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Identification of potential vaccine target antigens by immunoproteomic analysis of a virulent and a non-virulent strain of the fish pathogen *Flavobacterium psychrophilum*

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ABSTRACT: *Flavobacterium psychrophilum* is the etiological agent of bacterial coldwater disease (CWD) and rainbow trout fry syndrome (RTFS). To identify antigens associated with virulence or host immunity, we compared total and immunogenic proteins of cellular and extracellular products (ECP) between a virulent (CSF-259-93) and non-virulent (ATCC 49418) strain of *F. psychrophilum*. One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis of total cellular proteins revealed only minor differences between the strains; however, separation of ECP showed that proteins were differentially expressed. Western blot analysis using rainbow trout (*Oncorhynchus mykiss*) anti-CSF-259-93 sera showed greater reactivity to proteins of the virulent strain, including many >50 kDa. Further analysis by 2-dimensional electrophoresis (2DE) identified numerous differences between the strains. Western blot analysis combined with 2DE identified several immunogenic proteins that reacted with the antisera and were shared between the 2 strains. However, at least 15 immunogenic proteins appeared to be unique to the virulent strain, while 4 such proteins were identified in the non-virulent strain; 8 proteins unique to the virulent strain and 6 shared proteins were further analyzed for identification by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis. Of these, 3 immunogenic proteins (heat shock proteins HSP 60 and HSP 70) and 2 other proteins (ATP synthase and thermolysin) were conclusively identified. The 2 highly immunogenic heat shock proteins were shown to share extensive homology with heat shock proteins of related bacteria. This approach for antigen identification may provide a basis for targeted vaccine development against CWD and RTFS.

KEY WORDS: *Flavobacterium psychrophilum* · Proteomics · Salmonid pathogen · Immunogenic antigens · Aquaculture vaccine

INTRODUCTION

Flavobacterium psychrophilum is a Gram-negative bacterium belonging to the *Cytophaga–Flavobacterium–Bacteroides* group and is the causative agent of coldwater disease (CWD) and rainbow trout fry syndrome (RTFS). This fish pathogen can infect a broad range of hosts including salmonids (Holt et al. 1993), ayu *Plecoglossus altivelis* (Wakabayashi et al. 1994), and a few other fish species (Lehman et al. 1991, Iida & Mizokami 1996, Amita et al. 2000). Fry and fingerlings are primarily affected and disease outbreaks typically

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occur at water temperatures between 3 and 15°C (Borg 1948, Rucker et al. 1953, Holt et al. 1989). High mortality rates and spinal deformities associated with CWD and RTFS may occur and result in large economic losses. Treatment for these diseases requires antibiotics, but success is often limited and there is concern that strains may develop resistance (Bruun et al. 2000, Michel et al. 2003). Therefore, vaccination is desired for the prevention of CWD and RTFS; however, there has been difficulty in developing a feasible and efficacious vaccine.

Immunization of rainbow trout *Oncorhynchus mykiss* with killed *Flavobacterium psychrophilum* preparations have, in general, yielded limited success for the prevention of CWD and RTFS unless the preparation is injected with adjuvant incorporation (Obach & Laurencin 1991, LaFrentz et al. 2002, Rahman et al. 2002, Madetoja et al. 2006). This, combined with the difficulties of economical large-scale production of *F. psychrophilum* (Crump et al. 2005) has prompted studies aimed at identifying specific virulence factors and protective antigens associated with the host immune response to target for vaccine development. Several proteins from *F. psychrophilum* have been purified and characterized, including 2 metalloproteases (Secades et al. 2001, 2003), a membrane glycoprotein (Merle et al. 2003), and a surface layer protein (Massias et al. 2004). The role these play in virulence and in eliciting a protective immune response is not known. Other studies have utilized rainbow trout antisera to identify components recognized by the host immune system. Crump et al. (2001) identified several protein and carbohydrate antigens of *F. psychrophilum* and subsequently characterized and expressed a ~20 kDa host-recognized protein antigen for use in a recombinant protein vaccine (Crump et al. 2005). LaFrentz et al. (2004) identified 3 immunogenic regions of the bacterium and demonstrated a high level of protection in rainbow trout immunized with antigens in the 70 to 100 kDa molecular mass range. Additionally, Rahman et al. (2002) demonstrated that outer membrane components of the bacterium were highly protective. These studies indicate that protective antigens of *F. psychrophilum* exist, and a more detailed characterization of these and specific virulence factors will allow the identification of antigens to target for vaccine development.

In the present study, our goal was to compare proteins of a virulent and non-virulent strain of *Flavobacterium psychrophilum* to identify possible factors associated with virulence, and to incorporate immunoproteomics to identify specific proteins recognized by the host immune response. The specific objectives of this study were to compare total proteins and extracellular products (ECP) of a virulent and non-virulent strain of *F. psychrophilum* using single and 2-dimensional electrophoresis (2DE) and Western blotting, and to identify proteins of interest by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis.

MATERIALS AND METHODS

Bacterial growth conditions. Bacterial strains used in this study included a virulent strain of *Flavobacterium psychrophilum* (CSF-259-93) and a non-virulent strain (ATCC-49418). The CSF-259-93 strain was isolated from moribund rainbow trout during an epizootic of CWD in Southern Idaho, and a subcutaneous challenge model has shown it to be highly virulent in rainbow trout (LaFrentz et al. 2002, 2003, 2004). The ATCC-49418 strain is the type strain of *F. psychrophilum* and intraperitoneal injection (Madsen & Dalsgaard 2000) and subcutaneous challenge models (K. D. Cain unpubl. results) have shown it to be non-virulent in rainbow trout. Bacteria were cultured in tryptone yeast extract salts broth (TYES, 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulfate, pH 7.2; Holt et al. 1993) or agar at 15°C for 72 h. Single colonies from agar plates were transferred to 10 ml TYES broth and grown at 15°C for 72 h to an optical density (OD) of 1.5 at 525 nm. Bacteria were harvested from broth cultures by centrifugation for 15 min at 3000 × *g* and washed twice in phosphate buffered saline (PBS, pH 7.2); the pellet was stored at –80°C until needed for electrophoretic analysis.

Preparation of ECP. The ECP from the virulent and non-virulent strains of *Flavobacterium psychrophilum* were obtained by a cellophane overlay method (Sudheesh & Xu 2001). This growth technique allows the bacteria to draw nutrients freely from below the cellophane sheet, and at the same time prevent mixing of the ECP proteins released by the bacteria with the proteins present in the underlying culture medium. Briefly, bacteria were grown on TYES agar plates and single colonies were transferred to TYES agar slants and cultured at 15°C for 72 h. Cells were then washed with sterile 0.01 M PBS (pH 7.2). Sterile cellophane sheet overlaid TYES plates were prepared onto which 200 µl of the bacterial cell suspension was spread and incubated at 15°C for 72 h. Bacteria grown on the cellophane sheet overlay were washed into 2 ml of PBS and centrifuged for 20 min at 15 000 × *g*. Following centrifugation, the supernatant containing ECP was filter sterilized with a 0.2 µm membrane filter and stored at –80°C until needed. The protein concentration of whole bacterial cells and ECP were determined using a Micro BCA protein assay kit (Pierce

Biotechnology) according to the manufacturer's instructions.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Bacterial whole-cell lysates and ECP were analyzed by SDS-PAGE according to the method of Laemmli (1970) with some modifications. Approximately 25 µg of each protein sample was diluted in sample buffer with 40 mM dithiothreitol (DTT) and boiled for 4 min. Samples were resolved on precast 10 to 20% polyacrylamide gradient gels (Bio-Rad) using a Mini-PROTEAN 3 electrophoresis cell (Bio-Rad). Initial gel running conditions were at 85 V for 15 min followed by 185 V until the dye front migrated just out of the gel. Precision Plus protein standards (Bio-Rad) were used as molecular mass standards. To determine if antigens were protein or carbohydrate in nature, Proteinase K digestion of whole bacterial and ECP protein samples was carried out in SDS-PAGE sample buffer. This simply required addition of Proteinase K (Sigma-Aldrich) at a 10 µg ml^{-1} final concentration followed by sample incubation at 37°C for 60 min. After Proteinase K digestion phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM to inhibit Proteinase K activity.

2DE. Whole bacterial cell lysate samples for 2DE were prepared by sonicating washed cells (4 times at 50 W for 30 s on ice) in 40 mM Tris-HCl. Sonicated cells were treated with 150 U of endonuclease (Sigma-Aldrich) and incubated for 30 min at room temperature. Bacterial cell lysates and ECP protein samples were extracted in sample extraction buffer, which contained 5 M urea, 2 M thiourea, 2 mM tributyl phosphene (TBP), 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2% sulfobetaine 3-10, 0.5% biolyte ampholyte, 10 mM Tris and 0.001% Orange G dye. Extracted protein samples (25 µg total protein) were applied to 7 cm immobilized pH gradient (IPG) strips (pH 4–7) (Bio-Rad) and rehydrated overnight in a humidified chamber at room temperature. First dimension isoelectric focusing was performed on IPG strips using a Mini Protean IEF cell (Bio-Rad). The strips were focused initially for 15 min at 250 V, 2 h at 4000 V and then 5 h at 4000 V to achieve 20 000 Vh. Focused strips were equilibrated in 5× Tris-HCl glycine gel buffer containing 6 M urea, 2% SDS, 20% glycerol, 5 mM TBP and 2.5% acrylamide monomer. Second dimension separation was carried out using precast 10 to 20% polyacrylamide gradient gels with a 2D miniprep well (Bio-Rad) on a Mini-PROTEAN 3 electrophoresis cell (Bio- Rad). Electrophoresis was performed using standard Laemmli buffer system at a constant current of 5 mA for 30 min and then at a constant current of 12 mA for 1.5 h. Triplicate gel strips were prepared for each sample with the same protein concentration, and isoelectric focusing was carried out simultaneously to minimize experimental variability. Multiple gels were prepared for the same protein samples from both strains to ensure consistency of protein profile on 2DE gels. The gels were either silver-stained and scanned or stained with Sypro Ruby (Bio-Rad) and imaged under UV transillumination.

Western blot analysis. Immunogenic antigens were identified by Western blot analysis of proteins separated by SDS-PAGE and 2DE. Different pools of immune sera were obtained from juvenile rainbow trout immunized with whole-cell *Flavobacterium psychrophilum* (CSF-259-93) with Freund's complete adjuvant (LaFrentz et al. 2002). Antigens separated by SDS-PAGE and 2DE were transferred onto nitrocellulose membranes by electrophoresis at a constant current of 100 V for 1 h in a Mini Trans-blot electrophoretic transfer cell (Bio-Rad). The nitrocellulose membranes were blocked for 1 h at room temperature in PBS containing 4% non-fat dry milk. Membranes were allowed to react with the rainbow trout antiserum to *F. psychrophilum* (1:50 in PBS containing 0.05% Tween-20, 0.05% sodium azide and 4% non-fat dry milk) overnight at 15ºC. The primary fish antibody was detected by incubation of membranes for 1 h at room temperature with a monoclonal mouse antibody raised against trout immunoglobulin (mAb 1.14) (DeLuca et al. 1983) diluted 1:40 in PBS containing 0.05% Tween-20 and 0.05% sodium azide (PBST-AZ). Membranes with proteins transferred from 1-dimensional SDS-PAGE gels were incubated for 1 h at room temperature with an alkaline phosphatase conjugated goat anti-mouse immunoglobulin (Bio-Rad) diluted 1:500 in PBST-AZ. Membranes were washed 3 times in PBST-AZ between each incubation. The immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolylphosphate toluidine salt and 4-nitro blue tetrazolium chloride (Bio-Rad). The Western blot analysis of membranes with proteins transferred from 2DE gels was visualized by incubating an anti-mouse IRDye–800 (Rockland Immunochemicals) conjugated rabbit antiserum (1:10 000) with the secondary, antibody bound membranes for 1 h in the dark. Blocking and antibody binding reactions of IRDye conjugated membranes were done in blocking buffer for near infrared fluorescent Western blotting (Rockland) according to the manufacturer's instructions. The membranes were scanned with an Odyssey infrared scanner (Li-COR Biosciences).

LC-MS/MS analysis. Individual protein spots were cut out from Sypro Ruby stained gels using sterile scalpels and in-gel tryptic digestion was carried out with slight modifications of previous protocols

(Shevchenko et al. 1996, Jensen et al. 1999, Rowley et al. 2000). Briefly, each 2DE gel piece was washed in 50 µl of 100 mM ammonium bicarbonate and dehydrated in 100% acetonitrile (ACN). The gels were rehydrated with 50 µl of 10 mM DTT in 100 mM ammonium bicarbonate. Cysteines were reduced by heating at 56°C for 30 min and alkylated by incubating the samples with 55 mM iodoacetamide in 100 mM ammonium bicarbonate. Samples were washed in 100 mM ammonium bicarbonate, treated with ACN once again and dried in a Speedvac (Thermo Savant). The samples were rehydrated in digestion buffer containing 50 mM ammonium bicarbonate and sequencing grade modified trypsin (13 ng μl^{-1}) (Promega), and incubated overnight at 37°C. The following day, samples were extracted with 60% ACN/1% trifluro-acetic acid (TFA) for 15 min and sonicated in an ultrasonic water bath (Branson Ultrasonics) for 10 min. The extraction and sonication was repeated with 3% TFA. LC-MS/MS of the peptide digests were performed on a Thermo Finnigan LTQ Fourier Transform Mass Spectrophotometer (Thermo Electron Corporation) at the proteomics laboratories of Washington State University (Pullman) and Michigan State University (East Lansing). For LC-MS/MS analysis the extracted peptides were injected onto a peptide trap (Agilent Zorbax 300 SB-C18 5 × 0.3 mm). The bound peptides were then eluted onto a 15 cm × 75 um column (New Objectives Picofrit) packed with Microm Magic C18 AQ packing material and eluted over 30 min with a gradient of 5% to 90% B, with constant 10% C in 24 min using a Michrom Paradigm LC (Buffer A = 100% water, Buffer B = 100% acetonitrile, Buffer C = 1% formic acid) into a Thermo Electron LTQ-FTICR mass spectrometer with a flow rate of 250 nl min^{-1}. Survey scans were taken at 100 000× resolution and the top 5 ions in each survey scan were then subjected to automatic low energy, collision-induced dissociation (CID) in the LTQ.

Sequencing and database searches. Sequence homology analysis was performed by using the BLAST and PSI-BLAST algorithms (www.ncbi.nlm.nih.gov/). Pair-wise sequence alignments and multiple sequence alignments were carried out using ClustalW (www.ebi.ac.uk/clustalw/). LC-MS/MS peptide sequence data were analyzed using Mascot search (www.matrixscience.com/) against Swiss-Prot (http://ca.expasy.org/sprot/), UniProt (www.ebi.uniprot.org/index.shtml), NCBI-GenBank (www.ncbi.nlm.nih.gov/Genbank/index.html) and other public databases. Significant hits were assigned probability-based Mowse scores based on ion score, which is $-10 \times \log(p)$, where p is the probability that the observed match is a random event. Individual ion scores >24 ($p < 0.05$) indicate identity or extensive homology. Protein scores were derived from ion scores as a non-probabilistic basis for ranking protein hits. The MS/MS spectra were converted to peak lists using BioWorks Browser Version 3.2 and searched against an appropriate database using the Mascot searching algorithm. Identifications were considered positive if 2 peptides per protein were identified with a significant Mascot score ($p < 0.05$). Estimation of theoretical molecular weights and isoelectric points were performed using the Compute pI/ Mw algorithm (http://ca.expasy.org/) (Bjellqvist et al. 1993). The peptide sequences were also compared to a *Flavobacterium psychrophilum* (CSF-259-93) draft genome sequence data (G. D. Wiens et al. unpubl. data).

RESULTS

SDS-PAGE

The electrophoretic patterns of whole-cell lysates from the CSF-259-93 and ATCC-49418 strains of *Flavobacterium psychrophilum* are similar and only minor variations in band intensity were observed (Fig. 1A). For example, the intensity of 2 bands with approximate molecular masses of 18 and 19 kDa were greater in the ATCC-49418 strain when compared visually with the CSF-259-93 strain.

SDS-PAGE analysis of the ECP did show differences between the 2 strains of *Flavobacterium psychrophilum* (Fig. 1B). At least 5 bands were identified as being unique to the ECPs of the CSF-259-93 strain, and at least 3 bands were present only in the ATCC-49418 strain. In addition to the presence and absence of the aforementioned bands, it should be noted that slight differences in the staining intensity of several shared bands were observed (Fig. 1B).

2DE

2DE analysis of the ECP of the CSF-259-93 and ATCC-49418 strains revealed differences in secreted proteins of these strains (Fig. 2). Using this method of separation, at least 5 proteins were identified as unique to the CSF-259-93 strain and at least 2 proteins were unique to the ATCC-49418 strain.

Whole-cell lysates extracted from the CSF-259-93 and ATCC-49418 strains of *Flavobacterium psychrophilum* were also separated and further analyzed by 2DE. This provided increased resolution over single dimension SDS-PAGE and demonstrated that differences exist between the strains (Fig. 3). In addition to numerous shared proteins, at least 8 proteins were identified as being unique to the CSF-259-93 strain (Fig. 3, circled spots), and at least 1 protein was unique to the ATCC-49418 strain.

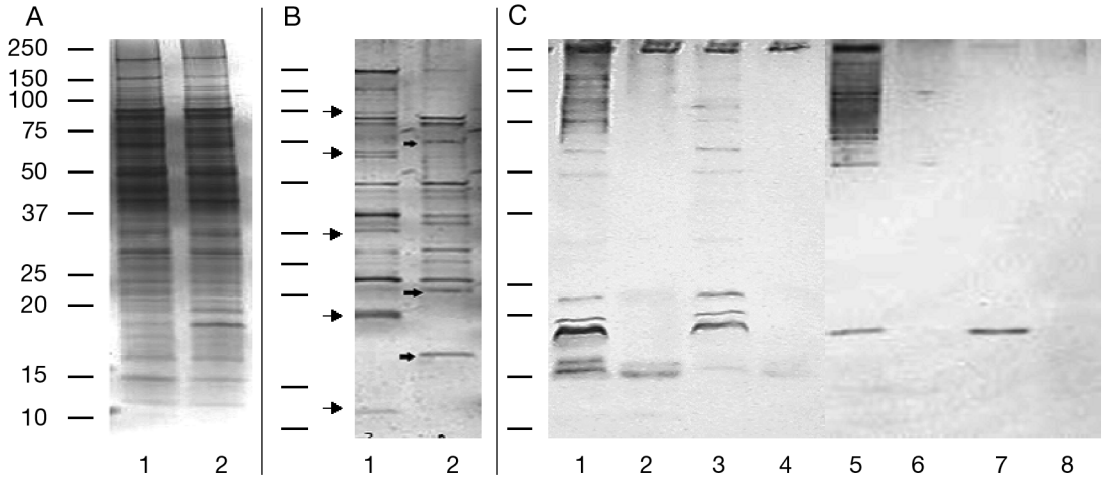

Fig. 1. *Flavobacterium psychrophilum.* (A) SDS-PAGE of whole cells: Lane 1, Strain CSF-259-93; Lane 2, Strain ATCC-49418. (B) SDS-PAGE of extracellular products (ECP) of *F. psychrophilum*: Lane 1, CSF-259-93; Lane 2, ATCC-49418; arrows indicate unique protein bands. (C) Western blot analysis of whole cells and ECP probed with trout anti-*F. psychrophilum* antiserum; Lane 1, CSF-259-93 whole cells; Lane 2, Proteinase K-digested CSF-259-93 whole cells; Lane 3, ATCC-49418 whole cells; Lane 4, Proteinase K-digested ATCC-49418 whole cells; Lane 5, ECP of CSF-259-93; Lane 6, Proteinase K-digested ECP of CSF-259-93; Lane 7, ECP of ATCC-49418; Lane 8, Proteinase K-digested ECP of ATCC-49418. Molecular mass markers (kDa) relate to (A), (B) and (C)

Western blot analysis: SDS-PAGE

Western blot analysis, using rainbow trout anti-CSF-259-93 serum, was utilized to probe whole-cell lysates and ECP of both the CSF-259-93 and ATCC-49418 strains. Antibodies reacted with numerous bands of the CSF-259-93 strain with high molecular masses of >50 kDa, as well as several low molecular mass bands ranging from 15 to 21 kDa (Fig. 1C). However, following Proteinase K digestion, only the 15 and 16 kDa bands were visualized (Fig. 1C). When used to probe the ATCC-49418 whole-cell lysate, antibodies primarily reacted with 3 low molecular mass bands ranging from 18 to 21 kDa and a small number of high molecular mass bands. Upon Proteinase K digestion, no bands were visualized (Fig. 1C).

Western blot analysis of the ECP of both strains revealed similar differences in immunoreactivity (Fig. 1C). The antiserum reacted with multiple high molecular mass proteins >50 kDa and an 18 kDa band in the CSF-259-93 strain, but antibodies only reacted with the 18 kDa band in the ATCC-49418 strain. Following Proteinase K digestion, no bands were visualized for either strain. It was particularly noted that very few bands in the 25 to 60 kDa region of all whole-cell and ECP samples reacted with the antiserum com-

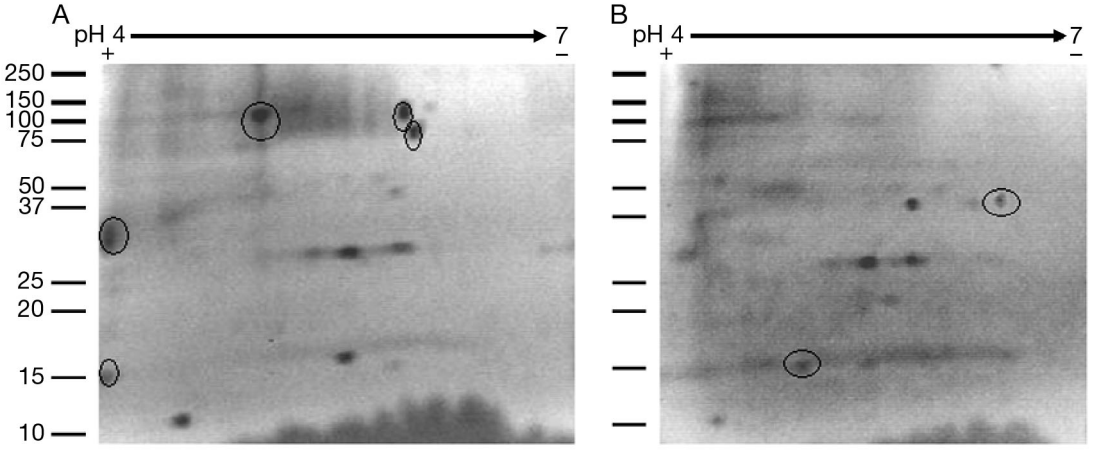

Fig. 2. *Flavobacterium psychrophilum.* 2DE analysis of ECP proteins (A) Strain CSF-259-93; (B) Strain ATCC-49418. Spots unique to each strain are circled. Molecular mass markers (kDa) relate to both (A) and (B)

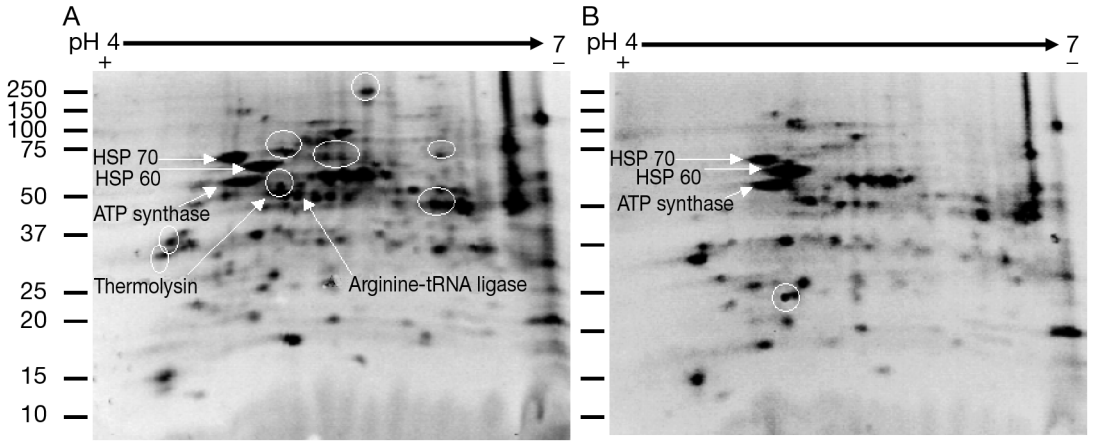

Fig. 3. *Flavobacterium psychrophilum.* 2DE analysis of whole-cell proteins. (A) Strain CSF 259-93; (B) Strain ATCC-49418. Spots unique to each strain are circled. Molecular mass markers (kDa) relate to both (A) and (B)

pared to other higher and lower molecular weight bands, and this was observed consistently in several repeated Western blot preparations.

Western blot analysis: 2DE

To allow further analysis and possible identification of protein antigens, Western blot analysis was also performed on whole-cell lysates separated by 2DE from the CSF-259-93 and ATCC-49418 strains of *Flavobacterium psychrophilum* using the anti-CSF-259-93 serum (Fig. 4). As observed with single dimension SDS-PAGE, antibodies reacted with more antigens in the CSF-259-93 strain than in the ATCC-49418 strain. Approximately 15 spots unique to the CSF-259-93 strain were shown to react with the trout antiserum, while 4 spots unique to the ATCC-49418 strain were recognized. Control antiserum showed no binding by Western blotting (data not shown).

LC-MS/MS analysis

The 8 proteins that appeared unique to the CSF-259-93 strain and 6 proteins shared between the 2 strains and isolated from the whole cell fractions were selected and analyzed by LC-MS/MS. This analysis followed by a Mascot search using peptide fragment sequences against public databases identified 5 proteins (Table 1). Two shared proteins of approximate molecular weights of 60 and 70 kDa were identified as heat shock proteins HSP 60 and HSP 70, respectively. The short peptide sequences obtained from LC-MS/MS analysis were further searched against a preliminary draft genome database of *Flavobacterium*

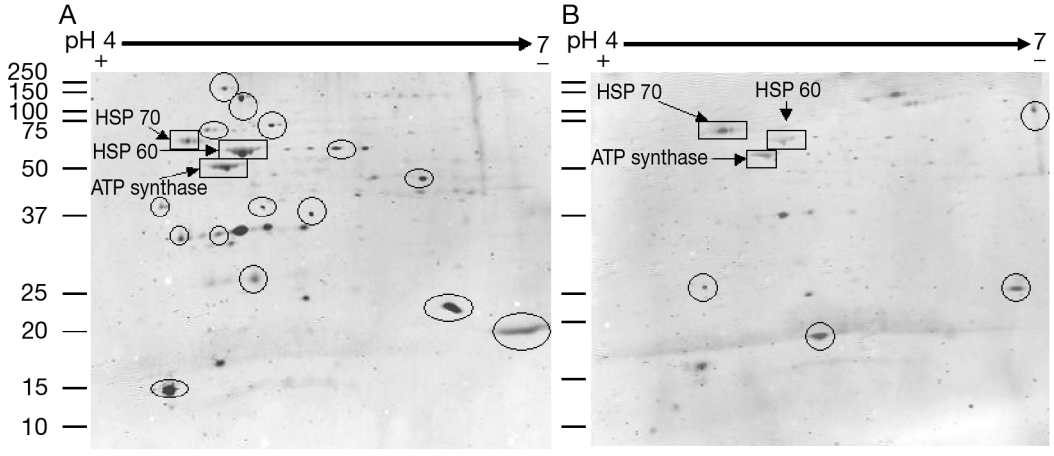

Fig. 4. *Flavobacterium psychrophilum.* Western blot analysis of whole-cell proteins separated by 2DE gels. (A) Strain CSF-259-93; (B) Strain ATCC-49418. Antigens unique to each strain are circled, highly immunogenic common antigens are enclosed in rectangles. Molecular mass markers (kDa) relate to both (A) and (B)

Table 1. *Flavobacterium psychrophilum.* LC-MS/MS analysis of selected proteins. Seq. cov.: sequence coverage; pI: isoelectric point

Protein spot no.	Description (GenBank Accession No.)	Queries matched	Seq. cov. (%)	Nominal mass (Da)	Calculated pI	Score
1[a]	60 kDa chaperonin (*F. psychrophilum*) (AAX56915)	NIKFDIEAR.D GVDALANAVKVTLGPK.G AAVEEGIVAGGGVALLR.A TIVENAGLEGSVVVAK.V GFTLENTTIEMLGTAK.R + Oxidation	15.5	57107	4.99	213
2[a]	70 kDa heat shock protein (*F. psychrophilum*) (AAX56916)	IADEVEKFFGK.K AKFEQLSDTLVK.R IEASSGLTSEEIER.M LYTAQELSAMTLQK.M + Oxidation IINEPTAAALAYGLDKK.G	13	67377	4.83	173
3[b]	Arginine–tRNA ligase (*Flavobacterium johnsoniae*) UW101 (ZP_01245348)	MSLQEILNPSIK.T + Oxidation (M) TQIINDRGIHICK.S	4.8	67899	5.63	36
4[a]	F0F1-type ATP synthase, alpha subunit (*Cytophaga hutchinsonii*) (ZP_00309791)	QPVTEPLQTGIK DTGRPALVVYDDLSK VGGNAQIK	9	56812	6.16	208
5[c]	Thermolysin (*Bacillus thermoproteolyticus*) (720316A)	LSYDGNNAAIR ALTQYLTPTSNFSQLR AAAVQSATDLYGSTSZZVASVK QAFDAVGVK	30	34313	5.03	476

[a]Protein spots reacted following Western blot analysis and were common to CSF-259-93 and ATCC-49418 strains
[b]Protein spot appears to be common to CSF-259-93 and ATCC-49418 strains
[c]Protein spot unique to CSF-259-93 strain

psychrophilum (CSF-259-93) and matched with open reading frames ORF RFPS02405 and ORF RFPS00215 in the database, corresponding to *groEL* (HSP 60) and *dnaK* (HSP 70) genes, respectively.

Three other proteins were identified by LC-MS/MS; an ATP synthase-alpha subunit homologue (shared by both strains), a thermolysin (unique to CSF-259-93), and an arginine–tRNA ligase that also appears shared by both strains (Table 1). The peptide fragment sequences of these proteins obtained by LC-MS/MS were also searched against the draft genome sequence of *Flavobacterium psychrophilum*. The ATP synthase-alpha subunit homologue and the arginine–tRNA ligase were confirmed to be ORF RFPS01834 and RFPS01165, respectively, but the thermolysin protein could not be matched with any ORF.

Nucleotide and translated protein sequences of both *groEL* and *dnaK* genes were compared with reported sequences of related bacteria, *Flavobacterium ferrugineum*, *F. hydatis, Cytophaga hutchinsonii* and other non-related bacteria using the ClustalW program, and were found to share many conserved regions and per-

Table 2. Sequence homology of *groEL* and *dnaK* genes of *Flavobacterium psychrophilum* to those of other bacteria. Aa: amino acid; % sim: percent similarity; na: not available

	groEL				*dnaK*			
	DNA		Aa		DNA		Aa	
	Length	% sim	Length	% sim	Length	% sim	Length	% sim
Flavobacterium hydatis	604	84	201	95	1083	61	361	65
Flavobacterium ferrugineum	589	69	196	77	1080	48	360	55
Cytophaga hutchinsonii	555	71	185	76	na	na	na	na
Escherichia coli	1644	62	548	66	2141	63	638	68
Brucella abortus	2404	61	544	65	na	na	na	na
Pseudomonas aeruginosa	1877	58	547	66	1914	58	637	68
Rhizobium leguminosarum	1697	56	542	66	1917	58	638	66

cent similarities ranging from 48 to 95% (Table 2). The complete DNA sequence of the *groEL* gene and the partial sequence of the *dnaK* gene of *F. psychrophilum* have been deposited in GenBank under Accession Nos. AY955285 and AY955286, respectively.

DISCUSSION

Development of an efficacious vaccine for CWD has been hindered by our lack of understanding of the pathogen and the host immune response. Research efforts in the past have focused on using vaccine preparations composed of either killed whole cells or groups of antigens of unknown identity. This study utilized standard SDS-PAGE and proteomics (2DE) to identify protein antigens possibly associated with virulence or host immunity. The application of 2DE to the study of fish pathogens is limited. Tan et al. (2002) and Srinivasa et al. (2004) used 2DE to identify virulence factors in the ECP of *Edwardsiella tarda*. In other studies, 2DE was used to differentiate expression of outer membrane proteins of *Aeromonas salmonicida* in response to low iron and *in vivo* growth conditions (Ebanks et al. 2004) and to identify the major outer membrane proteins of this pathogen (Ebanks et al. 2005). In addition, 2DE techniques have been combined with Western blotting and gene mining to identify antigens from *Helicobacter pylori* (Nilsson et al. 2000), *Staphylococcus aureus* (Vytvytska et al. 2002), *Bacillus anthracis* (Ariel et al. 2003), *Haemophilus influenzae* (Langen et al. 2000), as well as *Flavobacterium psychrophilum* (Crump et al. 2005).

The results of this study show that following initial separation of whole cell proteins and ECPs of *Flavobacterium psychrophilum* by single dimension SDS-PAGE, potential differences exist between a virulent (CSF-259-93) and non-virulent (ATCC 49418) strain (Fig. 1A,B). Further characterization by Western blotting revealed several unique immunogenic bands in whole cell and ECP products of the CSF-259-93 strain compared to the ATCC 49418 strain. Western blot analysis of total cellular proteins using anti-CSF-259-93 sera clearly showed that many proteins of >50 kDa molecular mass and a few low molecular mass proteins of 15 to 21 kDa were highly immunogenic in the virulent strain (Fig. 1C, Lane 1). This is supported by earlier studies investigating the immunogenic nature of this bacterium (LaFrentz et al. 2004).

In both strains, 3 bands in the 18 to 21 kDa region were equally reactive. Previously, it was shown that rainbow trout convalescent antiserum recognized a predominant 20 kDa protein (Crump et al. 2001) and rabbit antiserum recognized an 18 kDa surface layer protein (Massias et al. 2004). Hence, the 18 to 21 kDa bands observed in our study are likely to have been the same proteins reported in these earlier studies. The 15 kDa and 16 kDa antigens of the CSF-259-93 strain identified by Western blotting of the whole-cell preparation may be a carbohydrate (possibly associated with lipopolysaccharide), since it was not susceptible to Proteinase K digestion. This agrees with findings of Crump et al. (2001) and LaFrentz et al. (2004), who partially characterized lipopolysaccharide (LPS) components. Although identification of these components is important, the possibility that more than 1 antigen occurs at a particular band position (molecular mass) makes interpretation of SDS-PAGE data difficult. This is apparent in Fig. 1B, where a band at 18 kDa was only observed in the ECP proteins of the ATCC 49418 strain, whereas when analyzed by Western blotting (Fig. 1C) a band at the 18 kDa position reacted with the antiserum in both strains.

LaFrentz et al. (2004) demonstrated that whole-cell antigens of *Flavobacterium psychrophilum* CSF-259-93 in the approximate molecular mass range of 70 to 100 kDa were immunogenic and conferred near complete protection following immunization. Based on this, proteins of higher molecular mass were our primary focus. Although ECPs from both strains were analyzed by 2DE and differences were found (Fig. 2), protein spots were not further characterized. Since total cellular proteins were analyzed in the LaFrentz et al. (2004) study, we decided to give these priority for further identification. When total cellular proteins were separated by 2DE, at least 8 protein spots appeared to be unique to the virulent strain. Based on this analysis and our desire to characterize proteins primarily in the higher molecular mass ranges, the 8 cell-associated proteins unique to CSF-259-93, and 6 shared proteins (some of which were immunogenic) were analyzed by LC-MS/MS. Among them, 5 proteins were conclusively identified (3 immunogenic and shared between strains, 1 shared, and 1 unique to CSF-259-93) (Table 1). Of these, the identity of the 3 immunogenic proteins (nos. 1, 2, and 4 in Table 1) was further confirmed by comparison with a preliminary draft genome of *F. psychrophilum*. PCR amplification identified the genes encoding HSP 60 and HSP 70 as *groEL* and *dnaK*, respectively, and nucleotide comparisons showed extensive homology with HSPs of related and unrelated bacteria (Table 2).

The 2 highly immunogenic proteins identified as heat shock proteins HSP 60 and HSP 70 were of particular interest. They were expressed in both the virulent and non-virulent strains of *Flavobacterium psychrophilum*, and HSPs are known to be important targets of the host immune response in other pathogens (Wilhelm et al. 2005). Heat shock proteins are considered molecular chaperones expressed by prokaryotes and

eukaryotes that bind polypeptide chains, prevent aggregation, and support protein folding (Bukau & Horwich 1998). Their expression in most organisms is increased during stress, and bacterial HSPs are up-regulated during the course of most bacterial infections. Bacterial HSPs have been recognized as immunodominant antigens that swamp the host immune system with epitopes (Buchmeier & Heffron 1990, Lee & Horwitz 1995, Qoronfleh et al. 1998, Zügel & Kaufmann 1999).

The protein spots identified here as HSP 60 and HSP 70 were highly reactive against trout antiserum by Western blot (Fig. 4) indicating that these are indeed immunodominant antigens of the bacteria. Several studies have shown that anti-HSP responses are protective against many bacterial pathogens (Kaufmann et al. 1991, Blander & Horwitz 1993, Noll et al. 1994, Suzue & Young 1996). In addition, studies have shown that in some bacteria HSPs are located in the periplasm, on the bacterial surface and in some cases secreted into the surrounding medium (Gillis et al. 1985, Scorpio et al. 1994, Garduño et al. 1998, Hoffman & Garduño 1999, Hennequin et al. 2001). They have been implicated as bacterial virulence factors playing a significant role in mediating attachment. There are also reports suggesting that bacterial HSPs are ubiquitous host-evasion factors countering oxidative killing mechanisms of the host (Kaufmann 1989). In addition, they may provide immuno-stimulatory activity (Barrios et al. 1992, Noll et al. 1994) and act as B cell mitogens (Aosai et al. 2002, Rico et al. 2002). They have novel functions associated with activation of professional antigen-presenting cells (Kol et al. 2000, Bulut et al. 2002) and stimulation of cytokine (Asea et al. 2000) and chemokine production (Wang et al. 2001). The combined roles of HSPs as antigens and immunomodulators have led to the suggestion that they might provide an important link between innate and acquired immune responses (Srivastava et al. 1998). Recently, it was shown that fish immunized using recombinant HSP 60 and HSP 70 from another salmonid pathogen, *Piscirickettsia salmonis*, elicited a humoral immune response when intraperitoneally injected in Atlantic salmon *Salmo salar*, and protection was also conferred to fish challenged with *P. salmonis* (Wilhelm et al. 2005). These findings are interesting and suggest that the HSP proteins identified in this study may provide possible vaccine targets.

This study has demonstrated the usefulness of proteomics in the discovery of vaccine candidates of *Flavobacterium psychrophilum.* The approach presented here is unique in that targets are selected based on their presence within the pathogen, and their potential relationship to virulence or host immunity. Although further characterization of a number of these proteins is needed, the identification and isolation of genes encoding HSP 60 and HSP 70 of *F. psychrophilum* is significant and provides targets for vaccine efficacy studies.

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