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# AI-3 Synthesis Is Not Dependent on *luxS* in *Escherichia coli*†

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**The quorum-sensing (QS) signal autoinducer-2 (AI-2) has been proposed to promote interspecies signaling in a broad range of bacterial species. AI-2 is spontaneously derived from 4,5-dihydroxy-2,3-pentanedione that, along with homocysteine, is produced by cleavage of *S*-adenosylhomocysteine (SAH) and *S*-ribosylhomocysteine by the Pfs and LuxS enzymes. Numerous phenotypes have been attributed to AI-2 QS signaling using *luxS* mutants. We have previously reported that the *luxS* mutation also affects the synthesis of the AI-3 autoinducer that activates enterohemorrhagic *Escherichia coli* virulence genes. Here we show that several species of bacteria synthesize AI-3, suggesting a possible role in interspecies bacterial communication. The *luxS* mutation leaves the cell with only one pathway, involving oxaloacetate and L-glutamate, for de novo synthesis of homocysteine. The exclusive use of this pathway for homocysteine production appears to alter metabolism in the *luxS* mutant, leading to decreased levels of AI-3. The addition of aspartate and expression of an aromatic amino acid transporter, as well as a tyrosine-specific transporter, restored AI-3-dependent phenotypes in an *luxS* mutant. The defect in AI-3 production, but not in AI-2 production, in the *luxS* mutant was restored by expressing the *Pseudomonas aeruginosa S*-adenosylhomocysteine hydrolase that synthesizes homocysteine directly from SAH. Furthermore, phenotype microarrays revealed that the *luxS* mutation caused numerous metabolic deficiencies, while AI-3 signaling had little effect on metabolism. This study examines how AI-3 production is affected by the *luxS* mutation and explores the roles of the LuxS/AI-2 system in metabolism and QS.**

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a human pathogen that colonizes the large intestine and causes outbreaks of bloody diarrhea and hemolytic-uremic syndrome throughout the world. Once inside the colon, EHEC forms attaching and effacing lesions on the intestinal epithelial cells and produces Shiga toxins (14, 25). The genes necessary for the formation of the attaching and effacing lesions are encoded by a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) (19). The LEE is composed of 41 genes, most of which are organized into five polycistronic operons (*LEE1-5*) (6, 7, 21). *LEE1* encodes a transcriptional activator, Ler, which is required for expression of the LEE genes (2, 6, 8, 12, 21, 29, 33). The LEE encodes a type III secretion system and effector proteins that are translocated into the epithelial cells and cause extensive cytoskeletal rearrangements leading to attaching and effacing lesion development (13).

The EHEC LEE has previously been shown to be regulated by a quorum-sensing (QS) mechanism (33). QS depends on hormone-like signal molecules, termed autoinducers, that interact with bacterial transcriptional factors to regulate gene expression when a critical threshold concentration is reached. The *cysK*, *astD*, *tnaB*, and *gabT* genes in *E. coli* have been shown to be regulated in a QS-dependent manner in response to indole produced by the bacteria (42). Another QS system involving *luxS* has been shown to regulate the *lsr* genes in *E. coli* (46). LuxS is involved in the production of autoinducer-2 (AI-2) and this system has been identified in over 55 bacterial species (23, 45). AI-2 is produced from *S*-adenosylmethionine (SAM) through a series of enzymatic steps (see Fig. 3A). SAM acts as a methyl donor and creates the toxic intermediate *S*-adenosylhomocysteine (SAH), which is hydrolyzed by the enzyme Pfs to *S*-ribosylhomocysteine (SRH) (30). The LuxS enzyme catalyzes the cleavage of SRH to form homocysteine and the AI-2 precursor, 4,5-dihydroxy-2,3-pentanedione (DPD) (30). DPD is an unstable compound that spontaneously cyclizes to form several furanone ring formations, including AI-2 (30).

The LuxS/AI-2 system was initially characterized in *Vibrio harveyi* (30). AI-2 is one of two signals that regulate the *lux* genes and light production in *V. harveyi* (Fig. 1A). The structure of AI-2 that *Vibrio* spp. recognizes has been determined to be a furanosyl-borate diester (3). The AI-2 signal is detected by the periplasmic protein LuxP that binds to LuxQ. At low cell densities, when a small amount of AI-2 is present, a phosphorylation cascade involving LuxQ, LuxU, and LuxO leads to the expression of small regulatory RNAs that, along with the chaperone Hfq, destabilize the mRNA that encodes LuxR, the protein required for transcription of the luciferase genes (16). When a high concentration of AI-2 is present, LuxQ becomes a phosphatase, and the system becomes dephosphorylated, allowing for activation of the luciferase operon. Homologues of this system have been found only in other *Vibrio* species (15).

In *Salmonella enterica* serovar Typhimurium and *E. coli*, the genes that comprise the *lsr* operon are the only genes demonstrated to be directly regulated by AI-2 (40, 46). The *lsr* operon encodes an ABC transporter that is responsible for AI-2 uptake (Fig. 1B). LsrB binds AI-2 and has been shown to interact

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† Supplemental material for this article may be found at http://jb.asm.org/.

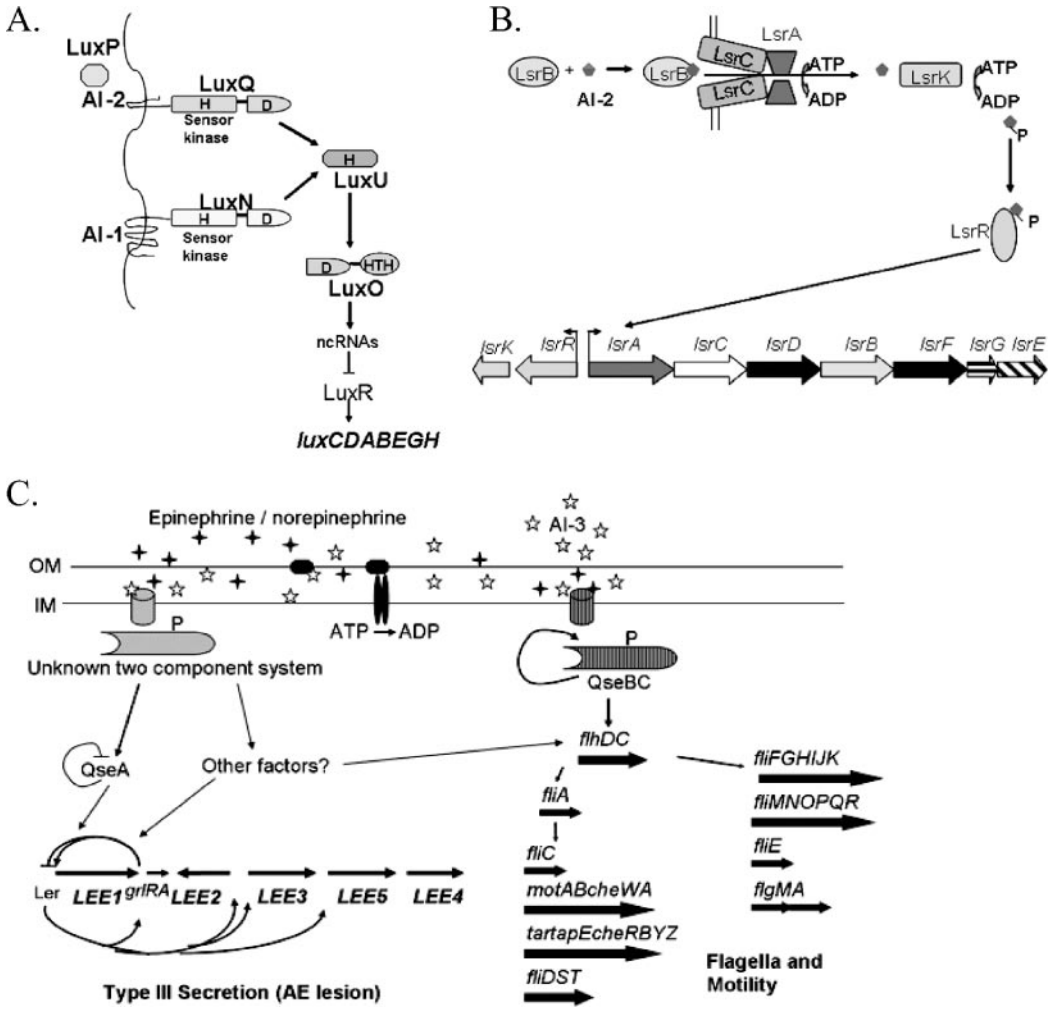


FIG. 1. AI-2 and AI-3 signaling pathways. (A) In *V. harveyi*, AI-2 is bound by LuxP. This signals LuxQ to become a phosphatase, leading to LuxU and LuxO dephosphorylation, which allows LuxR expression and activation of the luciferase operon. (B) Uptake of AI-2 by the Lsr ABC transporter system in *E. coli* and *Salmonella*. The *lsrACDBFGE* genes are transcribed as an operon, while *lsrK* and *lsrR* are transcribed divergently. Once AI-2 is bound, it is transported into the cell through the Lsr ABC transporter, phosphorylated by LsrK, and is then thought to interact with LsrR and relieve repression of the *lsr* operon. (C) Model of AI-3-mediated QS signaling cascade in EHEC. AI-3 and epinephrine/norepinephrine seem to be recognized by the same receptor and interact with sensor kinases once inside the periplasm. The QseC sensor kinase is part of the QseBC two-component system that regulates the flagellum regulon. Another two-component system is hypothesized to recognize the signal and activate transcription of the LEE. QseA regulates the LEE by activating transcription of *LEE1*.

with a chemically distinct form of the AI-2 signal, (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran, that does not contain boron (23). Directly upstream of the *lsr* operon are two divergently transcribed genes, *lsrR* and *lsrK*. The *lsrR* gene encodes a repressor of the *lsr* operon, while *lsrK* encodes a kinase that phosphorylates internalized AI-2 (Fig. 1B). Phosphorylated AI-2 is then hypothesized to indirectly induce expression of the *lsr* operon by binding to the LsrR repressor and inactivating it, leading to higher expression of the *lsr* operon and increased uptake of AI-2 from the environment (39).

*S. enterica* serovar Typhimurium and *E. coli lsr* transporter mutants maintain the ability to slowly take up AI-2 from the environment, suggesting the presence of an additional low-affinity transporter involved in AI-2 uptake (39, 46). It has been suggested that the *luxS*/AI-2 system may be more involved in cell metabolism than in QS signaling in enteric bacteria (43, 44). Winzer et al. have proposed that AI-2 may be toxic to the cell during exponential growth and is internalized at a later stage of growth during which controlled amounts can be degraded (43). This process would be metabolically beneficial to the bacteria since they are no longer losing one "ribose-equivalent" unit per methyl-group transfer (43). It remains unclear whether the primary role of AI-2 uptake in enteric bacteria is central metabolism or whether it is a mechanism of regulating gene expression by monitoring cell population density as well as a method of interspecies communication.

The mutation of *luxS* has pleiotropic effects on the production of autoinducer-3 (AI-3), which serves as the QS signal for EHEC virulence genes (35). This compound was shown to be chemically distinct from AI-2 and able to activate transcription of the genes encoding the LEE type III secretion system in EHEC (35). AI-3 cannot activate bioluminescence in *V. harveyi*, but it is

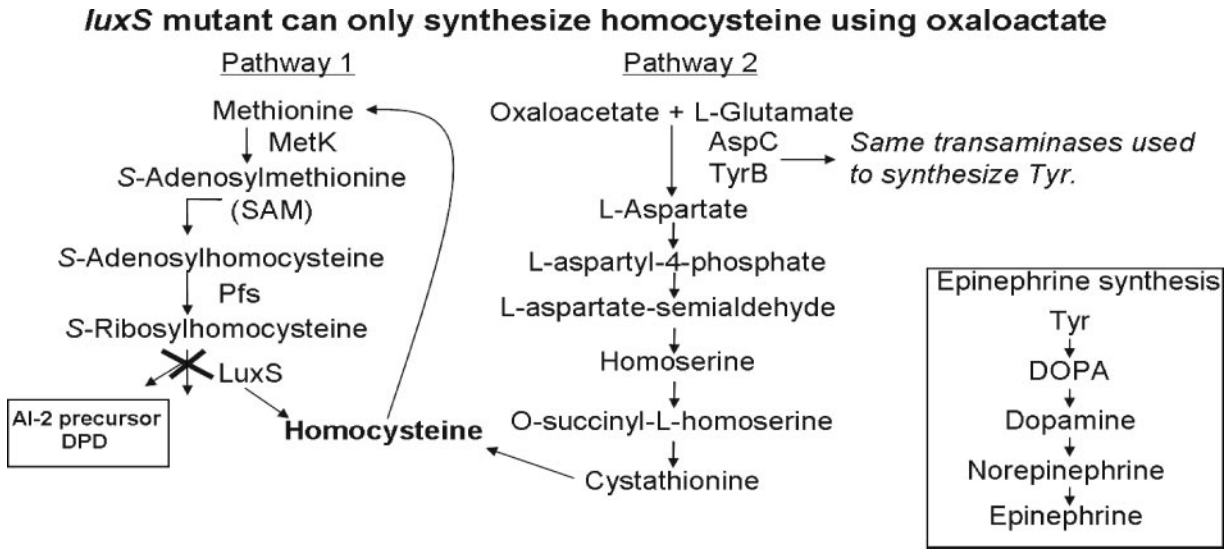


FIG. 2. Pathways for homocysteine synthesis in *E. coli*. Homocysteine is needed in the cell for de novo synthesis of methionine, and methionine is required for the production of the vital metabolic enzyme SAM. SAM is an important methyl donor in the cell, involved in the methylation of lipids, proteins, RNA, and DNA. The *luxS* mutant cannot produce homocysteine through SRH hydrolysis, leaving only one pathway involving the use of oxaloacetate to generate homocysteine. Oxaloacetate, L-glutamate, and the AspC and TyrB transaminases are used to produce aspartate, which can then proceed through a series of reactions resulting in the synthesis of homocysteine. Exclusive use of this pathway may lead to altered metabolism and amino acid content in the *luxS* mutant, resulting in reduced AI-3 synthesis.

able to activate transcription of *LEE1*, which encodes the Ler regulator that activates the other LEE genes (35). In contrast, AI-2 activates bioluminescence in *V. harveyi* but does not affect transcription of *LEE1* (35).

The mammalian hormones epinephrine and norepinephrine have been shown to cross talk with the AI-3 QS system (35). Both epinephrine and norepinephrine can substitute for AI-3 in the regulation of EHEC virulence gene expression (35). The effects of AI-3 and epinephrine/norepinephrine can be blocked with adrenergic receptor antagonists, suggesting that these compounds may share a similar structure (35). A model of QS virulence signaling in EHEC is shown in Fig. 1C.

AI-3 and epinephrine/norepinephrine are thought to be recognized by the same receptor(s). One of the receptors known to detect these signals is the two-component system QseBC (M. B. Clarke and V. Sperandio, submitted for publication). QseBC responds to AI-3 and epinephrine/norepinephrine and regulates transcription of the flagella regulon, as well as its own transcription (4, 35, 36) (Clarke and Sperandio, submitted). Fecal filtrates containing autoinducers from human intestinal flora are also able to activate transcription from the *LEE1* promoter, as well as *V. harveyi* light production, suggesting that the human gastrointestinal flora produces both AI-3 and AI-2 (35).

LuxS does not produce AI-3 directly, suggesting that the *luxS* mutation disrupts another pathway involved in AI-3 synthesis. The *luxS* mutant is unable to convert SRH to homocysteine (Fig. 2). Homocysteine is needed for de novo synthesis of methionine in the cell. Methionine is an essential nonpolar amino acid in living cells and is required for the production of SAM, an important methyl donor used in many critical cellular functions. The two cellular pathways in *E. coli* that produce the homocysteine needed for de novo synthesis of methionine are depicted in Fig. 2 (http://www.ecosal.org/ecosal/index.jsp). The *luxS* mutant can synthesize homocysteine only from the pathway that involves the use of oxaloacetate, which may disrupt normal amino acid synthesis and cellular metabolism. A previous gene array study indicated that the *luxS* mutation resulted in altered transcription of genes involved in amino acid biosynthesis and metabolism, nucleotide biosynthesis and metabolism, and carbon compound catabolism in addition to the effects seen on the LEE and motility genes (34). One of the genes downregulated in the *luxS* mutant was *aroP*, which produces a protein that transports aromatic amino acids into the cell (34). The *luxS* mutant may be unable to efficiently transport aromatic amino acids into the cell, leading to further disruption of normal amino acid biosynthesis. In the present study, we examine the affected pathways leading to diminished AI-3 production and altered metabolism in the *luxS* mutant and further distinguish the roles of AI-2 and AI-3 in EHEC.

## MATERIALS AND METHODS

**Strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. Primers used in this study are shown in Table 2. All strains were grown aerobically at 37°C in Luria-Bertani (LB) medium or Dulbecco's modified Eagle's medium (DMEM) (Invitrogen). Antibiotics for selection were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 25 μg/ml. The EHEC Δ*lsrR* knockout was created by chromosomal gene replacement with a chloramphenicol marker generated by PCR using the pKD3 plasmid as a template and the lsrR P1l Red and lsrR P2l Red primers using methods described by Datsenko and Wanner (5). Strain MW192 was created by amplifying *aroP* from strain 86-24 with *Pfx* polymerase (Invitrogen) using primers AroPF1 and AroPR1, subcloned into pCR-Blunt II-TOPO (Invitrogen), digested with KpnI and BamHI restriction enzymes, cloned into pQE30 (QIAGEN), and transformed into VS94. Strain MW196 was created by amplifying *sahH*, including its native promoter, from *Pseudomonas aeruginosa* PA01 with *Taq* polymerase (Invitrogen) using primers SAHFA and SAHRA, subcloned into pCR 2.1-TOPO (Invitrogen), digested with HindIII and EcoRV restriction enzymes, cloned into pACYC177 (New England Biolabs), and transformed into VS94. Strain MW199 was created by amplifying *tyrP* from EHEC with JumpStart KlenTac Lr polymerase (Sigma) using prim-

TABLE 1. Plasmids and strains used in this study

| Plasmid or strain | Relevant genotype | Reference or source |
|---|---|---|
| Plasmids | | |
| pACYC177 | Cloning vector | New England Biolabs |
| pQE30 | Cloning/expression vector | Qiagen |
| pVS212 | *luxS* cloned in pQE30 | 35 |
| pVS214 | *pfs* cloned in pQE30 | 35 |
| pMW191 | *aroP* cloned in pQE30 | This study |
| pMW195 | *sahH* from *P. aeruginosa* cloned in pACYC177 | This study |
| pKD3 | λRed template plasmid | 5 |
| pKM201 | λRed helper plasmid | 24 |
| pCP20 | λRed resolvase plasmid | 5 |
| PBAD33 | Low-copy-number expression vector | 11 |
| pRS551 | *lacZ* reporter gene fusion vector | 31 |
| Strains (no. of strains tested) | | |
| 86-24 | Stx2+ EHEC (serotype O157:H7) | 10 |
| VS94 | 86-24 *luxS* mutant | 34 |
| *lsr* mutant | 86-24 Δ*lsrR* mutant | This study |
| MW90 | VS94 p*luxS* | This study |
| MW192 | VS94 p*aroP* | This study |
| MW196 | VS94 p*sahH* | This study |
| MW199 | VS94 p*tyrP* | This study |
| E2348/69 | EPEC (serotype O127:H6) | James B. Kaper |
| VS102 | E2348/69 *luxS* mutant | 32 |
| VS104 | VS102 p*luxS* | 32 |
| TEVS232 | *LEE1*::*lacZ* reporter strain | 33 |
| BB170 | *V. harveyi* (sensor 1, sensor $2^+$) | 37 |
| EHEC O26:H11 | EHEC clinical isolate | Luis R. Trabulsi |
| EPEC O111lac:H9 | EPEC clinical isolate | Luis R. Trabulsi |
| *E. coli* commensal (1) | | Hospital Sao Paulo |
| *Shigella* sp. (5) | | Hospital Sao Paulo |
| *Salmonella* sp. (1) | | Hospital Sao Paulo |
| *K. pneumoniae* (17) | | Hospital Sao Paulo |
| *E. cloacae* (1) | | Hospital Sao Paulo |
| *Citrobacter diversus* (1) | | Hospital Sao Paulo |
| DH5α | $\lambda^-$ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*($r_K^-m_K^-$) *supE44 thi-1 gyrA relA1* | Promega |

ers tyrP F1 and tyrP R1, subcloned into pCR 2.1-TOPO (Invitrogen), digested with HindIII and PstI restriction enzymes, cloned into pQE30 (QIAGEN), and transformed into VS94.

**Recombinant DNA techniques.** Plasmid purification, PCR, ligation, restriction, transformation, and DNA gel electrophoresis were performed using standard methods (28). DNA sequence analysis was carried out at the University of Texas Southwestern Medical Center Sequencing Core Facility using an ABI automated sequencer.

TABLE 2. Primers used in this study

| Primer | Sequence |
|---|---|
| lsrR P1l Red | ATAAATGCGCAAGAACTGAACAATT GCATTAAAGATTTAAATATGTTCA AGTGTAGGCTGGAGCTGCTTC |
| lsrR P2l Red | TCTGTTCCTCTATACGTTCTCCATCAT TCCCGGTAATAAGGTCTGCAAACA TATGAATATCCTCCTTA |
| AroPF1 | CGGGCACCCGCATTATTCTTGATCTG |
| AroPR1 | GGGGTACCCCGGCGTAGAGAGATTA |
| SAHFA | CGCTATAATCGCCCGCTCAG |
| SAHRA | DTGGTTGTAGTGATCGGCGA |
| tyrP F1 | CAGGACAGAAGAAAGCGTGA |
| tyrP R1 | CGTTAATTCTGGCACCCAAT |
| LerRT F1 | CGACCAGGTCTGCCCTTCT |
| LerRT R1 | GCGCGGAACTCATCGAAA |
| RpoART F1 | GCGCTCATCTTCTTCCGAAT |
| RpoART R1 | CGCGGTCGTGGTTATGTG |

**RNA extraction and real-time RT-PCR studies.** RNA was extracted from three biological replicate cultures of strains 86-24, VS94, MW90, MW192, MW196, MW199, and strain VS94 supplemented with either 0.5 mM aspartate dipeptides (BACHEM), 50 mM sodium fumarate dibasic, and or 0.2% ammonium sulfate grown in DMEM (Invitrogen) aerobically at 37°C to an optical density at 600 nm ($OD_{600}$) 0.5. RNA was extracted using a RiboPure bacteria RNA isolation kit (Ambion) according to the manufacturer's guidelines. The primers used in the real-time assays were designed using Primer Express, version 1.5 (Applied Biosystems) (Table 2). Real-Time reverse transcription-PCR (RT-PCR) was performed in a one-step reaction using an ABI 7500 sequence detection system (Applied Biosystems).

For each 20-μl reaction mixture, 10 μl of 2× SYBR master mix, 0.1 μl of Multiscribe reverse transcriptase (Applied Biosystems), and 0.1 μl of RNase inhibitor (Applied Biosystems) were added. The amplification efficiency of each of the primer pairs was verified using standard curves of known RNA concentrations. Melting curve analysis was used to ensure template specificity by heating products to 95°C for 15 s, followed by cooling to 60°C and heating to 95°C while monitoring fluorescence. Once amplification efficiency and template specificity were determined for each primer pair, relative quantification analysis was used to analyze the unknown samples using the following conditions for cDNA generation and amplification: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. The *rpoA* (RNA polymerase subunit A) gene was used as the endogenous control.

**Detection, quantification, and statistical analysis.** Data collection was performed using the ABI Sequence Detection 1.3 software (Applied Biosystems). Data were normalized to levels of *rpoA* and analyzed using a comparative cycle threshold method previously described (26). The expression level of *ler* in the different strains and under different conditions was compared using the relative quantification method (26). Real-time data are presented as the relative change

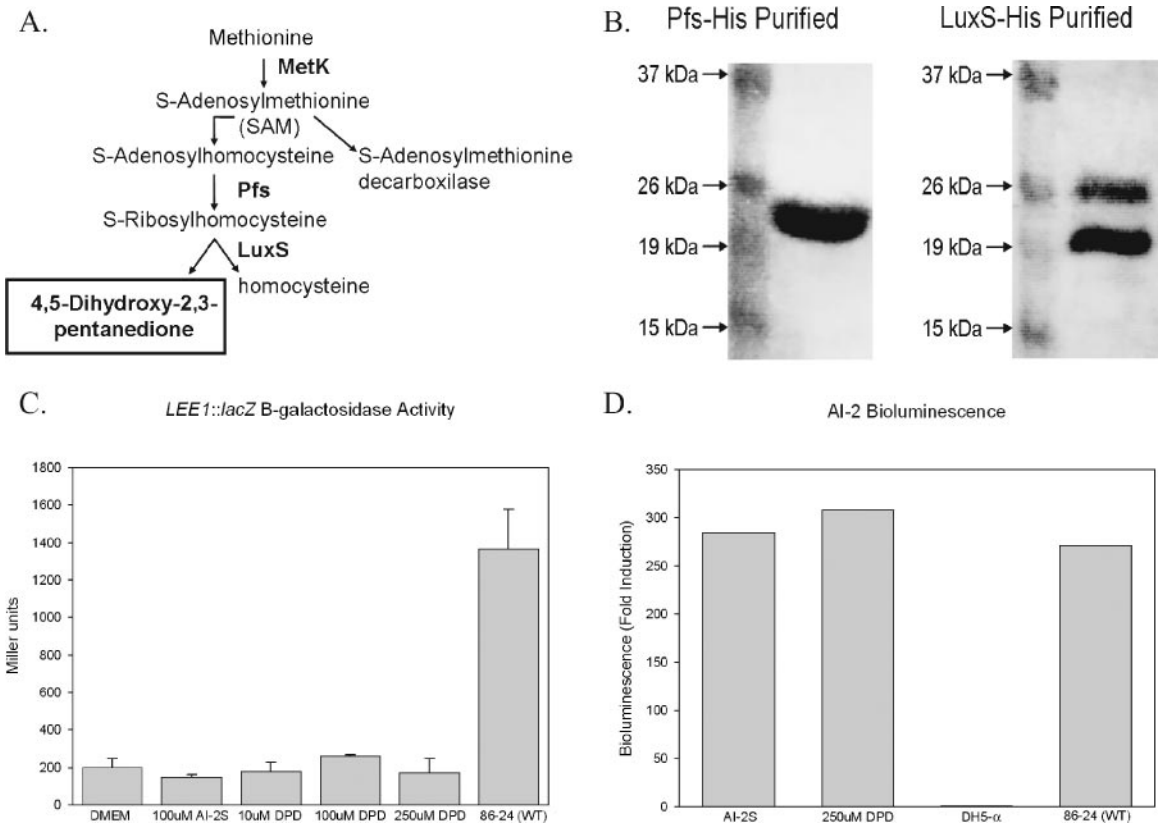


FIG. 3. Enzymatically and chemically synthesized AI-2 activated *V. harveyi* bioluminescence and does not activate transcription from the *LEE1* promoter. (A) Biosynthetic pathway leading to AI-2 production. (B) His-tagged purified Pfs and LuxS used to enzymatically synthesize AI-2 in vitro. (C) Neither AI-2 produced enzymatically (AI-2S) nor chemically synthesized AI-2 (DPD) can activate the transcription of *LEE1* in the *luxS* mutant, as shown by the β-galactosidase detection assay. (D) *V. harveyi* bioluminescence test to determine AI-2 production demonstrating that AI-2S, as well as DPD, activates bioluminescence. DH5α does not produce AI-2 and was used as a negative control.

compared to strain 86-24 (wild type [WT]). Error bars represent the standard deviation of the $\Delta\Delta C_T$ value (26).

**In vitro synthesis of AI-2.** In vitro synthesis of AI-2 was carried out as previously described (30). His-tagged Pfs and LuxS were purified from pVS212 and pVS214 (Table 1) by using a nickel resin (QIAGEN) according to the manufacturer's protocol. In vitro synthesis of AI-2 was performed with 1 mM SAH (Sigma), 1 mg/ml His-LuxS, and 1 mg/ml His-Pfs in 10 mM sodium phosphate buffer, pH 7.5, at 37°C for 1 h. The AI-2 was separated from the Pfs and LuxS proteins by a Centrifuge Biomax-5 size exclusion column (Millipore). The amount of AI-2 was indirectly quantified by measuring homocysteine production using Ellman's test for the sulfhydryl group as previously described (30).

***V. harveyi* bioluminescence assay.** AI-2 activity in preconditioned (PC) medium, enzymatically derived AI-2, and chemically synthesized DPD AI-2 precursor (a gift from Michael Meijler and Kim D. Janda, The Scripps Research Institute) (17) was assayed by using the *V. harveyi* BB170 reporter strain, which responds only to AI-2 (17, 37). The assays were performed as previously described (37) and read using a Bio-Rad Lumimark microplate reader. In order to test the EHEC Δ*lsrR* mutant, the following protocol was used. Strain 86-24 and the Δ*lsrR* mutant were grown in LB to an $OD_{600}$ of 1.0, pelleted by centrifugation, washed three times with LB, and then incubated with synthetic AI-2 for 1 h at 37°C. The bacteria were again pelleted, and the supernatants were filter sterilized and assessed for the amount of remaining AI-2 using the *V. harveyi* bioluminescence assay, as previously described by Taga et al. (40).

**Western blotting.** Secreted proteins from strains 86-24, Δ*lsrR*, VS94, and VS94 supplemented with 0.5 mM aspartate dipeptide (BACHEM) or aspartate-alanine dipeptide (BACHEM) were grown in DMEM to an $OD_{600}$ of 1.0, and secreted proteins were prepared as described by Jarvis et al. (13). Western blotting procedures were performed as previously described (28), and blots were probed with polyclonal antisera against EspA and EspB (kindly provided by James Kaper, University of Maryland School of Medicine).

**β-Galactosidase assays.** The TEVS232 reporter strain containing a chromosomal transcriptional fusion between the *LEE1* promoter and *lacZ* was used to assay AI-3-dependent transcription of *LEE1*. TEVS232 was grown in fresh medium or in medium supplemented with PC medium and grown as previously described (33). Cultures were diluted 1:10 in Z buffer, and β-galactosidase activity was measured by using o-nitrophenyl β-D-galactopyranoside as a substrate as previously described (22).

**Biolog PM.** Strains 86-24 and VS94 were used in phenotype microarrays (PMs). Four conditions were compared and assayed in duplicate: VS94 versus 86-24, VS94 plus enzymatically synthesized AI-2 versus VS94, 86-24 plus 5 μM epinephrine versus 86-24, and VS94 plus 5 μM epinephrine versus VS94. PM tests were performed in 96-well microplates with each well containing a different nutrient source or inhibitor. Cell respiration was measured using a tetrazolium dye that produces a strong color when cells are actively respiring. All assays were performed by Biolog, Inc. (Hayward, Calif.) as previously described (1).

## RESULTS

**The LuxS/AI-2 QS system does not activate the LEE genes.** It has been previously shown that the *luxS* mutation leads to decreased LEE expression and that LEE activity cannot be restored by addition of either purified or enzymatically synthesized AI-2 (35). Figure 3A illustrates the metabolic pathway leading to the formation of the AI-2 precursor DPD. LuxS is involved in converting SRH into DPD and homocysteine. To confirm that AI-2 does not play a role in LEE activation, we tested the ability of two different sources of AI-2 to activate

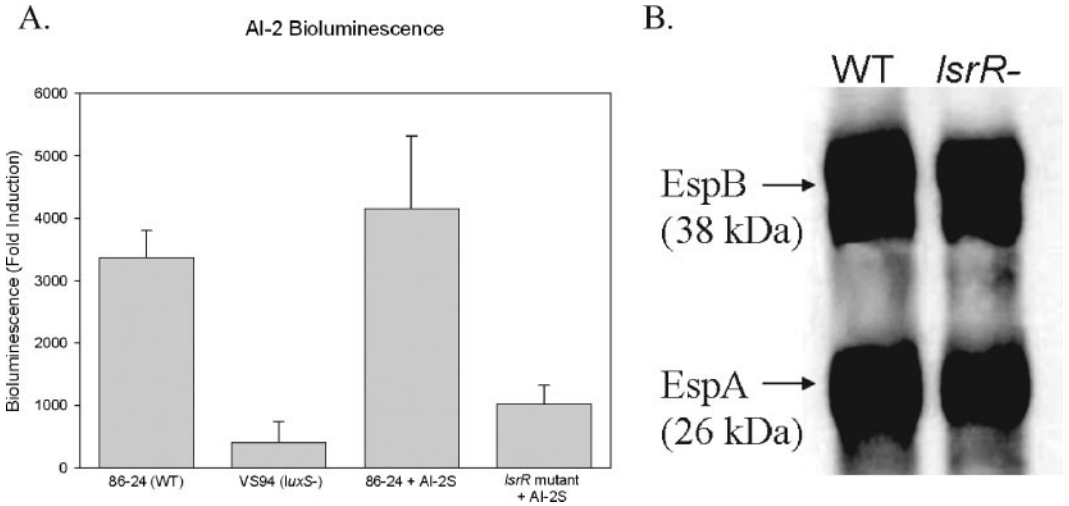


FIG. 4. An EHEC *lsr* mutant imported more AI-2 from the supernatant but displays normal LEE function. (A) Enzymatically synthesized AI-2 was added to late exponential cultures of either the WT or an *lsr* EHEC mutant for 1 h. The *V. harveyi* bioluminescence assay was used to determine the amount of AI-2 left in the supernatants. Less AI-2 was left in the *lsr* mutant supernatant, indicating increased AI-2 uptake compared to the WT. PC media from strains 86-24 (WT) and VS94 (Δ*luxS*) were used as controls. (B) Immunoblot analysis of the amount of EspA and EspB secreted into culture supernatants did not reveal any differences in LEE expression and function between the WT and *lsr* mutant.

*LEE1* transcription. The first form of AI-2, designated AI-2S, was generated using His-tagged purified Pfs and LuxS enzymes in vitro (Fig. 3B). Chemically synthesized AI-2 precursor, designated DPD (20), was also tested for its ability to activate transcription from the *LEE1* promoter.

A β-galactosidase reporter system containing the *LEE1* promoter and a promoterless *lacZ* gene was used to assess the effect of AI-2 on LEE activation. Neither AI-2S nor DPD was able to activate transcription from the *LEE1* promoter (Fig. 3C). PC medium from 86-24 (WT), containing AI-3, was able to activate transcription from the *LEE1* promoter (Fig. 3C). In order to demonstrate that both sources of AI-2 were biologically functional, we tested each source for its ability to activate bioluminescence in *V. harveyi* strain BB170 (37). Supernatant from 86-24 (WT), AI-2S, and 250 μM DPD was able to activate bioluminescence in *V. harveyi* (Fig. 3D). PC medium from *E. coli* strain DH5α, which does not produce AI-2 (37), was used as a negative control.

**LsrR mutant.** An EHEC Δ*lsrR* deletion mutant (Fig. 1B) was created in order to further examine if AI-2 plays a role in the pathogenesis of EHEC. Exogenous AI-2S was added to the WT and the Δ*lsrR* mutant, and the supernatants were examined for the AI-2 remaining in the supernatant. Taga et al. have previously demonstrated that a Δ*lsrR* mutant no longer represses transcription of the Lsr ABC transporter and that the mutant imports AI-2 from supernatants into the cell more efficiently than the WT (39). As expected, the *lsr* mutant was found to import more AI-2 from the medium than WT, thus leaving less AI-2 signaling molecule in the PC medium (Fig. 4A). The Δ*lsrR* mutation caused higher expression of the Lsr ABC transporter, which resulted in less AI-2 in the culture supernatant. Next, we assessed the effects of the *lsrR* mutation on the function of the LEE pathogenicity island. The EspA and EspB proteins are encoded by *LEE4* and secreted through the LEE type III secretion system. Proper expression of *ler* (*LEE1*) is required for transcription of the *espA* and *espB* genes and the secretion of these proteins through the type III secretion apparatus. To examine LEE function as a whole in the Δ*lsrR* mutant, we examined the amount of EspA and EspB secreted into culture supernatants by Western blot analysis. There was no detectable difference in secretion of these two proteins by the WT or the Δ*lsrR* mutant (Fig. 4B), further suggesting that AI-2 does not regulate the LEE.

**Commensal bacteria and other pathogens synthesize both AI-2 and AI-3.** The signaling cascade for AI-3 detection is present in many bacterial species. In order to examine which bacterial species are capable of producing AI-2 and AI-3, supernatants from many different bacterial cultures (strains and number tested are listed in Table 1) were tested for their ability to activate *V. harveyi* bioluminescence and transcription of the *LEE1* promoter using the *LEE1*::*lacZ* β-galactosidase reporter system. All of the strains tested, except for strains without a functional *luxS* gene (DH5α and the *luxS* mutant), were able to produce AI-2 (Fig. 5A). Supernatants from all species activated bioluminescence at least 10-fold higher than the *luxS* mutant and DH5α. Many bacterial supernatants were also able to activate transcription from the AI-3-dependent *LEE1* promoter, suggesting that these bacterial species also make AI-3 (Fig. 5B). Commensal *E. coli*, as well as several other intestinal bacterial species (enteropathogenic *E. coli* [EPEC] E2348/69, EHEC O26:H11 a clinical isolate, EPEC O111lac:H9 a clinical isolate, *Klebsiella pneumoniae*, *Shigella* sp., *Salmonella* sp., and *Enterobacter cloacae*), were found to produce both AI-2 and AI-3. The wide variety of enterobacteria able to produce AI-3 suggests that it may serve as another interspecies QS signal.

**Aspartate restores *LEE1* transcription and protein secretion in the *luxS mutant*.** In order to explore the hypothesis that the *luxS* mutation causes a metabolic shift and that exclusive use of the oxaloacetate pathway may lead to decreased AI-3 synthesis, we first studied the effects of the addition of aspartate to the growth medium. The DMEM used in our EHEC virulence assays did not contain aspartate; thus, all aspartate

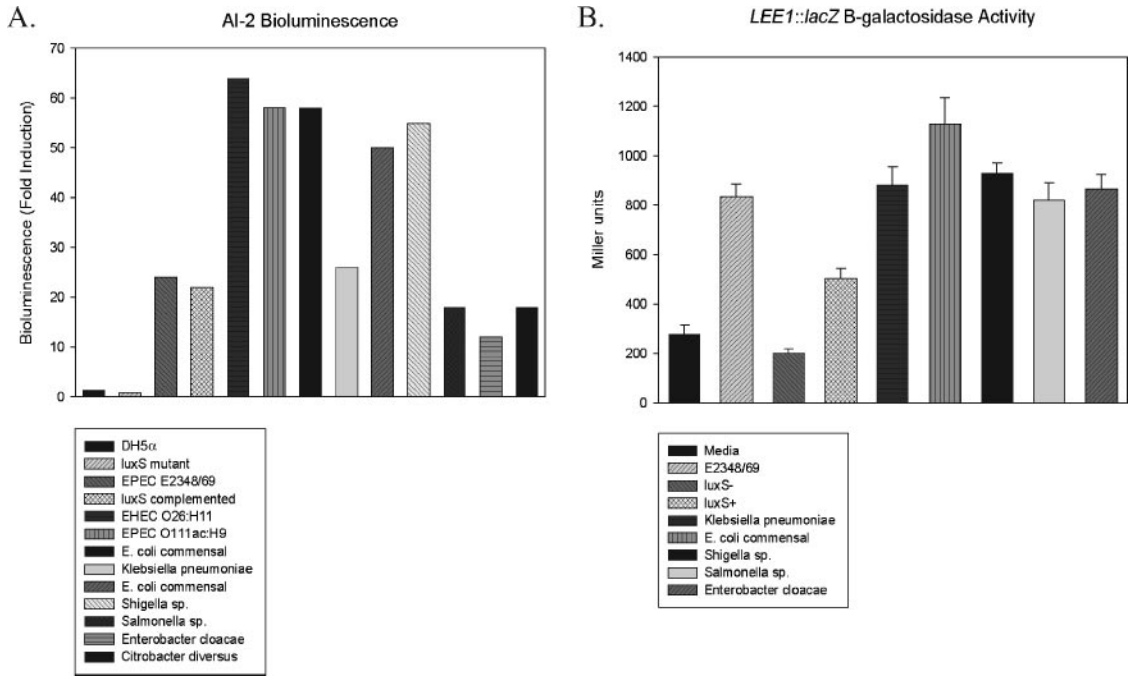


FIG. 5. Many commensal and pathogenic bacterial strains produce both AI-2 and AI-3. (A) *V. harveyi* bioluminescence test to determine AI-2 production. All strains containing a functional *luxS* gene produced AI-2, and culture supernatants from these strains activated bioluminescence in *V. harveyi*. Strain DH5α and the *luxS* mutant do not produce AI-2. (B) A *LEE1*::*lacZ* β-galactosidase assay was used to detect AI-3 in PC medium. All strains tested produced AI-3, which activated transcription from the *LEE1* promoter, except the *luxS* mutant.

must be synthesized endogenously by the cell. L-Aspartate is the second product in the pathway that utilizes oxaloacetate to produce homocysteine (Fig. 2). This reaction involves the AspC and TyrB transaminases, which are also required for tyrosine production. By adding exogenous aspartate to DMEM, we attempted to decrease the requirement of AspC and TyrB transaminases to synthesize aspartate, allowing them to play other roles in cellular metabolism. Restoration of AI-3 synthesis was assessed by monitoring AI-3-dependent phenotypes, such as transcription of *LEE1* and secretion of EspA and EspB.

The addition of 0.5 mM aspartate dipeptide, a concentration similar to the other amino acids present in DMEM, restored transcription from the *LEE1* promoter in the *luxS* mutant to near WT levels using an *LEE1*::*lacZ* reporter system (Fig. 6A). These results were further characterized by measuring the amount of *ler* (*LEE1*) transcription in response to aspartate by real-time RT-PCR. The *luxS* mutation resulted in a decrease of *ler* transcription, which was complemented when *luxS* is expressed from a plasmid (Fig. 6B). The addition of aspartate restored *ler* transcription in the *luxS* mutant to greater than WT levels (Fig. 6B). Growing the *luxS* mutant in the presence of aspartate also increased the secretion of the EspB and EspA proteins, which is diminished in the *luxS* mutant (35) (Fig. 6C). The addition of aspartate complemented a defect in the *luxS* mutant, restoring transcription of *ler* and function of the LEE type III secretion system.

In order to test whether the effects of aspartate addition were due to an increase in the cellular nitrogen levels, we supplemented the DMEM with 0.2% ammonium sulfate to increase nitrogen levels in the cell. The addition of 0.2% ammonium sulfate did not restore *ler* transcription in the *luxS* mutant, suggesting that nitrogen limitation was not responsible for the decrease in AI-3 production (Fig. 6B). We also explored the idea that the decreases in *ler* transcription and AI-3 production may result from altered carbon metabolism in the *luxS* mutant. The addition of 50 mM fumarate, which increases available carbon, may have partially restored transcription of *ler*, although the increase in transcription was not significantly different from that of the *luxS* mutant (Fig. 6B).

The effect of aspartate on AI-2 production was assessed using culture supernatants from the WT, *luxS* mutant, and *luxS* mutant plus the addition of aspartate dipeptides in the *V. harveyi* bioluminescence assay for AI-2. As expected, it was found that the addition of aspartate to the *luxS* mutant had no effect on AI-2 production, and the *luxS* mutant did not produce AI-2 (Fig. 6D).

**SahH restores *ler* transcription but not AI-2 production in the *luxS* mutant.** When SAM is used as a methyl donor in the cell, SAH is formed. SAH is a potent feedback inhibitor of SAM-dependent methyltransferases, and its hydrolysis is necessary to avoid toxic effects on the cell. Organisms utilize one of two pathways to further process SAH and inhibit its lethal effects on the cell. *E. coli* uses a 5′-methylthioadenosine-SAH nucleosidase (Pfs) and an SRH cleavage enzyme (LuxS) to convert SAH to homocysteine (Fig. 7A) (30). *P. aeruginosa* does not contain Pfs or LuxS and uses an SAH hydrolase to convert SAH to homocysteine in a single-step reaction (Fig. 7A) (43). Low concentrations of homocysteine added to minimal medium, such as DMEM, have been shown to be inhibitory to growth of *E. coli* (27, 41). To increase homocysteine levels in the cell while avoiding cell toxicity and interference

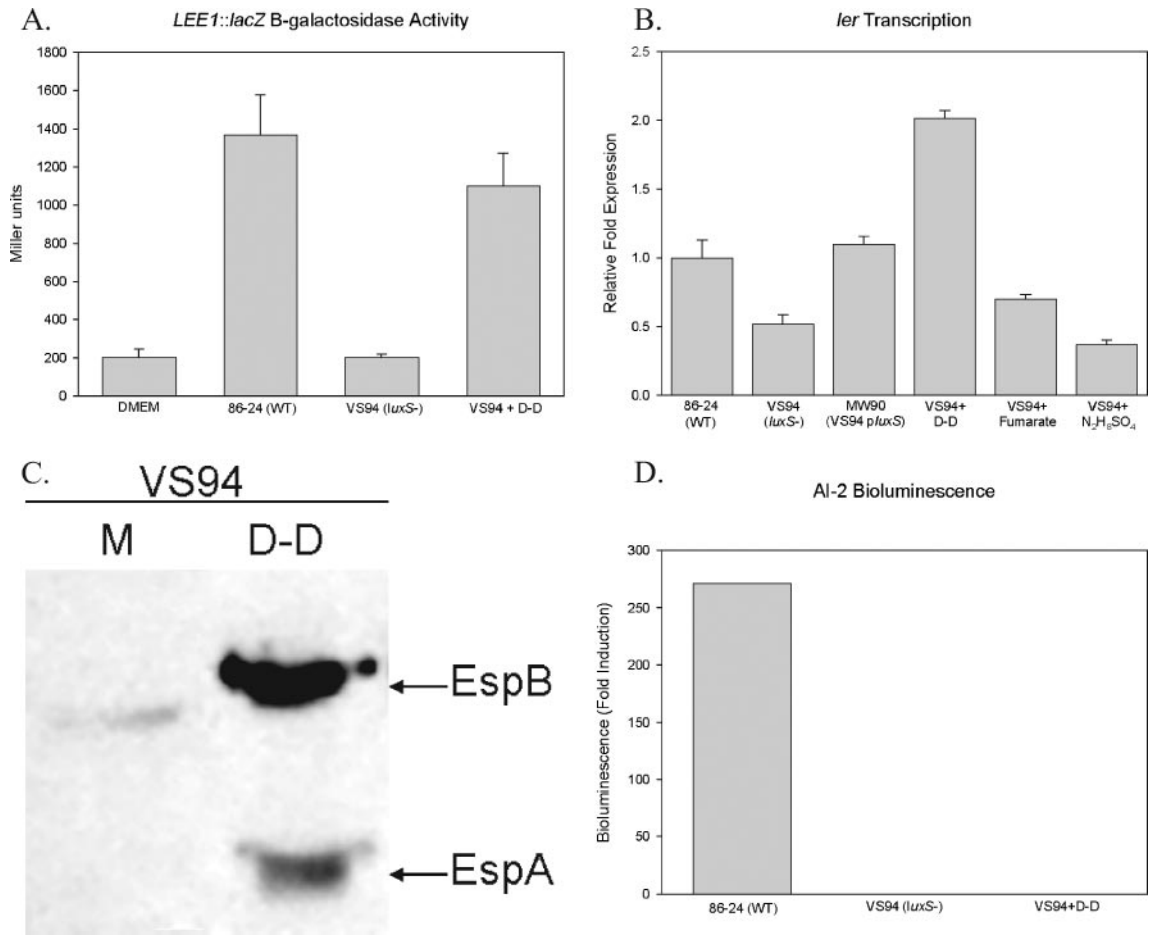


FIG. 6. The addition of aspartate restored AI-3 production, *ler* (*LEE1*) transcription, and EspA and EspB secretion in the *luxS* mutant. (A) The addition of 0.5 mM aspartate dipeptide to the *luxS* mutant restored the AI-3-dependent activation of *LEE1* in an *E. coli* K-12 background. Only supernatants from the WT and *luxS* mutant with the addition of aspartate were able to activate transcription from the *LEE1* promoter in this system. (B) Real time RT-PCR revealed that *ler* transcription in the *luxS* mutant was restored to greater than WT by the addition of aspartate. Complementing *luxS* on a plasmid also restored *ler* transcription levels. (C) Aspartate increased secretion of the LEE-encoded EspA and EspB proteins in the *luxS* mutant, as seen by immunoblotting. (D) *V. harveyi* bioluminescence assay showing that the addition of aspartate to the *luxS* mutant did not restore the mutant's ability to produce AI-2.

with growth, we complemented the EHEC *luxS* mutant's inability to produce homocysteine through SAM detoxification by expressing *sahH* (SAH hydrolase) from *P. aeruginosa* in the EHEC *luxS* mutant.

The SAH hydrolase restored the *luxS* mutant's ability to produce homocysteine from SAM, restoring normal metabolism in the cell and AI-3 production. Expression of the *P. aeruginosa* SahH in the EHEC *luxS* mutant restored the ability of the *luxS* mutant to produce AI-3. AI-3 was present in PC medium from WT, the *luxS* mutant expressing *sahH*, and the *luxS* complemented strain (Fig. 7B). SahH also restored the AI-3-dependent transcription of *ler* as measured by real-time RT-PCR to greater than WT levels (Fig. 7C). To confirm that expressing SahH in the *E. coli* background had no effect on AI-2 production, we tested this strain's ability to produce AI-2 using the *V. harveyi* bioluminescence assay. As expected, SahH expression did not restore AI-2 production in the *luxS* mutant (Fig. 7D).

**AroP and TyrP complement the AI-3 defect of the *luxS* mutant.** The results of the previous experiments suggest that the decreased AI-3 production could occur as a result of the exclusive use of the oxaloacetate pathway to produce homocysteine. Under normal cell metabolism conditions, the major biosynthetic pathway to aspartate is through transamination between oxaloacetate and L-glutamate involving the AspC and/or TyrB amino acid transaminases. These are the same transaminases involved in the biosynthesis of tyrosine. Increased use of this pathway to produce homocysteine could lead to altered amino acid levels in the cell, including tyrosine, since the AspC and TyrB transaminases would be used to synthesize aspartate and not tyrosine (Fig. 2). Tyrosine is a component of DMEM at a concentration of 0.398 mM. AroP is responsible for transporting aromatic amino acids, such as tyrosine into the cell. However, a gene array revealed that *aroP* is downregulated in the *luxS* mutant (34). A decrease in AroP production may impair the ability of the *luxS* mutant to import aromatic amino acids.

To verify the results of the array study indicating *aroP* downregulation in the *luxS* mutant, real time RT-PCR was used to measure *aroP* transcript levels in the WT and *luxS* mutant. The

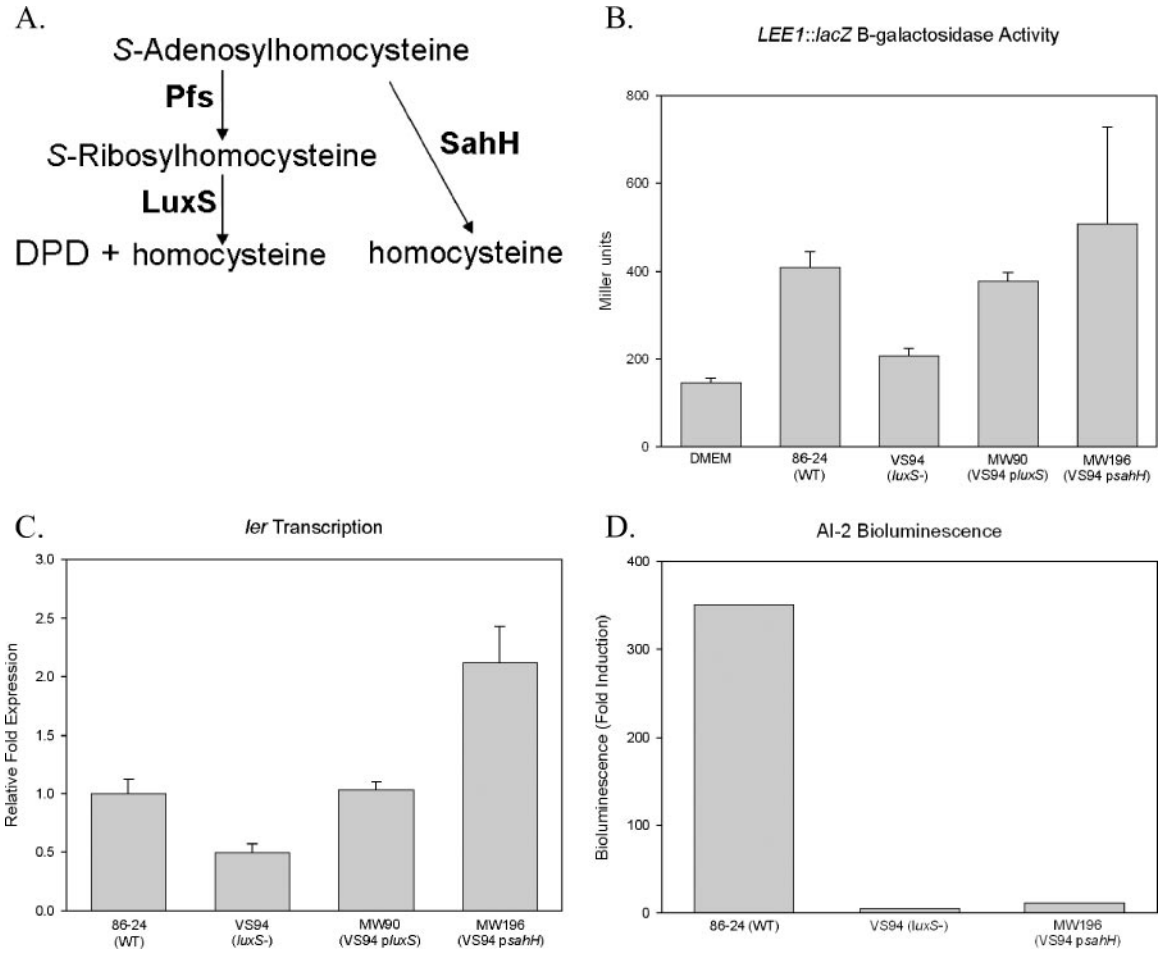


FIG. 7. Expressing the *P. aeruginosa* gene *sahH* in the EHEC *luxS* mutant restored AI-3-dependent phenotypes but not AI-2-dependent phenotypes. (A) Pathways leading to homocysteine production from SAH. *E. coli* uses a two-step mechanism involving the Pfs and LuxS enzymes to produce the AI-2 precursor (DPD) and homocysteine. *P. aeruginosa* produces homocysteine from SAH in a one-step reaction involving the SahH enzyme. (B) The expression of *sahH* in the *luxS* mutant restored AI-3-dependent activation of the *LEE1* promoter in an *E. coli* K-12 background. PC medium from the WT, the *luxS* mutant expressing *sahH*, and the *luxS* complemented strain activated transcription from the *LEE1* promoter, as measured by β-galactosidase activity. (C) Real time RT-PCR was used to demonstrate that *ler* transcription is restored in the *luxS* mutant by expressing *P. aeruginosa sahH* from a plasmid. (D) Expression of *P. aeruginosa sahH* did not restore the EHEC *luxS* mutant's ability to produce AI-2, as determined by the *V. harveyi* bioluminescence assay.

transcription of *aroP* was significantly reduced in the *luxS* mutant compared to the WT (Fig. 8A). To further study the effects of *aroP* on AI-3 production and LEE activation, we expressed *aroP* in the *luxS* mutant under an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter and measured the amount of AI-3 in culture medium using the *LEE1*::*lacZ* reporter assay. Inducing the expression of AroP, and presumably increasing the intracellular concentration of aromatic amino acids, complemented the AI-3 defect observed in the *luxS* mutant (Fig. 8B). When *aroP* was expressed in the *luxS* mutant, transcription of *ler* was also restored (Fig. 8C). To more specifically address the role of tyrosine in AI-3 synthesis, the tyrosine-specific transporter TyrP was expressed from an IPTG-inducible promoter in the *luxS* mutant, and the amount of AI-3 in culture supernatants was determined using the *LEE1*::*lacZ* reporter assay. Inducing *tyrP* expression in the *luxS* mutant restored AI-3 activity in culture supernatants to WT levels (Fig. 8B). TyrP also restored transcription of *ler* in the *luxS* mutant to greater than WT levels as measured by real-time RT-PCR (Fig. 8C). The increased import of aromatic amino acids and tyrosine from the growth medium appears to have allowed for more AI-3 production, suggesting that these molecules are important in AI-3 synthesis. As expected, expression of AroP had no effect on AI-2 production as measured by the *V. harveyi* bioluminescence test (Fig. 8D).

**PM analysis.** The exact roles of the *luxS* AI-2 QS in EHEC and other enteric bacteria remain unclear. The previous results from this study suggested that the reduced AI-3 production by the *luxS* mutant was a result of altered cellular metabolism. In order to examine the metabolic roles of the *luxS*/AI-2 QS system, PMs were used to globally examine the effects of the *luxS* mutation on metabolism. These arrays screen nearly 2,000 cellular phenotypes (1). We examined four different conditions, in duplicate, comparing the WT and *luxS* mutant and the effects of adding the QS signals AI-2S and epinephrine (which can substitute for AI-3) (35). Pure AI-3 was not used due to the

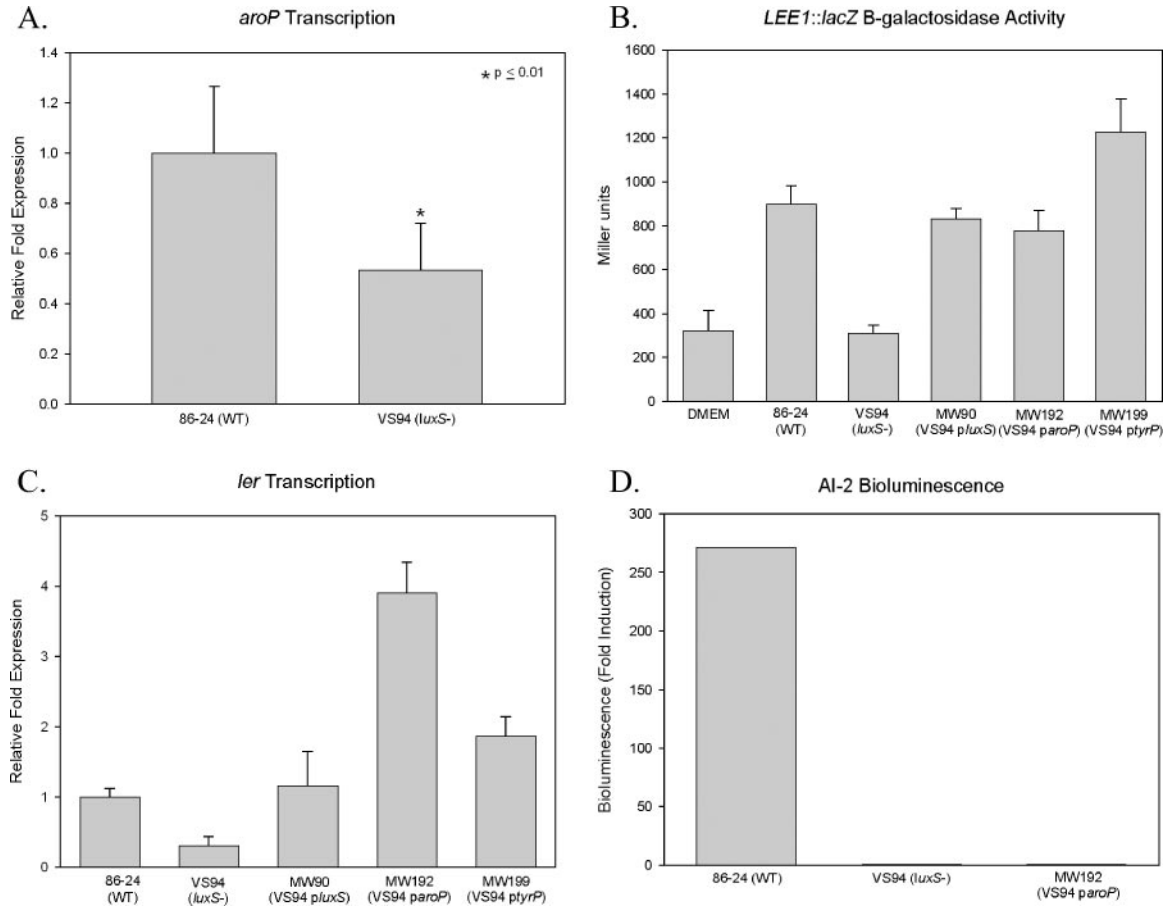


FIG. 8. The *luxS* mutant *ler* transcriptional defect can be complemented by overexpressing *aroP* and *tyrP*. (A) Real time RT-PCR was used to show that *aroP* is downregulated in the *luxS* mutant. (B) The expression of *aroP* and *tyrP* in the *luxS* mutant restored AI-3-dependent activation of the *LEE1* promoter in an *E. coli* K-12 background. PC medium from the WT, MW192, MW199, and MW90 strains activated transcription from the *LEE1* promoter as measured by β-galactosidase activity, while the *luxS* mutant and medium alone did not. (C) Expressing *aroP* or *tyrP* on an inducible plasmid in the *luxS* mutant restored transcription of *ler*, as measured by real time RT-PCR. (D) The expression of *aroP* did not affect AI-2 production in the *luxS* mutant, as determined by the *V. harveyi* bioluminescence test.

difficulty in obtaining sufficient quantities of the purified AI-3 needed for these studies. A summary of results from the four conditions is shown in Table 3.

The first condition compared the *luxS* mutant to WT (see Table S1 in the supplemental material). The *luxS* mutant gained 45 phenotypes compared to the WT. Of these 45 phenotypes, 37 were related to increased antimicrobial resistance, most likely the result of the efflux pump encoded by the tetracycline cassette that was used to inactivate the *luxS* gene in this strain. The *luxS* mutant lost 172 growth phenotypes compared to the WT. Forty-two of these conditions involved the utilization of nitrogen sources. The *luxS* mutant also lost the ability to utilize 15 carbon sources, 5 phosphate sources, and 5 sulfur sources. Ninety-four phenotypes involved nutrient stimulation. All of these nutrient stimulation phenotypes occurred on the same PM array plate in a minimal medium with strict metabolic sources. These results may suggest that minimal medium does not support efficient growth of the *luxS* mutant. The effects observed may not be due to the different compounds in each well but, rather, to the inability of the *luxS* mutant to grow in this medium.

We next examined the effects of the addition of enzymatically synthesized AI-2 to the *luxS* mutant (see Table S2 in the supplemental material). AI-2 synthesis was performed for 1 h at 37°C under conditions previously described (30). Carrying out this reaction for 1 h allows for the oxidation of the homocysteine produced by the synthesis reaction (30). Homocysteine levels were undetectable using Ellman's test for the sulfhydryl group (data not shown). It was found that 62 growth phenotypes were gained by the addition of synthesized AI-2 to the *luxS* mutant. Many of these involved the utilization of metabolic compounds, such as 26 nitrogen sources, 16 phosphate sources, and 10 carbon sources. Twenty-two of these phenotypes were the same ones lost in the *luxS* mutant compared to the WT. Addition of AI-2 resulted in the loss of 17 phenotypes, including the ability to utilize seven sulfur sources. Three of the phenotypes lost by the addition of AI-2 were gained by the *luxS* mutant compared to the WT.

The effects of adding 5 μM epinephrine to the WT strain versus WT without the addition of epinephrine were also tested (see Table S3 in the supplemental material). Seven phenotypes were gained by the addition of epinephrine. Three

TABLE 3. Phenotype microarray results

| Phenotype | Results (arbitrary units) for strains compared and conditions | | | |
|---|---|---|---|---|
| | VS94 vs 86-24 | VS94+AI-2 vs VS94 | 86-24+EPI[a] vs 86-24 | VS94+EPI vs VS94 |
| Gained phenotypes | | | | |
| Chelator, lipophilic | 1 | | 1 | |
| Cholinergic antagonist | 1 | | | |
| C source | | 10 | | |
| Cyclic nucleotide phosphodiesterase | 1 | | | |
| DNA intercalator | 1 | | | |
| DNA polymerase | 1 | | | |
| DNA topoisomerase | 5 | 1 | | |
| Folate antagonist | 1 | | | |
| Ion channel, K+ | 1 | | | |
| Membrane, detergent | 3 | | | |
| Membrane, transport | 1 | | | |
| N source | | 26 | 3 | |
| Phenothiazine | | 1 | | |
| Protein synthesis | 15 | 1 | 2 | |
| P source | | 16 | | |
| RNA polymerase | | 1 | 1 | |
| Wall, cephalosporin | 5 | 2 | | 1 |
| Wall, lactam | 9 | 2 | | 3 |
| Lost phenotypes | | | | |
| Anti-capsule, anti-inflammatory | | 1 | | |
| Anti-tuberculosic | 1 | | | |
| C source | 15 | | | |
| DNA polymerase | | 1 | | |
| DNA topoisomerase | | 1 | 1 | |
| Folate antagonist | 1 | | | |
| Fungicide | | | 1 | |
| Membrane | 2 | | | |
| Membrane, detergent | | | 1 | |
| N source | 42 | | 1 | |
| Nutrient stimulation | 94 | | | |
| Oxidizing agent | 1 | 2 | | |
| pH, deaminase | 2 | | | |
| Protein synthesis | 1 | 1 | | |
| P source | 5 | | | |
| Respiration | | 2 | | |
| S source | 5 | 7 | | |
| Transport, toxic anion or cation | 3 | | | |
| Wall, cephalosporin | | 1 | | |

[a] EPI, epinephrine.

were involved in antimicrobial resistance, while three others were involved in nitrogen metabolism. Four phenotypes were lost due to the addition of epinephrine. The last condition examined was the addition 5 μM epinephrine to the *luxS* mutant versus the *luxS* mutant with no epinephrine added (see Table S4 in the supplemental material). No phenotypes were lost under this condition. Four phenotypes were gained when epinephrine was added. These phenotypes involved cell wall modifications which resulted in increased antimicrobial resistance. Consensus PMs and the correlation between replicates are shown in Fig. S1 and S2, respectively, in the supplemental material.

## DISCUSSION

In the present study, we have addressed the role of the *luxS* gene in the production of the AI-2 and AI-3 QS signals produced by EHEC. Several EHEC virulence factors, such as motility and the LEE, are under QS control (33, 34). QS relies on signals that are secreted by bacteria and regulate gene expression when a critical threshold is reached. The greatest density of signaling molecules occurs at high bacterial densities, and the largest population of bacterial species in the human body occurs in the gastrointestinal tract.

The human gastrointestinal flora produces both AI-2 and AI-3 (35), and this study specifically demonstrates that many other commensal and enteric pathogens are also capable of producing both AI-2 and AI-3. Given the large numbers of bacteria in the gastrointestinal tract and the ability of many different species to produce both AI-2 and AI-3 (Fig. 5), it seems possible that EHEC may use one or both of these signals to recognize that it is within a host. The QS signal which has been shown to activate motility and the LEE is AI-3 (35). The low infectious dose of EHEC, estimated to be as few as 50 to 100 organisms, may be a result of its ability to detect the high concentration of autoinducers in the gastrointestinal tract and regulate its virulence genes accordingly. This may be advantageous to EHEC because it could activate expression of the virulence genes required for infection quickly without the need to grow to a high cell density and produce its own autoinducers.

The existence of QS gene regulation in EHEC was initially observed in an EHEC *luxS* mutant (33). It was originally assumed that the lack of AI-2 produced by the *luxS* mutant was responsible for the reduced virulence phenotypes, but the decrease in virulence was later shown to be a result of the absence of another autoinducer, termed AI-3 (33). AI-3 is chemically distinct from AI-2. It is less polar, binds to $C_{18}$ columns, and elutes only with methanol, while AI-2 is a polar furanone that does not bind $C_{18}$ columns and elutes with buffer alone (35). To date, the only *E. coli* and *Salmonella* genes known to be regulated in response to AI-2 are in the *lsr* operon (40, 46). This study further demonstrates that AI-2 does not activate the transcription of *ler* and expression of the LEE using both enzymatically and chemically synthesized AI-2. Our previous work used enzymatically synthesized AI-2 to demonstrate that AI-2 does not affect *LEE1* transcription (35). We have shown that chemically synthesized DPD (20), which is purer than enzymatically prepared AI-2, also does not affect transcription of *LEE1*. LsrR has been suggested to be the transcription factor that interacts with AI-2 (46). Here, we have demonstrated that a Δ*lsrR* mutant displays normal expression and function of the LEE-encoded type III secretion system, despite this mutant's ability to import AI-2 more efficiently into the cell (Fig. 4).

These observations lead to the question of why the EHEC *luxS* mutant has decreased AI-3 production and subsequently decreased activation of the LEE and motility genes. This study examines the possible metabolic defects present in the *luxS* mutant which lead to reduced AI-3 synthesis. The *luxS* mutation leaves only one pathway to produce homocysteine. The *luxS* mutant can use only the pathway involving oxaloacetate to generate homocysteine (Fig. 2). Homocysteine is an important compound in the cell and is required for the de novo synthesis of methionine in the cell. The *E. coli* MetK enzyme uses methionine to produce SAM. SAM is a multipurpose essential growth compound that plays a role in many key metabolic

aspects of the cell such as polyamine biosynthesis and that serves as a primary methyl donor in many biosynthetic reactions such as the methylation of DNA, RNA, lipids, and proteins (18, 38).

To examine whether the reduced AI-3 production by the *luxS* mutant was due to altered metabolism, we assessed restoration of AI-3-dependent phenotypes by complementing the defects in the *luxS* mutant at different levels in the oxaloacetate-homocysteine pathway. The homocysteine biosynthesis pathway thought to be employed by the *luxS* mutant uses oxaloacetate and L-glutamate to generate L-aspartate that is converted to homocysteine in a series of reactions (Fig. 2). The medium used in all of our virulence assays does not contain aspartate. The addition of aspartate to the *luxS* mutant was able to restore production of AI-3, transcription of *LEE1*, and secretion of EspA and EspB (Fig. 6). The addition of aspartate to the growth medium could change the nitrogen and carbon levels in the *luxS* mutant. When free aspartate is available in the growth medium, the need for aspartate biosynthesis in the cell will diminish. L-Glutamate and oxaloacetate will no longer be required for synthesis of aspartate, leading to increased availability of these compounds within the cell. L-Glutamate is an important factor in the nitrogen assimilation cycle, and an increase in the levels of L-glutamate may lead to an increase in the nitrogen levels in the cell. It is possible that the restoration of tyrosine synthesis may have resulted from the higher nitrogen levels or precursor molecules from the aspartate-glutamate pathways in the cell. Altering nitrogen levels with the addition of ammonium sulfate did not restore transcription of *ler* in the *luxS* mutant, suggesting that the aspartate-induced transcription of *ler* was not a result of altered nitrogen levels within the cell.

If the exclusive use of the oxaloacetate pathway to produce more homocysteine in the *luxS* mutant is responsible for the decreased AI-3 production, correcting the defect of the pathway leading to homocysteine production from SAM should restore normal AI-3 synthesis. *E. coli* uses the two-step reaction involving Pfs and LuxS to hydrolyze SAH and SRH, respectively, to produce DPD and homocysteine, while *P. aeruginosa* uses the SahH enzyme that produces adenosine and homocysteine as a result of SAH hydrolysis in a one-step reaction. Accordingly, *P. aeruginosa* is not capable of producing DPD or, consequently, AI-2. We were able to restore production of AI-3 in the *luxS* mutant without restoring AI-2 production by expressing *sahH* from *P. aeruginosa*. These experiments suggest that SahH expression in the *luxS* mutant lessens the need for oxaloacetate to be used for homocysteine synthesis, restoring some metabolic defects in the *luxS* mutant and resulting in AI-3 production. These experiments allowed us to uncouple AI-2 and AI-3 production in *E. coli*. The *luxS* mutation seems to alter cellular metabolism, leading to decreased AI-3 production, possibly by reducing the tyrosine levels in the cell.

The eukaryotic hormones epinephrine and norepinephrine are able to activate transcription from the *LEE1* promoter, restore type III secretion of the EspB and EspA proteins in the *luxS* mutant, and restore the motility of the *luxS* mutant to WT levels (35). The synthesis of both hormones begins with a tyrosine molecule (9). The effects of epinephrine and norepinephrine on LEE activation can be blocked by the use of adrenergic receptor antagonists, such as propranolol and phentolamine (35). These adrenergic receptor antagonists also decrease *LEE1* transcription and secretion of EspB and EspA in the WT strain, both of which are controlled by AI-3 signaling (35). AI-3 and epinephrine/norepinephrine are all recognized by the QseC sensor kinase (Clarke and Sperandio, submitted), suggesting that they share many similar structural features. Increased use of the oxaloacetate-homocysteine pathway in the *luxS* mutant may lead to higher production of aspartate (Fig. 2). This could lead to a role for AspC and TyrB in the synthesis of aspartate, making them less available for the production of tyrosine. If AI-3 synthesis begins with a tyrosine molecule, as with epinephrine and norepinephrine, a decrease of tyrosine in the cell would lead to decreased synthesis of AI-3 and to the virulence defects observed in the *luxS* mutant.

Tyrosine is present in the DMEM used in all of our assays, but the *luxS* mutant may be unable to import tyrosine as efficiently as the WT because of a decrease in *aroP* transcription. AroP is a transporter protein responsible for transporting aromatic amino acids, such as tyrosine, into the cell. Increasing AroP levels in the *luxS* mutant, by expressing AroP from an IPTG-inducible promoter, was able to restore transcription from the *LEE1* promoter. These results further suggest that aromatic amino acids, including tyrosine, are important for AI-3 synthesis. In order to examine the effect of tyrosine in a more direct manner, the tyrosine-specific TyrP transporter was expressed from a multicopy plasmid in the *luxS* mutant. Induction of *tyrP* expression with IPTG restored *ler* transcription in the *luxS* mutant to above WT levels and restored AI-3 levels in culture supernatants. Increasing cellular tyrosine levels seems to have allowed for more AI-3 to be produced, complementing the defects in LEE transcription observed for the *luxS* mutant.

The decreased AI-3 production in the *luxS* mutant seems to be the result of metabolic defects created by the mutation. PM studies were performed to gain an understanding of the role that *luxS* and autoinducers play in cell metabolism. We used epinephrine to study the effects of AI-3 signaling on cell growth because of the difficulty in purifying large enough amounts of AI-3 required for the PMs. Very few phenotypes in either the WT or *luxS* mutant were altered by the addition of 5 μM epinephrine. Several of the phenotypes affected by the addition of epinephrine in the WT and all of those in the *luxS* mutant involved cell wall modifications which resulted in increased antimicrobial resistance. Metabolism was not greatly affected by the addition of epinephrine.

The *luxS* mutation resulted in numerous metabolic changes compared to the WT. Sixty-seven of the phenotypes lost involved the inability to use carbon, nitrogen, phosphate, and sulfur sources that the WT strain could utilize for growth. The *luxS* mutant lost the ability to use 42 nitrogen sources, suggesting that nitrogen metabolism is significantly altered by the *luxS* mutation. However, the altered nitrogen metabolism does not seem to affect AI-3-dependent phenotypes, as increased nitrogen levels did not restore AI-3-dependent transcription of *ler* in the *luxS* mutant (Fig. 6B). The results from the PM suggest that the *luxS* mutation drastically alters the metabolism of the cell. These growth phenotype assays revealed that although the *luxS* mutant is able to grow at the same rate as WT in laboratory medium, it exhibits a variety of defects when grown in minimal medium with select compounds available to the cell.

This work helps to establish that the *luxS* mutation not only results in the loss of AI-2 production but also significantly alters the metabolism of the cell.

In *E. coli*, AI-2 has only been shown to regulate the expression of the *lsr* operon that controls uptake of AI-2 (46). To try to further understand the function of AI-2, we assessed the effects of adding enzymatically synthesized AI-2 to the *luxS* mutant and examining the consequences using PMs. The addition of AI-2 resulted in a gain of 62 phenotypes compared to the *luxS* mutant with no AI-2 present. The majority of the phenotypes gained involved the ability to use different carbon, nitrogen, and phosphate sources. It is unclear if the ability to use these compounds is a result of the metabolizing of AI-2 or a product of AI-2 signaling. The addition of AI-2 also resulted in the loss of several phenotypes compared to the *luxS* mutant. These phenotypes may represent metabolic pathways that were active in the *luxS* mutant and are no longer required when AI-2 is present. In addition, three of these phenotypes lost by the addition of AI-2 were phenotypes gained by the *luxS* mutant compared to the WT. All three phenotypes involved antibiotic resistance, with the addition of AI-2 making the *luxS* mutant more sensitive. The addition of AI-2 will activate the Lsr ABC transporter which imports AI-2 into the cell. One possible explanation for the increased antibiotic sensitivity is that the expression of the Lsr ABC transporter may disrupt the expression of other efflux pumps in the cell that normally remove the antibiotics from inside the cell, or the Lsr transporter may bring antibiotics into the cell.

In summary, our results suggest that the *luxS* mutation affects the production of AI-3 by altering cellular metabolism. The *luxS* mutation leaves the cell with only one pathway to produce homocysteine, which is required for de novo synthesis of methionine. Exclusive use of this pathway may change metabolism and alter amino acid levels in the cell, possibly leading to reduced tyrosine levels and decreased AI-3 production, based on the assumption that epinephrine and AI-3 share similar structures and synthesis pathways. The PM studies revealed that the *luxS* mutation alters many metabolic aspects of the cell and that addition of AI-2 to the medium can affect different growth phenotypes, either by signaling or being metabolized. The work presented here further distinguishes the role of AI-3 signaling from that of AI-2 signaling and begins to explore how the *luxS* mutation affects AI-3 production.

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