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Proteomic analysis of *Flavobacterium psychrophilum* cultured *in vivo* and in iron-limited media

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ABSTRACT: *Flavobacterium psychrophilum* is the etiologic agent of bacterial coldwater disease, but the pathogenic mechanisms of this important fish pathogen are not fully understood. Identifying bacterial genes of *F. psychrophilum* differentially expressed *in vivo* may lead to a better understanding of pathogenesis and provide targets for vaccine development. Therefore, the present study used a proteomic approach to identify and quantify proteins of *F. psychrophilum* following growth *in vivo* and under iron-limited growth conditions. As determined by 2D polyacrylamide gel electrophoresis (2D-PAGE), numerous proteins exhibited different spot intensities following culture of the bacterium *in vivo*, and of these, 20 were selected and identified by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis and Mascot searches of the *F. psychrophilum* genome. Eighteen proteins exhibited increased spot intensities *in vivo*, and these included: several chaperone and stress proteins, gliding motility protein GldN, outer membrane protein OmpH, 2 probable outer membrane proteins (OmpA family), probable aminopeptidase precursor, probable lipoprotein precursor, 3-oxoacyl-[acyl-carrier-protein]-reductase, and several proteins with unknown function. Two proteins exhibited decreased spot intensities *in vivo* and were identified as ferritin FtnA and outer membrane protein OmpA (P60). Culture of *F. psychrophilum* in iron-limited media resulted in similar protein spot intensity changes for 6 of the 20 proteins identified following growth *in vivo*. Results from the present study suggest a role of upregulated proteins in the pathogenesis of *F. psychrophilum* and these may represent potential vaccine candidate antigens.

KEY WORDS: *Flavobacterium psychrophilum* · Pathogenesis · Vaccine candidate · Bacterial coldwater disease · Proteomic · *In vivo* growth · Iron-limited growth

INTRODUCTION

Flavobacterium psychrophilum is a Gram-negative bacterium that causes bacterial coldwater disease (CWD) and rainbow trout fry syndrome. This pathogen represents a significant problem for aquaculture due to its ability to cause high mortality and subsequent economic losses, a wide geographic distribution, and an ability to infect a large number of fish species (Nematollahi et al. 2003). Consequently, there is a need for an effective vaccine that can be easily administered to large numbers of susceptible fish.

The success of vaccine development against a pathogen is enhanced by knowledge of host–pathogen interactions, such as the host mechanisms involved in eliciting a protective immune response and the pathogenic mechanisms or virulence factors of the pathogen. In recent years, a number of such mechanisms have

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begun to be elucidated for *Flavobacterium psychrophilum*. Research has demonstrated that specific antibodies are important for protection, but non-specific immune components also appear to be involved in stimulating an effective immune response (LaFrentz et al. 2002, 2003, Wiklund & Dalsgaard 2002). It has been suggested that the pathogenicity of *F. psychrophilum* may be linked to adherence to host tissue, production of proteolytic enzymes, iron acquisition mechanisms, secretion systems, and production of lipopolysaccharide and a glycocalyx capsular layer (Nematollahi et al. 2003, Duchaud et al. 2007, Alvarez et al. 2008). However, the exact mechanisms are not fully understood.

Although an increased understanding of host–pathogen interactions related to *Flavobacterium psychrophilum* has been gained, the development of an efficacious vaccine has not been successful to date. There are reports of success via oral immunization of fish against CWD (Aoki et al. 2007). Nevertheless, most vaccines based on killed whole-cell preparations have been ineffective when administered using mass delivery methods that are practical for most species affected by *F. psychrophilum* in aquaculture (Obach & Laurencin 1991, LaFrentz et al. 2002). Therefore, recent efforts have been aimed at identifying specific bacterial antigens to target for vaccine development. Such research has resulted in the identification of numerous proteins or specific fractions of *F. psychrophilum* (Rahman et al. 2002, Merle et al. 2003, LaFrentz et al. 2004, Massias et al. 2004, Crump et al. 2005, 2007, Dumetz et al. 2007, 2008, LaFrentz 2007, Sudheesh et al. 2007), some of which have been shown to be protective (Rahman et al. 2002, LaFrentz et al. 2004, Dumetz et al. 2006, Crump et al. 2007). These studies demonstrate the potential for identifying vaccine candidates; however, the work was performed using *in vitro* cultured bacteria, and our knowledge of differential gene expression by *F. psychrophilum* grown *in vivo* is lacking.

The *in vitro* culture environment is artificial and does not necessarily represent *in vivo* conditions. One example of this relates to the availability of iron, which is an essential element for the growth of bacteria. Iron is readily available in most bacteriological media; however, iron is limited *in vivo* due to host production of iron-binding proteins such as transferrin and lactoferrin (Otto et al. 1992). Brown et al. (1988) suggested that bacteria grown *in vivo* should be analyzed in studies aimed at identifying virulence factors and vaccine candidate antigens, because expression of these factors may be altered in the *in vitro* environment. Several studies have taken this approach and identified differences in bacterial pathogens by comparing cells grown *in vitro* and *in vivo*, including differences in carbohydrate components (Thornton et al. 1993, Wang et al. 2004, Poobalane et al. 2008) and protein regulation (Thornton et al. 1993, Colquhoun & Sorum 1998, Bakopoulos et al. 2004, Ebanks et al. 2004, Poobalane et al. 2008).

A possible explanation for the lack of protection following immunization with killed whole-cell *Flavobacterium psychrophilum* preparations is that important virulence determinants and/or protective components of the bacterium are not produced during *in vitro* culture in standard bacteriological media. This has been suggested for fish pathogens such as *Aeromonas salmonicida* (Thornton et al. 1993) and *Photobacterium damsela* subsp. *piscicida* (Bakopoulos et al. 2004). *In vitro* culture of *F. psychrophilum* can be difficult, and the media currently used is not optimal (Cepeda et al. 2004). Numerous attempts have been made to enhance bacterial growth *in vitro* (Daskalov et al. 1999, Michel et al. 1999, Cepeda et al. 2004); however, available media does not accurately reflect the environment to which *F. psychrophilum* is exposed *in vivo*. An analysis of specific protein changes following growth of *F. psychrophilum in vivo* and in iron-limited conditions may identify virulence factors and provide targets for vaccine development. The objective of the current study was to identify proteins of *F. psychrophilum* that increase or decrease in spot intensity (as assessed by 2D polyacrylamide gel electrophoresis [2D-PAGE]) following growth *in vivo* and *in vitro* under iron-limited conditions.

MATERIALS AND METHODS

Fish and rearing conditions. Fifteen rainbow trout with a mean weight of 150 g were obtained from the Aquaculture Research Institute (University of Idaho, Moscow). Prior to implantation, fish were acclimated for 1 wk in a 190 l tank supplied with de-chlorinated municipal water at 15°C. Following implantation, fish were held in a 190 l tank supplied with 15°C water. The fish were not fed during the trial. All procedures utilizing fish were approved by the University of Idaho Animal Care and Use Committee (Protocol # 2006-31).

Bacterial culture. A previously frozen glycerol stock of a virulent strain of *Flavobacterium psychrophilum*, CSF-259-93 (Sudheesh et al. 2007), was plated for isolation on tryptone yeast extract salts (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% $MgSO_4$, 0.05% $CaCl_2$, pH 7.2) agar (Holt et al. 1993) and incubated at 15°C for 4 d. Several colonies were transferred to 20 ml TYES broth and incubated at 15°C for 72 h. The 72 h cultures were used in the following growth conditions.

Growth in iron-limited conditions: A trial experiment was implemented to determine the optimal concentration of the iron chelator 2,2-dipyridyl (DPD;

Sigma-Aldrich) to obtain inhibited growth of *Flavobacterium psychrophilum*. Duplicate culture tubes of 20 ml TYES broth containing 0, 50, 75, and 100 µM DPD were inoculated with 50 µl of a 72 h culture of *F. psychrophilum* and incubated statically at 15°C. Static growth conditions were used due to the tendency of this strain to auto-agglutinate when cultured on an orbital shaker. The growth was monitored daily by measuring the optical density at 525 nm.

Flavobacterium psychrophilum was cultured in TYES broth with or without 50 µM DPD to obtain cells for proteomic analyses. Two milliliters of a 72 h culture were inoculated into 200 ml TYES broth with or without 50 µM DPD and incubated statically at 15°C for 72 h. Cells were harvested by centrifugation at 4300 × *g* for 15 min at 4°C and washed twice with sterile phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.2).

Growth in vivo: A 72 h culture of *Flavobacterium psychrophilum* (2 ml) was inoculated into 200 ml TYES broth and incubated at 15°C for 72 h. Cells were harvested by centrifugation at 4300 × *g* for 15 min at 4°C, washed twice with sterile PBS, and resuspended to an optical density of 0.5 at 525 nm in PBS.

Dialysis tubing chambers (n = 30), with a molecular weight cutoff of 12 kDa, were prepared as described by Colquhoun & Sorum (1998) and each chamber was inoculated with 1 ml of the resuspended *Flavobacterium psychrophilum*. Fifteen rainbow trout were implanted (peritoneal cavity) with 1 dialysis tubing chamber each as described by Colquhoun & Sorum (1998), with the exception that 90 mg l^{-1} tricaine methanesulfonate (MS-222; Argent Chemicals) was used for anesthetization and gill flushing. Following surgery, fish were immediately returned to the culture tank and monitored for recovery. Control dialysis tubing chambers (n = 15) were inoculated as described at the beginning of this paragraph and incubated in 500 ml TYES broth at 15°C.

Harvest and quantification of bacteria from dialysis tubing chambers. At 2, 4, 6, 8, and 10 d post-implantation, 3 fish were killed with an overdose of MS-222 (250 mg l^{-1}), and the dialysis tubing chambers were removed. Additionally, 3 control chambers were removed from TYES broth at each sampling time. The external surface of each chamber was washed with sterile PBS, and the contents were extracted using a sterile 21-gauge needle and placed into sterile 1.5 ml microcentrifuge tubes. Twenty microliters of each culture were streaked for isolation onto tryptic soy agar and TYES plates and incubated at 15°C for 72 h to check for bacterial contamination. The number of colony-forming units (CFUs) of *Flavobacterium psychrophilum* ml^{-1} of each culture was determined by plating 10-fold serial dilutions on TYES agar plates. The remaining culture was centrifuged at 5000 × *g* for 20 min at 4°C, the supernatant was removed, and bacterial pellets were stored at –20°C.

Preparation of whole-cell lysates. Whole-cell lysates of *Flavobacterium psychrophilum* cultured in TYES and TYES with 50 µM DPD were prepared by resuspending 100 mg (wet wt) of cells into 800 µl deionized water containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich). The cell suspensions were sonicated on ice 8 times for 30 s each at 20% amplitude (Model 500 Sonic Dismembrator, Fisher Scientific). The suspensions were cooled on ice for 2 min between each 30 s sonication step. The lysates were centrifuged at 16 000 × *g* for 30 min at 4°C, and then the supernatants were removed and frozen at –80°C.

For each sampling period, the *Flavobacterium psychrophilum* cell pellets obtained from triplicate dialysis tubing chambers incubated *in vivo* or *in vitro* were combined according to treatment and resuspended into 200 µl deionized water containing 0.1 mM PMSF. Whole-cell lysates were prepared by sonicating the resuspended bacteria for 8 min in a cup sonicator using chilled deionized water (Sonicator Ultrasonic Processor XL, Misonix). Following sonication, the lysates were centrifuged at 16 000 × *g* for 30 min at 4°C, and then supernatants were removed and frozen at –80°C. The protein concentration of each lysate was determined using a Micro BCA Protein Assay (Pierce) according to the manufacturer's directions.

2D-PAGE. Whole-cell lysate proteins were diluted to a concentration of 0.48 µg $µl^{-1}$ in rehydration buffer containing 8 M urea, 50 mM dithiothreitol (DTT), 4% 3-[(3-cholamidopropyl)dimethylammonia]-1-propanesulfonate, and 0.25% Bio-Lyte 3/10 ampholyte (Bio-Rad). Protein samples were vortexed for 1 min, incubated at room temperature for 10 min, then centrifuged at 16 000 × *g* for 10 min at room temperature. Immobilized pH gradient (IPG) strips (7 cm, pH 3 to 10; Bio-Rad) were passively rehydrated with 60 µg protein (125 µl $strip^{-1}$) for 1 h, covered with 2 ml mineral oil, and then rehydrated overnight at room temperature. First-dimension isoelectric focusing was performed using a PROTEAN IEF Cell (Bio-Rad). IPG strips were focused at 250 V for 15 min, ramped up to 4000 V over 2 h, held at 4000 V for 20 000 volt hours (Vh), and then held at 500 V until frozen at –80°C. The total Vh applied to the IPG strips was approximately 25 000. Focused strips were thawed and equilibrated for 20 min in equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, 20% glycerol) containing 130 mM DTT and then equilibrated for an additional 20 min in equilibration buffer containing 135 mM iodoacetamide. Second-dimension separation of equilibrated IPG strips was performed in 10 to 20% linear gradient polyacrylamide gels (Bio-Rad) using a Mini-PROTEAN 3 electrophore-

sis cell, and Precision Plus (Bio-Rad) protein standard plugs were included. Gels were electrophoresed using the standard Laemmli buffer system (Laemmli 1970) for 30 min at 5 mA gel^{-1} and then at 12 mA gel^{-1} until the dye front migrated out of the gels.

To determine the reproducibility of this method, whole-cell lysates from 2 biological replicates of *Flavobacterium psychrophilum* cultured in TYES broth were analyzed. Duplicate IPG strips were focused with proteins from each biological replicate and 2D-PAGE was performed as described in the previous paragraph. Gels were analyzed as described below ('Image analysis of 2D-PAGE gels') and a scatter plot and regression line was generated to determine the degree of similarity between the gels obtained from both biological replicates. A correlation coefficient of 0.93 was obtained, demonstrating the reproducibility of the method (data not shown).

Image analysis of 2D-PAGE gels. Gels were stained with SYPRO Ruby (Bio-Rad) according to the manufacturer's directions, digitally imaged using a FLUOR-S MultiImager (Bio-Rad), and analyzed with PDQuest version 8.0.1 (Bio-Rad). Automated spot detection and matching were used followed by a manual inspection for accuracy, and all gels were normalized using the local regression model. For each comparison (normal TYES versus iron-limited TYES and *in vivo* versus *in vitro* at 2, 4, 6, 8, and 10 d), 4 gels were analyzed representing 1 biological replicate and duplicate gels for each culture condition. Gels were analyzed for qualitative differences (protein spots unique to each culture condition) and quantitative differences (protein spot intensity changes ≥2.0-fold). For each protein identified by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), the spot intensities at each sampling time were quantified and reported as the ratio of spot intensity of *in vivo* culture to *in vitro* culture to determine if the spot intensities changed over the course of the 10 d experiment.

Identification of differentially expressed proteins. Proteins of interest were excised from SYPRO Ruby-stained gels, placed in 5% acetic acid, and submitted to the Proteomics Core Facility at Michigan State University (East Lansing) for LC-MS/MS analysis. Proteins within each excised gel piece were subjected to in-gel tryptic digestion (Shevchenko et al. 1996, Jensen et al. 1999). The extracted peptides were automatically injected by a Michrom Paradigm Endurance Bio-Cool Autosampler onto a Michrom Paradigm Platinum Peptide Nanotrap (C18, 0.15 × 50 mm) and washed for 5 min. The bound peptides were then eluted onto a 10 cm × 75 µm New Objective Picofrit column packed with Michrom Magic C18 AQ packing material. Peptides were eluted from this column over 30 min with a gradient of 5% B to 90% B, with constant 10% C in 24 min using a Michrom Paradigm MDLC (Buffer A = 100% water; Buffer B = 100% acetonitrile; Buffer C = 1% formic acid) into a ThermoFisher LTQ linear ion trap mass spectrometer at a flow rate of 250 nl min^{-1}. The top 5 ions in each survey scan were subjected to data-dependent zoom scans followed by low-energy collision-induced dissociation and the resulting MS/MS spectra were converted to peak lists using BioWorks Browser Version 3.2 (ThermoFisher). The peak lists were searched against the *Flavobacterium psychrophilum* (ATCC 49511) genome (Duchaud et al. 2007) (EMBL database accession no. AM398681) using the Mascot searching algorithm Version 2.2 (Matrix Science). For all searches, peptide tolerance was set to 200 ppm, fragment tolerance was 0.8 Da, 2 tryptic missed cleavages were allowed, and the carbamidomethyl (C) and oxidation (M) peptide modifications were considered. All spectral assignments were then validated using Scaffold Version Scaffold-01_07_00 (Proteome Software). Identifications were considered positive if 2 or more peptides from the same protein were identified and the confidence level of the Scaffold analysis was >95%. Estimation of the theoretical molecular mass (M_r) and isoelectric points (pI) of the proteins identified were determined using the Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html).

RESULTS

Growth in iron-limited media

Growth curves of *Flavobacterium psychrophilum* (CSF-259-93) were determined in TYES broth containing 0, 50, 75, and 100 µM of DPD at 15°C (Fig. 1). There was a dose response between the concentration of DPD and bacterial growth. Growth was partially inhibited at 50 µM DPD and almost completely inhibited at 100 µM DPD (Fig. 1). A DPD concentration of 50 µM was chosen for the subsequent growth of *F. psychrophilum* in iron-limited conditions to identify proteins with different spot intensities.

In vivo growth in dialysis tubing chambers

Pure *Flavobacterium psychrophilum* cultures were obtained from all dialysis tubing chambers with the exception of 1 chamber recovered from a fish on Day 8, and this culture was removed from the analysis. The number of CFUs ml^{-1} was determined for *F. psychrophilum* cultured *in vivo* and *in vitro* in dialysis tubing chambers at each sampling period (Fig. 2). *F. psychrophilum* cultured *in vivo* exhibited an initial decrease in viable cells. Growth then increased rapidly from

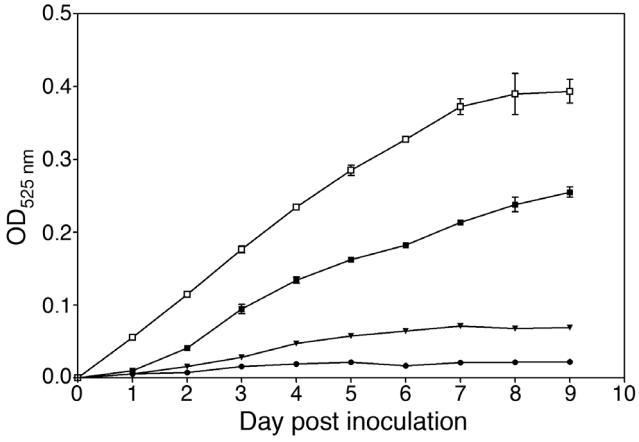

Fig. 1. *Flavobacterium psychrophilum*. Growth curves of *F. psychrophilum* (CSF-259-93) cultured in tryptone yeast extract salts broth containing 0 (□), 50 (■), 75 (▼), or 100 (●) µM 2,2-dipyridyl (DPD). Duplicate cultures were grown under each condition at 15°C and growth was monitored daily by determining the optical density (OD) at 525 nm. Error bars indicate SD

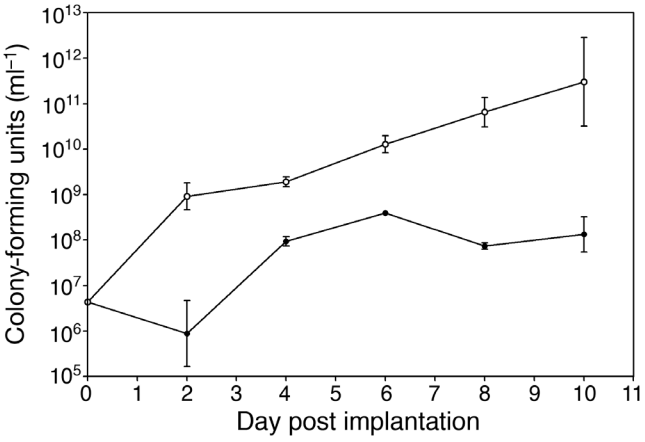

Fig. 2. *Flavobacterium psychrophilum*. Growth curves of *F. psychrophilum* (CSF-259-93) cultured in dialysis tubing chambers *in vitro* (○) or *in vivo* (●). Triplicate cultures were removed at each sampling period, and the number of colony-forming units ml^{-1} was determined by plating serial dilutions onto tryptone yeast extract salts agar plates. Error bars indicate SD

Days 2 to 6, and then cell numbers decreased slightly through 10 d post-implantation. *F. psychrophilum* cultured *in vitro* exhibited linear growth throughout the trial (Fig. 2).

2D-PAGE profile following iron-limited conditions

Whole-cell lysate proteins of *Flavobacterium psychrophilum* cultured in TYES broth and iron-limited TYES broth were separated by 2D-PAGE, stained, and analyzed to identify proteins with different spot intensities (Fig. 3). Thirty-two proteins exhibited a

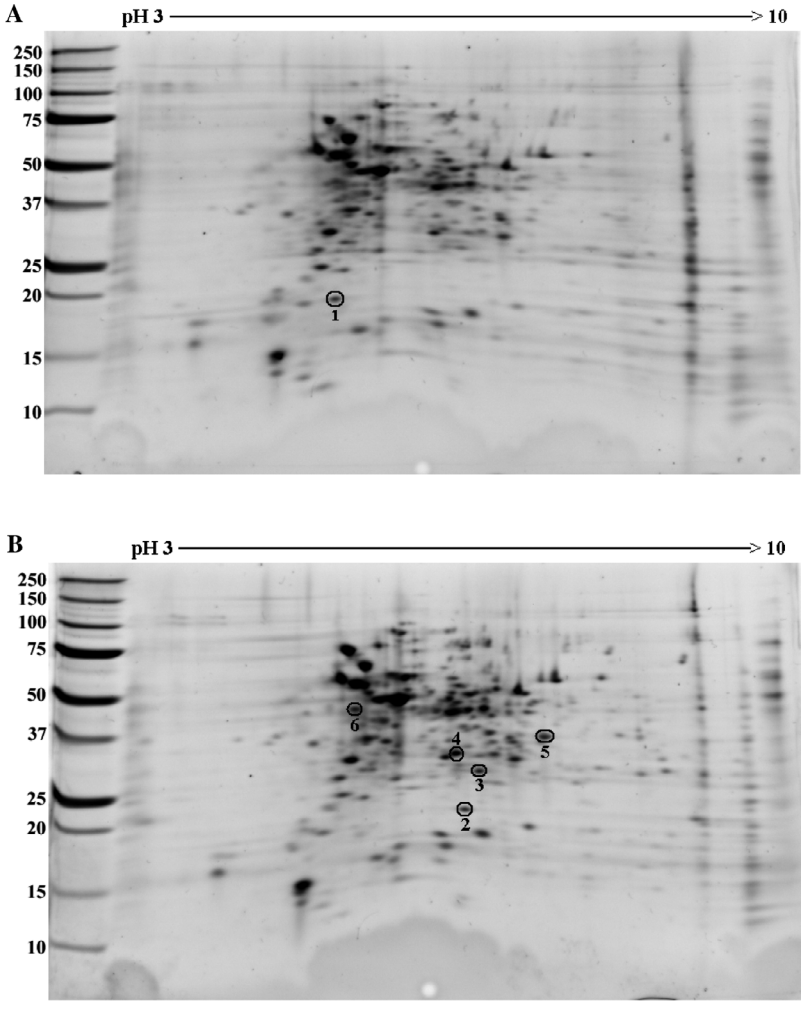

Fig. 3. *Flavobacterium psychrophilum*. 2D polyacrylamide gel electrophoresis analysis of whole-cell lysate proteins of *F. psychrophilum* cultured in (A) tryptone yeast extract salts (TYES) broth or (B) iron-limited TYES broth. Proteins were visualized by SYPRO Ruby staining. Proteins with increased spot intensities for each growth condition are circled, and protein spot numbers correspond to proteins identified by liquid chromatography-mass spectrometry/mass spectrometry (see Table 1). First-dimension separation was performed using immobilized pH gradient strips with a pH range of 3 to 10, and second-dimension molecular mass markers (kDa) are indicated to the left of each gel

≥2.0-fold change in spot intensity, 15 or 17 of which were increased or decreased in iron-limited media, respectively. Additionally, 13 proteins were identified as unique to iron-limited media and 8 proteins were unique to normal TYES media. Five proteins exhibiting increased spot intensities following culture in iron-limited media were selected for identification (Fig. 3B). Two of these proteins were identified as proteins with unknown function, whereas the other 3 were identified as universal stress protein UspA, 3-oxoacyl-[acyl-carrier-protein]-reductase, and gliding motility protein GldN (Table 1). One protein with decreased spot intensity in iron-limited media (Fig. 3A) was selected and identified as ferritin FtnA (Table 1).

2D-PAGE profile following *in vivo* growth

A comparative analysis of *Flavobacterium psychrophilum* proteins following *in vivo* and *in vitro* growth in dialysis tubing chambers revealed numerous differences, and this was best exemplified at 8 d post-implantation (Fig. 4). Fifty-two proteins exhibited a ≥2.0-fold change in spot intensity, 27 or 25 of which were increased or decreased following *in vivo* growth, respectively. Additionally, 31 proteins were identified as unique to bacteria cultured *in vivo* and 12 proteins were unique to *in vitro* cultured cells. Eighteen proteins exhibiting increased spot intensities *in vivo* were selected for identification (Fig. 4, Table 1). Single unique proteins were positively identified for 16 of these and included several chaperone and stress proteins, gliding motility protein GldN, outer membrane protein OmpH, probable outer membrane protein (OmpA family), probable aminopeptidase precursor, probable lipoprotein precursor, 3-oxoacyl-[acyl-carrier-protein]-reductase, and 5 proteins with unknown function (Fig. 4, Table 1). For one spot (Protein spot no. 10), 2 proteins were identified (FP1486, protein of unknown function; FP0770, probable acetyl-CoA acetyltransferase), and for another spot (Protein spot no. 17) a single protein sequence was identified but 2 identical genes (FP0321 and FP1169, probable outer membrane protein OmpA family) were present in the genome. Two proteins exhibiting decreased spot intensities *in vivo* were selected and identified as ferritin FtnA and

Table 1. *Flavobacterium psychrophilum*. Proteins of *F. psychrophilum* identified by liquid chromatography-mass spectrometry/mass spectrometry. Protein spot numbers refer to proteins in Figs. 3 & 4. M_r: molecular mass; pI: isoelectric point

Spot no.	Identified protein	NCBI accession no. (locus tag)	Theoretical M_r (kDa) / pI	No. peptides matched	Sequence coverage (%)	Mascot score
1	Ferritin FtnA	FP1782	20.3 / 4.94	9	55	519
2	Protein of unknown function	FP1493	22.7 / 8.61	7	54	538
3	3-oxoacyl-[acyl-carrier-protein]-reductase	FP0964	26.3 / 5.95	3	15	267
4	Universal stress protein UspA	FP0467	30.5 / 5.73	3	17	326
5	Protein of unknown function	FP1496	36.2 / 8.77	18	60	1213
6	Gliding motility protein GldN	FP1970	39.1 / 5.82	7	28	595
7	Probable lipoprotein precursor	FP2424	15.3 / 5.10	3	37	216
8	Protein of unknown function	FP0012	16.1 / 7.83	4	59	366
9	Outer membrane protein OmpH	FP2098	18.7 / 8.58	6	42	995
10	Protein of unknown function Probable acetyl-CoA acetyltransferase	FP1486 FP0770	44.7 / 5.61 40.8 / 5.39	9 4	34 17	701 465
11	Chaperone protein HtpG	FP1509	71.6 / 5.07	16	26	1039
12	Probable lipoprotein of unknown function	FP0110	60.7 / 6.35	6	16	444
13	ATPase with chaperone activity ATP-binding subunit ClpB	FP1765	96.9 / 5.33	23	32	1927
14	Probable aminopeptidase precursor	FP1888	84.0 / 6.01	8	15	620
15	Probable outer membrane protein, OmpA family	FP2411	69.9 / 6.6	4	12	407
16	Probable outer membrane protein, OmpA family	FP2411	69.9 / 6.6	20	35	1156
17	Probable outer membrane protein, OmpA family	FP0321[a] FP1169[a]	72.5 / 6.52	17	36	1552
18	Peptidyl-prolyl *cis-trans* isomerase precursor SurA	FP1167	52.4 / 7.04	8	20	726
19	Protein of unknown function	FP2205	63.3 / 8.78	6	13	428
20	Outer membrane protein OmpA (P60)	FP0156	49.4 / 4.87	6	19	602

[a]Protein sequences are identical and thus the gene cannot be definitely determined

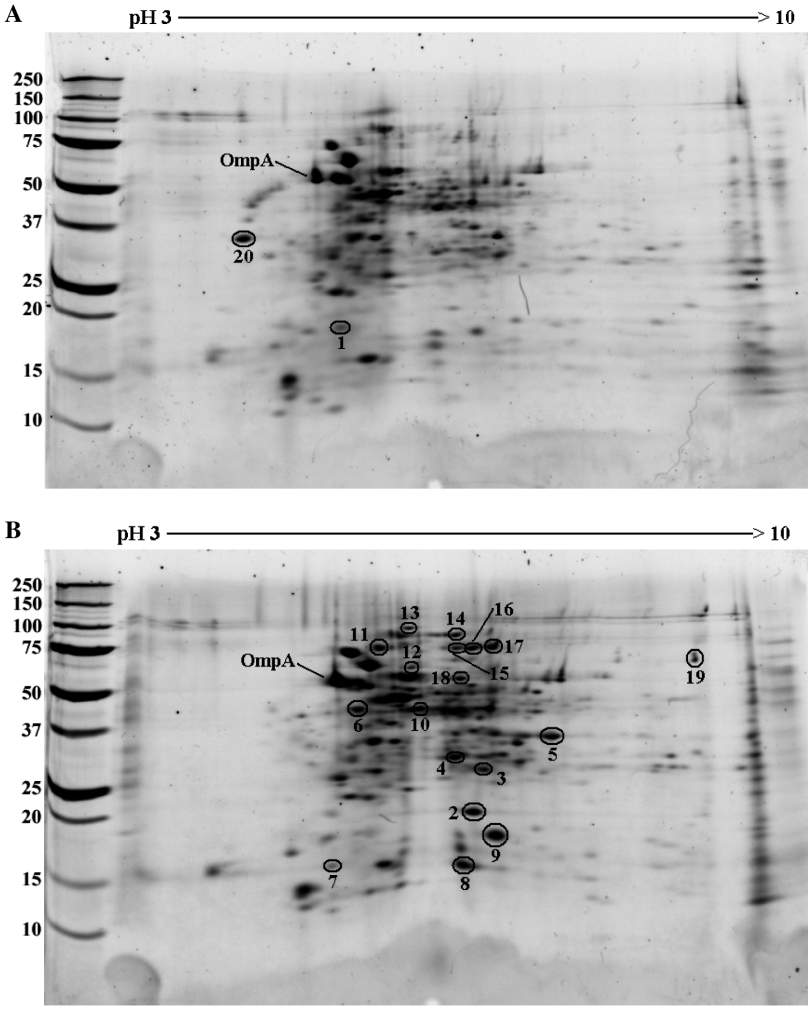

Fig. 4. *Flavobacterium psychrophilum*. 2D polyacrylamide gel electrophoresis analysis of whole-cell lysate proteins of *F. psychrophilum* cultured in dialysis tubing chambers (A) *in vitro* or (B) *in vivo* for 8 d. Proteins were visualized by SYPRO Ruby staining. Proteins with increased spot intensities for each growth condition are circled, and protein spot numbers correspond to proteins identified by liquid chromatography-mass spectrometry/mass spectrometry (see Table 1). The protein spot labeled as OmpA (P60) was previously identified (LaFrentz 2007). First-dimension separation was performed using immobilized pH gradient strips with a pH range of 3 to 10, and second-dimension molecular mass markers (kDa) are indicated to the left of each gel

outer membrane protein OmpA (P60), respectively (Fig. 4, Table 1).

The ratio of spot intensities between *in vivo* and *in vitro* cultured *Flavobacterium psychrophilum* cells for each protein identified by LC-MS/MS was calculated to determine if there were trends over the course of the 10 d experiment (Table 2). Protein spot nos. 2, 5, 8, 13, and 19 were consistently present at higher spot intensities *in vivo* at each sampling time. Protein spot nos. 9, 10, and 11 exhibited a general trend of increasing spot intensities *in vivo* over the course of the experiment. Protein spot nos. 3, 6, 12, 15, 16, and 18 exhibited a general trend of increasing spot intensities *in vivo* from Days 2 to 8, and then decreased spot intensities *in vivo* on Day 10. Protein spot no. 1 was consistently present at lower spot intensities *in vivo* from Days 2 to 6 and was absent *in vivo* on Days 8 and 10. At each sampling time, Protein spot no. 7 was only present in *in vivo* cultured cells, and Protein spot no. 20 was only present in *in vitro* cultured cells. There were no major differences in the protein spot intensities of *F. psychrophilum* cultured *in vitro* in the dialysis tubing chambers over the 10 d experiment (data not shown).

DISCUSSION

Stress resistance is an important mechanism for survival in the host environment (Vorob'eva 2004). Bacteria produce a number of heat shock and chaperone proteins that are important for this adaptation (Vorob'eva 2004). Similarly, several chaperone and stress proteins of *Flavobacterium psychrophilum* exhibited increased spot intensities following *in vivo* growth. These included the chaperone protein HtpG, peptidyl-prolyl *cis-trans* isomerase precursor SurA, ATPase with chaperone activity ClpB, and universal stress protein UspA. Additionally, a protein identified as one with unknown function (Protein spot no. 19; Table 1) contained a tetratricopeptide repeat motif that is common in chaperone proteins or proteins involved in protein–protein interactions (Blatch & Lassle 1999). This suggests that these proteins may be important for *in vivo* survival and pathogenesis of *F. psychrophilum*, and there is evidence for this based on homologous proteins in other pathogenic bacterial species.

The HtpG chaperone protein plays an important role in providing bacterial cells with protection from oxidative and temperature stress (Tanaka & Nakamoto 1999, Okano et al. 2006), and the production of this protein in *Porphyromonas gingivalis* is upregulated following contact with human epithelial cells (Hosogi & Duncan 2005). Further, this protein may play an important role in the pathogenesis of *Francisella tularensis* because an *htpG* mutant was highly attenuated and unable to sustain intracellular growth (Weiss et al. 2007).

Table 2. *Flavobacterium psychrophilum*. Quantification of *F. psychrophilum* proteins identified by 2D polyacrylamide gel electrophoresis from bacteria cultured *in vivo* for 10 d. Protein spot intensity ratio: *in vivo* to *in vitro* cultured cells. vit: protein absent from *in vivo* cultured cells; viv: protein only present for *in vivo* cultured cells; neith: protein absent from both growth conditions

Spot no.	Identified protein	Protein spot intensity ratio				
		Day 2	Day 4	Day 6	Day 8	Day 10
1	Ferritin FtnA	0.24	0.38	0.19	vit	vit
2	Protein of unknown function	viv	3.05	3.22	2.53	8.56
3	3-oxoacyl-[acyl-carrier-protein]-reductase	0.82	1.12	1.72	4.2	1.48
4	Universal stress protein UspA	2.08	1.72	1.55	2.43	0.67
5	Protein of unknown function	6.84	5.82	5.03	8.27	5.72
6	Gliding motility protein GldN	0.67	0.98	1.74	2.85	1.29
7	Probable lipoprotein precursor	viv	viv	viv	viv	viv
8	Protein of unknown function	2.99	2.67	1.95	2.12	2.54
9	Outer membrane protein OmpH	1.54	1.7	1.68	1.95	3.18
10	Protein of unknown function Probable acetyl-CoA acetyltransferase	neith	neith	0.99	viv	2.04
11	Chaperone protein HtpG	neith	1.67	1.33	2.74	2.46
12	Probable lipoprotein of unknown function	1.03	0.51	3.4	viv	1.45
13	ATPase with chaperone activity ATP-binding subunit ClpB	2.03	viv	1.86	viv	5.17
14	Probable aminopeptidase precursor	viv	3.0	1.26	3.52	1.53
15	Probable outer membrane protein, OmpA family	viv	viv	2.13	4.6	1.38
16	Probable outer membrane protein, OmpA family	0.91	1.21	1.64	3.18	1.82
17	Probable outer membrane protein, OmpA family	1.35	1.38	1.3	1.68	1.76
18	Peptidyl-prolyl *cis-trans* isomerase precursor SurA	1.5	1.5	1.97	1.57	1.17
19	Protein of unknown function	viv	viv	2.37	6.10	2.12
20	Outer membrane protein OmpA (P60)	vit	vit	vit	vit	vit

A primary role of the SurA chaperone protein is to assist in the folding of outer membrane proteins (Lazar & Kolter 1996). SurA interacts with proteins involved in pilus production and adhesion (Justice et al. 2005). Mutations in *surA* have been associated with attenuation in *Salmonella enterica* and *Klebsiella pneumoniae* (Sydenham et al. 2000, Struve et al. 2003). Interestingly, the SurA protein of *Brucella abortus* has been identified as a protective antigen (Delpino et al. 2007). Immunization of mice with recombinant SurA from *B. abortus* conferred a protective immune response as determined by a significant reduction in the number of CFUs in the spleen (Delpino et al. 2007).

ClpB protein is a chaperone that belongs to the Clp ATPase family, and it is involved in protein disaggregation (Squires & Squires 1992) and stress responses (Squires & Squires 1992, Ekaza et al. 2001). Research suggests that this protein may be involved in the virulence of bacterial pathogens (Chastanet et al. 2004, Yuan et al. 2007). The ClpB protein of *Francisella tularensis* is immunogenic (Havlasova et al. 2005), and similarly the ClpB protein of *Flavobacterium psychrophilum* is immunogenic (LaFrentz 2007).

The expression of the gene encoding the stress protein UspA increases following numerous stressors (Nystrom & Neidhardt 1992) and during *in vivo* growth (Twine et al. 2006, Hughes et al. 2007). Additionally, there is evidence that the UspA protein of *Salmonella typhimurium* is involved in pathogenesis because a mutant strain with a defective *uspA* gene was attenuated in mice when administered orally at low doses (Liu et al. 2007). Research is needed to further define the role of these chaperone and stress proteins in the pathogenesis of *Flavobacterium psychrophilum* and their potential as vaccine candidate antigens.

The *Flavobacterium psychrophilum* gliding motility protein GldN exhibited increased spot intensity during growth *in vivo* and in iron-limited media. The role of gliding motility in the pathogenesis of *F. psychrophilum* is not known, but in general, motility is assumed to be an important factor in host–pathogen interactions (Ottemann & Miller 1997). Martinez et al. (2004) suggested that *F. psychrophilum* uses a combination of proteolytic activity and gliding motility as a mechanism to gain entry into host tissues. The GldN of *F. johnsoniae* has been characterized and is involved in motility and chitin utilization (Braun et al. 2005). The upregulation of the GldN protein of *F. psychrophilum in vivo* suggests that it may play a role in pathogenesis, and host-related nutrients appear to turn on the associ-

ated gene. Further, this protein is immunogenic in rainbow trout, exhibiting enhanced protection against experimental *F. psychrophilum* challenge (LaFrentz 2007), and may therefore be a promising vaccine candidate antigen.

The outer membrane protein OmpH of *Flavobacterium psychrophilum* has been extensively characterized (Massias et al. 2004, Dumetz et al. 2006) and is immunogenic (Dumetz et al. 2006, 2008). Initial studies demonstrated that this protein is continuously produced and the production is not affected by temperature and aeration (Massias et al. 2004). In the present study, an increased spot intensity of OmpH was observed *in vivo* suggesting that certain environmental factors stimulate this response. Antibodies specific for the OmpH protein were demonstrated to have bacteriostatic and bacteriocidal activities, and immunization of rainbow trout with semi-purified OmpH stimulated protective immunity against experimental *F. psychrophilum* challenge (Dumetz et al. 2006). Brown et al. (1988) suggested that vaccines based on surface proteins will only be successful if the proteins are produced *in vivo* ; thus, the present results provide further support for the use of the OmpH protein of *F. psychrophilum* as a vaccine candidate.

Two probable outer membrane proteins of *Flavobacterium psychrophilum* with similarity to the OmpA family were identified with increased spot intensity *in vivo*. Protein spot nos. 15 and 16 were both identified as a probable outer membrane protein, OmpA family (FP2411), which suggests this protein is modified post-translation because they differ in pI, but have the same molecular mass. Protein spot no. 17 was identified as an OmpA-family outer membrane protein. Interestingly, there are 2 identical copies of this gene in the *F. psychrophilum* genome (FP0321 and FP1169). OmpA and OmpA-like proteins are well documented as bacterial virulence factors (Smith et al. 2007), and some studies have suggested roles in adhesion (Dabo et al. 2003, Serino et al. 2007). It is possible that these proteins play a similar role in the pathogenesis of *F. psychrophilum*. Considering the surface exposure and increased spot intensities *in vivo*, these proteins are promising candidates for further investigation. OmpA and OmpA-like proteins have been identified as vaccine candidate antigens (Ochoa-Reparaz et al. 2004, Kurupati et al. 2006) and appear protective in other systems (Ross et al. 2004).

Several proteins that exhibited increased spot intensity *in vivo* were positively identified, but these proteins did not exhibit similarity to any known proteins (Table 1). Two of these proteins are of interest for vaccine development. These include the probable lipoprotein of unknown function (Protein spot no. 12) and a protein with unknown function (Protein spot no. 10). These proteins were recently identified in an outer membrane extraction of *Flavobacterium psychrophilum* and were demonstrated to be immunogenic by Western blot analysis with CWD-convalescent rainbow trout serum (Dumetz et al. 2008). Although the functions of these proteins are not known, they may be good candidates for vaccine development due to their potential surface exposure, immunogenicity, and increased spot intensity *in vivo.*

Two proteins exhibited decreased spot intensity *in vivo*. One of these was identified as ferritin FtnA, and it was also downregulated in iron-limited growth conditions. FtnA is an iron-storage protein, and it functions to bind intracellular iron that can be toxic and stores iron for use if needed in an iron-limited environment (Andrews et al. 2003). The results of the present study suggest that the FtnA of *Flavobacterium psychrophilum* functions in a similar manner. The spot intensity of this protein was increased when *F. psychrophilum* was cultured in TYES media, presumably for binding excess iron to avoid toxicity and to serve as a reserve for future use. However, culture of the bacterium in iron-limited and *in vivo* growth conditions resulted in decreased spot intensity of this protein, presumably because there was no need to store iron in these conditions. The mechanism by which *F. psychrophilum* regulates its iron metabolism is not known, but it may be mediated by the ferric-uptake regulator (Fur) protein as in *Escherichia coli* (Andrews et al. 2003), and there is a putative Fur homologue (FP1903) present in the *F. psychrophilum* genome.

OmpA (P60) also exhibited decreased spot intensity *in vivo*. This was an interesting identification because the approximate experimental molecular mass (~35 kDa) was much lower than expected for this protein (Table 1). Previous research in our laboratory has identified another protein spot at ~58 kDa as OmpA (P60) (Fig. 4), which was immunogenic and exhibited experimental values that are closer to the theoretical molecular mass and pI of the protein (LaFrentz 2007). Similarly, Dumetz et al. (2008) identified multiple protein spots with approximate molecular masses of <30 kDa as OmpA (P60), and they suggested that these represented degraded OmpA (P60) products from sample preparation. In the present study, this seems unlikely because the whole-cell lysates were prepared identically for *Flavobacterium psychrophilum* cultured *in vivo* and *in vitro*. Using the same sample preparation methods, the ~35 kDa OmpA (P60) protein was consistently absent from *F. psychrophilum* cultured *in vivo*, and the ~58 kDa OmpA (P60) protein was present in similar quantities between cells cultured *in vitro* and *in vivo*. This suggests that the presence of the ~35 kDa OmpA (P60) protein is not an artifact due to sample preparation, but there may be processing or degradation occurring in *in vitro* culture. The OmpA (P60) protein of *F. psychrophilum* has been extensively

characterized and suggested to play a potential role in protective immunity (Merle et al. 2003, Dumetz et al. 2007). In these studies, rabbit polyclonal serum was prepared against purified OmpA (P60), and Western blot analysis of crude *F. psychrophilum* extracts revealed a single band in 1-dimensional electrophoresis (Dumetz et al. 2007). Whether the identification of 2 distinct protein spots exhibiting different spot intensities as OmpA (P60) is a noteworthy finding remains to be determined, but is of importance as this protein has been suggested for vaccine development against *F. psychrophilum*.

The culture of bacteria in iron-limited media has been shown to mimic the *in vivo* environment (Ebanks et al. 2004). The results of the present study suggest that the culture of *Flavobacterium psychrophilum* in such conditions mimics the *in vivo* environment to some extent, but not completely because only 6 of 20 identified proteins shared similar spot intensity changes. It is possible that the 50 µM DPD employed in the present study was insufficient to chelate all iron present in the media and certainly, the availability of host nutrients is lacking in artificial media. Growth of the bacterium was inhibited at this concentration, which suggests that iron was limiting; however, the use of higher concentrations of DPD may more accurately mimic the iron availability *in vivo*. Another explanation is that the analysis was limited to one time point (3 d culture) for iron-limited growth, and it is possible that other changes in the proteome may have occurred at later time points. This was evident in the analysis of *F. psychrophilum* cultured *in vivo* as several trends in protein spot intensity were identified over the course of the 10 d experiment (Table 2).

Results from *in vivo* and iron-limited culture of *Flavobacterium psychrophilum* did not show increased spot intensities of recognized iron-regulated outer membrane proteins, as has been demonstrated in other bacterial species following culture under these conditions (Colquhoun & Sorum 1998, Ebanks et al. 2004). Moller et al. (2005b) studied the iron-acquisition mechanisms of *F. psychrophilum* and suggested that it produces siderophores and is capable of using iron from transferrin and hemoglobin. The *F. psychrophilum* genome contains 5 TonB-dependent outer membrane iron receptors and 2 proteins exhibiting similarity to an iron uptake system of *Campylobacter jejuni* (Duchaud et al. 2007). Research has also demonstrated that the TonB system of *F. psychrophilum* is important for virulence (Alvarez et al. 2008). It is possible that the quantities of these proteins were not detectable in the whole-cell lysate preparations and/or that the rehydration buffer used for 2D-PAGE was not effective at solubilizing these outer membrane proteins (Molloy et al. 1998). Alternatively, it is also possible that *F. psychrophilum* relies on other mechanisms to obtain iron, such as the release of proteolytic enzymes as suggested by Moller et al. (2005b).

The present study examined the proteome of *Flavobacterium psychrophilum* when grown *in vivo* or under iron-limited conditions as a first step to understand the pathogenesis of this bacterium. Future research should focus on the outer membrane proteins, because these are involved in host–pathogen interactions and may also be important for pathogenesis and vaccine development. By enriching for outer membrane proteins, other proteins may be identified that were not present in whole-cell lysates at sufficient quantities for detection in the present study. Additionally, the membrane vesicles, lipopolysaccharide, and glycocalyx produced by *F. psychrophilum* should be examined following growth *in vivo*. Previously, it was demonstrated that some strains of *F. psychrophilum* exhibit increased production of membrane vesicles when cultured in iron-limited media and media containing rainbow trout serum (Moller et al. 2005a). The authors further demonstrated that cells exhibit a slight increase in the amount of glycocalyx when cultured in media supplemented with rainbow trout serum (Moller et al. 2005a).

In summary, the combination of growth in iron-limited conditions, use of dialysis tubing chambers for *in vivo* culture, and proteomics have proven to be useful techniques for identifying *Flavobacterium psychrophilum* proteins that exhibit changes in protein spot intensities. It is likely that at least some of these proteins are involved in the pathogenesis of this fish pathogen. Future research should be aimed at determining the precise roles of these proteins in the pathogenesis of *F. psychrophilum* and evaluating their potential to provide protection against CWD when used in immunization trials.

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