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RpoS Affects Gene Expression in *Salmonella enterica* serovar Typhi Under Early Hyperosmotic Stress

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Abstract During the infectious procedure of *Salmonella enterica* serovar Typhi (*S*. Typhi), *Salmonella* would suffer from some severe environmental stresses, such as gastric acid stress, enteric hyperosmotic stress, bile acids stress, and oxidative stress. *S*. Typhi must regulate the expression of numerous genes through the complex regulatory network to adapt strict stresses. *RpoS*, which encodes sigma factor σ^s, was reported to be a very important regulator in the maximal survival of enteric pathogens including *S. enterica* in the stress conditions. However, the role of RpoS in *S*. Typhi under early hyperosmotic stress is not clear. In this study, we prepared the *RpoS*-deleted mutant (Δ*RpoS*) and compared the growth of Δ*RpoS* and wild-type strain. In addition, we analyzed its global transcription profile under early hyperosmotic stress by a microarray. The results showed that Δ*RpoS* grew significantly slower than wild-type strain and 24 genes displayed differential expression between the wild-type strain and Δ*RpoS* upon 30-min exposure in the high osmolarity. Most of these genes are associated with enzymes of metabolism. Taken together, our study firstly demonstrated that *RpoS* affects gene expression in *S*. Typhi under early hyperosmotic stress and may impact the growth of bacteria by regulating bacterial metabolic enzymes.

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Introduction

Salmonella enterica serovar Typhi (*S*. Typhi) is a gram-negative human-restricted bacterium that causes severe typhoid fever [1]. Due to the development and use of antibiotics, harm caused by *S*. Typhi has been greatly reduced [2]. However, typhoid fever remains a common disease in tropical and subtropical regions where many drug-resistant strains have been isolated [3, 4]. *S*. Typhi is capable of survival in a variety of environments, including hyperosmotic stress. This adaptation to the hyperosmotic stress environment is carried out by a series of global regulatory networks.

It is well established that the housekeeping sigma factors control the transcription of essential genes [5]. However, bacteria also possess alternative sigma factors that contribute to initiate the transcription of a specific set of genes by recognizing the promoters of these genes [5, 6]. RpoS is one of the most important alternative sigma factors and required for maximal survival of enteric pathogens in the stress conditions, including *Salmonella enterica* [7]. Many studies of RpoS were focused on its roles in survival of several stress conditions [8–10]. In the previous study, it was shown that RpoS impacts the lag phase of *S. enterica* during osmotic stress [11], and RpoS is involved in the cross-talk with another sigma factor RpoE under hyperosmotic stress [12]. However, the role of RpoS in *S*. Typhi under early hyperosmotic stress is unclear. In this study, we prepared the *RpoS*-deleted mutants and compared its global transcription profile with the wild-type under early hyperosmotic stress.

Materials and Methods

Bacterial Strains and Culture Conditions

Salmonella enterica serovar Typhi Ty2 strain was used in this study. Mutants and plasmids generated and used in this study are listed in Table 1. Bacteria were grown in Luria–Bertani (LB) broth (pH 7.0) containing 50 mM NaCl under shaking at 37 °C, representing low-osmolarity environment. For early hyperosmotic stress, NaCl was added to cultures to a final concentration of 300 mM and cultured for 30 min.

Construction of *RpoS*-Deleted Mutant and Complementation Strains

A mutant of *RpoS* was generated by homologous recombination mediated by the suicide plasmid pGMB151 as described previously [12]. The primers used in this study are listed in Table 2. The *RpoS*-homologous fragment in which 591 bp of the *RpoS* gene was absent was amplified and inserted into the *Bam*HI site of the suicide plasmid pGMB151, which carries the sucrose-sensitivity gene *sacB*. The suicide plasmid carrying the deleted *RpoS* gene was transferred into the wild-type strain by electroporation. Then the mutant strain was selected by PCR, verified by sequencing, and designated as the mutant strain Δ*RpoS*.

The complementary strain of *RpoS* in Δ*RpoS* was prepared as described previously [13]; the CDS of *RpoS* was amplified with *pfu* DNA polymerase by PCR. The amplicon was inserted into the vector pACYC184 to form the recombinant plasmid pACYC184-*RpoS*. The mutant train Δ*RpoS* was transformed by the recombinant plasmid pACYC184-*RpoS* and designated as the *RpoS* complementary strain Δ*RpoS* (pACYC184-*RpoS*). As a control, Δ*RpoS* strain was also transformed with the empty vector pACYC184 and named Δ*RpoS* (pACYC184).

Table 1 Strains and plasmids used in the study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
S. Typhi Ty2	Wild-type strain	Laboratory collection
SY372λpir	*E. coli* host strain of suicide plasmid	Laboratory collection
Δ*RpoS*	*S*. Typhi Ty2 (Δ*RpoS*)	This work
Δ*RpoS* (pACYC184)	Δ*RpoS* containing pACYC184 empty vector	This work
Δ*RpoS* (pACYC184-*RpoS*)	Δ*RpoS* containing pACYC184-*RpoS* recombinant plasmid	This work
Plasmids		
pGMB151	Suicide plasmid; *sacB*; Amp^r	Laboratory collection
pGMB151-*RpoS*	pGMB151 containing *RpoS*-deleted homologous fragments	This work
pACYC184	Complementary vector; Chl^r, Tet^r	Laboratory collection
pACYC184-*RpoS*	pACYC184 containing *RpoS* gene	This work

Table 2 Primers used in this study

Primers	Sequence (5′-)	Purpose
F1A (*Bam*HI)	TTAGGATCCCGTAAACCCGCTGCGTTATT	*RpoS* mutant construction
F1B (*Bgl*II)	TGAGATCTCTTCAATCAGGTCCAGCAA	
F2A (*Bgl*II)	GCAGATCTTTATCCGTGCAGTCGAGAA	
F2B (*Bam*HI)	CTAGGATCCGATGATTTGTCCACGCTGTG	
C-*RpoS*-A (*Xba*I)	CGTCTAGAATGAGTCAGAATACGCTGA	pACYC184-*RpoS* construction
C-*RpoS*-B (*Hind*III)	GCAAGCTTTTACGCCGTACCAGCCGCG	
F-*guaA*	ACGATATGAATACCTCGGA	qRT-PCR
R-*guaA*	TCCAGTCTTTCACGTCCAG	
F-*narG*	GCAGGAAAATAACCCGGAA	
R-*narG*	CACGTCAGCAATCTCATCC	
F-*dmsB*	GTGACTGGCAGGAAGATAA	
R-*dmsB*	GCCGTCGCACTTGGTCATA	
F-*yihV*	GGGGCAAATATGTGGCGAA	
R-*yihV*	TGGCATCCACCATAATCGC	

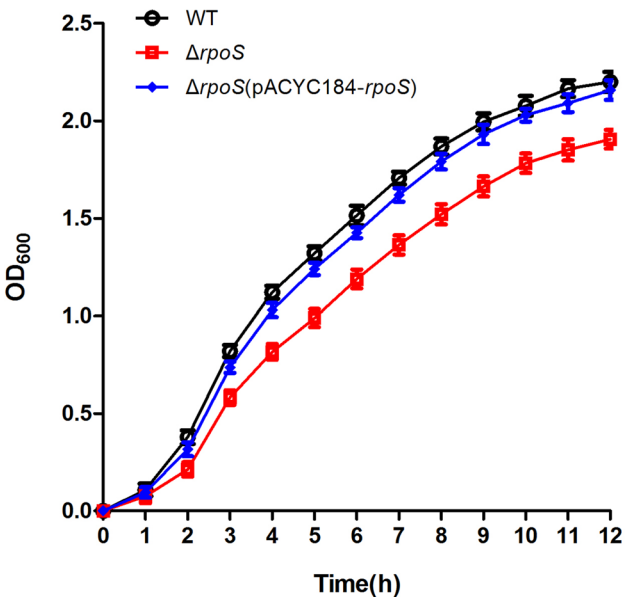

Fig. 1 Growth curve of *Salmonella*

Monitoring the Bacterial Growth Curve Under Hyperosmotic Stress Conditions

A single colony of wild-type, Δ*RpoS* and *RpoS* complementary strain from a LB agar plate was inoculated in 2 ml of LB broth and incubated at 37 °C shaking (250 rpm) overnight. The culture was diluted 1/100 in fresh LB broth containing 50 mM NaCl. For osmotic stress, NaCl was added into cultures to a final concentration of 300 mM. The bacterial growth curve was measured at OD_{600} every hour using a BioPhotometer (Eppendorf, Hamburg, Germany). The experiments were repeated at least three times.

Investigation of the Global Transcription Profile by Microarray

Wild-type and Δ*RpoS* strains were cultured overnight at 37 °C with shaking (250 rpm) in LB broth containing 50 mM NaCl. After dilution into fresh medium, cultures were incubated to exponential growth (OD 0.5 at 600 nm). For simulating the early hypeosmotic stress condition, NaCl was added to a final concentration of 300 mM and bacteria were incubated with shaking for a further 30 min at 37 °C. Bacteria were collected by centrifugation and total RNA was extracted using a RNeasy kit according to the manufacturer's instructions. 2 µg of the total RNA was used for fluorescence labeling of cDNA probes. A DNA microarray designed for *S*. Typhi was provided by Jiangsu University in this study. The hybridization, microarray scanning, and data analysis were performed as described previously [14].

Quantitative Real-Time PCR (qRT-PCR) Assay

Total RNA of wild-type and Δ*RpoS* and *RpoS* complementary strains extracted after 30 min of hyperosmotic stress as above was subjected to qRT-PCR as described previously [15, 16]. Each experiment was performed with three RNA samples from three independent experiments. Student's *t* test was used for the statistical analysis. Differences were considered statistically significant when *P* was <0.05 in all cases.

Statistical Analysis

Each experiment was conducted with at least three independent experiments. All *P* values were calculated in Microsoft Excel using a two-tailed unpaired Student's *t* test.

Table 3 Genes regulated by *RpoS* in *S*. Typhi under early hyperosmotic stress by microarray-based profile assay

Gene name	Product	Log2 (Δ*RpoS*/wild)
yaaI	Hypothetical protein t0011	−1.16
nhaR	Transcriptional activator protein NhaR	−1.35
mutT	7,8-Dihydro-8-oxoguanine-triphosphatase	−1.02
btuF	Vitamin B12-transporter protein BtuF	−1.11
rof	ROF protein	−1.21
asrC	Anaerobic sulfite reductase subunit C	−1.21
guaA	Bifunctional GMP synthase/glutamine amidotransferase protein	−1.22
dedA	DedA protein	−1.02
napF	Ferredoxin-type protein NapF	−2.40
aadA	Aminoglycoside-resistance protein	−1.09
narH	Respiratory nitrate reductase 1 beta chain	−1.33
narG	Respiratory nitrate reductase 1 alpha chain	−2.33
narK	Nitrite extrusion protein	−2.21
exo	Exonuclease	−1.01
yajC	Preprotein translocase subunit YajC	−1.06
sodA	Manganese superoxide dismutase	−1.25
hslT	Heat shock protein A	−1.01
nrfA	Cytochrome c552 precursor	−1.16
dmsB	Anaerobic dimethyl sulfoxide reductase chain B	2.24
dmsA	Anaerobic dimethyl sulfoxide reductase chain A	1.89
invF	Possible AraC family regulatory protein	1.63
yihV	Putative sugar kinase	3.04
deoC	Deoxyribose-phosphate aldolase	1.21
deoA	Thymidine phosphorylase	1.02

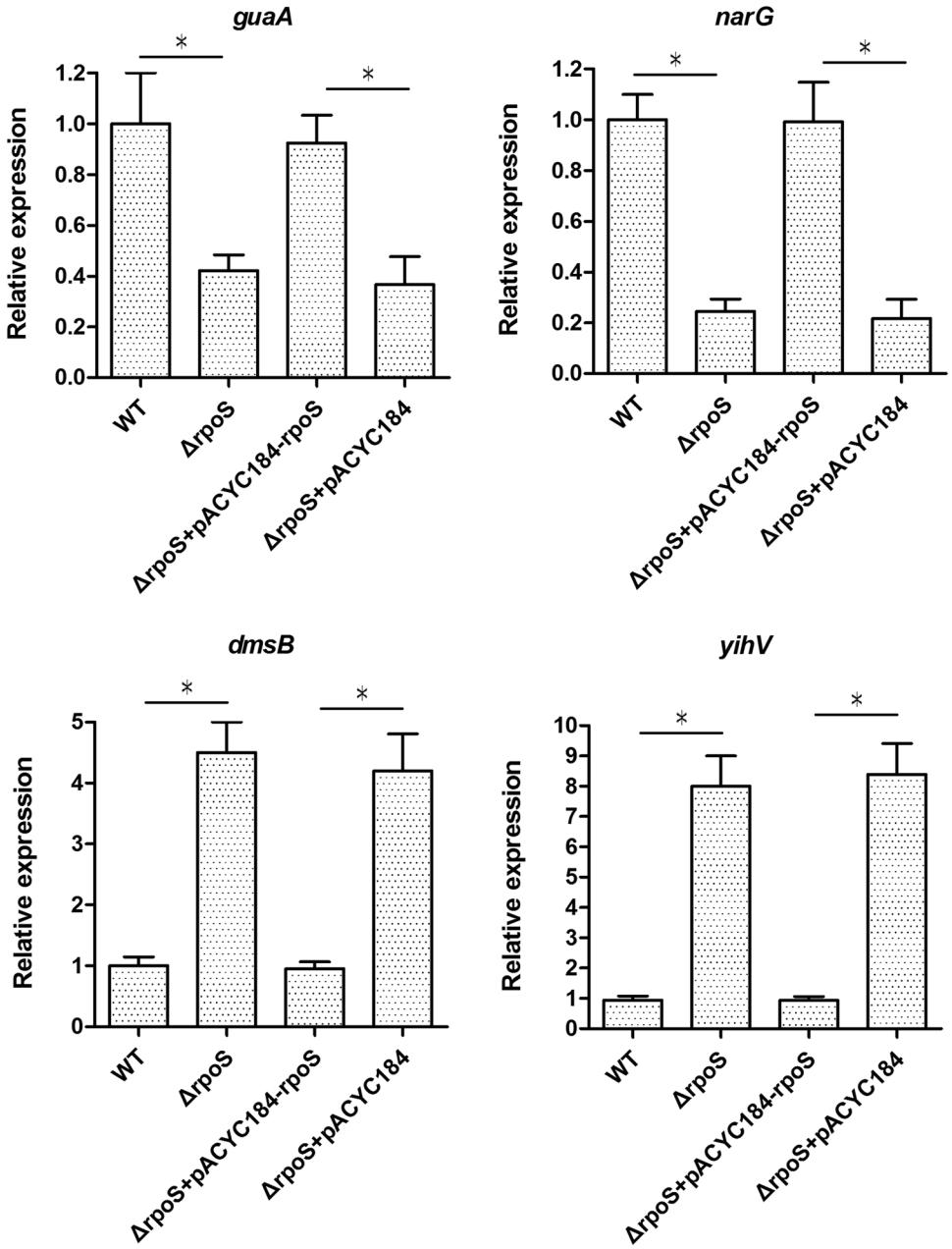

Fig. 2 qRT-PCR validation of the microarray results. $^{*}P < 0.05$

Results and Discussion

Deletion of *RpoS* Affects Bacterial Growth in the Hyperosmotic Condition

Under the hyperosmotic stress condition, the growth of wild-type and Δ*RpoS* strain was measured and the result showed that Δ*RpoS* strain grew significantly slower than wild-type strain ($P < 0.05$) (Fig. 1). In addition, the growth of complementary strain Δ*RpoS* (pACYC184-*RpoS*) restored to the wild-type level (Fig. 1). While the growth of Δ*RpoS* strain and the wild-type strain at low osmotic conditions (50 mM NaCl) had no significant difference (data not shown), RpoS seems to extend the lag phase of Δ*RpoS* under high osmotic stress. In 2014, Shiroda et al. reported that the expression level of RpoS influences the length of lag phase and the growth of *S*. Typhimurium in hyperosmotic growth conditions [11]. In this study, it was shown that deletion of *RpoS* repressed the growth of *S*. Typhi in the hyperosmotic condition.

Transcriptional Profile Assay of the *RpoS* Mutant and Wild-Type Strain Under Early Hyperosmotic Stress

To investigate the regulatory role of *RpoS*, the global transcriptional differences between wild-type strain and Δ*RpoS*

were analyzed using a genomic DNA microarray of *S.* Typhi under early hyperosmotic stress. The results showed that 24 genes displayed differential expression (twofold or greater) between the wild-type strain and Δ*RpoS* by maintaining the high osmolarity for 30 min. As shown in Table 3, 18 genes of Δ*RpoS* were downregulated under early hyperosmotic stress and most of these genes are associated with enzymes involved in metabolism. Six genes were upregulated and these upregulated genes encode metabolic enzymes as well except gene *invF* which encodes a possible AraC family regulatory protein. It was respeculated that *invF* might regulate the downstream genes involved in bacterial metabolism. All these results showed that *RpoS* may impact the growth of bacteria by regulating bacterial metabolic enzymes.

Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) Validation

qRT-PCR was employed to verify the microarray results, and expression of several representative genes including *guaA, narG, dmsB*, and *yihV* was further examined. As shown in Fig. 2, expressions of *guaA* and *narG* in Δ*RpoS* were significantly decreased compared to that of the wild-type strain, while *dmsB* and *yihV* expression levels of Δ*RpoS* were significantly higher than that of wild-type strain. In addition, expression level of these representative genes in Δ*RpoS* (pACYC184-*RpoS*) was restored to the wild-type strain. The qRT-PCR results were consistent with the microarray analysis described above.

We propose that RpoS affects gene expression in *S.* Typhi under early hyperosmotic stress and may impact the growth of bacteria by regulating bacterial metabolic enzymes. Our future work will focus on RpoS-regulated genes that contribute to infectious procedure during osmotic stress in *S.* Typhi.

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