Identification and expression of a host-recognized antigen, FspA, from Flavobacterium psychrophilum

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Flavobacterium psychrophilum is the aetiological agent of rainbow trout fry syndrome, an economically important disease of immature salmonid fish for which there is no vaccine. Convalescent serum from the host, rainbow trout (Oncorhynchus mykiss), reacted strongly with a ~20 kDa, Flavobacterium-specific protein antigen (subsequently named FspA) from F. psychrophilum. Protein-enriched, detergent-partitioned samples were separated by two-dimensional gel electrophoresis and the protein target was excised, proteolytically cleaved and the resulting peptides analysed by MS. Quadrupole-time-of-flight MS was used to generate a fragmented peptide spectrum. The resulting peptide sequences were then used to design degenerate PCR primers to amplify the gene (fspA) of interest: 612 bp encoding 203 aa, including a putative 19 aa N-terminal signal sequence which predicted a processed 19 303-6 Da protein. FspA proved to be unique and only homologous to two unspecified sequences reported from Flavobacterium johnsoniae, although weakly homologous to a Yersinia pseudotuberculosis adhesin. An amplified gene fragment (537 bp, encoding 179 aa) was further cloned into an expression vector, expressed as a ~ 30 kDa N-terminal fusion protein and found to retain its strong reactivity with host serum antibodies. These results suggest that the surface-localized FspA may be an important subunit vaccine candidate antigen against F. psychrophilum.

INTRODUCTION

Flavobacterium psychrophilum (syn. Cytophaga psychrophila, syn. Flexibacter psychrophilus) is a psychrophilic, yellow-pigmented, filamentous Gram-negative bacterium belonging to the family Flavobacteriaceae. Members of the Flavobacteriaceae infect a wide range of hosts, including mammals (Hsueh et al., 1996; Siegman-Igra et al., 1987), birds (Vancanneyt et al., 1994) and fish (Inglis et al., 1993). First isolated by Borg in 1948 (Borg, 1960), F. psychrophilum (Cytophaga psychrophila) was named as the aetiologic agent of bacterial cold water disease in fish in the Pacific Northwest, USA (Davis, 1946). F. psychrophilum has since emerged as a causative agent of severe rainbow trout (Oncorhynchus mykiss) fry mortality (rainbow trout fry syndrome, RTFS) throughout Europe (Baudin-Laurencin et al., 1989; Bernardet et al., 1988; Bruno, 1992; Lorenzen et al., 1991; Santos et al., 1992; Sarti et al., 1992; Toranzo & Barja, 1993) and is now known to affect numerous fish species worldwide, mainly salmonid species (Iida & Mizokami, 1996; Lehman et al., 1991; Ostland et al., 1997; Schmidtke & Carson, 1995; Wakabayashi et al., 1991; Wiklund et al., 1994). The most serious losses occur in trout fry of approximately 0.2–2 g, where 30–90% cumulative mortality can result (Lorenzen, 1994). No vaccine is commercially available to protect against RTFS. F. psychrophilum strains have been difficult to speciate although there has been some recent progress in the preliminary identification and characterization of bacterial surface antigens (Crump et al., 2001; Massias et al., 2004; Merle et al., 2003) as well as in the development of facile diagnostics (Crump et al., 2003). As a further complication, the slow and fastidious growth of F. psychrophilum does not lend itself to the economical production of whole-cell bacterins or derivatives thereof as a cost-effective approach to vaccine production. However, whereas F. psychrophilum bacterins have been shown not to be protective (Rahman et al., 2002), outer-membrane preparations did confer significant protection against lethal challenges, indicating that immunoprotective antigens were present at the bacterial cell surface (Rahman et al., 2002). Likewise, LaFrentz et al. (2004) showed distinctive molecular mass fractions of F. psychrophilum to be more protective than whole-cell preparations. Consequently, recombinant subunit vaccine development is a rational objective.
The aim of this study was to identify host-recognized protein antigens from *F. psychrophilum* as potential vaccine candidates. Here we describe the use of convalescent sera from the host, rainbow trout, and proteomics technologies to identify a 20 kDa protein antigen, FspA. We report the expression of this 20 kDa antigen as a fusion protein in *Escherichia coli*. Further investigation is required to assess whether FspA will elicit a protective response in rainbow trout, as part of a recombinant vaccine against *F. psychrophilum*.

**METHODS**

**Bacterial strains and growth conditions.** *F. psychrophilum* (259-93) was routinely grown at 15 °C in MAT medium (0-4 % tryptone, 0-04 % yeast extract, 0-05 % CaCl2, 0-05 % MgSO4, 0-02 % sodium acetate, 1 % maltose; Crump *et al.*, 2001), adding 1.5-1 % agar when necessary. *E. coli* strains BL21(DE3) and XL-1 Blue (Stratagene) were grown in Luria broth (LB) or terrific broth (TB) (Sambrook *et al.*, 1989). When required, supplements were added at the following concentrations: ampicillin (Ap) 50 μg ml⁻¹, tetracycline (Tet) 12-5 μg ml⁻¹, IPTG, 1 mM, X-Gal, 30 μg ml⁻¹.

**Generation of antisera.** Rainbow trout convalescent serum was obtained from rainbow trout that survived challenge with *F. psychrophilum* (259-93). Rainbow trout (15 g) were injected with live *F. psychrophilum* (2·4×10⁷ cells) from a 24 h MAT broth culture. Pooled rainbow trout serum from 45 surviving fish was obtained 5 weeks post-challenge. Sera were also obtained from unexposed fish injected with saline for use as a negative control.

**SDS-PAGE.** Protein analyses of whole-cell lysates and inclusion body samples were carried out by SDS-PAGE according to the method of Laemmli (1970) as modified by Ames (1974). Samples were resolved in 12 % polyacrylamide separating gels with 5 % stacking gels. Molecular mass was estimated according to the apparent molecular mass of prestained protein markers. SDS-PAGE gels were washed for 15 min in distilled H₂O and stained with GelCode Blue G-250 based stain.

**Immunoblotting.** Bacterial cell proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes by blottransfer at 50 mA per gel for 1 h in a semidry transblot apparatus (LKB Multiphor II, Pharmacia) as described by Towbin *et al.* (1979). The membrane-immobilized immunogenic proteins were detected using rainbow trout serum raised against *F. psychrophilum*. Primary fish antibody (1/40 dilution) was incubated with the membrane overnight at 4 °C. The primary fish antibody was detected by rabbit anti-salmon IgG polyclonal antibody (Immuno-Precise Antibodies) followed by a goat anti-rabbit IgG (1/4000 dilution) conjugated to alkaline phosphatase (Caltag Labs) as outlined previously (Collinson *et al.*, 1991). Negative controls were carried out using naïve sera as the primary antibody. The immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium chloride (Sigma) as previously described (Müller *et al.*, 1989).

**Triton X-114 phase partitioning of *F. psychrophilum*.** Phase partitioning of *F. psychrophilum* cells was carried out by the method of Cunningham *et al.* (1988) with the following modifications. Wet cell pellets were suspended in Triton X-114 (Sigma) solubilization buffer (1 % Triton X-114/10 mM Tris pH 8/5 mM EDTA pH 8) to 20 mg ml⁻¹. The hydrophobic fraction was solubilized by rotat-ting the cell suspension in a tube at 4 °C for 2–3 h. The suspension was centrifuged at 4 °C (5 min, 12 100 g) and the clear supernatant (detergent-soluble fraction) transferred to a new tube, leaving behind the insoluble ‘cold pellet’. The suspension was then incubated at 37 °C for 15 min to precipitate the detergent and the phases separated by centrifugation at room temperature (15 min, 12 100 g). This resulted in a top aqueous phase, a lower detergent phase and a translucent ‘warm pellet’. The aqueous and detergent phases were transferred to new tubes and washed three times each as follows: to the aqueous phase, Triton X-114 was added to a final concentration of 1 % and to the detergent phase, an equal volume of TE (10 mM Tris/5 mM EDTA pH 8) was added. Both Triton X-114 suspensions were dissolved by incubating on ice for 10 min and incubated at 37 °C for 10 min for phase partitioning prior to centrifugation at room temperature (15 min, 12 100 g) to separate the phases. The washed detergent and aqueous phases were transferred to new tubes. Acetone precipitations were performed to remove detergent from the Triton phase and to concentrate proteins in the is, the phases ten volumes of cold acetone was added to the sample, which was mixed by vortexing briefly and incubated at −20 °C overnight. The precipitated protein mixture was centrifuged (17 400 g, 15 min, 4 °C) and the protein pellet air-dried.

**Two dimensional (2D) gel electrophoresis.** High-resolution 2D SDS-PAGE was performed using the ISO-DALT multiple 2D system (Anderson & Anderson, 1978a, b). Protein samples were solubilized in 30 μl urea mix (9 M urea, 4 %, v/v, NP-40, 2 %, v/v, Pharmalyte 3–10 amphiines, 2 %, v/v, DTT) and loaded onto pre-focused tube gels containing pH range 3–10 amphiines (Pharmalyte 3–10, Amersham Pharmacia). First-dimension IEF was conducted at 800 V for 18 h (14 400 V h). Following electrophoresis, the tube gels were equilibrated for 15 min at room temperature in equilibration buffer and immediately mounted onto 10–16 % gradient SDS-PAGE slab gels with the acidic end positioned to the left. Electrophoresis was performed at 4 °C at 1 A, until the dye front was about 1 cm from the bottom of the gel (~5 h). Alternatively, mini-2D gels were run using the Mini-Protean II tube cell module (Bio-Rad) as follows. Capillary IEF tube gels were pre-focused by running at 200 V for 10 min, 300 V for 15 min and 400 V for a further 15 min. Tube gels were run at 500 V for 10 min followed by 750 V for 3·5 h. Mini-slab gels were run at 10 mA through the stacking gel (5 %) and 20 mA through the separating gel (12 %) until the dye front reached the bottom of the gel (~90 min). After electrophoresis, gels were fixed and stained with colloidal Coomassie brilliant blue G-250 or electroblotted onto nitrocellulose membranes for immunoblot analysis.

**Staining of 2D gels with Coomassie brilliant blue G-250.** Gels were agitated gently in fixative (50 %, v/v, ethanol, 3 %, v/v, orthophosphoric acid) for 1–4 days at room temperature, washed 3 × 30 min in distilled H₂O and allowed to equilibrate in Neuhoff’s solution (16 %, w/v, ammonium sulphate, 25 %, v/v, methanol, 5 %, v/v, orthophosphoric acid; Neuhoff *et al.*, 1988) for 1 h with gentle agitation. Coomassie brilliant blue G-250 (1 g) (EM Science) was added to the Neuhoff’s solution and staining continued for 3–5 days. Digital images of Coomassie-stained gels were captured by scanning at 300 d.p.i. using a colour scanner (UMAX Astra 3400). Protein spots of interest were then cored from the gel for further analysis.

**Reduction, alkylation and tryptic digests of 2D gel spots.** Protein spots were excised from 2D gels using a 4 mm plastic straw and either transferred to 1·5 ml microcentrifuge tubes (previously autoclaved and rinsed with 50 % methanol to remove any contaminants) for digestion or to 96-well sterile tissue culture plates (one spot per well in 10 μl 20 %, v/v, ammonium sulphate; Neuhoff *et al.*, 1988) for storage at −20 °C. For analysis by mass spectrometry, 2D gel protein spots were destained (50 %, v/v, methanol/5 %, v/v, acetic acid), reduced with 10 mM dithiothreitol and alkylated with 100 mM iodoacetamide as described by Kinter & Sherman (2000). The carboxyamidomethylated protein spots were digested overnight at 37 °C with 20 ng ml⁻¹ modified, sequence-grade, porcine trypsin
according to the manufacturer’s directions (Promega). Peptides were extracted from the gel pieces using a series of elutions with 50% (v/v) acetonitrile/5% (v/v) formic acid. The resulting pooled eluates were each reduced to a final volume of 20 μL in a Speed Vac Concentrator (Savant) and processed for mass spectrometry.

Nanospray tandem mass spectrometry (MS/MS) and peptide sequencing. Tryptic peptides were desalted using glass capillary needles (Protana) packed with C18 resin (Perceptive POROS R2, 50 μm beads) and were extracted into sample needles using 1-0 μL 50% (v/v) methanol/1% (v/v) formic acid. Nanospray electroospray ionization was used to introduce ions into a PE-SCIEX Q-STAR quadrupole time-of-flight mass spectrometer (Q-TOF) (Applied Biosystems). Data were managed with Bioanalyst Software (PE-SCIEX) and peptides sequenced by following Kinter’s nine-step strategy for interpretation of product ion spectra (Kinter & Sherman, 2000). Peptide fragmentation data searching was performed using the Mascot MS/MS Ions Search algorithm (Matrix Science; http://www.matrixscience.com/).

Determination of the fspA gene sequence. Based on peptide sequences obtained from MS analysis, pairs of degenerate oligonucleotide primers were designed to amplify a segment of the fspA gene. Use of the following primers amplified a gene sequence: AAY GTD GCH GGW ACH GTD GG (NVAGTVG forward, 324-fold degeneracy); GGN GCN AAY GAY TTY GA (AANDF reverse, 128-fold degeneracy). Each 50 μL reaction contained the following: 20 mM Tris/HCl, pH 8.4; 50 mM KCl; 1·5 mM MgCl2; 0·2 mM deoxyribonucleoside; 50 pmol of each degenerate primer; 1·0 μL Taq DNA polymerase (Roche); 500 ng template DNA. Amplification was performed as follows: 2 min at 94°C, 30 cycles of 30 s at 94°C, 1 min at 52°C, and 1 min at 72°C; followed by 5 min at 72°C.

Uneven PCR was carried out to amplify the unknown 5’ end of the fspA gene, according to the method of Chen & Wu (1997) with minor modifications. Each 50 μL reaction contained the following: 20 mM Tris/HCl, pH 8·4; 50 mM KCl; 1·5 mM MgCl2; 0·2 mM deoxyribonucleoside; 12·5 pmol specific primer; 2·5 pmol degamer random primer; 1·0 μL Taq DNA polymerase (Roche). The template in the first set of cycles (round 1) was 50 ng genomic DNA, amplified with primer 1 (GCT ACC TTC AAT ACC TAG TGT CAT G) and the random primer GTT TCG CTC C. One microlitre of the final amplified reaction mix from round 1 was used as the template for round 2 of reactions, using a nested primer (primer 2, GCC AAC CAA CTT TGT GAG AAA AAT CGA AAC C) and the same random primer. The cycling reactions were performed as described by Chen & Wu (1997) with changes in the specific primer annealing temperatures. Annealing temperatures in round 1 were cycled between 48/50°C and 42/45°C. Round 2 annealing temperatures tested were 54°C and 45°C. PCR products were cloned into pGEM-T Easy (Promega) according to the manufacturer’s instructions and sequenced.

Automated DNA sequencing and sequence analysis. Plasmids submitted for DNA sequencing were purified using a Qiagen Spin Miniprep Kit (Qiagen) according to the manufacturer’s instructions. Pure double-stranded plasmids were submitted to the Centre for Biomedical Research, University of Victoria, Victoria, BC, Canada, and automated dideoxyxynucleotide sequencing was conducted using a NEN Global IR2 DNA Sequencer (LI-COR) using dye-labelled primers. DNA traces were visually examined for errors and ambiguous regions, and aligned using ContigExpress from Vector NTI Suite 7 (InforMax) for derivation of the final consensus sequence. Coding predictions were made to identify ORFs using the National Center for Biotechnology Information (NCBI) program ORF finder (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Sequences were subjected to BLAST 2 (Altschul et al., 1997) and FASTA 3 (Pearson, 1998) analysis to determine if any similar sequences were known. Multiple sequence alignments were performed using AligN from Vector NTI Suite 7 (InforMax). Translated DNA sequences were analysed using software to predict N-terminal signal sequences (IPSOReB; Bannai et al., 2002) and signal peptide cleavage sites (SignalP; Nielsen et al., 1997).

PCR amplification of fspA. Primers used to amplify the 537 bp fspA gene segment (named fspA’) and insert restriction sites for cloning purposes were as follows: forward, CGA CGG ATC GTG CTC ATA TQA ACG TGG CAG GTA CAG TGG GTT TTA ACT C; reverse, TGC GAA GCT TAA TTA ATG AAA TTT TTA GAG ATA CCA AGC CTG CCT ACT TGT TTT CCG. The forward primer encoded BamHI and Ndel restriction sites (underlined). The reverse primer encoded HindIII and VspI restriction sites (underlined). Each 50 μL reaction contained the following: 20 mM Tris/HCl, pH 8·4; 50 mM KCl; 1·5 mM MgCl2; 0·2 mM deoxyribonucleoside; 20 pmol primer 1 μL Taq DNA polymerase (Roche); and 50 ng template DNA. Amplification was performed as follows: 1 cycle at 94°C for 2 min, 30 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 30 s followed by 1 cycle at 72°C for 5 min.

Creation of fusion construct CFspA and protein expression. fspA’ (partial sequence, 537 bp encoding 179 aa) was amplified by PCR, digested with EcoRI and BamHI and cloned into the expression vector pETC, a pET21a (+) (Novagen) derived expression vector encoding a 10 kDa, non-specific N-terminal fusion protein, protein C (Microtek International). C-protein fusions readily form inclusion bodies to aid isolation of the expressed product. Induction experiments were carried out in E. coli BL21(DE3). Overnight 37°C cultures in LBa, or TBa were used to inoculate fresh LB medium. Cultures were grown at 37°C to an OD{600} of 0.6–0.8, at which point they were induced by the addition of 1 mM IPTG for 2 h. Inclusion bodies were isolated from cells by sonication as follows: an induced culture was centrifuged at 10 000 g for 10 min and resuspended in 10 000 g, 15 min); pellets were resuspended in distilled H2O and the procedure repeated. The final inclusion body samples were resuspended in distilled H2O and stored at −20°C.

RESULTS

Convalescent rainbow trout serum strongly recognizes a hydrophobic, 20 kDa protein antigen

To discover which antigens promoted a humoral response in the salmonid host, immunoblots of F. psychrophilum whole-cell lysates were carried out using convalescent rainbow trout serum. A strong band at ~20 kDa was revealed as well as several faint bands (Fig. 1a). The negative control carried out with sera from saline-injected fish showed numerous but faint protein bands (Fig. 1a). To further characterize the ~20 kDa antigen recognized by convalescent fish serum, cells were fractionated with Triton X-114, and the two resulting phases (aqueous and detergent) were examined for protein bands. The negative control showed numerous but faint protein bands (Fig. 1b). The ~20 kDa antigen recognized by fish serum was separated by 2D gel electrophoresis. To minimize the
protein load on the gels and aid resolution, *F. psychrophilum* cells were first fractionated with Triton X-114 to enrich the hydrophobic components, and the Triton-soluble, ‘warm pellet’ phase was loaded onto the 2D gels, instead of whole-cell preparations. Gels were either stained with Coomassie G-250 or electroblotted onto nitrocellulose for immunoblot analysis (Fig. 2). The specificity of the fish antiserum made it an excellent tool, reacting strongly with only one protein within the sample (Fig. 2b). The corresponding protein in a Coomassie-stained 2D gel was therefore easy to identify (Fig. 2a, arrows). The immunoblot of the 2D gel revealed a protein smear at ~20 kDa (Fig. 2b, arrow z), as did the Coomassie-stained gel (Fig. 2a, arrow y). Protein in the centre of the smear was excised from the gel (Fig. 2a, arrow y) as well as the spot formed at the end of the smear (Fig. 2a, arrow x) for MS analysis.

**Q-TOF MS analysis of the ~20 kDa host-recognized antigen**

By comparing the Coomassie-stained 2D gel of detergent-phase *F. psychrophilum* with an immunoblot, a protein smear corresponding to the ~20 kDa antigen was identified. Two samples, termed x and y (Fig. 2a, arrows), along the smear were excised and proteolytically cleaved. The two samples, FspA x and y, were analysed by Q-TOF MS. The initial TOF MS survey scans showed that the peaks occurred at the same *m/z* values, although the peak intensities were lower for spot x than spot y (data not shown); therefore, the two samples were shown to contain the same protein. The peptide ions generated from nanospray ionization of each sample were analysed for doubly charged ions by looking for peaks that differed by half a mass unit. The doubly charged peptide ions obtained from spots x and y were then fragmented to produce a spectrum of collision-induced product ions from which the peptide sequences were determined.
Table 1. Product ions sequenced from protein FspA

Tryptic peptides were analysed by Q-TOF MS and sequenced. Spots x and y refer to two protein samples excised from the gel (Fig. 2a, arrows). Underlined amino acids were found to be incorrect when compared to the translated dPCR sequence. Numbers in parentheses denote masses of unknown amino acid sequences flanking known sequence.

<table>
<thead>
<tr>
<th>Product ion (m/z)</th>
<th>Peptide sequence</th>
<th>Spot</th>
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<tbody>
<tr>
<td>923-4</td>
<td>(278-20) GFYLGVT (712-21)</td>
<td>y</td>
</tr>
<tr>
<td>923-5</td>
<td>(278-11) GFYLGVT (712-35)</td>
<td>x</td>
</tr>
<tr>
<td>923-5</td>
<td>(1183-55) LGVDMK</td>
<td>x</td>
</tr>
<tr>
<td>692-3</td>
<td>(158-06) FADLGVM (345-09)</td>
<td>y</td>
</tr>
<tr>
<td>486-2</td>
<td>SSGEDFKPK</td>
<td>x</td>
</tr>
<tr>
<td>1022-5</td>
<td>(342-20) QFSENMTVGLE (447-14)</td>
<td>y</td>
</tr>
<tr>
<td>1030-5</td>
<td>(243-08) VQFSENFTVLEGFSR</td>
<td>y</td>
</tr>
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<td>985-5</td>
<td>(286-21) NVATGVDGST (615-29)</td>
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</tr>
<tr>
<td>613-3</td>
<td>AANDFELNGK</td>
<td>x</td>
</tr>
</tbody>
</table>

Identification of the fspA gene sequence by degenerate PCR

To identify the gene sequence encoding FspA, degenerate PCR (dPCR) primers were designed from peptide sequences to amplify a portion of the gene. A reproducible 852 bp product was amplified by dPCR, cloned into pGEM-T Easy and electroporated into E. coli XL-1 Blue. Transformants were selected by blue/white screening and ampicillin resistance. Three positive clones were confirmed by restriction digest analysis and sequenced. The consensus sequence of the dPCR product was translated into amino acid sequence and searched for the presence of the peptide sequences obtained from MS analysis of FspA. All the peptide sequences (Table 1) were found within the translated dPCR product (Fig. 3), thus confirming that the partial gene sequence obtained by dPCR was derived from the protein of interest excised from the 2D gel. Analysis of the translated sequence revealed 10 stop codons in the C-terminal portion, starting at position 537 bp (179 aa) (Fig. 3). All of the peptides sequenced (Table 1) were found upstream of the first stop codon, including the peptide used to design the antisense primer (AANDE). Closer inspection of the dPCR sequence showed that the 3’ end was compatible with the degenerate, sense strand primer at the 5’ end. Fortunately, another form of the sense primer, which had 324-fold degeneracy, performed as an anti-sense primer, and resulted in sequencing beyond the C-terminus of the protein. The partial protein sequence obtained, from position 1 to the first stop codon (537 bp, 179 aa), was found to have a theoretical mass of 18-8 kDa and pI of 8-70, which was in close enough agreement with the observed mass and pI from 2D gel electrophoresis (~ 20 kDa, pI > 7), and suggested that the vast majority of the gene sequence had been obtained by dPCR.

Fig. 3. Sequence of the dPCR product. dPCR was carried out with primers designed from peptide sequences [NVAGTVG (sense) and AANDFEL (antisense) (Table 1)] and the product sequenced. The translated amino acid sequence is represented in single-letter code. Peptide sequences obtained by Q-TOF MS are boxed. The underlined sequence at the 3’ end was found to be compatible with the dPCR sense strand primer. Asterisks denote stop codons.
Uneven PCR was used to amplify the unknown, upstream sequence and thus identify the N-terminal portion of protein FspA. A further 72 bases of the ORF were discovered, identifying the missing 24 N-terminal amino acids. The entire fspA gene sequence was found to be 612 bp in length, encoding a 203 aa protein, including a putative 19 aa N-terminal signal sequence. The theoretical molecular mass of the whole FspA protein is 21 298±0 Da and its pI 9.35. The amino acid composition was found to be 36% hydrophobic, 17% charged and 31% polar. Omission of the putative 19 aa sequence results in a 184 aa protein with a theoretical molecular mass of 19 303±6 Da and pI of 9.02.

**The FspA sequence is specific to Flavobacterium species**

Database searching for similar sequences (performed March 2005) resulted in only two significant matches, which were hypothetical proteins from Flavobacterium johnsoniae (Fig. 4). The two neighbouring F. johnsoniae genes were 72% identical and encoded hypothetical proteins of 193 aa and 194 aa that shared 66% identity, and for the purpose of this study were arbitrarily named Fj1 and Fj2 respectively. At the protein level, FspA was found to be 53% and 56% similar to Fj1 and Fj2 respectively, and 43% identical. Alignment of the translated amino acid sequences of the hypothetical proteins and FspA showed overall 81% similarity and 36% homology between the three sequences (Fig. 4). All three Flavobacterium sequences were found to contain a putative N-terminal signal sequence cleavage site (ANA^Q^QK) that occurred at position 18 in Fj1 and Fj2, and at position 19 in FspA.

**Expression of FspA fusion in E. coli**

PCR primers were designed with flanking restriction enzyme sites to amplify the 537 bp sequence encoding the 179 aa portion of the protein antigen FspA identified by dPCR. The amplified gene segment was gel-purified, digested with BamHI and HindIII and ligated into the expression vector pETC. Plasmid constructs were confirmed by restriction digest analysis, as well as DNA sequencing. E. coli BL21(DE3) was electroporated with pETCFspA’ or pETC and transformants were selected overnight on LB_ap at 37 °C. For expression experiments, overnight LB_ap 37 °C cultures were used to inoculate fresh LB_ap. Once cultures had reached an OD_{600} of 0.6–0.8, they were induced with 1 mM IPTG for 2 h. Whole-cell lysates were then separated by SDS-PAGE and visualized by Coomassie staining and immunoblotting. Coomassie-stained gels showed that the FspA’ sequence was expressed as a C-protein fusion, termed CFspA’, with an apparent molecular mass of ~30 kDa (Fig. 5a). Immunoblotting with convalescent rainbow trout serum showed CFspA’ to be antigenic, whereas C protein alone did not react (Fig. 5b).

**DISCUSSION**

There are no cost-effective, efficacious vaccines currently available to prevent F. psychrophilum infections. As a rational approach to vaccine design, F. psychrophilum antigens were sought as potential vaccine candidates. For routine antigenic analysis, rabbit serum is relatively easy to obtain and provides insight as to the potential antigenic nature of F. psychrophilum in the host. Rabbit antiserum has been successfully employed in the past to identify protective antigens in the production of vaccines for aquaculture (Kuzyk et al., 2001). However, rainbow trout are evolutionarily far removed from mammals and so the more refined goal was to identify antigens recognized by the host. Consequently, convalescent rainbow trout serum was raised against sublethal concentrations of live F. psychrophilum cells. Western blot analysis of F. psychrophilum whole cells

![Fig. 4. Multiple sequence alignment of the translated fspA gene. The 203 aa FspA sequence from F. psychrophilum is shown here with two hypothetical proteins from F. johnsoniae, arbitrarily labelled Fj1 and Fj2. Black shading indicates identity between the three sequences, dark grey indicates identity in two sequences and light grey indicates similar residues. The three sequences are 81% similar, 36% identical. Multiple sequence alignments were constructed using AlignX (Vector NTI Suite 7, InforMax).](https://www.microbiologyresearch.org/)
showed numerous bands reacting with both convalescent and naïve serum. Although many of the same bands were detected by both sera, the relative antibody binding of one protein (20 kDa) was greatly enhanced in the convalescent serum compared to the ‘background’ reaction of the other bands (Fig. 1). The nature of fish immunoglobulin, being high avidity low affinity (for a review see Kaattari & Piganelli, 1996), probably accounts for the observed binding of naïve trout sera to many of the lesser reacting proteins.

The ~20 kDa protein antigen partitioned into the detergent phase following extraction with Triton X-114, indicating that the protein is, at least in part, hydrophobic and most likely membrane associated; although the gene sequence (predicted 36% hydrophobic amino acids) suggests the protein is hydrophobic as well, but not strongly. In 2D gel electrophoresis, the ~20 kDa antigen tended to smear across the basic region of the second-dimension slab gel. The poor resolution of basic proteins in 2D gel electrophoresis is a well-known artifact, and can be indicative of partial insolubility, over/underfocussing or high protein load (Berkelman & Stenstedt, 1998). Insolubility of basic proteins and their poor resolution can be caused by migration of the reducing agent (dithiothreitol) towards the anode during IEF, resulting in a depletion of reducing agent at the cathode (Pennington et al., 2004). Despite the poor resolution, we were able to identify the protein by Q-TOF MS analysis, and showed that the samples taken from both the smear and the resolved spot were of the same protein.

The results obtained from dPCR analysis were initially surprising, representing an apparent gene fragment somewhat larger than the expected size for the observed protein. However, these results would be expected were one of the degenerate primers acting as both a sense and an anti-sense primer. This fortunate match led to the sequencing of the protein’s C-terminus, leaving only the N-terminal sequence unknown, which was later obtained by uneven PCR.

Database searching with the translated 203 aa FspA sequence resulted in only two significant matches. The two similar sequences found were hypothetical proteins from a closely related fish pathogen F. johnsoniae. The two predicted proteins were 193 and 194 aa in length and are encoded by consecutive genes on the F. johnsoniae chromosome (Agarwal et al., 1997). Database searching revealed that the 194 aa F. johnsoniae protein is 27% identical and 46% similar to Ail from Yersinia pseudotuberculosis (Yang et al., 1996), a 17 kDa outer-membrane protein that mediates adhesion to mammalian cells and contributes to serum resistance. It is therefore possible that the surface-localized FspA is an adhesin and an important pathogenesis factor of F. psychrophilum. A ~18 kDa, immunogenic (rabbit) protein was shown to be readily released from F. psychrophilum with aqueous buffers at different pHs and by chelators and was speculated to be an S-layer protein (Massias et al., 2004). While of similar molecular mass to FspA these proteins appear to differ in their solubilization properties. Two genes representing immunogenic (rabbit) membrane proteins of the related fish pathogen Flavobacterium columnare, a metalloprotease and an oligopeptidase, have also been characterized but are clearly different from FspA based on molecular mass (Xie et al., 2004).

A 179 aa fragment of FspA could be abundantly expressed in E. coli as an N-terminal fusion protein, CFspA’, and retained its immuno-reactivity with convalescent serum. Not surprisingly, the antibody reaction with the native protein (Figs 1 and 2b) is apparently stronger than the reaction observed with the C-protein fusion (CFspA’) (Fig. 5b). This may be due to the removal of the N-terminal residues and conformational change following fusion with C-protein.

In summary, a ~20 kDa (SDS-PAGE) F. psychrophilum protein recognized strongly by convalescent rainbow trout serum was characterized by 2D gel electrophoresis, immunochemistry, MS, dPCR and uneven PCR, as a 184 aa (19.3 kDa) surface-localized protein (FspA). A 179 aa portion of FspA was overexpressed in E. coli and retained immunoreactivity with convalescent rainbow trout serum. The FspA antigen of F. psychrophilum is currently the only well-characterized prospective subunit vaccine candidate against bacterial cold water disease (rainbow trout fry syndrome).

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