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The Effect of Protein Expression of *Streptococcus pneumoniae* by Blood

Song-Mee Bae[1], Sun-Mi Yeon[2], Tong-Soo Kim[2] and Kwang-Jun Lee[1,*]

[1]Division of Bacterial Respiratory Infections, Center for Infectious Disease,

[2]Lab. of Pathogenic Proteomics, Center for Immunology & Pathology, National Institute of Health,

Korea Center for Diseases control and Prevention, 5 Nokbun-Dong, Eunpyung-Ku, Seoul, 122-701, Korea

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During infection, the common respiratory tract pathogen *Streptococcus pneumoniae* encounters several environmental conditions, such as upper respiratory tract, lung tissue, and blood stream, etc. In this study, we examined the effects of blood on *S. pneumoniae* protein expression using a combination of highly sensitive 2-dimensional electrophoresis (DE) and MALDI-TOF MS and/or LC/ESI-MS/MS. A comparison of expression profiles between the growth in THY medium and THY supplemented with blood allowed us to identify 7 spots, which increased or decreased two times or more compared with the control group: tyrosyl-tRNA synthetase, lactate oxidase, glutamyl-aminopeptidase, L-lactate dehydrogenase, cysteine synthase, ribose-phosphate pyrophosphokinase, and orotate phosphoribosyltransferase. This global approach can provide a better understanding of *S. pneumoniae* adaptation to its human host and a clue for its pathogenicity.

Keywords: Blood, *Streptococcus pneumoniae*, Two-dimensional gel electrophoresis

Introduction

Streptococcus pneumoniae (Pneumococci) is a major pathogen causing very serious and often fatal diseases such as meningitis, bacteremia, sepsis, or pneumonia and less serious diseases such as sinusitis or otitis media (Schuchat *et al.*, 1997; Tuomanen, 1999). This pathogen commonly colonizes the nasopharynx asymptomatically in healthy children and adults and is also a transient commensal, colonizing upper respiratory tract in 40% of humans (Dinieal *et al.*, 2000; Hoskins *et al.*, 2001; Nakamura *et al.*, 2004). The breadth of invasive diseases caused by *S. pneumoniae* implies that this microorganism has to adapt to the environmental conditions encountered at several sites of infection (Charpentier *et al.*, 2000).

During infection, invasive strain of *S. pneumoniae* penetrates into the blood stream and undergoes spontaneous phase variation switching from transparent to opaque colony morphotype. The switch of phenotype might regulate the protein expression and improve survival in the bloodstream of host (Tuomanen, 1999; Novak *et al.*, 1999; Novak *et al.*, 2000). Understanding the mechanisms regulating the expression of genes in response to environmental changes is great important for comprehending the host-pathogen interaction during infection.

In this study, we established *S. pneumoniae* proteome and examined the effects of blood on *S. pneumoniae* protein expression using a combination of highly sensitive 2-dimensional electrophoresis (DE) and matrix-assisted laser desorption-/ionization-time of flight mass spectrometry (MALDI-TOF MS) and/or liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS).

Materials and Methods

Bacterial strain and growth conditions. A virulent *S. pneumoniae* D39 strain (capsular type 2) was routinely maintained on blood agar plate supplemented with 5% (v/v) defibrinated sheep blood (KOMED Co. Ltd, Korea). For a reference map, *S. pneumoniae* was grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) in 5% CO_2 at 37°C. Bacteria growth was monitored by measuring the absorbance at 600 nm using a Beckman DU 530 spectrophotometer. For compare the effect of blood, *S. pneumoniae* was inoculated on the Todd-Hewitt agar plate supplemented with 5% sheep blood (THA-BA plate) or Todd-Hewitt agar plate (THA plate) supplemented with 5,000 U catalase (SIGMA) per plate, respectively. All cultures were incubated in a controlled atmosphere chamber (37°C, 5% CO_2; Bellco) for 16 h. The resulting serial passages of cultures were avoided and stocks were maintained at −70°C until they were required for further experiments.

*To whom correspondence should be addressed.
Tel: 82-2-380-1474; Fax: 82-2-385-8043
E-mail: Kwangjun@yahoo.com

Protein extraction for 2-DE. Colonies grown on each plate were washed twice in 30 mL of prechilled phosphate buffered saline (0.01 M PBS, pH 7.4). Bacteria were resuspended in an appropriate volume of TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid, pH 8.0) and sonicated six times for 30 s each, using a sonicator model XL (Heat Systems). The cell lysates were treated with 100 μg/mL DNase I and 50 μg/mL RNase A on ice for 30 min and clarified by centrifugation at 12,000 × *g* for 10 min at 4°C. The protein concentration was measured using the Bradford assay (Bio-Rad). The resulting supernatants were stored in aliquots at –70°C until they were required for further experiments.

2-DE and image analysis. Approximately 80 μg (for silver staining) of protein samples was brought to a final volume of 500 μL with rehydration solution containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethyl-amonio]-1-propanesulfonate (CHAPS), 40 mM Tris-Cl, 0.5% immobilized pH gradient (IPG) buffer, 2 mM tributyl phosphine (TBP), and 0.002% bromophenol blue (BPB) and applied on 18 cm IPG strips (4-7 linear, Amersham Pharmacia Biotech) by in-gel rehydration for 16 h at 20°C (Rabilloud, 1996; Herbert *et al.*, 1998). After rehydration, isoelectric focusing (IEF) was carried out using the IPGphore Isoelectric Focusing System (Amersham pharmacia Biotech). Proteins were focused at 500 V for 1 h, 1000 V for 1 h, and then 8000 V for 4 h. The IPG strips were then equilibrated in 50 mM Tris-Cl, 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), 1% BPB, and 5 mM TBP. Protein separation in the second dimension was performed in 10% SDS-polyacrylamide gels in a Protean II xi electrophoresis kit (Bio-Rad). Protein spots were visualized by silver staining (Amersham pharmacia Biotech) (Neuhoff, 1988; Heukeshoven *et al.*, 1991). The abundance of protein spots on the 2-DE gels was analyzed by scanning the spots with a PowerLook 1100 scanner (UMAX, Taiwan). The intensity of each protein spot was measured using Progenesis software V.1.01 (PerkinElmer, nonlinear Dynamics). For the same sample, 2-DE was repeated at least five times to confirm reproducibility.

Protein identification

MALDI-TOF. Following the selection of the spots of interest, they were manually excised from the stained 2-DE gels and subjected to in-gel tryptic digestion (Dowds *et al.*, 1991). The MALDI-TOF spectra of the peptides were obtained with a Voyager-DE STR Biospectrometry Workstation mass spectrometer (PE Biosystems) by delayed extraction in reflector mode. The peptide mass fingerprints obtained for each protein digestion were analyzed using the NCBI nonredundant protein database against *S. pneumoniae* with MASCOT software (Matrix Science, London, UK, at http://www.matrixscience.com). Criteria for positive identification of the proteins were set as follows: (1) at least four matched peptide masses, (2) mass accuracy of 50 ppm or higher, (3) 50 scores in MASCOT, and (4) 10% of sequence coverage. All proteins were identified within expected molecular weights, and pI ranges were based on their positions in the gels.

LC/ESI-MS/MS. For LC/ESI-MS/MS analysis of tryptic-digested peptides, protein spots of interest were excised and digested in-gel with sequencing-grade and modified trypsin (Promega) (Dowds *et al.*, 1991; Herbert *et al.*, 1998). The digested peptides were separated and analyzed using reversed-phase capillary HPLC directly coupled to a Finnigan LCQ ion trap mass spectrometer (LC MS/MS). Peptides were bound and preconcentrated in the trapping column using 5% (v/v) acetonitrile in 0.1% (v/v) formic acid. The eluting gradient was 5%-80% (v/v) acetonitrile in 0.5% (v/v) for 50 min at a flow rate of 0.15 μL/min. Eluent from the capillary column was directly sprayed into the ion trap mass spectrometer. All data were collected in centroid mode using the "triple play" mode: a full mass scan at a mass range of 395-2000 Da (m/z), determination of the charge state of an ion on zoom scan, and then acquisition of the MS/MS spectrum of each ion on a full MS/MS scan, whose collision energy was preset at a value of 55%. Sequences of noninterpreted MS/MS spectra were identified by correlation with peptide sequences present in the MSDB, OWL or NCBI nonredundant protein databases using MASCOT search program (http://www.matrixscience.com).

Results

***S. pneumoniae* D39 proteome map.** For the reference proteome map, *S. pneumoniae* D39 was grown to exponential growth phase (OD_{600nm} of 0.3) in THY broth, and 80 μg of protein was separated on the IPG strips with a p*I* range of 4-7 and 10% SDS-PAGE. A total of 466 protein spots were detected in the p*I* range 4 to 7, as shown in Fig. 1, and then, 49 spots were identified by MALDI-TOF MS or LC-ESI MS/MS (Table 1). Interestingly, several proteins are expressed the multiple spots, in most cases two or three forms, exhibited variability in p*I* values (horizontal spot patterns).

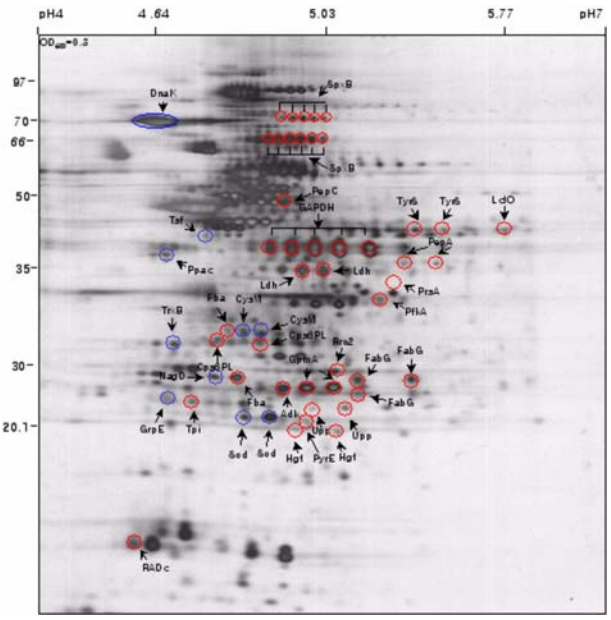

Fig. 1. The reference map of *S. pneumoniae* D39 at an OD_{600nm} of 0.3 in THY broth. Extracted proteins were separated by isoelectric focusing in the p*I* range of 4 to 7 in the first dimension (18 cm) and by 10% SDS-PAGE in the second dimension. Resolved proteins were visualized by silver staining.

Table 1. Summary of identified pneumococal 49 protein spots in the reference map

No.	Identified protein	Top score	Matched peptides	Sequence coverage (%)	Theoretical Mr(KDa)/pI	Measured Mr(KDa)/pI
1	Fructose-bisphosphate aldolase (Fba)	62	5	31	31.4/5.0	33.4/5.0
2	Cysteine synthase	466	21	34	32/5.0	36/5.0
3	3-oxoacyl-[acyl-carrier protein] reductase (FabG)	55	5	23	22.9/5.31	28/5.5
4	3-oxoacyl-[acyl-carrier protein] reductase (FabG)	124	9	49	25.7/5.49	28/5.5
5	Uracil phosphate dehydrogenase (Upp)	56	5	37	22.9/5.42	29/5.5
6	Uracil phosphate dehydrogenase (Upp)	66	8	44	22.9/5.42	26/5.4
7	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	47	6	14	38.7/5.78	37/5.2
8	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	58	8	15	32.4/5.16	37/5.3
9	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	60	6	20	35.8/5.29	37/5.4
10	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	95	9	30	35.8/5.29	37/5.5
11	Pyruvate oxidase (SpxB)	73	11	25	65.2/5.03	80/5.4
12	Lactate dehydrogenase (Ldh)	70	9	28	35/5.02	43/5.5
13	ABC transporter, ATP-binding protein (ABC-NBD)	81	6	36	28.4/4.67	28/4.5
14	Heat shock protein GrpE	163	3	7	20/4.6	25/4.3
15	Cysteine synthase	431	14	27	32/5.0	36/5.2
16	Thioredoxin reductase	171	5	12	33/4.8	31/4.2
17	Fructose-bisphosphate aldolase (Fba)	62	6	33	31.4/5.0	31/5.2
18	Adenylate kinase (Adk)	78	8	41	23.6/4.96	28/5.0
19	Manganese co-factored superoxide dismutase	353	14	31	22/5.0	24/4.9
20	rRNA(adenine-N6)-methyltransferase (RADc)	40	6	24	28.8/9.8	19/4.2
21	Pyruvate oxidase (SpxB)	75	7	35	41.6/5.77	60/5.2
22	Aminopeptidase C (PepC)	97	13	33	50.2/5.09	50/5.2
23	Tyrosyl-tRNA synthetase (TyrS)	99	10	29	47.5/5.46	44/5.4
24	Tyrosyl-tRNA synthetase (TyrS)	56	7	29	47.5/5.4	64.4/5.5
25	Glutamyl-aminopeptidase (PepA)	71	7	34	38.2/5.46	40/6
26	L-lactate dehydrogenase (Ldh)	93	8	28	35.3/5.09	35/5.1
27	6-phosphofructokinase (PfkA)	78	8	34	35.3/5.33	37/5.5
28	Glucose-1-phosphate thymidylytransferase (Cps6PL)	131	14	50	32.3/4.79	32/4.6
29	Glucose-1-phosphate thymidylytransferase (Cps6PL)	84	9	37	32.3/4.79	32/4.8
30	N-acetyl-glucosamine metabolism (NagD)	373	8	34	28/4.7	30/4.8
31	Glucose-1-phosphate thymidylytransferase (Cps6PL)	65	6	35	26.7/4.97	29/5.2
32	Cysteine synthase (CysM)	120	9	33	32/4.9	33/5.0
33	Orotate phosphoribosyltransferase (PyrE)	74	6	31	22/5.0	23/5.0
34	Lactate oxidase (LctO)	75	7	35	41.6/5.77	45/6.4
35	Glutamyl-aminopeptidase (PepA)	108	10	50	38.2/5.46	40/5.9
36	DNA-binding response regulator (Rr02)	65	6	351	35/5.02	40/4.9
37	L-lactate dehydrogenase (Ldh)	73	6	24	35.3/5.09	40/5.1
38	Ribose-phosphate pyrophosphokinase (PrsA)	94	9	38	35.6/5.68	38/5.6
39	Glucosamine-6-phosphate isomerase (NagB)	78	5	33	25.5/4.56	31/4.2
40	Glucosamine-6-phosphate isomerase (NagB)	42	4	24	25.5/4.56	31/4.3
41	Phosphoglycerate mutase (GpmA)	165	11	58	26/5.14	28/5.2
42	Phosphoglycerate mutase (GpmA)	186	13	62	26/5.14	28/5.3
43	Triosephosphate isomerase (Tpi)	62	7	27	26.8/4.75	27/4.8
44	Hypoxanthine phosphoriosyltransferase (Hgt)	58	5	26	20.3/5.26	24/5.2
45	Hypoxanthine phosphoriosyltransferase (Hgt)	66	7	31	20.3/5.26	24/5.3
46	Heat shock protein 70 (DnaK)	714	31	24	65/4.6	70/4.5
47	Manganese-co-factored superoxide dismutase	161	2	14	22/5.0	25/4.8
48	Elongation factor TS	98	3	9	37/4.8	40/4.8
49	Manganese-dependent inorganic pyrophosphatase (Ppac)	484	20	27	33/4.6	40/4.5

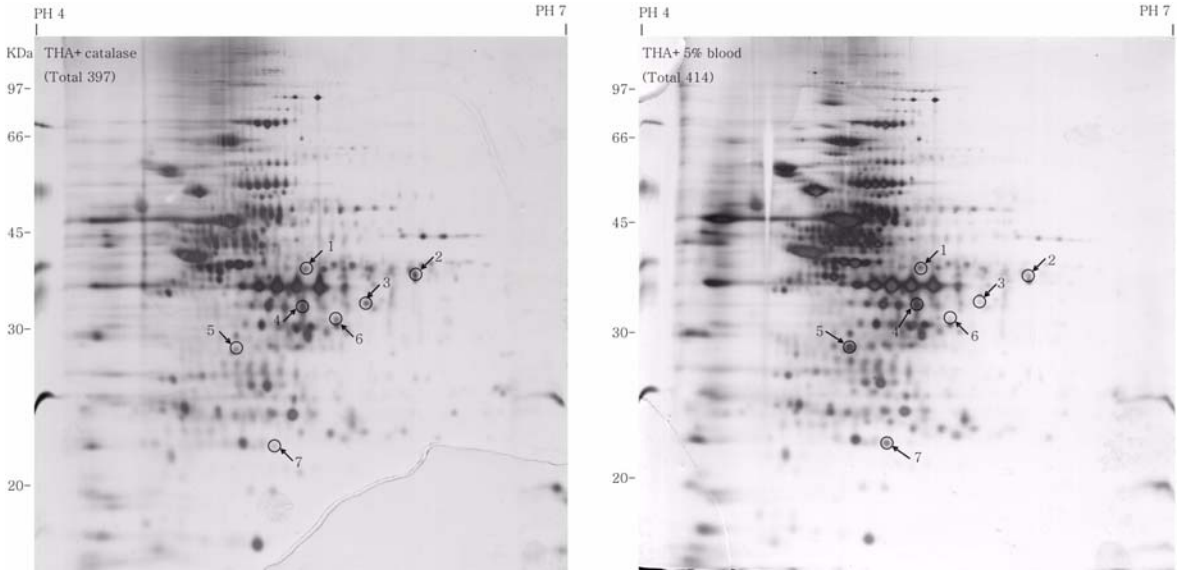

Fig. 2. Comparison of protein expression of *S. pneumoniae* D39 grown in THA containing without (A) and with 5% sheep blood (B). Extracted proteins were separated by isoelectric focusing in the p*I* range of 4 to 7 in the first dimension (18 cm) and by 10% SDS-PAGE in the second dimension. Resolved proteins were visualized by silver staining. Spot numbers indicate proteins with altered expression.

Spot No.	THA	THA+5%blood	Fold change
Spot 1.			2.9 down
Spot 2.			3.5 down
Spot 3.			8.0 up
Spot 4.			4.2 down
Spot 5.			6.0 up
Spot 6.			3.0 down
Spot 7.			3.4 up

Fig. 3. Comparison of selected protein spots with different abundance in THA and THA supplemented with blood.

Effect of blood on *S. pneumoniae* D39 protein expression. To investigate the effect of blood on *S. pneumoniae* protein expression, *S. pneumoniae* D39 was grown on the THA plate with or w/o blood at 37°C under 5% CO_2, respectively. Since the growth of *S. pneumoniae*, a catalase-deficient species, is inhibited under aerobic conditions, we added 5,000 U of catalase per THA plate without blood. The relative protein expression levels of *S. pneumoniae* D39 grown with or w/o blood were compared using two-dimensional protein gel electrophoresis followed by computerized comparison of the silver-stained gels. A total of 414 protein spots were observed when grown in THA plate supplemented with blood, and 397 spots were founded when grown on THA plate (Fig. 2). The results describe that *S. pneumoniae* alters its protein expression when exposed to blood, compared to growth without blood. Among the differentially expressed protein spots, we selected the most extensively changed seven protein spots: 3 proteins (Spot no. 4, 5, and 7) were increase by a factor of 3.4 times and 4 proteins (Spot no. 1, 2, 3, and 6) were down-regulated by a factor of at least 2.9 times in the presence of blood (Fig. 3).

Identification of differentially expressed proteins. The selected seven protein spots were identified using MALDI-TOF MS or LC-ESI MS/MS and MASCOT search program (Seong *et al*., 2002; Vandahl *et al*., 2004; Zhao *et al*., 2004). The identified seven proteins are summarized in Table 2. These included tyrosyl-tRNA synthetase, lactate oxidase, glutamyl-aminopeptidase, L-lactate dehydrogenase, cysteine synthase, ribose-phosphate pyrophosphokinase, and orotate phosphoribosyltransferase. These proteins involved in several cellular metabolisms such as protein biosynthesis, carbohydrate metabolism, and DNA/RNA synthesis, etc.

Table 2. Identification of differentially expressed proteins

Functional category	Spot no.	Identified protein	Top score	Mached peptides	Sequence coverage (%)	Mr (KDa)	p*I*	Fold change
Aminoacyl tRNA synthetases and their modification	1	Tyrosyl-tRNA synthetase	99	10	29	47.5	5.46	2.9 down
Central and intermediary metabolism	2	Lactate oxidase	75	7	35	41.6	5.77	3.5 down
	4	L-lactate dehydrogenase	73	6	24	35.3	5.09	4.2 up
Degradation of proteins, peptides and glycoproteins	3	Glutamyl-aminopeptidase	108	10	50	38.2	5.46	8.0 down
Amino acid biosynthesis	5	Cysteine synthase	120	9	33	31.9	4.96	6.0 up
Purines, pyrimidines, nucelosides, and nucleotides metabolisms	6	Ribose-phosphate pyrophosphokinase	94	9	38	35.6	5.68	3.0 down
	7	Orotate phosphoribosyltransferase	74	6	31	22.8	5.07	3.4 up

Discussion

Streptococcus pneumoniae is a major respiratory pathogen of humans, causing pneumonia, septicemia, otitis media, and meningitis. This pathogen colonizes the mucosal surface of the nasopharynx and can spread into other sites such as bloodstream or lung tissues, resulting in the most serious forms of pneumococcal disease such as sepsis, meningitis, and pneumonia (Weiser *et al*., 2001). *S. pneumoniae* alters its protein expression in response to environmental changes which the bacteria encounter during infection.

In this study, the proteomic approach was used to assess the differences in *S. pneumoniae* protein expression between the growth in THY medium and THY supplemented with blood. We selected seven spots shown significant changes in spot intensity between the two conditions. The identified proteins are involved in various cellular metabolisms. Among them, the glutamyl-aminopeptidase dissolved proteins, peptides, and glycoproteins in order to use nutrients from the surroundings under nutrient-deficient conditions (Hoskins *et al*., 2001; Tettelin *et al*., 2001). Cysteine synthase is probably related both to homeostasis of the intracellular thiol-disulfide and to quick adaptation to sudden changes in the environment (Newton *et al*., 1996; Leichert *et al*., 2003).

Recently, Seki *et al*. (2004) reported lactate oxidase was responsible for H_2O_2 production in *S. pyogenes* during aerobic growth. In a number of species of lactic acid bacteria, it has been known that cells growing aerobically produce and excrete high amounts of H_2O_2. In *S. pneumoniae*, *in vitro* and *in vivo* studies have suggested that endogenous H_2O_2 production may exceed 1 mM in the surrounding media under aerobic growth, and act as a potential virulence factor by exerting direct damage to host tissues, as well as by inhibiting the growth of other respiratory tract flora (Hirst *et al*., 2000; Pericone *et al*., 2000). To date, it has been considered that pyruvate oxidase is the major enzyme responsible for H_2O_2 production in *S. pneumoniae*. The actual contribution of lactate oxidase for high levels of H_2O_2 by *S. pneumoniae* remains unknown. Thus, further characterization of lactate oxidase may provide a foundation for evaluating the role in the physiology and pathogenesis of pneumococcus.

In conclusion, this global approach revealed that *S. pneumoniae* altered its metabolism when exposed to blood. The results can also provide a better understanding of *S. pneumoniae* adaptation to its human host and a clue for its pathogenicity.

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