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Partial two-dimensional gel electrophoresis (2-DE) maps of *Streptococcus iniae* ATCC29178 and *Lactococcus garvieae* KG9408

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ABSTRACT: To examine the proteomes of 2 important causative agents of fish streptococcosis, *Streptococcus iniae* ATCC29178 and *Lactococcus garvieae* KG9408, we used 2-dimensional gel electrophoresis (2-DE) followed by mass spectrometry to generate 2-DE maps of these type strains. Silver-stained 2-DE gels of *S. iniae* ATCC29178 and *L. garvieae* KG9408 revealed ~320 and 300 spots, respectively, and immobilized pH gradient strips (13 cm, pH 4 to 7) revealed that the majority of the detected spots were concentrated in the pH range of 4.5 to 5.5. The spots were randomly selected from the 2-DE profiles and identified by peptide mass fingerprinting using matrix-assisted laser desorption/ionization time of flight mass spectrometry. The majority of the identified proteins were functionally related to energy and carbohydrate metabolism (e.g. enolase ATPase, glyceraldehyde-3-phosphate dehydrogenase) or translation and translocation (e.g. elongation factor G, elongation factor Tu, DNA-directed RNA polymerase alpha chain). These data, along with our partial 2-DE maps of *S. iniae* ATCC29178 and *L. garvieae* KG9408, may help suggest antigenic proteins for the development of effective diagnostic tools and vaccines against *S. iniae* and *L. garvieae*.

KEY WORDS: *Streptococcus iniae* · *Lactococcus garvieae* · Two-dimensional gel electrophoresis · Mass spectrometry · Proteome

INTRODUCTION

Bacterial, viral, fungal and parasitic diseases have become major biological threats for the development and sustainability of the world fish farming industry. Fish streptococcosis, one of the most important bacterial diseases in farmed and wild fishes, occurs primarily during the warmer months in association with other contributing causes such as poor water quality (Roberts 2001, Seng & Colorni 2002). *Streptococcus iniae* and *Lactococcus garvieae* have been identified as causative agents of streptococcosis in various species of fishes (Kusuda et al. 1976, Nakatsugawa 1983, Perera et al. 1994, Bromage et al. 1999, Eldar et al. 1999, Yuasa et al. 1999, Chang et al. 2002, Colorni et al. 2003, Kang et al. 2004). In addition, both bacteria have been isolated from human clinical samples, indicating that they may be important emerging zoonotic agents (Weinstein et al. 1997, Fefer et al. 1998). *S. iniae* ATCC29178 was originally isolated from skin lesions of the freshwater dolphin *Inia geoffrensis* (Peir & Madin 1976), while *L. garvieae* KG9408 was isolated from yellowtail *Seriola quinqueradiata* (Ooyama et al. 2002).

Proteomics, which is widely used to study the global changes in protein expression (Blackstock & Weir 1999), involves the separation and identification of individual proteins from cell, tissue, or organism lysates. The proteins are initially resolved by 2-dimensional gel electrophoresis (2-DE), in which isoelectric focusing (IEF) and sodium dodecyl sulfate polyacryl-

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amide gel electrophoresis (SDS-PAGE) are used to separate proteins according to their isoelectric point (pI) and molecular weight (MW), respectively (O'Farrell 1975). In spite of available promising technologies for studying proteins, such as multidimensional protein identification, stable isotope labeling, and protein or antibody arrays, the 2-DE is the only technique that can be routinely applied for quantitative expression profiling of a complex mixture of proteins according to pI, MW, solubility, and relative abundance (Görg et al. 2004). The proteins separated by 2-DE are then identified by immunoblotting with specific antibodies (Winterhoff et al. 2002), Edman sequencing with N-terminal sequencing (Anglade et al. 2000, Lei et al. 2000), or peptide mass fingerprinting (PMF) using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (Cash et al. 1999, Thongboonkerd et al. 2002, Lee et al. 2003, Len et al. 2003, Lin et al. 2003, Mortz et al. 2003). The identified proteins may then be assembled into a 2-DE map or reference map of the sampled cell, tissue, or organism (Cash 2000, Cordwell et al. 2001, Champomier-Verges et al. 2002, Hughes et al. 2002, Lee et al. 2003, Len et al. 2003). Such maps have been widely used to compare differences in protein expression among strains of the same species or among the same strains under different experimental conditions. In this way, researchers have been able to study the mechanisms involved in pathogenicity, antibiotic resistance, environmental adaptation and responses to host immune systems (Cash et al. 1999, Jungblut et al. 1999, Cash 2000, Lei et al. 2000, Cordwell et al. 2001, 2002, Thongboonkerd et al. 2002, Vytvytska et al. 2002). In addition, antigenic proteins have been identified by comparing 2-DE maps and immunoblot profiles (combination of 2-DE and immunoblot assay), using computational image analysis, from various pathogenic bacteria. Computational image analysis of 2-DE maps has allowed identification of antigenic proteins in a variety of pathogenic bacteria (Cash 1999, Jungblut et al. 1999, Lei et al. 2000, Cordwell et al. 2001, Vytvytska et al. 2002).

The complete genome sequences have been established for a number of bacterial species, including the pathogenic streptococci *Streptococcus pyogenes* (Ferretti et al. 2001), *S. agalactiae* (Tettelin et al. 2002), *S. pneumoniae* (Tettelin et al. 2001), *S. thermophilus* (Bolotin et al. 2004) and *S. mutans* (Ajdic et al. 2002), and the lactococcus *Lactococcus lactis* (Bolotin et al. 2001). These sequences have given important insights into the pathogenic mechanisms of these organisms. *S. iniae* and *L. garvieae* belong to the genera *Streptococcus* and *Lactococcus*, respectively, and proteins of these species were identified using the available database for the related species/genera. Recently, several reports have been published on the application of the proteomics approach for identifying diagnostic markers and candidate proteins for vaccine development from various fish pathogenic bacteria, e.g. *Edwardsiella tarda* and *Vibrio anguillarum* (Kawai et al. 2004, Rao et al. 2004, Rasch et al. 2004). To our knowledge, there have been no published reports on the proteomes of *S. iniae* and *L. garvieae*, both highly important fish pathogenic bacteria found in a wide variety of fish species. In the present study we have used 2-DE, followed by PMF using MALDI-TOF MS, to construct partial proteome reference maps for *S. iniae* ATCC 29178 and *L. garvieae* KG9408. The results of this study may be very useful in differentiating these pathogens at the protein level and in understanding the pathogenesis of both bacteria by identifying the major proteins involved in pathogenesis.

MATERIALS AND METHODS

Preparation of bacteria. *Streptococcus iniae* type strain ATCC29178 was purchased from the ATCC (Manassas, USA) and *L. garvieae* KG9408 was kindly provided by Dr. Yoshida (Miyazaki University, Japan). Bacteria were stored in Todd-Hewitt broth (THB) containing 10% glycerol at –70°C. For experiments, bacteria were subcultured in triplicate in 50 ml THB at 30°C, grown to an optical density $(OD)_{610\ nm}$ of 1.0 (10^9 CFU ml^{-1}), and then harvested by centrifugation at 2000 × *g* for 30 min at 4°C. The pellets were washed 3 times with phosphate-buffered saline (PBS; 3 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.4), and resuspended in 5 ml of PBS. The bacterial suspensions were transferred to new tubes (1 ml each) and centrifuged at 2000 × *g* for 10 min at 4°C, and the resulting pellets were stored at –20°C until use.

Extraction of bacterial proteins. A 2-DE sample was prepared using chemical followed by mechanical extraction methods to obtain a high abundance and good solubility of whole cell proteins (Lehner et al. 2003, Görg et al. 2004). Firstly, for chemical extraction, bacterial pellets were resuspended in 100 µl of Lysis Buffer A (12 mM Tris, 5% glycerol, 0.4% SDS, and 200 mM dithiothreitol [DTT]) and, for mechanical extraction, were sonicated 8 times (XL-2020, Misonix) at 5.5 W for 30 s in ice slurry. Cells were then disrupted by boiling for 10 min, cooled in ice and lysed in 2-DE Lysis Buffer B consisting of 2 M thiourea, 7 M urea, 40 mM Tris, 1% (w/v) DTT, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 0.5% (v/v) immobilized pH gradient (IPG)-buffer pH 4 to 7. The lysates were incubated on ice for 30 min, pelleted by centrifugation at 16 000 × *g* for 30 min at 4°C, and stored at –70°C until use. Protein concentrations were

estimated with the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as standard. The lysed protein was diluted with double distilled water (dDW) in order to measure accurate concentration of protein and, at the same time, avoid any inhibition by chemicals including urea and SDS in Bradford assay.

2-DE. *IEF* and *SDS-PAGE:* IEF was performed using the IPGphor™ system (Amersham Bioscience) with IPG strips (Immobiline DryStrip™, pH 4 to 7, 13 cm; Amersham Bioscience), according to the previously reported method (Lee et al. 2003). The protein loading volume was adjusted to 0.6 mg ml^{-1} with rehydration buffer (9 M urea, 2% CHAPS, 0.4% DTT, 0.5% IPG buffer, and 0.002% bromophenol blue). The prepared sample was loaded onto the IPG strips and focusedto 86.1 kVh using an automated programmer at 20°C as follows: rehydration for 14 h (7 h at 30 V followed by 7 h at 60 V), and focusing for 17 h (2 h at 200 V, 1 h at 500 V, 1 h 1000 V, 1 h at 2000 V, 2 h at 4000 V and 10 h at 8000 V). After IEF, the IPG strips were equilibrated with 10 mg ml^{-1} of DTT in equilibration buffer (6 M urea, 2% SDS, 30% glycerol, 0.002% bromophenol blue, and 50 mM Tris-HCl, pH 8.8) for 15 min, and then with 40 mg ml^{-1} of iodoacetamide in the same buffer for another 15 min. Equilibrated IPG strips were placed onto 12.5% SDS-polyacrylamide gels (18 × 16 × 0.1 cm), sealed with 0.5% w/v agarose, and electrophoresed at 10 mA gel^{-1} for 15 min followed by application of 20 mA gel^{-1} until the dye reached the bottom of the gel.

Protein visualization and image analysis: Electrophoresed gels were silver stained as previously described (Mortz et al. 2001). Briefly, each 2-DE gel was fixed with Solution A (50% methanol, 12% acetic acid, and 0.05% [v/v] of 37% formaldehyde) for 1 h, washed twice with 50% ethanol and sensitized with sensitizing solution (0.01% sodium thiosulfate) for 1 min. The sensitized gels were rinsed with 3 changes of dDW and then incubated with Solution B (0.1% silver nitrate and 0.1% of 37% formaldehyde) for 30 min. Gels were then washed twice with dDW for 30 s and treated with developing solution (12% sodium carbonate and 0.05% of 37% formaldehyde) until the desired level of staining was obtained, whereupon the developing solution was removed and the reaction was stopped by addition of Solution A without formaldehyde. The stained gels were stored in 50% methanol at 4°C until analysis. The gels were digitalized with an Agfa Arcus 1200 image scanner (Agfa-Gevaert), and the acquired images were analyzed using the Phoretix 2D software (Ver. 5.01, NonLinear Dynamics).

PMF for protein identification. *In-gel digestion:* The protein spots were in-gel digested as previously described (Lee et al. 2003). In brief, stained spots of interest were picked using a micropipette tip (1 mm diameter), washed with DW followed by 50% acetonitrile and dried completely in a vacuum centrifuge. The dried gel pieces were rehydrated at room temperature (RT) for 45 min in reducing buffer (100 mM NH_4HCO_3 and 10 mM DTT) and then incubated for 30 min in alkylation solution (100 mM NH_4HCO_3 and 55 mM iodoacetamide) in the dark at RT. The gel pieces were dried, rehydrated in digestion buffer (50 mM NH_4HCO_3 and 12.5 ng ml^{-1} of porcine trypsin; Promega) and incubated for 45 min on ice. The excess liquid was removed, and 10 µl of digestion buffer without trypsin was added. Samples were incubated at 37°C overnight (approximately 16 h). The supernatant was recovered and extracted twice with a 1:1 mixture of 5% formic acid and acetonitrile, and the extracts were pooled and dried in a vacuum centrifuge.

MALDI-TOF MS and database searches: Dried tryptic peptides were redissolved in 2 µl sample solution (93:5:2 ratio of DW, acetonitrile and trifluoroacetic acid [TFA]), and the targeting on MALDI plate was performed using the solution-phase nitrocellulose method described by Lee et al. (2003). The α-cyano-4-hydroxycinamic acid (40 mg ml^{-1}) and nitrocellulose (20 mg ml^{-1}) were prepared separately in acetone and mixed with isopropanol in a ratio of 2:1:1. The internal standards, des-Arg-Bradykinin (monoisotopic mass, 904.4681) and Angiotensin I (1296.6853) (Sigma-Aldrich) were added to the mixture to generate the matrix solution, which was then mixed with the sample peptide prepared by trypsin digestion. The mixed solution (1 µl) was spotted onto target circles on the MALDI plate and dried. The dried samples were sequentially washed with 5% formic acid and dDW, dried and analyzed using a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Bio-systems). The masses of parent ions were measured in the positive ion reflection/delayed extraction mode, with an accelerating voltage of 20 kV, a grid voltage of 76%, a grid wire voltage of 0.01% and a delay time of 150 ns. Mass spectra were obtained by irradiating the target with 128 laser pulses. Monoisotopic peptide masses were selected in the mass range of 800 to 2500 Da. Proteins were identified from the National Center for Biotechnology Information (NCBI) and SwissProt protein sequence databases using the MS-Fit program (http://prospector.ucsf.edu).

RESULTS

Proteomic analysis of *Streptococcus iniae* ATCC29178

In a preliminary study using IPG strips with a pH of 3 to 10, 2-DE profiles of *Streptococcus iniae* and *Lactococcus garvieae* showed that most spots detected by

silver staining were concentrated in the range pH 4 to 7 of the 2-DE gels. In addition, it was very difficult to distinguish between spots, due to overlapping. Therefore, 2-DE reference maps for both bacteria were constructed by using an IPG strip with a pH of 4 to 7 in the present study. An average of 320 spots was observed in the 2-DE profile of *S. iniae* ATCC29178 (n = 3) after silver staining (Fig. 1). The number of spots distributed in the pH ranges of 4 to 5, 5 to 6 and 6 to 7 were 99, 140 and 81, respectively. Approximately 50% of the spots (152 spots) were distributed in the pH range of 4.5 to 5.5. In terms of molecular masses, 34, 120, 98, 35 and 15 spots were detected in the ranges of 10 to 20, 21 to 30, 31 to 40, 41 to 60 and 61 to 80 kDa, respectively.

For protein identification, the spots were randomly selected and subjected to PMF analysis. Twenty-two protein spots, corresponding to 17 different proteins, were successfully identified (Table 1). Among these, glyceraldehyde-3-phosphate dehydrogenase (GapC; Spot 11) was the only protein identified from the *Streptococcus iniae* database. The others were identified by comparison to protein databases of related species, yielding 2 matching proteins from *S. mutans*, 11 from *S. pyogenes*, 3 from *S. agalactiae*, and 1 from *S. pneumonia*. The 17 identified proteins were then classified into 7 functional groups (see Table 3): translation, ribosomal structure and biogenesis (4 proteins), transcription (2 proteins), posttranslational modification (2 proteins), nucleotide transport and metabolism (1 protein), carbohydrate transport and metabolism (5 proteins), energy production and conversion (2 proteins) and lipid transport and metabolism (1 protein) (www.jgi.doe.gov).

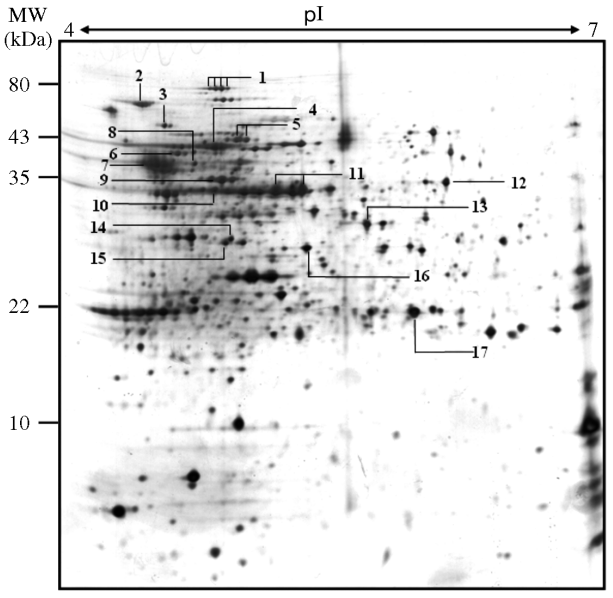

Fig. 1. *Streptococcus iniae* ATCC29178. A partial 2-dimensional gel electrophoresis (2-DE) map. Whole bacterial cells were lysed, and isoelectric focusing (IEF) was carried out using an immobilized pH gradient (IPG) dry strip (pH 4 to 7, 13 cm) followed by 2-DE and silver staining. Spots (1–17) were randomly selected, numbered and identified by peptide mass fingerprinting (PMF) analysis. MW: molecular weight; pI: isoelectric point

Table 1. *Streptococcus iniae*. Proteins identified from the *S. iniae* ATCC29178 proteome by 2-DE followed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis and database searching. MOWSE score: molecular weight search score, scoring based on peptide frequency distribution of protein searched by the MS-Fit program (http://prospector.ucsf.edu) in protein sequence databases of the NCBI and SwissProt. pI: isoelectric point

Spot no.	Protein	MOWSE score	Masses matched	Coverage (%)	Da/pI	Database species identification
1	Translation elongation factor G (EF-G)	6.01E + 09	19	36	76 665/4.8	*S. mutans*
2	Chaperone protein dnaK (Heat Shock Protein 70; HSP 70)	6.65E + 04	8	19	64 921/4.6	*S. pyogenes*
3	GroEL protein (60 kDa chaperonin; HSP 60)	2.29E + 04	6	16	57 332/4.7	*S. agalactiae*
4	Translation elongation factor TU (EF-Tu)	1.92E + 08	15	39	43 856/4.9	*S. pyogenes*
5	Pyruvate kinase	2.94E + 07	15	28	54 536/5.0	*S. pyogenes*
6	Ribosomal protein S1-like DNA-binding protein	1.88E + 04	8	23	43 850/4.9	*S. pyogenes*
7	Enolase	1.04E + 06	10	33	47 357/4.7	*S. pyogenes*
8	Proton-translocating ATPase, beta subunit	7.51E + 04	8	23	51 056/4.7	*S. pyogenes*
9	Phosphoglycerate kinase	1.18E + 04	7	27	42 130/4.8	*S. pyogenes*
10	DNA-directed RNA polymerase alpha chain (RNA polymerase alpha subunit)	1.12E + 05	8	29	34 530/4.9	*S. pyogenes*
11	Glyceraldehyde-3-phosphate dehydrogenase (GapC) *S. iniae*	1.35E + 05	8	27	35 724/5.2	*S. agalactiae*
12	Transcription antiterminator	2.73E + 04	7	40	32 529/6.2	*S. pyogenes*
13	6-phosphofructokinase	1.04E + 04	6	22	35 749/5.3	*S. pyogenes*
14	Formate acetyltransferase (pyruvate formate-lyase)	1.41E + 04	7	10	87 607/5.2	*S. mutans*
15	Acetoin dehydrogenase (TPP-dependent) beta chain	5.34E + 04	11	30	35 837/4.9	*S. pyogenes*
16	30S ribosomal protein S2	1.87E + 06	9	36	28 387/5.1	*S. pneumoniae*
17	Uracil phosphoribosyltransferase (UMP pyrophosphorylase; UPRTase)	1.96E + 04	6	33	22 736/5.4	*S. agalactiae*

Proteome analysis of *Lactococcus garvieae* KG9408

The 2-DE profiles of *Lactococcus garvieae* KG9408 (n = 3) revealed approximately 300 spots (Fig. 2), ~121, 123 and 55 of which were distributed in the pH ranges of 4 to 5, 5 to 6 and 6 to 7, respectively. More than 50% of the spots (170 spots) were concentrated in the pH range of 4.5 to 5.5. In terms of molecular mass, the spots showing relatively low intensities were distributed in the range of 15 to 32 kDa, while more intense spots were found in the range of 30 to 70 kDa. Two long horizontal streaks were found at molecular weights of 43 and 35 kDa.

For protein identification, the spots were randomly selected and subjected to PMF analysis. A total of 30 protein spots corresponding to 28 different proteins were successfully identified (Table 2). Of these, only Heat Shock Protein 60 (HSP 60, Spot 3) and 6-phosphofructokinase (Spot 20) were identified from the *Lactococcus garvieae* database, while the remainders were identified based on similarities to known proteins of other species in genus *Lactococcus* or *Streptococcus*. Functional analysis allowed us to divide these 28 proteins into 9 functional groups (Table 3): translation, ribosomal structure and biogenesis (6 proteins), transcription (1 protein), posttranslational modification (2 proteins), nucleotide transport and metabolism (3 proteins), carbohydrate transport and metabolism (7 proteins), energy production and conversion (2 proteins), amino acid transport and metabolism (2 proteins), cell wall/membrane/envelope biogenesis (4 proteins) and lipid transport and metabolism (1 protein) (www.jgi.doe.gov).

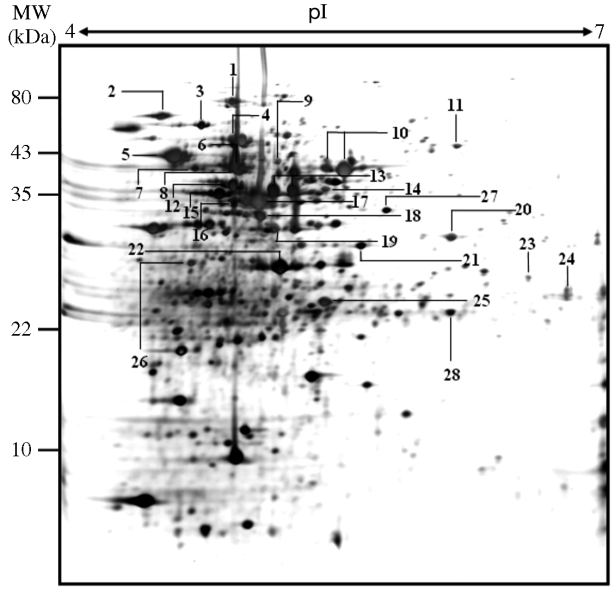

Fig. 2. *Lactococcus garvieae* KG9408. A partial 2-DE map. IEF was performed on whole cell lysates with an IPG dry strip (pH 4 to 7, 13 cm), followed by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. Spots (1–17) were randomly selected, numbered and identified by PMF analysis. MW: molecular weight; pI: isoelectric point

DISCUSSION

Although complete genomic sequences are available for many organisms, this information is of limited use in predicting gene and protein functions (Blackstock & Weir 1999, Cash 2000, Cordwell et al. 2001, Champomier-Verges et al. 2002, Lin et al. 2003). Thus, proteomics has emerged as a valuable method for analyzing the genome-encoded proteins, and has proven useful for studying protein functions in cellular architecture, metabolic regulation and disease pathology (Cash 2000, Cordwell et al. 2001, Lin et al. 2003). A core method for proteomic analysis involves the use of 2-DE to separate the protein complement of a cell or tissue based on MW and pH (O'Farrell 1975), followed by identification of the separated proteins by PMF using MALDI-TOF MS and subsequent database searching (Cash et al. 1999, Thongboonkerd et al. 2002, Lee et al. 2003, Len et al. 2003, Lin et al. 2003, Mortz et al. 2003). Here, we used this technique to construct partial 2-DE reference maps for *Streptococcus iniae* ATCC29178 and *Lactococcus garvieae* KG9408.

In the 2-DE profiles of *Streptococcus iniae* and *Lactococcus garvieae* KG9408, the majority of the protein spots were distributed at pH 4.5 to 5.5 and MW 20 to 60 kDa. These findings are consistent with a previous report on *S. mutans*, *S. pnumoniae* and lactic acid bacteria (LAB). Len et al. (2003) detected 800, 1300, 650 and 200 spots at pH 4.0 to 5.0, 4.5 to 5.5, 5.5 to 6.7 and 6.0 to 11.0 of IPG strips (18 cm), respectively, in 2-DE profiles of *S. mutans* The majority of proteins were found below pH 6.2 in 2-DE profiles of *S. pnumoniae* (Cash et al. 1999), and at pH 4.5 to 5.5 in the LABs *L. lactis*, *S. thermophilus*, *Lactobacillus delbrueckii* and *L. sakei* (Champomier-Verges et al. 2002). Moreover, the majority of proteins predicted from the *L. lactis* genome were concentrated in 2 groups, the major group consisting of 56% of proteins in the pI range 3.4 to 7.0 and the minor group consisting of 33% of proteins at pI > 9 (Champomier-Verges et al. 2002). A similar pattern was also reported in theoretical proteome for *S. thermophilus* genomes (Arena et al. 2006). In addition, the preliminary study showed that when a pH 3 to 10 IPA strip was used both bacterial proteins were concentrated in the range of pH 4 to 7 of the 2-DE gels using the IPG strip with a pH of 3 to 10. The above studies show that the majority of proteins in *Streptococcus* spp. and LAB seem to distribute around pH 4 to 7, particularly, at pH range 4.5 to 5.5 of the 2-DE gels

Table 2. *Lactococcus garvieae.* Proteins identified from *L. garvieae* KG9408 using 2-DE followed by MALDI-TOF MS and database analysis. MOWSE: molecular weight search score, scoring based on peptide frequency distribution of protein searched by the MS-Fit program (http://prospector.ucsf.edu) in protein sequence databases of the NCBI and SwissProt. pI: isoelectric point

Spot no.	Protein	MOWSE score	Masses matched	Coverage (%)	Da/pI	Database species identification
1	Elongation factor G (EF-G)	1.62E + 07	12	21	77 957/4.7	*L. lactis*
2	Chaperone protein dnaK (Heat Shock Protein 70; HSP 70)	2.00E + 04	6	17	64 949/4.6	*L. lactis*
3	60 kDa chaperonin (HSP 60)	1.46E + 04	7	46	19 924/4.8	*L. garvieae*
4	GMP synthase (glutamine amidotransferase)	7.77E + 04	8	17	56 781/4.9	*L. lactis*
5	Enolase	2.01E + 05	9	29	46 912/4.7	*L. lactis*
6	Elongation factor Tu (EF-TU)	1.58E + 08	14	33	43 212/4.9	*L. lactis*
7	ATP synthase alpha subunit	8.37E + 06	11	26	50 911/4.7	*L. lactis*
8	30S ribosomal protein S1	1.06E + 03	5	12	44 710/5.0	*L. lactis*
9	Glutamyl-tRNA synthetase	3.15E + 04	7	15	55 462/5.1	*L. lactis*
10	Arginine deiminase (arginine dehydrolase; AD)	1.50E + 06	10	21	45 981/5.1	*L. lactis*
11	Inosine-5′-monophosphate dehydrogenase (IMP dehydrogenase)	3.83E + 05	12	29	52 827/5.9	*L. lactis*
12	Phosphoglycerate kinase	1.43E + 04	8	26	41 909/4.9	*S. pneumoniae*
13	Glyceraldehyde-3-phosphate dehydrogenase (GapC)	4.91E + 04	8	23	35 907/5.3	*S. uberis*
14	dTDP-glucose 4,6-dehydratase	8.90E + 04	10	30	39 461/5.3	*L. lactis*
15	DNA-directed RNA polymerase alpha chain (RNA polymerase alpha subunit)	8.41E + 06	12	30	34 194/4.9	*L. lactis*
16	Phosphomannomutase	3.60E + 03	8	17	63 112/5.1	*S. pyogenes*
17	Ornithine carbamoyltransferase (OTCase)	1.79E + 04	8	17	39 456/5.4	*L. lactis*
18	L-lactate dehydrogenase (L-LDH)	1.49E + 03	6	16	35 272/5.1	*S. pyogenes*
19	UDP-galactose 4-epimerase	4.58E + 05	9	35	36 292/5.1	*L. lactis*
20	Similar to *Lactococcus lactis* 6-phosphofructokinase	2.29E + 04	7	56	20 013/5.2	*L. garvieae*
21	30S ribosomal protein S2	1.25E + 05	11	35	28 539/5.1	*L. lactis*
22	Fructose-bisphosphate aldolase	3.70E + 03	7	40	31 990/5.0	*L. lactis*
23	Glu-tRNA amidotransferase subunit A	1.12E + 04	8	19	52 072/5.5	*L. lactis*
24	NADH-dependent enoyl-ACP reductase	1.59E + 04	7	25	26 383/6.4	*L. lactis*
25	Phosphoglycerate mutase	1.13E + 04	7	36	26 330/5.3	*L. lactis*
26	Glucose-1-phosphate thymidylyltransferase	7.72E + 04	7	34	32 131/4.7	*L. lactis*
27	UDP-glucose pyrophosphorylase	1.66E + 04	8	26	35 003/5.6	*L. lactis*
28	Uracil phosphoribosyltransferase (UMP pyrophosphorylase) (UPRTase)	1.27E + 04	6	20	23 230/6.5	*L. lactis*

(Cash et al. 1999, Champomier-Verges et al. 2002, Len et al. 2003, Arena et al. 2006). Hence, the present study was conducted for constructing 2-DE reference maps of *S. iniae* and *L. garvieae* using an IPG strip with a pH of 4 to 7 (13 cm). 2-DE maps using pH 4 to 7 in the present study, however, detected fewer spots compared with those of Len et al. (2003). This could be due to differences in protein loading volume according to the pH range, or the length of the IPG strip used for 2-DE.

Our PMF analysis revealed that several proteins involved in energy production and the glycolytic pathway were detected in the acidic region of the 2-DE maps, particularly at pH 4.5 to 5.5. Similarly, the majority of LAB glycolytic enzymes were reported in the same range (Champomier-Verges et al. 2002). This may indicate that the distribution of specific proteins might be similar among the species and/or that it might be a specific characteristic of the family Streptococcaceae.

Because of the limitation of *Streptococcus iniae* and *Lactococcus garvieae* databases, we used the protein databases established from the complete genome sequences of 5 species of the genus *Streptococcus*, *S. pyogenes*, *S. agalactiae*, *S. pneumoniae*, *S. mutans* and *S. thermophilus*, and 1 species of the genus *Lactococcus*, *L. lactis* (Bolotin et al. 2001, 2004, Ferretti et al. 2001, Tettelin et al. 2001, 2002, Ajdic et al. 2002). We identified 17 and 28 proteins in *S. iniae* and *L. garvieae* from the conservative proteins of closely related species, respectively. However, it is likely that other, nonconserved proteins could be present in *S. iniae* ATCC29178 and *L. garvieae* KG9408.

Table 3. Functional classification of proteins identified by PMF using MALDI-TOF MS. Classification of proteins was performed by the integrated microbial genomes (IMG) system of the Joint Genome Institute (www.jgi.doe.gov). –: unidentified protein

Functional categories	Spot no. in 2-DE	
	Streptococcus iniae	*Lactococcus garvieae*
Translation, ribosomal structure and biogenesis		
Elongation factor G (EF-G)	1	1
Elongation factor Tu (EF-Tu)	4	6
30S ribosomal protein S1	–	8
30S ribosomal protein S2	16	21
Glutamyl-tRNA synthetase	–	28
Ribosomal protein S1-like DNA-binding protein	6	–
Glu-tRNA amidotransferase subunit A	–	23
Transcription		
Transcriptional antiterminator	12	–
DNA-directed RNA polymerase alpha chain	10	16
Posttranslational modification, protein turnover, chaperones		
Heat Shock Protein 70 (HSP 70)	2	2
HSP 60	3	3
Nucleotide transport and metabolism		
Glutamine amidotransferase (GMP synthase)	–	4
IMP dehydrogenase	–	11
Uracil phosphoribosyltransferase (UPRTase)	17	28
Carbohydrate transport and metabolism		
Enolase	7	5
Phosphoglycerate kinase (PGK)	9	12
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	11	13
6-phosphofructokinase	13	20
Putative phosphomannomutase	–	15
Phosphoglycerate mutase	–	25
Pyruvate kinase	5	–
Fructose-bisphosphate aldolase	–	22
Energy production and conversion		
ATP synthase alpha subunit	8	7
Putative L-lactate dehydrogenase	–	18
Formate acetyltransferase	14	–
Amino acid transport and metabolism		
Arginine deiminase (ADI)	–	10
Ornithine carbamoyltransferase	–	17
Cell wall/membrane/envelope biogenesis		
dTDP-glucose 4,6-dehydratase	–	14
UDP-galactose 4-epimerase	–	19
Glucose-1-phosphate thymidylyltransferase	–	27
UDP-glucose pyrophosphorylase	–	28
Lipid transport and metabolism		
Acetoin dehydrogenase	15	–
NADH-dependent enoyl-ACP reductase	–	24

Of the identified proteins, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase, HSP 70 and HSP 60 were identified in the 2-DE map of *Streptococcus iniae* ATCC29178. These proteins have previously been associated with virulence and antibiotic resistance (Pancholi & Fischetti 1992, Polla et al. 1995, Cash et al. 1999, Cash 2000, Cunningham 2000, Cordwell et al. 2001, Thongboonkerd et al. 2002). Both GAPDH and enloase are known plasminogen-binding proteins that are involved in the adherence of *S. pyogenes* (Pancholi & Fischetti 1992, Cunningham 2000), while 2-DE profiling revealed that 1 GAPDH isoform was significantly upregulated in an erythromycin-resistant strain of *S. pneumoniae* (Cash et al. 1999). In addition, HSP 70 and HSP 60 have been associated with invasion and immunodominant antigens of pathogenic bacteria (Polla et al. 1995). Similarly, 10 out of the 28 proteins identified from the 2-DE map of *Lactococcus garvieae* KG9408 have been previously associated with virulence, antibacterial targeting and capsule synthesis in species of Streptococcaceae. Seven of these proteins, GAPDH, enolase, HSP 70, HSP 60, arginine deiminase (AD), ornithine carbamoyltransferase (OTCase) and phosphoglycerate kinase (PGK), have been associated with virulence (Pancholi & Fischetti 1992, Polla et al. 1995, Cash et al. 1999, Jungblut et al. 1999, Cash 2000, Cunningham 2000, Cordwell et al. 2001, Hughes et al. 2002, Thongboonkerd et al. 2002). OTCase and PGK have also been reported to act as protective antigens in *S. agalactiae* (Hughes et al. 2002). Inosine-5′-monophosphate dehydrogenase (IMP dehydrogenase) and NADH-dependent enoyl-ACP reductase have been shown to act as antibacterial targets (Hedstrom 1999, Marrakchi et al. 2003), while UDP-glucose pyrophosphorylase is a capsule synthesis enzyme (Crater & van de Rijn 1995). Thus, our partial 2-DE maps of *L. garvieae* KG9408 and *S. iniae* ATCC 29178 allowed us to successfully identify a number of proteins potentially responsible for the virulence and function of these bacteria.

Proteomic approaches have been widely used to study the pathogenic *Streptococcus* species for their pathogenicity mechanisms, antibiotic resistances and abilities to adapt to adverse conditions and host immune responses (Cash et al. 1999, Cash 2000, Lei et al. 2000, Cordwell et al. 2001, Hughes et al. 2002, Thongboonkerd et al. 2002, Len et al. 2003). However, while *S.*

iniae and *Lactococcus garvieae* are serious biological threats to the development and sustainability of fish farming, little is known about the pathogenesis of these bacteria. Here, we report preliminary partial 2-DE reference maps of *S. iniae* ATCC29178 and *L. garvieae* KG9408. These proteomes may provide useful information for the development of effective diagnostic tools and vaccines against *S. iniae* and *L. garvieae.*

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