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Cyclic AMP (cAMP) and cAMP Receptor Protein Influence both Synthesis and Uptake of Extracellular Autoinducer 2 in *Escherichia coli*

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Bacterial autoinducer 2 (AI-2) is proposed to be an interspecies mediator of cell-cell communication that enables cells to operate at the multicellular level. Many environmental stimuli have been shown to affect the extracellular AI-2 levels, carbon sources being among the most important. In this report, we show that both AI-2 synthesis and uptake in *Escherichia coli* are subject to catabolite repression through the cyclic AMP (cAMP)-CRP complex, which directly stimulates transcription of the *lsr* (for "*luxS* regulated") operon and indirectly represses *luxS* expression. Specifically, cAMP-CRP is shown to bind to a CRP binding site located in the upstream region of the *lsr* promoter and works with the LsrR repressor to regulate AI-2 uptake. The functions of the *lsr* operon and its regulators, LsrR and LsrK, previously reported in *Salmonella enterica* serovar Typhimurium, are confirmed here for *E. coli*. The elucidation of cAMP-CRP involvement in *E. coli* autoinduction impacts many areas, including the growth of *E. coli* in fermentation processes.

Bacteria have evolved complex genetic circuits to modulate their physiological states and behaviors in response to a variety of extracellular signals. In a process termed quorum sensing, or density-dependent gene regulation, bacteria produce, release, and respond to signaling molecules (autoinducers), which accumulate as a function of cell density. Quorum sensing allows bacteria to communicate with each other and coordinate their activities at a multicellular level. The autoinducers of many gram-positive bacteria are secreted peptides (30, 42), while gram-negative bacteria use small chemical molecules (60). Among gram-negative bacteria, the LuxI/LuxR signal synthase-signal receptor system is the most studied at the molecular level, with the signaling species being a family of *N*-acylhomoserine lactones. However, the cross-species autoinducer, autoinducer 2 (AI-2), has received intense interest recently because the gene for its terminal synthase, *luxS*, is present in over 55 bacteria and its activity can be readily assayed biologically (61). It is known that quorum sensing regulates diverse cellular processes, including bioluminescence (19, 34), spore formation (33), motility (18, 22), competence (35), conjugation (20), antibiotic synthesis (2, 17), virulence (38, 44, 50), and biofilm maturation (13, 45).

Our laboratory is interested in understanding and controlling microbial behavior in bioreactors in order to enhance recombinant protein synthesis and yield. Since quorum sensing is emerging as a global regulator of many intracellular processes, including those that influence protein synthesis, efforts to understand this "tunable" controller are essential. In our previous work using chemostat cultures (14), many stimuli were found to affect the level of AI-2. Among these, the pulsed addition of glucose, a common carbon source for recombinant *Escherichia coli* fermentations, resulted in increased AI-2 levels, but with the dynamic response dependent on the steady-state growth rate (e.g., dilution rate) of the culture. Indeed, AI-2 production on a per-cell basis was linearly proportional to the growth rate of the cells (14). Also, the level of AI-2 in extracellular fluids was reduced relative to that in controls in direct response to protein overexpression. This was attributed to the metabolic burden commonly associated with the requisite redirection of metabolites and resources (15).

The level and possible role of AI-2 as a mediator of intra- and intercellular coordination have been the subject of varied interpretations, as recently reviewed by Ahmer (1). Outside of the *Vibrio* genus, only the *lsr* operon of *Salmonella* has been found to directly respond to AI-2, although mutation of its synthase, LuxS, has far-reaching effects (16, 51), but this evidence is muddled by the coincident effects on cell growth (1) and the potential accumulation of *S*-ribosylhomocysteine (SRH). Hence, it is important to delineate pleiotropic effects of *luxS* gene knockout and of varying the glucose level and growth rate from molecular events directly attributed to AI-2. In the present work, we investigated the mechanistic effects of glucose on the synthesis and uptake of AI-2 in *E. coli* W3110.

Schauder et al. demonstrated that AI-2 is produced from *S*-adenosylmethionine (SAM) in three enzymatic steps and that LuxS is an AI-2 synthase (48) (Fig. 1). SAM serves as methyl donor in a variety of methylation reactions and as a propylamino donor in polyamine biosynthesis (24). The methyl group of SAM is transferred by several methyltransferases to its acceptors, resulting in production of *S*-adenosylhomocysteine (SAH). Accumulation of SAH is toxic to the cell, and it

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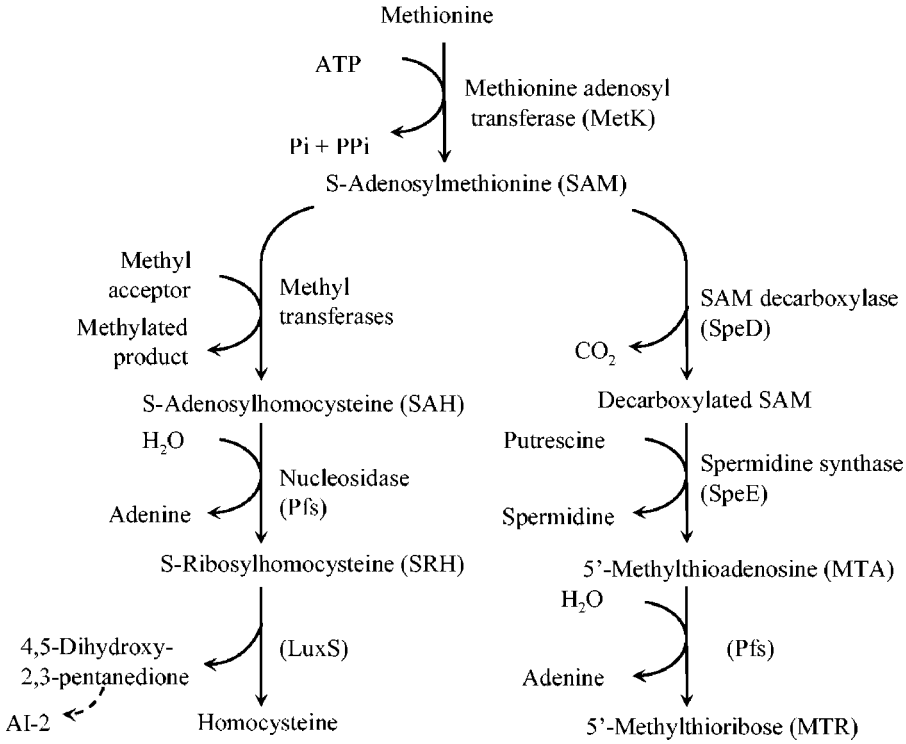

FIG. 1. Pathways for AI-2 biosynthesis and SAM utilization in *E. coli.*

is rapidly degraded by a nucleosidase, Pfs, into adenine and SRH. LuxS acts on SRH to produce homocysteine, which can be recycled to methionine (24) and 4,5-dihydroxy-2,3-pentanedione, which likely undergoes cyclization and further rearrangement to yield AI-2 (10, 39, 48). Interestingly, the same nucleosidase, Pfs, participates in the SpeD-directed SAM decarboxylation pathway that ultimately produces methylthioribose during synthesis of polyamines. In some bacteria, Pfs is also involved in the pathway responsible for *N*-acylhomoserine lactone production (40, 43, 56).

The uptake of AI-2 was recently elucidated by Taga et al. in *Salmonella enterica* serovar Typhimurium (55). A genetic screen for *luxS*-regulated genes in serovar Typhimurium resulted in identification of the *lsr* operon (55). The *lsrACDBFGE* operon encodes an AI-2 uptake and modification system that has similarity to the ribose transport system of *E. coli* and serovar Typhimurium. The AI-2 transport apparatus is encoded by the first four genes (*lsrA*, *lsrC*, *lsrD*, and *lsrB*). Upon entry into cells, AI-2 is phosphorylated by a kinase encoded by *lsrK* (54). The phospho–AI-2 appears to be the inducer that releases LsrR-mediated repression of *lsr* transcription (54). LsrR is homologous to SorC, a DNA-binding transcriptional regulator involved in sorbose metabolism in *Klebsiella pneumoniae* (55). At this time, LsrR has not been shown to bind to the promoter region of the *lsr* operon, and it is not clear whether the effect of phospho–AI-2 on LsrR is direct, although these are likely, based on the available data. LsrF and LsrG are responsible for further processing of phospho–AI-2 (54). In *E. coli*, there exists a similar set of genes, including an *lsr* operon (b1513 operon) and homologues to *lsrK* (*ydeV*) and *lsrR* (*ydeW*); there is no *lsrE* homologue. To date, there have been no studies regarding the function and/or regulation of the *E. coli lsr* operon.

It has long been known that the presence of glucose (or other phosphotransferase system [PTS] sugars) in the growth medium of *E. coli* and serovar Typhimurium cultures affects the level of extracellular AI-2 (25, 52). Moreover, maximal AI-2 activity is typically observed during mid- to late exponential phase, and this extracellular activity is removed when glucose becomes depleted (52). These findings suggest a linkage between catabolite repression and AI-2 production and transport. In this study, we show that catabolite repression influences AI-2 accumulation through the cyclic AMP (cAMP)-cAMP receptor protein (CRP) complex, which directly stimulates transcription of the *lsr* operon and indirectly represses *luxS* expression. cAMP-CRP is shown to bind to the upstream region of the promoter of the *lsr* operon and works with the LsrR repressor to regulate AI-2 uptake. A working model describing the appearance and disappearance of AI-2 in *E. coli* cultures is presented, along with our interpretation of AI-2 regulation.

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains used in this study are listed in Table 1. Luria-Bertani broth (LB) contained 5 g of yeast extract (Sigma) $liter^{-1}$, 10 g of Bacto tryptone (Difco) $liter^{-1}$, and 10 g of NaCl $liter^{-1}$. Glucose and cAMP, when present, were added at 0.8% and 10 mM, respectively. The autoinducer bioassay (AB) and Luria-marine (LM) media are described in detail elsewhere (6, 23). When necessary, media were supplemented with antibiotics at the following concentrations: ampicillin, 60 $\mu g\ ml^{-1}$; kanamycin, 50 $\mu g\ ml^{-1}$.

Plasmid construction. The plasmids used in this study are listed in Table 1 and were generated using standard procedures (47). Restriction enzymes, T4 DNA

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	Laboratory stock
LW1	W3110 Δ*crp*::Kan	This study
LW5	W3110 Δ(*lsrACDBFG*)::Kan	This study
LW6	W3110 Δ*lsrR*::Kan	This study
LW10	W3110 Δ*lsrK*::Kan	This study
HT28	W3110 Δ*cya*::Kan	29
ZK126	W3110 Δ*lacU169 tna-2*	11
ZK1000	ZK126 Δ*rpoS*::Kan	8
LW2	ZK126 Δ*crp*::Kan	This study
LW7	ZK126 Δ*luxS*::Kan	This study
LW8	ZK126 Δ*lsrR*::Kan	This study
LW9	ZK126 Δ(*lsrACDBFG*)::Kan	This study
LW11	ZK126 Δ*lsrK*::Kan	This study
V. harveyi strains		
BB152	BB120 *luxL*::Tn5 (AI-1$^-$, AI-2$^+$)	52
BB170	BB120 *luxN*::Tn5 (sensor 1$^-$, sensor 2$^+$)	5
Plasmids		
pFZY1	*galK′-lacZYA* transcriptional fusion vector, Ap^r	32
pLW10	pFZY1 derivative, containing *luxS* promoter region, Ap^r	This study
pLW11	pFZY1 derivative, containing *lsrACDBFG* promoter region, Ap^r	This study
pLW12	pFZY1 derivative, containing mutated *lsrACDBFG* promoter region, Ap^r	This study
pYH10	pFZY1 derivative, containing *pfs* promoter region, Ap^r	This study
pLW9	pCR-Blunt (Invitrogen) derivative, containing *luxS* promoter region, Kan^r	This study
pHA7E	pBR322 derivative, *crp*$^+$ Ap^r	27
pIT302	pACYC184 derivative, *cya*$^+$ Cm^r	29

ligase, and Vent DNA polymerase were used as specified by the manufacturer (New England Biolabs, Beverly, Mass.). An *E. coli* W3110 chromosomal DNA preparation was performed using the QIAGEN DNeasy tissue kit (QIAGEN, Valencia, Calif.). Extractions of DNA from agarose gels were performed using the QIAGEN Qiaquick gel extraction kit. Oligonucleotides were from Gene Probe Technologies (Gaithersburg, Md.). 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.

Plasmid pFZY1 is a mini-F derivative (average copy number, one to two per cell) with a polycloning site upstream of a promoterless *galK′-lacZYA* reporter segment (32). To create pLW10, the *luxS* promoter region was amplified by PCR using primers luxSpF and luxSpR (Table 2). This DNA fragment was cloned into the pCR-Blunt vector (Invitrogen). The resulting plasmid, pLW9, was digested with EcoRI; the fragment containing the *luxS* promoter was inserted into the EcoRI site of pFZY1, producing plasmid pLW10. The *luxS* promoter region (−104 to +36 relative to the *luxS* start codon) contains the native sequence up to the transcriptional terminator of the upstream gene *gshA*. To create pYH10, the *pfs* promoter region (−337 to +113 relative to the *pfs* start codon) was amplified by PCR using the primers pfspF and pfspR (Table 2). The purified

TABLE 2. Oligonucleotide primers used in this study

Name	Sequence[a]	Relevant description
luxSpF	CCACTCGTGAGTGGCCAA	Upstream primer for cloning *luxS* promoter
luxSpR	GGTATGATCGACTGTGAAGCTATCTAA	Downstream primer for cloning *luxS* promoter
pfspF	CCGGAATTCAAAATTTCTTTGGCGATGTAGCG	Upstream primer for cloning *pfs* promoter
pfspR	CGCGGATCCAGTTGGCCGGTATAGATTTCGC	Downstream primer for cloning *pfs* promoter
lsrpF	CCGGAATTCGCGACCTGTTCTTCTTCACACATT	Upstream primer for cloning *lsr* operon promoter
lsrpR	CTCGGATCCTCGATGCCTTTCAGGACATTG	Downstream primer for cloning *lsr* operon promoter
lsrpM	ATAGCATAAATCGATCTCTATTCGTCGGAAATATGTGCAATG	Primer for making mutation of *lsr* operon promoter
crpHP1	ACTCTCGAATGGTTCTTGTCTCATTGCCACATTCATAAGTGTAGGCTGGAGCTGCTTC	Primer for deletion of *crp* gene
crpHP2	AAACGACGATGGTTTTACCGTGTGCGGAGATCAGGTTCTGCATATGAATATCCTCCTTAG	Primer for deletion of *crp* gene
luxSHP1	ATGCCGTTGTTAGATAGCTTCACAGTCGATCATACCCGGAGTGTAGGCTGGAGCTGCTTC	Primer for deletion of *luxS* gene
luxSHP2	CTAGATGTGCAGTTCCTGCAACTTCTCTTTCGGCAGTGCCCATATGAATATCCTCCTTAG	Primer for deletion of *luxS* gene
lsrRHP1	ATGACAATCAACGATTCGGCAATTTCAGAACAGGGAATGTGTGTAGGCTGGAGCTGCTTC	Primer for deletion of *lsrR* gene
lsrRHP2	TGATCGGTAACCAGTGCGTTGATATAACCGCCTTTCATTGCATATGAATATCCTCCTTAG	Primer for deletion of *lsrR* gene
lsrKHP1	GCGTGAAGGCATTGTTTTATATAACAATGAAGGAACACCGTGTAGGCTGGAGCTGCTTCG	Primer for deletion of *lsrK* gene
lsrKHP2	TCCGCCTGCAAAGACTAACGATGAAGGATGAATAGTCGAATTCCGGGGATCCGTCGACC	Primer for deletion of *lsrK* gene
lsrHP1	CGCTCGGTTTATAAACAGTATTCAGGGGTCAATGTCCTGAGTGTAGGCTGGAGCTGCTTC	Primer for deletion of *lsrACDBFG* operon
lsrHP2	AGATTCCAGTTTCGCGACACAGGTTTTGTAGTGGGGCGTGCATATGAATATCCTCCTTAG	Primer for deletion of *lsrACDBFG* operon

[a] The underlined sequences anneal to the template plasmid, while the remaining sequences correspond to the ends of the indicated genes.

PCR product was digested with EcoRI-BamHI and was inserted into EcoRI-BamHI-digested pFZY1. To create pLW11, the promoter region of the *lsrACDBFG* operon [−307 to +92 relative to the start codon of *lsrA* (b1513)] was amplified by PCR using primers lsrpF and lsrpR (Table 2). The purified PCR product was digested with EcoRI-BamHI and was inserted into EcoRI-BamHI-digested pFZY1.

To create pLW12, the method of site-directed mutagenesis by PCR (21, 28) was used and modified as follows. Two subsequent PCR steps were carried out. In the first round of PCR, lsrPM and lsrPR were used as primers with pLW11 as template. The PCR products were purified with a QIAGEN MinElute PCR purification kit and used as a megaprimer in the second round of PCR with pLW11 as the template and lsrPF as another primer. The products in the second round of PCR were purified using the QIAGEN Qiaquick gel extraction kit and cloned into pCR-Blunt II TOPO vector (Invitrogen), which was transformed into Top10 competent cells (Invitrogen). The plasmids were prepared from the transformants and sequenced to confirm the mutation of the *lsr* promoter region. The plasmid with correct insertion was digested with EcoRI-BamHI, and the DNA fragment containing a mutated *lsr* promoter was inserted into EcoRI-BamHI-digested pFZY1.

Chromosomal deletions of *crp*, *luxS*, *lsrR*, *lsrK*, and the *lsrACDBFG* operon. The one-step replacement method described by Datsenko and Wanner (12) was used to construct a *crp* deletion in *E. coli* W3110 and ZK126. We used the phage λ Red recombination system to replace *crp* with a *crp*::*kan* PCR fragment. PCR was done using pKD4 as template and the primers crpHP1 and crpHP2 (Table 2). The PCR products were treated with DpnI and introduced by electroporation into *E. coli* W3110 or ZK126 containing plasmid pKD46, which expresses the Red recombinase and was cured later by growth at 37°C. Recombinants were selected on LB supplemented with kanamycin. Deletions of *luxS*, *lsrR*, and the *lsrACDBFG* operon were constructed similarly by PCR amplification of pKD4 with primers luxSHP1 and luxSHP2, lsrRHP1 and lsrRHP2, and lsrHP1 and lsrHP2, respectively, except that in making the deletion of *lsrK*, pKD13 was used as template with primers lsrKHP1 and lsrKHP2 for PCR (Table 2). The deletion of genes was verified by PCR tests.

Preparation of cell-free fluids. Unless otherwise stated, an overnight culture grown in LB or LB plus 0.8% glucose was diluted 100-fold to an optical density at 600 nm (OD_{600}) below 0.03 in LB or LB plus 0.8% glucose. Cells were incubated at 30°C with shaking at 250 rpm in Erlenmeyer flasks. Samples were removed at regular intervals. Cell-free culture fluids were prepared by centrifugation of the *E. coli* culture at 12,000 rpm for 10 min in a microcentrifuge. Cleared supernatants were filtered (0.2-μm-pore-size HT Tuffryn filters; Pall Corp., Ann Arbor, Mich.) and stored at −20°C.

AI-2 activity assay. *E. coli* cell-free culture fluids were tested for the presence of AI-2 by inducing luminescence in *Vibrio harveyi* reporter strain BB170. The assays were performed as outlined by Surette and Bassler (52). Briefly, BB170 was grown for 16 h with shaking at 30°C in AB medium, diluted 1:5,000 in fresh AB medium, and aliquoted to sterile 12- by 75-mm tubes (Fisher Scientific Co., Inc., Pittsburgh, Pa.). Cell-free culture fluids were added to a final concentration of 10% (vol/vol) to these tubes. Positive controls contained 10% (vol/vol) cell-free culture fluid from BB152, while negative controls contained 10% (vol/vol) sterile LB or LB plus 0.8% glucose. Tubes were shaken at 175 rpm and 30°C in an air shaker (New Brunswick Scientific), and hourly measurements of luminescence were taken. Luminescence was measured as a function of *V. harveyi* cell density by quantitating light production with a luminometer (EG&G Berthold, Gaithersburg, Md.). *V. harveyi* cell density was determined by spreading identical dilutions used for luminescence measurements onto solid LM medium and counting colonies after overnight growth. Relative light units (RLU) were defined as follows: [(counts per minute per milliliter) (10^3)]/(CFU per milliliter). AI-2 activities were obtained by dividing the RLU produced by the reporter after addition of *E. coli* culture fluid by the RLU of the reporter when growth medium alone was added (52, 53). The obtained values were in a linear range.

β-Galactosidase assays. Cultures of *E. coli* were grown overnight in LB, diluted 100-fold into fresh LB, grown to mid-exponential phase, and then diluted into different medium 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 intervals for determination of the OD_{600} and β-galactosidase activity. Specific activity of β-galactosidase is expressed in Miller units (37).

Gel mobility shift assay. The 140-bp EcoRI fragment containing the promoter region of the *luxS* gene was prepared from pLW9. The 42-, 89-, and 120-bp DNA fragments containing the wild-type or mutated promoter regions of the *lsr* operon were synthesized by Integrated DNA Technologies (Coralville, Iowa). 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 of bovine serum albumin/ml, 15 μg of sonicated salmon sperm DNA/ml, 100 μM cAMP). Following incubation at 37°C for 10 min, 5 μl of gel loading buffer (0.25× 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.

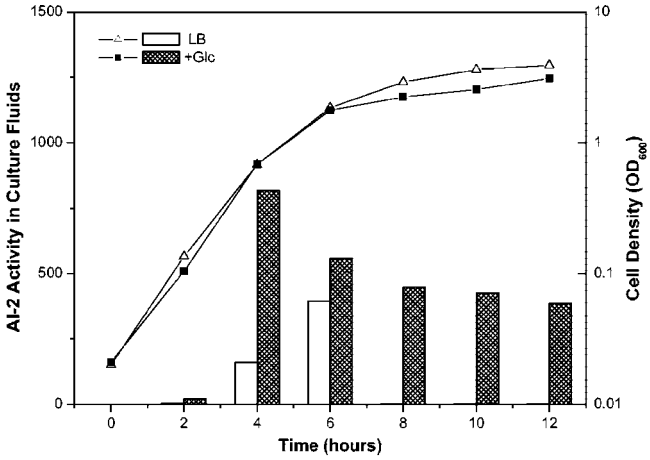

FIG. 2. Effects of glucose on extracellular AI-2 activity. Overnight cultures of *E. coli* W3110 were diluted in LB or LB plus 0.8% glucose to an OD_{600} below 0.03. At different time points during cell growth, aliquots were collected for measurement of the OD_{600} (triangles and squares) and AI-2 activity (bars). AI-2 activity in the culture fluids was measured using the *V. harveyi* BB170 AI-2 bioassay, and the values shown are representative of three independent experiments (some values were very small, but measured, as indicated). Replicate assays agreed to within 10%.

RESULTS

Extracellular AI-2 activity is increased by mutation of *crp* and *cya*. Extracellular AI-2 activity is produced when *E. coli* is grown with glucose or other PTS saccharides (52). The levels of extracellular AI-2 in the cell-free culture fluids of *E. coli* W3110 grown in LB and LB containing 0.8% glucose are shown in Fig. 2. AI-2 was produced under both growth conditions but at different levels. In the presence of glucose, AI-2 activity increased during the exponential phase, reached the maximum at 4 h, and then declined slowly. This result is consistent with a previous report (52). However, we also found that when grown in LB in the absence of glucose, the bacteria still produced AI-2 activity during exponential phase, but at a much lower level. Furthermore, no AI-2 activity in cell-free culture fluids was detected after the cells entered stationary phase.

Glucose is known to affect gene expression through the cAMP-CRP complex, and the presence of glucose in the growth medium results in decreased levels of cAMP and CRP (26, 36). To check whether cAMP and CRP are involved in the production of extracellular AI-2 activity, we compared the extracellular AI-2 activity produced by W3110 with that of isogenic *crp* and *cyaA* mutants grown in LB (Fig. 3). Deletion of the *crp* gene or the *cya* gene in W3110 resulted in much higher AI-2 activity throughout the cell cultures (peak levels

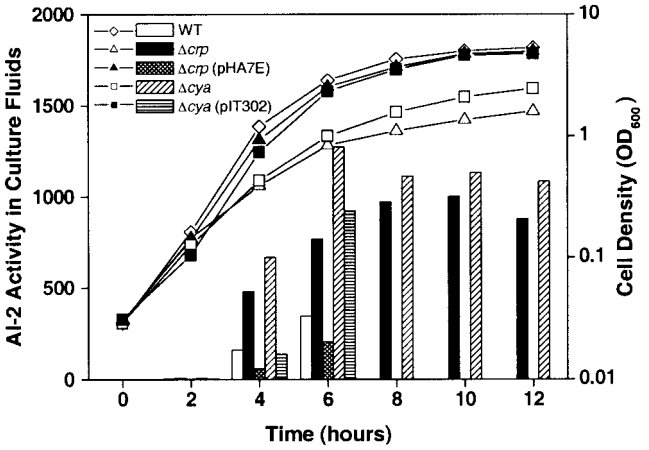

FIG. 3. *crp* and *cya* mutations increase extracellular AI-2 activity. Overnight cultures of *E. coli* W3110 (wild type) and strains containing deletion of *crp* and *cya* were diluted in LB to an OD_{600} below 0.03. At different time points, aliquots were collected for measurement of the OD_{600} (diamonds, triangles, and squares) and AI-2 activity (bars). Plasmids pHA7E and pIT302 carry wild-type *crp* and *cya* genes, respectively. AI-2 activities shown are representative of three independent experiments. Replicate assays agreed to within 10%.

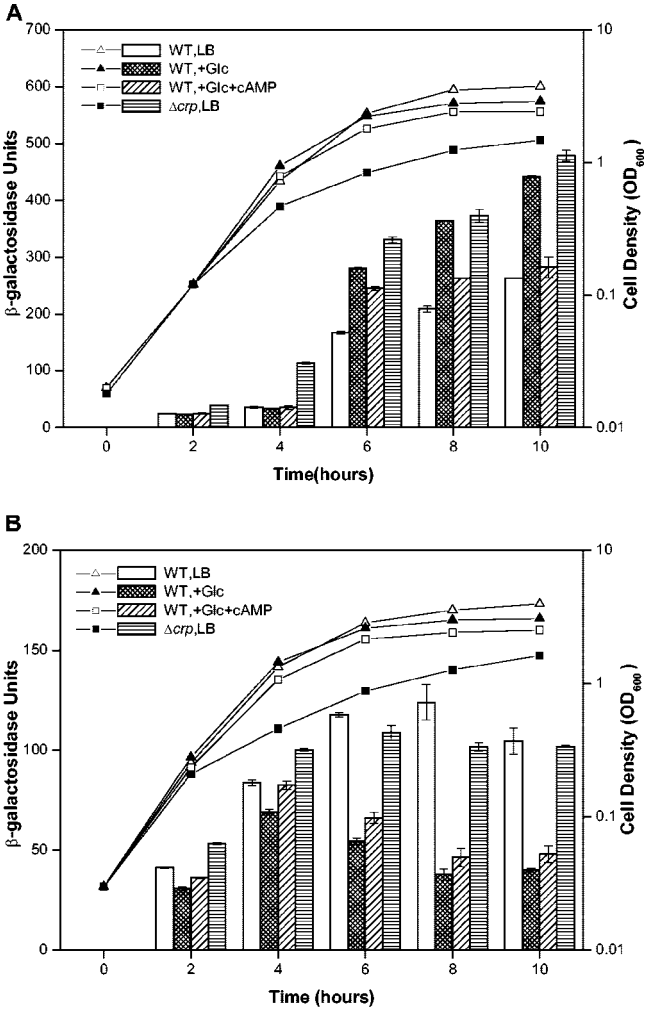

FIG. 4. Effects of cAMP and CRP on the transcription of *luxS* and *pfs*. Conditions for cell growth and β-galactosidase activity are described in Materials and Methods. *E. coli* ZK126 (wild type) and isogenic *crp* mutant carrying plasmid pLW10 (*luxS-lacZ*) (A) and pYH10 (*pfs-lacZ*) (B) were grown in LB, LB plus 0.8% glucose, or LB plus 0.8% glucose plus 10 mM cAMP. At different time points during cell growth, aliquots were collected for measurement of the OD_{600} (triangles and squares) and β-galactosidase activity (bars).

increased ~4-fold). In addition, the AI-2 activity remained high during stationary phase, suggesting the involvement of cAMP and CRP in regulating biosynthesis and/or removal of AI-2 from the medium. The differences in AI-2 activity of cell-free fluids from the wild type and *crp* and *cya* mutants grown with glucose (data not shown) were not as marked as the differences observed when cells were grown without glucose, probably due to the already very low level of cAMP and CRP in the cells in the presence of glucose.

The introduction of plasmid-borne *crp* or *cya* reduced the AI-2 activity to levels closer to that of W3110. In fact, the Δ*crp* mutant LW1 transformed with crp^+ plasmid pHA7E resulted in even lower AI-2 activity than that produced by the wild type. This may be due to the use of multicopy pHA7E, which increased the concentration of CRP above that in W3110.

cAMP and CRP negatively regulate the expression of *luxS* but not *pfs*. Since deletion of either *crp* or *cya* greatly enhanced extracellular AI-2 activity, we tested whether this was due to modulation of some genes involved in AI-2 biosynthesis. *lacZ* fusions were constructed to check the regulation of *luxS* and *pfs*, which encode enzymes responsible for AI-2 synthesis. We found that addition of 0.8% glucose to the growth medium increased the β-galactosidase activity from the *luxS* promoter (<2-fold), while addition of 10 mM cAMP partly offset this glucose effect (Fig. 4A). Moreover, deletion of the *crp* gene increased the expression of *luxS* (Fig. 4A). These results suggest that the expression of the *luxS* gene is negatively regulated by cAMP and CRP.

In contrast to the effect of glucose on *luxS* transcription, the presence of glucose lowered the level of β-galactosidase activity expressed from the *pfs* promoter, principally in the stationary phase (Fig. 4B). The addition of cAMP to the cells grown in LB with glucose did not restore β-galactosidase activity to the level when the cells were grown in LB alone. Furthermore, deletion of *crp* had no effect on β-galactosidase expression from the *pfs* promoter relative to that in the wild-type control. While these results suggest that glucose plays a role in *pfs* expression, the control is likely to be through a mechanism other than the cAMP-CRP complex

We tested whether the action of CRP and cAMP on *luxS* transcription was mediated by cAMP-CRP binding near the promoter of *luxS*. CRP binding sites contain a palindromic sequence in which two conserved motifs, TGTGA and TCACA, are separated by a spacer (31). The length of the spacer is usually 6 bp, but spacers of 7 or 8 bp, and possibly 9 bp, have been observed (3, 4, 31, 46). Although a potential CRP binding site, 5′-TGGGAagaaagagtTCAGA-3′, was present (spacer shown in lowercase letters), a gel mobility shift assay showed no binding of the cAMP-CRP complex to the *luxS* promoter region (data not shown), suggesting that the

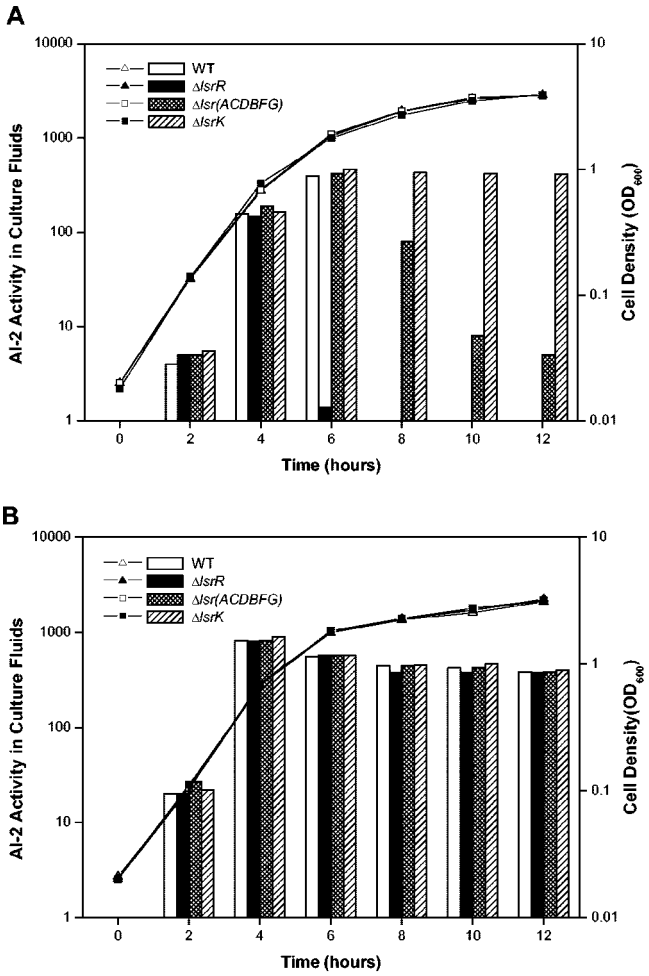

FIG. 5. AI-2 activity profiles of *E. coli lsr* mutants. Overnight cultures of *E. coli* W3110 (wild type) and strains containing deletion of *lsrR*, *lsrK*, or *lsrACDBFG* were diluted in LB (A) or LB plus 0.8% glucose (B) to an OD_{600} below 0.03. At different time points during cell growth, aliquots were collected for measurement of the OD_{600} (triangles and squares) and AI-2 activity (bars). AI-2 activities shown are representative of three independent experiments. Replicate assays agreed to within 10%.

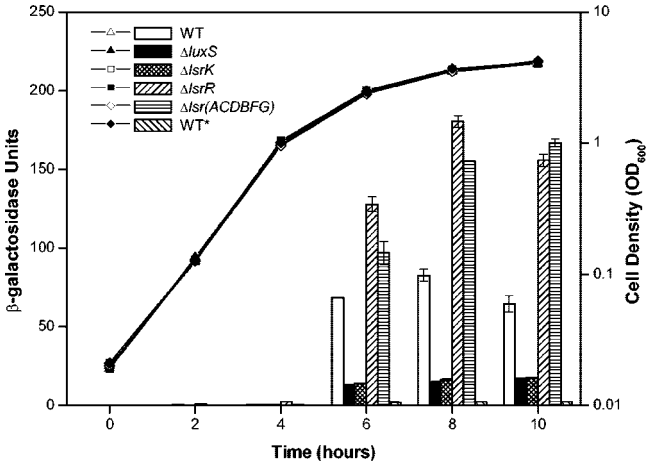

FIG. 6. Transcriptional regulation of the *E. coli lsr* operon. *E. coli* ZK126 (wild type) and strains containing deletions of *luxS*, *lsrK*, *lsrR* and *lsrACDBFG* carry plasmid pLW11 (*lacZ* fusion containing wild type *lsrA* promoter region). ZK126 (WT*) carries plasmid pLW12 (*lacZ* fusion containing mutated *lsrA* promoter region with base substitutions in CRP binding motif). Cells were grown in LB medium. At different time points during cell growth, aliquots were collected for measurement of the OD_{600} (triangles, squares, and diamonds) and β-galactosidase activity (bars).

effect of CRP and cAMP on *luxS* transcription was probably indirect.

cAMP-CRP stimulates expression of the *lsr* operon by directly binding to the upstream region of its promoter. Although deletion of the *crp* gene resulted in an increase of *luxS* expression (<2-fold) in the absence of glucose (Fig. 4A), this seemed insufficient to explain the dramatic differences in extracellular AI-2 activities in the wild type and *crp* mutant, particularly during the stationary phase (Fig. 3). Thus, we investigated the role of cAMP and CRP in AI-2 uptake. First, to confirm the role of the *lsr* operon in *E. coli*, we made a deletion of the entire $lsrACDBFG_{E.c.}$ operon (see Materials and Methods). The $\Delta lsr(ACDBFG)_{E.c}$ mutant showed much slower removal of AI-2 from extracellular fluids relative to the wild type when grown in the absence of glucose (Fig. 5A), although the removal of AI-2 was not completely blocked. It is likely that there is an alternative mechanism for AI-2 removal from the *E. coli* extracellular medium. To explain a similar observation in serovar Typhimurium, Taga et al. suggested that there may exist another low-affinity transporter for AI-2 uptake (54). Deletion of $lsrR_{E.c.}$ resulted in accelerated removal of AI-2 from extracellular fluids (Fig. 5A) relative to the wild type when grown in LB, similar to a serovar Typhimurium $lsrR_{S.t.}$ mutant (54). Finally, deletion of $lsrK_{E.c}$ caused a severe defect in AI-2 removal (Fig. 5A). This is also consistent with the serovar Typhimurium $lsrK_{S.t.}$ mutant, where it was suggested that the absence of LsrK prevents sequestration of AI-2 in cytoplasm in the form of phospho–AI-2 (54). In summary, the function and control of the $lsr_{E.c.}$operon seems to operate similarly as in serovar Typhimurium in its role as an AI-2 autoregulated transporter and processing system.

Although the Δ*lsrR*, Δ*lsrK*, and Δ*lsr(ACDBFG)* mutants and the wild-type cells displayed different rates of AI-2 removal when grown in LB without glucose, all of the mutants and the wild type showed very similar extracellular AI-2 levels when glucose was present in the growth medium. The levels of AI-2 were relatively high throughout the stationary phase (Fig. 5B). These results suggest that the presence of glucose may affect the regulation of AI-2 uptake.

To investigate involvement of glucose-mediated catabolite repression in *lsr* regulation, we constructed a *lacZ* fusion under control of the promoter region of the *lsrACDBFG* operon. When the wild-type cells (ZK126) were grown in LB medium, *lsr* expression remained very low until the cells entered the stationary phase, consistent with the accumulation of AI-2 as observed earlier (Fig. 3 and 6 and Table 3). The addition of 0.8% glucose to the growth medium strongly decreased transcription from the *lsr* promoter in the wild type and in all of the *lsr* mutants (Table 3). These results are consistent with the significantly higher extracellular AI-2 activities of these cells (Fig. 5B). Deletion of the *crp* gene decreased *lsr* expression

TABLE 3. Regulation of expression of the *lsrACDBFG* operon[a]

Strain genotype	Plasmid genotype	β-Galactosidase activity (Miller units)[c]			
		Late exponential[b]		Early stationary[b]	
		LB	LB + Glc	LB	LB + Glc
Wild type	pLW11	0.5	0.1	68	0.4
Δ*crp*	pLW11	0.2	—	0.4	—
Δ*luxS*	pLW11	0.6	0.2	13	0.4
Δ*lsrK*	pLW11	0.5	0.2	14	0.4
Δ*lsrR*	pLW11	2.4	0.3	128	0.7
Δ*lsr(ACDBFG)*	pLW11	0.4	0.2	97	0.5
Δ*rpoS*	pLW11	4.0	—	119	—
Wild type	pLW12	0.3	—	2	—

[a] *E. coli* ZK126 (wild type) and strains containing deletion of *crp*, *luxS*, *lsrK*, *lsrR*, *lsr(ACDBFG)*, and *rpoS* carry plasmid pLW11 (*lacZ* fusion containing wild-type *lsrA* promoter region) or plasmid pLW12 (*lacZ* fusion containing mutated *lsrA* promoter region with base substitutions in CRP binding motif). Cells were grown in LB or LB plus 0.8% glucose (Glc). Growth conditions were the same as for Fig. 6.

[b] Late exponential growth phase is at 4 h of growth (Fig. 6); early stationary phase is at 6 h of growth (Fig. 6).

[c] All measurements were within ±10% of the standard deviation. —, not determined.

(Table 3), indicating that CRP is needed to activate transcription from the *lsr* promoter. Deletion of *lsrR* caused a significant increase in *lsr* expression (Fig. 6 and Table 3), confirming the role of the LsrR protein as a repressor protein. These transcription results are consistent with the rapid AI-2 removal observed in the Δ*lsrR* mutant (Fig. 5A). The much lower *lsr* expression levels in the exponential phase in the Δ*lsrR* mutant and other strains were likely caused by the low levels of glucose and other PTS sugars initially present in the LB medium. It is also noteworthy that deletion of either *luxS* or *lsrK* resulted in a much lower level of *lsr* expression during late-exponential and stationary phases (Fig. 6 and Table 3), supporting the AI-2/phospho–AI-2–dependent regulation, as shown in serovar Typhimurium (54). It was further shown here that LsrR-mediated repression of *lsr* expression is not complete, as noted by significant expression levels in the Δ*luxS* or Δ*lsrK* mutants (Fig. 6). Finally, deletion of the entire *lsrACDBFG* operon resulted in a significant increase in transcription from the *lsr* promoter (Fig. 6 and Table 3). This was, at first, unexpected since the absence of the Lsr transporter decreases uptake of AI-2, which is recruited to enhance *lsr* transcription. Thus, in the absence of AI-2 we would expect effective LsrR-mediated repression. We suggest that LsrK phosphorylates endogenous AI-2 and/or AI-2 imported by the alternative transporter, which then derepresses transcription from the *lsr* promoter through LsrR (see Discussion). Moreover, since LsrF and LsrG are not present, which are reported to promote AI-2 degradation (54), the inducer phospho–AI-2 may persist.

To evaluate whether cAMP-CRP directly modulates *lsr* transcription, we performed gel mobility shift assays. The promoter region of the *lsr* operon contains two potential CRP binding sites (Fig. 7A). Site 1 is located 19 to 34 bp upstream of the transcription start site (predicted via the website http://www.fruitfly.org/seq_tools/promoter.html), while site 2 is located at residues −60 to −77. Our results revealed that cAMP-CRP binds to the DNA fragment containing both site 1 and site 2, but not the fragment containing only site 2 (Fig. 7B). A shorter DNA fragment containing only site 1 was bound by cAMP-CRP (Fig. 7C), and there was no binding of cAMP-CRP to a DNA fragment that contained only site 1 with substitutions in 4 bp in one of the CRP binding motifs (Fig. 7C). These data confirm that cAMP-CRP specifically recognizes and binds to the CRP binding site located in site 1, and not site 2. Interestingly, the spacer for this binding site is 8 bp, while most of the known CRP binding sites contain a conventional 6-bp spacer (3, 4, 31). It has been demonstrated that cAMP-CRP binds to many sites with an 8-bp spacer in vitro (3, 4, 9, 49), although we have found no reports showing such CRP binding sites are functional in vivo. In this study, in vivo experiments were performed with wild-type ZK126 cells carrying pLW12, which contains the identical *lsr* promoter region as pLW11 but with the four base substitutions in the CRP binding site as used in the gel shift assay (Fig. 7A, seq4). Significantly decreased transcription from the mutated *lsr* promoter was found (Fig. 6 and Table 3), indicating a requirement for this site in cAMP-CRP-mediated activation. These results demonstrate that cAMP-CRP stimulates transcription of the *lsrACDBFG* operon by directly binding to the upstream region of the *lsr* promoter and that mutation in the CRP binding site abolishes this stimulation.

DISCUSSION

AI-2 was first discovered in *V. harveyi* as a quorum signal to regulate bioluminescence (5, 6). In addition to communicating cell density, it is suggested that AI-2 may relay information pertaining to the growth phase and metabolic potential of the bacterial cells (14, 62). In this study, we investigated the regulation of AI-2 production and uptake. We showed that cAMP and CRP negatively regulate *luxS* transcription. However, the action of CRP and cAMP on *luxS* transcription is not mediated by cAMP-CRP binding to the promoter region of *luxS*, as indicated by the gel shift assay (data not shown). It is possible that cAMP-CRP may control another transcriptional regulator(s) that modulates *luxS* expression. Interestingly, we found that the expression of *pfs* was reduced by the presence of glucose, but not through the cAMP-CRP complex. Considering that Pfs is involved in both AI-2 synthesis and polyamine formation pathways (Fig. 1), regulation of AI-2 production through LuxS appears more practical and efficient, although other factors, such as the concentrations of precursors, may also affect AI-2 synthesis. Beeston and Surette found that the extracellular AI-2 activities were correlated with the transcription profile of *pfs* in serovar Typhimurium (7), which is similar to our result in the presence of glucose (Fig. 2 and 4). Despite the correlation, the earlier (54, 55) and present works suggest that the extracellular activities of AI-2 are controlled by a dynamic regulatory process involving many factors that affect its synthesis and transport. In the stationary phase the AI-2 levels in culture medium appear to be decided by the rate of its import rather than the rate of synthesis.

Since *E. coli* possesses almost all genes homologous to those in the *lsr* operon of serovar Typhimurium, we were curious whether the function and regulation of the *E. coli* Lsr transporter were similar to those of serovar Typhimurium and whether cAMP and CRP were involved in regulation of AI-2

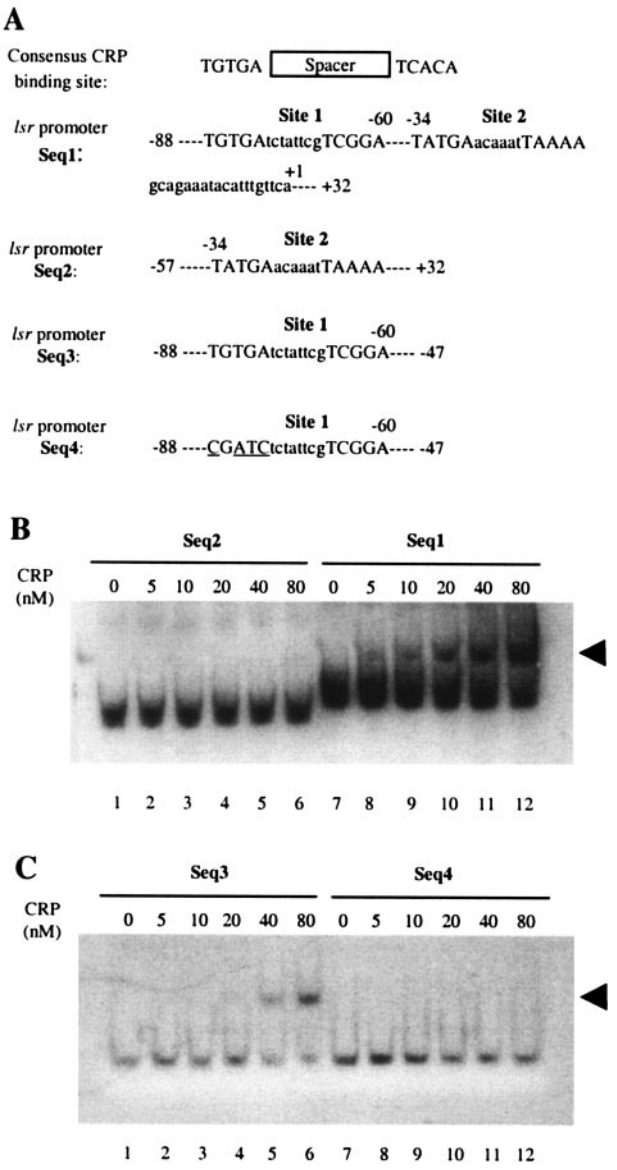

FIG. 7. cAMP-CRP binds to an upstream region of the *lsr* promoter. (A) CRP consensus sequence and DNA fragments used for the CRP binding assay. Consensus and potential CRP recognition sites are shown in capital letters. The underlined bases in seq4 show substitutions eliminating CRP binding. The numbers indicate the nucleotide position relative to the predicted *lsrA* transcription start site. (B and C) Gel mobility shift assays were performed as described in Materials and Methods. Digoxigenin-labeled DNA fragments of seq1, seq2, seq3, and seq4 were incubated with 0 to 80 nM 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.

transport. Our data are consistent with both, although some differences were noted. For example, *lsrE* is apparently absent in *E. coli*. This gene is homologous to *rpe*, which encodes the ribulose phosphate epimerase, suggesting the possibility that an additional step(s) is required for AI-2 modification in serovar Typhimurium. Examination of the upstream region of the serovar Typhimurium *lsr* operon reveals one potential CRP binding site (spacer shown in lowercase letters): 5′-TGAGAg tttttTGACC-3′ (−32 to −47 relative to the predicted transcriptional start site of the *lsr* operon). This site has a 6-bp spacer, and its function has yet to be confirmed. While the molecular basis for the cAMP-CRP-mediated regulation may be slightly different, the function and control of the Lsr transporter are similar. In a genetic screen for the regulator of the *lsr* operon in serovar Typhimurium, mutation of either *cya* or *ptsI* caused reduced transcription of the *lsrC-lacZ* reporter (54). Since Cya is directly involved in the production of cAMP, and since PtsI is enzyme I of the PTS and is required for the activation of Cya, it is not surprising that mutation of *cya* or *ptsI* results in lower expression of the *lsr* operon.

We noted that deletion of the whole *lsr* operon in *E. coli* did not completely block AI-2 import (Fig. 5A), which was also the case for the Lsr transport mutants of serovar Typhimurium (54, 55). Taga et al. proposed the existence of another low-affinity Lsr-independent transport mechanism(s) (54). Interestingly, while the transport of AI-2 is slower in this mutant, there is much higher transcription from the *lsr* promoter relative to that in both the wild type and the Δ*luxS* mutant (Fig. 6) (all in LB medium). In other words, transcription from the *lsr* promoter can still be induced even without AI-2 import by the Lsr transporter. Perhaps endogenous AI-2 and/or AI-2 imported from an alternative transporter can serve as the substrate for the LsrK kinase to produce the inducer, phospho–AI-2. Consistent with this, *lsr* expression in a serovar Typhimurium Δ*lsrB* mutant with a defective Lsr transporter is higher than that in a Δ*luxS* mutant, although lower than that in the wild type (54). The reduced *lsr* expression in the Δ*lsrB* mutant relative to that in the wild type probably results from a lower rate of AI-2 phosphorylation due to the absence of AI-2 uptake from a functional Lsr transporter. The higher induction from the *lsr* promoter in the *E. coli* Δ*lsrACDBFG* mutant is probably also influenced by the loss of the phospho–AI-2 degradation by LsrF and LsrG. Hence, LsrF and LsrG may function as a signal terminator in the wild-type *E. coli* cells, as shown in serovar Typhimurium (54).

There are, therefore, several regulators that influence the expression level of the Lsr transporter. Since σ^s is very important in sensing stress during the transition to stationary phase, we investigated the possibility that σ^s plays an additional role in controlling AI-2 transport. Our results showed that mutation of *rpoS* (encoding σ^s) causes an eightfold increase in the transcription of the *E. coli lsr* operon during the late exponential growth phase when cells are grown in the absence of glucose (Table 3), and extracellular AI-2 activities in Δ*rpoS* mutant are significantly decreased (data not shown). Notley-McRobb et al. reported that the mutation of *rpoS* induces expression of certain transporter genes under glucose limitation, probably due to loss of competition between σ^s and σ^{70} for core RNA polymerase (41). Also, it may be possible that σ^s controls other regulators that affect *lsr* expression. In addition to σ^s, Taga et al. reported that transcription of the *lsrC-lacZ* reporter in serovar Typhimurium is lowered eightfold by mutation of the *lon* gene, which encodes the Lon protease (54). How Lon is related to the regulation of *lsr* expression is unclear.

Our working model for AI-2 synthesis and transport in *E. coli* (Fig. 8) is that cAMP-CRP acts as a global controller, while LsrR functions as a specific controller. When glucose or other

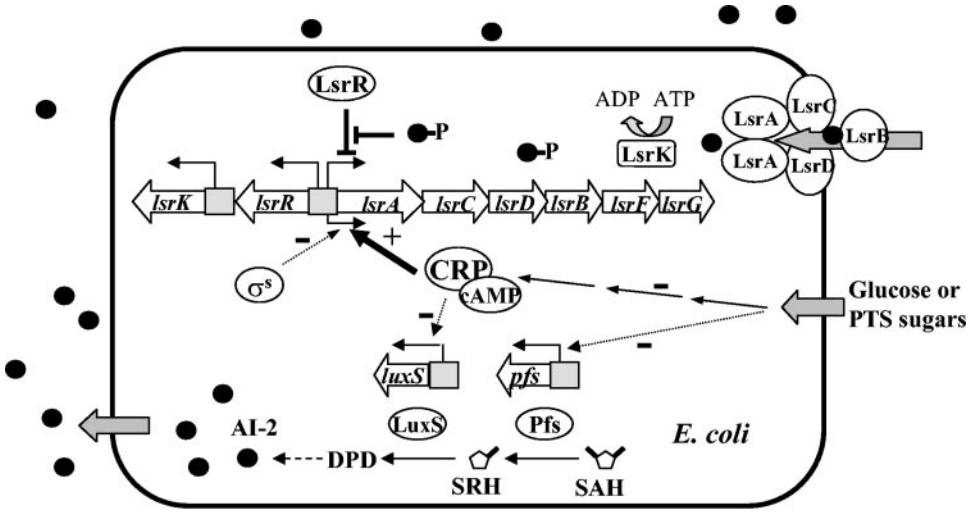

FIG. 8. Conceptual model of AI-2 synthesis and uptake in *E. coli*. In the presence of glucose, low levels of cAMP and CRP result in almost no expression of the *lsr* operon. Indirect upregulation of *luxS*, and likely increased precursor flux, increases AI-2 synthesis. Both enable rapid accumulation of AI-2 in the extracellular medium. In the absence of glucose, cAMP-CRP is needed to stimulate *lsr* expression, while LsrR represses its expression in the absence of the inducer phospho–AI-2. As AI-2 accumulates, *lsr* transcription is de-repressed, enabling more AI-2 uptake. In addition, σ^s negatively affects *lsr* expression, especially during the late exponential phase. As noted above, the expression of *pfs* is negatively influenced by the presence of glucose; the effects of this are unclear but might be complicated by the polyamine pathways also utilizing Pfs and SAM. Transcriptional regulation is shown by solid arrows (direct) or dashed arrows (indirect or unclear mechanisms). Plus and minus signs indicate positive and negative regulations, respectively. DPD, 4,5-dihydroxy-2,3-pentanedione. See text for additional details.

PTS sugars are present in the growth medium, low intracellular levels of cAMP and CRP result, and there is almost no transcription of the *lsr* operon. Instead, the level of *luxS* transcription increases, and it is likely that the metabolic flux of precursors (SAM, SAH, etc.) also increases concomitant with an increased demand for biosynthesis. It is also likely that other cellular activities requiring methylation (through SAM) will impact the level of the substrate for AI-2 synthesis. Thus, with sufficient glucose, increased carbon and energy storage may result and the net effect is an acceleration in AI-2 synthesis and export. This is consistent with the exponential-phase results shown in Fig. 5 and 6, where there was minimal *lsr* transcription and the AI-2 levels were significantly higher (~3 times) in both the wild type and the Δ*lsrACDBFG* mutant (having drastically impaired AI-2 uptake) in the presence of glucose.

However, when glucose or other PTS sugars are absent, the cAMP-CRP complex binds to the upstream region of the *lsr* promoter and stimulates its transcription. It is under this condition that LsrR, LsrK, LsrF, and LsrG play a greater role in regulating and "tuning" AI-2 uptake. If there is no inducer (phospho–AI-2), LsrR represses transcription of the *lsr* operon. During the late exponential phase, a basal level of *lsr* expression likely results in uptake and phosphorylation of a small amount of AI-2. Endogenous AI-2 and AI-2 imported by a low-affinity transporter may also provide substrate for LsrK. The phospho–AI-2 inactivates the repressor LsrR and increases transcription of the *lsr* operon. Then, more AI-2 is transported into the cells and the cycle continues until LsrF and LsrG provide feedback control.

In summary, we have shown that *E. coli* cells synthesize and secrete AI-2 in the early growth phase and take it up during the stationary phase under glucose limitation. The simplest interpretation is that the bacterial cells use AI-2 as a carbon source in the absence of the preferred glucose, just like they use lactose, arabinose, and galactose, which are similarly regulated. This interpretation does not address its role as a signal molecule, however. Taga et al. reported that serovar Typhimurium cannot grow in minimal medium containing AI-2 as the sole carbon source (55). Winzer et al. suggested that utilization of AI-2 as the sole carbon source might require additional conditions (59). Yet, they suggested further that AI-2 may be a toxic by-product of SAM metabolism, which is excluded during early growth and taken up and metabolized at a later stage (for detoxifying and recycling the energetically expensive "ribose equivalent" unit) (58, 59). We found that the Δ*luxS* mutant of *E. coli* ZK126 grows as well as the wild type in LB medium (Fig. 6), but Sperandio and coworkers found that mutation of *luxS* in enterohemorrhagic *E. coli* O157:H7 resulted in a faster growth of the mutant when grown in Dulbecco's modified Eagle's medium and that there was a global effect on gene expression (51). It is therefore possible that the specific growth condition and/or specific genotype might affect or determine the role of AI-2 as a signal molecule. Indeed, there are many other reports suggesting that AI-2 may participate in the control of certain genes and physiological activities (reviewed in reference 61). Thus, while the role of AI-2 as a signal molecule remains unclear in *E. coli* W3110, it is clear that these cells have a complex hierarchical regulatory system for its control that is in part based on AI-2, suggesting potential involvement of AI-2 in the regulation of additional cellular processes.

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