A Protective Immune Response Is Generated in Rainbow Trout by an OmpH-Like Surface Antigen (P18) of Flavobacterium psychrophilum

Fabien Dumetz,1 Eric Duchaud,2 Scott E. LaPatra,3 Claire Le Marrec,4 Stéphane Claverol,5 Maria-C. Urdaci,1 and Michel Le Hénañ1*

Laboratoire de Microbiologie et de Biochimie Appliquées, Ecole Nationale d’Ingénieurs des Travaux Agricoles de Bordeaux, 1 Cours du Général de Gaulle, CS40201, F-33175 Gradignan, France;1 Unité de Virologie et Immunologie Moléculaires, INRA, Domaine de Vilvert, F-78352 Jouy-en-Josas, France;2 Clear Springs Foods, Inc., Research Division, Buhl, Idaho;3 Laboratoire de Biotechnologie et Microbiologie Appliquée, Université Victor Segalen Bordeaux 2 UMR INRA, Faculté d’Oenologie, 351 Cours de la Libération, F-33405 Talence, France; and Plateforme Génomique Fonctionnelle, Université Victor Segalen Bordeaux 2, 146 Rue Léo Saignat, F-33076 Bordeaux, France5

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Investigations of the surface characteristics of Flavobacterium psychrophilum, an important pathogen of fish, assisted us in identifying a surface protein termed P18. In the current study, we developed a simple and efficient procedure for the purification of this protein by a two-step method. First, P18 was selectively released from flavobacteria by a heat-HEPES treatment of the cells and then subjected to anion-exchange high-performance liquid chromatography. De novo sequencing was used to generate a fragmented peptide spectrum from purified P18. Comparison of two obtained peptide sequences with a partial genome sequence of F. psychrophilum (INRA, Jouy-en-Josas, France) identified one gene encoding a 166-amino-acid OmpH-like protein that mostly likely undergoes N-terminal cleavage of the 23-residue signal peptide. The susceptibility of the OmpH-like protein to proteinase K treatment and the bacteriostatic/bactericidal activities of anti-OmpH-like protein antibodies indicated that this protein is actually exposed on the surface of F. psychrophilum. Vaccination trials showed that the OmpH-like protein can induce a high titer of anti-OmpH-like protein antibodies which are protective. Taken together, these results suggest that this surface protein produced by F. psychrophilum could be used in future vaccine development as a promising candidate antigen.

Flavobacterium psychrophilum (syn. Cytophaga psychrophila or Flexibacter psychrophilus) is a gram-negative, motile, nonspore-forming bacterium (7). This eubacterium belongs to the family Flavobacteriaceae in the Cytophaga-Flavobacterium-Bacteroides (CFB) phylum (5, 6). F. psychrophilum is the etiologic agent of “rainbow trout fry syndrome” and “bacterial cold water disease” (CWD), the two most significant systemic infections of primarily freshwater-reared salmonid fish. These diseases cause serious problems in public and private aquaculture (for a review, see reference 35). F. psychrophilum probably affects all species of salmonid fish, but coho salmon (Oncorhynchus kisutch), rainbow trout (Oncorhynchus mykiss), and ayu (Plecoglossus altivelis) (17) appear to be particularly susceptible (23). In addition, F. psychrophilum has been isolated from diseased nonsalmonid fish species (17, 26).

The molecular pathogenesis of F. psychrophilum is not well understood. However, the virulence of this flavobacterium might be related to the production of exoenzymes and/or endotoxins causing direct tissue damage in fish or enhanced invasiveness (13, 29, 43, 44). Little knowledge about the surface immunogenic factors of F. psychrophilum exists. What is known are the structures of the lipopolysaccharide O antigen (28) and some surface components, such as sialic acid-binding lectin (33) or surface blebs (34), which have been shown (or suspected) to interact with the immune system of the host. No commercial vaccine is yet available, although several studies dealing with the effects of vaccination have been done. Vaccines based on whole formalin-killed cells (19), a Sarkosyl-insoluble membrane fraction (38), or distinct molecular mass fractions (25) of F. psychrophilum were shown to confer significant protection against disease. To date, the prevention of disease outbreaks and therapeutic treatments rely on the use of approved antibiotics. However, such an approach is limited by the cost of the treatments and the potential for resistant mutants (9, 18, 42).

The identification of the major immunogenic components of an infectious agent is essential for understanding the molecular mechanism of virulence and the route of the infection, for proposing serological diagnosis of the disease, and for developing strategies for efficient immune protection. Bacterial surface components and, more particularly, the outer membrane proteins are generally very immunogenic and play a key function in virulence and the immune responses to bacterial diseases. In this context, investigations of the surface architecture of F. psychrophilum led to the identification of several dominant membrane antigens (11, 12, 31, 38) which might be used as antigenic subunits for vaccines. Because of the important role that membrane proteins play in attachment of many pathogenic bacteria to their hosts, we hypothesized that flavobacterial outer membrane proteins are likely candidates for mediating the specific attachment of these pathogens to their fish tissues. Previously, we identified two proteins produced by F. psychrophilum, termed P60 (31) and P18 (30) according to their relative molecular masses. In this study, we report the purification and characterization of the immunogenic and pro-

* Corresponding author. Mailing address: ENITAB, LMBA, 1 Cours du Général de Gaulle, CS40201, F-33175 Gradignan Cedex, France. Phone: 33 (0)557 350 738. Fax: 33 (0)557 350 739. E-mail: m-lehenaff @enitab.fr.
tective 18-kDa outer membrane-associated OmpH-like protein. This surface protein seems to be a promising candidate for the development of a future vaccine.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and isolation of surface proteins. The different F. psychrophilum strains used in this study and their origins are listed in Table 1. Cells were cultured in a modified Anacker and Orladi's (AOAE) liquid medium, 0.5% (wt/vol) tryptone, 0.05% (wt/vol) yeast extract, 0.02% (wt/vol) beef extract, and 0.02% (wt/vol) sodium acetate (pH 7.2) (2). Bacteria were incubated at 18°C under aerobic conditions (orbital shaking, 150 rpm). When the bacteria were harvested (optical density at 600 nm, 0.8), purity was checked by examination of Gram-stained smears, and the cells were pelleted for 10 min at 6,000 × g at 4°C and washed twice with phosphate-buffered saline (PBS; 50 mM sodium phosphate buffer and 150 mM NaCl, pH 7.4). Isolation of surface proteins from F. psychrophilum was accomplished as previously described (30). Protein content was estimated in the presence of sodium dodecyl sulfate (SDS), using bovine serum albumin as the standard (27).

Chromatographic techniques. Anion-exchange high-performance liquid chromatography (HPLC) was performed in an Amersham Biosciences HiTrap DEAE column (diameter, 0.7 cm; length, 2.5 cm), using a Pharmacia Biocytom GradientFrac system. The sample was 1 ml of HEPS extract. The flow rate was set at 0.5 ml/min, and the eluate was collected in 0.5-ml fractions. Buffer A was used for column equilibration and for the elution of proteins (10 mM HEPS-NaOH buffer [pH 8.0]-10 mM NaCl), and buffer B was buffer A with 1 M NaCl. Elution conditions were the following: (i) 0 to 10 min with 100% buffer A and (ii) 10 to 35 min with a linear gradient to 50% buffer B. The elution profile was spectro-photometrically monitored at 280 nm, and selected eluates were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and immunoblotting. SDS-PAGE analyses were performed as described by Laemmli (22). Protein samples were solubilized in a reduced SDS-PAGE sample buffer and separated in SDS-polyacrylamide gels (10 by 8 by 0.075 cm; 12.5% acrylamide and 0.26% bisacrylamide; 200 V, 2 h). Protein bands were stained with silver (45) or Coomassie brilliant blue R250 or were immunolabeled. Following electrophoresis, the proteins were electroblotted onto a nitrocellulose filter (Bio-Rad) by use of a Bio-Rad TransBlot electrophoretic transfer cell as specified by the manufacturer. The blots were blocked with 5% bovine serum albumin in PBS for 1 h at room temperature and then incubated for 1 h with rabbit antisera directed against P18 purified from F. psychrophilum G4 (dilution, 1:1,000) in 0.05% Tween 20 in PBS. The washing steps were performed three times with PBS. Immunolabeled proteins were visualized using alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (IgG) antibodies (dilution, 1:2,000; DakoCytomation, Glostrup, Denmark), followed by a color reaction with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride. Primary antibodies were elicited in two rabbits (female New Zealand White rabbits) by subcutaneous inoculation of HPLC-purified P18 twice a month over a 3-month period (inoculum, 50 μg of P18 in 0.5 ml of PBS; emulsified with 0.7 ml of Freund's incomplete adjuvant [or complete adjuvant for the two first inoculations]). The two sera were pooled at the end of the immunization schedule. When trout antisera from (i) CWD-convalescent trout taken from an in-fective French farm or (ii) experimentally immunized trout (see below) were used as primary antibodies, an additional incubation was done with rabbit anti-trout immunoglobulins (dilution, 1:2,000).

De novo P18 sequencing. Purified P18 was electrophoresed and stained with Coomassie brilliant blue R250, and the spots were excised from a gel. After a washing step with H2O-methanol-acetic acid (47:5:47.5) and acetonitrile, the gel pieces were dried in a vacuum centrifuge and rehydrated in 8 ng/μl trypsin (Sigma-Aldrich, St. Louis, MO) in 50 mM NH4HCO3. After incubation and washing steps, peptide mixtures were analyzed by online capillary chromatography (C18 PepMap column [75-μm inner diameter by 15 cm]; LC Packings, Amsterdam, The Netherlands) coupled to nanospray LCO ion-trap mass spectrometry (MS; ThermoFinnigan, San Jose, CA). The mass spectrometer was operated in positive-ion mode at a 2-kV needle voltage and a 46-V capillary voltage. Data acquisition was performed in a data-dependent mode alternating full-scan MS over the range m/z 50 to 2,000 and a MS/MS scan of the most intense ion in the preceding MS spectrum. MS/MS data were acquired using a 2-μm-unit ion isolation window and a 35% relative collision energy. Every MS/MS spectrum was submitted to the DeNovoX sequencing program (ThermoFinnigan, San Jose, CA). Sequences with an absolute probability and relative probability higher than 20% and 75%, respectively, and consisting of more than four amino acids were selected for further analysis. Corresponding spectra were manually checked.

In situ protease treatment. Intact mid-log-phase F. psychrophilum cells were washed once with PBS and dispersed in PBS with 10 mM MgCl2 to a final concentration of 2 × 108 bacteria/ml. Examination of bacterial suspensions by phase-contrast light microscopy did not indicate detectable lysis of the bacteria. Cells were then incubated with 40 μg/ml soluble protease K for 30 min or 1 h at 25°C, after which digestion was terminated by the addition of 5 mM phenylmethylsulfonyl fluoride followed by sample boiling. As a negative control, the above procedure was repeated except that cells were incubated in buffer without protease K. Cells were disrupted by sonication (24 W), and about 50 μg of proteins was prepared from each sample. Bacterial lysates were subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose membranes. The susceptibilities of individual proteins to protease K digestion were assessed by immunoblotting with the appropriate rabbit polyclonal antibodies. To exclude the possibility of outer membrane damage, the lysates were also immunoblotted with antibodies directed against GldJ, a lipoprotein involved in the gliding mobility of Flavobacteium johnsoniae which is not exposed at the cell surface and is thus protected against protease digestion in intact bacteria (8). To assess the protease susceptibilities of the proteins when not in situ, an additional control was added. Cells were treated with protease K as described above in the presence of Triton X-100 to a final concentration of 0.05%, which disrupts F. psychrophilum membranes.

Vaccination, challenge trials, and trout antibody quantification. Immunization trials were conducted on rainbow trout groups (20 fish per group; mean weight, 2.8 g). Fish were anesthetized with 100 μg/ml tricaine methane sulfonate (MS-222; Argent, Redmond, WA) and immunized intraperitoneally with 50 μl of P18-enriched fraction (P18-EF), with or without Freund's complete adjuvant (FCA) (approximately 7 μg and 14 μg of protein, respectively). The remaining treatments included fish injected with 50 μl of buffer (PBS) with and without FCA. At 9 and 14 weeks postimmunization, rainbow trout were challenged with...
live *F. psychrophilum* 259-93 cells as described previously (24). A single 20-fish group for each treatment was mock infected and served as a negative control group. The fish were monitored for 28 days after challenge for mortality. A minimum of 20% of the fish that died each day were cultured on tryptic yeast extract agar in an attempt to reisolate *F. psychrophilum* and confirm the cause of death. The cumulative percent mortality (CPM) was determined after 28 days, and the relative percent survival (RPS) was calculated using the following equation: RPS = [1 – (CPM of immunized trout)/(CPM of PBS-injected trout)] x 100.

**Sera from the mock-infected control rainbow trout were collected at the end of the challenge, and the *F. psychrophilum*-specific antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (23). An analysis of differences in serum ELISA antibody titers between treatment groups was performed by one-way ANOVA on log_{10}-transformed titer data, and pairwise comparisons were made using Dunnett’s multiple comparison test. The mean cumulative percent mortality following bacterial challenge was analyzed by ANOVA using Tukey’s test. Differences were considered significant at P values of <0.05.**

**DNA manipulation and nucleic acid sequencing.** Standard procedures were used to isolate genomic DNA and to clone and analyze DNA fragments (39). Restriction enzymes, T4 DNA ligase, extensor Hi-Fidelity PCR enzyme, and deoxynucleotides were obtained from Promega or ABgene and used according to the instructions of the suppliers. Nucleic acid sequencing was performed by a standard protocol (Genome Express, Meylan, France). All reported DNA sequence data were confirmed by sequencing both DNA strands from at least two independent cloned PCR products. Comparisons to database sequences were made by using the BLAST (1) and FASTA (37) algorithms. Multiple sequence alignment was performed by the ClustalW program. A prediction of membrane-spanning regions and their orientations was performed using the TMpred program (16). The method of Kyte and Doolittle was used to analyze hydrophobicity (21). Signal peptide prediction was done with SignalP 3.0 software (4, 36). Protein homology searches were carried out with the SWISS-PROT database using the BLAST and EMBL BLAST and EMBL FASTA servers.

**Nucleotide sequence accession numbers.** The sequences reported in this paper have been deposited in the EMBL database under accession no. AM161038, AM161039, AM161040, AM161041, and AM161042.

**RESULTS**

**Purification of P18.** P18 was first selectively released from a cell suspension by treatment with 10 mM HEPES-NaOH buffer (pH 7.5) for 10 min at 65°C. The supernatant collected after centrifugation contained mainly P18 (>73%), which was weakly contaminated by several low-molecular-weight components and P22 as the major contaminants (Fig. 1, lane 2). Silver-stained SDS-PAGE did not reveal any lipopolysaccharide in this preparation. Thereafter, the P18-enriched fraction was subjected to anion-exchange HPLC. P18 was eluted from the column with 0.25 mM NaCl, as revealed by SDS-PAGE analysis (Fig. 1, lane 3). The protein was recovered with a yield of about 80%. The whole purification procedure is summarized in Table 2.

**In situ protease sensitivity of P18.** Protein surface exposure was studied by incubation of intact *F. psychrophilum* G4 with protease K on the premise that surface-exposed proteins would be degraded while subsurface proteins would be protected against proteolysis. First, we verified that protease K did not penetrate or destroy *F. psychrophilum* cells, as the A_{280}-to-A_{260} ratio of the supernatant obtained was not different before or after the protease treatment. Western blot analysis of protease K-treated and untreated whole cells (Fig. 2) revealed that P18 disappeared completely after protease K treatment, indicating that it was located on the outside surfaces of the cells. Analysis of the signal intensities revealed that >80% of the P18 signal disappeared after 30 min of such treatment. The bacterial outer membrane remained intact during the in situ proteolysis treatments since there was no detectable degradation of a 65-kDa component recognized with antibodies directed against *F. johnsoniae* GldJ. As a periplasmic exposed outer membrane lipoprotein, GldJ could only be digested with protease K when the cells were solubilized with Triton X-100 prior to protease addition.

**FIG. 2.** Proteinase K sensitivities of P18 and GldJ in 2 × 10^9 intact *F. psychrophilum* G4 cells in the absence (−) or presence (+) of 0.05% Triton X-100. After incubation with protease K for 0, 30, or 60 min at 25°C, cells were lysed in 1% SDS. Protein samples were then analyzed by SDS-PAGE, and immunoblots were developed with anti-P18 or anti-*F. johnsoniae* GldJ rabbit serum.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>30</th>
<th>60</th>
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<tbody>
<tr>
<td>Triton X-100</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Anti-P18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-GldJ</td>
<td></td>
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</table>

**TABLE 2. Purification of P18 from intact cells of F. psychrophilum G4**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Amt of P18 (mg)</th>
<th>Purification yield (%)</th>
<th>Purification factor (fold)</th>
</tr>
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<tr>
<td>Cellular extract</td>
<td>522.08</td>
<td>10.96</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>P18-enriched fraction</td>
<td>13.05</td>
<td>9.51</td>
<td>86.8</td>
<td>34.7</td>
</tr>
<tr>
<td>Anion-exchange HPLC eluate</td>
<td>8.74</td>
<td>8.70</td>
<td>79.4</td>
<td>47.4</td>
</tr>
</tbody>
</table>
The specificity of rabbit anti-P18 serum was checked by Western blotting of the whole membrane protein extract of F. psychrophilum G4. The results clearly indicated that the antibodies prepared against P18 were specific because a single band was detected (Fig. 3, lane 2). The immunologic reactions of whole-cell extracts of the F. psychrophilum strains listed in Table 1 were analyzed with anti-serum against the purified P18 protein of F. psychrophilum G4. An immune reaction was observed with proteins of similar sizes (i.e., apparent molecular mass of 18 kDa) and in the same range (Fig. 4). This supports the high conservation of P18 among F. psychrophilum strains. Additionally, a serum collected from a naturally infected trout (i.e., a CWD-convalescent fish) was also used to identify major immunogens. P18 was one of the major antigens recognized by the trout antibodies (Fig. 3, lanes 3 and 4). The reactivity of P18 was also assayed with sera derived from rainbow trout immunized with P18-EF. As expected, immunoblots exhibited a strong reaction of fish anti-P18 antibodies with the protein (Fig. 5). However, sera from fish immunized without FCA supplementation also showed weak immune reactions, with two minor components with apparent molecular masses of 22 and 19 kDa (Fig. 5, lanes 1 to 4 [four representative samples]). When FCA was added to the antigenic preparation, fish sera contained higher antibody titers (Table 3), which were mainly directed against P18 (dilution of 1:2,000 versus 1:200 for sera from fish immunized with P18-EF alone). Additionally, sera from this group (Fig. 5, lanes 5 to 8 [four representative samples]) showed weak reactions, with three different contaminants with apparent molecular masses of 37, 33, and 15 kDa. Antibodies did not react with an aliquot of the whole flavobacterial cells which had been incubated with proteinase K prior to loading in the SDS-PAGE gel (Fig. 5, lane 9), while they strongly reacted with HPLC-purified P18 (Fig. 5, lane 11). Sera from fish which had been inoculated with PBS supplemented or not with FCA were used as negative controls (one representative sample is shown in Fig. 5, lane 10). They did not contain any antibodies capable of recognition of F. psychrophilum antigens.
Bacteriostatic and bactericidal activities of anti-P18 antibodies. As a component located on the surface of F. psychrophilum G4, P18 should lead to the production of specific antibodies which could be deposited on the bacterial cell surface and trigger a set of biological phenomena related to this deposition (i.e., activation of complement, bacteriostasis, and bacteriolysis). Growth inhibition tests were performed with anti-P18 antibodies to confirm the position of P18 with respect to the lipid bilayer of the flavobacterial outer membrane. The anti-P18 serum exhibited a threshold inhibiting dilution of 1:200, while the preimmune serum did not inhibit the growth of F. psychrophilum G4. This observation confirmed that P18 is exposed at the surface of the flavobacterial cell. To assess the bacteriostatic and bactericidal activities of anti-P18 antibodies, F. psychrophilum cells were thereafter grown in the presence of heat-treated anti-P18 serum (dilution, 1:200) supplemented or not with fetal bovine serum containing complement. Preliminary experiments showed that the concentration of fetal calf serum that was used was not bactericidal for F. psychrophilum in the absence of flavobacterium-specific antibodies (Fig. 6). In the absence of complement, no significant F. psychrophilum growth was observed at 4 days when a 1:200 dilution of anti-P18 antibodies was added compared to that of cells treated with PBS, fetal bovine serum, or a similar dilution of preimmune serum. The growth inhibition that was observed was bacteriostatic, since the number and viability of bacterial cells were almost constant over this time period (Fig. 6). In the presence of fetal calf serum (which contained bovine complement), anti-P18 antibodies were bacteriostatic on the first day, since the cell number did not increase and the cell viability was constant. On the other hand, a drastic decrease in cell viability was observed between the second and fourth day compared to that in control samples containing only anti-P18 antibodies and no complement. Viable flavobacteria were estimated to comprise $<16\%$ of initially inoculated cells. Moreover, bacterial agglutination was seen in the presence of anti-P18 but not with fetal calf serum (data not shown).

Protection studies. Duplicate groups of 20 rainbow trout were used in the study to evaluate protection, including groups receiving the P18-enriched fraction supplemented (P18-EF + FCA) or not (P18-EF) with FCA. Additionally, PBS- and PBS + FCA-injected groups of fish were included in the evaluation. No significant protection was observed at 9 weeks postvaccination.

The results of protection studies performed at 14 weeks postvaccination are summarized in Table 3. With FCA, P18-EF gave an RPS value of 88.5, which was significantly ($P < 0.05$) larger than that of the control group (PBS), while P18-EF alone elicited a decrease in mortality which was not statistically significant ($P > 0.05$). F. psychrophilum was reisolated from 74% of the fish that died in the study and were checked for the presence of the bacterium by culture. The highest F. psychrophilum-specific antibody titers were observed in sera from P18-EF + FCA-vaccinated fish, followed by sera from PBS + FCA-injected trout. Fish injected with P18-EF only generated a weaker antibody response than P18-EF + FCA-injected fish (about 10-fold less) but a significantly different response (3-fold rise) than PBS-injected controls. Together, these data indicate that P18 is capable of inducing antibody synthesis by fish and that such antibodies are protective.

P18 sequencing and identification of the ompH-like gene sequence. For further characterization of P18, the protein was subjected to de novo sequencing. Several peptides were identified, among which the following two sequences were found: (i) TEYGMTVTYE and (ii) ATTATEAI. These peptides were searched against an ongoing genome sequence of F. psychrophilum strain JIP02.86 (INRA, Jouy-en-Josas, France). Both peptides matched (100% identity) an OmpH-like protein encoded by one gene belonging to a cluster of six genes, including two genes for additional probable outer membrane proteins (Fig. 7A). Protein identity was ascertained by submitting MS/MS data obtained with P18 to a search against a database harboring the OmpH-like protein sequence. Two

<table>
<thead>
<tr>
<th>Antigen</th>
<th>ELISA titer (mean ± SEM)$^{a,c}$</th>
<th>% Protection efficacy</th>
<th>CPM (mean ± SEM)$^{a}$</th>
<th>RPS$^{d}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>360$^a$ ± 83.3</td>
<td>21.8$^a$ ± 2.6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>PBS + FCA</td>
<td>2,060$^b$ ± 578.2</td>
<td>28.4$^a$ ± 3.2</td>
<td>−30.5</td>
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</tr>
<tr>
<td>P18-EF alone</td>
<td>1,060$^b$ ± 279.8</td>
<td>10.0$^a$ ± 1.9</td>
<td>54.1</td>
<td></td>
</tr>
<tr>
<td>P18-EF + FCA</td>
<td>9,600$^a$ ± 1,141.6</td>
<td>2.5$^a$ ± 1.0</td>
<td>88.5</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Rain trout (mean weight, 16 g) were challenged at 14 weeks postvaccination with 6.25 × 10$^7$ CFU/fish. $^b$ F. psychrophilum-specific antibody titers were measured after vaccination in mock-infected rainbow trout sera. $^c$ Mean CPM and titer values of different superscripts indicate significant differences, with $P$ values of <0.05. $^d$ RPS ($n = 2$ per treatment) was determined relative to PBS treatment.
highly conserved genes within the CFB phylum (uppS [di-
trans.poly-cis-decaprenyltransferase] and murI [glutamate
racemase]) belonging to the locus/operon were used to
design primers for amplification of the whole locus. They
were (i) uppS_fw (CATTATTATGGACGGGAATGG) and (ii)
murI_rev (GCCGCCTATTCCTGAATCGAA). Using PCR,
the ompH gene and surrounding regions were amplified (5 kb)
and sequenced. Analysis of the deduced amino acid sequence
of the F. psychrophilum G4 OmpH-like protein revealed that it
is a 166-amino-acid protein (Fig. 7B) that is almost totally
conserved (except for a single Ile25/Thr25 substitution) among
the F. psychrophilum strains used in this study (Table 1). Se-
quences producing significant alignments were (i) the cationic
outer membrane OmpH-like protein from Porphyromonas gin-
givalis and (ii) the putative outer membrane proteins from
Bacteroides fragilis, Bacteroides thetaiotaomicron, and Cyto-
phaga hutchinsonii, all of which belong to the CFB phylum
(Fig. 7B). However, the best hit found was with P. gingivalis
OmpH (163 amino acids long) and was only 32%. A search of
the NCBI conserved domain database revealed significant
alignments with almost the whole sequence of the pfam03938
domain of OmpH, an outer membrane protein (OmpH-like)
(E value = 3 × 10^{-39}), and with the COG2825 domain of the
histone-like protein (HipA), another outer membrane protein
(E value = 1 × 10^{-17}), confirming the similarity to the
OmpH analogs. The program SignalP does in fact predict
that the OmpH-like protein is an outer membrane protein
with an N-terminal signal peptide that may be cleaved be-
tween Ala23 and Gln 24 (signal peptide probability, 0.999).
The leader sequence shows a typical transmembrane helix
structure with a significant outside-to-inside helix formation
score of 1,422 on the transmembrane prediction scale, sug-
ggesting strongly that OmpH is a surface-located protein.
The mature protein (143 amino acids) gives rise to a pre-
dicted 16,248.6-Da protein, which is in very close agreement
with the observed mass (18 kDa).

DISCUSSION
This study was undertaken to characterize P18 and to assess
the potential relevance of such a protein in eliciting a protec-
tive immune response in fish. First, we developed a purification
method allowing quantitative recovery of the native protein by
applying the following simple two-step purification protocol:
heat-HEPES extraction of P18 from the cells followed by an-
ion-exchange HPLC. SDS-PAGE analysis of the purified P18
revealed a high degree of purity (Fig. 1; Table 2). This is the
first report of the purification of an F. psychrophilum OmpH signal peptide is indicated (▼). Peptides sequenced de novo are underlined
and shown in bold.

FIG. 7. (A) ompH locus organization in F. psychrophilum JIP02.86. (B) Multiple sequence alignment of F. psychrophilum G4 P18 with OmpH-like homologues from the following strains belonging to the CFB phylum: Bacteroides fragilis NCTC 9343 (BF_NCTC), Bacteroides thetaiotaomicron VPI-5482 (BT_VPI), and Porphyromonas gingivalis W83 (PG_W83). Identical amino acids, conservative substitutions, and semiconservative substitutions are indicated by stars, stacked double dots, and single dots, respectively. Dashes are gaps introduced to maximize
alignment. The predicted cleavage site of the F. psychrophilum OmpH signal peptide is indicated (▼). Peptides sequenced de novo are underlined
and shown in bold.
homologous to the ompH genes of some bacteria belonging to the CFB phylum and some other gram-negative and -positive bacteria. The DNA sequence of the cloned F. psychrophilum ompH gene was determined, and the deduced amino acid sequence revealed significant identity with the OmpH-like protein of Porphyromonas gingivalis. The amino acid sequence shows a typical tripartite “positive-hydrophobic-polar” sequence for a precursor signal sequence, with the presence of a cleavage site for signal peptidase I, used to remove the signal peptide for exported proteins (46). The final location of the OmpH-like protein at the cell surface had been suspected since the protein is released from F. psychrophilum by heat-HEPES treatment (30). The surface exposure of the OmpH-like protein was confirmed here by the following two approaches: first, the protein was totally digested in intact cells by proteinase K, while the outer membrane lipoprotein GldJ, as expected from its periplasmic exposition, was not digested (Fig. 2); second, anti-OmpH-like protein antibodies were capable of bacteriostatic and bactericidal activities toward F. psychrophilum in the absence and presence of complement, respectively (Fig. 6). The processed OmpH-like protein in F. psychrophilum has an apparent molecular mass of 18 kDa (30; this study) (Fig. 1), while the molecular mass for the amino acid sequence of the protein after cleavage of the probable signal peptide was calculated to be 16.2 kDa. This might be due to the experimental conditions used for molecular mass determination. However, we cannot rule out the possible presence of an alternative signal cleavage site in F. psychrophilum.

We previously hypothesized that P18 might be an S-layer protein (30) since it is actually recovered from flavobacterial cells by treatments known to release such components. However, the protein appears to be a true member of the OmpH family (also known as Skp or HlpA) (15, 20, 32). The OmpH-like protein produced by F. psychrophilum is surface located and immunoinaccessible and induces neutralizing antibodies, as recently reported for Chlamydia pneumoniae (14). In Escherichia coli, OmpH has been described as a molecular chaperone required for efficient release of translocated proteins from the plasma membrane. It interacts with unfolded proteins emerging from the sec translocation machinery and contributes to the correct folding of some outer membrane proteins and their insertion into the outer membrane (e.g., OmpA [10, 40]) as well as to preventing the aggregation of soluble proteins such as lysozyme (47). Such a key function in the periplasmic space for OmpH (41) is not consistent with the surface exposition observed in F. psychrophilum (this study) as well as in Chlamydia trachomatis (3) and C. pneumoniae (14). The flavobacterial OmpH protein might have different functions, including the chaperone periplasmic function generally assigned to the OmpH family and an interaction function with the environment, which might be an adaptation to some peculiar aspect of flavobacterial physiology. Further studies are needed to determine whether surface-exposed P18 has chaperone-like activities similar to those of periplasmic OmpH proteins from other bacteria.

Proteins belonging to the OmpH family have been described for a wide variety of bacterial species. Some studies have provided results indicating that members of the OmpH family may be immunogenic antigens of some bacteria (e.g., Chlamydia trachomatis [3]). This was also observed with F. psychrophilum. Indeed, sera from convalescent fish that were naturally infected were used successfully in this study to identify the OmpH-like protein in a set of other antigenic flavobacterial proteins recognized predominantly in the context of infection (Fig. 3). Consequently, the native OmpH-like protein was involved in serological and immunological experiments. High-titer anti-OmpH-like protein antibodies were raised in rabbits. The monospecific antiserum reacted with the OmpH-like protein only since one single band of 18 kDa was detected on immunoblots of F. psychrophilum lysates resolved by SDS-PAGE (Fig. 3 and 4), and the OmpH-like protein was shown to be highly conserved. Moreover, such antibodies binding the OmpH-like protein were capable of inducing protein and/or membrane distortions, resulting in the lysis of F. psychrophilum in the presence of complement. However, it is well known that the correlation is limited between protective immunity and the level of antibodies in fish after vaccination and that fish elicit a nonspecific immune response upon injection of non-antigen-containing agents (e.g., Freund’s adjuvant). Here we observed good protective immunity against F. psychrophilum, suggesting that specific immunity was obtained. Indeed, vaccination trials with a fraction highly enriched with OmpH-like protein induced significant protective immunity in fish, with this protection very probably being a result of the relatively high titers of antibodies produced against the flavobacterial OmpH-like protein (Fig. 5; Table 3). The bacteriostatic/bactericidal actions of the monospecific anti-OmpH-like protein rabbit serum observed in the present study also suggest that a sufficiently strong immune response against this protein could be related to protection.

Taking these results as a whole, we concluded that the OmpH-like protein in F. psychrophilum presents the capability to induce high antibody titers in rainbow trout and results in significant protective immunity. While additional studies are needed to assess the possible involvement of the OmpH-like protein in the pathogenesis of this bacterium, this work should be a prelude to the development of recombinant vaccines against F. psychrophilum, using the antigen characterized here as a possible immune target.

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