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luxS-Dependent Gene Regulation in *Escherichia coli* K-12 Revealed by Genomic Expression Profiling

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The bacterial quorum-sensing autoinducer 2 (AI-2) has received intense interest because the gene for its synthase, *luxS*, is common among a large number of bacterial species. We have identified *luxS*-controlled genes in *Escherichia coli* under two different growth conditions using DNA microarrays. Twenty-three genes were affected by *luxS* deletion in the presence of glucose, and 63 genes were influenced by *luxS* deletion in the absence of glucose. Minimal overlap among these gene sets suggests the role of *luxS* is condition dependent. Under the latter condition, the *metE* gene, the *lsrACDBFG* operon, and the flanking genes of the *lsr* operon (*lsrR*, *lsrK*, *tam*, and *yneE*) were among the most significantly induced genes by *luxS*. The *E. coli lsr* operon includes an additional gene, *tam*, encoding an *S*-adenosyl-L-methionine-dependent methyltransferase. Also, *lsrR* and *lsrK* belong to the same operon, *lsrRK*, which is positively regulated by the cyclic AMP receptor protein and negatively regulated by LsrR. *lsrK* is additionally transcribed by a promoter between *lsrR* and *lsrK*. Deletion of *luxS* was also shown to affect genes involved in methionine biosynthesis, methyl transfer reactions, iron uptake, and utilization of carbon. It was surprising, however, that so few genes were affected by *luxS* deletion in this *E. coli* K-12 strain under these conditions. Most of the highly induced genes are related to AI-2 production and transport. These data are consistent with the function of LuxS as an important metabolic enzyme but appear not to support the role of AI-2 as a true signal molecule for *E. coli* W3110 under the investigated conditions.

Bacteria can respond to a variety of chemical and physical changes in their environment by regulating gene expression. Changes such as heat shock, nutrient limitation, and high osmolarity can cause multigenic cellular responses in transcription and translation. Some changes which cause similar responses are traced to the bacteria themselves. For example, some bacteria produce metabolites that are released into the environment as the cell density increases. These molecules could be metabolic wastes, which are toxic to the normal physiological activities of the cells and are therefore secreted. However, some metabolic products may serve as a signaling molecule, which can be perceived by the cells to control the expression of specific genes as the population increases. It has been pointed out that such a chemical molecule can only be considered as truly signaling if the cellular response extends beyond physiological activities required to catabolize the signal molecule (52). This type of signaling molecule-dependent regulation confers upon bacteria the capability to communicate with each other and coordinate their activities and has been termed "quorum sensing."

For example, many bacteria produce and secrete a freely diffusible signaling molecule, acyl-homoserine lactone (AHL). With an increase in cell density, the concentration of AHL can increase and reach a threshold stimulatory level, at which the signal molecule binds to a LuxR-like protein, the transcriptional regulator, to control gene expression and cell activity. AHL-mediated quorum sensing is well documented in gram-negative bacteria (28, 53).

A large number of gram-negative and gram-positive bacteria have been found to produce and release another type of signaling molecule, autoinducer 2 (AI-2), which can act via a phosphorelay cascade to stimulate production of bioluminescence in *Vibrio harveyi*. Schauder et al. showed that AI-2 is produced from *S*-adenosylmethionine in three enzymatic steps, wherein LuxS is the enzyme most directly linked to AI-2 production (38). More than 55 bacterial species possess a gene homologous to *luxS* (22, 43, 49, 54), and many have been shown to produce AI-2-like activities by using a *V. harveyi* BB170 reporter strain (44, 45). Recent advances have indicated that the AI-2 molecules from various bacterial species may differ in their structure, although all of them are derived from the product of the LuxS reaction, 4,5-dihydroxy-2,3-pentanedione (DPD) (29). DPD is a highly reactive molecule which likely undergoes cyclization and further arrangements to form a mixture of varied chemical molecules (38). The AI-2 molecules from *Vibrio harveyi* and *Salmonella enterica* serovar Typhimurium have been reported to be (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*S*-THMF-borate) and (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF), respectively (6, 29). It was also suggested that DPD, *R*-THMF, and *S*-THMF-borate are in an equilibrium which is affected by the presence of borate (29).

Evidence accumulated during the last several years suggests that AI-2/*luxS*-mediated regulation may be important in controlling different cell activities in a variety of bacterial species (54). Some of these include biofilm production in *Streptococcus mutans*, *S. enterica* serovar Typhimurium, and *Vibrio cholerae* (17, 26, 35, 56); motility in *Campylobacter jejuni*, enterohem-

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orrhagic *Escherichia coli* (EHEC), and enteropathogenic *E. coli* (12, 15, 42); iron acquisition in *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, and *V. harveyi* (8, 13, 23); and expression of virulence factors in *A. actinomycetemcomitans*, *E. coli* EHEC, *P. gingivalis*, *V. cholerae*, and *Clostridium perfringens* (8, 13, 30, 41, 57). These studies have contributed to our understanding of the AI-2/*luxS*-mediated regulation of gene expression and cell activity but questions remain, as few genes appear to be directly influenced by AI-2. That is, many studies have depended upon comparison of a *luxS* mutant and its parent strain, and so there are questions regarding whether *luxS*-dependent phenotypes are caused by an AI-2 signaling defect, by metabolic perturbation, or by both (49, 52, 54).

In a search for *luxS*-regulated genes in *S. enterica* serovar Typhimurium, the *lsrACDBFGE* operon and the methionine synthase gene *metE* were identified by Taga et al. (47). They found that the *lsrACDBFGE* operon encodes an AI-2 uptake and modification system. In *E. coli*, there exists a similar *lsr* operon (b1513 operon), except that it does not have the *lsrE* homolog. It was shown recently that the functions of the *E. coli lsr* operon and its regulators, LsrR and LsrK, are similar to those in *S. enterica* serovar Typhimurium, and cyclic AMP (cAMP)-cAMP receptor protein (CRP) are involved in regulation of the *lsr* operon (50, 55).

In this study, we have attempted to identify the *luxS*-controlled genes by comparing the wild type and Δ*luxS* mutant under two different growth conditions using DNA microarrays. In the first case, we examined cells in the presence of glucose at low cell density (late exponential phase). Then, we examined cells in the absence of glucose at high density (early stationary phase). Profiles of gene regulation were very different under these two conditions, and many more genes were significantly affected by *luxS* deletion in the latter case. Importantly, we have shown new regulatory and structural characteristics for the *E. coli lsr* and *lsrRK* operons and demonstrated that both *lsrRK* and *lsr* operons are subject to two controllers: a repressor, LsrR, and an activator, cAMP-CRP. This study serves to enhance our understanding of the regulation of AI-2 transport and the growth conditions by which AI-2/*luxS* modulates gene expression in *E. coli* K-12.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* K-12 strain W3110 [F^- λ^- in(*rrnD-rrnE*)] was obtained from the Genetic Stock Center (New Haven, Conn.). Δ*luxS*::*kan* was moved into W3110 from LW7 (ZK126; Δ*luxS*::*kan*) (50) via $P1_{vir}$ transduction. Luria-Bertani broth (LB) contains 5 g liter^{-1} yeast extract (Difco), 10 g liter^{-1} Bacto tryptone (Difco), and 10 g liter^{-1} NaCl. Minimal media have been described previously (32, 39). Cultures of *E. coli* (wild type and the Δ*luxS* mutant) that had been grown overnight in LB or LB plus 0.8% glucose were diluted to an optical density at 600 nm (OD_{600}) of about 0.02 in LB or LB plus 0.8% glucose. The cultures were then incubated at 30°C with shaking at 250 rpm in 50-ml flasks. When the cultures reached the appropriate OD_{600} (1.0 or 2.4), the cells were harvested for RNA extraction.

Plasmid construction. The plasmids used in this study are listed in Table 1 and were generated using standard procedures (37). Restriction enzymes, T4 DNA ligase, and Vent DNA polymerase were used as specified by the manufacturer (New England BioLabs, Beverly, MA). The *E. coli* W3110 chromosomal DNA preparation was performed using the QIAGEN DNeasy tissue kit (QIAGEN, Valencia, CA). Extractions of DNA from agarose gels were performed using the QIAGEN QIAEX II gel extraction kit. Oligonucleotides were from Integrated DNA Technologies (Coralville, IA). DNA sequencing was performed at the DNA core facility of the Center of Biosystems Research (University of Maryland Biotechnology Institute). All constructs made by PCR were sequenced to verify their integrity.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and property	Source or reference
E. coli strains		
W3110	Wild type	*E. coli* Stock Center
LW12	W3110 Δ*luxS*::Kan	This study
ZK126	W3110 Δ*lacU169 tna-2*	9
ZK1000	ZK126 Δ*rpoS*::Kan	2
LW2	ZK126 Δ*crp*::Kan	50
LW7	ZK126 Δ*luxS*::Kan	50
LW8	ZK126 Δ*lsrR*::Kan	50
LW9	ZK126 Δ(*lsrACDBFG*)::Kan	50
LW11	ZK126 Δ*lsrK*::Kan	50
Plasmids		
pFZY1	*galK′-lacZYA* transcriptional fusion vector, Ap^r	21
pJLlsrR	pFZY1 derivative, containing *lsrR* promoter region, Ap^r	This study
pJLlsrK	pFZY1 derivative, containing *lsrK* promoter region, Ap^r	This study

Plasmid pFZY1 is a mini-F derivative (average copy number, 1 to 2 per cell) with a polycloning site upstream of a promoterless *galK′-lacZYA* reporter segment (21). To create pJLlsrR, the *lsrR* promoter region [−340 to +59 relative to the start codon of *lsrR* (b1512)] was amplified by PCR using primers lsrRpF (CCGGAATTCTCGATGCCTTTCAGGACATTG) and lsrRpR (CTCGGATCCGCGACCTGTTCTTCTTCACACATT). The purified PCR product was digested with EcoRI-BamHI and was inserted into EcoRI-BamHI-digested pFZY1. To create pJLlsrK, the *lsrK* promoter region [−367 to +53 relative to the start codon of *lsrK* (b1511)] was amplified by PCR using primers lsrKpF (CCGGAATTCTCGCTCCGGTTATATCAGCCAGGGCGAACA) and lsrKpR (CTCGGATCCTCCAGCGCCATCAGGTAGTACTTT). The purified PCR product was digested with EcoRI-BamHI and was inserted into EcoRI-BamHI-digested pFZY1.

Total RNA isolation. Total RNA was isolated from the cultures using an RNeasy Mini kit (QIAGEN, Inc., Valencia, CA) according to the manufacturer's instructions. RNAprotect bacteria reagent (QIAGEN, Inc., Valencia, CA) was added to the cultures to stabilize RNA before isolation. The RNase-free DNase set (QIAGEN, Inc., Valencia, CA) was used for on-column DNase digestion to remove residual DNA; removal of contaminant DNA was confirmed by PCR. RNA quality was examined spectrophotometrically and with gel electrophoresis.

cDNA synthesis and labeling. cDNA was synthesized and labeled according to the manufacturer's suggestions for the Affymetrix *E. coli* antisense genome array (Affymetrix, Inc., Santa Clara, CA). Briefly, in 60 μl of reaction mixture, 10 μg of total RNA was used for cDNA synthesis by random primers (12.5 ng/μl) and SuperScript II reverse transcriptase (25 U/μl) (both from Invitrogen Corp., Carlsbad, CA). RNA was removed by addition of 20 μl of 1 N NaOH and incubation at 65°C for 30 min. cDNA was purified with a Qiaquick PCR purification kit (QIAGEN, Inc., Valencia, CA) and then fragmented using DNase I (0.6 U/μg of DNA; Amersham Pharmacia Biotech, Piscataway, NJ) at 37°C for 10 min. The Enzo BioArray terminal labeling kit with biotin-ddUTP (Affymetrix, Inc., Santa Clara, CA) was used to label the 3′ termini of the fragmented cDNA. A gel shift assay with NeutrAvidin (Pierce Biotechnology, Inc., Rockford, IL) was performed to estimate the labeling efficiency based on the instructions from Affymetrix.

Microarray hybridization, washing, and scanning. Hybridization solution mix was made with the labeled cDNA according to the manufacturer's instructions (Affymetrix, Inc., Santa Clara, CA), and the mixture was hybridized to the *E. coli* antisense genome arrays at 45°C for 16 h. A GeneChip fluidics station (Affymetrix, Inc., Santa Clara, CA) was used to automate the washing and staining of the arrays. Sequentially, the arrays were stained with ImmunoPure streptavidin (Pierce Biotechnology, Inc., Rockford, IL), antistreptavidin goat antibody (Vector Laboratories, Inc., Burlingame, CA), and R-phycoerythrin streptavidin (Molecular Probes, Inc., Eugene, OR). Finally, the probe arrays were scanned using the Affymetrix GeneArray scanner.

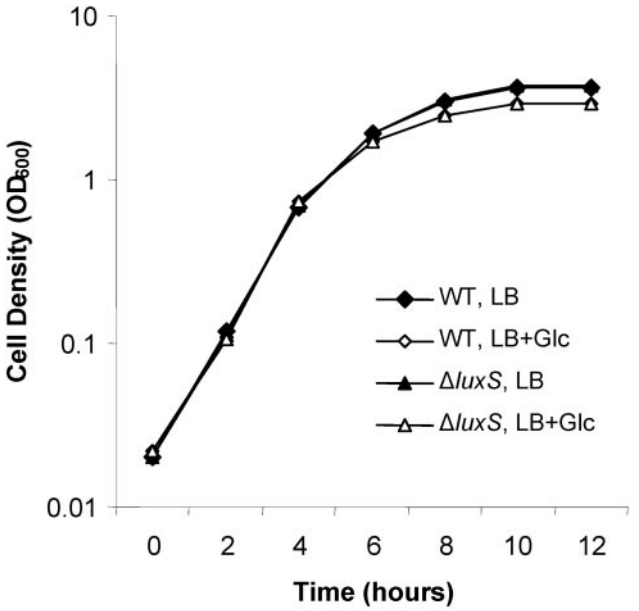

FIG. 1. Growth of the wild type and Δ*luxS* mutant of *E. coli* W3110. Overnight cultures of *E. coli* W3110 and the Δ*luxS* mutant were diluted in LB or LB plus 0.8% glucose to an OD_{600} of about 0.02. At different time points during cell growth, aliquots were collected for measurement of the OD_{600}.

Data analysis. Microarray data were analyzed with the Affymetrix Microarray Suite software version 5.1 (Affymetrix, Inc., Santa Clara, CA) and the four-comparison survival method (7). The fluorescence of each array was normalized by scaling total chip fluorescence intensities to a common value of 500. For each growth condition, two independent experimental cell cultures (wild type) were compared with two independent control groups (Δ*luxS* mutant), and four comparisons were made. The fold change for each gene was calculated as the ratio of signal intensity for the wild type to the signal intensity for the Δ*luxS* mutant. The reported value for the fold change is the average of the four comparisons. Genes with a consistent increase or decrease in all comparisons were determined and used for the analysis. However, the induced genes with absent calls of the array signal in the experimental groups (wild type) and the repressed genes with absent calls of the array signal in the control groups (Δ*luxS* mutant) were eliminated from the analysis. Genes were considered to be statistically significantly over- or underexpressed based on the following criteria: average change of at least 1.8-fold and P values of <0.05 (t test). Our full microarray data are available at our website (http://www.umbi.umd.edu/%7Ecbr/lab_web/home.htm).

RT-PCR and real-time RT-PCR. cDNA was synthesized from total RNA and random hexamers using the SuperScript III first-strand synthesis system for reverse transcription-PCR (RT-PCR; Invitrogen) according to the manufacturer's instructions. Real-time RT-PCR was performed in 50 μl of reaction mixture containing the Platinum SYBR Green qPCR Supermix UDG (Invitrogen), 0.2 μM of primers, and cDNA (50°C for 2 min, 95°C for 2 min, 95°C for 15 s, and 60°C for 30 s). The dye-labeled PCR products were detected with a GeneAmp 5700 sequence detection system (Applied Biosystems). Regular RT-PCR was used to check for the existence of the *lsrRK* and *lsr-tam* operons, and data presented are from reactions using 22 amplification cycles. Primers were designed and purchased from Integrated DNA Technologies (Coralville, IA) (primer sequences are available upon request). Controls were always used to ensure absence of genomic DNA in the DNase I-treated RNA samples. *clpB* was used as the normalizing gene for all reactions, since its transcript levels were not significantly different between the wild type and the *luxS* mutant (data not shown).

β-Galactosidase assays. Cultures of *E. coli* were grown overnight in LB, diluted 100-fold into fresh LB, and grown to mid-exponential phase and then diluted into different media with the OD_{600} below 0.03. The cultures were incubated at 30°C with shaking at 250 rpm in flasks. Samples were removed at regular intervals for determination of the OD_{600} and β-galactosidase activity. Specific activity of β-galactosidase is expressed in Miller units (27).

Gel mobility shift assay. The 46-bp DNA fragments containing the wild-type or mutated *lsrR* promoter regions were synthesized by Integrated DNA Technologies (Coralville, IA). A digoxigenin gel shift kit (Boehringer Mannheim) was used for labeling of DNA fragments and detection of signals according to the manufacturer's instructions. Binding reactions were performed by incubating the labeled DNA fragments with various amounts of purified CRP (generously provided by Fred Schwarz, University of Maryland Biotechnology Institute) in 20 μl of binding buffer (10 mM Tris-HCl [pH 8.0], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 50 μg ml^{-1} bovine serum albumin, 15 μg ml^{-1} sonicated salmon sperm DNA, 100 μM cAMP). Following incubation at 37°C for 10 min, 5 μl of gel loading buffer (0.25× Tris-borate-EDTA [TBE], 60%; glycerol, 40%; bromphenol, 0.2% [wt/vol]) was added, and mixtures were electrophoresed in a 6% native polyacrylamide gel in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) containing 100 μM cAMP. DNA bands were detected according to the manufacturer's instructions.

Motility assays. The medium used for the motility swimming assay was tryptone broth (10 g/liter tryptone [Difco], 5 g/liter NaCl) containing 0.3% Difco agar. Cultures of *E. coli* were grown overnight in liquid tryptone broth, diluted 100-fold into the same fresh medium, and grown to mid-exponential phase. Swim plates were inoculated at the center with 5 μl of cell culture and incubated at 30°C in a humid environment for 11 h.

Biofilm assays. Biofilm assays were performed as described previously (34) with modifications. *E. coli* cells were grown in polypropylene tubes in LB at room temperature without shaking for 24 h and subcultured at a 1:100 dilution into different media: LB, LB and glucose, glycerol minimal, glucose minimal, glycerol minimal with Casamino Acids (CAA), glucose minimal with CAA, or minimal medium with CAA. CAA was used at 5%. These cultures were grown for 48 h at room temperature without shaking and then rinsed with distilled water and stained with 1.0% crystal violet. After 20 min, the tubes were rinsed. The biofilm-associated crystal violet was solubilized by dimethyl sulfoxide, and the OD_{570} of the suspension was measured.

RESULTS

Deletion of the *E. coli* W3110 *luxS* gene does not affect growth, motility, and biofilm formation. Previous reports showed that deletion of *luxS* resulted in increased growth rate and reduced motility in EHEC (42). Similarly, a *luxS* mutant of *Campylobacter jejuni* had reduced motility (12). In addition, it was shown that the *luxS* mutant of *S. enterica* serovar Typhimurium and *Streptococcus mutans* has a defective ability to form biofilms (26, 35, 56). We investigated whether the mutation of *luxS* of *E. coli* K-12 strain W3110 has similar effects on these phenotypes. Figure 1 shows that the Δ*luxS* mutant grows as well as its isogenic parent when the cells are grown in LB or in LB plus glucose. We further tested the motility of the Δ*luxS* mutant. On the 0.3% agar swim plate of tryptone broth, there was no apparent difference of the swimming, as measured by the ability to form halos, between the mutant and the wild type (data not shown). Finally, we tested biofilm formation of both strains in various growth media (see Materials and Methods). No significant differences were observed between the wild type and the Δ*luxS* mutant; biofilm formation was supported in both strains in LB (or with glucose) or various minimal media containing Casamino Acids but not in minimal media without Casamino Acids (see Materials and Methods) (data not shown), consistent with a previous study with *E. coli* 2K1056 (34). In summary, there were no apparent phenotypic differences between the wild-type and the Δ*luxS* mutant cells under the investigated conditions, although we did not rule out the possibility that some specific conditions for these assays may affect such differences. To investigate how *luxS* deletion affects cellular activities, we further performed microarray analysis of these two strains (see below).

TABLE 2. Genes regulated by *luxS* at OD 2.4 in LB

B no.	Gene	Gene product[a]	Fold change (WT/Δ*luxS*)
b1518	*lsrG*	ORF, hypothetical protein	12.64
b1515	*lsrD*	Putative transport system permease protein	11.9
b1513	*lsrA*	Putative ATP-binding component of a transport system	11.16
b1516	*lsrB*	Putative LACI-type transcriptional regulator	10.14
b3829	*metE*	Tetrahydropteroyltriglutamate methyltransferase	9.74
b1517	*lsrF*	Putative aldolase	6.54
b1511	*lsrK*	Putative kinase	5.52
b1520	*yneE*	ORF, hypothetical protein	3.49
b2236	*yfaE*	ORF, hypothetical protein	3.19
b1519	*tam*	*trans*-aconitate 2-methyltransferase	2.82
b4308	*yjhR*	Putative frameshift suppressor	2.46
b1512	*lsrR*	Putative transcriptional regulator, SorC family	2.22
b1514	*lsrC*	Putative transport system permease protein	2
b4395	*gpmB*	Phosphoglyceromutase 2	1.99
b3796	*argX*	Arginine tRNA3	1.97
b3852	*ileT*	Isoleucine tRNA1, triplicate	1.91
b4017	*arpA*	Putative regulator of acetyl CoA synthetase	1.89
b2087	*gatR*	Split galactitol utilization operon repressor, interrupted	−1.81
b0974	*hyaC*	Probable Ni/Fe-hydrogenase 1 b-type cytochrome subunit	−1.83
b1127	*pepT*	Putative peptidase T	−1.85
b1019	*ycdB*	ORF, hypothetical protein	−1.85
b1550	*gnsB*	ORF, hypothetical protein, GnsB protein	−1.86
b4196	*sgaH*	Probable hexulose-6-phosphate synthase	−1.87
b0630	*lipB*	Protein of lipoate biosynthesis	−1.87
b1022	*ycdQ*	ORF, hypothetical protein	−1.88
b1437		ORF, hypothetical protein	−1.88
b4186	*yjfC*	Putative synthetase/amidase	−1.92
b2406	*xapB*	Xanthosine permease	−1.93
b3939	*metB*	Cystathionine gamma-synthase	−1.93
b3945	*gldA*	Glycerol dehydrogenase (NAD)	−1.95
b0648	*ybeU*	Putative tRNA ligase	−1.98
b1680	*sufS*	ORF, hypothetical protein, selenocysteine lyase, PLP dependent	−1.98
b0823	*ybiW*	Putative formate acetyltransferase	−2
b1112	*ycfR*	ORF, hypothetical protein	−2
b4288	*fecD*	Citrate-dependent iron transport, membrane-bound protein	−2.03
b4310	*yjhT*	ORF, hypothetical protein	−2.05
b3103	*yhaH*	Putative cytochrome	−2.07
b0621	*dcuC*	Transport of dicarboxylates	−2.09
b1407	*ydbD*	ORF, hypothetical protein	−2.11
b0076	*leuO*	Probable transcriptional activator for *leuABCD* operon	−2.14
b2723	*hycC*	Membrane-spanning protein of hydrogenase 3 (part of FHL complex)	−2.14
b3683	*glvC*	PTS system, arbutin-like IIC component	−2.2
b3028	*mdaB*	NADPH-quinone reductase (modulator of drug activity B)	−2.22
b2968	*yghD*	Putative secretion pathway protein	−2.33
b0579	*ybdF*	ORF, hypothetical protein	−2.34
b3220	*yhcG*	ORF, hypothetical protein	−2.35
b0260	*ykfD*	Putative amino acid/amine transport protein	−2.38
b3046	*yqiG*	Putative membrane protein	−2.39
b2919	*ygfG*	Putative enzyme	−2.4
b3906	*rhaR*	Positive regulator for *rhaRS* operon	−2.47
b2060	*wzc*	ORF, hypothetical protein, tyrosine-protein kinase	−2.49
b0790	*ybhP*	ORF, hypothetical protein	−2.51
b1720		ORF, hypothetical protein	−2.52
b1010	*ycdK*	ORF, hypothetical protein	−2.53
b2797	*sdaB*	L-serine dehydratase (deaminase), L-SD2	−2.57
b2993	*hybD*	Probable processing element for hydrogenase-2	−2.59
b0042	*fixB*	Probable flavoprotein subunit, carnitine metabolism	−2.7
b1001	*yccE*	ORF, hypothetical protein	−2.87
b2549	*yphG*	ORF, hypothetical protein	−2.97
b1311	*ycjO*	Putative binding protein-dependent transport protein	−3.06
b3141	*agaI*	Putative galactosamine-6-phosphate isomerase	−3.15
b1012	*ycdM*	ORF, hypothetical protein	−3.24
b1571	*ydfA*	ORF, hypothetical protein	−6.85

[a] Abbreviations: ORF, open reading frame; CoA, coenzyme A.

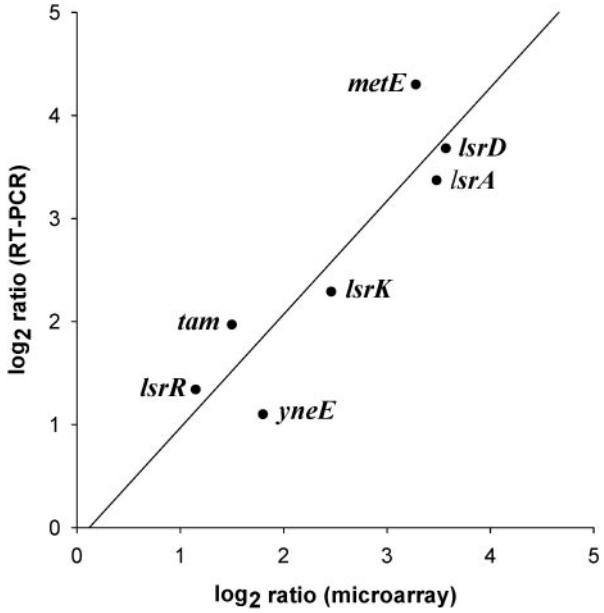

FIG. 2. Correlation of microarray and real-time RT-PCR results. The differences in expression of seven *luxS*-controlled genes (in LB at high cell density) were $\log_2$ transformed and plotted against each other, microarray versus real-time RT-PCR.

Genomic transcriptional analyses of the *luxS* deletion. Using DNA microarrays, we compared genomic transcript levels of the wild type and Δ*luxS* mutant of *E. coli* W3110 under two different growth conditions. One condition (I) was in LB medium when the cells reached an OD_{600} of 2.4 (early stationary phase), while the other (II) was in LB plus 0.8% glucose when the cells reached an OD_{600} of 1.0 (late exponential phase). In the first case, the wild type had low levels of AI-2 and the mutant had none. In addition, the expression level of the *lsrACDBFG* operon is much higher in wild-type cells than in the Δ*luxS* mutant, as CRP is required for its activation (50). It is possible that there exist additional genes regulated by *luxS* under this condition. The second case is characterized by high extracellular AI-2 activity in cultures of the wild type (50) and no AI-2 activity in cultures of the Δ*luxS* mutant. This is another condition under which AI-2 signaling may be important; the wide disparity in AI-2 for these cultures should be revealing (11).

To report the number of genes differentially expressed, we used a 1.8-fold induction ratio as a cutoff limit. Although a 2-fold cutoff is commonly used for analysis of microarray data (20), we have used a slightly less stringent cutoff, as previous studies indicated that even a 1.5-fold difference in transcript level can be biologically significant (3, 19, 40). Table 2 shows that, under condition I (no glucose, OD of 2.4), there were 17 and 46 genes that were induced and repressed at least 1.8-fold by *luxS*, respectively. To verify our microarray data, we further performed real-time RT-PCR on a selected number of the identified *luxS*-regulated genes. Figure 2 shows that there was a strong positive correlation ($r^2 = 0.90$) between the two techniques, validating our microarray profiles.

Under condition II (with glucose, OD of 1.0), there were fewer genes significantly regulated by *luxS* (Table 3). With the 1.8-fold cutoff, only 15 and 8 genes were up- and down-regulated, respectively, by the presence of the *luxS* gene, indicating expression of most of the genes were not affected markedly by the *luxS* deletion. In addition, the genes regulated by *luxS* were different from those observed under the first condition (I). These results indicate that *luxS*-controlled gene expression varies with conditions, suggesting that both (i) careful experimental designs are important in identifying the *luxS*-controlled genes among various bacteria, and (ii) the role of *luxS* may vary with conditions.

Genes controlled by *luxS* in the absence of glucose at OD of 2.4. There were more genes down-regulated than up-regulated by *luxS* when cells were grown to an OD of 2.4 in the absence of glucose. Table 2 shows that 17 and 46 genes were induced and repressed at least 1.8-fold by *luxS*, respectively. The most significantly induced genes belong to the *lsrACDBFG* operon. This result is consistent with the previous *lsr-lacZ* fusion studies performed in *E. coli* (50, 55), which showed the *lsr* operon was differentially expressed between the wild type and the *luxS* mutant mainly in stationary phase. A relatively lower fold change of *lsrC* compared to the other genes in the *lsr* operon might have resulted from interfering effects of certain cDNA fragments, which masked the hybridization of *lsrC* to its probes. Further analysis of *lsrC* expression by RT-PCR indicated a similar induction level by the *luxS* gene (~12-fold change) (data not shown). Surprisingly, expression of *lsrR*, *lsrK*, *tam*, and *yneE*, which flank the *lsrACDBFG* operon, were significantly induced

TABLE 3. Genes regulated by *luxS* at OD 1.0 in LB plus glucose

B no.	Gene	Gene product[a]	Fold change (WT/Δ*luxS*)
b1561	*rem*	ORF, hypothetical protein	2.67
b1700	*ydiT*	Putative ferredoxin	2.58
b4186	*yjfC*	Putative synthetase/amidase	2.51
b3711	*yidZ*	Putative transcriptional regulator, LYSR type	2.42
b3580	*lyxK*	L-xylulose kinase, cryptic	2.32
b1567	*ydfW*	ORF, hypothetical protein	2.25
b3004		ORF, hypothetical protein	2.19
b4002	*zraP*	ORF, hypothetical protein, Zn-binding periplasmic protein	2.04
b0805	*ybiL*	Putative outer membrane receptor for iron transport	1.97
b0667		Putative RNA	1.93
b1834	*yebT*	ORF, hypothetical protein, putative membrane protein	1.92
b0671		Putative RNA	1.84
b4367	*fhuF*	ORF, hypothetical protein, ferric hydroxamate transport protein	1.82
b3829	*metE*	Tetrahydropteroyltriglutamate methyltransferase	1.81
b0872	*hcr*	Putative enzyme, NADH oxidoreductase for HCP	1.8
b2597	*yfiA*	Putative YhbH sigma 54 modulator	−1.83
b1482	*osmC*	Osmotically inducible protein	−1.91
b1461	*ydcE*	ORF, hypothetical protein	−1.92
b3267	*yhdV*	ORF, hypothetical protein	−1.92
b3110	*yhaO*	Putative transport system permease protein	−1.98
b2715	*ascF*	PTS system enzyme II ABC (asc), cryptic, transports specific beta-glucosides	−2.02
b3108	*yhaM*	ORF, hypothetical protein	−2.14
b3109	*yhaN*	ORF, hypothetical protein	−2.87

[a] ORF, open reading frame.

by *luxS* (2.2-, 5.5-, 2.8-, and 3.5-fold, respectively). The *lsrR* and *lsrK* genes encode the *lsr* regulator and the AI-2 kinase, respectively. We investigated their regulation in more detail later. The *tam* gene encodes an *S*-adenosyl-L-methionine-dependent methyltransferase, which catalyzes the methyl esterification of *trans*-aconitate (4). The *trans*-aconitate appears to be formed spontaneously from the citric acid cycle intermediate *cis*-aconitate (4). The benefit of methylation of the *trans*-aconitate to the *E. coli* cells is not clear. The other *luxS*-dependent gene, *yneE*, is within close proximity to the *lsr* operon and encodes a protein with unknown function. This gene is transcribed in the opposite direction to the *tam* gene and the *lsr* operon, and so its up-regulation is potentially important and worthy of further investigation.

metE, which encodes methionine synthase, has much lower expression in the Δ*luxS* mutant than in the wild-type strain (9.7-fold decrease). This gene was identified before by Taga et al. in their search for *luxS*-controlled genes in *S. enterica* serovar Typhimurium (47). MetE catalyzes the last step of methionine synthesis in a vitamin B_{12}-independent pathway from homocysteine which, in turn, can be recycled from the LuxS-catalyzed reaction with *S*-ribosylhomocysteine. It was previously reported that homocysteine is required for the full induction of *metE* expression (5, 25, 48). Lack of homocysteine in the Δ*luxS* mutant was suggested to result in lower transcription of *metE* (47). On the other hand, homocysteine was shown to play an inhibitory role in the expression of MetA, which catalyzes the first reaction unique to the homocysteine synthetic pathway from homoserine (24). Consistent with this, we found that *metA* expression was slightly higher in the Δ*luxS* mutant (1.43-fold), which does not synthesize homocysteine via SAH detoxification. In addition, the expression level of MetB, the enzyme immediately downstream of MetA in homocysteine synthesis, was also increased by *luxS* deletion (1.93-fold).

Our results also showed that deletion of the *luxS* gene results in induction of several genes involved in utilization of various carbohydrates. The *rhaBAD* operon, which encodes enzymes responsible for utilization of L-rhamnose, and its regulatory gene *rhaSR* (51) all have increased expression in the *luxS* mutant (1.44-, 1.96-, 1.65-, 1.58-, and 2.47-fold, respectively). Expression of the *glvCBG* operon, which encodes putative proteins involved in utilization of arbutin, is also induced by 2.2-, 1.68-, and 1.78-fold, respectively. In addition, the *luxS* mutant has higher expression of *dcuC* and *xapB*, which encode proteins involved in transport of dicarboxylates and xanthosine (2.09- and 1.93-fold, respectively). It is not clear why the *luxS* deletion increases expression of these carbohydrate utilization genes.

In addition to the genes mentioned above, many of the *luxS*-regulated genes have unknown functions, which are worthy of further investigation.

Transcriptional regulation of *lsrR* by LsrR and CRP. It is interesting that *lsrR* expression is induced by *luxS*, as shown by the microarray experiment and quantitative RT-PCR. This was initially unexpected, because the produced LsrR cannot repress the *lsr* operon (or *lsr* regulon) in the presence of the inducer phospho–AI-2 under this condition. To investigate the control of *lsrR* transcription in greater detail, we constructed a *lacZ* fusion plasmid containing the *lsrR* promoter region and checked its expression levels in different mutant strains and under different growth conditions (Fig. 3). The overall regulatory pattern of the *lsrR* gene is similar to that of the *lsr* operon (50). When the wild-type ZK126 cells (*lsrR-lacZ*) were grown in LB medium, transcription from the *lsrR* promoter remained low until the cells entered the stationary phase (Fig. 3). Deletion of *lsrR* significantly increased *lsrR* expression, indicating that the *lsrR* transcription was autorepressed. Since phospho–AI-2 is an inducer that releases LsrR repression (46), it is reasonable to see that deletion of either *luxS* or *lsrK* reduced the *lsrR* expression, because there is no phospho–AI-2 available in these two deletion strains. Figure 3 also showed that deletion of the *lsrACDBFG* operon increased the *lsrR* transcription. The presence of an alternative AI-2 transport mechanism, and the absence of phospho–AI-2 degradation enzymes (LsrF and LsrG), may account for this increase, as suggested for *lsr* expression in the same mutant (50).

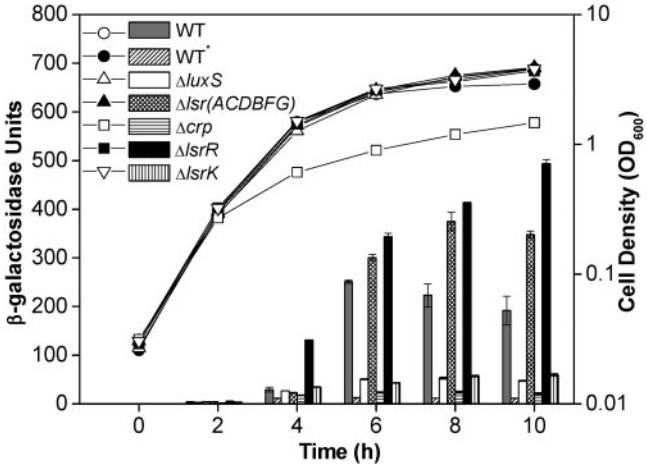

FIG. 3. Transcriptional regulation of *lsrR* expression. *E. coli* ZK126 (wild type) and strains containing deletion of *crp*, *luxS*, *lsrK*, *lsrR*, and *lsrACDBFG* carry plasmid pJLlsrR (*lsrR-lacZ*). All strains were grown in LB medium except for ZK126 (WT*), which was grown in LB plus 0.8% glucose. At different time points during cell growth, aliquots were collected for measurement of the OD_{600} (circles, triangles, and squares) and β-galactosidase activity (bars).

Finally, we found that addition of glucose to the growth medium significantly reduced *lsrR* transcription and that deletion of the *crp* gene had effects on *lsrR* expression similar to the addition of glucose (Fig. 3). These results suggested that *lsrR* expression was subject to catabolite repression, and CRP was needed for stimulation of *lsrR* transcription. In our previous paper (50), we identified one CRP binding site (CRP I) located upstream of the *lsr* promoter region which is necessary for activation of the *lsr* operon (Fig. 4A). Examination of the intergenic region between *lsrR* and the *lsr* operon, which are divergently transcribed, revealed another CRP binding site (CRP II), which has a typical 6-bp spacer between two conserved motifs (Fig. 4A). Gel mobility shift assay results (Fig. 4B) demonstrated that cAMP-CRP binds to a 46-bp DNA fragment in the intergenic region containing this site. CRP did not bind the identical DNA fragment with substitutions in 4 bp of one of the CRP-binding motifs. These findings positively confirm the CRP binding capability to the *lsrR* regulatory region. Whether the two CRP binding sites are independent or cooperate in stimulation of transcription of *lsrR* and the *lsr* operon needs further investigation; however, our results clearly indicate that the

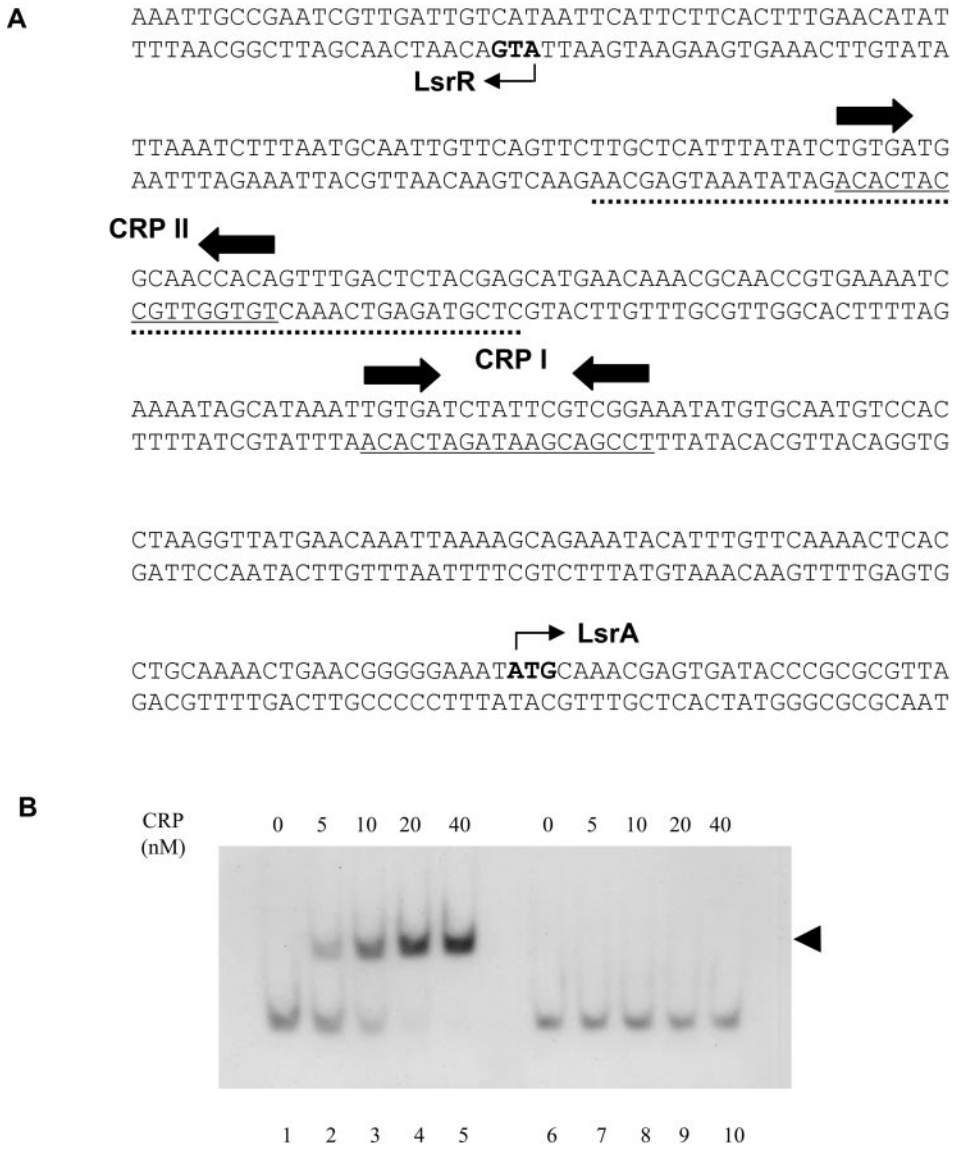

FIG. 4. CRP binding to regulatory regions of *lsrR* and the *lsr* operon. (A) Sequences of the promoter and regulatory regions of *lsrR* and the *lsr* operon. The underlined sequences indicate locations of the CRP binding site (CRP I and CRP II). The inverted arrows denote the conserved CRP binding motifs. The dotted DNA sequence was used in gel mobility shift assays. The translation start sites of LsrR and LsrA are shown by small arrows. (B) Gel mobility shift assays were performed as described in Materials and Methods. Digoxigenin-labeled DNA fragments which contained CRP II (the dotted sequence) or changed CRP II (with substitutions in 4 bp in the left CRP binding motif) (see Materials and Methods) were incubated with 0 to 40 nM of purified CRP, as indicated. cAMP was included in all reaction mixtures at a final concentration of 100 μM. The arrow denotes the CRP-DNA complex.

promoters of *lsrR* and the *lsr* operon are both subject to LsrR repression and CRP activation.

***lsrK* and *lsrR* belong to the same operon.** Microarray and quantitative RT-PCR results showed that *lsrK* expression was increased by the presence of *luxS*. This is consistent with its role as a kinase that phosphorylates AI-2 newly taken up. To confirm regulation of *lsrK* expression, we constructed another *lacZ* fusion plasmid with the *lsrK* promoter region (−367 to +53 relative to the start codon of *lsrK*). Surprisingly, deletion of *luxS*, *lsrR*, *lsrK*, or the *lsr* operon did not affect *lsrK* expression compared to the wild type (Fig. 5). In addition, the β-galactosidase activities for the *lsrK-lacZ* fusion were much lower than those of the *lsrR-lacZ* fusion (Fig. 3 and 5). However, we observed similar hybridization signals for *lsrK* and *lsrR* in the wild-type cells during the microarray experiments (data not shown). Further, we found that the *luxS* mutant cells had *lsrK* hybridization signals that were much lower than that for *lsrR* (data not shown). We speculated that *lsrK* could be transcribed together with *lsrR* under control of the *lsrR* promoter. To test this idea (*lsrRK* operon), we performed regular RT-PCR (differential display). Figure 6 shows that there exists a transcript spanning the coding sequences of both *lsrR* and *lsrK*. In addition, the level of this transcript was much lower in the *luxS* deletion mutant compared to the wild type. These results indicated that *lsrR* and *lsrK* belong to one operon. It is possible that

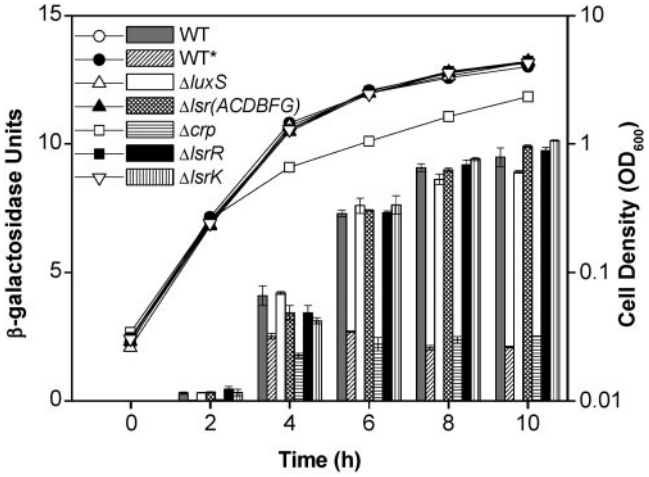

FIG. 5. Transcriptional regulation of *lsrK* expression. *E. coli* ZK126 (wild type) and strains containing deletions of *crp*, *luxS*, *lsrK*, *lsrR*, and *lsrACDBFG* carry plasmid pJLlsrK (*lsrK-lacZ*). All strains were grown in LB medium except ZK126 (WT*), which was grown in LB plus 0.8% glucose. At different time points during cell growth, aliquots were collected for measurement of the OD_{600} (circles, triangles, and squares) and β-galactosidase activity (bars).

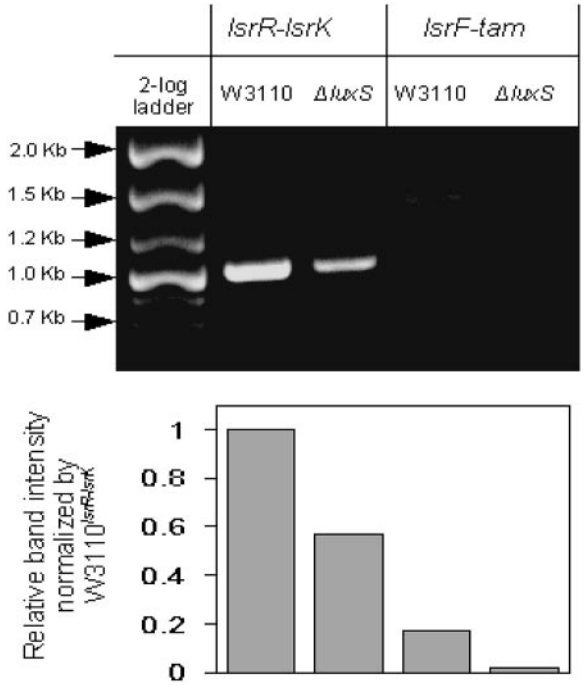

FIG. 6. Transcriptional analysis of *lsr* and the *lsrRK* operon. The agarose gel was run to show DNA fragments obtained from RT-PCR of total RNA prepared from the OD 2.4 cell cultures of the wild type (WT) and the Δ*luxS* mutant grown in LB. Specific primers were used to amplify the fragments that span coding sequences of the *lsrR-lsrK* and *lsrF-tam* genes. The 2-log DNA ladder (New England BioLabs) and intensity results (NIH Image J) are depicted.

weak transcription from the *lsrR* promoter in the *luxS* mutant, which is therefore repressed by LsrR, accounts for the polar effect of transcription.

Intriguingly, we also found that addition of glucose (0.8%) to the growth medium or deletion of the *crp* gene reduced transcription of the *lsrK-lacZ* fusion (Fig. 5), suggesting additional control of *lsrK* by catabolite repression and CRP, although there are no apparent CRP binding sequences in the *lsrK* promoter region. Whether CRP directly acts on this promoter awaits further study.

The *E. coli lsr* operon includes an additional gene, *tam*. The *E. coli lsrACDBFG* operon is similar to the *S. enterica* serovar Typhimurium *lsrACDBFGE* operon, except that it does not contain an *lsrE* homolog. However, the *tam* gene is located immediately downstream of the $lsr_{E.c}$ operon. Although several programs predict the existence of a potential *tam* promoter, there appears to be no obvious transcription terminator between *lsrG* and *tam*. Both microarray and quantitative RT-PCR showed that *tam* expression was increased by *luxS* (about 3.4-fold). Although this induction was lower than those for the other *lsr* genes, additional transcriptional controls may exist that cause this difference. To check whether the *tam* gene belongs to the *lsr* operon, we again used RT-PCR to see whether we could amplify a region spanning coding sequences of *lsrF*, *lsrG*, and *tam*. The results in Fig. 6 support our hypothesis, showing amplification of this region from wild type, but not from Δ*luxS* (with limited PCR cycles). It should be noted that higher numbers of PCR cycles resulted in the appearance of *lsrF-tam* products from both strains (data not shown).

DISCUSSION

In our previous search for AI-2-regulated genes in *E. coli* (11), conditioned media (with or without AI-2) from the same wild-type cells (W3110) and a *luxS* mutant (MDAI2) were added to *luxS* mutant cultures, revealing 242 genes that were significantly affected by the resultant 300-fold differential in AI-2. Our study provided useful information on the global effects of AI-2 and *luxS*; however, because the addition of AI-2 was accompanied by conditioned media to the mutant, there remains ambiguity as to whether the observed effects were caused by AI-2, other compounds in the conditioned medium, or *luxS*. In the current study, we directly compared the transcriptional profiles of W3110 and the *luxS* mutant and identified the *luxS*-controlled genes under two different growth conditions. Some of these genes, such as *metE* and the *lsrACDBFG* operon, were identified previously in *S. enterica* serovar Typhimurium as *luxS* regulated by using a different method (47). The identified *luxS*-controlled genes in *E. coli* K-12 strain W3110 are different from those identified in EHEC O157:H7 (42); there are significantly fewer genes regulated by *luxS* in W3110. In addition, unlike the EHEC strain, there were no apparent phenotypic differences between the W3110 wild-type and the Δ*luxS* mutant cells under the investigated conditions.

Sperandio et al. (42) reported that 404 genes were regulated by *luxS* at least fivefold in the EHEC strain, in which the flagellum and motility genes were highly induced by *luxS*. In that study, the EHEC wild-type and *luxS* deletion cells were grown to an OD_{600} of 1.0 in Dulbecco's modified Eagle's medium at 37°C. One phenotypic difference between the *luxS* mutants of W3110 and EHEC is that the *luxS* mutant of EHEC grows much faster than its parent strain (42), while the growth of the W3110 *luxS* mutant and its parent strain are indistin-

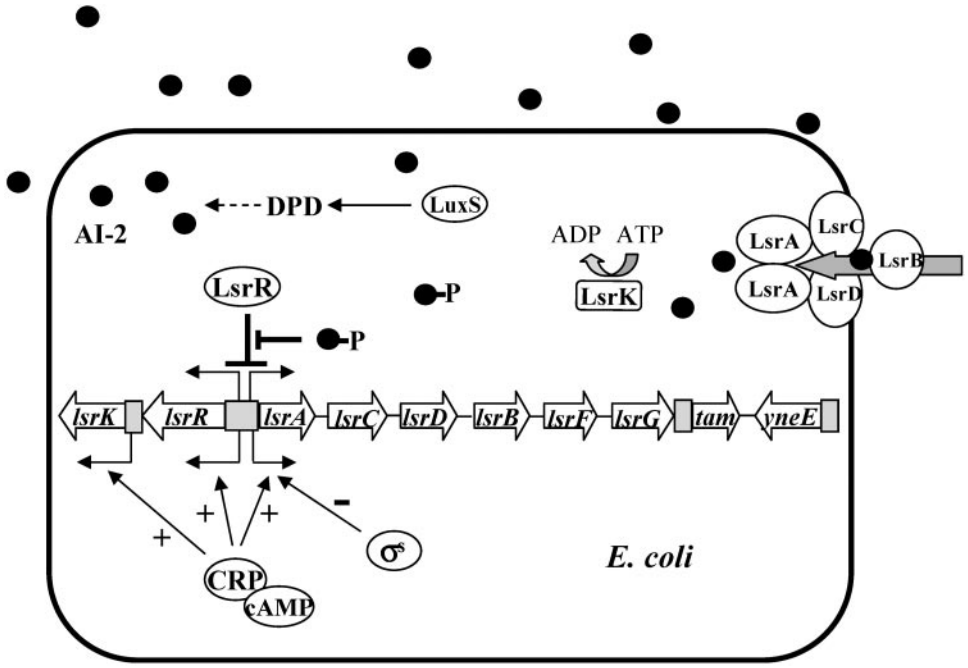

FIG. 7. Schematic of *lsr* and *lsrRK* operon regulation. cAMP-CRP and LsrR are involved in transcriptional control of *lsr* and *lsrRK* operons. cAMP-CRP, a positive regulator, stimulates expression of both operons in the absence of glucose, while LsrR prevents their expression. The function of LsrR is inhibited when cell density increases and inducer phospho–AI-2 accumulates. In addition, cAMP-CRP stimulates transcription from the *lsrK* promoter, and σ^s negatively regulates *lsr* expression (50). Also included in the schematic is the *yneE* gene, whose expression is increased by *luxS*. Gray boxes denote promoter. Plus and minus signs indicate positive and negative transcriptional regulation. DPD, 4,5-dihydroxy-2,3-pentanedione.

guishable under the investigated conditions. Although we do not understand the reason for the *luxS*-mediated growth stimulation in EHEC, we suspect that the faster growth rate in the EHEC *luxS* mutant may have distorted the effects reported for the *luxS* mutation. A second phenotypic difference between the two strains was that the EHEC *luxS* mutant had reduced motility relative to the wild type (42), while the W3110 *luxS* mutant does not.

Genomic comparisons of K-12 and EHEC O157:H7 strains revealed that they share a 4.1-Mb backbone sequence, which is punctuated by hundreds of strain-specific genomic regions (K-islands and O-islands) (14, 18, 33). These genetic differences as well as the difference in growth conditions between the studies may account for the divergence in identified genes controlled by *luxS*. That the genes identified under the two growth conditions in our work were mostly of orthogonal sets supports the latter of these hypotheses.

Both the current and previous studies (47) indicated that methionine metabolism and regulation were affected by deletion of *luxS*. At an OD of 2.4 without glucose, the expression of *metE* genes was most repressed in the Δ*luxS* mutant, while expression levels of other methionine synthesis and regulation genes were either unaffected or to a lesser degree. Consistent with this, the *metJ* gene, which encodes a major repressor of the *met* regulon (16), did not exhibit significant differences in expression level. It should be noted that the stable expression of MetH, the B_{12}-dependent methionine synthase, might ensure that the reduced MetE level in the Δ*luxS* mutant did not decrease methionine synthesis significantly under the investigated conditions. That is, relative stability in the biosynthesis and utilization of methionine and *S*-adenosylmethionine might be important for the cells to function normally, i.e., to preserve peptide synthesis, methyl donation, and spermidine synthesis (16). We do not understand the reason for increased expression of *metB* in the Δ*luxS* mutant (1.93-fold), although it may suggest potential relatedness between *luxS* and *metB*. It is interesting that the two genes are transcribed in a 4.9-kb operon (*ycgJ-metB-cysK-luxS*) in the gram-positive pathogen *Clostridium perfringens* (30, 31), although they are transcribed separately in *E. coli*. Our results also indicated that the growth conditions influenced the degree of repression of *metE*. When grown in glucose at an OD of 1.0, the repression of *metE* expression in the Δ*luxS* mutant was much lower (1.81-fold). It is not clear whether the global effect of glucose on cell metabolism caused this difference.

Importantly, our microarray and RT-PCR results revealed an unexpected regulatory mode for the *E. coli lsr* operon (Fig. 7). It was shown that *lsrR* and *lsrK* belong to the same *lsrRK* operon, and the *E. coli lsr* operon includes an additional gene, *tam*. Although we do not understanding the advantages conferred upon *E. coli* cells by Tam-mediated methylation of *trans*-aconitate, it appears linked to the AI-2 biosynthesis pathway through the reaction of the *S*-adenosylmethionine-dependent methyl transfer. Transcriptional control of *tam* may be complicated due to the potential promoter/regulatory sequence between *lsrG* and *tam* in addition to the *lsrA* promoter. It is interesting that another *luxS*-induced gene, *yneE*, which is

adjacent to *tam* and is transcribed in the opposite direction as the *lsr* operon, was significantly affected. We do not know the function of *yneE*, or whether it is involved in *lsr* regulation. It was shown earlier that the *S. enterica* serovar Typhimurium *lsr* operon does not contain *tam* or *yneE* (47). Instead, it includes the *lsrE* gene at the end of the *lsr* operon, which is homologous to the *rpe* gene that encodes a ribulose phosphate epimerase (47). Whether these genetic differences between these two organisms cause differences in *luxS*-regulated genes and related cellular activities awaits further investigation.

It was unexpected that the *lsr* and *lsrRK* operons, which are divergently transcribed, are controlled by the same transcriptional regulators: CRP and LsrR (Fig. 7). CRP, as a global regulator, is needed for activation of these two operons through direct binding to two different sequences within the operator regions. LsrR, as a repressor protein for both operons, undergoes dynamic control of the transcription network through a negative autoregulation feedback loop, which has been shown to speed up the transcription response and reduce cell-to-cell fluctuations in the steady-state level of the transcription repressor (1, 10, 36). In the absence of active inducer phospho–AI-2, the LsrR proteins not only repress expression of the *lsr* operon but also repress transcription of the *lsrRK* operon. However, when inducers are available, repression by LsrR is released, and transcription of the two operons is increased rapidly. The effect of this autoregulation of LsrR is a more severe or amplified "switch" that responds to high levels of AI-2. Increased levels of LsrR provide a mechanism for quickly shutting down expression of the *lsr* and *lsrRK* operons when the inducer phospho–AI-2 is not available later due to the degradation by LsrF and LsrG (46).

It was shown in this study that deletion of *luxS* affects genes associated with different cell activities in *E. coli* K-12, such as AI-2 transport, biosynthesis of methionine, transfer of methyl groups, iron uptake, and the utilization of different carbon sources. Many of the differentially expressed proteins have unknown functions. For this study, we had expected to identify genes whose expression might respond to AI-2 signaling and to the metabolic effects of the *luxS* deletion, such as accumulation of *S*-ribosylhomocysteine and a reduced level of homocysteine. It was shown that the most highly induced genes (such as the *lsr* operon, *lsrR*, *lsrK*, *tam*, and *metK*) are related to AI-2 production and transport, while the genes involved in other activities are induced to a lesser degree. These data are consistent with the function of LuxS as an important metabolic enzyme in *E. coli* K-12 and suggest that its role as a signal molecule might require additional cellular factors not revealed in our study. In a previous study (50), we showed that the presence of glucose strongly inhibits expression of the *lsr* operon through control of cAMP-CRP. That the *lsr* operon is induced only in the absence of glucose is consistent with the notion that AI-2 is used here as a carbon source. Although Taga et al. reported that *S. enterica* serovar Typhimurium could not grow on AI-2 as the sole carbon source (47), additional conditions may be needed for AI-2 utilization as a carbon source, as suggested by Winzer et al. (52). It is possible that AI-2, which is synthesized by many species, is perceived as a true signal only by specific bacterial species or under specific conditions.

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