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Repressed Quorum Sensing by Overexpressing LsrR Hampers *Salmonella* Evasion from Oxidative Killing Within Macrophages

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Bacterial cell-to-cell communication, termed quorum sensing (QS), leads to coordinated group behavior in a cell-density-dependent fashion and controls a variety of physiological processes including virulence gene expression. The repressor of the *lsr* operon, LsrR, is the only known regulator of LuxS/AI-2-mediated QS in *Salmonella*. Although lack of *lsrR* did not result in noticeable differences in *Salmonella* survival, the down-regulation of QS as a result of *lsrR* overexpression decreased *Salmonella* survival within macrophages. We found that impaired growth of *Salmonella* overexpressing *lsrR* within macrophages was due largely to its hypersensitivity to NADPH-dependent oxidative stress. This, in turn, was a result of decreased expression of genes involved in the oxidative stress response, such as *sodA*, *sodCI*, and *sodCII*, when *lsrR* was overexpressed. These results suggest that down-regulation of QS by excess LsrR can lower *Salmonella* virulence by hampering *Salmonella* evasion from oxidative killing within macrophages.

Keywords: *Salmonella* Typhimurium, LsrR, oxidative stress response

Bacteria can control gene expression in response to changes in their population density through the quorum sensing process. In the course of QS, small signaling molecules (*i.e.*, autoinducers) are synthesized and released outside of the cells as the bacterial population grows [22]. When the cell density reaches a certain level and autoinducers are produced above a minimal threshold, the autoinducers bind to cognate receptors to promote changes in gene expression [22]. By using QS systems, bacteria can control various physiological processes such as biofilm formation, virulence factor production, bioluminescence, sporulation, motility, and antibiotic production [4, 21].

Several QS systems are known in various species of bacteria. Many Gram-negative bacteria employ acyl homoserine lactones (AHLs) for intraspecies communication [21], whereas peptides are used as autoinducers in Gram-positive bacteria [8]. In addition, another QS signaling, mediated by the LuxS-produced autoinducer-2 (AI-2), is known to be shared by both Gram-negative and Gram-positive bacteria [21]. The LuxS protein is found in over 55 bacterial species and catalyzes the conversion of *S*-ribosylhomocysteine to 4,5-dihydroxy-2,3-pentanedione (DPD) as a precursor of the signaling molecule, AI-2 [23].

The LuxS/AI-2 QS system has been found in *Salmonella enterica* serovar Typhimurium, and the AI-2 molecule has been identified as (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (R-THMF) [21]. This signaling molecule is transported by the Lsr transporter, an ATP-binding-cassette transporter composed of LsrA, LsrB, LsrC, and LsrD, encoded by the *lsr* operon. The internalized AI-2 is phosphorylated by LsrK and further modified by LsrF and LsrG [19]. Phosphorylated AI-2 (phospho-AI-2) binds to the LsrR protein (*i.e.*, a repressor of the *lsr* operon), which inactivates the regulatory function of LsrR, resulting in increased transcription of the *lsr* operon [24].

Salmonella Typhimurium causes a typhoid-like disease in mice. During systemic infection of animal hosts, *Salmonella* resides within macrophages, evading the host's defenses. This lifestyle of *Salmonella* requires multiple systems to endure or overwhelm the host defense mechanisms. One of the important host defense mechanisms to overcome by *Salmonella* is the production of microbicidal reactive oxygen species (ROS) during oxidative burst, induced by invading bacteria [10]. The reactive oxygen intermediates, including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), are known

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to cause severe damage to DNA, RNA, proteins, and lipids [10].

Although several studies have used a *luxS*-deleted mutant to study the role of QS in bacterial physiology, the role of *luxS* in *Salmonella* pathogenesis is controversial owing to the pleiotropic nature of LuxS [4, 15]. LsrR is the only known regulatory protein that serves as a repressor of the *lsr* operon in *Salmonella* [19]. It is known that *lsrR* is autoregulated and LsrR is active in the absence of phospho-AI-2. Therefore, *Salmonella* overexpressing *lsrR* is expected to have a high level of active LsrR not associated with AI-2, if LsrR levels exceed the intracellular level of AI-2. We found that *Salmonella* overexpressing *lsrR* was defective for survival within macrophages owing to their high sensitivity to ROS, a result of down-regulation of the oxidative stress response genes, such as *sodA*, *sodCI*, and *sodCII.*

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *Salmonella enterica* serovar Typhimurium strains used in this study were derived from strain SL1344 [12]. Phage P22-mediated transductions were performed as previously described [2]. All *Salmonella* strains were grown aerobically at 37°C in Luria–Bertani (LB) broth or M9 minimal media. Antibiotics were used at the following concentrations: ampicillin 50 μg/ml, chloramphenicol 25 μg/ml, and kanamycin 50 μg/ml.

Construction of Strains

We constructed *Salmonella* strains harboring chromosomal deletions or *lacZ*-fusions as described previously [7, 9]. The primers used for construction of bacterial strains are listed in Table 2.

Construction of Plasmids

Plasmids overexpressing *lsrR* were constructed by cloning the *lsrR* gene from *S.* Typhimurium strain SL1344 into the pUHE21-2*lacI*^{q} plasmid vector [18]. The *lsrR* gene was amplified by PCR using primers lsrR-pF1 and lsrR-pR1, and the PCR product was cloned between the *Eco*RI and *Bam*HI sites of pUHE21-2*lacI*^{q}; the resulting plasmid was named as pJH1. To construct pJW1 for complementation of *soxS*, the *soxS* gene was amplified by PCR using primers soxS-pF1 and soxS-pR1, and the PCR product was cloned between the *Hin*dIII and *Sph*I sites of vector pACYC184 [3]. The primers used for those plasmid constructions are listed in Table 2.

β-Galactosidase Assay

β-Galactosidase assays were carried out in triplicate, and activity was determined as described previously [14].

Hydrogen Peroxide Resistance Assay

Sensitivity to hydrogen peroxide (H_2O_2; Sigma) was determined as follows. To assess the sensitivity of *Salmonella* strains to H_2O_2, overnight cultures of *S.* Typhimurium strains were inoculated (1:100) into fresh LB broth containing 2 mM H_2O_2 and incubated at 37°C. The sensitivity was determined by plating dilutions of cultures

Table 1. Bacterial strains and plasmids used in this study.

Strain	Description	Reference
Salmonella enterica serovar Typhimurium		
SL1344	Wild type, Sm^R	[11]
SR4558	SL1344, pUHE21-2*lacI*^{q}	This study
SR4559	SL1344, pJH1	This study
SR4743	P*sodA*::*lacZ*, pUHE21-2*lacI*^{q}	This study
SR4572	P*sodA*::*lacZ*, pJH1	This study
SR4773	P*sodA*::*lacZ*, Δ*soxS*, pUHE21-2*lacI*^{q}	This study
SR4774	P*sodA*::*lacZ*, Δ*soxS*, pJH1	This study
SR4787	P*sodA*::*lacZ*, Δ*soxS*, pJW1, pUHE21-2*lacI*^{q}	This study
SR4795	P*sodA*::*lacZ*, Δ*soxS*, pJW1, pJH1	This study
SR4729	P*sodCI*::*lacZ*, pUHE21-2*lacI*^{q}	This study
SR4730	P*sodCI*::*lacZ*, pJH1	This study
SR4731	P*sodCII*::lacZ, pUHE21-2*lacI*^{q}	This study
SR4732	P*sodCII*::*lacZ*, pJH1	This study
Plasmids		
pKD46	Ap^R P_{BAD}-*gam-beta-exo oriR101 repA101*^{ts}	[6]
pKD13	Ap^R FRT Km^R FRT PS1 PS4 *oriR6Kγ*	[6]
pCP20	Ap^R Cm^R *cI*857 λP_R*flp oripSC101*^{ts}	[6]
pCE70	Km^R FRT *tnpR lacZY*$^+$ *oriR6Kγ*	[8]
pUHE21-2*lacI*^{q}	rep_{pMB1}, Ap^R, *lacI*^{q}	[15]
pACYC184	p15A *ori*, Tet^R, Cm^R	[3]
pJH1	pUHE21-2*lacI*^{q}::*lsrR*	This study
pJW1	pACYC184::*soxS*	This study

Table 2. Primers for construction of strains and plasmids.

Primers	Sequences (5'→3')
For construction of mutant strains	
sodA-RED-F	GGC GTT AAC ACT GTG CCG CTC GAC AAT AAT GGA GAT GAT TTG TAG GCT GGA GCT GCT TCG
sodA-RED-R	CAG GCC CAG GAT CGG GAA GCC GGA AGC GCC GGA AAT GGC TAT TCC GGG GAT CCG TCG ACC
sodA-F1	GGT TTT TTT CAG CGG ATG CCG TAA C
sodA-R1	CGC TTC TAC AGA CGT GCA ATG CAA A
sodCI-RED-F	TTA ATG GTA TTT ACG ATA CAA CCA AAA AAC GAG GTA ACT ATG TAG GCT GGA GCT GCT TCG
sodCI-RED-R	ATG GGT AAA ACG AAA TTA TGA CGA TAT GGC TAT GTT GCT GAT TCC GGG GAT CCG TCG ACC
sodCI-F1	GGG TTT CCT GTG GTA TAT CCT G
sodCI-R1	TAG GTG CTT GGC GTA GGT TAC A
sodCII-RED-F	CGG CGG AAT GCG TTA CGC CTG CGG CGT CAT TAA ATA TCC GTA TGA TGT TCC TGA TTA TGC TAG CCT CTA ATA ATG TAG GCT GGA GCT GCT TCG
sodCII-RED-R	AGC TGG AAC AGG CTC CAG CGC AGG GAA CAC TGG CTC CGG GAT TCC GGG GAT CCG TCG ACC
sodCII-F1	CGT ACG CGC CGC ACT AGA AG
sodCII-R1	CGA TGT GAT GAG CGA GCG CG
For construction of plasmids	
lsrR-pF1	GTA AAG CCA GAA TTC GAC AAT GAG
lsrR-pR1	CGT TAC ATA GGA TCC TGT CAG TTA
soxS-pF1	GAG TGC ATG TTA AGC TTC TTC AAG
soxS-pR1	AAC AGG TTA GCT GCA TGC TAA AAC

on LB agar. Alternatively, aliquots of overnight cultures containing approximately 10^7 CFU and serially diluted samples of the culture were dropped on the surface of LB agar containing 200 mM H_2O_2. The colonies that formed from different dilution factors were compared.

Gentamicin Protection Assay

J774A.1 macrophage cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50 U/ml). Confluent monolayers were prepared in 24-well tissue culture plates for infection with bacteria. Each well was seeded with 2×10^5 cells suspended in DMEM/10% FBS without antibiotics and incubated for 1 h at 37°C under 5% CO_2. The wells were washed three times with phosphate-buffered saline (PBS) before bacterial cells were added. Bacterial cells were washed with PBS, suspended in prewarmed DMEM, and then added to the cell monolayer with a multiplicity of infection (MOI) of 10. Following 30 min of incubation, the wells were washed three times with prewarmed PBS to remove extracellular bacteria and then incubated with prewarmed medium supplemented with 100 μg/ml gentamicin to kill extracellular bacteria. If necessary, diphenyleneiodonium chloride (DPI; Sigma) was added to a final concentration of 10 μM. Next, the wells were washed three times with PBS, lysed with 1% Triton X-100 for 30 min, and then diluted with PBS. A dilution of the suspension was plated on LB agar medium to count colony forming units (CFUs).

RESULTS

Overexpression of *lsrR* Causes Defects in *Salmonella* Survival Within Macrophages

Because QS has been reported to serve various functions in bacteria including virulence gene regulation [21], we tested whether LsrR has any effect on *Salmonella* virulence in macrophages. Whereas the *lsrR* mutant showed no difference in survivability compared with the wild type (data not shown), *lsrR* overexpression caused a significant defect in *Salmonella* survival within macrophages (Fig. 1A).

When *Salmonella* cells are engulfed by macrophages, one of the major early attack mechanisms used to kill the bacteria is the production of ROS [10]. To test whether the impaired proliferation of *Salmonella* overexpressing *lsrR*

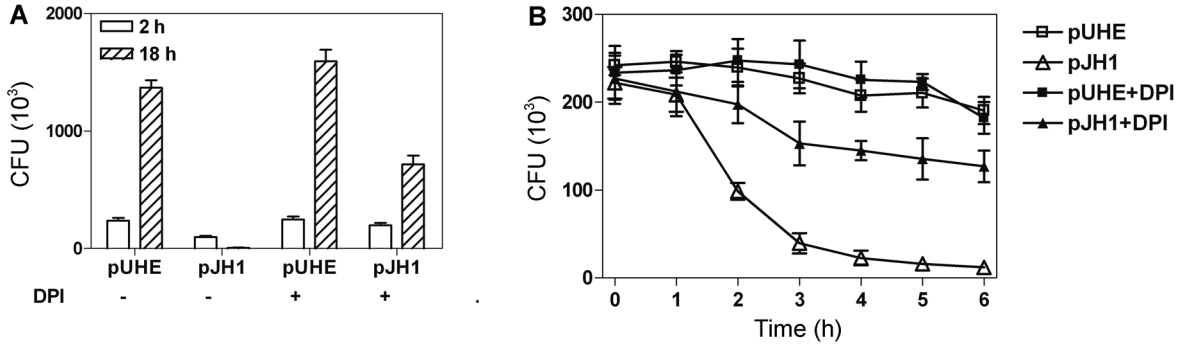

Fig. 1. Overexpression of LsrR reduced survival of *Salmonella* within macrophages.
Macrophages J774A.1 were infected with wild-type (WT) *Salmonella* strains harboring pUHE21-2*lacI*^{q} (pUHE) or pJH1. The numbers of intracellular bacteria were determined at designated time points. Infected macrophages were incubated in the presence of 100 μM of IPTG and in the presence or absence of 10 μM of DPI. The intracellular bacterial number was determined at 2 and 18 h (**A**), or at every hour for 6 h (**B**).

was due to oxidative stress generated by phagocyte NADPH oxidase, we infected macrophages in the presence of DPI, an inhibitor of this enzymatic complex. The number of intracellular *Salmonella* overexpressing *lsrR* increased in the presence of DPI in the early stages of infection (Fig. 1B); additionally, the bacteria were able to replicate better within macrophages in the presence of DPI (Fig. 1A). These results suggested that impaired QS due to excess *lsrR* resulted in lower survival of *Salmonella* within macrophages, probably due to a weakened response to the NADPH-dependent oxidative stress.

Salmonella Strains Overexpressing *lsrR* Display High Sensitivity to ROS

We next tested whether the sensitivity to ROS was influenced by the LsrR level in *Salmonella*. The survival of LsrR-overexpressing *Salmonella* was compared with the wild-type strain after exposure to 2 mM hydrogen peroxide. The viability of *Salmonella* was significantly reduced when *lsrR* was overexpressed (Fig. 2). In agreement with these results, the number of *Salmonella* overexpressing *lsrR* declined by three-logs compared with wild type on LB agar containing 0.2 mM hydrogen peroxide (Fig. 2). The redox-cycling agent paraquat generates increased levels of intracellular superoxide radicals (O_2^-). We determined the susceptibility of *Salmonella* overexpressing *lsrR* to paraquat in LB broth and on agar, and found that this strain was also more sensitive to paraquat (data not shown). These results suggest that *Salmonella* overexpressing *lsrR* shows a higher susceptibility to ROS.

Overexpression of the *lsrR* Reduces Transcription of Genes Involved in the Oxidative Stress Response

In *Salmonella*, it is known that oxidative stress response genes, such as *sodA* and *sodC,* are important for detoxification of ROS [1, 16]. Thus, we studied the

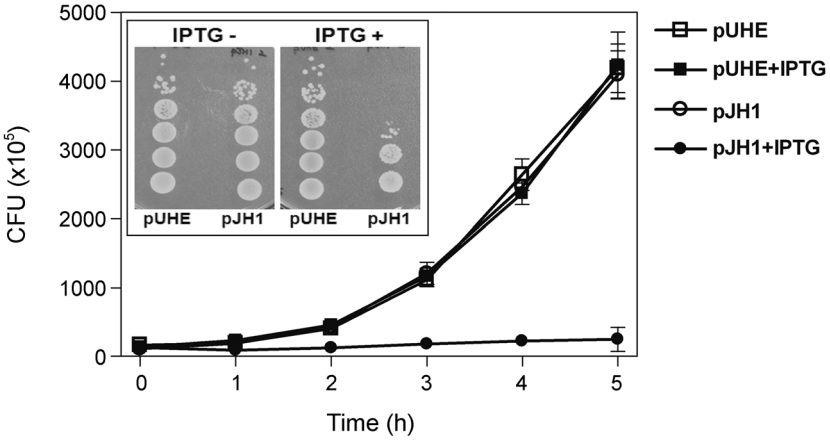

Fig. 2. LsrR-overexpressing *Salmonella* is hypersensitive to hydrogen peroxide.
Overnight cultures of wild-type (WT) *Salmonella* harboring pUHE21-2*lacI*^{q} (pUHE) or pJH1 were diluted (1:100) into fresh LB broth containing 2 mM of hydrogen peroxide. Aliquots of cultures were used to determine the viable bacterial numbers. The overnight cultures of wild-type *Salmonella* harboring pUHE21-2*lacI*^{q} or pJH1 were diluted serially, and 10 μl of each dilution was dropped on LB agar containing 200 μM of hydrogen peroxide. If necessary, IPTG was added at the final concentration of 100 μM.

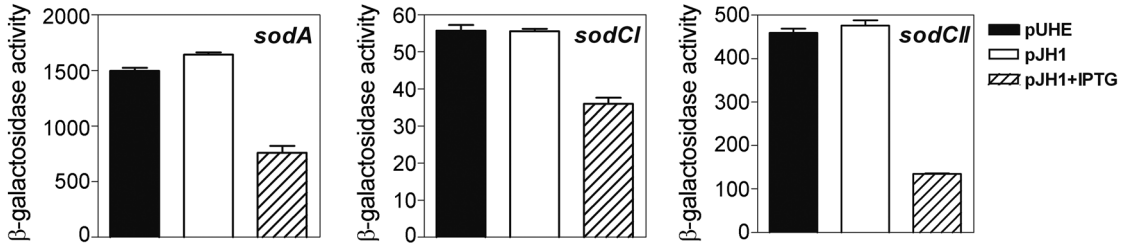

Fig. 3. LsrR overexpression reduces transcription levels of the oxidative-stress-related genes.
Wild-type (WT) *Salmonella* strains carrying a transcriptional fusion of *sodA-lacZ*, *sodCI-lacZ*, or *sodCII-lacZ* were introduced by the backbone plasmid, pUHE21-2*lacI*^{q} (pUHE), or pJH1. These bacteria were grown in M9 minimal media. To induce the production of LsrR from the *lac* promoter, 100 μM of IPTG was added to the medium. The mean values and standard deviation of three independent experiments are shown.

transcription levels of several genes related to the oxidative-stress response in *Salmonella* strains carrying a chromosomal *lacZ* fusion to *sodA*, *sodCI*, or *sodCII.* As expected, the transcription levels of *sodA*, *sodCI*, and *sodCII* were 2- to 4-fold lower when *lsrR* was overexpressed (Fig. 3).

LsrR-Mediated Regulation of Oxidative Stress Response Genes Requires SoxS Protein

The *sodA* gene is a member of the SoxSR regulon, and the oxidative induction of *sodA* expression is dependent on SoxS [16]. Thus, it is possible that SoxS could be involved in the regulatory cascade of *sodA* by LsrR. To test this idea, we constructed a *soxS* deletion mutant with a chromosomal *sodA–lacZ* fusion and examined the effects of *lsrR* overexpression. The deletion of *soxS* did not influence the expression level of *sodA* in M9 or LB media (data not shown). In contrast, *sodA* expression was not increased in the presence of paraquat in the *soxS* mutant, as expected (Fig. 4). Reduction of *sodA* expression was observed upon *lsrR* overexpression in the wild-type background but not in the *soxS* mutant. These results indicate that LsrR may alter the expression of the *sodA* gene *via* SoxS.

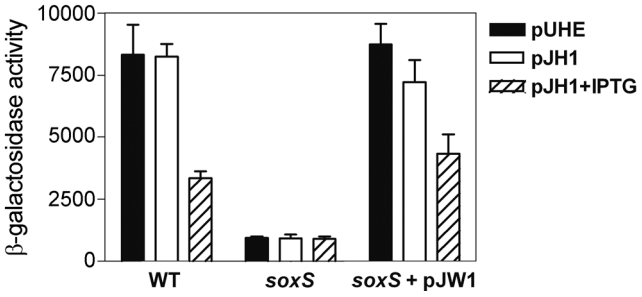

Fig. 4. SoxS is involved in the LsrR-mediated regulation of the *sodA* gene.
Wild-type (WT) and *soxS*-deleted mutant *Salmonella* strains carrying a chromosomal transcriptional fusion of *sodA–lacZ* were introduced by the backbone plasmid, pUHE21-2*lacI*^{q} (pUHE), or pJH1. For complementation of the *soxS* gene, pJW1 was introduced into the *soxS* mutant strain. The bacteria were grown in LB broth containing 250 μM of paraquat to mid-exponential phase. If necessary, IPTG was added at a final concentration of 100 μM.

DISCUSSION

Although various roles of LuxS/AI-2 QS have been reported in bacteria, few mechanisms have been elucidated in *Salmonella*. LsrR is the only known transcriptional regulator involved in *Salmonella* QS, although a recent study reported that LsrR and LsrK serve as global regulators of gene expression in *E. coli* [11]. In the present study, we demonstrated the effects of LsrR on the oxidative stress response and consequently on replication of *Salmonella* Typhimurium within macrophages.

Activated macrophages use a complex array of oxygen-dependent antimicrobial molecules to inhibit or kill intracellular bacteria. The initial killing of intracellular bacteria depends on the production of superoxide anions and hydrogen peroxide [20]. Thus, *Salmonella* must quickly adapt to or evade the hostile intracellular environment of the phagocyte. To scavenge and degrade ROS, *Salmonella* has evolved numerous defense mechanisms to avoid oxidative stress [10]. In a strain overexpressing LsrR, we found that the expression of genes involved in resistance to oxidative stress was decreased (Fig. 2), and that this regulatory effect required SoxS, an activator of *sodA* (Fig. 4). For *Salmonella* to survive within macrophages, especially at the initial phase of infection, resistance to oxidative stress must occur. Therefore, the number of intracellular *Salmonella* overexpressing *lsrR* was rapidly decreased within macrophages in the initial stage of infection (Fig. 1B). This is consistent with previous studies that showed that a SOD mutant was less virulent than wild type [6]. Similar effects of QS on the regulation of oxidative stress response were reported in the cases of *Burkholderia pseudomallei*, *Burkholderia glumae*, and *Sodalis glossinidius* [5, 13, 17].

DPI, an inhibitor of NADPH oxidase, reduced the rapid killing of *Salmonella* overexpressing *lsrR* and allowed the bacteria to proliferate within macrophages by suppressing production of ROS by NADPH oxidase (Fig. 1B). However,

the addition of DPI did not allow *Salmonella* overexpressing *lsrR* to reach the replication efficiency of wild type, whose growth was not affected by DPI (Fig. 1A). These results indicated that there may be another LsrR regulon involved in *Salmonella* virulence.

In summary, we found that the overexpression of LsrR decreased the expression of oxidative stress response genes in *S.* Typhimurium. As a consequence, *Salmonella* overexpressing *lsrR* showed increased sensitivity to ROS and reduced survival within macrophages. Although the exact mechanism of this regulation is not clear, these findings imply that *Salmonella* may require LsrR-mediated QS to evade bactericidal oxidative killing in the macrophage environment.

Acknowledgments

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