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Proteomic Analysis of Membrane Proteins from *Streptococcus pneumoniae* with Multiple Separation Methods Plus High Accuracy Mass Spectrometry

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Abstract

Streptococcus pneumoniae is a Gram-positive human pathogen that causes a variety of serious mucosal and invasive diseases in human. Bacterial membrane proteins play crucial roles in host–pathogen interactions and bacterial pathogenesis, and thus are potential drug targets or vaccine candidates. In this study, membranes from *Streptococcus pneumoniae* D39 were enriched by mechanical grinding and ultracentrifugation, and then the membrane proteins were extracted with trifluroethanol and chloroform. Around 60% of the extracted proteins were identified to be membrane proteins with 2-DE coupled with MALDI-MS/MS and 2D-LC-ESI-MS/MS. These identified membrane proteins can be functionally categorized into various groups involved in nutriment transport, signal transduction, protein folding or secretion, oxidation, carbohydrate metabolism, and other physiological processes. A protein interaction network was constructed for understanding the regulation relationship of the membrane proteins. This study represents the first global characterization of membrane proteome from Gram-positive streptococcus species of bacteria, providing valuable clues for further investigation aiming at identifying drug/vaccine targets for the bacterial infection.

Introduction

Bacterial membrane proteins play fundamental roles in the interaction between the bacterial cell and its environment. These proteins perform essential physiological functions, including adhesion to and invasion in host cells, sensing the chemical and physical conditions of the external environment, sending appropriate signals to the cytoplasmic compartments, transportation of small molecules, defense against host responses, export of metabolic end products and toxic substances (Poetsch and Wolters, 2008; Rabilloud, 2009; Rodriguez-Ortega et al., 2006; Santoni et al., 2000). Hence, membrane proteins are potential targets of drugs or vaccine candidates in preventing bacterial infections and diseases (Pasini et al., 2009; Rodriguez-Ortega et al., 2006). Despite around 30% of all genes in bacteria encoded as membrane proteins, membrane proteins are rarely detected and identified in two-dimensional electrophoresis (2-DE) (Poetsch and Wolters, 2008; Zuobi-Hasona and Brady, 2008). This is originated from the special properties of membrane proteins, that is, low abundance and limited aqueous solubility. Proteins with high hydrophobicity tend to self-aggregate during isoelectric focusing (IEF), leading to low protein resolution and streaking in 2-DE gels (Santoni et al., 2000). Despite the inherent difficulties to obtain the membrane proteome, reference maps with limited membrane proteins have been published for some bacterial species, including *Escherichia coli* (Molloy et al., 2000), *Bacillus subtilis* (Antelmann et al., 2002), *Pseudomonas aeruginosa* (Nouwens et al., 2000), and *Staphylococcus aureus* (Nandakumar et al., 2005).

Streptococcus pneumoniae (*S. pneumoniae*) is an important human pathogen, responsible for high levels of morbidity and mortality worldwide. This organism is the causative agent of various important diseases including pneumonia, bacteremia, bacterial meningitis, sinusitis, and otitis media (Bae et al., 2006; Mitchell, 2000). The burden of the pneumococcal diseases is highest in children and the elderly, or individuals with immature/compromised immune systems, such as those with diabetes or acquired immuno-deficiency syndrome (Hsieh et al., 2008). Recently, proteomic technologies have been applied to study the regulatory networks in pathogenicity and the protein expression in *S. pneumoniae* (Encheva et al., 2006; Ling et al., 2004; Soualhine et al., 2005; Sun et al., 2010).

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However, proteomic studies focusing on *S. pneumoniae* membrane proteins have not yet been reported.

With a goal to globally characterize the bacterial membrane proteins, we applied mechanical grinding and ultracentrifugation to enrich membranes of *S. pneumoniae* D39, and then extracted the membrane proteins using trifluroethanol/chloroform mixture, solubilized using highly chaotropic solutions containing ASB-14 and Triton X-100. The membrane proteins were separated and efficiently identified through the combined use of 2-DE plus MALDI-MS/MS and 2D-LC-ESI-MS/MS. The identified membrane proteins were further analyzed through functional categorization and network construction using bioinformatics tools. In view of the substantial membrane proteins involved in various physiological and pathogenetic processes being identified, these data may represent a valuable resource for further studying the pathogenesis of streptococcus species, and identifying potential targets for drug or vaccine development against the streptococcus species infections.

Materials and Methods

Bacterial strains and culture

S. pneumoniae D39 strains were grown in Todd-Hewitt broth (THB) supplemented with 0.3% yeast extract at 37°C and 5% CO_2, until an OD_{600} of $\sim$0.6 (exponential phase) reached. Bacteria were harvested by centrifugation at 5,000 $\times g$ for 10 min at 4°C, and washed twice with TM buffer (50 mM maleate buffer plus 20 mM $MgCl_2$, pH 6.0) containing protease inhibitors at 1:30 v/v ratio. Finally, the cell pellets were resuspended with TM buffer and stored at -80°C until further use.

Isolation of S. pneumoniae *cell membrane proteins*

Membrane proteins were obtained by adopting a successful procedure previously reported (Zuobi-Hasona and Brady, 2008) with minor modifications. Briefly, the membrane-containing fractions were enriched by ultracentrifuge after cell suspensions being ground with alumina, and then were resuspended in 300 μL of 50 mM ammonium bicarbonate containing 30 μL protease inhibitor cocktail. Trifluroethanol/chloroform mixture (2:1 v/v, 1 mL) was added and maintained on ice for 1 h with periodical vortex (Deshusses et al., 2003). This was followed with centrifugation at 10,000 $\times g$ for 5 min to separate the mixture into three phases: aqueous upper phase, insoluble interphase, and lower chloroform-solubilized phase. Each phase was dried by vacuum centrifuge for about 2 h, and then solubilized with 200 μL solubilization buffer (7 M urea, 2 M thiourea, 2% Triton X-100, 0.5% ASB-14, 50 mM dithiothreitol, and 0.2% IPG pH4-7). Protein samples were purified with 2-D Clean-Up Kit (GE Healthcare, Uppsala, Sweden) and resolubilized again in solubilization buffer. The protein concentration was determined using the Bradford assay.

2-DE

A previously reported method was applied to perform 2-DE with Amersham Biosciences IPGphor IEF System and Hoefer SE 600 (GE Healthcare) electrophoresis (13 cm) units (He et al., 2003). In brief, 50 μg of proteins (mixture of aqueous upper phase and lower chloroform-solubilized phase fractions) was diluted in a rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT, and 0.5% IPG buffer). IEF was carried out with precast IPG strips (13 cm, pH 4–7, NL). After reduction and alkylation, IPG strips were transferred onto a 12.5% polyacrylamide gel. Gels were visualized using silver-staining in accordance with a previously described protocol (Sun et al., 2009).

In-gel digestion and peptide sequencing

Tryptic in-gel digestion was performed in accordance with our previously described protocol (Wang et al., 2006). Mass spectra of peptides were obtained on an ABI 4800 plus MALDI TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). Both the MS and MS/MS data were processed and interpreted by the GPS Explorer software (Version 3.6, Applied Biosystems), and then the peptide masses were searched against the NCBI database using the MASCOT search engine. The search parameters were as follows: a maximum of two missed cleavages, variable modification of cysteine carboamido methylated and methionine oxidized, none fixed modifications, MS tolerance of 100 ppm, MS/MS tolerance of 0.2 Da. Proteins exceeding 95% confidence are considered. Transmembrane regions for proteins were predicted using the program TMPred (http://www.ch.embnet.org/software/TMPRED_form.html) (Nandakumar et al., 2005). The SwissProt IDs of proteins were input into the TMPred program where the minimal and maximal length of the hydrophic part of the transmembrane helix was set as 14 and 41, respectively.

In-solution digestion and 2D-LC-MS/MS analysis

Tryptic in-solution digestion was performed according to our previously described method (Sun et al., 2010). The peptide mixtures were analyzed by a Finnigan Surveyor HPLC system coupled with a nanospray LTQ-Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) using the experimental parameters applied in our previous study with minor modifications (Ge et al., 2010). Briefly, peptides were separated into three fractions with a series of concentrations of NH_4Cl (0, 50, and 1,000 mM) using strong cation exchange (SCX) column. The three eluted fractions from the SCX column were loaded onto a C18 column. The peptide mixtures were eluted from the C18 column with an increasing 0$\sim$35% [B/(A + B)] acetonitrile gradient solution (Buffer A, 0.1% formic acid and 5% ACN; Buffer B, 0.1% formic acid and 95% ACN) over the time of 120 min, and were monitored online with the mass spectrometer via a data-dependent TOP10 method (Haas et al., 2006). A spray voltage of 1.85 kV was used in this approach. All the raw data files were processed using BioWorks 3.3.1 (Thermo Finnigan, San Jose, CA) and the derived peak lists were searched against the forward and reversed NCBI *S. pneumoniae* D39 protein database using the MASCOT search engine. The search criteria were as follows, full tryptic specificity, two missed cleavages, carbamidomethylation as fixed modification, and oxidation (M) as variable modification. The false-positive rate based on the decoy database search was $<$2%.

Protein categorization and network construction

Identified membrane proteins were classified based on the Gene Ontology (GO) database of *S. pneumoniae* D39 according

to their functions (Sun et al., 2010). Some proteins were annotated manually based on literature searches or closely related homologues. The interaction network of membrane-associated proteins was built automatically by the STRING (http://string.embl.de) (Search Tool for the Retrieval of Interacting Genes/Proteins) system with default setting except that organism, confidence (score), and interactors shown were set to "*S. pneumoniae* D39," "0.40," and "no more than 10 interactors," respectively (von Mering et al., 2005, 2007). The interaction network was represented with Cytoscape, a versatile open source environment for visualizing and analyzing molecular interaction networks (Shannon et al., 2003).

Results and Discussion

2-DE profile of enriched membrane proteins

Cellular membrane proteins extracted from the bacterium were subjected to 2-DE separation. Figure 1 shows a representative profile of three repetitions of the 2-DE experiments for the extracted membrane proteins. Proteins as spots were nicely distributed throughout the gel in the range of pH 4–7 where more proteins were concentrated in the lower pH areas. Protein spots were cut from the gels and subjected to in-gel tryptic digestion and MALDI-MS/MS analysis. Totally, 68 unique proteins were identified. After literature searching and TMPred analysis, 47 proteins were determined to belong to the family of membrane proteins. Among these proteins, 31 proteins contain at least one TMR (TMpred), 16 proteins are membrane-associated proteins (Table 1). All the relevant information of the identified proteins, including their protein NCBI accession numbers, gene names, and the number of transmembrane region are listed in Supplementary Table S1. Although only 114 protein spots were visible in the membrane protein profile compared to approximately 800 spots appearing in its total protein profile, many hydrophobic proteins or proteins with low abundance were only observed in the current map of membrane proteins. These include cell division protein DivIVA, YlmF protein, some ABC transporters and conserved hypothetical proteins.

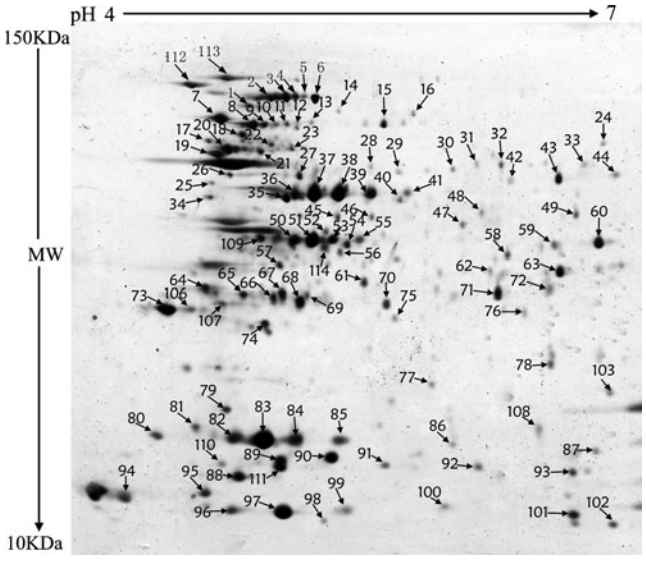

FIG. 1. 2D-PAGE of *S. pneumoniae* D39 cell membrane proteins. A total of 50 μg of membrane proteins were extracted from *S. pneumoniae*, separated by 2D-PAGE, and stained with silver. More than 120 proteins were visualized on the gel.

Identification of membrane proteins with 2D-LC-MS/MS

To increase the coverage of membrane proteins, extracted membrane fraction (1 mg) was directly in-solution digested with trypsin. After analysis with the high-accuracy LTQ-Orbitrap mass spectrometer, 249 proteins were identified. By comparison, almost all of the proteins identified above with the traditional 2-DE coupled with MALDI-MS/MS method can be found in the protein dataset produced by the 2D-LC-MS/MS method. The only three exceptions are oxidoreductase (aldo/keto reductase family protein), 6,7-dimethyl-8-ribityllumazine synthase, and tRNA (guanine-N (7)-)-methyltransferase. Among these identified proteins, 163 proteins were acknowledged as membrane proteins (Table 1). After TMPred prediction, 133 proteins were recognized as integral membrane proteins containing at least one transmembrane region, 30 proteins belong to membrane-associated proteins. All the identified proteins with their protein NCBI accession numbers and gene names based on 2D-LC-MS/MS analysis are listed in Supplementary Table S2. In comparison with membrane protein profiles of other streptococcus strains, only 13 membrane proteins were commonly identified in both *S. pneumoniae* and *S. pyogenes* (Severin et al., 2007), whereas another six membrane proteins common in both *S. pneumoniae* and *S. aureus* (Nandakumar et al., 2005). This may be due to the different enrichment and identification methods used in different studies.

Functional classification of membrane proteins

Classification of the membrane proteins according to the Gene Ontology (GO) database revealed that several important categories including membrane transporters/binding proteins (18%), cell adhesion (1%), oxidation (10%), proteolysis (3%), and carbohydrate metabolism (10%) were well covered (Fig. 2). In addition, some membrane proteins are also involved in the translation, nucleotide metabolism and DNA replication or transcription process in *S. pneumoniae.*

Among the 164 membrane-associated proteins, 37 belong to the ABC (ATP-binding-cassette) transporter superfamily. Found in all species from the microbe to human, ABC transporters represent one of the largest families of paralogous transmembrane proteins (Higgins, 1992). In bacteria, ABC transporters are central in many physiological processes, including the uptake of nutriments, the extrusion of noxious substances, the secretion of extracellular toxins and signaling molecules, and antibiotic resistance (Higgins, 2001; Schmitt and Tampe, 2002). Despite the large number and overwhelming diversity of substrates, an ABC transporter consists of four core domains, two hydrophobic transmembrane domains (TMDs) as a functional transporter and two ATP or nucleotide-binding domains (NBDs) (Higgins et al., 1986; Hyde et al., 1990).

Six proteins (AtpA, AtpC, AtpD, AtpF, AtpG, AtpH) in the F_1F_0 ATP synthase family were detected. Widely distributed from prokaryotic to eukaryotic cells, F_1F_0 ATP synthase produces ATP from ADP in the presence of a proton or sodium

Table 1. Identification of Membrane Proteins with 2-DE Coupled with MALDI-TOF-MS/MS Method and in Solution Digestion with 2D-LC-MS/MS

Accession No.	*Protein name*	*Gene_symbol*	*Molecular_weight*	*PI*	*TMR*	*2-DE*	*2D-LC-MS/MS*
Translation							
gi|116076623	ribosomal protein S13	rpsM	13413	10.63	1	–	+
gi|116076281	ribosomal protein S8	rpsH	14745	9.58	1	–	+
gi|116076101	ribosomal protein S1	rpsA	43869	5.05	1	+	+
gi|116077197	ribosomal protein L7/L12	rplL	12435	4.42	Ref [1]	–	+
gi|116075932	translation elongation factor Tu	tuf	43943	4.86	Ref [3-5]	+	+
gi|116076652	translation elongation factor Ts	tsf	37338	4.84	Ref [3-5]	+	+
gi|116076755	translation elongation factor P	efp	20587	4.86	Ref [3]	–	+
gi|116076360	aspartate–ammonia ligase	asnA	37641	5.05	1	–	+
Protein folding or secretion							
gi|116077567	peptidyl-prolyl *cis-trans* isomerase, cyclophilin-type	SPD_0672	29048	5.47	1	–	+
gi|116077659	protease maturation protein, putative	prsA	34476	5.04	1	–	+
gi|116077212	chaperonin GroEL	groL	57060	4.77	2	+	+
gi|116077405	chaperone protein DnaJ	dnaJ	40487	6.80	1	–	+
gi|116077074	chaperone protein DnaK	dnaK	64772	4.63	Ref [2]	+	+
Proteolysis							
gi|116077146	lipoprotein, putative	SPD_0549	26579	5.13	1	–	+
gi|116076163	cell division protein FtsH	ftsH	71210	5.43	3	–	+
gi|116076257	signal peptidase I	lepB	23483	5.84	1	–	+
gi|116077489	aminopeptidase C	pepC	50281	5.09	Ref [2]	+	+
gi|116077300	peptidase, U32 family protein	SPD_1258	48193	5.23	1	–	+
gi|116076970	conserved hypothetical protein	SPD_0704	41246	5.34	1	–	+
Cell cycle or regulation							
gi|116077314	heat-inducible transcription repressor HrcA	hrcA	39457	5.12	2	–	+
gi|116076185	Hpr (Ser) kinase/phosphatase	hprK	34774	5.29	1	–	+
gi|116076859	cell division protein FtsZ	ftsZ	44389	4.70	2	+	+
gi|116077419	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	murA-2	45923	5.38	1	–	+
gi|116076748	UDP-N-acetylglucosamine pyrophosphorylase	glmU	49454	5.30	1	–	+
gi|116077572	cell division protein FtsA	ftsA	49444	5.12	1	+	+
DNA replication or transcription							
gi|116077314	heat-inducible transcription repressor HrcA	hrcA	39457	5.12	2	–	+
gi|116076869	DNA-directed RNA polymerase, alpha subunit	rpoA	34243	4.64	1	+	+
gi|116077405	chaperone protein DnaJ	dnaJ	40487	6.8	1	–	+
Amino acid metabolism							
gi|116076444	methylenetetrahydrofolate dehydrogenase/ methenyltetrahydrofolate cyclohydrolase	folD	31592	6.25	1	–	+
gi|116077077	carbamoyl-phosphate synthase, large subunit	carB	116499	4.8	1	–	+
gi|116076820	homoserine dehydrogenase	hom	46091	5.05	3	–	+
gi|116077013	aspartate-semialdehyde dehydrogenase	asd	39128	5.09	2	–	+
gi|116077050	GMP synthase, C-terminal domain	guaA	57664	4.94	2	+	+
gi|116076666	glutamine synthetase, type I	glnA	50461	4.93	1	–	+

(*continued*)

Table 1. (Continued)

Accession No.	Protein name	Gene_symbol	Molecular_weight	PI	TMR	2-DE	2D-LC-MS/MS
Carbohydrate metabolism							
gi|116077344	phosphopyruvate hydratase	eno	47131	4.7	1	+	+
gi|116077018	glyceraldehyde-3-phosphate dehydrogenase, type I	gap	35947	5.29	Ref [1,2]	+	+
gi|116077186	glucose-6-phosphate isomerase	pgi	49847	5	3	+	+
gi|116077571	(NAD(P)+)glycerol-3-phosphate dehydrogenase	gpsA	36754	5.19	2	–	+
gi|116076185	Hpr(Ser) kinase/phosphatase	hprK	34774	5.29	1	–	+
gi|116077615	chain length determinant protein/polysaccharide export protein, MPA1 family protein	cps2C	25504	8.68	2	–	+
gi|116076178	L-lactate dehydrogenase	ldh	35390	5.09	2	–	+
gi|116076912	UDP-glucose 4-epimerase	galE-1	37630	4.82	1	–	+
gi|116076436	fructose-1,6-bisphosphate aldolase, class II	fba	31496	5	1	+	+
gi|116077388	dTDP-glucose 4,6-dehydratase	rfbB	39077	5.26	1	–	+
gi|116076180	phosphoglucomutase/phosphomannomutase family protein	pgm	62746	4.74	1	–	+
gi|116077161	phosphoglycerate kinase	pgk	41913	4.92	Ref [2]	–	+
gi|116077356	thioredoxin	trx	11584	4.75	Ref [2]	–	+
gi|116077374	triosephosphate isomerase	tpiA	26818	4.75	2	–	+
gi|116077101	L-serine dehydratase, iron-sulfur-dependent, alpha subunit	sdhA	30644	4.65	5	–	+
gi|116077604	glucokinase	gki	33630	4.85	4	–	+
gi|116076770	glucosamine-fructose-6-phosphate aminotransferase, isomerizing	glmS	65609	5.04	1	–	+
gi|116076462	alcohol dehydrogenase, iron-containing	SPD_1834	97909	6.11	4	–	+
Nucleotide metabolism							
gi|116076444	methylenetetrahydrofolate dehydrogenase/ methenyltetrahydrofolate cyclohydrolase	folD	31592	6.25	1	–	+
gi|116077732	penicillin-binding protein 2B	penA	74500	5.15	2	–	+
gi|116076157	CTP synthase	pyrG	59505	5.45	2	–	+
gi|116077077	carbamoyl-phosphate synthase, large subunit	carB	116499	4.8	1	–	+
gi|116077395	aspartate carbamoyltransferase	pyrB	34742	5.1	Ref [2]	–	+
gi|116075934	uracil phosphoribosyltransferase	upp	23698	5.6	2	+	+
gi|116077388	dTDP-glucose 4,6-dehydratase	rfbB	39077	5.26	1	–	+
gi|116075886	uridylate kinase	pyrH	26417	5.25	1	–	+
gi|116076110	orotate phosphoribosyltransferase	pyrE	22861	5.07	1	–	+
gi|116076833	adenine phosphoribosyltransferase	apt	19631	5.31	1	–	+
gi|116076348	adenylosuccinate synthetase	purA	47712	5.49	1	–	+
gi|116077738	ribonucleoside-diphosphate reductase, beta subunit	nrdF	36939	4.42	1	–	+
Oxidation							
gi|116076444	methylenetetrahydrofolate dehydrogenase/ methenyltetrahydrofolate cyclohydrolase	folD	31592	6.25	1	–	+
gi|116077541	NADP-specific glutamate dehydrogenase	gdhA	48937	5.43	Ref [2]	+	+
gi|116077018	glyceraldehyde-3-phosphate dehydrogenase, type I	gap	35947	5.29	Ref [1,2]	+	+
gi|116076695	pyruvate oxidase	spxB	65327	5.06	3	+	+
gi|116077571	(NAD(P)+) glycerol-3-phosphate dehydrogenase	gpsA	36754	5.19	2	–	+
gi|116076228	dihydrolipoamide dehydrogenase	lpdA	59892	4.88	3	–	+
gi|116077245	NADH oxidase	nox	50452	4.99	1	–	+

(*continued*)

Table 1. (Continued)

Accession No.	Protein name	Gene_symbol	Molecular_weight	PI	TMR	2-DE	2D-LC-MS/MS
gi|116076178	L-lactate dehydrogenase	ldh	35390	5.09	2	−	+
gi|116076123	superoxide dismutase, manganese-dependent	sodA	22383	4.93	1	−	+
gi|116076033	conserved hypothetical protein	SPD_0249	38861	5.44	1	−	+
gi|116076462	alcohol dehydrogenase, iron-containing	SPD_1834	97909	6.11	4	−	+
gi|116076820	homoserine dehydrogenase	hom	46091	5.05	3	−	+
gi|116077013	aspartate-semialdehyde dehydrogenase	asd	39128	5.09	2	−	+
gi|116076541	inosine-5′-monophosphate dehydrogenase	guaB	52526	5.18	1	+	+
gi|116077095	alcohol dehydrogenase, zinc-containing	SPD_0265	36184	4.95	1	−	+
gi|116077673	alcohol dehydrogenase, zinc-containing	SPD_1865	38695	5.24	1	−	+
gi|116076362	oxidoreductase, aldo/keto reductase family protein	SPD_0693	35114.1	5.83	1	+	−
gi|116077488	thioredoxin-disulfide reductase	trxB	33186	4.76	1	−	+
tRNA aminoacylation							
gi|116077391	TrmH family RNA methyltransferase group 3	SPD_1286	26549	8.52	1	−	+
gi|116076343	dimethyladenosine transferase	rsmA	32200	6	1	−	+
gi|116077050	GMP synthase, C-terminal domain	guaA	57664	4.94	2	+	+
gi|116077438	phenylalanyl-tRNA synthetase, alpha subunit	pheS	39335	5.48	1	+	+
gi|116077424	tRNA modification GTPase TrmE	mnmE	50560	4.8	1	−	+
gi|116077485	SpoU rRNA Methylase family protein	SPD_1771	26871	5.38	1	−	+
Cell adhesion							
gi|116076549	ABC transporter, substrate binding lipoprotein	SPD_1463	34630	5.3	1	−	+
gi|116076636	zinc ABC transporter, zinc-binding lipoprotein	adcA	56351	5.05	1	−	+
Membrane transporters/binding proteins							
gi|116076368	PTS system, mannose-specific IIAB components	manL	35422	5.15	1	+	+
gi|116077748	maltose/maltodextrin ABC transporter, maltose/maltodextrin-binding protein	malX	45396	5.1	2	−	+
gi|116075931	oligopeptide ABC transporter, oligopeptide-binding protein AmiA	amiA	72448	4.92	2	+	+
gi|116076035	oligopeptide ABC transporter, ATP-binding protein AmiE	amiE	39579	5.09	1	+	+
gi|116077744	iron-compound ABC transporter, iron compound-binding protein	SPD_0915	37712	5.38	2	−	+
gi|116077131	oligopeptide ABC transporter, ATP-binding protein AmiF	amiF	34748	6.21	MA	+	+
gi|116077661	phosphate ABC transporter, phosphate-binding protein, putative	SPD_1232	31296	4.77	1	−	+
gi|116077674	ABC transporter, substrate-binding protein	SPD_0150	30666	5.14	1	−	+
gi|116075981	sugar ABC transporter, sugar-binding protein	SPD_1495	48413	5.31	1	−	+
gi|116076002	choline transporter	proV	27082	4.97	MA	+	+
gi|116075915	oligopeptide ABC transporter, oligopeptide-binding protein	SPD_1170	61226	5.86	1	−	+
gi|116076548	ABC transporter, ATP-binding protein	SPD_1525	25565	5.48	MA	+	+
gi|116077179	amino acid ABC transporter, amino acid-binding protein	SPD_1226	29360	4.96	1	−	+
gi|116076332	branched-chain amino acid ABC transporter, amino acid-binding protein	livJ	40446	5.29	2	−	+
gi|116075923	iron-compound ABC transporter, iron-compound-binding protein	SPD_1652	34898	5.55	1	−	+
gi|116077249	amino acid ABC transporter, ATP-binding protein	SPD_0411	27856	5.84	MA	+	+
gi|116076321	ABC transporter, ATP-binding protein	SPD_0464	27109	5.02	2	+	+
gi|116076424	potassium uptake protein, Trk family protein	SPD_0077	24059	4.61	1	−	+
gi|116077108	amino acid ABC transporter, ATP-binding protein	SPD_1099	27391	5.1	1	+	+

(*continued*)

Table 1. (Continued)

Accession No.	Protein name	Gene_symbol	Molecular_weight	PI	TMR	2-DE	2D-LC-MS/MS
gi|116077285	amino acid ABC transporter, ATP-binding protein	SPD_1289	27350	6.01	MA	+	+
gi|116076310	amino acid ABC transporter, ATP-binding protein	SPD_0720	27100	5.05	MA	+	+
gi|116076082	sugar ABC transporter, ATP-binding protein	SPD_1409	41979	5.83	1	+	+
gi|116076430	sugar ABC transporter, ATP-binding protein	SPD_0740	55029	5.47	MA	+	+
gi|116076108	amino acid ABC transporter, amino acid-binding protein	aatB	31057	4.83	1	−	+
gi|116076558	oligopeptide ABC transporter, oligopeptide-binding protein AliA	aliA	73015	5	2	−	+
gi|116076287	efflux transporter, RND family protein, MFP subunit	SPD_0686	41839	5.12	2	−	+
gi|116076086	ATP synthase F0, B subunit	atpF	17976	5.31	1	−	+
gi|116077082	penicillin-binding protein 3/D-alanyl-D-alanine carboxypeptidase	dacC	45192	4.87	1	−	+
gi|116077732	penicillin-binding protein 2B	penA	74500	5.15	2	−	+
gi|116076282	ABC transporter, ATP-binding protein	SPD_1167	75814	5.86	1	−	+
gi|116077578	ABC transporter, ATP-binding protein	SPD_2024	27502	5.7	1	−	+
gi|116076812	branched-chain amino acid ABC transporter, ATP-binding protein	livG	28262	5.61	MA	−	+
Others							
gi|116077725	lactate oxidase	lctO	41577	5.67	1	+	+
gi|116076429	1,4-beta-N-acetylmuramidase, putative	lytC	57424	5.94	1	−	+
gi|116076433	acetate kinase	ackA	43429	5.09	1	−	+
gi|116077624	malonyl CoA-acyl carrier protein transacylase	fabD	33200	4.74	1	−	+
gi|116077289	phosphocarrier protein HPr	ptsH	8934	4.74	Ref [3]	−	+
gi|116077120	ATP synthase F1, beta subunit	atpD	50872	4.85	MA	+	+
gi|116077785	ATP synthase F1, alpha subunit	atpA	54695	4.97	1	+	+
gi|116076809	N-acetylglucosamine-6-phosphate deacetylase	nagA	41899	5.11	1	+	+
gi|116077353	autolysin/N-acetylmuramoyl-L-alanine amidase	lytA	36663	5.14	1	+	+
gi|116076897	phosphopantothenoylcysteine decarboxylase	coaC	19911	6.36	2	+	+
gi|116076305	transketolase	tkt	71125	5.05	3	−	+
gi|116076780	TPP-dependent acetoin dehydrogenase alpha-subunit	acoA	35259	5.27	2	+	+
gi|116075983	acetoin dehydrogenase complex, E2 component, dihydrolipoamide acetyltransferase, putative	SPD_1026	38074	6.13	1	+	+
Unknown							
gi|116076765	ABC transporter, substrate-binding protein, putative	SPD_2025	37810	5.41	2	−	+
gi|116076997	phosphate ABC transporter, ATP-binding protein, putative	SPD_1228	27993	5.36	MA	+	+
gi|116076614	phosphate ABC transporter, ATP-binding protein, putative	SPD_1229	30402	6.39	MA	−	+
gi|116075979	lipoprotein, putative	SPD_0179	21084	5.83	1	−	+
gi|116076613	ABC transporter, ATP-binding protein	SPD_0554	37867	5.5	MA	−	+
gi|116076737	amino acid ABC transporter, ATP-binding protein	SPD_1329	23129	4.98	MA	−	+
gi|116076062	iron-compound ABC transporter, ATP-binding protein	SPD_0918	29815	5.89	MA	−	+
gi|116077307	ATPase, AAA family protein	SPD_1580	46940	5.69	1	+	+
gi|116076452	pneumococcal surface protein A	pspA	68563	5.12	4	−	+
gi|116076681	lipoprotein, putative	SPD_0792	32954	5.32	1	−	+
gi|116076960	ABC transporter, ATP-binding protein	SPD_0614	24111	7.55	MA	−	+
gi|116076909	preprotein translocase, YajC subunit	yajC-2	11040	5.39	1	−	+
gi|116076973	ATP synthase F1, epsilon subunit	atpC	15628	5.94	MA	+	+
gi|116076719	ATP synthase F1, delta subunit	atpH	20585	7.77	MA	−	+

(*continued*)

Table 1. (Continued)

Accession No.	*Protein name*	*Gene_symbol*	*Molecular_weight*	*PI*	*TMR*	*2-DE*	*2D-LC-MS/MS*
gi|116076481	ATP synthase F1, gamma subunit	atpG	32418	5.13	MA	−	+
gi|116076604	PhoH family protein	SPD_0838	36305	5.7	1	−	+
gi|116077635	thioredoxin family protein	SPD_0886	20904	6.37	1	−	+
gi|116076448	type I restriction-modification system, M subunit, putative	SPD_0782	56627	4.99	1	−	+
gi|116076250	FeS assembly protein SufB	sufB	52757	4.94	1	−	+
gi|116076635	3-oxoacyl-[acyl-carrier-protein] synthase II	fabF	44075	5.44	3	−	+
gi|116077023	lipoprotein	SPD_0151	31185	5.46	1	−	+
gi|116077527	probable membrane lipoprotein TmpC precursor	SPD_0739	36820	5.17	3	−	+
gi|116077089	trans-2-enoyl-ACP reductase II	fabK	34156	5.33	3	−	+
gi|116076043	purine nucleoside phosphorylase, family protein 2	SPD_0726	29000	5.15	2	−	+
gi|116076578	branched-chain amino acid aminotransferase	ilvE	37649	4.97	2	−	+
gi|116076234	Uncharacterized BCR, putative	SPD_1346	60760	5.07	1	−	+
gi|116076579	LemA protein	lemA	20621	5.66	1	−	+
gi|116077170	cmp-binding-factor 1	cbf1	36278	6.07	1	−	+
gi|116076129	iojap-related protein	SPD_1554	12845.5	4.59	1	+	+
gi|116077679	2-C-methyl-D-erythritol-4-phosphate cytidylyltransferase	ispD	26340	5.19	1	−	+
gi|116077621	formate–tetrahydrofolate ligase	fhs	59821	5.27	1	+	+
gi|116077436	3-dehydroquinate synthase	aroB	39176	5.28	1	−	+
gi|116077029	glycosyl transferase, group 1 family protein, putative	cps2T	45366	5.86	1	−	+
gi|116076919	immunoglobulin A1 protease precursor	iga	218438	5.23	4	−	+
gi|116077633	dihydroorotate dehydrogenase, catalytic subunit	pyrDb	33374	5.12	2	−	+
gi|116076760	conserved hypothetical protein	SPD_0310	55030.5	5.51	1	+	+
gi|116077593	conserved hypothetical protein	SPD_0873	30064	6.18	1	−	+
gi|116077698	conserved hypothetical protein	SPD_0954	36417	7.74	3	−	+
gi|116076125	conserved hypothetical protein	SPD_1928	11376	6.6	1	−	+
gi|116077031	conserved hypothetical protein	SPD_1395	36150	5.64	1	−	+
gi|116077084	conserved hypothetical protein	SPD_0373	19974	4.79	3	−	+
gi|116075987	conserved hypothetical protein	SPD_1389	14488	4.39	1	−	+
gi|116077203	conserved hypothetical protein TIGR01440	SPD_0576	20327	6.35	1	−	+
gi|116076519	conserved hypothetical protein	SPD_1417	29232	4.77	1	−	+

MA, membrane-associated protein; +, detected; −, undetected.
Ref [1]: Zuobi-Hasona et al. (2008); Ref [2]: Ling et.al. (2004); Ref [3]: Wilkins et al. (2003); Ref [4]: Kesimer et al. (2009); Ref [5]: Severin et al. (2007).

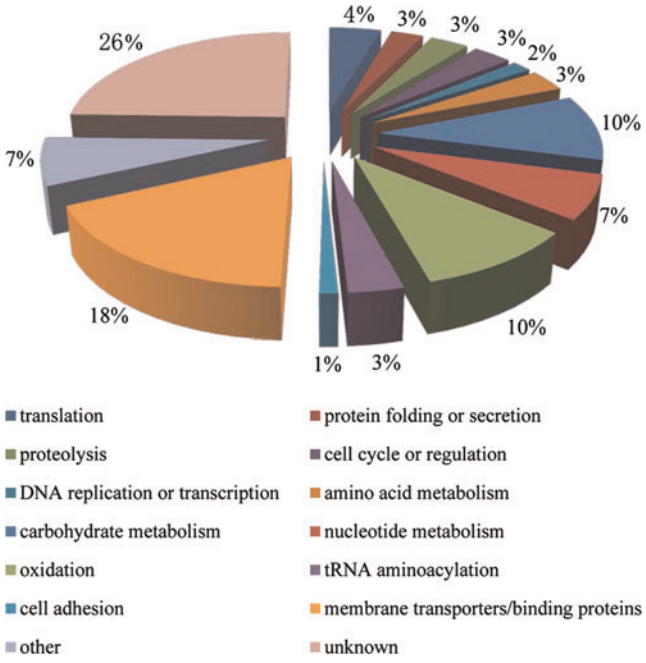

FIG. 2. Assignment of the identified membrane proteins of *S. pneumoniae* into functional categories according to Gene Ontology (GO) database.

gradient (Hong and Pedersen, 2003; Maeda, 2008). This group of proteins consists of a transmembrane F0 with an $ab_2c_{10\sim15}$ subunit composition and a membrane peripheral F1 with a $\alpha_3\beta_3\gamma\delta\varepsilon$ subunit composition. Localized in the bacterial plasma membrane, F_1F_0 ATP synthase plays a central role in oxidative phosphorylation and photophosphorylation and thus could be drug targets for specific bacterial species (Maeda, 2008).

Several virulence-related proteins, including pneumococcal surface protein A (PspA), autolysin, manganese-dependent superoxide dismutase (MnSOD), phosphopyruvate hydratase, and pyruvate oxidase, Hpr (Ser) kinase/phosphatase (HprK) were also successfully identified. As a key surface protein of all *pneumococci*, PspA is required for the full virulence of the bacteria (Crain et al., 1990; McDaniel et al., 1991). Autolysin is a cell wall-degrading enzyme found in the cell envelope of the *pneumococcus* (Berry et al., 1989; Mitchell, 2000; Ng et al., 2002). Autolysin has been implicated to play a role in the early pathogenesis of pneumococcal endophthalmitis as attenuated virulence was found in autolysin-deficient *S. pneumoniae* (Ng et al., 2002). HprK is a bifunctional enzyme that regulates carbon metabolism and virulence development in Gram-positive bacteria and thus may be a new antimicrobial target (Deutscher et al., 2005; Nessler, 2005).

Interestingly, there were several proteins with dual localizations in the bacterial cells, both in the cytoplasm and on the bacterial membrane. These proteins include DNA-directed

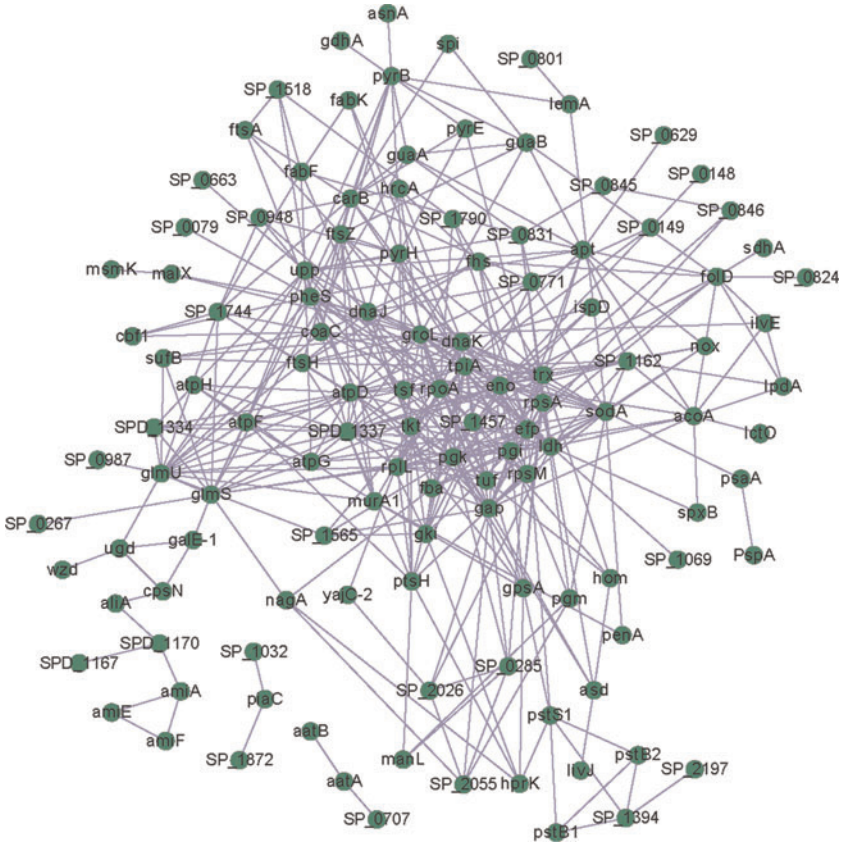

FIG. 3. The protein–protein interaction network of the identified membrane proteins in *S. pneumoniae* D39. The network containing proteins that links with others of 164 membrane proteins was mapped using the STRING system (http://string.embl.de/) based on evidences with different types. The links between proteins represent possible interactions.

RNA polymerase, elongation factors EF-G, EF-Ts, EF-P, and EF-Tu that may interact with extracellular proteins through some transport mechanisms (Beckmann et al., 2002; Kesimer et al., 2009; Pancholi et al., 1998; Severin et al., 2007; Wilkins et al., 2003) and cell division proteins FtsH, FtsZ, FtsA, and chaperonin GroEL that have capacities to play multiple functions (Bergonzelli et al., 2006; Lara et al., 2005). Furthermore, it has been demonstrated by immunological and biochemical evidences that chaperonin GroEL from *Escherichia coli* may cycle between the cytosol and membrane compartments due to its multiple roles in scavenging degradable proteins and in maintaining newly synthesized secretory or membrane proteins in a state competent for interaction with the membrane (Bochkareva et al., 1998). Multiple functions may be expected for these proteins with dual localizations in *S. pneumoniae* D39.

Association network of membrane proteins

Construction of protein interaction networks of the identified membrane proteins can help understand the regulatory mechanisms and the relationship of the proteins in *S. pneumoniae*. Based on the prediction results of STRING system (Fig. 3), most of the 164 membrane proteins can be linked through direct interactions into a protein–protein interaction network. In this map, seven proteins, that is, glyceraldehyde-3-phosphate dehydrogenase (Gap), L-lactate dehydrogenase (Ldh), phosphopyruvate hydratase (Eno), pyruvate oxidase (SpxB), UDP-glucose4-epimerase (GalE-1), glucose-6-phosphate isomerase (Pgi), and fructose-1,6-bisphosphate aldolase, class II (Fba), are involved in glycolysis (Ling et al., 2004). Among them, Eno was shown to be the major plasminogen-binding protein in *S. pyogenes* (Derbise et al., 2004) and *S. pneumoniae* (Bergmann et al., 2003), and was found to be involved in the invasion and adherence of bacteria to human pharyngeal cells (Pancholi et al., 2003). In addition, the multifunctional surface protein Gap recognizes the pharyngeal membrane-bound urokinase plasminogen activator receptor CD87, contributing to bacterial adherence and thus playing a significant role in bacterial pathogenesis (Jin et al., 2005). Moreover, Gap and Fba have been found to be effective in eliciting protection against *S. pneumoniae* (Ling et al., 2004), *Schistosoma mansonii* (Argiro et al., 2000) and *Onchocerca volvulus* (McCarthy et al., 2002) upon vaccination.

Interestingly, five members of the oligopeptide ABC transporter family, including oligopeptide-binding proteins AmiA (AmiA), AliA (AliA), and SPD_1170, and ATP-binding proteins AmiE (AmiE) and AmiF (AmiF), form a small close network. The contents of this small network play key roles in oligopeptide transport, signal transduction and competence triggering (Alloing et al., 1994; Corratge-Faillie et al., 2010). The Ami-AliA/AliB complex is involved in the colonization of the nasopharynx, and mutations in either AmiA or AliA from *S. pneumoniae* have been suggested to diminish adherence to eucaryotic cells (Cundell et al., 1995; Kerr et al., 2004). In addition, several proteins including chaperonin GroEL, chaperone protein DnaJ, and DnaK involved in protein folding or secretion are in the core nodes, implicating their critical roles in the interaction network.

Conclusions

Membrane proteins are important to the successful host colonization and establishment of infections for bacteria. In this study, we efficiently separated bacterial membranes and globally identified membrane proteins of *S. pneumoniae* D39 by complementarily using 2DE plus MALDI-MS/MS and 2D-LC-ESI-MS/MS characterizations. More than 60% of the identified proteins were determined to be membrane proteins through the combined prediction by GO, literature searching, and TMPred analysis. Bioinformatics tools were used to categorize these membrane proteins and to construct the protein interaction network, implicating important protein targets and regulation pathways for related functional studies. The data present in the current report may serve as a valuable resource or reference map for further investigations into the infectious mechanisms correlated to the bacterial membrane in *S. pneumoniae*.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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