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Proteomic Analysis of Protein Expression in *Streptococcus pneumoniae* in Response to Temperature Shift

Myoung-Ro Lee[2], Song-Mee Bae[1], Tong-Soo Kim[2] and Kwang-Jun Lee[1,2,*]

[1]Division of Bacterial Respiratory Infections, Center for Infectious Disease, [2]Lab. of Pathogenic Proteomics, Center for Immunology and Pathology, National Institute of Health, Korea Center for Diseases control and Prevention, 5 Nokbun-Dong, Eunpyung-Ku, Seoul 122-701, Republic of Korea

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From its initial colonization to causation of disease, *Streptococcus pneumoniae* has evolved strategies to cope with a number of stressful *in vivo* environmental conditions. In order to analyze a global view of this organism's response to heat shock, we established a 2-D electrophoresis proteome map of the *S. pneumoniae* D39 soluble proteins under *in vitro* culture conditions and performed the comparative proteome analysis to a 37 to 42°C temperature up-shift in *S. pneumoniae*. When the temperature of an exponentially growing *S. pneumoniae* D39 culture was raised to 42°C, the expression level of 25 proteins showed changes when compared to the control. Among these 25 proteins, 12 were identified by MALDI-TOF and LC-coupled ESI MS/MS. The identified proteins were shown to be involved in the general stress response, energy metabolism, nucleotide biosynthesis pathways, and purine metabolism. These results provide clues for understanding the mechanism of adaptation to heat shock by *S. pneumoniae* and may facilitate the assessment of a possible role for these proteins in the physiology and pathogenesis of this pathogen.

Keywords: heat shock, two-dimensional gel electrophoresis, *Streptococcus pneumoniae*

Streptococcus pneumoniae is one of the most important bacterial pathogens of the respiratory tract in adults and children resulting in significant morbidity and mortality (Klugman and Fieldman, 2001; Kyaw *et al.*, 2002). *S. pneumoniae* is the leading cause of bacteremia, bacterial meningitis, otitis media, and pneumonia in the human (Musher, 1992).

The preliminary step in clinical disease caused by *S. pneumoniae* is colonization of the nasopharynx (Mizrachi-Nebenzahl *et al.*, 2003). This may result in carriage without an inflammatory response, or spread into the middle ear, lungs, or the bloodstream within a host (Dinieal *et al.*, 2002). From the initial colonization to the causation of disease, pneumococci have evolved strategies to cope with a number of stressful environmental conditions *in vivo*. Thus, a changed of environmental condition of a temperature shift may significantly influence the survival and growth of the pneumococcus during infection.

Despite some limitations in resolving complex proteins, the combination of two-dimensional gel electrophoresis (2-DE) with the latest advanced techniques (MALDI-TOF and ESI-MS/MS) plus knowledge of the whole *S. pneumoniae* genome sequence could provide a powerful tool for the investigation of a large number of protein spots, simultaneously, on 2-DE gels (Pappin *et al.*, 1993; Tettelin *et al.*, 2001; Boyce *et al.*, 2004).

In this study, we present a global 2-DE proteome map of the *S. pneumoniae* soluble proteins for common *in vitro* culture conditions. We also describe in detail comparative 2-DE proteome analyses used to investigate the effects of a temperature shift on the *S. pneumoniae* proteome.

Materials and Methods

Bacterial strain and growth conditions

The *S. pneumoniae* D39 (capsular type 2) strain was grown on blood agar plates containing 5% (v/v) defibrinated sheep blood (KOMED Co. Ltd, Korea) or in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) in 5% CO_2 at 37°C. Bacterial growth was monitored by measuring the absorbance at 600 nm using a Beckman DU 530 spectrophotometer.

* To whom correspondence should be addressed.
(Tel) 82-2-380-1474; (Fax) 82-2-385-8043
(E-mail) kwangjun@nih.go.kr

In vitro heat shock experiments

Colonies grown on blood agar plates were re-suspended in 100 ml of THY broth and incubated to an optical density at 600 nm of 0.2. Five ml of seed culture aliquots were inoculated into 100 ml fresh THY broth at 37°C and then up shifted to 42°C and incubated for 15 min (heat shock). Each culture experiment was repeated three times and gels were produced for each of these preparations.

Preparation of S. pneumoniae cellular proteins

After sampling by centrifugation (3,500 × g, 20 min), cell pellets were washed twice with 30 ml pre-cooled PBS buffer and resuspended in an appropriate volume of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The bacteria were disrupted six times for 60 sec each by a Sonicator XL (Heat system, USA). The samples were treated with 100 g/ml DNase I and 50 g/ml RNase A for 30 min on ice and the soluble protein fractions were separated from cell debris by centrifugation (7,500 × g for 20 min at 4°C). Protein quantification was performed by Bradford assay (Bio-Rad, USA). And the protein samples were stored in aliquots at −80°C until needed.

Two-dimensional electrophoresis (2-DE)

Approximately 80 g (for silver staining) or 300 g (for CBB staining) of the protein samples were resuspended in rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris-HCl, 0.5% IPG buffer, 2 mM TBP, 0.002% bromophenol blue) and loaded into 18 cm immobilized pH gradient (IPG) strips (4-7 linear, Amersham Pharmacia Biotech, Sweden) by in-gel re-hydration for 16 h at 20°C (Rabilloud, 1996; Herbert *et al.*, 1998). After rehydration, isoelectric focusing was carried out using the IPGphore Isoelectric Focusing System (Amersham Pharmacia Biotech). Proteins were focused at 500 V for 1 h, 1,000 V for 1 h, and 8,000 V for 4 h. The the IPG strips were then equilibrated in 50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, 1% bromophenol blue, 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). For mass spectrometric analysis, the gels were stained with colloidal Coomassie blue (CBB G-250; ProteomeTech Inc., Korea); otherwise, the gels were visualized by silver staining (Amersham Pharmacia Biotech) (Heukeshoven and Dernick, 1985; Neuhoff *et al.*, 1988). The gels were scanned by a PowerLook 1100 scanner (U-MAX), and the protein spots were analyzed using Progenesis software (PerkinElmer). For each sample, the 2-DE was repeated at least three times to establish reproducibility.

MALDI-TOF and LC-coupled ESI-MS/MS

The protein spots of interest were manually excised from the Coomassie-stained gels and subjected to in-gel tryptic digestion with sequencing grade, modified trypsin (Promega, USA) (Dowds and Hoch, 1991; Shevchenko *et al.*, 1996; Herbert *et al.*, 1998). 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 digest were analyzed using the NCBI non-redundant protein database against *S. pneumoniae* using MASCOT software (Matrix Science, UK, at http://www.matrixscience.com). The criteria for the positive identification of the proteins were: (i) at least four matching peptide masses, (ii) a 50 ppm or better mass accuracy, (iii) score ≥ 50 in MASCOT, and (iv) sequence coverage ≥ 10%. All protein identifications were in the expected molecular weight and p*I* range based on their position in the gel.

For LC-coupled ESI-MS/MS analysis, the separation and analysis of tryptic peptides were performed using reversed phase capillary HPLC directly coupled to a Finnigan LCQ ion trap mass spectrometer (LC MS/MS). All data were collected in centroid mode using a "triple play" mode; a full mass scan at mass range of 395-2,000 Da (m/z), determination of the charge states of an ion on zoom scan, and then acquisition of MS/MS spectrum of the ion on a full MS/MS scan, with the collision energy preset at 55%. Sequences of uninterrupted MS/MS spectra were identified by correlation with peptide sequences present in the MSDB, OWL or NCBI non-redundant protein database using Mascot software (http://www.matrixscience.com).

Results and Discussion

Characterization of protein profiles of exponentially growing S. pneumoniae

The whole protein profile of exponentially growing *S. pneumoniae* D39 (OD_{600} of 0.3) in THY medium were characterized to generate a reference map. We initially performed 2-DE under a broad range of p*I* and molecular weight conditions with proteins extracted from exponentially growing *S. pneumoniae* D39 (data not shown). These preliminary studies allowed us to select the optimal conditions to perform analysis at high resolution in the regions in which the main differences in the protein patterns were detected. The 2-DE reference map of *S. pneumoniae* showed that the most proteins are located in the weak acidic and/or neutral portion. Therefore, we focused on this p*I* range (pH of 4-7) as this also resulted in excellent spot resolution. For the current study, 80 g of protein from exponentially growing cells was separated by

2-DE and visualized with silver staining. Using Progenesis software, approximately 431 protein spots were detected in the p*I* range of 4 to 7, as shown in Fig. 1. For each protein sample, the reproducibility of the 2-DE gels was assessed by repeated analysis (more than six times) under the same conditions.

Interestingly, several proteins showed multiple spots, in most cases two or three forms but as many as eight spots in some cases (Fig. 1 and Table 1). Individual isoforms are characterized by differences in the observed Mw and/or p*I* values. Several proteins, for example cysteine synthase, pyruvate oxidase, uracil phosphate dehydrogenase, glyceraldehydes 3-phosphate dehydrogenase, superoxide dismutase, and lactate dehydrogenase are expressed as multiple isoforms. Most of the multiple spots exhibited variability in p*I* values (horizontal spot patterns), and some proteins showed additional changes in their Mw, which probably reflect differential post-translational modifications. The nature of these modifications is currently under investigation.

Comparison of 2-D S. pneumoniae proteome before and after temperature shift

During the disease process, bacterial pathogens are exposed to various stimuli, for example, a sudden increase in temperature upon entering a host. In recent years, several studies have reported that some stress-induced proteins may contribute to the pathogenic process of infections (Goulhen *et al.*, 2003). Thus, virulence factor synthesis is influenced by nutrient availability, pH, growth phase and temperature. Many types of proteolytic and heat shock proteins have been identified as fundamental elements in controlling envi-

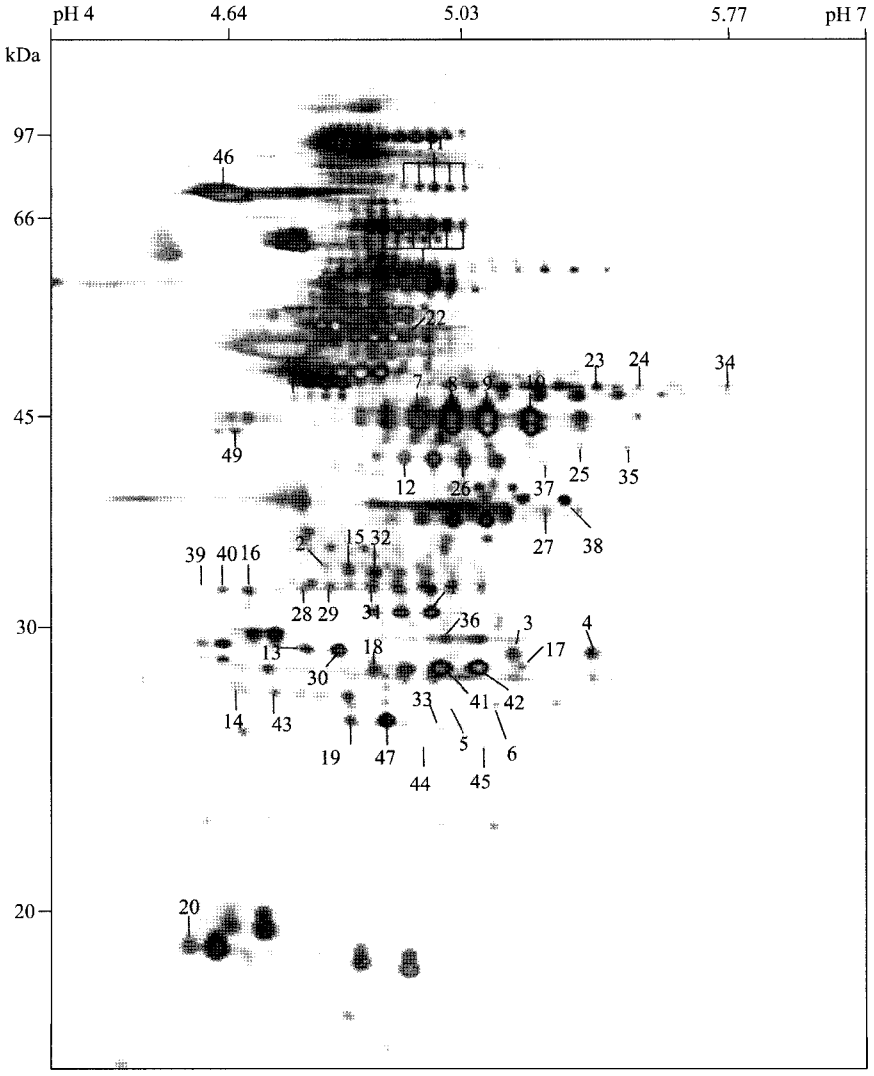

Fig. 1. Proteome map of exponentially growing *S. pneumoniae* D39 on an 18 cm pH 4-7 IPG strip in the first dimension followed by the second dimension on a 10% SDS-PAGE gel. Protein spots were visualized by silver staining. Protein spots are labeled as indicated in the *legend for Table 1.*

Table 1. List of proteins identified by MALDI-TOF MS in *S. pneumoniae*

Spot no.	Identified protein	Top score	Matched peptides	Sequence coverage(%)	Theoretical *Mr* (kDa)/p*I*	Measured *Mr* (kDa)/p*I*
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	44/5.2
8	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	58	8	15	32.4/5.16	44/5.3
9	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	60	6	20	35.8/5.29	44/5.4
10	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	95	9	30	35.8/5.29	44/5.5
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-1-1phosphate 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

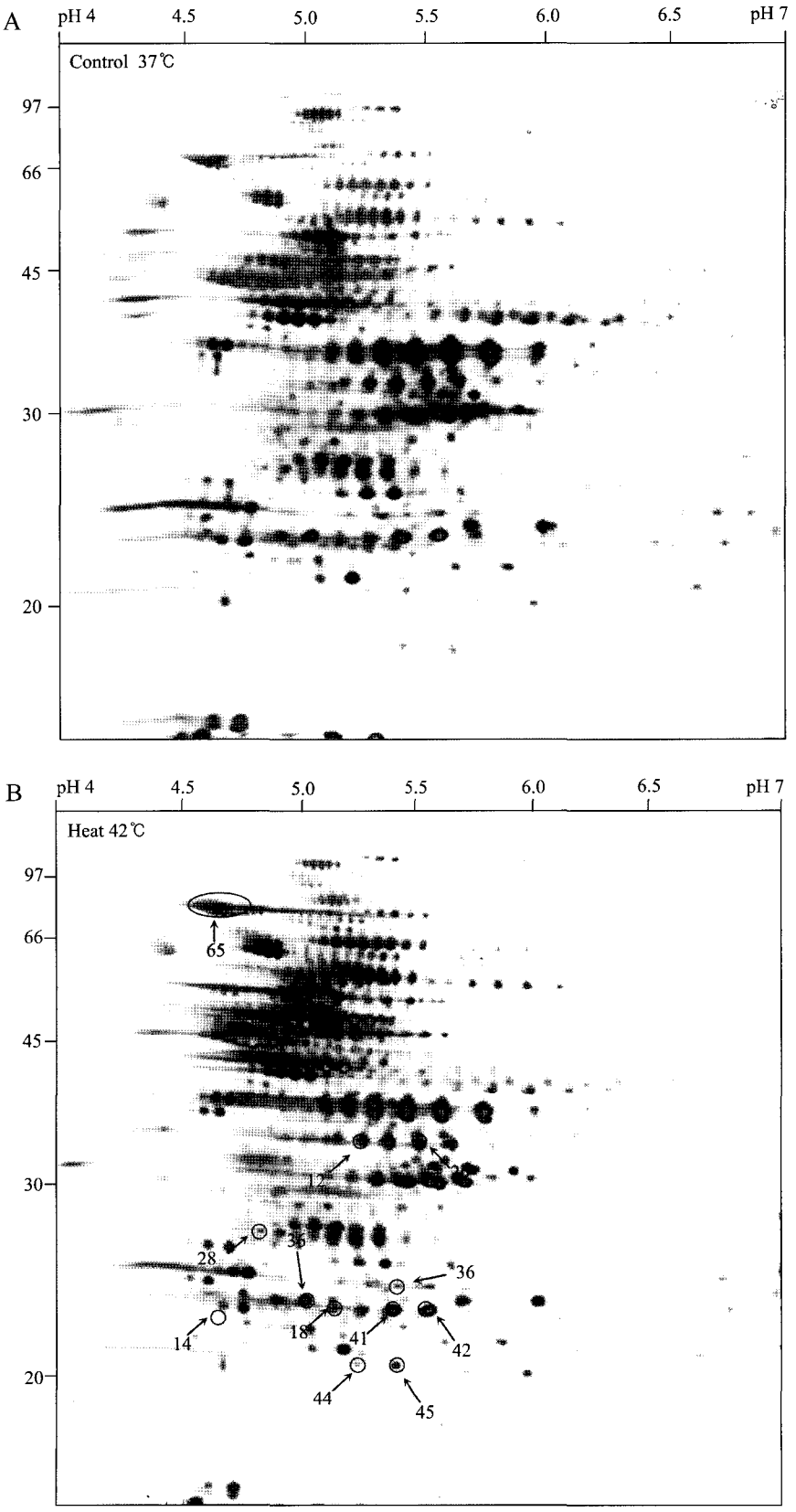

Fig. 2. Effect of the 5°C temperature shift from 37°C (A) to 42°C (B) on *S. pneumoniae* D39. Open circles and spot numbers indicates those proteins with altered expression compared with the controls (under heat shock) and listed in Table 2.

Table 2. Identification of differentially expressed proteins after heat shock (42°C)

Functional category	Spot No.	Identified proteins	*Mr* (kDa)	p*I*	Fold change
Central and intermediary metabolism	26	L-lactate dehydrogenase	35.3	5.09	2.8 down
	41	Phosphoglycerate mutase	26	5.14	2.8 up
	42	Phosphoglycerate mutase	26	5.14	1.4 up
	12	L-lactate dehydrogenase	35.3	5.09	1.5 down
	28	Glucose-1-phosphate Thymidyltransferase	32.3	4.79	2.3 down
	36	N-acetyl glucosamine metabolism	32.4	4.86	2.2 up
Heat shock proteins	14	Heat shock protein GrpE	20	4.6	1.5 up
	65	Heat shock protein 70	70	4.5	1.3 up
Regulatory proteins	36	DNA-binding response regulator	26.7	4.97	1.6 down
Nucleotide metabolism	44	Hypoxanthine phosphoribosyltransferase	20.3	5.26	1.8 up
	45	Hypoxanthine phosphoribosyltransferase	20.3	5.26	2.1 up
	18	Adenylate kinase	23.6	4.96	3.0 up

ronmental adaptations. Since the stress response of *S. pneumoniae* is not fully understood, we sought to establish a global view of this organism's response to elevated growth temperature by using proteomics tools in our analysis.

Cells grown at 37°C to their mid-exponential phase (OD_{600}=0.2) were shocked by shifting the growth temperature upward by 5°C to 42°C. As shown by 2-DE analysis (Fig. 2), the whole-cell protein extracts of mid-exponential phase cells heated to 42°C for 15 min indicate a change threshold of 1.3-fold or greater in the level of expression of at least 25 proteins compared to the level of expressions in control cells. Among the 25 proteins, 14 spots were up-regulated 1.4- to 3.0-fold and 11 spots were down-regulated 1.5- to 2.8-fold after the upshift in growth temperature. We selected 12 spots for excision and further characterization.

Identification of differentially expressed proteins by temperature shift

Peptide mass fingerprinting using MALDI-TOF-MS was performed to identify the proteins that demonstrated altered expression on 2-DE. Spectra from MALDI-TOF were analyzed by MASCOT. Since MALDI-TOF analyses were unreliable due to low coverage in homologous sequence matching, LC-coupled ESI-MS/MS analysis was also applied to improve the accuracy of identification. Results obtained from MALDI-TOF and MS/MS analysis are listed in Table 2 and indicate detection of the stress proteins DnaK and GrpE, adenylate kinase, hypoxanthine phosphoribosyltransferase, phosphoglycerate mutase, N-acetyl glucosamine metabolism protein, L-lactate dehydrogenase, and glucose-1-phosphate thymidyltransferase.

In this study, a temperature shift from 37°C to 42°C *in vitro* triggered a 3-fold induction of adenylate kinase (AK) in *S. pneumoniae*. No comparable data on the role of AK in the stress response and pathogenesis of *S. pneumoniae* has to date been reported. Generally, nucleotides are metabolic intermediates essential for bacterial growth and multiplication. AK is one of the enzymes involved in nucleotide synthesis and catalyzes a reversible high-energy phosphoryl transfer reaction between adenine nucleotides. The enzyme, therefore, would appear to contribute to the homeostasis of cellular adenine nucleotide composition in addition to the nucleotide biosynthesis. The AK-driven maintenance of adenine nucleotide homeostasis was previously shown to be essential for bacterial growth *in vitro* to be crucial for growth *in vivo* and for the virulence of *Yersinia pestis* and *Pseudomonas aeruginosa* (Munier *et al.*, 2003).

Among the proteins up-regulated by heat shock, hypoxanthine phosphoribosyltransferase (HPRT) is responsible for salvaging the purine bases in nucleotide metabolism. Bacteria have evolved salvage pathways that enable recovery of purine and pyrimidine bases and nucleosides, which can then be recycled into nucleotides or used as a source of energy, carbon or nitrogen. Recently, at high-salt concentrations, a defect in nucleotide recycling has been shown to result in a decreased growth rate which suggests an important role for nucleotide recycling under these stressful environmental conditions. Also, HPRT was shown to be required for virulence in *Listeria monocytogenes* and to be essential for the growth of *Bacillus subtilis*

(Lithgow *et al.*, 2004).

The heat shock proteins (HSP), HSP70 (DnaK) and GrpE, previously characterized in other bacteria, were up-regulated by exposure to 42°C for 15 min. The production of these intracellular proteins have been found to play a role in maintaining cell homeostasis under stress conditions in several bacteria by functioning as chaperones (Mujacic *et al.*, 2004). In *Escherichia coli*, the *dnaK-dnaJ-grpE* chaperone systems interact with misfolded polypeptides and promote refolding by using ATP-driven conformational changes (Mogk *et al.*, 2003). Also, HSPs appear to be important biological molecules, since they have been implicated in thermotolerance, pathogenicity, immunodominance, and autoimmune phenomena (Goulhen *et al.*, 2003). In *S. pneumoniae*, the structure of the *dnaK* operon comprises *hrcA-grpE-dnaK-dnaJ*, and the complex regulatory pathways for the transcription of *dnaK* operon have been partially investigated under stress environments. Although the major HSP, DnaK, is highly immunogenic in *S. pneumoniae* (Hamel *et al.*, 1997), the role of HSPs in the pathogenesis of *S. pneumoniae* remainslargely unknown.

Through a proteomic study based on 2-DE and MS, we have determind that the expression of some proteins was modulated in response to heat stress. Some of these proteins may be general stress response proteins, while others may play various roles in cell physiology, including carbohydrate metabolism, purine metabolism, and nucleotide metabolism. The results of this study in *S. pneumoniae* offer clues for understanding the mechanism that leads to an alteration in protein synthesis for bacteria adaptation under heat shock stress. It is suggested that gene inactivation or functional studies on the differentially expressed proteins may provide a foundation for further evaluation of their role in the physiology and pathogenesis of infectious pneumococcus.

References

Boyce, J.D., P.A. Cullen, and B. Adler. 2004. Genomic-scale analysis of bacterial gene and protein expression in the host. *Emerg. Infect. Dis.* 10, 1357-1362.

Dinieal, M.M., F.B. Robert, and T. Alexander. 2002. *Streptococcus pneumoniae*: molecular biology and mechanism of disease. Alexander Tomasz (eds). NY: Mary Ann Liebert, p. 485-491.

Dowds, B.C.A. and J.A. Hoch. 1991. Regulation of the oxidative stress response by the *hpr* gene in *Bacillus subtilis*. *J. Gen. Microbiol.* 137, 1121-1125.

Goulhen, F., D. Grenier, and D. Mayrand. 2003. Oral microbial heat-shock proteins and their potential contributions of infections. *Crit. Rev. Oral. Biol. Med.* 14, 399-412.

Hamel, J., D. Martin, and B.B. Brodeur. 1997. Heat shock response of *Streptococcus pneumoniae* : identification of immunoreactive stress proteins. *Microb. Pathog.* 23, 11-21.

Herbert, B.R., P.M. Mark, A.G. Andrew, J.W. Bradley, G.B. Warren, and L.W. Keith. 1998. Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent. *Electrophoresis* 19, 845-851.

Heukeshoven, J. and R. Dernick. 1985. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* 6, 103-112.

Klugman, K.P. and C. Feldman. 2001. *Streptococcus pneumoniae* respiratory tract infections. *Curr. Opin. Infect. Dis.* 14, 173-179.

Kyaw, M.H., S. Clarke, I.G. Jones, and H. Campbell. 2002. Incidence of invasive pneumococcal disease in Scotland, 1988-99. *Epidemol. Infect.* 128, 139-147.

Lithgow, J.K., E. Ingham, and S.J. Foster. 2004. Role of the *hprT-ftsH* locus in *Staphylococcus aureus*. *Microbiology* 150, 373-381.

Mizrachi-Nebenzahl, Y., S. Lifshitz, R. Teitelbaum, S. Novick, A. Levi, D. Benharroch, E. Ling, and R. Dagan. 2003. Differential activation of the immune system by virulent *Streptococcus pneumoniae* strains determines recovery or death of the host. *Clin. Exp. Immunol.* 134, 23-31.

Mogk, A., C. Schlieker, K.L. Friedrich, H.-J. Schönfeld, E. Vierling, and B. Bukau. 2003. Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most efficiently by ClpB/DnaK. *J. Biol. Chem.* 278, 31033-31042.

Mujacic, M., M.W. Bader, and F. Baneyx. 2004. *Escherichia coli* Hsp31 functions as a holding chaperone that cooperates with the DnaK-DnaJ-GrpE system in the management of protein misfolding under severe stress conditions. *Mol. Microbiol.* 51, 849-859.

Munier-Lehmann, H., V. Chenal-Francisque, M. Ionescu, P. Christova, J. Foulon, E. Carniel, and O. Barzu. 2003. Relationship between bacterial virulence and nucleotide metabolism: a mutation in the adenylate kinase gee renders *Yersinia pestis* avirulent. *Biochem. J.* 373, 515-522.

Musher, D.M. 1992. Infections caused by *Streptococcus pneumoniae*: clinical spectrum, pathogenesis, immunity and treatment. *Clin. Infect. Dis.* 14, 801-809.

Neuhoff, V., N. Arold, D. Taube, and W. Ehrhardt. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 9, 255-262.

Pappin, D.J.C., P. Hojrup, and A.J. Bleasby. 1993. Rapid identification of proteins by peptide-mass fingerprinting. *Curr. Biol.* 3, 327-332.

Rabilloud, T. 1996. Solubilization of proteins for electophoretic analyses. *Electrophoresis* 17, 813-829.

Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* 68, 850-858.

Tettelin, H., K.E. Nelsion, I.T. Paulsen, J.A. Eisen, T.D. Read, S. Peterson, J. Heidelberg, R.T. DeBoy, D.H. Haft, R.J. Dodson, A.S. Durkin, M. Gwinn, J.F. Kolonay, W.C. Nelson, J.D. Peterson, L.A. Umayam, O. White, S.L. Salzberg, M.R. Lewis, D. Radune, E. Holtzapple, H. Khouri, A.M. Wolf, T.R. Utterback, C.L. Hansen, L.A.

McDonald, T.V. Feldblyum, S. Angiuoli, T. Dickinson, E.K. Hickey, I.E. Holt, B.J. Loftus, F. Yang, H.O. Smith, J.C. Venter, B.A. Dougherty, D.A. Morrison, S.K. Hollingshead, and C.M. Fraser. 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. *Science* 293, 498-506.