The Journal of Microbiology (2010) Vol. 48, No. 6, pp. 872-876
DOI 10.1007/s12275-010-0220-9

NOTE

Analysis of Cytoplasmic Membrane Proteome of *Streptococcus pneumoniae* by Shotgun Proteomic Approach[§]

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(Received June 15, 2010 / Accepted September 8, 2010)

In this study, cytoplasmic membrane proteins of *S. pneumoniae* strain R6 (ATCC BBA-255) were effectively separated from cell wall or extracellular proteins by sodium carbonate precipitation (SCP) and ultracentrifugation. Forty seven proteins were analyzed as cytoplasmic membrane proteins from the 260 proteins identified by the shotgun proteomic method using SDS-PAGE/LC/MS-MS. ABC transporters for metabolites such as metals, oligopeptides, phosphate, sugar, and amino acids, and membrane proteins involved in phosphotransferse systems, were identified as the predominant and abundant, cytoplasmic membrane proteins that would be essential for nutrient uptake, antibiotic resistance and virulence mechanisms. Our result supports that gel-based shotgun proteomics combined with sodium carbonate precipitation and ultracentrifugation is an effective method for analysis of cytoplasmic membrane proteins of *S. pneumoniae*.

***Keywords*:** *Streptococcus pneumoniae*, membrane proteome, 1-DE/LC/MS-MS

Streptococcus pneumoniae is one of the major Gram-positive pathogens of the respiratory tract and causes a variety of diseases such as bacterial pneumonia, meningitis, otitis media, and septicemia, particularly in infants and the elderly (Toumanen, 2004). *S. pneumoniae* isolated from hospitals or in the community are frequently drug resistant. More than 20% of *S. pneumoniae* isolates are multidrug resistant; more than 50% of isolates are penicillin-resistant (Soualhine *et al.*, 2005). Genome sequencing of several *S. pneumoniae* strains has recently been completed (Hoskins *et al.*, 2001; Tettelin *et al.*, 2001; Lanie *et al.*, 2007), which has made proteomic studies of *S. pneumoniae* possible. In general, proteomic profiling includes the identification of differentially expressed cytosolic proteins by two-dimensional gel electrophoresis (2DE)/mass spectrometry (MS), resulting in the postulation of their metabolic characteristics and possible role in pathogenicity (Encheva *et al.*, 2006; Lee *et al.*, 2006). There has been special interest in the surface proteins of *S. pneumoniae* due to their virulent effects and their potential role in the interaction with host cells; e.g. choline binding proteins (CBP), LPxTG proteins, and lipoproteins are well known surface proteins (Rigden *et al.*, 2003; Bergmann and Hammerschmidt, 2006; Garcia-Suarez Mdel *et al.*, 2006). To date, 270 cell-wall-associated proteins have been identified by three different proteomic strategies (SDS-PAGE LC-MS/MS, 2-DE/MALDI TOF/TOF, and in-solution digestion/LC-MS/MS) for screening protein vaccine candidates (Morsczeck *et al.*, 2008). In contrast to the study of surface proteins, studies of cytoplasmic membrane proteins are not well advanced (Soualhine *et al*., 2005) because of the difficulties in preparation and identification of membrane proteins that are insoluble and present in low abundance. However, cytoplasmic membrane proteins are essential for the transport of antibiotics and for energy metabolism. Genome sequencing of *S. pneumoniae* annotated many proteins as membrane proteins involved in the transport of drugs, nutrients, ions, and metals. For example, fourteen genes have been identified as possible antibiotic efflux pumps in the *S. pneumoniae* R6 strain (Hoskins *et al*., 2001). Proteomic analysis of such cytoplasmic membrane proteins is essential to prove their proposed functions.

In this study, we enriched cytoplasmic membrane fractions from the *S. pneumoniae* strain R6/BAA-255 using ultracentrifugation and sodium carbonate precipitation and identified proteins by 1-DE/LC/MS-MS. *S. pneumoniae* R6/BAA-255 was selected for our studies because strain BAA-255 is an R6 strain and thus we could use the genomic sequence of *S. pneumoniae* strain R6 as a reference database. Although sodium carbonate precipitation was originally devised for the preparation of cell walls and cell membranes of Gram-negative

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§ Supplemental material for this article may be found at http://www. springer.com/content/120956

bacteria, this approach proved to be useful also for fractionation of cytoplasmic membrane proteins of *S. pneumoniae* strain R6 /BAA-255.

Enrichment of membrane proteins by ultracentrifugation

The *S. pneumoniae* strain R6/BAA-255 was grown in Todd-Hewitt broth supplemented with 0.5% yeast extract in 5% CO_2 at 37°C to an optical density (OD) of 0.8. Bacterial growth was monitored every hour by measuring absorbance at 600 nm using a Beckman DU 800 spectrophotometer. Strain R6/BAA-255 was harvested at OD 0.8. Harvested bacteria were suspended in 20 mM Tris-HCl buffer (pH 8.0) and disrupted twice by a French pressure cell (SLM AMINCO, USA) at 20,000 lb/in^2 for the preparation of membrane proteins. Supernatants (crude cell extracts) were collected by centrifugation at 15,000×g for 20 min. Because ultracentrifugation was used successfully for enrichment of membrane proteins of a similar Gram-positive species, *Staphylococcus aureus*, (Nandakumar *et al*., 2005; Soualhine *et al*., 2005) we also utilized it in this study. In addition, sodium carbonate precipitation has been used for preparation of cell wall proteins in Gram-negative strains, such as *E. coli* and *Acinetobacter baumannii* (Molloy *et al.*, 2000; Yun *et al.*, 2008). In our recent experience, sodium carbonate precipitation also proved to be useful for obtaining cytoplasmic membrane proteins in *A. baumannii*. Therefore, we employed sodium carbonate precipitation to determine whether this approach is useful for preparation of membrane proteins of *S. pneumoniae*. The resultant proteins enriched by either ultracentrifugation or the sodium carbonate precipitation method were separated on SDS-polyacrylamide gels according to molecular weight and divided into five fractions for MS/MS analysis (Fig. 1). All MS and MS/MS analyses were performed using a LCQ-Deca ESI ion trap mass spectrometer (Thermo Scientific). Each full MS (*m*/*z* range of 400 to 2,000) scan was followed by three MS/MS scans of the most abundant precursor ions in the MS spectrum. For protein identification, MS/MS spectra were searched by MASCOT version 2.1 (www.matrixscience.com). The genome sequence of *S. pneumoniae* strain R6 was used as the reference database for protein identification (Hoskins *et al*., 2001). Cabamidomethylation of cysteine and oxidation of methionine were considered in MS/MS analysis as variable modifications of tryptic peptides. Proteins detected more than twice from a total of three analyses were considered as identified proteins. Total numbers of proteins identified by ultracentrifugation and sodium carbonate precipitation were 201 and 211, respectively (Supplementary data Tables 1 and 2). The list of identified membrane proteins is given in Table 1. Cellular localizations of identified proteins were predicted by the PSORTb program. Nearly 17-18% (35 and 38 proteins) of the total identified proteins were sorted as cytoplasmic membrane proteins. Forty seven cytoplasmic membrane proteins were identified by these two methods (Table 2). Nine and twelve proteins were exclusively identified by only one or the other of the two approaches. The exponentially modified protein abundance index (emPAI) calculation showed that psaB, manN, msmK, and pstB were highly expressed cytoplasmic membrane proteins in *S. pneumoniae* strain R6/BAA-255. These proteins are associated with transport of essential nutrients or metal ions. Unexpectedly, proteins assigned to the cell wall or extracellular proteins were very low; only PspC, AmiA, BgaA, PspA, PbpA, and PpiA were identified as minor proteins (Table 2). PspC and PspA are well known vaccine candidates (Briles *et al.*, 2000). Our results showed that membrane proteins of *S. pneumoniae* R6/BAA-255 can be effectively enriched and separated from the cell wall or extracellular proteins by sodium carbonate precipitation and ultracentrifugation. We speculate that due to the thick and rigid cell wall structure of *S. pneumoniae*, cell wall debris was easily removed by centrifugation after cell disruption and cytoplasmic membrane proteins were effectively separated and enriched by ultracentrifugation or sodium carbonate precipitation.

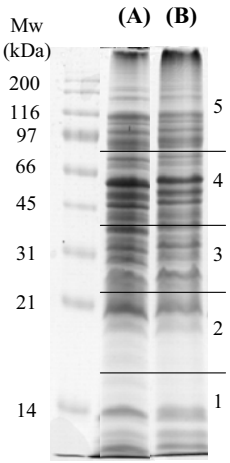

Fig. 1. 12% SDS-PAGE of 10 μg samples (A) ultracentrifugation, (B) sodium carbonate precipitation. The gels were stained with Coomassie Brilliant Blue R-250 and in-gel digestion was conducted in accordance with a previously described method (Kim *et al.*, 2006). Gels were fractionated into five parts according to molecular weight. Each part was digested with trypsin (1.2 μg) for 16 h at 37°C after reduction and alkylation of cysteines in the proteins. Digested peptides were extracted into extraction solution (50 mM ammonium bicarbonate, 50% acetonitrile, and 5% trifluoroacetic acid). Digested peptides were resolved using 15 μl of sample solution containing 0.02% formic acid and 0.5% acetic acid.

Table 1. Summary of protein identification for the enriched membrane fraction of *S. pneumoniae* R6/ BAA-255

Location[a]	Ultra-centrifugation	Sodium carbonate precipitation	Sum of two methods
Cell wall	2	1	2
Cytoplasmic membrane	35	38	47
Extracellular	3	1	4
Cytoplasmic	116	122	151
Unknown	45	49	56
Total protein number	201	211	260

[a] Prediction of cell location of identified proteins was performed by PSORTb v2.0 (http://www.psort.org/psortb/).

Table 2. List of identified membrane proteins of S. pneumoniae R6/BAA-255

Accession No.	Description of identified protein	MW (Da)	pI	No. of Trans-membrane domains[a]	Gene name	emPAI[b]	Fraction No. on SDS-PAGE
gi|15903296	Phosphate ABC transporter ATP-binding protein	27954	5.36	0	pstB	0.84	3
gi|15903387	Glycerol uptake facilitator protein, putative	30671	9.02	6	glpF	0.76	3
gi|15902303	Phosphotransferase system, mannose-specific EIID	33785	8.79	4	manN	1.19	3
gi|15902136	CapD protein, required for the biosynthesis of type 1 capsular polysaccharide	69305	8.81	4	capD	0.37	5
gi|15903535	Manganese ABC transporter, ATP-binding protein	26830	7.05	0	psaB	0.94	3
gi|15903480	Sugar ABC transporter, ATP-binding protein	41834	5.83	0	msmK	0.98	4
gi|15903602	ABC transporter ATP-binding protein - unknown substrate	26680	5.71	0	ABC-NBD	0.44	2
gi|15902663	ABC transporter ATP-binding protein	23840	7.55	0	ABC-NBD	0.27	2
gi|15903619	Serine/threonine protein kinase	72292	8.45	1	pkn2	0.38	5
gi|15902056	Cell division protein FtsH	71253	5.43	2	ftsH	0.35	5
gi|15902824	PTS system, fructose specific IIABC components	66934	5.31	9	fruA	0.30	5
gi|15902711	Cell division ABC transporter, permease protein FtsX	36581	8.87	4	ftsX	0.31	3
gi|15903982	Hypothetical protein spr1941	36330	5.02	2	-	0.24	3
gi|15903264	Hypothetical protein spr1221	38676	7.67	2	-	0.99	3
gi|15903745	Oligopeptide ABC transporter, ATP-binding protein AmiF	36752	7.08	0	amiF	0.67	3
gi|15903746	Oligopeptide ABC transporter, ATP-binding protein AmiE	39490	5.09	0	amiE	0.28	4
gi|15902978	Iron-compound ABC transporter, iron compound-binding protein	37506	5.38	0	ABC-SBP	0.11	4
gi|15902452	Amino acid ABC transporter ATP-binding protein	27873	5.84	0	glnQ	0.15	2
gi|15902608	PTS system, IIC component, putative	52961	9.02	9	PTS-EII	0.47	4
gi|15904076	ABC transporter ATP-binding protein	60870	4.72	0	ABC-NBD	0.25	5
gi|15903163	Amino acid ABC transporter permease/amino acid-binding protein	78367	8.39	3	glnP	0.32	5
gi|15902130	Hypothetical protein spr0086	40335	4.74	5	-	0.33	4
gi|15903960	Maltodextrin ABC transporter, permease protein	48302	9.45	8	malC	0.13	3
gi|15903741	Phosphotransferase system, trehalose-specific IIBC component	75716	5.28	10	treP	0.06	5
gi|15902253	Preprotein translocase subunit SecY	47353	9.68	10	secY	0.09	3
gi|15902457	Undecaprenyl pyrophosphate phosphatase	31810	9.52	7	uppP	0.13	2
gi|15903062	Hypothetical protein spr1018	31281	7.77	5	-	0.44	4
gi|15902712	PTS system, IIABC components	78021	5.94	9	ptsG	0.08	5
gi|15902423	Enoyl-acyl carrier protein(ACP) reductase	34177	5.33	0	fabK	0.18	3
gi|15903297	Phosphate ABC transporter ATP-binding protein	30363	6.39	0	pstB	0.13	3
gi|15903719	ABC transporter membrane-spanning permease - choline transporter	55543	6.36	6	proWX	0.08	4
gi|15902304	PTS system, mannose-specific IIC component	27230	4.84	7	manM	0.24	5
gi|15903226	ABC transporter ATP-binding protein	23486	8.96	0	ABC-NBD-truncation	0.18	4
gi|15903155	Hypothetical protein spr1112	20272	10.38	6	-	0.21	2
gi|15902959	Large conductance mechanosensitive channel protein MscL	13616	9.78	2	mscL	0.31	1
gi|15902549	PTS system, beta-glucosides-specific IIABC components	65852	5.97	9	PTS-EII	0.06	4
gi|15902601	ABC transporter ATP-binding protein	37776	5.5	0	ABC-NBD	0.34	3
gi|15903245	ABC transporter ATP-binding protein	27756	9.08	0	ABC-NDB	0.14	3
gi|15903720	Choline transporter	27099	4.97	0	proV	0.31	2
gi|15903748	Oligopeptide ABC transporter, permease protein AmiC	55622	9.11	6	amiC	0.43	4
gi|15903837	Hypothetical protein spr1795	49503	5.22	1	-	0.08	4
gi|15903844	Hypothetical protein spr1802	75876	5.3	8	-	0.05	5
gi|15902648	N-acetylglucosaminyl transferase	39448	6.64	0	murG	0.10	3
gi|15903832	OxaA-like protein precursor	34021	9.72	6	-	0.17	3
gi|15903398	Amino acid ABC transporter permease	26140	9.73	4	glnP	0.15	3
gi|15902804	Hypothetical protein spr0760	25851	8.75	5	-	0.15	2
gi|15902771	Amino acid ABC transporter permease	25203	9.71	3	glnP	0.15	2

a Prediction of transmembrane domains in proteins was performed by the TMHMM program (http://www.cbs.dtu.dk/ services/TMHMM)
[b] The emPAI was applied for the estimation of each amount of identified protein (Ishihama *et al.*, 2005).

ABC transporter of *S. pneumoniae*

In general, ABC transporters consist of two transmembrane domains (TMD) and two nucleotide-binding domains (NBD). Many ABC transporters were reported to function as multidrug transporters (Putman *et al.*, 2000). One of the ABC transporters identified in the present study, ABC-NBDs (gi|15903226) was previously reported as a drug efflux pump in *S. pneumoniae* R6 (Hoskins *et al*., 2001). In this study, fourteen ABC transporters, including four ABC-NBDs, were identified (Table 2). Representative ABC transporters identified in this study are Oligopeptide ABC transporters and phosphate ABC transporter. The major role of oligopeptide transporter systems is to supply bacteria (in the case of lactic acid bacteria) with essential amino acids as nutrients, and it is known to be fundamental for optimal growth (Garault *et al.*, 2002). Recently, one of the oligopeptide transporter systems (Ami) of *S. thermophilus* was reported to be involved in the import of several pheromones and triggering competence (Gardan *et al.*, 2009). The Ami oligopeptide transporter system of *S. pneumoniae* is also involved in the colonization of the nasopharynx (Kerr *et al.*, 2004). Our result revealed that AmiC (permease), AmiE (ATP-binding protein), AmiF (ATP-binding protein), and AmiA (substrate-binding protein) are abundantly present in the membrane fraction (Table 2), suggesting that these proteins may function as major transporters for nutrient uptake. In general, the resistance to β-lactam antibiotics in clinical isolates of *S. pneumoniae* is mediated by altered penicillin binding proteins (Hakenbeck *et al.*, 1999). Recent studies have shown that PstS, a subunit of the phosphate ABC transporter, is correlated with penicillin resistance (Soualhine *et al*., 2005). Interestingly, PstS and PstB (phosphate ABC transporter ATP-binding protein) of *S. pneumoniae* R6/BBA-255 were identified in this study. The high emPAI value of PstB suggests that β-lactam antibiotic resistance of *S. pneumoniae* R6/BBA-255 is due to overexpression of this transporter system.

Membrane proteins with multi-transmembrane domains

SDS-PAGE-based shotgun proteomic analysis was useful for identification of membrane proteins having multi-transmembrane domains. Twenty four membrane proteins (more than 51% of identified membrane proteins) have more than three transmembrane domains. The phosphotransferase (PTS) system and SecY are the major proteins. Four proteins having more than nine transmembrane domains (FruA, TreP, and two PTS-EII) are included in the PTS system (Table 2). In general, the PTS system and ABC transporter are components of sugar transporters in *S. pneumoniae* (Tettelin *et al*., 2001). Detected PTS systems were known to have specificities to fructose, trehalose, and glucosides. Additionally, a mannose-specific PTS system (GlpF), with four transmembrane domains, was identified as an abundantly expressed membrane protein. It suggests that PTS systems may be essential for the uptake of sugars from the environment and bacterial survival in *S. pneumoniae*.

SecY, with ten transmembrane domains, is a subunit of the preprotein translocase, known as a translocon. Other subunits such as SecA and SecG were also identified, and were sorted as cytoplasmic and undetermined proteins by PSORTb, respectively (Supplementary data Tables 1 and 2). Detailed function of the Sec system in *S. pneumoniae* remains unclear.

Putative pathogenic membrane, cytoplasmic, and unknown proteins

CapD is one of the putative pathogenic membrane proteins. CapD generates amide bonds with peptidoglycan cross-bridges to anchor capsular material in capsule synthesis. This anchoring interferes with the host defense mechanism (Richter *et al.*, 2009). More than 70% of identified proteins are grouped as cytoplasmic or unknown proteins. To date, it is not clear whether these proteins are interrelated with cytoplasmic proteins or simply contaminating proteins. However, considering that 30% of unknown proteins are predicted to have more than one TM region and that cytosolic and membrane components of membrane proteins (PstB/PstS and SecY/SecA/SecG) were detected in the same sample batch, our result suggests that many cytosolic or unknown proteins may interact with membrane proteins or be located in the cytoplasmic membrane.

Through a combination of ultracentrifugation and sodium carbonate precipitation, forty seven cytoplasmic membrane proteins of *S. pneumoniae* R6/BAA-255 were identified by gel-based shotgun proteomics. To the best of our knowledge, this is the largest number of cytoplasmic membrane proteins identified in *S. pneumoniae*. Thirty seven membrane proteins were identified in the process of surface proteomic analysis from a similar Gram-positive stain, *Streptococcus pyogenes* (Rodriguez-Ortega *et al.*, 2006). They were prepared by the cell surface shaving method using trypsin and proteinase K digestion. However, we found that those thirty seven membrane proteins of *S. pyogenes* were not included in our results. This discrepancy may be explained by the fact that the two proteomic experiments used different sample preparation methods. In contrast to the membrane proteins found for *S. pyogenes*, most of the cytoplasmic membrane proteins from *S. pneumoniae* R6/BAA-255 were essential for nutrient uptake, antibiotic resistance, virulence, and quorum sensing. This result showed that the enrichment by sodium carbonate precipitation and ultracentrifugation would be useful for the isolation of the cytoplasmic membrane proteome of Gram-positive *S. pneumoniae*. We applied this method to clinical *S. pneumoniae* stain 7663 and successfully enriched cytoplasmic membrane proteins. Fifty six and fifty seven cytoplasmic membrane proteins were identified from ultracentrifugation and sodium carbonate precipitation, respectively (data not shown). The result supported the conclusion that this approach can be generally used for the identification of cytoplasmic membrane proteins of *S. pneumoniae*. Moreover, these proteomic approaches can be further employed to elucidate the differential expression of cytoplasmic membrane proteins in bacteria subjected to different stress conditions.

Acknowledgements

This study was supported by a Grant from the Korea Basic Science Institute K-Mep (Grant T30100).

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