RESEARCH LETTER

Immunoproteomic assay of extracellular proteins in *Streptococcus equi* ssp. *zooepidemicus*

Ying Mao, Hongjie Fan & Chengping Lu

Key Lab Animal Disease Diagnostic and Immunology, Ministry of Agriculture, Nanjing Agricultural University, Nanjing, China

**Correspondence:** Hongjie Fan, Key Lab Animal Disease Diagnostic and Immunology, Ministry of Agriculture, Nanjing Agricultural University, Nanjing 210095, China. Tel.: +86 025 84395328; fax: +86 025 84396517; e-mail: fhj@njau.edu.cn

Received 21 January 2008; accepted 5 June 2008.
First published online 9 July 2008.
DOI:10.1111/j.1574-6968.2008.01259.x

**Editor:** Mark Enright

**Keywords**
*Streptococcus equi* ssp. *zooepidemicus*; extracellular proteins; two-dimensional gel electrophoresis (2-DE); immunoproteomic; MALDI-TOF/TOF-MS.

**Abstract**

A proteomic approach combining two-dimensional electrophoresis, Western blot and matrix-assisted laser desorption tandem time-of-flight mass spectrometry has been used to map the extracellular proteins of *Streptococcus equi* ssp. *zooepidemicus* (*S. zooepidemicus*) strain ATCC 35246. These bioinformatic technologies facilitated the identification of novel *S. zooepidemicus* vaccine candidate antigens and therapeutic agents. Despite the limitations posed by the unavailability of complete genome and proteome data for *S. zooepidemicus*, seven of 15 chosen immunogenic spots were successfully identified as streptococcal proteins (AE1 and AE4 c. 10) from homologous *Streptococcus* species. Among these, AE6 and AE7 were identified as *S. zooepidemicus* UDP-N-acetyl-glucosamine pyrophosphorylase and UDP-glucose pyrophosphorylase proteins. In addition, AE4 was determined to be glyceraldehyde-3-phosphate dehydrogenase from *Enterococcus faecalis*. Following SIGNALIP 3.0 (http://www.cbs.dtu.dk/services/SignalIP) prediction, data suggested that AE5, AE7 and AE9 contained signal peptides. BLAST (http://www.sanger.ac.uk) results found that nucleotide sequences of all identified proteins shared high homology (≥65%) with *S. zooepidemicus*. The majority of proteins identified in our study remain formally unreported in *S. zooepidemicus*. However, these proteins serve a vital role in the immune system and reproduction of host species. Therefore, we further evaluated the proteins as vaccine candidates in this study.

**Introduction**

*Streptococcus equi* ssp. *zooepidemicus* (*S. zooepidemicus*) is classified in Lancifield’s group C and causes respiratory disease in a variety of mammals. In Europe and the United States, *S. zooepidemicus* is the primary cause of equine respiratory tract infections in foals and infertility in mares (Causey et al., 2006). However, in China, *S. zooepidemicus* is the main pathogen causing disease in swine. In the summer of 1975, a pandemic swine streptococcosis outbreak occurred in western China, resulting in substantial economic losses. The pathogen was identified as *S. zooepidemicus* (Feng & Hu, 1977). From the 1990s to the present, sporadic cases and regional epidemics are still impacting the pig industry. Humans can also be infected through contact with sick animals, unpasteurized milk or other dairy products (World Health Organization, 2005). Therefore, this zoonotic bacterium poses a health risk to both swine and humans.

The development of an effective vaccine is vital to circumvent the widespread economic losses in the event of a pandemic. Subunit vaccine is preferred in our vaccine development strategies due to its high efficiency and safety, while the killed whole-cell vaccines and the living vaccines both have severe defects (Zhang & Lu, 2007a, b). Previous researches have reported several virulent factors of *S. zooepidemicus*, including M-like protein, IgG-binding proteins and fibronectin (Jonsson et al., 1995; Timoney et al., 1995; Lindmark et al., 1996), but no effective subunit vaccine against *S. zooepidemicus* has yet been reported. Therefore, novel immunogens need identification, which may become potential vaccine candidates.

Extracellular bacterial proteins fulfill a variety of functions to ensure bacterial survival in their respective environments. Pathogenic bacteria, however, secrete an additional arsenal of strategies for cell adhesion and invasion, as well as survival and proliferation in hosts (Trost et al., 2005). Some extracellular bacterial proteins or their byproducts have...
been used successfully as vaccines, such as the tetanus (Clostridium tetani) and the pertussis toxins (Bordetella pertussis) (Pichichero & Casey, 2005).

This paper describes an immunoproteomic approach to finding new antigenic extracellular proteins in S. zooepidemicus strain ATCC 35246. The powerful techniques of immunoproteomics are based on isoelectric focusing (IEF) dominating two-dimensional gel electrophoresis (2-DE). In addition, 2-DE is followed by immunoblotting and subsequently combined with protein identification by matrix-assisted laser desorption tandem time-of-flight mass spectrometry (MALDI-TOF/TOF-MS). We used these novel approaches to rapidly survey the extracellular proteins of S. zooepidemicus, to recognize new immunogens and direct vaccine-selecting research.

Materials and methods

Strain and culture conditions

Streptococcus equi ssp. zooepidemicus strain ATCC 35246 was isolated from a diseased pig in Sichuan Province, China, in 1976 (Feng & Hu, 1977). The strain was purchased from the American Type Culture Collection (ATCC) and preserved by Key Lab Animal Disease Diagnostic and Immunology, Ministry of Agriculture, Nanjing Agricultural University. Streptococci were cultured overnight at 37 °C in serum-free Todd–Hewitt broth (THB) (Difco Laboratories, Detroit, MI) on a gently rocking shaker.

Preparation of extracellular proteins by TCA-acetone

Extracellular proteins were prepared using the method of Bumann et al. (2002). The precipitated proteins were air-dried and stored at −20 °C until use. THB medium was treated employing the same protocol and used as a blank control.

Preparation of convalescent serum

Swine origin convalescent sera were obtained from specific pathogen-free (SPF) minipigs artificially infected with ATCC 35246. Professor Xiougou Hua at the College of Agriculture and Biology, Shanghai Jiao Tong University kindly provided two SPF minipigs.

Before immunization, we collected sera as a negative control. Immunogens preparation and immune procedure were followed according to a protocol of Zhang & Lu (2007a, b), and the dose was 1.0 × 10⁸ cells per pig. Swine sera were collected after the second immunization, and titers of sera were evaluated by whole-cell enzyme-linked immunosorbent assay (ELISA) using the method of W. Zhang et al. (unpublished data). Formaldehyde-inactive streptococci (1.0 × 10⁸ cells) were coated in every well of the ELISA plate (Costar, Corning Incorporated), and subsequently blocked with 0.5% bovine serum albumin (BSA) at 37 °C. The detected positive sera were loaded at dilutions from 1:20 to 1:12 800, and the negative sera were diluted to 1:200. Staphylococcal Protein A–horseradish peroxidase (SPA–HRP, Boster; 1:2000 dilution) was used as the second antibody, which was developed with 3,3′,5,5′-tetramethylbenzidine, and the results detected at OD450 nm. The detected (positive) sera of OD450 nm exceeded that of the negative sera (OD450 nm = 0.15) by 2.1 times. Finally, we chose the sera with high titer for the below experiments (OD450 nm = 1.13; negative sera OD450 nm = 0.15).

2-DE

The rehydration buffer and equilibration solution were obtained from a 2-D Starter Kit (Bio-Rad). Proteins were treated with 2-D Clean-up Kit (GE Healthcare) and quantified (2-D Quant Kit, GE Healthcare) before loading the strips. Two Immobiline DryStrip gel (IPG) strips were rehydrated at once, and each strip was loaded with up to a maximum of 169 μg of protein. IEF was conducted with 7-cm IEG (pH 4–7; Bio-Rad) and Zhang’s program parameters (Zhang & Lu, 2007a, b). Laemmli’s (1970) protocol was employed to perform the 2-DE (SE 600 Ruby, GE Healthcare). One gel was visualized by Coomassie G-250 staining and the other was used for Western blotting. Five replications were conducted to reduce or eliminate contingent spots.

Western blotting

The transfer procedures followed Donoghue et al. (2006) with few modifications. Briefly, the transferred membrane was then blocked overnight at 4 °C with 0.5% (w/v) BSA in PBST (0.05% w/v Tween-20 in PBS, pH 7.4) and subsequently incubated with sera (1:1000 dilution) at 37 °C for 1.5 h. The membrane was washed three times with TTBS (0.1% Tween-20, 20 mM Tris, 150 mM NaCl, pH 7.4), incubated with HRP–SPA (Boster; 1:10 000 dilution) at 37 °C for 1 h and washed again with PBST. The enhanced chemiluminescent (ECL) detection was performed with Western blotting detection reagents (GE Healthcare) according to the manufacturer’s recommendations. Finally, the treated membrane was exposed on films (X-Ray Omit BT Film, Kodak) for optimum image visualization. The blank control and the protein samples were similarly treated. Because of the high variance potential, five replications of the Western blot were performed.

Identification of antigenic proteins and MALDI-TOF/TOF-MS analysis

The gels and exposed films were both scanned into the computer. IMAGEMASTER 2D PLATINUM software (version 6.0,
GE Healthcare) was used to assist in matching gel and film spots. The spots identified as immune sera on a 2-D sodium dodecyl sulfate polyacrylamide gel electrophoresis duplicate gel were excised for tryptic in-gel digestion and MALDITOF/TOF-MS peptide fragmentation fingerprint (PFF) analysis (ABI Voyager DE Pro, Applied Biosystems) (Wang et al., 2005). PFF data were analyzed using the Mascot Sequence Query server (http://www.matrixscience.com). The parameters were set for trypsin digestion, one missed cleavage, [M+H]+ and monoisotopic peptide masses, oxidation modification option, peptide mass tolerance (± 100 mg L⁻¹) and fragment mass tolerance (± 0.8 Da). Only peptides in the Mascot Sequence Query search with a rank of 1.0 were considered significant. Consequently, the significant sequences were used for the combined peptide score, which were analyzed for homology on the Sanger Centre database (http://www.sanger.ac.uk). Low-scoring proteins were either verified manually or rejected. The criteria used to accept protein identifications were based on PFF data, including the extent of sequence coverage, number of peptides matched, and score of probability.

**Bioinformatics analysis**

The online software Compute pI/MW (http://www.expasy.org/tools/pi_tool.html) tool predicted the molecular weight (MW), and isoelectric point (pI). The online software SignalIP 3.0 (http://www.cbs.dtu.dk/services/SignalIP) was applied to predict protein signal peptides. The species option for gram-positive bacteria was selected and the default was accepted for other all other options.

**Results**

**2D gel profiles of ATCC 35246 extracellular proteins**

ImageMaster 2D Platinum software detected a total of c. 263 spots on the 2-DE profile of ATCC 35246 (Fig. 1a) extracellular proteins compared with the blank control (Fig. 1c). Five replicates of 2-DE were conducted, and the patterns were consistently reproducible. It is of interest that the five linear spots on the blank control gel (shown in the oblong area in Fig. 1c) were all identified as BSA (data not shown).

**Identification of immunoreactive proteins and PFF analysis**

Fifteen immunoreactive proteins with high repeatability (AE1 to AE15) were identified by immunoproteomics (Fig. 2a and b), and the blank control was not detected (Fig. 1d). Typically, abundant spots do not possess strong immunogenicity. Spots corresponding to protein spots in the preparative 2D gel were analyzed by tryptic digestion and PFF analysis. For microorganisms, PFF results are highly reliable when putative proteins are homologous with proteins in the National Center for Biotechnology Information (NCBI) database. AE1 and AE4 c. 10 were successfully identified via BLAST queries (Table 1), while AE2, AE3 and AE11 c. 15 were determined to be novel proteins lacking homologues from the PFF database searching.

**Bioinformatics analysis**

The theoretical MW, pI and putative signal peptides of the identified proteins are summarized in Table 1.

**Discussion**

Our study found that the protein most similar to AE4 was glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; GAPDH) from Enterococcus faecalis (identities = 765/1008, 75%). Previous reports indicated GAPDH is located on the cell wall in Streptococcus species, and transported to the cell surface without recognizable signal sequences (Lottenberg et al., 1994), the results consistent with SignalIP 3.0 predictions (Table 1). In addition, streptococcal surface GAPDH was determined to serve as the streptococcal plasmin receptor, involved in bacterial adhesion and signal transduction to host cells (Cunningham, 2000). In bacterial microorganisms, GAPDH has been either shown to be a protective antigen or proposed as a potential target for vaccine development (Fontaine et al., 2002; Rosinha et al., 2002). Perez-Casal found GAPDH have the ability to elicit immune response in cattle exposed to Mycoplasma bovis, which make it a potential candidate for a vaccine (Perez-Casal & Prysliak, 2007).

AE1 [3-phosphoglycerate kinase (EC 2.7.2.3; 3-PGK)] is one of the oldest ‘housekeeping’ enzymes (Ciccarese et al., 1989). Signal peptide prediction indicated that AE1 did not possess the typical LPXTG cell-anchoring sequence motif in gram-positive cocci (Fischetti et al., 1990). A recent study found that 3-PGK from group B streptococci had the ability to bind actin, and the 3-PGK–actin interaction played a role in bacterial internalization (Burnham et al., 2005). Hughes et al. (2002) reported that 3-PGK was a successful protective antigen against various serotypes of Streptococcus agalactiae (Hughes et al., 2002). This glycolytic enzyme exhibited 82% sequence identity with S. zooepidemicus. It is possible that 3-PGK also works in S. zooepidemicus, and thus warrant further investigation as vaccine candidate.

Group C streptococci have been shown to possess a hyaluronic acid capsule identical to the hyaluronic acid in human connective tissue (Kim et al., 1996), which may be the cause of its poor immunogenicity in human hosts. AE7 and AE6 were separately identified as UDP-glucose pyrophosphorylase (EC 2.7.7.9; UDPG: PP) and UDP-N-acetylglucosamine pyrophosphorylase (EC 2.7.7.23; UDP-GlcNAc...
PP), which are required for capsular polysaccharide synthesis. Both of these enzymes shared the highest sequence homology (97%) with S. zooepidemicus. In addition, AE7 contained a recognizable signal peptide, indicating that it is secreted to the extracellular environment. Both AE6 and AE7 possessed no additional annotations before now, and it needs further study to confirm whether they are protective antigens.

AE8 was identified as 50S ribosomal protein L1 and exhibited 87% sequence similarity with S. zooepidemicus. The protein has a dual function and serves as both a ribosomal structural protein that binds rRNA and a translational repressor that binds its own mRNA (Nevskaya et al., 2006). Rapid protein synthesis adaptations to varied environmental conditions are afforded by this translational regulation (Williams et al., 2007). This trait may increase

Fig. 1. (a) Coomassie brilliant blue (CBB) G-250-stained 2-DE gel of extracellular proteins of Streptococcus equi ssp. zooepidemicus. All protein spots identified were excised from the preparative gel and characterized by MALDI-TOF/TOF-MS analysis. The spots were encoded using an abbreviation of ATCC 35246 extracellular protein (AE) followed by protein number. (b) Western blot analysis of proteins on the 2D duplicate gel, which were transferred to a polyvinylidene difluoride (PVDF) membrane. The primary antibody was a convalescent sera raised in S. zooepidemicus-immunized SFP minipigs. (c) CBB G-250-stained 2-DE gel of the blank control (THB medium extraction). (d) ECL image of Western blot blank control.
the pathogen's adaptation to the host, and therefore provides an obvious advantage to the microorganism (Williams et al., 2007), but there have been no reports about whether 50S ribosomal protein L1 is a protective antigen till now.

AE5 was putatively identified as the secreted Glucan-binding protein B (GbpB) antigen, whose gene encodes a peptidoglycan hydrolase, presumed important in cell wall integrity and glucan binding. Previous data indicated that

![Fig. 2. Amplified oblong areas of (a) and (b) in Fig. 1. A total of 15 immunoreactive proteins with high repeatability, from AE1 to AE15, were identified and chosen for further MALDI-TOF/TOF-MS analysis using the duplicate gel.](image)

<table>
<thead>
<tr>
<th>Spot number</th>
<th>MASCOT matches*</th>
<th>BLAST results*/species*/identity score¹</th>
<th>SIGNALP 3.0 prediction³</th>
<th>Experimental Mr/p</th>
<th>Theoretic Mr/p⁶</th>
<th>MASCOT score⁷</th>
<th>Sequence coverage (%)⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE4</td>
<td>gi</td>
<td>29376486</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase/Enterococcus faecalis V583/identities = 765/1008 (75%)</td>
<td>–</td>
<td>35749/5.03</td>
<td>35771.5/5.03</td>
<td>150</td>
</tr>
<tr>
<td>AE1</td>
<td>gi</td>
<td>21911160</td>
<td>3-Phosphoglycerate kinase/S. pyogenes MGA5315/identities = 986/1198 (82%)</td>
<td>–</td>
<td>42104/4.86</td>
<td>42129.9/4.86</td>
<td>262</td>
</tr>
<tr>
<td>AE7</td>
<td>gi</td>
<td>24940624</td>
<td>UDP-glucose pyrophosphorylase/S. zooepidemicus/identity = 876/903 (97%)</td>
<td>+</td>
<td>33186/5.94</td>
<td>33206.3/5.94</td>
<td>207</td>
</tr>
<tr>
<td>AE8</td>
<td>gi</td>
<td>21909861</td>
<td>50S ribosomal protein L1/S. pyogenes MGA5315/identities = 604/690 (87%)</td>
<td>–</td>
<td>24336/9.22</td>
<td>24351.9/9.22</td>
<td>107</td>
</tr>
<tr>
<td>AE6</td>
<td>gi</td>
<td>33318646</td>
<td>UDP-N-acetyl-glucosamine pyrophosphorylase/S. zooepidemicus/identities = 1350/1383 (97%)</td>
<td>–</td>
<td>49373/5.55</td>
<td>49402.8/5.55</td>
<td>117</td>
</tr>
<tr>
<td>AE5</td>
<td>gi</td>
<td>24378550</td>
<td>Putative secreted antigen GbpB; putative peptidoglycan hydrolase/S. mutans UA159/identities = 480/734 (65%)</td>
<td>+</td>
<td>44594/4.83</td>
<td>44620.4/4.83</td>
<td>78</td>
</tr>
<tr>
<td>AE9</td>
<td>gi</td>
<td>2326862</td>
<td>Streptodornase/S. pyogenes/identities = 759/1161 (65%)</td>
<td>+</td>
<td>43450/6.09</td>
<td>43476.4/6.09</td>
<td>77</td>
</tr>
<tr>
<td>AE10</td>
<td>gi</td>
<td>15675236</td>
<td>6-Phosphofructokinase/S. pyogenes M1 GAS/identities = 853/1013 (84%)</td>
<td>–</td>
<td>35726/5.33</td>
<td>35748.3/5.33</td>
<td>550</td>
</tr>
</tbody>
</table>

*Search results from the Matrix Science MASCOT server (http://www.matrixscience.com), and the genomic sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov).

¹Search results from the Sanger Centre (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_zooepidemicus).

³Signal peptides were predicted from SIGNALP 3.0 server (http://www.cbs.dtu.dk/services/SignalP).

⁶Theoretical MW and pI were calculated using the online software COMPUTE PI tool (http://www.expasy.ch/tools/pi_tool), with the average resolution option.

⁷The threshold of significance was 77 for all values in this study.

⁸Nonsignificant results of spots AE2, AE3 and AE11 according to MASCOT Sequence Query, with a lack of sufficient protein information and genome data from Streptococcus equi ssp. zooepidemicus in NCBI.

Table 1. Summary of protein spots analysis identified by MALDI-TOF/TOF-MS

——

FEMS Microbiol Lett 286 (2008) 103–109 © 2008 Federation of European Microbiological Societies Published by Blackwell Publishing Ltd. All rights reserved
GbpB played an important role in bacterial adherence and pathogenesis as a pacemaker and space-maker enzyme for cell wall growth, although its glucan-binding properties are still obscure (Mattos-Graner et al., 2006; Lynch et al., 2007). Other research demonstrated some peptidoglycan hydrolases trigger cell lysis, which suggested several important implications for cell wall turnover, cell wall modification, cell separation and motility (Perkins, 1980). Our study indicated that a signal sequence was contained in the N-terminal region of GbpB. However, it was reported to exist in both secreted and cell-associated forms (Yamaguchi et al., 2004), and surface antigen A is the secreted form of GbpB, and binds to various extracellular matrix proteins (Yamaguchi et al., 2004; Mattos-Graner et al., 2006). In addition, GbpB was reported to induce protective immunity against dental caries caused by Streptococcus mutans in humans and other animal models (Smith et al., 2003). Therefore, it may be a suitable target in the development of vaccines to S. zooepidemicus.

AE9 was identified as streptodornase, also known as streptococcal deoxyribonuclease (SD). It is one of the group C streptococci extracellular nuclease, which endonucleolytically degrade native and double-stranded DNA (Locke et al., 1997). Preliminary studies showed streptodornasases allow bacteria to spread and invade deep host tissues by increased evasion of the innate immune response (Sumby et al., 2005). Moreover, it may have a role in nutrition (Patil et al., 2005). SD has also been reported as a virulence factor of Streptococcus equi (Tiwari et al., 2006), but there are no reports about SD as a vaccine candidate according to the available documents.

Spot AE10 [6-phosphofructokinase (PFK)/Streptococcus pyogenes M1 GAS] had the highest MASCOT score (550) and sequence coverage (47%) in our study. Therefore, homology between PFK in S. pyogenes M1 GAS and that of S. zooepidemicus was high. PFK, a bifunctional enzyme in the classical sense, catalyzes the first essentially irreversible reaction of glycolysis, but in many cases, the physiological role of PFK is not obvious (Siebers et al., 1998). Loo et al. (2003) found a PFK from Streptococcus thermophilus that provided a regulatory link between glycolytic activity and signal transduction regulation involving biofilm formation (Loo et al., 2003). In addition, this enzyme is evolutionarily constrained (Bapteste et al., 2003); hence, we tentatively determined that PFK in S. zooepidemicus possesses similar protective antigen functions.

The accuracy of results can be improved if the following conditions are met: the entire genome of S. zooepidemicus is represented in the NCBI nr database (where the MASCOT server was originally linked) and S. zooepidemicus polypeptide databases are developed from Expressed Sequence Tag (EST) sequences (Wang et al., 2005). The eight putative extracellular proteins newly identified from S. zooepidemicus are of great interest to elucidate pathogen–host interactions and as novel vaccine candidates. However, immunogens are not protective antigens. Protective immunity and neutralizing ability evaluation of all immunogens is vital in vaccination strategy development (Chen et al., 2004). Research to generate additional data from S. zooepidemicus is ongoing in our laboratory, and the results of further studies will be reported in the near future.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (No. 30771605), and the Key Program of Ministry of Education, China (No. 106091).

References


