Analysis of Soluble Protein Complexes in *Shigella flexneri* Reveals the Influence of Temperature on the Amount of Lipopolysaccharide*

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*Shigella flexneri*, which is closely related to *Escherichia coli*, is the most common cause of the endemic form of shigellosis. In this study, 53 homomultimeric protein complexes and nine heteromultimeric protein complexes from *S. flexneri* 2a strain 2457T were separated and identified. Among these, three potential homomultimeric protein complexes had not been previously described. One complex, thought to be composed of 12 PhoN1 subunits, is a periplasmic protein with an unknown physiological role encoded on the virulence plasmid of *S. flexneri*. The abundance of the protein complexes was compared following growth at 37 or 30°C, and the abundance of three protein complexes (PyrB-PyrI, GlmS, and MgiB) related to the synthesis of lipopolysaccharides (LPS) appeared to be temperature-dependent. Many studies have shown that LPS is essential to the virulence of *S. flexneri*. Here, we report the influence of temperature on the amount of LPS. *Molecular & Cellular Proteomics* 12: 10.1074/mcp.M112.025270, 1250–1258, 2013.

Recently, the number of reports focused on microbial proteomics has rapidly increased. The technologies used in most of these studies can be classified into two groups as follows: gel-based systems, such as two-dimensional electrophoresis (2-DE) and difference gel electrophoresis, followed by protein identification using MALDI-TOF/TOF; and gel-free systems, in which protein separation and identification are achieved by multidimensional liquid chromatography coupled with tandem mass spectrometry. However, proteins must be denatured in all of these systems, resulting in the loss of information regarding protein-protein interactions. In living cells, interactions with other proteins are vitally important for the majority of proteins to carry out their biological functions. Therefore, it would be desirable to separate and identify the components of protein complexes in cells at a global level.

The methods most widely used to study protein-protein interaction include affinity purification, co-immunoprecipitation, yeast two-hybrid systems, and blue native PAGE (BN-PAGE). The first three of these techniques require cloning and expression of bait proteins. Thus, large scale screening using these methods is very labor-intensive. Another obvious shortcoming is that these methods are not suitable for analyzing homomultimeric protein complexes. In comparison with the first three methods, native electrophoresis is the most convenient and robust method to generate large scale protein-protein interaction maps. In addition, the most important advantage of BN-PAGE is that the complexes separated by this method remain associated and are folded in a manner similar to how they would be in the cell. Recently, a comprehensive study (2) addressed the advantages and disadvantages of this technique. In general, native electrophoretic techniques are highly useful for isolating moderately stable protein complexes, although “false-positives” and “false-negatives” can still occur.

In this study, we aimed to separate and identify protein complexes in *Shigella flexneri* 2a, the most common cause of the endemic form of shigellosis. An important approach for studying the pathogenic mechanism of a bacterium is to compare the protein profiles of cells grown under normal and virulence gene-inducing conditions (3). It was previously reported that *S. flexneri* cultivated at 37°C caused keratoconjunctivitis in guinea pigs and could penetrate and replicate in intestinal epithelial cells, whereas the same strain grown at 30°C was phenotypically avirulent and noninvasive (4). Thus, we used a comparative proteomic approach to analyze the expression of protein complexes from *S. flexneri* grown at 37 and 30°C. We identified changes in the abundance of three

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protein complexes involved in the synthesis of lipopolysaccharide (LPS) at different temperatures. These results offer new insights for the study of the pathogenic mechanism of Shigella spp.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—Escherichia coli strain DH5α, used for plasmid construction, was grown on LB (Luria-Bertani) agar or in LB broth (Difco) at 37°C. Wild-type S. flexneri serotype 2a strain 2457T was grown in tryptic soy agar (TSA) (Difco) containing 0.01% Congo Red or in LB broth at 30 and 37°C.**

A vector containing a Myc-tagged SodB clone was constructed by adding a Myc tag (TTAGATACCTCTTACAGATGTTCTGCT) to the C terminus of the coding sequence of the SodB protein from S. flexneri 2457T. The Myc-tagged sequence was then cloned into plasmid pAK (5). The plasmid sequence was verified by restriction enzyme analysis and DNA sequencing.

**Preparation of Protein Complex Samples—Wild-type S. flexneri 2457T was grown aerobically in 100 ml of LB medium. Bacteria were harvested at stationary phase (A600 nm = 3.2) by centrifugation at 4°C for 5 min at 4,000 × g. The pellet was washed once with cold phosphate-buffered saline (PBS) and resuspended in 5 ml of lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA, pH 8.0, 10% glycerol, pH 7.4, with 1 mg/ml lysozyme, 1,000 units of DNase I, 0.1% Triton X-100, and protease inhibitor added prior to use). Cells were treated at 37°C for 2.5 h, and debris was removed by centrifugation at 20,000 × g for 20 min. The protein concentration of samples was measured using a PlusOne 2-D Quant kit (GE Healthcare).

**2-D BN/SDS-PAGE—BN-PAGE** was carried out as described previously (6). Acrylamide gels with linear 6–11 and 10–16% gradients were used for separating whole cell protein complexes. A gel with a linear 4–15% gradient was used to compare the abundance of protein complexes in cells grown at different temperatures. A stacking gel of 3.2% was used. Anode and cathode electrophoresis buffers were prepared as described by Swamy et al. (7). Anode and cathode buffers contained 15 mM BisTris and 50 mM Tricine, and the cathode buffer was supplemented with 0.02% (w/v) Coomassie Brilliant Blue G-250. Prior to loading, 1.5 (v/v) sample loading buffer (750 mM 6-amino-caproic acid, 5% (w/v) Coomassie Brilliant Blue G-250, and 20% (v/v) glycerol) was added to the sample. The gel was electrophoresed at 100 V for 1 h at 4°C. The cathode electrophoresis buffer was then exchanged for cathode buffer without Coomassie Brilliant Blue G-250 and run for a further 16 h at 300 V. For the second dimensional SDS-PAGE, a lane was cut out of the first dimensional gel and dipped into equilibrating buffer (8). SDS-PAGE was performed using a 12.5% separating gel according to standard protocols. Gels were stained with Coomassie Brilliant Blue G250 (9).

**Protein Identification by MALDI-TOF/TOF—** All of the protein spots separated by electrophoresis were analyzed by MALDI-TOF/TOF mass spectrometry. The protein spots were carefully excised from the Coomassie-stained 2-DE gel, washed with destaining solution (50% acetonitrile, 25 mM ammonium carbonate), and then digested for 13 h with sequencing grade modified trypsin (Roche Applied Science). Peptides from digested proteins were used for MALDI-TOF/TOF analysis. The MALDI-TOF MS measurement was performed on a Bruker Ultraflex III MALDI-TOF-MS apparatus (Bruker Daltonics) in reflectron mode with 20 kV accelerating voltage and 23 kV reflecting voltage. A saturated solution of 3,5-dihydroxybenzoic acid as a matrix was used for MALDI analysis. Data were acquired with a mass range of 700–4,000 m/z. The subsequent MS/MS analysis was performed in a data-dependent manner, and the five most abundant ions were subjected to high energy CID analysis. The collision energy was set to 1 keV, and nitrogen was used as the collision gas.

**Data Interpretation and Database Searching—** To analyze one peptide mass fingerprinting and multiple TOF/TOF spectra from one sample as a single combined dataset, raw data were first merged into one mascot generic format file using the Biotools Version 3.0 software (Bruker Daltonics). Mascot 2.1 (Matrix Science) was then used to compare the merged files with the S. flexneri 2457T database (including all predicted chromosomal ORFs, the virulence plasmid pCP301 from S. flexneri 2a 301, and the large IncHI plasmid R27 from Salmonella typhi (GenBank accession numbers GI:30043918, GI: 18462515, and GI:7800243, respectively) to eliminate redundancy resulting from multiple members of the same protein family. Results were checked against the NCBI's database (version 20061021, 4,072,503 sequences). For those proteins identified in the NCBI database, proteins from S. flexneri species were selected as the best hits from the homologous protein lists. The search parameters were as follows: trypsin digestion with one missed cleavage; carbamidomethyl modification of cysteine as a fixed modification and oxidation of methionine as a variable modification; peptide tolerance maximum ±0.2 Da; MS/MS tolerance maximum ±0.6 Da; peptide charge +1; monoisotopic mass. Scores greater than 21 were considered significant (p < 0.05) for the local MS/MS search. Scores greater than 49 were considered significant (p < 0.05) for the local peptide mass fingerprinting search. For unambiguous identification of proteins, more than five peptides must be matched for a peptide mass fingerprinting search.

**Western Blot—** The BN- and SDS-polyacrylamide gels were transferred to PVDF membranes at 15 V for 1.5 h. PVDF membranes were blocked with 10% (w/v) skim milk powder in TBS (100 mmol/liter Tris-HCl, pH 7.5, 0.9% (w/v) NaCl) containing 0.1% (v/v) Tween 20 (TBST) for 1 h. Membranes were incubated in anti-Myc and anti-IPAβ antibody (diluted in TBST) for 1–2 h at room temperature or 4°C overnight at the recommended concentration, followed by detection using ECL reagents (Thermo) and manual film development. Anti-Myc tag (HRP-conjugated) was purchased from Abmart. Anti-IPAβ antibody was a generous gift from Prof. Armelle Phalipon (Institut Pasteur, Paris, France).

**RNA Collection and Extraction—** For quantitative real time-PCR (qRT-PCR) analysis, bacteria were harvested in stationary phase. Total RNA was isolated from the cultures using RNeasy mini spin columns (Qiagen) and treated with RNase-free DNase I (New England Biolabs). cDNA was generated from 3 μg of each RNA sample using a RevertAid first strand cDNA synthesis kit (Thermo Scientific).

**Quantitative Real Time PCR Analysis—** qRT-PCR was carried out in an iCycler iQ real time PCR system (Bio-Rad) in 50-μl reaction mixtures containing 1 μl of cDNA, 600 nM forward and reverse primers, and 1 × iQ SYBR Green supermix (Bio-Rad) according to the manufacturer’s instructions. Reactions were carried out under the following conditions: 30 s at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at the specific annealing temperature, and 30 s at 72°C. The qRT-PCRs were performed in triplicate for each of the three biological replicates tested. Data were analyzed using CFX Manager 2.1 (Bio-Rad). Gene-specific primers (supplemental Table 1) were designed using Primer Premier 5.0 software (Premier Biosoft). Relative amounts of cDNA were normalized to the amounts of 16 S rRNA cDNA in each sample.

**LPS Preparation—** Wild-type S. flexneri 2457T, grown aerobically in 5 ml of LB medium to stationary phase, was harvested by centrifugation at 4°C for 5 min at 4,000 × g. The cell pellet was washed once with cold PBS, resuspended in 10 ml of lysis buffer (0.1 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, and protease inhibitor added prior to use). Cells were treated at 37°C for 2.5 h, and debris was removed by centrifugation at 20,000 × g for 20 min. The protein concentration of samples was measured using a PlusOne 2-D Quant kit (GE Healthcare).

**MALDI TOF/TOF** was used to select the 150 most prominent peaks in a mass range of 700–4,000 m/z. The subsequent MS/MS analysis was performed in a data-dependent manner, and the five most abundant ions were subjected to high energy CID analysis. The collision energy was set to 1 keV, and nitrogen was used as the collision gas.

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6.8, 2% SDS, 4% β-mercaptoethanol, and 10% glycerol), and then boiled for 10 min. Proteinase K (50 mg, Sigma-Aldrich) was added, and the mixture was incubated for 1 h at 60°C. Samples were electrophoresed on an SDS-polyacrylamide gel and visualized by silver staining (10).

Transmission Electron Microscopy—Bacteria grown overnight in LB at either 37 or 30°C were harvested by centrifugation (3,000 g, 5 min) and washed in 0.1 M cacodylate buffer, pH 7.2. Pellets were fixed in 3% (w/v) glutaraldehyde for 1 h at room temperature. To reveal bacterial surface sugars with the highest possible resolution, glutaraldehyde was diluted in two different concentrations of cacodylate buffer (0.1 and 1 M). Samples were then washed five times in cacodylate buffer, post-fixed for 2 h in 1% (w/v) osmium tetroxide, and washed five times in 0.1 M cacodylate buffer. Samples were then dehydrated by passage through a graded ethanol series (50, 75, 85, and 95%), for 15 min at each concentration and then washed three times for 15 min in pure ethanol. Samples were then embedded in Epon resin. Blocks were thinly sectioned and post-stained with uranyl acetate and Reynold’s lead citrate (11). Sections were visualized on a JEOL 1200EX transmission electron microscope at 80 kV.

RESULTS AND DISCUSSION

Whole Cell Protein Complexes of S. flexneri Serotype 2a

Strain 2457T

Known Protein Complexes—To improve protein complex separation in the first dimension, two different acrylamide gradients (6–11% (A) and 10–16% (B)) were used in the first dimension. Two different acrylamide gradients (6–11% (A) and 10–16% (B)) were used in the first dimension.

Fig. 1. Whole cell soluble protein complexes separated by BN-SDS-PAGE. Two different acrylamide gradients (6–11% (A) and 10–16% (B)) were used in the first dimension.

6.8, 2% SDS, 4% β-mercaptoethanol, and 10% glycerol), and then boiled for 10 min. Proteinase K (50 mg, Sigma-Aldrich) was added, and the mixture was incubated for 1 h at 60°C. Samples were electrophoresed on an SDS-polyacrylamide gel and visualized by silver staining (10).

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RESULTS AND DISCUSSION

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Known Protein Complexes—To improve protein complex separation in the first dimension, two different acrylamide gradients (6–11% and 10–16%) were used for BN-PAGE (Fig. 1). According to the principle proposed by Lasserre et al. (2) regarding the differentiation of protein complexes, nine heteromultimeric protein complexes and 53 homomultimeric protein complexes were separated and identified in this study. Given the high level of similarity between S. flexneri and E. coli, information on E. coli protein complexes from a publicly available bioinformatics database (12) was used as a reference in this study. All nine of the heteromultimeric protein complexes and 49 of the homomultimeric protein complexes have been previously described. For example, the FadAB fatty acid oxidation complex is composed of two α subunits (FadB, 79 kDa, S146 in Fig. 1A) and two β subunits (FadA, 40 kDa, S147 in Fig. 1A) (13). We observed this heterotetramer on the gel as a protein complex of ~230 kDa, which is similar to its theoretical molecular weight. These validated protein complexes are listed in supplemental Tables 2 and 3.

Because parts of the different acrylamide gradient gels overlapped, the same protein was often identified in different acrylamide gradient gels, for example, S332 (Fig. 1A) and S443 (Fig. 1B); S097 (Fig. 1A) and S442 (Fig. 1B); S162 (Fig. 1A) and S419 (Fig. 1B); S155 (Fig. 1A) and S413 (Fig. 1B); and S317 (Fig. 1A) and S425 (Fig. 1B).

Interestingly, we found that the relative position of a protein spot was different when separated with different acrylamide gradient gels. This phenomenon reiterated that care must be taken in the determination of different components of a protein complex, particularly that the results of the analysis of heteromultimeric protein complexes based on only one kind of gradient gel might be questionable. The different protein complex components should always be arranged in a straight line in the different acrylamide gradient gels.

We identified some protein complexes whose experimental molecular weights differed from those previously described. For example, the glutathione synthetase complex (GshB, S322 in Fig. 1A) was reported as a homotetramer in E. coli, with a molecular mass of 152 kDa (14). However, its experimental molecular mass calculated from the current BN-PAGE analysis was 110 kDa. Similarly, the experimental molecular mass of the purine nucleoside phosphorylase complex was 120 kDa.
Novel Protein Complexes—We identified three potentially novel homomultimeric protein complexes, composed of the proteins UshA, YghZ, and PhoN1, respectively. Detailed information on these complexes is given in Table I.

The UshA protein from E. coli is a bifunctional periplasmic protein with both 5′-nucleotidase and UDP-sugar hydrolase activity (19). UshA was more recently found to have a novel, highly efficient CDP-alcohol hydrolase activity (20), and it may have a general role in utilization of external UDP-glucose and extracellular nucleotides (21). X-ray structures have shown that UshA is a monomer (22); however, in our analyses it appeared as a complex with a molecular mass of ~177 kDa. Thus, UshA might be a homotrimer or may have other potential subunits unidentified in this work.

YghZ belongs to the aldo-keto reductase 14 protein family. It is a putative L-glyceraldehyde-3-phosphate reductase (23), involved in the detoxification of the unnatural metabolite L-glyceraldehyde 3-phosphate. According to our findings, the molecular masses of the YghZ monomer and complex are about 39 and 325 kDa, respectively. Thus, YghZ may be an octamer or may have other potential subunits that were again not identified in this work.

Virulence-related Protein Complexes—We identified one potential virulence-related protein complex, composed of 12 PhoN1 subunits, which was presumed to be homomultimeric (S097 in Fig. 1A and S442 in Fig. 1B). PhoN1, encoded on the virulence plasmid of S. flexneri, is a periplasmic nonspecific acid phosphatase, whose physiological role remains to be determined (24). We constructed a phoN1 deletion mutant using the λ-red recombination system, and we tested the virulence of this mutant by HeLa cell invasion experiments. Results indicated that the presence or absence of PhoN1 had little or no impact on the invasion of S. flexneri (data not shown). Protein sequence homology analysis showed that it was highly homologous (51% identity) to another periplasmic nonspecific acid phosphatase, PhoN2, which has been reported as a virulence-related protein essential for proper unipolar IcsA localization and for efficient intercellular spread (25). The identification of the PhoN1 protein complex in our analysis suggests that its function contributes to the virulence of S. flexneri and that it might play a similar role to PhoN2.

Interestingly, known key virulence proteins, such as IpaBCD, were not identified in this study. According to previ-
uous reports, the IpaBC proteins are associated with their specific chaperone to form complexes in the cytoplasm prior to secretion into the host cells (3). The proteome reference map of *S. flexneri*, constructed by classic 2-DE using IEF in the first dimension, indicated that these proteins should be detected under the experimental conditions used here (7). Thus, we speculated that the sample preparation method used in this study may have prevented the detection of these proteins. However, Western blotting detected IpaB in our protein complex samples (Fig. 3). Estimated from the non-denatured gel, the molecular mass of the complex composed of IpaB was about 600 kDa (Fig. 3B). The presence of key virulence proteins was also validated by LC-MS/MS analysis (data not shown). Together, these findings indicated that virulence protein complexes were not lost during the sample preparation process. Therefore, we hypothesize that the lack of identification of known virulence complexes in this study may be a result of the limitations of current methods of non-denaturing electrophoresis or protein detection. For example, the complex composed of IpaB (~600 kDa, Fig. 3B) may be too large to accurately run on the gel, or its structure might be too tightly bound to be denatured in the second dimensional electrophoresis.

### Table I

| Complex no. | Spot ID | Score* | GI     | Theoretical Mr | Peptides identified by MS/MS | Sequence coverage| Matched/searched | Gene | Product                                      |
|-------------|---------|--------|--------|----------------|-----------------------------|----------------|-----------------|------|---------------------------------------------|
| 1           | S306    | 145    | 30042612 | 38,904 | EDFAAYRELJSTKA LYGISSYSYPER IVGSAIVgLHSNPVF OAQLQK | 33 | 14/56 | yghZ | Putative reductase |
| 2           | S097    | 165    | 18462539 | 27,309 | IVGSAIVgLHSNPVF OAQLQK | 26 | 4/55 | phoN1 | Periplasmic nonspecific acid phosphatase |
| 3           | S442    | 236    | 18462539 | 27,309 | LLTNMIEDAGDLATR | 56 | 8/24 | phoN1 | Periplasmic nonspecific acid phosphatase |
| 3           | S441    | 253    | 30042224 | 60,915 | IGNLEYFTDIEFR | 32 | 14/23 | ushA | UDP-sugar hydrolase (5-nucleotidase) |

*A score of greater than 21 is significant for a MS/MS ion search.*

**Fig. 3. Detection of IpaB in protein complex samples using Western blot.**

A, protein complex samples were separated by 12% denatured SDS-PAGE and immunoblotted with anti-IpaB antibody. B, protein complex samples were separated by 4–15% native SDS-PAGE and immunoblotted with anti-IpaB antibody.

### Abundance Analyses of Protein Complexes from *S. flexneri* Cultured at Two Different Temperatures

Analysis of changes in abundance of bacterial protein complexes at temperatures similar to the host environment allows identification of virulence-related complexes (3). We identified four complexes that showed temperature-dependent changes in abundance. A protein spot was regarded as temperature-dependent if the spot density at one temperature was significantly different (greater than 2-fold) from that at the other temperature. The temperature-dependent protein spots are shown in Fig. 4C, with detailed information listed in Table II.

Glycine decarboxylase is composed of two GcvP subunits (S151 in Fig. 4B). This complex is one component of a loosely associated multienzyme complex named the glycine-cleavage system. The function of the glycine-cleavage system was to catalyze glycine oxidation, producing 5,10-methylene-tetrahydrofolate. In this reaction, tetrahydrofolate receives one-carbon units generated during glycine cleavage (26). One-carbon units are essential to the biosynthesis of both purines and pyrimidines in bacteria. Together with serine hydroxymethyltransferase, the glycine-cleavage system provides the largest share of the one-carbon units available to bacteria. We
determined that the abundance of the glycine decarboxylase complex was higher at 30°C than at 37°C (Fig. 4C).

The abundance of the aspartate transcarbamylase complex was also upshifted at 30°C (Fig. 4C). This complex is made up of two catalytic trimers (encoded by the gene pyrB, S197 in Fig. 4B) and three regulatory dimers (encoded by the gene pyrI, S208 in Fig. 4B). It is the first committed enzyme in the pathway of pyrimidine nucleotide biosynthesis, catalyzing the condensation of aspartate and carbamyl phosphate to form N-carbamyl-L-aspartate and inorganic phosphate (27). Thus, both of these complexes that are up-regulated at 30°C are related to pyrimidine nucleotide biosynthesis. These findings agree with a previous report that the abundance of some enzymes involved in purine nucleotide biosynthesis was up-regulated at 30°C (7). One possible explanation is that the synthesis of bacterial virulence proteins was increased at 37°C, which consumed a large amount of energy and material resources, and thus the abundance of those proteins involved in nucleotide biosynthesis decreased.

L-Glutamine: d-fructose-6-phosphate aminotransferase (GFAT) is a homomultimeric protein complex composed of two GlmS subunits (S095 in Fig. 4A) (28). In this study, the abundance of the GFAT complex was higher at 37°C than at 30°C (Fig. 4C). GFAT catalyzes the first step in UDP-N-acetyl-d-glucosamine (UDP-GlcNAc) biosynthesis from fructose 6-phosphate. UDP-GlcNAc is an essential precursor of cell wall peptidoglycan, LPS, and common enterobacterial antigen. The enzymes of this pathway are targets for development of novel antibacterial compounds (29).

The abundance of MglB (S160 in Fig. 4B), one of the components of the galactose ABC transporter MglABC (30), was greater at 30°C than at 37°C (Fig. 4C). MglABC is a β-methylgalactoside transport system and a member of the ATP-binding cassette superfamily of transporters. This complex is

**Fig. 4.** Analysis of soluble protein complexes from *S. flexneri* grown at 37°C (A) and 30°C (B) by BN-SDS-PAGE. An acrylamide gradient of 4–15% was used in the first dimension. Enlarged images of differential protein spots are shown in the lower panel of C. Transcript levels of pyrB, pyrI, gcvP, mglB, glmS, ipaB, and mxiH were determined by qRT-PCR normalized against the levels of the 16 S rRNA gene in each sample (D).
Soluble Protein Complexes of Shigella flexneri

TABLE II
Complexes differentially expressed at the two experimental temperatures

| Complex no. | Spot ID | Scorea | GI | Theoretical M₀ | Peptides identified by MS/MS | Sequence coverage | Matched/searched | Gene | Product |
|------------|---------|--------|----|----------------|-----------------------------|------------------|-----------------|------|---------|
| 1          | S197    | 279    | 30043732 | 34,463 | HKVIASCFFEASTR VIASCFEFESTR ERLDPSEYANVK GTVIDHPAQIGFK ITGIQLPSGEMGR YCEKEFHSNWVLN FTSYQMGYAVQLPPVRER NMLENPGWYTATTPY QPEVSQGR LTQYDAVMQPNSGQA GGEYAGLLAIR | 33 | 8/52 | pyrB | Aspartate carbamoyltransferase, catalytic subunit |
| 2          | S208    | 372    | 30043732 | 17,352 | GTVIDHPAQIGFK ITGIQLPSGEMGR YCEKEFHSNWVLN FTSYQMGYAVQLPPVRER NMLENPGWYTATTPY QPEVSQGR LTQYDAVMQPNSGQA GGEYAGLLAIR | 70 | 10/40 | pyrI | Aspartate carbamoyltransferase, regulatory subunit |
| 3          | S151    | 372    | 30042471 | 105,041 | FTSYQMGYAVQLPPVRER NMLENPGWYTATTPY QPEVSQGR LTQYDAVMQPNSGQA GGEYAGLLAIR | 47 | 25/74 | gcvP | Glycine decarboxylase, P protein of glycine cleavage system |
| 4          | S160    | 236    | 30041846 | 35,690 | GQNVPVFFNKPSR | 56 | 15/28 | mglB | Galactose-binding transport protein; receptor for galactose taxis |
| 5          | S095    | 618    | 30043227 | 67,053 | VQMLAQAAEEHPHGG TGIAHTR RFIFLEEGDIAETR FIFLEEGDIAETR ELGYLSLAICNVPGSSLVR IEALAEFDSKKHALFLGR | 55 | 24/56 | glmS | L-Glutamine: D-fructose-6-phosphate aminotransferase |

a A score greater than 21 is significant for a MS/MS ion search.

As mentioned above, three of the four differentially expressed protein complexes were related to LPS biosynthesis (Fig. 5). UTP and GlcNAc-1-phosphate are the products of the pathways involving aspartate transcarbamylase complex and GFAT, respectively. These two compounds can be catalyzed to produce UDP-GlcNAc, which participates in LPS biosynthesis. Galactoside can be directly used as a sugar moiety in LPS biosynthesis. However, there is a discrepancy between the abundance patterns of aspartate transcarbamylase complex/MglABC and that of GFAT. Further experiments are required to determine the influence of temperature on LPS biosynthesis in S. flexneri.

Analysis of the Amount of LPS

qRT-PCR Validation—To verify proteome data, the transcript levels of five differentially expressed genes (pyrB, pyrI, gcvP, mglB, and glmS) were quantitated using qRT-PCR (Fig. 4D). Virulence genes ipaB and mxiH, whose expression levels are regulated by temperature (3), were selected as a positive control. The expression of four of the five genes measured by qRT-PCR showed changes similar to those measured by BN-PAGE, despite quantitative differences in the magnitudes of abundance changes (Fig. 4C), whereas the last gene showed changes contrary to our previous findings. Protein Glms was down-regulated at 30°C according to the results of BN-PAGE, whereas the corresponding gene was up-regulated in the 30°C sample by qRT-PCR analysis. Although this inverse correlation between the proteomics and qRT-PCR data is somewhat surprising, it is important to keep in mind that protein stability, modifications, and turnover might alter the protein abundance in comparison with that expected from the qRT-PCR data (31). In fact, the regulation of glmS is very complicated.

It was previously reported that the expression of glmS is controlled at both the transcriptional and post-transcriptional levels (32). In addition, the abundance of GlmS, as analyzed by classic 2-DE, showed no significant changes at two different temperatures (5). These results indicate that glmS is regulated at a post-transcriptional level to maintain the total amount of this protein. We hypothesize that the amount of GlmS used to form a complex is only a part of the total amount of GlmS protein in the cells and that overall GlmS levels decreased at 30°C. The monomeric GlmS not used to form complexes might be degraded. Another possible explanation might be that there are various complexes formed by GlmS, and the detectable complex by BN-PAGE was only one of these.

LPS Tests—Because the majority of differentially expressed protein complexes were related to LPS synthesis, the LPS of bacteria grown at two different temperatures was prepared and analyzed. Experimental results showed that the relative intensities of LPS ladders of bacteria grown at 30°C were slightly higher than those grown at 37°C (Fig. 6A), although there were no significant differences in the length of LPS. To validate silver staining results, the cell walls of bacteria grown at the two different temperatures were visualized by trans-
Mission electron microscopy (Fig. 6B). In comparison with cells grown at 37°C, cells grown at 30°C had an exterior composed of more diffuse, filamentous material from the outer membrane, i.e., the LPS of bacteria grown at 30°C appeared to be thicker than that of cells grown at 37°C. For a clearer observation of the cell wall structure, we used 1 M cacodylate buffer to dilute glutaraldehyde, creating a high osmotic pressure environment, thus causing the cytoplasm to shrink. Similar results were obtained (data not shown).

The results of the above two experiments agreed with our hypothesis that the relative intensity of LPS is higher at 30°C, as deduced from the abundance changes of protein complexes (Fig. 5). LPS is composed of a lipid A region, core oligosaccharide, and an O-antigen polysaccharide side chain that is exposed to the bacterial surface. West et al. (33) determined that glucosylation of Shigella O-antigen, the basis of different serotypes, shortens the LPS molecule by around 50%, because glucosylated O-antigen is more compact and shorter than unglucosylated LPS. Because the average length of the protruding needles of the Shigella type three secretion system is 60 nm (34), a shorter LPS molecule would optimize the exposure of the needle complex, thus enhancing bacterial invasion. Martinic et al. (35) also demonstrated that the length, distribution, and glucosylation of the O-antigen contribute to acid resistance. They also showed that pH affects the length of LPS, suggesting that there is a balance in the length of LPS O-antigen side chains to provide resistance to extreme acidity or against innate immune effectors, and also influences the function of the type three secretion system needle. Our study shows another regulatory mechanism of bacterial LPS synthesis, suggesting that the relative intensity but not the length of LPS might be regulated by temperature. One possible explanation is that the assembly of the type three secretion system needle at 37°C might occupy more space at the cell wall, and thus reduce the abundance of LPS.

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