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A comparative proteomic analysis of *Salmonella typhimurium* under the regulation of the RstA/RstB and PhoP/PhoQ systems

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ABSTRACT

In pathogenic bacteria, the two-component regulatory systems (TCSs) play important roles in signal transduction and regulation of their pathogenesis. Here, we used quantitative proteomic methods to comparatively analyze functional networks under the control of the RstA/RstB system versus the PhoP/PhoQ system in *Salmonella typhimurium*. By comparing the proteomic profile from a wild-type strain to that from a Δ*rstB* strain or a Δ*phoPQ* strain under a condition known to activate these TCSs, we found that the levels of 159 proteins representing 6.92% of the 2297 proteins identified from the Δ*rstB* strain and 341 proteins representing 14.9% of the 2288 proteins identified from the Δ*phoPQ* strain were significantly changed, respectively. Bioinformatics analysis revealed that the RstA/RstB system and the PhoP/PhoQ system coordinated with regard to the regulation of specific proteins as well as metabolic processes. Our observations suggested that the regulatory networks controlled by the PhoP/PhoQ system were much more extensive than those by the RstA/RstB system, whereas the RstA/RstB system specifically regulated expression of the constituents participating in pyrimidine metabolism and iron acquisition. Additional results also suggested that the RstA/RstB system was required for regulation of *Salmonella* motility and invasion.

1. Introduction

Salmonella enterica serovar *typhimurium* (*S. typhimurium*) is a facultative intracellular Gram-negative bacterium and the causative agent of gastroenteritis and septicemia in humans. To survive in the host tissues, bacteria require the ability to sense host environmental signals and adapt accordingly by regulating various gene expression. This sensing and adaptation process often involves the two-component regulatory systems (TCSs), which are the signal transduction systems widely spread in various bacteria [1]. A typical TCS consists of two proteins, a histidine sensor kinase, and a cognate response regulator. The sensor protein is located in the inner membrane, which senses certain environmental signals directly or indirectly, autophosphorylates and then transfers the phosphoryl group to activate its cytoplasmic cognate regulator, which in turn regulates transcription of specific genetic loci [2].

The PhoP/PhoQ system is a master TCS to control *Salmonella* virulence. The known environmental signals that can activate PhoP/PhoQ include low Mg^{2+}, acidic pH, and cationic antimicrobial peptide (CAMP) [3,4]. The PhoP/PhoQ system also controls the expression of some other TCSs such as SsrA/SsrB [5], PmrA/PmrB [6], and RstA/RstB [7]. When the PhoP/PhoQ system is activated, it regulates the transcription of >100 genes [8]. It has been recently reported that in *Salmonella*, RstB, the histidine sensor kinase of RstA/RstB TCS, enhances the activity of PhoQ by increasing the transcription of PhoP-activated genes [9]. RstA, the cognate response regulator, is activated by the PhoP/PhoQ system under the condition of either low Mg^{2+} or acidic pH [10]. These studies suggest a coordinated regulation between the 'master regulator' PhoP/PhoQ and the less known RstA/RstB system.

The PhoP/PhoQ system has been the focus of many studies using system biology tools [11–13]. However, no systematic profiling of the RstA/RstB-dependent regulatory network has been reported. To better understand the RstA/RstB regulation and its coordinated regulation with PhoP/PhoQ, we compared alterations of the proteome landscape caused by Δ*rstB* and Δ*phoPQ* mutations in *S. typhimurium* cultured under a known condition that activates PhoP/PhoQ and RstA/RstB (i.e., 10 μM Mg^{2+}) [10,14–16]. To carry out these analyses of quantitative proteomics, we used stable isotope dimethyl labeling and tandem mass spectrometry techniques. A total of 2297 and 2288 proteins were quantitated, and 159 and 341 proteins were found to be

Abbreviations: TCSs, two-component regulatory systems; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; IPG, immobilized pH gradient; LC–MS/MS, liquid chromatography–mass spectrometry/mass spectrometry; MALDI-TOF/TOF, matrix assisted laser desorption ionization-time-of-flight/time-of-flight; PRM, parallel reaction monitoring.

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significantly changed in the Δ*rstB* and Δ*phoPQ* strains, respectively. When comparing the RstA/RstB- and PhoP/PhoQ-regulated proteins, we identified similarities and differences between the two TCSs. Two, seven and two proteins related to pyrimidine metabolism, iron acquisition and cell motility ability, respectively, were found to be specifically regulated in the Δ*rstB* strain. Thus, our quantitative proteomic analysis provides a novel view on *Salmonella* pathogenesis regulation by means of TCS coordinated regulation.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A *Salmonella enterica* serovar *typhimurium* wild-type strain ATCC 14028 was used for the study. The *S. typhimurium* Δ*phoPQ* strain was constructed as previously described [14,17]. Briefly, the chloramphenicol (Cm)-resistant cassette from pKD3 was amplified using primer 5′-AACGCTAGACTGTTCTTATTGTTAACACAAGGGAGAAGAGGTGTAGGCTG GAGCTGCTTC-3′ and 5′-GGATGCTTAACGAGATGCGTGGAAGAACGCAC AGAAATGTATGGGAATTAGCCATGGTCC-3′. The resulting PCR product was electroporated into a *S. typhimurium* WT strain harboring pKD46 and integrated into the chromosome by homologous recombination. The Δ*phoPQ* strain was then selected by the acquired chloramphenicol resistance and confirmed by colony PCR.

The Δ*rstB*::Cm mutant was constructed similarly to the Δ*phoPQ* strain using the primers as follows: 5′-AGCTAATGTATTGAGATCCGGT GGGCGTTGCATATGAATATCCTCCTTAG-3′ and 5′-TCCCGCCGCTCAACCA GCGTGCAAAATGCGGTGTAGGCTGGAGCTGCTTC-3′.

The Δ*udp*::Km mutant was constructed using pKD4 as the template to amplify the kanamycin (Km)-resistant cassette with primers 5′-ATGT CCAAGTCTGATGTTTTTCATCTCGGCCATATGAATATCCTCCTTAG-3′ and 3-TTACAGCAGACGACGGGCCGCTTCCACGACGTGTAGGCTGGAGCTGCT TC-5′. P22 phage lysate was used to mediate the transfer of the Δ*rstB*::Cm region into the Δ*udp*::Km mutant to construct the Δ*rstB*::Cm Δ*udp*::Km double mutant.

2.1.1. Plasmid construction

To construct plasmid pUHE21, in which the RstB protein was expressed from the lac promoter, the *rstB* gene was amplified by PCR using primers pRstB-F/(CGGGATCCGGTGGGCGTTGATGAAAAAGCTG), pRstB-Flag-R (CCCCTGCAGTCACTTGTCATCGTCGTCCTTGTAGTCGGCA GCGGTCATGTCGG) and chromosomal DNA from the *S. typhimurium* strain as a template. After digestion with *Bam*HI and *Pst*I, the PCR products and plasmid pUHE21 were taped with T4 ligase at the corresponding restriction sites. Sequences of the cloned regions of the recombinant plasmids were confirmed by nucleotide sequencing.

2.1.2. Bacterial culture

The bacteria were grown at 37 °C in either Luria-Bertani (LB) medium (10 g/l NaCl, 5 g/l yeast extract, and 10 g/l tryptone) or N-minimal medium (5 mM KCl, 7.5 mM $(NH4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 0.1 M Tris-base, 38 mM glycerol, and pH 7.4). For the "low Mg^{2+} medium", N-minimal medium was supplemented with 10 μM $MgCl_2$. The bacteria were cultured at 9 h.

2.2. Protein extraction

The bacterial cells from the late log growth phase were harvested by centrifugation at 8000 ×*g* for 8 min and resuspended in the lysis buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM ethylenediaminetetraacetic acid calcium disodium salt (EDTA) and protease inhibitor cocktails (Roche Applied Sciences). The cells were lysed by sonication in an ice water bath and then subjected to centrifugation at 12,000 ×g for 20 min at 4 °C. To precipitate the proteins, three volumes of a mixture of 50% acetone, 50% ethanol, and 0.1% acetic acid were added to the supernatant and incubated for 2 h at −40 °C. The protein pellet was collected by centrifugation at 4000 ×*g* for 20 min and resuspended in lysis buffer. The resuspended protein concentration was determined using the Bradford assay. The extracted proteins were stored at −80 °C.

2.3. Two-dimensional polyacrylamide gel electrophoresis

A two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed as we previously described [11]. Briefly, dry polyacrylamide gel strips with an immobilized pH gradient (IPG) in the pH range of 4–7 and a size of 13 cm were used. A total of 450 μg protein was resuspended in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.0002% bromophenol blue, 0.5% pharmalyte, 20 mM dithiothreitol) to a total volume of 250 μl. The resuspended proteins were applied to an IPG strip and allowed to rehydrate followed by isoelectric focusing for 18 h using the IPGphor system (GE Healthcare, Uppsala, Sweden). Subsequently, the IPG-strips were equilibrated with rotation in buffer A (50 mM Tris-HCl, pH 8.8; 6 M urea, 30% glycerol; 2% SDS and 1% DL-dithiothreitol (DTT) for 10 min) and then in buffer B (the same as buffer A but with 2.5% iodoacetamide replacing DTT) for an additional 10 min. Each strip was then transferred on top of a 12% polyacrylamide gel for the second dimension of the gel electrophoresis. Protein spots were visualized using Coomassie blue G-250 stain and then scanned.

2.4. Protein digestion

The protein extract was resuspended in 8 M urea/0.2 M Tris, pH 8/4 mM $CaCl_2$, reduced with 10 mM DTT at 50 °C for 30 min and alkylated with 40 mM iodoacetamide at room temperature in the dark for 30 min. After diluting the urea concentration to 2 M with water, trypsin (Promega, Madison, WI) was added (1:50; w/w, trypsin/protein) and the proteins were digested at 37 °C overnight.

The protein-containing 2D gel spots were excised and cut into small pieces. The gel slices were washed three times with 200 μl of 50% acetonitrile/100 mM NH_4HCO_3 for 10 min each and dried by a SpeedVac. Then, the gel pieces were rehydrated with 100 μl of 10 mM NH_4HCO_3, reduced with DTT, alkylated with iodoacetamide, and digested in gel with trypsin (Promega, Madison, WI) as previously described [11].

2.5. Peptide stable isotope dimethyl labeling and strong cation exchange fractionation

The tryptic peptide mixtures were desalted using C18 Sep-Pak (Waters) before dimethyl labeling. The dimethyl labeling was performed as previously described [18].

Strong cation exchange (SCX) fractionation was performed using a PolySULFOETHYL Column (2.1 mm ID × 50 mm, 5 μm particle size, 200 Å, PolyLC, Columbia, MD). SCX buffer A contained 5 mM KH_2PO_4, pH 2.7/20% acetonitrile/80% H_2O, and buffer B contained 5 mM KH_2PO_4, 0.5 M KCl pH 2.7/20% acetonitrile/80% H_2O. The peptide mixtures were dissolved in buffer A. The SCX was performed at a flow rate of 0.2 ml/min. Six fractions were collected during the 55 min gradient. The peptides in each fraction were desalted using a C18 Ziptip (Millipore) before the mass spectrometry analysis.

2.6. Mass spectrometry analysis and data processing

LC–MS/MS experiments were performed using a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) equipped with an EASY-nLC 1000 UHPLC system (Thermo Fisher Scientific) via a nanoelectrospray ion source. A chromatographic peptide separation was performed with a 25 cm column packed in-house with Uchrom 3 μm C18 resin in a 120 min gradient. The mobile phase consisted of two components: component A was 0.1% formic acid, and component B was 0.1% formic acid in acetonitrile.