Separation and Comparison of Proteins from Virulent and Nonvirulent Strains of the Fish Pathogen *Flavobacterium psychrophilum,* Using a 2-D Electrophoretic Approach

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Introduction

Coldwater disease (CWD) is a worldwide problem in commercial (foodfish) aquaculture as well as public resource enhancement hatcheries, and is caused by the gram-negative bacterium *Flavobacterium psychrophilum*. Juvenile trout and salmon infected with this disease experience high mortalities, and surviving fish often exhibit skeletal deformities associated with spinal compression. Aquaculture vaccines have become important tools in preventing fish diseases, but a commercial vaccine is not currently available for CWD. Development in this area has likely been hampered by altered expression of virulence factors among different strains of *F. psychrophilum* and an incomplete understanding of the immune response of fish to this pathogen. It has been noted that protease differences exist between strains (Dalsgaard 1993, Bertolini et al. 1994, Madsen and Dalsgaard 1999), but the identities of these components and roles they play in the fish immune response are unknown. Significant protection has been observed under laboratory conditions following immunization with *F. psychrophilum* (Holt 1988, Obach and Laurencin 1991), but antigenicity appears to be strain dependent.

A study is currently underway in our laboratory using two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) to identify antigens and virulence factors of this bacterium that may be targeted for the development of an efficacious vaccine for the aquaculture industry. 2-D gel profiles of virulent and nonvirulent strains are being compared to identify and further characterize antigens of interest. The role these play in pathogen virulence and immunoprotection is currently being evaluated using the PROTEAN® IEF cell and ReadyStrip™ IPG strips to isolate and excise specific bacterial proteins. Sequential extraction procedures are being utilized along with biotin labeling to distinguish between cytoplasmic, membrane-associated, and cell surface proteins. Since fish are capable of mounting a protective immune response against *F. psychrophilum*, it is speculated that a 2-D approach will be valuable in allowing identification and characterization of immunodominant and virulence-associated proteins in relation to specific bacterial strains.

Methods

Bacterial Culture

Virulent (CSF-259-93) and nonvirulent (ATCC 49418) isolates of *F. psychrophilum* were cultured in tryptone yeast extract salts broth at 15°C. Bacteria used for 2-D PAGE were harvested from log-phase cultures and adjusted to an absorbance of 0.4 at 525 nm. This corresponds to an approximate bacterial concentration of 5 x 10^7 cfu/ml.

Sequential Extraction of Bacterial Proteins

To identify hydrophilic (primarily cytoplasmic nonmembrane proteins) and hydrophobic proteins associated with cell or organelle membranes, a method for sequential extraction was developed using the ReadyPrep™ sequential extraction kit. Briefly, proteins were extracted from *F. psychrophilum* (strain CS-259-93) following harvest from log phase cultures. Samples were washed twice in PBS and resuspended to an approximate protein concentration of 12 mg/ml in reagent 1 (40 mM Tris, pH 8.0). Bacteria were lysed by rapid freeze-thawing (10 cycles), and were vortexed between cycles for 1–2 min. Endonuclease was added to a concentration of 150 U/ml and samples were incubated at room temperature for 30 min to digest DNA. Samples were then centrifuged (12,000 x g) and supernatant containing hydrophilic proteins was retained (E1). To separate hydrophobic proteins, reagent 2 (8 M urea, 4% CHAPS, 40 mM Tris, and 0.2% Bio-Lyte® 3/10 carrier ampholytes) containing 2 mM tributylphosphine (TBP) was added to the pellet. The sample was vortexed for 2 min and centrifuged as above. The supernatant, containing hydrophobic proteins (corresponding primarily to loosely bound membrane proteins), was retained (E2). Reagent 3 (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3-10, 40 mM Tris and 0.2% Bio-Lyte 3/10 carrier ampholytes) with 2 mM TBP was then added to the pellet and the process repeated to obtain highly hydrophobic proteins generally associated with tightly bound membrane proteins (E3). Samples were either subjected to isoelectric focusing immediately following extraction or stored at -80°C until needed.

Biotinylation of Cell Surface Components

In order to detect cell surface membrane proteins and allow comparison to sequentially extracted proteins, viable bacteria were biotinylated prior to protein extraction. Bacteria were harvested in log-phase growth at a concentration of approximately 5.0 x 10^7 viable bacteria/ml (0.4 absorbance) and washed twice in ice-cold PBS (pH 8.0) taking care not to disrupt or lyse cells. The bacteria were resuspended to an absorbance of 0.2 at 525 nm and 1.7 mg of sulfo-NHS-LC-biotin was added per ml of bacteria. This mixture was allowed to rock for 30 min at room temperature. The bacteria were then washed in ice-cold PBS and resuspended to an approximate protein concentration of 12 mg/ml in reagent 1. Washed cells were subjected to the initial extraction step (see above) and an equal volume of reagent 3 was added to obtain total proteins for 2-D PAGE separation. Following 2-D PAGE and transfer of separated proteins to a nitrocellulose membrane, biotin-labeled proteins were detected by probing blots with ExtrAvidin-alkaline phosphatase (Sigma) and visualized colorimetrically by the addition of NBT/BCIP.

IEF and Second-Dimension Separation of Proteins

Sequentially extracted or total protein samples were applied to ReadyStrip IPG Strips pH 4–7 or pH 3–10 and passively rehydrated overnight in a humidity chamber. Once fully rehydrated, isoelectric focusing of proteins was carried out with the PROTEAN IEF cell using a preset method that allowed a minimum of 20,000 V-hr to be obtained while maintaining a constant temperature of 17°C. Focused strips were stored at -80°C until second-dimension PAGE could be performed.

For second-dimension separation, IPG strips were equilibrated in an equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris, 4% glycerol, 5 mM TBP, 2.5% acrylamide monomer) for 20 min at room temperature. Strips were then transferred to the prep wells of Ready Gel® 10–20% Tris-HCl precast gels and electrophoresed in the Mini-PROTEAN® 3 cell for 20 min at 4 mA/gel and then 1.5 hr at 12 mA/gel. Separated proteins were silver stained or transferred to nitrocellulose for detection of biotin-labeled cell surface proteins.

Results

Sequential Extraction and Biotin Labeling of Bacterial Proteins

Using the CSF-259-93 reference strain, the ability to separate cellular proteins based on their hydrophobicity was examined. The relationship of extracted proteins to cell surface proteins was then determined by comparison of 2-D gel profiles of biotin-labeled proteins. The results clearly demonstrate the ability of the extraction procedure to isolate proteins associated with cytoplasmic or cell membrane compartments of the bacteria (Figure 1).

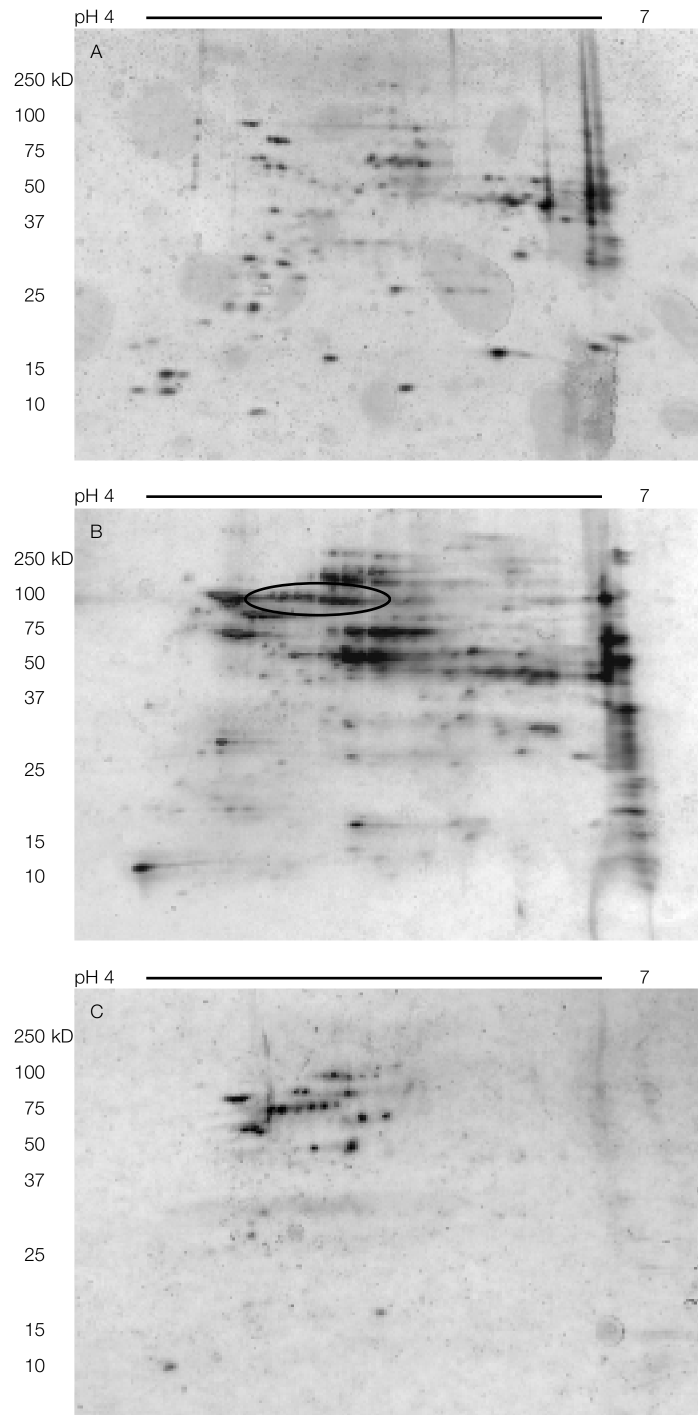


Fig. 1. Sequential extraction of bacterial proteins from *F. psychrophilum* strain CSF-259-93 and separation by 2-D PAGE. A, E1 fraction, hydrophilic (generally cytoplasmic) proteins; B, E2 fraction, hydrophobic proteins (generally associated with loosely bound membrane proteins); C, E3 fraction, highly hydrophobic proteins (associated with tightly bound membrane proteins). The circled region represents cell surface proteins as confirmed by comparison to biotinylated protein (Figure 2).

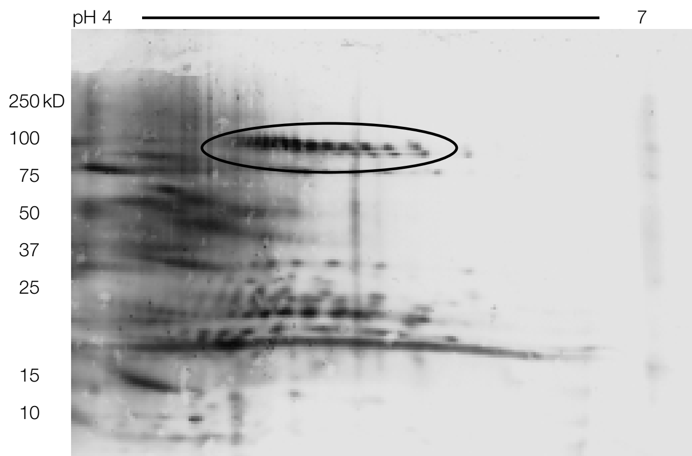
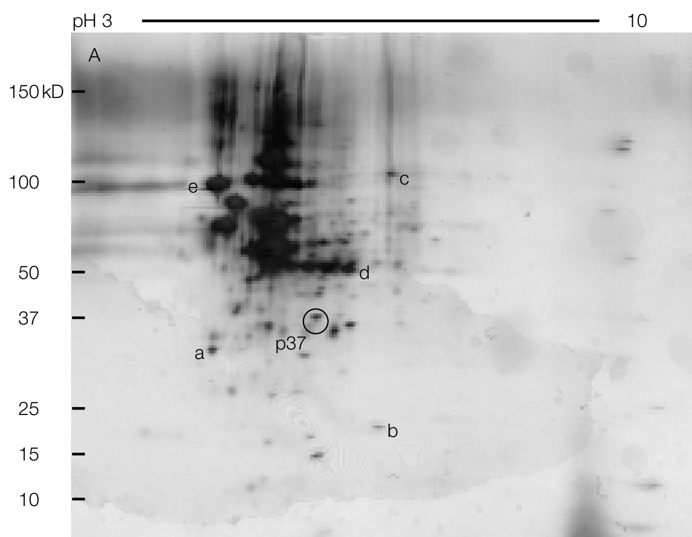


Fig. 2. Cell surface-associated proteins from CSF-259-93 were biotin-labeled and separated by 2-D PAGE then transferred to a nitrocellulose membrane. Proteins were visualized colorimetrically. The string of proteins (circled region) corresponds primarily to membrane-associated proteins separated in the E2 fractions (Figure 1).



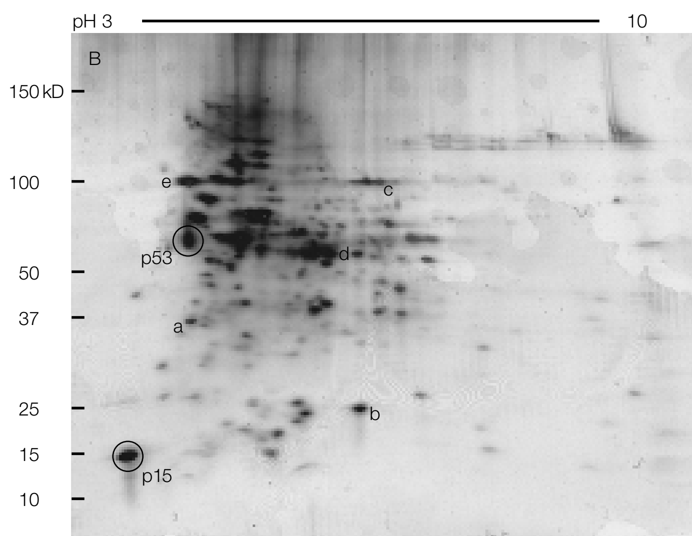


Fig. 3. Separation and comparison of total bacterial proteins. A, total proteins from *F. psychrophilum* (ATCC 49418); B, total proteins from the CSF-259-93 strain. Lowercase letters correspond to common proteins identified in each bacterial strain. Additional protein spots (p37, gel A, and p53 and p15, gel B) represent proteins that are differentially expressed between strains. Proteins were visualized following 2-D PAGE separation and silver staining.

Comparison of gel profiles shows that a large number of proteins are extracted in the E1 cell lysate fraction. Membrane-associated (less soluble) fractions were then obtained following E2 and E3 separation. Subsequent comparison to biotin-labeled proteins (Figure 2) shows that a dominant string of proteins (circled) at an approximate molecular weight of 100 kD and pI values between 5.2 and 5.8 represents a cell surface component and appears to primarily correspond to proteins in extraction E2 of Figure 1. Additional biotin-labeled proteins are present in the 20 kD range (pI approximately 5.1–5.6) but are not readily identified in the sequentially extracted fractions.

Comparison of Total Proteins from Virulent and Nonvirulent Strains of *F. psychrophilum*

Separation and comparison by 2-D PAGE of total proteins over the pH 3–10 range from strain CSF-259-93 (virulent) and ATCC 49418 (nonvirulent) demonstrate that proteins are differentially expressed between bacterial strains (Figure 3). Initial qualitative analysis reveals that the majority of bacterial proteins separate in the molecular weight ranges of approximately 40–150 kD and have pI ranges of approximately 4.5–6.5. It appears that the nonvirulent ATCC strain has a higher concentration of proteins in this range, but distinct proteins at lower molecular weight did not resolve as well or were less concentrated in the ATCC strain than the CSF strain. Two major proteins (p15 and p53) are represented in the virulent (CSF) strain but do not appear in the ATCC strain, while at least one protein (p37) appears to be differentially expressed in the ATCC strain.

Conclusions

The techniques outlined here demonstrate the effectiveness of sequential extraction in obtaining bacterial proteins from different compartments of the cell. Combined with 2-D PAGE techniques, the ReadyPrep sequential extraction kit allowed cell-associated and cytoplasmic proteins to be effectively separated. In addition to the development of techniques to separate different bacterial protein components, we have successfully separated proteins from both virulent and nonvirulent strains of *F. psychrophilum* by 2-D PAGE. The use of the PROTEAN IEF cell allowed rapid throughput of samples and exceptional control over focusing conditions. The separation of distinct proteins using these methods has allowed the comparison of potential virulence factors between two bacterial isolates grown under identical conditions. Further characterization is needed and will focus on comparison of differentially expressed proteins to active proteases (potential virulence components) and immunodominant bacterial antigens. Protein sequencing and eventual gene isolation may lead to the development of an efficacious recombinant vaccine for aquaculture, or may allow screening of the many *F. psychrophilum* isolates for potential vaccine candidates. This approach has the potential to substantially advance our current understanding of this pathogen and may lead to better methods of disease control.

References

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