterium}-specific protein (FspA; Crump et al., 2005). Indeed, a protective immune
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 under-representation 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 ionomic detergent sodium laurel 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 (OD600=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 10000 g for 10 min at 4 °C. Cell envelopes were recovered by an ultracentrifugation step (260000 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 (215000 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 (Rhomerberg 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 (Kashara & 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-myristoylaminopropyl)ammonium]propanesulfonate (ASB-14), 25 mM dithiothreitol, 0.01% bromophenol blue, 1%, v/v, carrier ampholytes 3-10 (Invitrogen)). After sonication, samples were centrifuged by centrifugation (5000 g, 5 min, 20 °C), loaded on 1-D 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 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 x 10⁶) 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 tryptic 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, ΔCn 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; Schäffer et al., 2001) and FASTA (Pearson, 1990) algorithms. Identification of peculiar domains in proteins was conducted using the HMMER 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 hydrophathy (GRAVY) value (ProtParam program; ExPASY proteomics tools, www.expasy.org/tools/protparam.html) as the sum of the hydrophytality 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,
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 $\leq 0.4$, 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. OspA 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 & Gripón, 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, FP0136). This might be due to post-translational modifications. Indeed, such modifications can modify the pl 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 pump 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...
(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 cytolsin 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 sulisysin 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 sulisysin (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

![2-D gel map of *F. psychrophilum* JIP02/86](http://mic.sgmjournals.org)
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.

<table>
<thead>
<tr>
<th>Spot no</th>
<th>NCBI acc. no</th>
<th>Protein description</th>
<th>Peptide matching</th>
<th>Sequence coverage</th>
<th>pI/molecular mass (kDa)</th>
<th>GRAVY score</th>
<th>Putative cleavage site</th>
<th>Subcellular location</th>
<th>Transmembrane helix</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FP1199</td>
<td>Probable OMP (Omp121 family)</td>
<td>136/22</td>
<td>25.8</td>
<td>8.94/115.1</td>
<td>−0.285</td>
<td>Spi 22–23</td>
<td>OM</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>FP0144</td>
<td>Probable TonB-dependent OM receptor</td>
<td>64/36</td>
<td>51.6</td>
<td>9.04/104.9</td>
<td>−0.431</td>
<td>Spi 18–19</td>
<td>OM</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>FP2096</td>
<td>Probable OMP</td>
<td>35/25</td>
<td>35.1</td>
<td>9.13/99.9</td>
<td>−0.437</td>
<td>Spi 21–22</td>
<td>OM</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>FP1500</td>
<td>Probable TonB-dependent OM receptor</td>
<td>27/22</td>
<td>37.1</td>
<td>9.21/92.2</td>
<td>−0.556</td>
<td>Spi 19–20</td>
<td>OM</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>FP2456</td>
<td>Probable TonB-dependent OM ferrichrome-iron receptor</td>
<td>13/13</td>
<td>21.1</td>
<td>8.98/86.5</td>
<td>−0.290</td>
<td>SpiI 19–20</td>
<td>OM</td>
<td>0</td>
</tr>
<tr>
<td>–</td>
<td>FP0690</td>
<td>Putative OMP involved in nutrient binding</td>
<td>17/19</td>
<td>27.9</td>
<td>9.22/89.5</td>
<td>−0.368</td>
<td>Spi 21–22</td>
<td>OM</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>FP1922</td>
<td>Probable TonB-dependent OM ferrichrome-iron receptor FhuA</td>
<td>19/18</td>
<td>35.7</td>
<td>9.19/83.2</td>
<td>−0.300</td>
<td>Spi 20–21</td>
<td>OM</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>FP0521</td>
<td>Probable TonB-dependent OM receptor</td>
<td>22/17</td>
<td>40.4</td>
<td>9.18/66.3</td>
<td>−0.324</td>
<td>Spi 18–19</td>
<td>OM</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>FP0110</td>
<td>Probable lipoprotein of unknown function</td>
<td>105/21</td>
<td>46.2</td>
<td>6.35/60.7</td>
<td>−0.225</td>
<td>SpiI 21–22</td>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>FP1198</td>
<td>Probable lipoprotein of unknown function</td>
<td>105/15</td>
<td>37.8</td>
<td>8.18/58.3</td>
<td>−0.066</td>
<td>SpiI 19–20</td>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>FP1402</td>
<td>Protein of unknown function</td>
<td>36/16</td>
<td>42.4</td>
<td>9.03/56.4</td>
<td>−0.462</td>
<td>Spi 19–20</td>
<td>OM</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>FP1346</td>
<td>Probable lipoprotein of unknown function</td>
<td>166/23</td>
<td>57.9</td>
<td>5.49/54.5</td>
<td>−0.346</td>
<td>SpiI 20–21</td>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>FP2239</td>
<td>Probable lipoprotein of unknown function</td>
<td>93/10</td>
<td>27.6</td>
<td>5.61/50.4</td>
<td>−0.155</td>
<td>SpiI 18–19</td>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>FP0156</td>
<td>OMP P60 (OmpA family)</td>
<td>364/32</td>
<td>68.6</td>
<td>4.57/44.2</td>
<td>−0.324</td>
<td>Spi 21–22</td>
<td>OM</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>FP0073</td>
<td>Protein of unknown function</td>
<td>15/13</td>
<td>40.2</td>
<td>6.68/46.3</td>
<td>−0.248</td>
<td>Spi 22–23</td>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>FP1486</td>
<td>Probable lipoprotein of unknown function</td>
<td>215/19</td>
<td>56.1</td>
<td>5.61/44.7</td>
<td>−0.168</td>
<td>SpiI 18–19</td>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>FP2369</td>
<td>Bleomycin hydrolase minopeptidase</td>
<td>68/22</td>
<td>63.8</td>
<td>5.97/44.2</td>
<td>−0.454</td>
<td>Spi 21–22</td>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>FP0175</td>
<td>Probable cell-surface protein (Lrr protein)</td>
<td>6/4</td>
<td>14.7</td>
<td>5.26/44.1</td>
<td>+0.321</td>
<td>Spi 16–17</td>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>18</td>
<td>FP2433</td>
<td>Protein of unknown function, putative OMP</td>
<td>17/11</td>
<td>27.0</td>
<td>9.58/43.6</td>
<td>−0.067</td>
<td>Spi 19–20</td>
<td>OM</td>
<td>1</td>
</tr>
<tr>
<td>19</td>
<td>FP2425</td>
<td>Probable lipoprotein of unknown function</td>
<td>20/12</td>
<td>42.0</td>
<td>5.70/43.4</td>
<td>−0.480</td>
<td>SpiI–17–18</td>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>FP1478</td>
<td>Probable lipoprotein IrpA</td>
<td>137/15</td>
<td>49.2</td>
<td>4.88/42.4</td>
<td>−0.132</td>
<td>SpiI 18–19</td>
<td>OM</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>FP0097</td>
<td>Flavomodulin</td>
<td>27/11</td>
<td>32.2</td>
<td>8.26/42.0</td>
<td>−0.185</td>
<td>SpiI 18–19</td>
<td>OM</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>FP1456</td>
<td>Probable lipoprotein (‘S-layer’)</td>
<td>106/10</td>
<td>32.6</td>
<td>5.33/37.8</td>
<td>+0.074</td>
<td>Spi 19–20</td>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>23*</td>
<td>FP0071</td>
<td>Probable cell surface protein (Lrr protein)</td>
<td>17/3</td>
<td>13.8</td>
<td>8.88/30.3</td>
<td>+0.239</td>
<td>Spi 18–19</td>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>–</td>
<td>FP0072</td>
<td>Probable cell-surface protein (Lrr protein)</td>
<td>17/3</td>
<td>11.1</td>
<td>8.04/37.2</td>
<td>+0.305</td>
<td>Spi 18–19</td>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>24</td>
<td>FP1439</td>
<td>Protein of unknown function (FKBP-type)</td>
<td>60/9</td>
<td>28.4</td>
<td>4.73/37.2</td>
<td>−0.506</td>
<td>SpiI 19–20</td>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>FP0169</td>
<td>Probable cell-surface protein (Lrr protein)</td>
<td>12/3</td>
<td>11.8</td>
<td>7.55/35.3</td>
<td>+0.284</td>
<td>Spi 16–17</td>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>26</td>
<td>FP0261</td>
<td>Protein of unknown function</td>
<td>136/15</td>
<td>54.5</td>
<td>7.82/34.0</td>
<td>−0.323</td>
<td>Spi 19–20</td>
<td>OM</td>
<td>1</td>
</tr>
<tr>
<td>27</td>
<td>FP0578</td>
<td>Protein of unknown function</td>
<td>57/9</td>
<td>34.7</td>
<td>6.10/29.4</td>
<td>−0.010</td>
<td>SpiI 20–21</td>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>28</td>
<td>FP0451</td>
<td>Protein of unknown function</td>
<td>38/10</td>
<td>28.7</td>
<td>8.69/26.0</td>
<td>−0.593</td>
<td>SpiI 18–19</td>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>29</td>
<td>FP2138</td>
<td>Protein of unknown function</td>
<td>6/6</td>
<td>31.6</td>
<td>5.73/26.2</td>
<td>−0.333</td>
<td>Spi 16–17</td>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>30</td>
<td>FP1507</td>
<td>Probable lipoprotein TalD precursor</td>
<td>12/5</td>
<td>29.4</td>
<td>9.06/23.9</td>
<td>−0.217</td>
<td>Spi 21–22</td>
<td>OM</td>
<td>3</td>
</tr>
<tr>
<td>31</td>
<td>FP1493</td>
<td>Protein of unknown function</td>
<td>111/9</td>
<td>65.9</td>
<td>8.61/22.7</td>
<td>−0.015</td>
<td>SpiI 19–20</td>
<td>Unknown</td>
<td>0</td>
</tr>
<tr>
<td>32</td>
<td>FP2019</td>
<td>Flavobacterial-specific protein antigen FspA</td>
<td>176/12</td>
<td>83.3</td>
<td>9.35/21.3</td>
<td>+0.017</td>
<td>Spi 19–20</td>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>33</td>
<td>FP0139</td>
<td>Protein of unknown function</td>
<td>164/7</td>
<td>32.7</td>
<td>7.87/18.4</td>
<td>+0.027</td>
<td>Spi 18–19</td>
<td>OM</td>
<td>0</td>
</tr>
<tr>
<td>34</td>
<td>FP2098</td>
<td>OMP P18 (OmpH family)</td>
<td>35/7</td>
<td>48.8</td>
<td>8.58/18.7</td>
<td>−0.456</td>
<td>Spi 23–24</td>
<td>Unknown</td>
<td>1</td>
</tr>
</tbody>
</table>

*The peptides identified here did not allow us to differentiate the probable cell-surface proteins FP0171 and FP0172.*
The first group of proteins identified in the study included strong immunogens which have been previously well-characterized in \textit{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 \textit{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, invasin), to participate in biofilm formation and to act as both an immune target and an evasion. 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 \textit{et al.}, 2007). Although the N-terminal amino acid sequence of \textit{F. psychrophilum} OmpA shows no similarity with any other OMPs of Gram-negative bacteria outside of the family \textit{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 \textit{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 \textit{CWD}-convalescent fish (Crump \textit{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 \textit{Flavobacteriaceae}. However, weak sequence homologies were found with a 17 kDa OMP from \textit{Yersinia pseudotuberculosis} that mediates adhesion to mammalian cells and contributes to serum resistance (Yang \textit{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 \textit{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 microorganisms outside the \textit{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 \textit{Tannerella forsythensis} and \textit{Treponema denticola}, respectively. Like BspA and LrrA proteins, we have reported here that Lrr proteins in \textit{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 \textit{et al.}, 1998; Ikegami \textit{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 \textit{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 \textit{et al.}, 2006). More interestingly, this study draws up an inventory of new \textit{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 \textit{F. psychrophilum} in fish.

**ACKNOWLEDGEMENTS**

We would like to thank Anne-Marie Richard from Enitab for excellent technical assistance, Daniel Jacob from INRA, Centre de BioInformatique de Bordeaux, for his help in the analysis of the \textit{F. psychrophilum} complete proteome database, and Scott E. LaPatra, Clear Springs Foods, Inc., Buhl, Idaho, USA, for the generous gift of antiserum collected from healthy trout. This work was partly sponsored by grants from the Région Aquitaine, from the Ecole Doctorale Université Victor Segalen Bordeaux 2/Ministère de la Recherche and from the Enita-Bordeaux.

**REFERENCES**

psychrophilum: approche protéomique et caractérisation de deux protéines (OmpA/P60 et OmpH/P18) immunoprotectrices


