LsrR-Mediated Quorum Sensing Controls Invasiveness of Salmonella typhimurium by Regulating SPI-1 and Flagella Genes

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Abstract

Bacterial cell-to-cell communication, termed quorum sensing (QS), controls bacterial behavior by using various signal molecules. Despite the fact that the LuxS/autoinducer-2 (AI-2) QS system is necessary for normal expression of Salmonella pathogenicity island-1 (SPI-1), the mechanism remains unknown. Here, we report that the LsrR protein, a transcriptional regulator known to be involved in LuxS/AI-2-mediated QS, is also associated with the regulation of SPI-1-mediated Salmonella virulence. We determined that LsrR negatively controls SPI-1 and flagella gene expressions. As phosphorylated AI-2 binds to and inactivates LsrR, LsrR remains active and decreases expression of SPI-1 and flagella genes in the luxS mutant. The reduced expression of those genes resulted in impaired invasion of Salmonella into epithelial cells. Expression of SPI-1 and flagella genes was also reduced by overexpression of the LsrR regulator from a plasmid, but was relieved by exogenous AI-2, which binds to and inactivates LsrR. These results imply that LsrR plays an important role in selecting infectious niche of Salmonella in QS dependent mode.

Introduction

Bacteria control gene expression patterns in response to changes in their population density through a process called quorum sensing (QS). In QS, small signaling molecules called autoinducers are synthesized and released from the bacterial cells, and accumulate in the external environment [1]. When cell density reaches a certain level, producing autoinducer concentrations over a minimal threshold, the autoinducers bind to cognate receptors to promote changes in gene expression [2]. QS systems regulate a large number of physiological processes in bacteria including biofilm formation, virulence factor production, bioluminescence, sporulation, motility, and antibiotic production [3,4,5,6,7].

Several types of QS systems have been described in various species of bacteria. Many Gram-negative bacteria employ acyl homoserine lactones for interspecies communication [8], whereas Gram-positive autoinducers are typically peptides [9]. Another QS pathway, in addition, in which signaling is mediated by the LuxS-produced autoinducer-2 (AI-2), has been found in both Gram-negative and Gram-positive bacteria [10]. The LuxS protein is found in over 55 bacterial species [11], and catalyzes the conversion of S-ribosylhomocysteine to 4,5-dihydroxy-2,3-pentanedione (DPP), which spontaneously cyclizes to form the signaling molecule AI-2 [10].

The LuxS/AI-2 QS system is present in Salmonella enterica serovar Typhimurium (S. Typhimurium), whose signal molecule has been identified as (2R,4S)-2-methyl-2,3,3,4-tetrahydroxystetrahydrofuran [12]. In S. Typhimurium, AI-2 is imported by the Lsr transporter, an adenosine triphosphate-binding-cassette transporter encoded by the lsr operon and comprised of LsrA, LsrB, LsrC, and LsrD. The internalized AI-2 is phosphorylated by LsrK and modified further by LsrF and LsrG [13]. Phosphorylated AI-2 (phospho-AI-2) binds to the repressor protein LsrR, inactivating it and derepressing transcription of the lsr operon (see Fig.S1) [13].

S. Typhimurium causes gastroenteritis and diarrhea in humans due to acute intestinal inflammation, and also causes a typhoid-like disease in mice. During infection of animal hosts, Salmonella invade epithelial cells of the small intestine. Invasion is mediated by a type three secretion system encoded in Salmonella pathogenicity island 1 (SPI-1). The SPI-1 type three secretion system forms a needlelike complex through which a number of effector proteins are translocated into host cells and mediate modification of the actin cytoskeleton [14]. Expression of SPI-1 is controlled by various environmental cues including oxygen tension, osmolarity, pH, and nutrients, reflecting the complex conditions in the intestinal lumen [15,16,17].

Previously, we reported that LuxS-mediated QS is required for normal expression of a subset of genes within SPI-1 and contributes to virulence of S. Typhimurium because deletion of...
the luxS gene decreased the transcription of SPI-1 genes and impaired invasion of Salmonella [18]. In contrast, another study recently reported that mutation of the luxS gene has no effects on the expression of SPI-1 and Salmonella virulence in a mouse infection model [19].

LuxS could have different effects on SPI-1 expression depending on the exact experimental conditions because of its pleiotropic functions [20]. Thus, the contradictory results may be resolved by a more complete understanding of LuxS effects on SPI-1 expression. We sought to clarify the role of LuxS by finding a factor that links LuxS/AI-2-mediated QS and SPI-1 expression. In the present study, we report that LsrR, a DNA-binding repressor of the inv operon [21], negatively controls expression of SPI-1 and flagella genes and also regulates the ability of Salmonella to invade host cells.

Results

Salmonella LuxS Mutant is Indeed Defective for InvF-dependent Expression of SPI-1 Genes and Attenuated for Virulence in Mice

luxS deletion mutants have widely been used to reveal the roles of QS in bacterial physiology. However, the role of LuxS in Salmonella pathogenesis is controversial. We reported that a Salmonella strain lacking the luxS gene displays defects in virulence phenotypes associated with SPI-1 expression [18], but these findings were not reproduced by others [19]. These contradictory results could be due to the pleiotropic effects of luxS mutation [20], or reflect a recently described non-quorum sensing function of LuxS [22]. To clarify the role of LuxS in Salmonella virulence, it is necessary to identify the factor that directly connects LuxS-dependent QS to regulation of SPI-1 virulence genes.

Before searching for such a factor, we confirmed that luxS is required for normal SPI-1-mediated Salmonella virulence. We showed that luxS deletion causes reduced expression of the invF gene, which in turn decreases expression of the InvF-regulated SPI-1 genes sicA, sigD, and sopE [18]. By conducting qRT-PCR, we determined that the mRNA levels of the sicA, sopB, and sopE genes decrease in both the ΔinvF strain and the ΔluxS ΔinvF double mutant strain (Fig. 1A). Expression of invF from a plasmid restored transcription of sicA, sopB and sopE to wild-type levels in the ΔluxS ΔinvF and the ΔluxS strains as well as the invF deletion strain (Fig. 1A). In contrast, the luxS expression from a plasmid failed to do so in both the ΔluxS ΔinvF and the ΔinvF strains (Fig. 1A and data not shown). These data indicate that the reduced transcription levels of a subset of SPI-1 genes in the luxS deletion mutant were due to a decrease in InvF levels.

Our previous finding showed that the luxS mutant is attenuated for virulence in mice based on bacterial numbers in liver and spleen [18], whereas Perrett et al. [19] reported that the luxS mutant did not display any significant differences in virulence compared to wild-type was based on number of live mice at day 12 post infection [19]. We decided to re-evaluate the virulence of the luxS mutant strain with a detailed monitoring (i.e. monitoring for every 12 or 24 h) of the survival of mice infected with Salmonella. Our data also show that all mice infected with either the wild-type or the luxS mutant strain died by 12 days after oral or intraperitoneal challenge (Fig. 1B and C), but the survival of mice infected with the luxS mutant was significantly delayed compared to those infected with wild-type Salmonella (Fig. 1B and C, p<0.0001). Taken together, these data emphasize that LuxS is necessary for normal expression of SPI-1 by controlling InvF and also for normal virulence of Salmonella.

LsrR Negatively Controls Expression of SPI-1 Genes in the Absence of LuxS

Because LuxS is not a DNA-binding protein, we hypothesize that there must be a transcription factor that links QS and SPI-1 regulation as described in other bacteria that regulate gene expression in response to QS [11]. In this sense, we focused on the LsrR protein, the repressor of the inv operon, because it is the only known regulator controlled by Salmonella QS [1].

Phospho-AI-2 binds to LsrR inactivating its ability to act as a repressor and thereby inducing expression of the inv operon [13]. Because the luxS deletion mutant cannot produce AI-2, LsrR is constitutively active. If LsrR negatively regulates the expression of SPI-1 genes, then deletion of the luxS gene would be expected to decrease expression of SPI-1 genes as we reported previously [18]. To test the hypothesis that LsrR lowers expression of SPI-1 genes, we constructed Δlsr and Δlsr ΔluxS strains carrying a chromosomal invF-lacZ fusion. β-galactosidase assay determined that deletion of lsr had no effect on transcription of the invF-lacZ fusion (Fig. 2A). However, the absence of LsrR compensated for the transcriptional defect in invF-lacZ expression in the luxS deletion mutant (Fig. 2A). The regulatory effects of LsrR in the absence of luxS gene were analyzed in cultures with (Fig.2A) or without shaking (data not shown). This result was confirmed to be due to the absence of LsrR function because expression of LsrR from a heterologous promoter reduced invF transcription in the Δlsr ΔluxS Δlsr double mutant strain (Fig. 2B). These results suggest that in the absence of LuxS-catalyzed AI-2 production, LsrR represses InvF expression, which in turn reduces transcription levels of the InvF-regulated genes within SPI-1.

The internalization of Salmonella into epithelial tissues of animal hosts is mediated by a number of gene products expressed from the SPI-1 locus [14], suggesting a role for LsrR in regulating internalization. We next examined whether LsrR regulates the invasiveness of Salmonella. Deletion of luxS impaired the ability of Salmonella to invade HEp-2 epithelial cells (Fig. 2B), as we reported previously [18]. If both lsr and luxS are deleted, however, invasion of HEp-2 cells is comparable to that of the parental Δlsr ΔluxS strain (Fig. 2B). These results are in good agreement with the finding that lsr or deletions restored expression levels of InvF in the luxS deletion strain (Fig. 2A). Taken together, our results suggest that, when activated by the absence of AI-2, the LsrR protein represses SPI-1 expression, which impairs Salmonella’s invasiveness.

Overexpression of the LsrR Protein Lowers Expression of SPI-1 Genes

The down-regulation of invF expression by LsrR was only observed in the absence of LuxS (Fig. 2). This may be because the levels of active LsrR protein (i.e., LsrR not associated with AI-2) were elevated in the absence of LuxS. In contrast, LsrR could be present in a phospho-AI-2-bound inactive form in a luxS strain, which could explain why deletion of lsrR gene alone did not alter invF expression (Fig. 2A). If this were the case, overexpression of the LsrR protein could increase levels of active LsrR protein to a level sufficient to suppress expression of SPI-1 genes even in a strain carrying the luxS gene.

To test this idea, we constructed a strain carrying a chromosomal invF-lacZ fusion and harboring the plasmid pJH11 in which expression of LsrR is under control of the lac promoter. β-galactosidase assays show that IPTG induction of LsrR protein lowers the transcription levels of invF by about 4-fold (Fig. 3A). qRT-PCR analysis confirms that overexpression of LsrR decreases transcription of several SPI-1 genes: the transcript levels of the invF, sicA, sopB, and sopE genes were reduced by approximately
3- to 4-fold when the wild-type strain harboring pJH1 was grown with IPTG (Fig. 3B). Next, we used Western blot analysis to investigate whether levels of a SPI-1 protein were affected by LsrR overexpression. Western blot analysis determined that overexpression of LsrR in the wild-type strain dramatically reduced levels of the SopB protein (Fig. 3C). Although the mRNA fold changes and protein levels were different in the LsrR-overexpression and wild-type strains, the negative regulatory effects of LsrR were consistent. The data presented here demonstrate that the LsrR regulator negatively controls expression of SPI-1 genes.

**Overexpression of LsrR Inhibits Invasion into Epithelial Cells**

We next investigated whether the reduced expression of SPI-1 genes resulting from LsrR overexpression affects the ability of *Salmonella* to invade mammalian cells. When HEp-2 epithelial cells

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**Figure 1.** The *luxS* gene is necessary for normal expression of SPI-1 and virulence. (A) Transcriptional levels of the SPI-1 genes *sicA*, *sopB*, and *sopE* were determined by qRT-PCR. Overnight cultures of wild-type (WT) and mutant strains were diluted in fresh LB and mRNA samples were prepared from stationary phase of static cultures. Values are means and standard deviations of three independent experiments. (B and C) Six-week-old BALB/c mice (n = 10) were infected orally with 10^7 CFU (B) or intraperitoneally (I.P.) with 10^3 CFU (C) *Salmonella* strains. Mice surviving after infection were monitored daily for two weeks.

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**Figure 2.** LsrR is involved in the regulation of SPI-1 expression and invasion of *S. typhimurium*. (A) Wild-type (WT) and mutant strains carrying a chromosomal invF-lacZ fusion were diluted in LB medium and β-galactosidase activity (Miller units) was determined at 4 h of cultures grown with shaking. Values are the means and standard deviation of three independent experiments. (B) Monolayers of HEp-2 epithelial cells were infected with the wild-type (WT) and mutant strains and numbers of internalized bacteria were determined (see Methods). Values represent the relative amount of internalized bacteria normalized to the level of internalization of the WT strain, which was set to 1.00. Values are the average and standard deviation from three independent experiments, each done in triplicate.

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were incubated with the wild-type strain carrying pJH1 that had been grown with IPTG to induce LsrR expression, invasion was greatly impaired (i.e., 17% invasion compared to that of the wild-type strain; Fig. 4).

Expression of SPI-1 and motility are both required for Salmonella cells to gain entry into epithelial cells [14,23], but the requirement for motility can be overcome through the use of mild centrifugation [23]. To examine whether the defective invasion phenotype of LsrR-overexpressing Salmonella was solely due to the decrease in SPI-1 expression, or whether motility might also be affected, a mild centrifugation was applied to promote bacterium-host cell contacts during the invasion assay [23]. Although invasion was still decreased in the LsrR overexpressing strain when adherence of Salmonella cells to the epithelial cells was promoted using centrifugation (Fig. 4), invasion was only 62% that of the wild-type strain (Fig. 4). Thus, centrifugation partially restored invasion of HEp-2 cells by Salmonella overexpressing LsrR.

**The LsrR Protein Negatively Regulates Expression of Flagella Genes**

Because the enhanced contact of bacterium-host cell by centrifugation partially recovered the defective invasiveness of LsrR-overexpressing Salmonella, we reasoned that motility of Salmonella might also be regulated by LsrR or LuxS. We examined the effect of luxS and lsrR deletions on expression of fliC which encodes flagellin, the major component of the flagellum [24], by using chromosomal fliC-lacZ fusion strains. β-galactosidase assays

![Figure 3. LsrR negatively controls the expression of SPI-1 genes.](image3)

(A) Wild-type (WT) Salmonella carrying a chromosomal invF-lacZ fusion and either the control plasmid, pUHE21-2lacIq or the lasR+ plasmid pJH1 were grown in LB with shaking for 4 h. Production of LsrR was induced by the addition of 100 μM IPTG. (B) WT Salmonella carrying pJH1 were grown LB or LB supplemented with 100 μM IPTG, with shaking for 4 h. The mRNA levels of SPI-1 genes were determined by qRT-PCR. Shown in (A) and (B) are the mean values and standard deviation of three independent experiments. (C) Western blot analysis was conducted on cell extracts prepared from the strain harboring either pUHE21-2lacIq or pJH1 grown in LB with 100 μM IPTG, with shaking for 4 h. These strains express the SopB protein tagged with a HA-epitope from the normal chromosomal location. doi:10.1371/journal.pone.0037059.g003

![Figure 4. The overexpression of LsrR decreased Salmonella invasion into HEp-2 epithelial cells, even when the requirement for motility is bypassed through centrifugation.](image4)

Monolayers of HEp-2 epithelial cells were infected with the wild-type (WT) Salmonella, WT harboring backbone plasmid, pUHE21-2lacIq, and WT harboring pJH1 strains in the presence or absence of 100 μM IPTG. To exclude the requirement of motility, mild centrifugation was employed (centri.). The numbers of internalized bacteria were determined as described in Methods. Values represent the relative amount of internalized bacteria and have been normalized to the level of internalization of WT strain, which was set at 1.00. Values are the average and standard deviation from three independent experiments, each done in triplicate.

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determined that flbC expression decreased in the luxS deletion mutant, unaffected by deletion of lsrR, and elevated in the ΔluxS ΔlsrR double mutant (Fig. 5A). These results indicate that LsrR represses transcription of the flbC gene in the absence of LuxS.

We next investigated the effect of LsrR overexpression on flagella gene expression. β-galactosidase assays revealed that in the flbC-lacZ fusion strain carrying pJH1, the transcription levels of flbC decreased by approximately 4-fold upon IPTG addition (Fig. 5B). Moreover, qRT-PCR determined that the mRNA levels of the flhD and flaA, which encode key regulators of flagella synthesis [24], were reduced by approximately 3-fold when the wild-type strain harboring pJH1 was grown with IPTG (Fig. 5D). Analysis of secreted proteins showed that LsrR overexpression in wild-type Salmonella greatly reduced levels of the flagella proteins, FlhC, FlhD, and FlgI (Fig. 5C). Levels of the SPI-1 effector proteins SipA, SipC, and SipD, were also lowered upon LsrR overexpression, consistent with the regulatory role of LsrR for SPI-1 genes (Fig. 5C). These results indicate that, in addition to regulating the SPI-1 genes, the LsrR protein also negatively regulates flagella genes.

The LsrR Protein Suppresses Motility of Salmonella

Because expression levels of flagella decreased in the LsrR-overexpressing Salmonella strain (Fig. 5B and C), we asked whether the flagella structure is affected by LsrR levels. Transmission electron microscopy using a negative stain allows visualization of flagella appendages on the surface of the S. Typhimurium cells [24]. Using transmission electron microscopy, we were able to observe flagella on the Salmonella cells harboring the vector pUHE21-2lacF (Fig. 5D), but not on cells carrying pJH1 grown in the presence of IPTG to induce LsrR overexpression (Fig. 5D).

We also compared the motility phenotypes of the wild-type Salmonella strains harboring pUHE21-2lacF and pJH1. We found that both strains were motile in the absence of IPTG; however, motility of the strain carrying pJH1 was abolished in the presence of IPTG (Fig. 5E). These results show that the overexpression of LsrR suppresses Salmonella motility.

LsrR-mediated QS Controls Expression of SPI-1 and Flagella Genes

As LsrR is a repressor of an operon comprising AI-2 transporter, overexpression of LsrR turns off the QS system of Salmonella and results in impaired invasiveness with reduced expression of SPI-1 and flagella as described above. Those effects may be associated with AI-2 signaling and QS or be an artifact resulting from ectopic expression of LsrR without any relevance to QS. We reasoned that we could distinguish between these two possibilities by modulating the levels of AI-2 in the overexpression strain because the phospho-AI-2 binds to LsrR, inactivating it [13]. If LsrR regulates SPI-1 and flagella genes in response to QS, addition of exogenous AI-2 should result in derepression of those genes. This experiment is complicated by the fact that the strain overexpressing LsrR cannot import and phosphorylate AI-2 due to repression of the csr operon and lsrK [25]. To circumvent this problem, the promoters of the csr operon and the lsrK gene were replaced with the promoter of the chloramphenicol resistance gene (Pat) which is constitutively expressed [26], and not repressed by LsrR (Fig. 6A).

To test whether LsrR regulates SPI-1 and flagella genes in response to QS, we examined the expression of chromosomal invF-lacZ and flbC-lacZ fusions in Pat-lsr strains (containing Pat-lsrA and Pat-lsrK) harboring pJH1 that were grown in the absence of IPTG. Values are means and standard deviations of three independent experiments. (A) Wild-type (WT) and mutant strains carrying flbC-lacZ fusion on chromosome were diluted in LB medium grown with shaking, and β-galactosidase activity (Miller units) was determined at 4 h. Production of LsrR was induced by addition of 100 μM IPTG. (B) Overnight cultures of the WT strain harboring either pUHE21-2lacF or the lasR+ plasmid pJH1 were grown in LB with shaking for 4 h. Production of LsrR was induced by addition of 100 μM IPTG. (C) Representative SDS-PAGE gel of secreted proteins. (D) Electron microscopic observation of flagella using negative stain. Samples were prepared from WT cells harboring either pUHE21-2lacF or pJH1 grown in LB with 100 μM IPTG. The scale bar indicates 0.5 μm. (E) Phenotypic assay for motility was performed to confirm the down-regulation of flagella genes in LsrR-overexpressing Salmonella cells. A 1 μl aliquot of washed WT cells harboring either pUHE21-2lacF or pJH1 was stab inoculated into 0.3% LB agar with or without 100 μM IPTG. The images were taken following 8 h of growth at 37 °C.

Figure 5. LsrR negatively controls the expression of flagella. (A) Wild-type (WT) and mutant strains carrying flbC-lacZ fusion on chromosome were stab inoculated into 0.3% LB agar with or without 100 μM IPTG, and β-galactosidase activity (Miller units) was determined at 4 h. Production of LsrR was induced by addition of 100 μM IPTG. (B) Overnight cultures of the WT strain carrying pJH1 was grown in LB or LB with 100 μM IPTG, with shaking for 4 h. The mRNA levels of flagella genes were determined by qRT-PCR. Values are means and standard deviations of three independent experiments. (C) Representative SDS-PAGE gel of secreted proteins. (D) Electron microscopic observation of flagella using negative stain. Samples were prepared from WT cells harboring either pUHE21-2lacF or pJH1 grown in LB with 100 μM IPTG. The scale bar indicates 0.5 μm. (E) Phenotypic assay for motility was performed to confirm the down-regulation of flagella genes in LsrR-overexpressing Salmonella cells. A 1 μl aliquot of washed WT cells harboring either pUHE21-2lacF or pJH1 was stab inoculated into 0.3% LB agar with or without 100 μM IPTG. The images were taken following 8 h of growth at 37 °C.
presence or absence of IPTG or exogenous AI-2. As expected, overexpression of LsrR reduced expression levels of the invF and fliC genes (Fig. 6B and C). Exogenous AI-2 restored expression of both the invF and fliC genes in dose dependent manner (Fig. 6B and C). In agreement with the transcriptional changes, the decreased production of flagella and the SopB protein observed upon LsrR overexpression was restored upon the addition of AI-2 (Fig. 6D and E). In WT with backbone plasmid, however, exogenous AI-2 with or without IPTG did not affect the expression of both invF and fliC genes (Fig. S3A and B) and the production of SopB protein (Fig. S3C).
We also asked whether addition of AI-2 to the *Pst-lsr* *Pst-lsrK* strain could restore the defective invasiveness resulting from LsrR overexpression. Overexpression of LsrR inhibited invasion of both the wild-type and the *Pst-lsr* strains into epithelial cells (17% and 25% invasion relative to the wild-type strain, respectively; Fig. 6F). However, addition of exogenous AI-2 restored the invasion phenotype of the *Pst-lsr* strain, but not the wild-type strain (Fig. 6F). Taken together, these findings suggest that the LsrR-mediated QS signaling system modulates expression of SPI-1 and flagella, and consequently invasiveness of *Salmonella* into host cells.

**Discussion**

QS systems have been proposed to play a role in virulence of several pathogens based on studies of luxS mutant phenotypes, including *Escherichia coli* O157:H7 [27,28], *Streptococcus pyogenes* [4,29], *Porphyromonas gingivalis* [30], *Neisseria meningitides* [31], and *Salmonella* Typhimurium [18]. However, because the luxS gene has also been proposed to have non-QS functions [22], it can be difficult to define a role for LuxS-mediated QS in bacterial pathogenesis. Indeed, although we have reported the role of LuxS/AI-2 QS in regulation of SPI-1 and flagella genes [30], we hypothesized that LsrR might connect LuxS/AI-2-mediated QS to SPI-1 regulation [19].

In *S. Typhimurium*, the regulatory network for AI-2 signaling is composed of an AI-2 transporter complex, LsrABCD, the LsrR regulator, and the LsrK signal kinase [13]. LsrR regulates AI-2 uptake by directly binding and repressing the promoter of the *lsr* operon [21]. Because LsrR is the only known transcription factor in *Salmonella*, we hypothesized that LsrR might connect LuxS/AI-2-mediated QS to SPI-1 regulation. Here, we report that the LsrR protein can negatively regulate expression of SPI-1 and flagella genes, and impair *Salmonella*'s invasion into mammalian cells.

Consistent with previous report [32], the deletion of *lsrR* gene in wild-type *Salmonella* did not alter *invF* expression. However, the deletion of *lsrR* in the *luxS* mutant strain restored the reduced expression of the *invF* and *flhC* genes in the *luxS* mutant strain to the wild-type levels (Fig. 2A and Fig. 3A). These results suggest that LsrR is involved in regulation of SPI-1 and flagella genes, in contrast to a recent proposal that the regulatory function of LsrR is limited to the *lsr* operon [32]. The *luxS* deletion mutant would produce constitutively active LsrR due to the lack of AI-2, which supports the idea that LsrR might link LuxS-mediated QS to SPI-1 regulation and allows us to reveal additional target of LsrR in *Salmonella* Typhimurium. Overexpression of LsrR in wild-type *Salmonella* reduced both the mRNA and protein levels of SPI-1 (Fig. 3) and flagella genes (Fig. 5A, B, and C). These results emphasize that LuxS-mediated QS controls SPI-1 and flagella expression with AI-2-regulated LsrR activity playing a pivotal role.

We tested whether LsrR might regulate expression of SPI-1 and flagella genes by directly binding to their promoters. However, electrophoretic mobility shift assays failed to reveal LsrR protein binding to DNA fragments carrying promoters of SPI-1 and flagella genes such as *invF*, *supR*, *flhDC*, and *flhC*, while LsrR did bind to the promoter DNA of *lsr* operon (data not shown). It is possible that additional factor(s) may be required for LsrR binding to the target promoter or that LsrR works indirectly. Although we do not understand the molecular basis of how the LsrR protein negatively controls transcription of SPI-1 and flagella genes, our findings clearly indicate that LsrR has regulatory targets than the *lsr* operon in *Salmonella*, as reported in *Escherichia coli* [23].

Overproduction of LsrR from a plasmid reduced entry into epithelial cells (Fig. 4), perhaps by generating increased levels of the active form of LsrR (i.e., LsrR not associated with phospho-AI-2). This result is consistent with our previous report that the *luxS* mutant was defective for invasion [10]. Moreover, decreased expression of flagella genes in bacterial cells possessing active form of LsrR has also been reported in a *E. coli* *luxS* mutant [7]. It is still possible that the altered expression of SPI-1 and flagella genes and reduced invasiveness resulting from LsrR overexpression might be artifacts unrelated with QS. However, that possibility is made less likely by the finding that treatment with exogenous AI-2 restored the behaviors of the *LsrR* overexpressing *Salmonella* (Fig. 6). Overall, the data are consistent with a role for LsrR in regulating SPI-1 and flagella in response to QS.

The LsrR regulator represses expression of the *lsr* operon and the *lsrk* gene [25]. Consistent with this, exogenous AI-2 only counteracted the effects of LsrR overexpression in the engineered *Salmonella* strain (*Pst-lsr*) in which the promoters of the *lsr* operon and *lsrk* were replaced with the constitutive *Pst* promoter. To further verify whether the regulatory functions LsrR on SPI-1 and flagella genes are controlled by phospho-AI-2, we constructed a *Salmonella* strain (*Pst-lsrK*) carrying only *Pst-lsr* and not *Pst-lsrK* (Fig. S4A). We reasoned that the *Pst-lsr* strain could uptake AI-2 but not phosphorylate it due to LsrR-mediated repression of the *lsrk* gene which encodes the AI-2 kinase [1,25]. The *Pst-lsr* strain failed to restore expression of the *invF* and *flhC* genes upon the addition of AI-2 (Fig. S4B), indicating that LsrK is necessary for the LsrR protein to respond to AI-2 and control expression of SPI-1 and flagella (see Fig. S1).

When ingested by a host animal, *Salmonella* reach the small intestine and penetrate the epithelial cells. LuxS is a highly conserved protein, and many species of bacteria produce AI-2 [5,11]. Based on our findings, we propose that *Salmonella* could recognize the site of infection by detecting and internalizing the AI-2 signaling molecules produced by microorganisms in the normal gut flora in addition to recognizing well-known environmental signals such as high osmolarity to induce the expression of SPI-1 [33]. Moreover, studies of the known QS signaling mechanisms employing AI-2 have revealed that most QS bacteria are sensing the extracellular AI-2 rather than importing it [9,11]. Thus, AI-2 molecules produced by normal flora would accumulate in the intestinal tract. The ability of *Salmonella* to consume AI-2 molecules could be beneficial for their virulence because the consumption of AI-2 could interfere with communication between other bacterial species and perhaps disrupt the flora in a way that is favorable for *Salmonella* pathogenicity.

In summary, we have presented evidence that active LsrR can negatively regulate expression of SPI-1 as well as flagella genes, and in doing so, reduce the ability of *Salmonella* to invade host cells. The effects of LsrR overexpression were relieved by exogenous treatment with the QS signal molecule, AI-2. These findings suggest that *S. Typhimurium* may require QS for successful invasion when they have reached the proper environment. QS may also enable *Salmonella* cells to modulate expression of virulence factors when they reached a sufficient population level in the right niche.

**Materials and Methods**

**Ethics Statement**

This study was carried out according to the recommended protocol for the care and use of laboratory animals from the Institute of Laboratory Animal Resource in Seoul National University, which is based on the Korean Animal Protection Law and Korea Food and Drug Administration regulation on the laboratory animals. The protocol was approved by the Committee.
Bacterial Strains, Plasmids and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Salmonella enterica* serovar Typhimurium strains used in this study are derived from strain SL1344. Phage P22-mediated transductions were performed as described [34,35]. All *Salmonella* strains were grown with (aerobically) or without shaking (anaerobically) at 37°C in Luria-Bertani broth (LB). For shaking cultures, 1.5 ml of media for glass tubes or 50 ml of media for flask tubes were used. For static cultures, 50 ml of media in sealed flasks were used. Antibiotics were used in the following concentrations: ampicillin 50 µg/ml, chloramphenicol 25 µg/ml, kanamycin 50 µg/ml.

Construction of Strains

The method of Datsenko and Wanner [36] was used for chromosomal gene deletion, chromosomal lacZ fusion, and epitope tagging. For construction of a *Salmonella* strain carrying a chromosomal flIC-lacZ fusion, the KanR cassette from plasmid pKD13 was amplified using primers flIC-lacZ-F and flIC-lacZ-R (see Table 2 for primer sequences) and the resulting polymerase chain reaction (PCR) products were introduced into the SL1344 strain containing the plasmid pKD46, followed by selection for flIC::kan transconjugants. The KanR cassette was removed using plasmid pCP20 [36] leaving an FRT site for introduction of lacZ*Y* using pCE70 [37]. Other chromosomal lacZ*fusions were constructed following these procedures, and deletion mutants or epitope tagging mutants were constructed similarly including removal of the KanR cassette.

Construction of Plasmids

An invF*+* plasmid for complementation was constructed as follows. The invF gene from SL1344 was amplified by PCR using primers invF-F2 and invF-R2, and the resulting product was cloned between HindIII and SphI sites of vector pACYC184 [38] to generate plasmid pJH1. To construct the lacZ*Y* fusion, the lacR gene from SL1344 was amplified by PCR using primers lacR-pF1 and lacR-pR1, and the resulting product was cloned between EcoRI and BamHI sites of vector pUHE21-2lacY* [39]. Primers used for plasmid construction are listed in Table 2.

β-galactosidase Assay

β-galactosidase assays were carried out in triplicate and enzyme activity (Miller units) was determined as described [40].

Quantitative Real-time Reverse Transcription (RT)-PCR Analysis

*Salmonella* strains were grown in LB media aerobically or anaerobically to desired growth phase, and total RNA was isolated using RNaesy Mini Kit (Qiagen). After DNase treatment of the RNA solution, cDNA was synthesized using Omnitranscript Reverse Transcription reagents (Qiagen) and random hexamers. Quantification of cDNA was carried out using 2×iQ SYBR Green Supermix (Bio-Rad Laboratories), and real-time amplification of PCR products was performed using the iCycler iQ real-time detection system (Bio-Rad Laboratories). The relative amount of cDNA was calculated using a standard curve obtained from PCR using serially diluted genomic DNA as templates. The mRNA expression level of the target gene was normalized to the 16S rRNA expression level. Finally the values were normalized by those of wild-type. The sequences of the primers used are presented in Table 2.

Secreted Protein Analysis by Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

The proteins secreted into media were analyzed by SDS-PAGE. Bacterial overnight cultures were diluted (1:100) in fresh LB and incubated aerobically or anaerobically. Cell-free spent media was obtained by centrifuging bacterial cultures (13,200 ×

### Table 1. Bacterial strains and plasmids.

| Strain | Genotype | Source |
|--------|----------|--------|
| *Salmonella typhimurium* strains |
| SL1344 | wild-type, SmR |
| SR3306 | ΔluxS |
| SR3326 | ΔluxS, PjJ2 |
| SR4506 | ΔluxS, PjJ13 |
| SR4507 | ΔluxSΔinvF |
| SR4508 | ΔluxSΔinvF, PjJ2 |
| SR4511 | ΔluxSΔinvF, PjJ13 |
| SR4558 | SL1344, pUHE21-2lacY* |
| SR4559 | SL1344, pJH1 |
| SR4656 | Pcat-lsrK, Pcat-lsrA, ΔlsrR, pJH1 |
| SR3390 | Pocc-lacZ |
| SR3391 | Pocc-lacZ, ΔlsrR |
| SR3392 | Pocc-lacZ, ΔluxS |
| SR3393 | Pocc-lacZ, ΔluxS ΔlsrR |
| SR3394 | Pocc-lacZ, pUHE21-2lacY* |
| SR3395 | Pocc-lacZ, pJH1 |
| SR4668 | Pocc-lacZ, Pcat-lsrK, Pcat-lsrA, ΔlsrR, pJH1 |
| SR4669 | Pocc-lacZ, Pcat-lsrA, ΔlsrR, pJH1 |
| SR4550 | Pocc-lacZ |
| SR4551 | Pocc-lacZ, ΔluxR |
| SR4575 | Pocc-lacZ, ΔluxS |
| SR4610 | Pocc-lacZ, ΔluxS ΔluxR |
| SR4576 | Pocc-lacZ, pJH1 |
| SR4652 | Pocc-lacZ, Pcat-lsrA, ΔlsrR, pJH1 |
| SR4553 | Pocc-lacZ, Pcat-lsrK, Pcat-lsrA, ΔlsrR, pJH1 |
| SR3598 | sopB-HA, pUHE21-2lacY* |
| SR3599 | sopB- HA, pJH1 |
| SR4658 | sopB- HA, Pcat-lsrK, Pcat-lsrA, ΔlsrR, pJH1 |
| Plasmids |
| pKD46 | ApR pocc-gam-beta-exo oriT101 repA101 |
| pKD3 | ApR FRT CmR FRT PS1 PS4 oriR6K |
| pKD13 | ApR FRT KmR FRT PS1 PS4 oriR6K |
| pCP20 | ApR CmR cES7 tPs rol oriPSCI01 |
| pCE70 | ApR FRT trnp lacZ*Y* oriR6K |
| pACYC184 | TetR CmR p15A ori |
| pUHE21-2lacY* | repA101 ApR lacY* |
| pJ2 | pACYC184- ΔluxS CmR |
| pJ13 | pACYC184- ΔinvF CmR |
| pJH1 | pUHE21-2lacY*ΔlsrK ApR |

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for 20 min), collecting the supernatant, and then removing bacterial cells from the supernatant by filtration (0.22-μm pore size, Millipore). Cell-free spent media were mixed with prechilled trichloroacetic acid (TCA) to final concentration of 10%, chilled on ice for 2 h, and then centrifuged at 15,000 g for 15 min. The pellets were washed with acetone twice to remove all the TCA from the precipitates. After air drying, the pellets were dissolved in SDS sample buffer [41], and SDS-PAGE was performed using a 15% acrylamide gels from a mini-gel kit (Bio-Rad Laboratories, Inc). Proteins were visualized by staining with colloidal blue.

Transmission Electron Microscopic Analysis

Bacterial cells grown in LB for 16 h at 37°C without shaking were deposited on carbon-film grids, partially dried, and stained with 2.0% uranyl acetate. The negatively stained samples were observed using a 2000EX transmission electron microscope (JEOL, Ltd) at an acceleration voltage of 100 kV.

Motility Assay

Bacterial motility was measured as described previously [42]. Briefly, 0.3% LB agar plates were stab inoculated with 1 μl of a washed overnight culture. The plates were incubated upright at 37°C, and the diameter of bacterial growth was measured every

| Table 2. Primers for construction of strains and plasmids, and qRT-PCR. |
|-----------------------------------------------|
| Primer name | Oligonucleotide (5’→3’) |
|-----------------------------------------------|
| **for bacterial strain construction**          |
| invF-F  | CTG TTA CAA AAA AGC GAG AGT TAC TGG TTA GGT GCC TAG TTG TAG GCT GGA GCT GCT TCG |
| invF-R  | AAA CGC CAT AGT CTT CTC CCA GCA TTA GCG GGA AAA TGA TGG GTG CAG TTA GAT CCG CAT GGC ACC |
| invF-lacZ-F | TCG CGG CCG AAA TTA TCA AAT ATT ATT CAA TTG GCA GAC AAA TGA TGG AGG CTT GAG CTG CTT CG |
| invF-lacZ-R | CGG GGC ACA TGC CAG CAC TCT GGC CAA AAG AAT ATG CTG CT AT TCC GAG GGT CCG TGC ACC |
| sopB-HA-F | GCA GTC AGG AAA AGG CAT TTC TTC ATT AAT CAC ATC TTA TCC GTA TGA TGC TGA TTA TGA ACC TAT CTA CTA ATG ATG TAG GCT GGA GCT GCT TCG |
| sopB-HA-R | TAA ACG ATT TAA TAG ACT TTC CAT ATA ACC TCA AGA ATC CAT TCC GGA GAT CCG TGC ACC |
| flic-lacZ-F | AGC CCA ATA ACA TCA AGT TGT AAT TGA TAA GGA AAA GAT CTG TAG GCT GGA GCT GCT TCG |
| flic-lacZ-R | CCG CAC CCA GGT CAG AAT GTC CTA CTC CCA CAA CCC TGC TCC GAG GAT CCG TGC ACC |
| Pcat-IsrR-F | GGG TAC AGA GTC GAG CCA TTT TTT TAT CCT CCG CTA TTT TCC CCG TAG TGA TAT TTT CTT TCA ATT GAT GTG AAA GAT GGA ACC TCT TAC TGT CCA GTA TAT GAA TAT CCT CTC TAC TAG TCC |
| Del _IsrR-R | CGA GCC GGG CCG GCA TAA TAC TCA CGC TAG CAT ATG AAT ATC CTC TCA AGT TCC |
| Pcat-IsrA-R | TAT TGT GAC TTA TTT CTA GTC TGC TTC CCG CTC CTC CTA ATT GGC CCG TAG TGA TCT CTT TAT TCC ATG GTG AAA GAT GGA ACC TCT TAC TGT CCA GG TGA GGC ACC GGC TAC GGC TGC TAG TCC |
| **for plasmid construction**                    |
| invF-F2 | GAG GGC CCA AGT TTA TAC ACA |
| invF-R2 | AAT GTC CCG ATG CTA TCG TCT |
| lsrR-pF1 | GTA AAG CCA GAA TTC GAC AAT GAG |
| lsrR-pR1 | CGT TAC ATG GCA GTC TGT CAG TTA |
| **for real-time RT PCR**                        |
| RT-sicA-F1 | ATTTGGGATGCCGTTAGTGAAG |
| RT-sicA-R1 | TAAACCGTCACTCATCATGAGG |
| RT-sopB-F1 | AACCGTTCCGGGTAACAAAGAC |
| RT-sopB-R1 | GGTCCGCTTTAACCTTGCTAAC |
| RT-sopE-F1 | CAAACACTTCCGGAGAGGAG |
| RT-sopE-R1 | GGTCTGCGTCCGTGATG |
| RT-invF-F1 | GCAGGATTAGTGGACACGAC |
| RT-invF-R1 | TTTACGATCTGCAAATAAGG |
| RT-flhD-F1 | CAACGGAAGAGTGGAAAACA |
| RT-flhD-R1 | GACGCGTGAAAGCATGATA |
| RT-flA-F1 | CCGCTGAAGGTGATAGGAGAT |
| RT-flA-R1 | CCGCATTATAAACCGGAG |
| RT-flic-F1 | AACGACCGTATCTCCATTGC |
| RT-flic-R1 | TACACGGTGATTTCTCTCA |
| RT-msh-F1 | CGGGGAGAGAGGTGTTTG |
| RT-msh-R1 | GACGCGCGGGAGATTCCAATC |

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hour. Where indicated, isopropyl-β-D-thiogalactoside (IPTG) was added to the LB agar at 100 μM.

Western Blot Analysis

Salmonella strains expressing a HA epitope tagged version of SopB (SopB-HA) from the chromosome were grown in LB broth at 37°C. Bacteria were collected by centrifugation, and cell lysates were prepared using B-PER solution (Pierce). Cell lysates were resolved in 12% SDS-PAGE and the SopB protein was detected using anti-HA antibody (Sigma). Western blot was developed using anti-mouse immunoglobulin G horseradish peroxidase-linked antibody and the enhanced chemiluminescence detection system (Amersham Biosciences).

Invasion Assay

HEp-2 cells (ATCC, CCL-23) were grown in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50 μg/ml). Confluent monolayers for infection with bacteria were prepared in 24-well tissue culture plates. 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% CO₂. The wells were washed three times with phosphate-buffered saline (PBS) before bacterial cells were added. Bacterial cells were washed with PBS, suspended in pre-warmed DMEM medium, and then added onto the monolayer at a multiplicity of infection of 10:1. After 1 h of incubation, the wells were washed three times with pre-warmed PBS to remove extracellular bacteria and then incubated for 1 h with pre-warmed medium supplemented with 100 μg/ml of gentamicin (Gm) to kill extracellular bacteria. The wells were then washed three times with PBS to remove the Gm. The HEp-2 cells were lysed in 1% Triton X-100 for 30 min, and then diluted with PBS. Dilutions of the lysed cell suspension were plated on LB agar at 100 μM. Water and food were withdrawn 4 h before infection, and re-supplied 2 h post-infection. Mouse mortality was recorded daily.

Animal Experiments

The experiment was conducted as described previously [43,44]. Six-week-old female BALB/c mice were purchased from the Institute of Laboratory Animal Resources at Seoul National University. The mice were kept under pathogen-free conditions in filter-topped cages in Individually Ventilated Cage Racks (MSRS-M70S; Orient Bio Inc.) containing sterile bedding, and were fed sterile food and water ad libitum. All mice were acclimatized for at least 1 week prior to experimentation. Cohorts of ten mice were infected by oral gavage or intraperitoneally with about 10^7 CFU or 10^5 CFU of Salmonella cells in 100 μL of PBS, respectively. Water and food were withdrawn 4 h before infection, and re-supplied 2 h post-infection. Mouse mortality was recorded daily.

Supporting Information

Figure S1 Schematic diagram of LsrR-mediated QS regulatory circuit in Salmonella typhimurium. AI-2 is synthesized by LuxS and accumulates extracellularly. Lsr transporter, encoded by lsr operon, internalizes the AI-2 into the cytoplasm, where it is phosphorylated to produce phospho-AI-2 by LsrK. LsrR represses the expression of lsr operon in the absence of phospho-AI-2, while it is de-repressed in the presence of phospho-AI-2 molecules that bind and inactivate LsrR [13]. In this study, we demonstrated that LsrR negatively controls the expression of SPI-1 and flagella genes and this regulation was abolished by phospho-AI-2. (TIF)

Figure S2 LsrR is required for the regulation of invF expression by LuxS. Wild-type (WT) and other mutant strains carrying invF-lacZ fusion on chromosome were diluted in LB medium and grown with shaking, and β-galactosidase activity (Miller units) was determined at 4 h. If necessary, lsrR expression was modulated by adding IPTG as the indicated concentrations. Values shown are the means and standard deviation of three independent experiments. (TIF)

Figure S3 Treatment of AI-2 molecule or IPTG does not affect the expression of SPI-1 and flagella genes. (A and B) The wild-type (WT) strains harboring backbone plasmid (pUHE) carrying a chromosomal invF-lacZ (A) or fliC-lacZ (B) transcriptional fusion were grown in LB for 4 h with shaking. IPTG or AI-2 was added at final concentrations of 100 μM and 144 μM, respectively. (C) Western blot analysis was conducted with cell extracts prepared from wild-type (WT) strains harboring pUHE grown in LB or LB containing 100 μM IPTG and/or 144 μM DPD, with shaking for 4 h. These strains express the SopB protein tagged with a HA-epitope (SopB-HA) from the normal chromosomal location. (TIF)

Figure S4 LsrR is required to inhibit the regulatory function of LsrK. (A) Schematic of the genomic context of an engineered Salmonella strain (Patt-lsrK). The constitutively expressed promoter, promoter of chloramphenicol resistant gene (Pcat) was used to substitute the original promoters of lsrK. (B and C) The engineered strains (Patt-lsrK) carrying a chromosomal invF-lacZ (B) or fliC-lacZ (C) transcriptional fusion harboring pJH1 were grown in LB for 4 h with shaking. To induce the production of LsrK from the lac-promoter, 100 μM of IPTG was supplemented to LB. If necessary, the signal molecule, AI-2 (DPD), was added at the final concentrations of 48 and 144 μM. (TIF)

Author Contributions

Conceived and designed the experiments: JC SR. Performed the experiments: JC MK JP. Analyzed the data: JC DS SL SR. Contributed reagents/materials/analysis tools: DS SL. Wrote the paper: JC DS SR.

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