

LuxS-Based Quorum Sensing Does Not Affect the Ability of *Salmonella enterica* Serovar Typhimurium To Express the SPI-1 Type 3 Secretion System, Induce Membrane Ruffles, or Invade Epithelial Cells[∇]

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Bacterial species can communicate by producing and sensing small autoinducer molecules by a process known as quorum sensing. *Salmonella enterica* produces autoinducer 2 (AI-2) via the *luxS* synthase gene, which is used by some bacterial pathogens to coordinate virulence gene expression with population density. We investigated whether the *luxS* gene might affect the ability of *Salmonella enterica* serovar Typhimurium to invade epithelial cells. No differences were found between the wild-type strain of *S. Typhimurium*, SL1344, and its isogenic *luxS* mutant with respect to the number and morphology of the membrane ruffles induced or their ability to invade epithelial cells. The dynamics of the ruffling process were also similar in the wild-type strain (SL1344) and the *luxS* mutant. Furthermore, comparing the *Salmonella* pathogenicity island 1 (SPI-1) type 3 secretion profiles of wild-type SL1344 and the *luxS* mutant by Western blotting and measuring the expression of a single-copy green fluorescent protein fusion to the *prgH* (an essential SPI-1 gene) promoter indicated that SPI-1 expression and activity are similar in the wild-type SL1344 and *luxS* mutant. Genetic deletion of *luxS* did not alter the virulence of *S. Typhimurium* in the mouse model, and therefore, it appears that *luxS* does not play a significant role in regulating invasion of *Salmonella* in vitro or in vivo.

Quorum sensing is an intercellular signaling mechanism used by bacteria to regulate gene expression in response to changes in cell population density (30). Quorum sensing relies on the production and secretion of a small signaling molecule during growth, so that as the size of a bacterial population grows, there is a corresponding increase in the extracellular concentration of the autoinducer. Once the population has reached a certain cell density, a critical threshold concentration of the autoinducer will be exceeded and a response will be triggered, typically leading to changes in gene transcription and ultimately, modulation of population behavior. This response is usually one that is productive only when it is carried out simultaneously by many cells and includes responses such as bioluminescence, antibiotic production, production of virulence factors, and biofilm formation (8, 11, 19, 29). By coordinating the gene expression of a bacterial community, quorum sensing enables bacteria to behave like multicellular organisms (24).

A diverse range of bacterial quorum-sensing signaling molecules have been identified. Many gram-negative species are known to produce *N*-acyl homoserine lactones. In many cases, these are generated by LuxI homologues and sensed by transcriptional regulators of the LuxR family (3). In contrast, most gram-positive bacteria use either linear or cyclic peptides which are generated from short precursor proteins (16). The only system currently known to exist in both gram-negative and gram-positive bacteria relies upon the production of a signal molecule termed autoinducer 2 (AI-2) by an AI synthase, encoded by the *luxS* gene (26). Since the *luxS* gene has been found in over 55 species of bacteria (32), it has been suggested that AI-2 may be used for interspecies signaling, rather than the intraspecies communication associated with other quorum-sensing signaling molecules. Hence, AI-2 may enable differential gene expression depending on whether bacteria exist in pure culture or in a consortium (2).

The *luxS* gene has been found in *Salmonella enterica* serovar Typhimurium (26), with the AI-2 signal being identified as 2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (20). This is a derivative of 4,5-dihydroxy-2,3-pentanedione (DPD), a by-product of the activated methyl cycle in which LuxS plays a role (31). An initial screen for genes regulated by *luxS* in *Salmonella enterica* serovar Typhimurium identified those of the *luxS*-reg-

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∇ Published ahead of print on 25 September 2009.

TABLE 1. *S. enterica* serovar Typhimurium strains used in this study

Strain	Description	Reference
SL1344	Wild type (our culture collection)	14
SL1344LS	SL1344 <i>luxS</i>	15
SL1344LS(pBR <i>luxS</i>)	SL1344 <i>luxS</i> with pBR <i>luxS</i>	15
JH3010	SL1344 with transcriptional gene fusion <i>prgH::gfp</i> ⁺	13
SL1344LS <i>prgH::gfp</i> ⁺	SL1344 <i>luxS</i> with <i>prgH::gfp</i> ⁺	This study
SL1344LS <i>prgH::gfp</i> ⁺ (pBR <i>luxS</i>)	SL1344 <i>luxS</i> with <i>prgH::gfp</i> ⁺ with pBR <i>luxS</i>	This study
JH3016	SL1344 with transcriptional gene fusion <i>rpsM::gfp</i> ⁺	13
SL1344LS <i>rpsM::gfp</i> ⁺	SL1344 <i>luxS</i> with <i>rpsM::gfp</i> ⁺	This study
SL1344LS <i>rpsM::gfp</i> ⁺ (pBR <i>luxS</i>)	SL1344 <i>luxS</i> with <i>rpsM::gfp</i> ⁺ with pBR <i>luxS</i>	This study
SL1344 (Choi et al.)	Wild type (culture collection of Ryu group)	4
SR3306	SL1344 <i>luxS</i>	4
SH008	SL1344 (Choi et al.) with transcriptional gene fusion <i>prgH::gfp</i> ⁺	This study
SH009	SR3306 (<i>luxS</i> mutant) with transcriptional gene fusion <i>prgH::gfp</i> ⁺	This study

ulated (*lsr*) operon (28). The *lsr* operon encodes a transporter for AI-2, homologous to the ribose ABC transporter, and enzymes that modify this signal upon its entrance into cells (27, 28, 33). The Lsr receptor/transporter complex has subsequently been found to predominate in pathogenic bacteria associated with endotherms (23), indicating that quorum sensing may play an important role in the interaction of such bacteria with their hosts.

The virulence of *Salmonella enterica* depends upon their abilities to enter and survive in host cells, and genes involved with these processes are tightly controlled, often in relation to environmental cues (10). Quorum sensing could potentially act as one such cue. The purpose of this study was to examine whether *Salmonella enterica* serovar Typhimurium may use quorum sensing to regulate virulence, specifically examining the effect of deletion of *luxS* on the ability of *S. Typhimurium* to induce membrane ruffles and invade epithelial cells. We found no evidence for a major role for LuxS in *Salmonella* invasion or in regulation of *Salmonella* pathogenicity island 1 (SPI-1) expression or secretion. Significantly, *luxS* had no significant impact on the virulence of the strain in vivo. These data contrast with a recent report that the *luxS* gene is required for expression of a subset of genes in SPI-1 and to optimize invasion (4).

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. A complete deletion of *luxS* from the start codon to the stop codon was constructed in *Salmonella enterica* serovar Typhimurium SL1344 using the lambda Red system (7) to generate strain SL1344LS. The deletion was confirmed by PCR, Western blotting with LuxS antibodies, and AI-2 detection using the well-established *Vibrio* bioluminescence reporter bioassay (25). Strain SL1344LS was complemented using the plasmid pBR*luxS* (15). SL1344 derivative strains JH3010 and JH3016 with chromosomal integration of single-copy fusions of *prgH::gfp*⁺ or *rpsM::gfp*⁺ (13) were kindly provided by I. Hautefort, together with plasmids pZEP10 and pZEP16 (13) that were used to make equivalent transcriptional gene fusions in strain SL1344LS using the lambda Red system. Strains SH008 and SH009 were constructed by P22 transduction of the *prgH::gfp*⁺ reporter construct into strains SL1344 and SR3306, respectively (both strains kindly provided by S. Ryu) (4). Colonies were screened by PCR for correct insertions, and the abilities of the colonies to induce ruffling and invade epithelial cells were checked and were comparable to the abilities of their isogenic parent. For standard invasion and green fluorescent protein (GFP) expression assays, strains were grown overnight at 37°C as a static culture in LB (Miller) broth supplemented with antibiotics as appropriate at the following concentrations: carbenicillin, 100 µg/ml; chloramphenicol, 15 µg/ml. Cultures were diluted 1:100 into LB (plus antibiotics) and grown for 3.5 h prior to

infection at 37°C in an orbital shaker. To examine the effect of anaerobic growth conditions, an overnight static culture was diluted 1:100 into LB and grown statically in a sealed 2-liter gas jar made anaerobic with an AnaeroGen pack (Oxoid, Basingstoke, United Kingdom) at 37°C for 20 h.

Infection of cultured cells. For experiments in which epithelial cells were infected with *S. Typhimurium* and subsequently fixed and stained, Madin-Darby canine kidney (MDCK) strain I and HeLa cells were seeded on 13-mm coverslips (2.5×10^4 cells per coverslip) and maintained at 37°C in a humidified atmosphere of 5% CO₂ for 3 days. Prior to infection, the medium was replaced with a modified Krebs' buffer (137 mM NaCl, 5.4 mM KCl, 1 mM MgSO₄, 0.3 mM KH₂PO₄, 0.3 mM NaH₂PO₄, 2.4 mM CaCl₂, 10 mM glucose, and 10 mM Tris, adjusted to pH 7.4 at 37°C with HCl). After equilibration of the cells in this medium at 37°C in air, *S. Typhimurium* was added at a multiplicity of infection (MOI) of approximately 50 to 100 for 15 min for standard invasion assays. Increased infection times and/or decreased MOI were also examined to confirm data obtained under standard infection assay conditions.

Quantification of invasion. *S. Typhimurium* invasion was quantified using differential immunofluorescence staining by the method of Perrett and Jepson (21). Coverslips were examined using a Leica DM LB2 upright microscope (Leica Microsystems, Mannheim, Germany). Quantification of MDCK cells (labeled with 4',6'-diamidino-2-phenylindole [DAPI]), adherent bacteria (labeled with fluorescein isothiocyanate), and total bacteria (labeled with tetramethyl rhodamine isothiocyanate [TRITC]) in 10 randomly selected areas of each coverslip was performed to calculate the number of invading bacteria per cell. The identity of the strains was hidden from the person assessing invasion to eliminate any possible bias. The total number of cells counted per coverslip was 250 or greater.

Analysis of membrane ruffling by cytochemical staining of F-actin. After the cells were infected with *S. Typhimurium* for the required time, the coverslips were washed thoroughly in phosphate-buffered saline (PBS), fixed in 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and sequentially incubated with goat anti-*Salmonella* CSA-1 antibody and fluorescein isothiocyanate-conjugated rabbit anti-goat immunoglobulin alongside TRITC-phalloidin, as described previously (21). The coverslips were then washed thoroughly in PBS and mounted in Vectashield containing DAPI and examined using a Leica DM LB2 upright microscope to assess the frequency and morphology of *Salmonella*-induced membrane ruffles in 10 randomly selected fields per coverslip. The identity of the strains was hidden from the person quantifying ruffling and assessing morphology to eliminate any possible bias. The total number of cells counted per coverslip was 250 or greater.

Phase-contrast time-lapse microscopy. MDCK strain I cells were seeded on 22-mm coverslips (2.5×10^4 cells per coverslip) and maintained at 37°C in a humidified atmosphere of 5% CO₂. Three to 4 days after seeding, the coverslip was washed twice with warm (37°C) modified Krebs' buffer and mounted in a coverslip holder with 1 ml of modified Krebs' buffer. The coverslip holder was then placed on the stage of a Leica DM IRB inverted epifluorescence microscope enclosed within a stage incubator (Solent Scientific) at 37°C. *S. Typhimurium* grown for 3.5 h in LB Miller was added to the chamber of the coverslip holder at an MOI of approximately 50. Phase-contrast images were obtained with a 40× oil immersion lens (numerical aperture of 1.0). Improvisation Openlab 4 software was used to automate the acquisition of images at each of three focal depths (1.5-µm steps) at 10-s intervals over a 20-min time course with a Hamamatsu ORCA ER cooled charge-coupled-device camera. Data on the time

interval between bacterial binding and induction of membrane ruffling were pooled from at least four experiments, which represents the study of 100 individual ruffles for each strain. All data are expressed as medians, with the range of values indicated. Significance of differences between median values was assessed using a Kruskal-Wallis test followed by Dunn's multiple-comparison test.

Analysis of T3S profiles. *Salmonella* bacteria were grown in LB broth overnight before being diluted 1:50 in fresh LB broth and grown under conditions that stimulate expression of the SPI-1 type III secretion system (9). The culture supernatants were collected and filter sterilized (pore size, 0.22 μ m), and proteins were precipitated with ammonium sulfate (4 g/10 ml of supernatant) overnight at 4°C. Precipitated secreted proteins were pelleted by centrifugation at 42,000 \times g and 4°C for 1 h. The secreted protein pellet was resuspended in 500 μ l of sterile distilled H₂O and stored at -20°C until required. Samples were electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to Protran nitrocellulose transfer membranes (Schleicher and Schuell) by using a wet transfer apparatus (Bio-Rad). Western blot analysis on type 3 secretion system (T3SS) components and effectors was performed using rabbit antibodies at a dilution of 1:2,000 coupled with a goat anti-rabbit horseradish peroxidase-labeled secondary antibody (Dako Cytomation). Detection was carried out by using 4-chloro-1-naphthol (Sigma) according to the manufacturer's instructions.

Flow cytometry. For measurement of GFP in *S. Typhimurium*, samples were taken after 3.5 h of growth in LB at 37°C and fixed in 2% paraformaldehyde for 15 min at 4°C. Fixed bacteria were subsequently washed and diluted in PBS to obtain a maximum of approximately 10⁷ particles per ml. Samples were kept in the dark at 4°C until analysis.

Flow cytometric analysis was performed on a FACSVantage SE flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with a 15-mW air-cooled argon ion laser as the excitation light source (488 nm). *S. Typhimurium* SL1344 without the single chromosomal *gfp*⁺ gene fusions was used as a negative control, and *rpsM::gfp*⁺ mutants, which constitutively express GFP, were used as a positive control. Approximately 50,000 events identified as *Salmonella* cells were collected per sample. GFP fluorescence intensity values are presented as means for the populations.

Mouse infection. Bacterial strains were grown in 10 ml of LB at 37°C for 16 h with shaking (200 rpm). Female BALB/c mice (six per group) were challenged orally for each strain with 10⁵, 10⁶, or 10⁷ CFU of *S. Typhimurium* SL1344, the isogenic *luxS* strain (SL1344LS), or sterile PBS as a control. For each strain, the number of mice reaching the clinical end point after 12 days was noted. The experiment was repeated twice with similar results.

Statistical analysis. Data were analyzed using PRISM 3.0 software. Data are expressed as means \pm standard errors, with statistical significance set at a *P* of \leq 0.05. Data were compared using a one-way analysis of variance, with a Tukey test applied post hoc.

RESULTS

LuxS does not affect membrane ruffling or invasion of epithelial cell lines. A complete deletion of *luxS* was constructed in *Salmonella enterica* serovar Typhimurium SL1344 to generate SL1344LS. AI-2 secretion assays confirmed that the *luxS* mutant did not produce AI-2, while the wild-type strain (SL1344) and plasmid-complemented SL1344LS [SL1344LS (pBR*luxS*)] exhibited the previously reported AI-2 peak during log-phase growth (4, 26; data not shown). To elucidate whether LuxS has an effect on the virulence of *S. Typhimurium*, we compared the abilities of the wild-type SL1344 strain and its isogenic *luxS* mutant (SL1344LS) to invade and induce ruffling in two cell lines, MDCK and HeLa. The ability to produce membrane ruffles was assessed using TRITC-phalloidin staining of F-actin, while invasion was quantified using differential immunocytochemical staining of adhered and total cell-associated *S. Typhimurium*. Infection assays examined cultures in late log phase (3.5 h), when the AI-2 concentration in wild-type *S. Typhimurium* culture medium was maximal (data not shown).

The numbers of ruffles propagated by the wild-type strain (SL1344) and strain SL1344LS were similar after a 15-min

infection in both cell types (Fig. 1A and C). Similarly, the mean number of SL1344LS bacteria invading cells during a 15-min infection was not significantly different from those of the wild type and the complemented strain, SL1344LS(pBR*luxS*) (Fig. 1B and D). Increasing the infection time (to 60 min) or decreasing the MOI (to 2:1 or 20:1) confirmed that the numbers of invading bacteria of the wild-type and SL1344LS strains were comparable (data not shown). Following anaerobic growth, *Salmonella* invasion of MDCK cells was also unaffected by *luxS* deletion (data not shown). Measurements of invasion using polarized Caco-2 cells also showed no difference between the wild-type and SL1344LS strains (data not shown). Thus, LuxS does not affect entry of *S. Typhimurium* into epithelial cells under a range of growth conditions and infection models.

LuxS does not affect ruffle morphology or propagation dynamics. Despite the fact that there was no measured difference in the number of ruffles induced or invading bacteria, it remained possible that subtle differences in the induction and/or propagation of ruffles may exist between the wild-type and SL1344LS strains, as has been shown with other *Salmonella* mutants (22). Images of the ruffles propagated by strains SL1344, SL1344LS, and SL1344LS(pBR*luxS*), generated using confocal microscopy, were compared in each cell line, but no obvious differences between the morphology of the ruffles and the position of the bacteria relative to the ruffles were identified (data not shown). This observation was confirmed with time-lapse phase-contrast microscopy where the kinetics of ruffle induction were also measured (Table 2). Strain SL1344LS had a median time interval that was 10 s greater than those of both the wild type and SL1344LS(pBR*luxS*), a difference that was not statistically significant. Therefore, ruffle induction and propagation appear to occur in the same manner in strains SL1344 and SL1344LS.

Contribution of LuxS to SPI-1 type 3 secretion. Although no difference was observed in the invasion behavior of the *S. Typhimurium luxS* mutant, we decided to assess whether the expression of *Salmonella* pathogenicity island 1 may be different, which may indicate that LuxS does play a role in *S. Typhimurium* virulence. The secretion profiles of wild-type SL1344 and SL1344LS strains were compared by Western blotting using antibodies directed against both the SPI-1 type 3 secretion system apparatus proteins InvG, InvJ, PrgH, and also the effector proteins SipA, SipB, SipC, and SipD (12) (Fig. 2). The levels of SPI-1 apparatus and secreted effector proteins were comparable in strains SL1344 and SL1344LS, indicating that SPI-1 T3S protein expression and secretion are independent of *luxS*.

SPI-1 T3SS gene expression was also examined using single-copy *prgH::gfp*⁺ fusions inserted into the chromosomes of strains SL1344 (wild type), SL1344LS, and SL1344LS(pBR*luxS*). *prgH* encodes a structural component of the SPI-1 T3SS-1 apparatus (17) and has previously been used as a reliable measure of SPI-1 expression (5, 13). Strains without *gfp*⁺ fusions and with single-copy *rpsM::gfp*⁺ fusions were used as negative and positive controls, respectively. When the wild-type SL1344 strain and *luxS* mutant carrying a single copy of the *prgH::gfp*⁺ fusion in their chromosome were compared by flow cytometry at 3.5 and 6 h, a slight reduction was found in the proportion of the population expressing GFP, and therefore PrgH, in the *luxS*

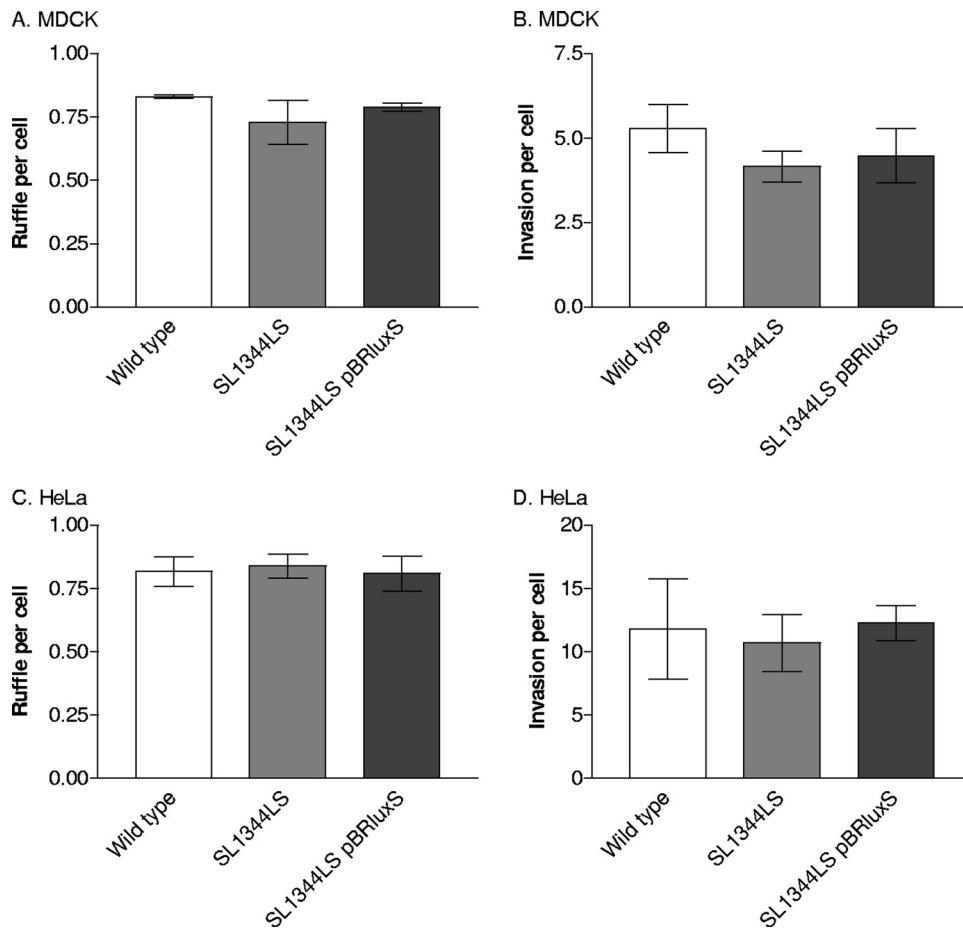


FIG. 1. Comparison of invasion and ruffle induction by *S. enterica* serovar Typhimurium strains SL1344 (wild type), SL1344LS, and SL1344LS(pBRluxS) in MDCK and HeLa cells infected for 15 min with each strain. Ruffle formation was measured using TRITC-phalloidin staining of F-actin (A and C), and the ability to enter cells was measured using differential staining of adhered and invaded bacteria (B and D). Values are the means of three independent experiments \pm standard errors of the means (error bars).

mutant, but this difference was not statistically significant (Fig. 3). The amount of PrgH expression per cell was also slightly lower in strain SL1344LS than in the wild type but again was not statistically different. The possibility that LuxS might influence the kinetics of *prgH* induction was explored further by quantifying the proportion of *S. Typhimurium* SL1344 *prgH::gfp*⁺ and SL1344LS *prgH::gfp*⁺ expressing GFP at hourly

intervals over a 6-h time course from overnight static culture through log to stationary growth phases. No difference was evident between the wild type and *luxS* mutant with respect to *prgH* expression at any point (data not shown), suggesting that

TABLE 2. Comparison of the time taken for membrane ruffle initiation after bacterial adherence by three *S. enterica* serovar Typhimurium strains^a

<i>S. Typhimurium</i> strain	<i>n</i> ^b	Ruffle induction time after adherence (s) ^c	
		Median	Range
SL1344	111	60	20–630
SL1344LS	119	70	20–490
SL1344LS(pBRluxS)	127	60	20–460

^a *S. enterica* serovar Typhimurium strains SL1344 (wild type), SL1344LS, and SL1344LS(pBRluxS) were grown for 3.5 h in LB.

^b The number of productive bacterium-cell interactions analyzed (*n*) is shown.

^c The time interval between bacterial binding to MDCK cells and induction of membrane ruffling was obtained from phase-contrast video microscopy. Data are expressed as median values, and the range in time intervals (minimum to maximum) was measured.

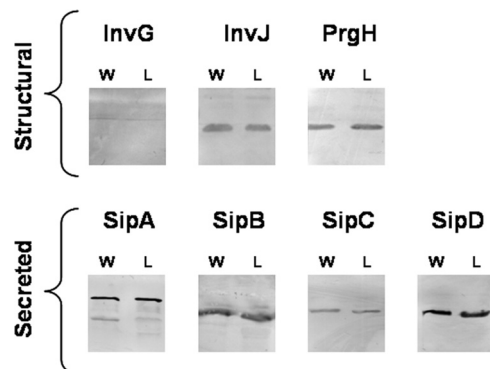


FIG. 2. SPI-1 secretion is not dependent upon *luxS*. Western blot analysis of whole-cell T3S structural proteins (InvG, InvJ, and PrgH) and extracellular secreted proteins (SipA to SipD) from the wild-type strain, SL1344 (W), and the isogenic *luxS* mutant, SL1344LS (L), shows no difference in the quantity of SPI-1 effectors secreted.

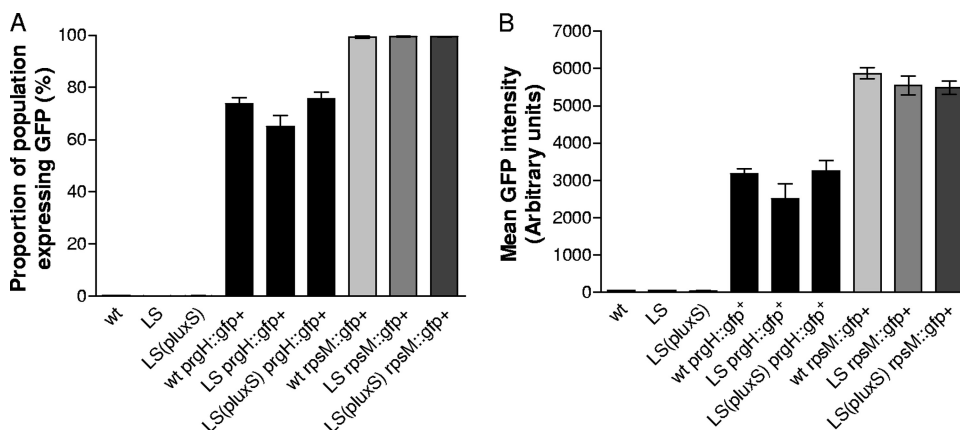


FIG. 3. Expression of SPI-1 in *S. enterica* serovar Typhimurium strains SL1344 (wild type), SL1344LS, and SL1344LS(pBR*luxS*) grown for 3.5 h in LB. Flow cytometry was used to measure the expression of GFP in the wild type (wt), *luxS* mutant (LS), and complemented mutant [LS(pluxS)] bacteria with no transcriptional *gfp*⁺ gene fusion, transcriptional gene fusion *rpsM*::*gfp*⁺ or transcriptional gene fusion *prgH*::*gfp*⁺. (A) Proportion of each bacterial population with detectable fluorescence in the GFP channel; (B) mean fluorescence intensity. Data represent means ± standard errors of the means (error bars) from three independent experiments. Of the population with no transcriptional *gfp*⁺ gene fusion, ≤0.3% have detectable fluorescence in the GFP channel (negative control), while approximately 99% of the *S. Typhimurium* cells containing the constitutive fusion (*rpsM*::*gfp*⁺) express GFP above the threshold intensity (positive control).

LuxS does not affect *prgH* expression kinetics. The similar levels of SPI-1 expression and protein secretion in the wild type and SL1344LS provide further support to our conclusion that *luxS* does not contribute to regulation of SPI-1 T3SS expression.

LuxS does not attenuate virulence in mice. To confirm our in vitro results, the contribution of *luxS* to virulence was tested in a mouse model. Female BALB/c mice were challenged orally with wild-type strain SL1344, SL1344LS, or PBS as a control. Table 3 shows no significant differences in the number of mice reaching the clinical end point 12 days after oral challenge with either the wild-type strain or *luxS* mutant. These results suggest that *luxS* has no significant impact on the pathogenicity of *Salmonella* in vivo.

DISCUSSION

The possession of the *luxS* gene, coding for autoinducer 2 synthase, by *Salmonella enterica* has led to the proposal that *Salmonella* may regulate gene expression in response to population size, i.e., quorum sense (25, 26). Initial screening of

TABLE 3. Virulence of *S. enterica* serovar Typhimurium in the mouse infection model^a

Strain or control	No. of mice (<i>n</i> = 6) that reached the clinical end point challenged with the following dose ^b :		
	10 ⁵ CFU	10 ⁶ CFU	10 ⁷ CFU
SL1344	5	6	6
SL1344LS	6	5	6
PBS (control)	0	0	0

^a Female BALB/c mice were challenged orally with 10⁵, 10⁶, or 10⁷ CFU of *S. Typhimurium* SL1344 (wild type), the isogenic *luxS* mutant SL1344LS, or sterile PBS as a control.

^b The number of mice that reached the clinical end point at 12 days postchallenge is shown. Mice that reached the clinical end point, i.e., the point of infection where severe symptoms appeared to be due to high bacterial burdens, were removed from the group. In the control group, all mice remained well when inoculated with PBS.

genes regulated by *luxS* and AI-2 in *S. enterica* serovar Typhimurium identified only those genes involved in the synthesis and detection of AI-2. These include *metE*, which encodes an enzyme with a role in the activated methyl cycle, from which AI-2 is a by-product, and those of the *luxS*-regulated (*lsr*) operon, which encode a transporter for AI-2 and enzymes which modify this signal upon its entrance into cells (28). Thus, it is not clear whether *luxS*-based quorum sensing per se regulates any biological activities in *Salmonella*, especially with the recent report that LuxS has been found to affect flagellar phase variation in *S. Typhimurium* independently of the AI-2 signal (15). However, one report has implicated *luxS* in the regulation of *S. Typhimurium* pathogenesis, with LuxS being necessary for optimal expression of a subset of type 3 secretion genes in SPI-1, under the control of InvF (4). We have used alternative methods to examine whether *S. Typhimurium* uses *luxS*-based quorum sensing to regulate its ability to invade host cells.

No differences were found between the wild-type SL1344 strain and its isogenic *luxS* mutant in the number of membrane ruffles propagated or the number of bacteria invading MDCK, HeLa, and Caco-2 cell lines. Visualization of the infection process in MDCK cells using phase-contrast time-lapse microscopy confirmed the results of the immunocytochemical staining assays, since no clear difference was found between the wild type (SL1344) and SL1344LS in the kinetics or morphology of membrane ruffles or in the positions of invading bacteria. Analysis of multiple host cell lines indicates that the similar behavior of the wild type and mutant in these assays is not cell line dependent. Similar to our in vitro observations, the *luxS* mutant was found not to differ from the wild type when comparing virulence via the oral route in the mouse model of infection. Thus, it appears that *luxS* is not essential for *S. Typhimurium* pathogenicity.

To complement the work examining the contribution of *luxS* to pathogenicity, the expression of SPI-1 T3SS in the wild type and the *luxS* deletion mutant were compared. SPI-1 encodes a type 3 secretion system (T3SS-1) and several effector proteins,

secreted through this macromolecular needle-complex, which are essential for invasion (6). Therefore, if *luxS*-based quorum sensing were to play a role in regulating *S. Typhimurium* invasion, a change in SPI-1 expression would be the expected mechanistic basis. In complete agreement with the results of the ruffling and invasion assays, the expression of the SPI-1 T3S apparatus proteins and the secreted effectors were found to be very similar in the wild type and SL1344LS. Choi et al. used real-time PCR to compare expression of SPI-1 genes in the wild type (SL1344) and *luxS* mutant grown under anaerobic conditions to stationary phase (4). Decreased expression of *invF* and several genes, *sicA*, *sigD*, and *sopE*, which are regulated by InvF, in the *luxS* mutant was reversed by introduction of the *luxS* gene in *trans* or by addition of 4,5-dihydroxy-2,3-pentanedione. DPD, a precursor of AI-2, or synthetic AI-2 is required to chemically complement the *luxS* gene in order to indicate that a phenotype is indeed regulated by quorum sensing and not related to the metabolic role of *luxS* (31). Choi et al. (4) also reported that, in contrast to our findings, *prgH* expression was approximately twofold lower in the *luxS* mutant than in the wild type. A smaller, and not statistically confirmed, reduction in *hilA* expression was also observed, suggesting that LuxS might regulate *prgH* via HilA, the predominant transcriptional activator of *prgH* expression (1). In contrast to their findings with *invF* and associated genes, expression of *prgH* and *hilA* in the *luxS* mutant was restored by pLuxS but not by DPD, indicating that any downregulation of *hilA* or *prgH* in the *luxS* mutant is independent of quorum sensing. Taken together with our findings that LuxS does not significantly affect *prgH* expression, SPI-1 type 3 secretion, invasion of a range of epithelial cell lines, and virulence in a mouse model, all these results support our conclusion that quorum sensing does not have a major role in either regulation of SPI-1 T3S or virulence.

We examined whether differences in the mutant strains tested and/or experimental conditions used might explain the disparity between our findings and those of Choi et al. with respect to our inability to detect any effect of LuxS on *Salmonella* virulence or SPI-1 gene expression as previously reported (4). We eliminated the possibility that the differences in invasion and SPI-1 regulation noted by Choi et al. (4) were related to some difference in the strains used by repeating our invasion, gene expression, and protein secretion assays with their *luxS* mutant and wild-type strains. These experiments revealed no difference in invasion under two alternative MOIs at either 15 or 60 min postinfection (data not shown). Similarly, we detected no difference in *prgH* promoter activity, expression of the SPI-1 proteins PrgH and InvJ in cellular fractions or in secretion of the SPI-1 effector proteins SipA, SipB, and SipC (regulated by InvF) between the wild type and *luxS* mutants examined by Choi et al. (4). It remained possible that differences in the bacterial growth phase examined are important, since our initial assays were performed at late log phase, conditions selected because extracellular AI-2 concentrations and SPI-1 expression are maximal (4, 18, 27), while Choi et al. (4) measured those genes regulated by HilA and InvF at stationary phase when SPI-1 expression is suboptimal and when AI-2 has been internalized and thus extracellular AI-2 concentrations are low. In addition, Choi et al. (4) grew *Salmonella* strains under anaerobic conditions, while most studies of invasion and SPI-1 expression use aerobic growth conditions. The differ-

ences reported by Choi et al. with respect to invasion and *prgH* gene expression in *luxS* mutant could not be replicated by growing either *luxS* mutant and their respective parent strains under anaerobic conditions to stationary phase (data not shown). Thus, we conclude that the previously reported role of *luxS* in these aspects of *Salmonella* biology are most likely related to the precise experimental conditions employed by Choi et al. (4) and are of negligible importance under the much more extensive range of growth conditions and infection protocols we have examined.

In summary, it was found that in *S. Typhimurium* SL1344, *luxS* has no measured effect on the expression of SPI-1 T3SS apparatus proteins or on its ability to secrete SPI-1 T3S effector proteins required for invasion. As a consequence, the epithelial cell invasion phenotype of the *luxS* mutant was similar to that of the wild type, with no difference being exhibited in either the time it takes to induce membrane ruffling after adherence to a host cell, the number of ruffles propagated, or on the rate of invasion. Additionally, the virulence of the *luxS* mutant was similar to that of the wild type in a mouse model of infection.

ACKNOWLEDGMENTS

We are grateful to Jay Hinton, Isabelle Hautefort, Sangryeol Ryu, and Jeongjoon Choi for providing bacterial strains. We thank Alan Leard for assistance with microscopy and Mick Bailey for help with the flow cytometry.

The Medical Research Council supported this work by providing a Ph.D. studentship to C.A.P. (G74/77) and funding microscopes in Bristol University's Cell Imaging Facility. Research in the laboratories of M.A.J., C.M.A.K., and P.W. was also supported by MRC and BBSRC research grants. S.H. is supported by BBSRC and Unilever under a CASE Ph.D. studentship, and H.S. was a recipient of a BBSRC DTA Ph.D. studentship.

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