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pfs-Dependent Regulation of Autoinducer 2 Production in *Salmonella enterica* Serovar Typhimurium

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Bacterial intercellular communication provides a mechanism for signal-dependent regulation of gene expression to promote coordinated population behavior. *Salmonella enterica* serovar Typhimurium produces a non-homoserine lactone autoinducer in exponential phase as detected by a *Vibrio harveyi* reporter assay for autoinducer 2 (AI-2) (M. G. Surette and B. L. Bassler, Proc. Natl. Acad. Sci. USA 95:7046-7050, 1998). The *luxS* gene product mediates the production of AI-2 (M. G. Surette, M. B. Miller, and B. L. Bassler, Proc. Natl. Acad. Sci. USA 96:1639-1644, 1999). Environmental cues such as rapid growth, the presence of preferred carbon sources, low pH, and/or high osmolarity were found to influence the production of AI-2 (M. G. Surette and B. L. Bassler, Mol. Microbiol. 31:585-595, 1999). In addition to LuxS, the *pfs* gene product (Pfs) is required for AI-2 production, as well as *S*-adenosylhomocysteine (SAH) (S. Schauder, K. Shokat, M. G. Surette, and B. L. Bassler, Mol. Microbiol. 41:463-476, 2001). In bacterial cells, Pfs exhibits both 5′-methylthioadenosine (MTA) and SAH nucleosidase functions. Pfs is involved in methionine metabolism, regulating intracellular MTA and SAH levels (elevated levels of MTA and SAH are potent inhibitors of polyamine synthetases and S-adenosylmethionine dependent methyltransferase reactions, respectively). To further investigate regulation of AI-2 production in *Salmonella*, we constructed *pfs* and *luxS* promoter fusions to a *luxCDABE* reporter in a low-copy-number vector, allowing an examination of transcription of the genes in the pathway for signal synthesis. Here we report that *luxS* expression is constitutive but that the transcription of *pfs* is tightly correlated to AI-2 production in *Salmonella* serovar Typhimurium 14028. Neither *luxS* nor *pfs* expression appears to be regulated by AI-2. These results suggest that AI-2 production is regulated at the level of LuxS substrate availability and not at the level of *luxS* expression. Our results indicate that AI-2-dependent signaling is a reflection of metabolic state of the cell and not cell density.

Bacterial intercellular communication provides a mechanism for the regulation of gene expression, resulting in coordinated population behavior. This phenomenon has been referred to as quorum sensing or cell-cell communication and has been reviewed recently (1, 12, 17, 30, 34). Gram-negative bacteria typically produce, release, and respond to acyl-homoserine lactone (HSL) molecules (autoinducers) that accumulate in the external environment as the cell population grows. HSLs are synthesized by the LuxI family of HSL synthases and, above threshold concentrations, bind to their cognate receptor proteins (the LuxR family of transcriptional regulators) to mediate changes in gene transcription.

Unlike other gram-negative quorum-sensing organisms, *Vibrio harveyi* mediates quorum sensing via two parallel signaling systems, and detection and response to either signal is mediated by a two-component phosphorylation-dephosphorylation cascade (3, 15). The first signaling system is comprised of autoinducer 1 (AI-1), a hydroxybutanoyl-l-HSL (synthesized by LuxLM), and its cognate sensor protein LuxN, whereas the second signaling system is composed of AI-2 (synthesized by LuxS) and the LuxPQ sensor complex (6, 35, 39). Both signaling systems regulate a phosphorelay signaling pathway through LuxU to the transcriptional regulator LuxO to relieve repression of the *lux* operon (15). High concentrations of either AI-1 or AI-2 regulate bioluminescence (3), siderophore production, colony morphology, and possibly the expression of other LuxO-σ^{54}-dependent genes in response to high cell density in *V. harveyi* (23).

V. harveyi reporter strains constructed to detect only AI-1 or AI-2 demonstrated that many species of bacteria, including *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Yersinia enterocolitica* (2) produce autoinducers which induce bioluminescence through the AI-2 system of *V. harveyi*. The gene whose product is responsible for AI-2 production was initially identified in *V. harveyi*, *Salmonella enterica* serovar Typhimurium, and *Escherichia coli* and was named *luxS* (44). The *luxS* family of genes are highly homologous to one another but not to any other identified gene and define a new family of autoinducer-producing genes. In the National Center for Biotechnology Information microbial genome database, 30 of 136 bacterial species contain a *luxS* homologue. The *luxS* family of genes has widespread distribution among gram-positive and gram-negative bacteria, including pathogenic and nonpathogenic species (41).

More recently, *luxS*-dependent AI-2 signaling activity has been reported in many other bacteria including: *E. coli* O157 (37), *Shigella flexneri* (9), *Helicobacter pylori* (14, 22), *Vibrio vulnificus* (27), *Streptococcus pyogenes* (25), *Mannheimia haemolytica* (26), and *Proteus mirabilis* (36), as well as in periodontal pathogens such as *Actinobacillus actinomycetemcomitans* (13), *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* (4, 7, 16). Recent studies with DNA arrays have implicated AI-2 in the regulation of a large number of genes in *E. coli* (10, 38). In *Salmonella* serovar Typhi-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
S. enterica serovar Typhimurium 14028	Wild type	ATCC 14028
S. enterica serovar Typhimurium CS132	Isogenic to 14028 containing *luxS*::*Mu*dJ	41
S. enterica serovar Typhimurium SS007	Isogenic to 14028 containing *luxS*::T-POP	35
V. harveyi BB170	Isogenic to wild-type BB120 containing *luxN*::Tn*5*Kn	3
Plasmids		
pCS26	Low-copy-number *luxCDABE* reporter vector	C. Southward
pAB12	pCS26 containing *luxS* promoter	This study
pAB13	pCS26 containing *pfs* promoter	This study
pBAD18	Arabinose-inducible expression vector	Invitrogen; 21
pMS234	pBAD18 containing *luxS*	35

murium, AI-2 regulates the expression of an outer membrane AI-2 transport protein (42).

A second protein (Pfs) is also required for AI-2 biosynthesis (35). Pfs catalyzes two reactions in bacterial cells: the formation of *S*-ribosylhomocysteine (SRH) from *S*-adenosylhomocysteine (SAH) to release adenine and the production of 5′-methylthioribose (MTR) from 5′-methylthioadenosine (MTA), also releasing adenine (11, 19, 28). Both SAH and MTA are potent inhibitors of *S*-adenosylmethionine (SAM)-requiring reactions, and the accumulation of these metabolites is avoided through the activities of Pfs (8). The absence of *pfs* in *E. coli* results in severe growth defects (5). A recent study by Schauder et al. has shown that purified Pfs and LuxS enzymes are necessary and sufficient for AI-2 production in vitro with SAH as a substrate (35).

The environmental regulation of signal (AI-2) production in *S. enterica* serovar Typhimurium LT2 has been previously reported (40). Maximal AI-2 activity is produced during mid-exponential phase when *Salmonella* serovar Typhimurium is grown in the presence of glucose or other preferred carbohydrates (40). Degradation of the signal is believed to occur toward the onset of stationary phase or when the carbohydrate is depleted from the medium (40). Maximal signaling activity is also observed if, after growth in the presence of glucose, *Salmonella* serovar Typhimurium is transferred to high-osmolarity (0.4 M NaCl) or low-pH (pH 5.0) conditions (40). High osmolarity and low pH are environmental conditions that *Salmonella* serovar Typhimurium may encounter during infection, suggesting that quorum sensing may have a role in the regulation of virulence in *Salmonella* serovar Typhimurium (40).

The purpose of this study is to determine how AI-2 production by *Salmonella* serovar Typhimurium 14028 is regulated at the genetic level by genes (*luxS* and *pfs*) whose products are directly involved in AI-2 generation. The analysis presented here demonstrates that the profile of *pfs* transcription is tightly correlated to the AI-2 production pattern in *Salmonella* serovar Typhimurium 14028 and that the transcription of *luxS* and *pfs* is not regulated by AI-2.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are listed in Table 1. All *Salmonella* strains were grown at 37°C with shaking at 200 rpm in Luria broth (LB; Gibco-BRL) containing 10 g of SELECT peptone 140, 5 g of SELECT yeast extract (low sodium), and 10 g of sodium chloride/liter with or without supplemented carbohydrate at 0.5% (wt/vol). *V. harveyi* was grown at 30°C with shaking at ca. 200 rpm in autoinducer bioassay (AB) medium as described previously (18). Antibiotics were used at the following concentrations: tetracycline, 15 μg/ml; ampicillin, 100 μg/ml; and kanamycin, 50 μg/ml.

DNA cloning. All cloning vectors and plasmid constructs used in this study are listed in Table 1. The *luxS* and *pfs* promoter regions were cloned into a low-copy-number vector (pCS26), creating promoter-*luxCDABE* reporter transcriptional fusions to allow sensitive high-throughput analysis of promoter activity during growth. pCS26 is derived from pZS21-luc (24), which contains a low-copy-number pSC101 origin of replication, and a kanamycin resistance cassette. Additional modifications of pZS21-luc included replacement of the luciferase gene (*luc*) and the $P_{LtetO-1}$ regulatory unit by the *luxCDABE* operon and a *Bam*HI cloning site, respectively.

pAB12 was created by cloning the *luxS* promoter region from *Salmonella* serovar Typhimurium 14028 into the *Bam*HI site of pCS26. The primers used to amplify the *luxS* promoter by PCR were luxS01 (CGGGG ATCCT TACCG TAATC TGTTA CGCG) and luxS02 (CGGGA TCCAA TAATG GCATT TAGTC ACCTC), generating a 405-bp fragment. pAB13 was created by cloning the *pfs* promoter region from *Salmonella* serovar Typhimurium 14028 into the *Bam*HI site of pCS26. The primers used to amplify the *pfs* promoter by PCR were pfs01 (CGGGA TCCTC CATTG CGCCA ATGAT GCC) and pfs02 (CGGGA TCCTG AACGA TAACG ACGAT GCC), generating a 167-bp fragment. The resulting pAB12 and pAB13 constructs contain considerable regions upstream of *luxS* and *pfs*, respectively. Although the transcription start sites are not known (or predicted) for these genes, the cloned regions are sufficient to contain RNA polymerase binding and regulatory protein-binding sites. Similar *luxS* expression profiles were observed between the chromosomal *luxS*::*Mu*dJ *lacZ* transcriptional fusion and the cloned *luxS-luxCDABE* construct (pAB12), indicating that the complete promoter region was cloned. A larger clone of the *pfs* promoter region that included an additional 161 bp upstream gave similar results.

pMS234 was created by cloning the entire *luxS* gene from *E. coli* O157:H7 into the *Eco*RI and *Hin*dIII cloning sites of pBAD18 (Invitrogen) (21). The primers used to amplify the *luxS* gene by PCR were ygag01 (GTGAA GCTTG TTTAC TGACT AGATG TGC) and ygag03 (GTGTC TAGAA AAACA CGCCT GA CAG).

PCR was perfomed in a Mastercycler gradient thermocycler (Eppendorf) with *Taq* polymerase (Gibco-BRL) and 55°C annealing temperature. Plasmid DNA was isolated by using QIA Spin Mini-Columns (Qiagen), and chromosomal DNA was isolated by the standard method of CTAB (cetyltrimethylammonium bromide) extraction (44).

Correct plasmid constructs were confirmed by PCR-based sequencing by using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI PRISM) at the University Core DNA Sequencing Services (University of Calgary).

AI-2 bioassays. The detection of AI-2 in culture supernatants by using *V. harveyi* BB170 was performed as previously reported (39). Briefly, the *V. harveyi* reporter strain was grown for ca. 16 h at 30°C at 200 rpm in AB medium (20). Cells were diluted 1/5,000 in fresh AB medium, and filter-sterilized culture supernatant samples were assayed at 10% (vol/vol) with the *V. harveyi* reporter strain BB170 (Δ*luxN*) to a final volume of 100 μl; the plates were then incubated with shaking at 30°C. Luminescence values were measured every hour in a Microbeta Liquid Scintillation and Luminescence Counter (Wallac model 1450) and reported as the fold induction of luminescence by the reporter strain above the negative medium control.

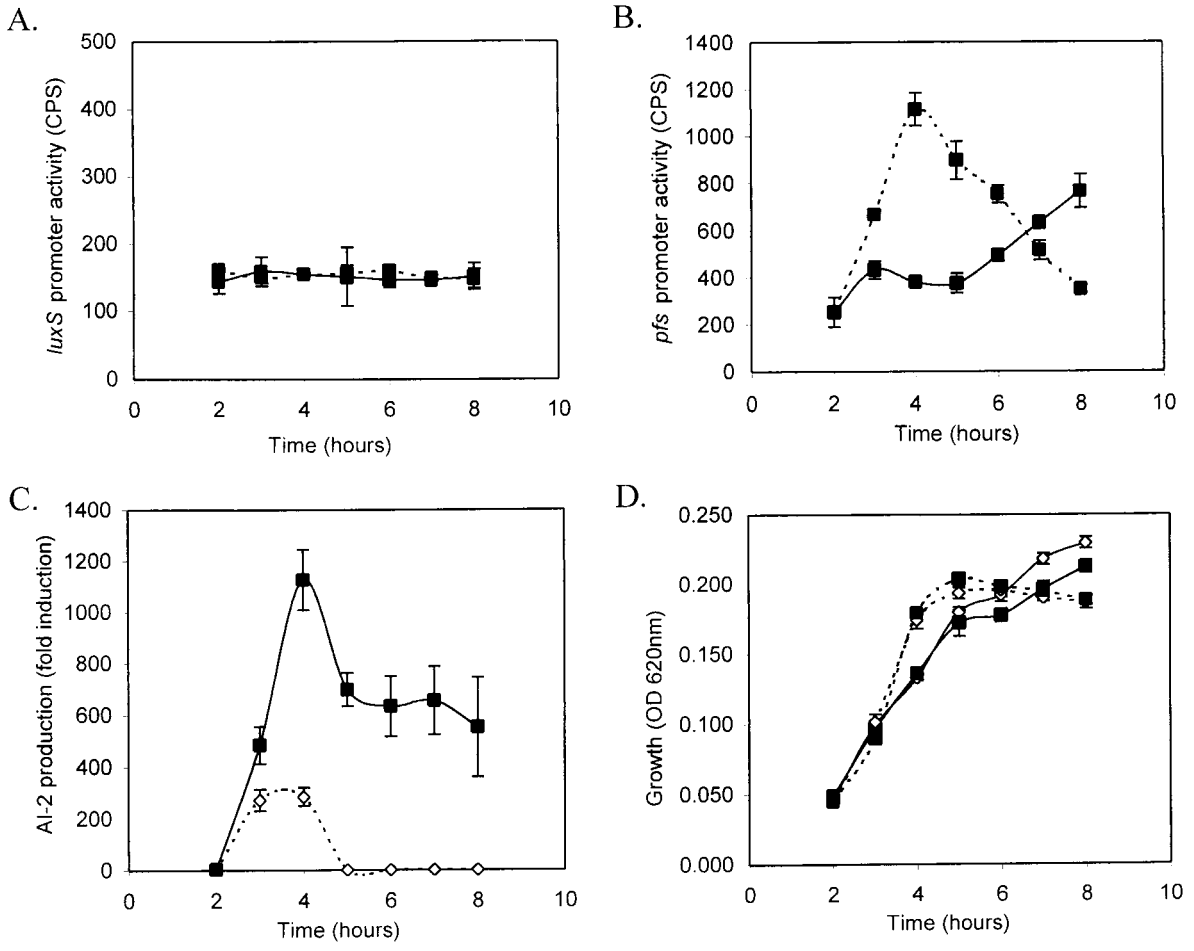

FIG. 1. Constitutive *luxS* expression and the correlation of *pfs* transcription to AI-2 production in *Salmonella* serovar Typhimurium during growth in LB medium with or without 0.5% glucose. *luxS* expression (A) and *pfs* expression (B) in *Salmonella* serovar Typhimurium during growth in LB medium with or without 0.5% glucose was measured by using low-copy-number *luxCDABE* reporter fusion to the *luxS* and *pfs* promoters. Solid line, LB; dashed line, LB with 0.5% glucose. (C) AI-2 production profile of *Salmonella* serovar Typhimurium during growth in LB media with or without 0.5% glucose. Supernatants were prepared from samples of growing cultures, filter sterilized, and assayed for AI-2 activity by using the *V. harveyi* BB170 AI-2 reporter strain. Samples collected from cultures grown in LB are shown by the broken line, and the dashed line represents AI-2 activity from cultures grown in LB with 0.5% glucose. (D) Growth curves of *Salmonella* serovar Typhimurium 14028 containing promoter-*luxCDABE* reporter vectors in LB or LB with 0.5% glucose are shown by solid and dashed lines, respectively. Symbols: ■, 14028/pAB12; ◇, 14028/pAB13.

Gene expression analysis. The promoter-*luxCDABE* fusions were used to measure promoter activity as counts per second (cps) of light in a Victor^2 Multilabel Counter (Wallac model 1420). Overnight cultures were diluted 1/100 in LB with the appropriate antibiotic(s), and measurements were taken every hour during the growth curve. The growth of the cultures was determined by measuring the optical density at 620 nm (OD_{620}) of 100-μl samples in 96-well plates (path length of 0.4 cm) with the Victor^2 Multilabel Counter. Gene expression was normalized per cell by dividing the luminescence value by the OD_{620} value of each sample.

luxS promoter activity was additionally examined with *luxS-lacZ* transcriptional fusions. β-Galactosidase activity was measured as described by Miller (29) and expressed as β-galactosidase activity per OD_{600} unit (in Miller units). For autoregulation experiments, cells were grown in LB with or without 0.5% L-arabinose, harvested after 4 h growth at 37°C, and assayed for β-galactosidase activity (OD measurements were taken at 420, 550, and 600 nm in a Beckman DU640 spectrophotometer). All experiments were performed in duplicate or triplicate.

RESULTS

Transcriptional analysis of *luxS*. A transcriptional fusion of the *luxS* promoter to the *luxCDABE* operon (pAB12) on a low-copy-number plasmid allowed sensitive, real-time, noninvasive monitoring of gene expression under a variety of growth conditions. By using this construct, we analyzed the transcription of *luxS* in conditions previously reported to result in minimal and maximal signaling activity by *Salmonella* serovar Typhimurium, namely, in LB or in LB supplemented with 0.5% glucose (40).

Salmonella serovar Typhimurium 14028/pAB12 (*luxS* promoter-*luxCDABE*) was grown in LB with or without 0.5% glucose, and samples were removed every hour for OD and light output (cps) measurements, as well as for measurements of AI-2 activity. *luxS* was constitutively transcribed at low levels throughout the measured time points, and the addition of glucose to the culture medium resulted in minimal changes in gene expression (Fig. 1A). Similar results were obtained with a chromosomal *luxS*::*Mu*dJ *lacZ* transcriptional fusion (data not shown). The close agreement between the plasmid reporter and the chromosomal *lacZ*

fusion validate the use of the plasmid-based reporter system in these studies. It should be emphasized that the use of low-copy-number vectors was important for this type of investigation.

Experiments were performed in an attempt to isolate mutations that resulted in changes in *luxS* transcription by random Tn*10* mutagenesis. No *luxS* regulatory mutants were recovered in these screens of >35,000 colonies, a finding which supports our observations that *luxS* is not regulated at the transcriptional level.

We also examined changes in *luxS* transcription with quantitative reverse transcription-PCR analysis. RNA from *Salmonella* serovar Typhimurium was isolated each hour during growth in LB with or without glucose and then subjected to quantitative reverse transcription-PCR with a commercially available β-actin RNA as a control standard. The amount of *luxS* RNA transcripts observed was independent of the availability of glucose in the growth medium (data not shown).

The decrease in AI-2 production by *Salmonella* serovar Typhimurium that occurs in late log phase may also be due to changes in the level of LuxS enzymes, LuxS stability, or LuxS enzymatic activity. Through Western blot analysis, we examined LuxS stability in *Salmonella* serovar Typhimurium during growth. Preliminary results show that LuxS protein levels in stationary-phase cells are not lower than cells in exponential phase (data not shown), indicating that regulation of AI-2 production is not due to changes in LuxS levels during growth.

Transcriptional analysis of *pfs*. Pfs catalyzes the production of SRH and MTR from SAH and MTA, respectively (11, 19, 28). The Pfs reaction product SRH is the substrate for LuxS. LuxS hydrolyzes SRH to homocysteine and AI-2 (35). A transcriptional fusion of the *pfs* promoter to the *luxCDABE* operon (pAB13) was used to analyze transcription of *pfs* in a manner similar to that described for the transcriptional analysis of *luxS*. Growth conditions were used that result in both minimal and maximal signal production by *Salmonella* serovar Typhimurium.

Salmonella serovar Typhimurium 14028/pAB13 (*pfs* promoter-*luxCDABE*) was grown in LB with or without 0.5% glucose, and samples were removed every hour for OD and cps measurements, as well as for measuring the AI-2 activity. In contrast to *luxS* transcription, *pfs* expression was sensitive to the presence of glucose. *pfs* expression in LB with 0.5% glucose increased dramatically to a maximal value during the exponential phase and decreased upon entry into stationary-phase growth (Fig. 1B). The *pfs* transcriptional profile correlates with the observed pattern of AI-2 production in *Salmonella* serovar Typhimurium, and maximal AI-2 production and *pfs* expression occurs at the same time. The AI-2 production profile by *Salmonella* serovar Typhimurium 14028 in LB with or without 0.5% glucose is shown in Fig. 1C. The increased presence of AI-2 activity at 3 and 4 h and the decrease beginning at 5 h correlates with the *pfs* expression pattern (Fig. 1B and C). Figure 1D shows that growth was unaffected by the presence of the reporter plasmids. These results indicate that the production of AI-2 by *Salmonella* serovar Typhimurium is partially regulated at the transcriptional level of the *pfs* gene in the AI-2 synthesis pathway. Transcriptional fusions to the *luxS* and *pfs* promoters were also constructed by using *lacZ* as a reporter,

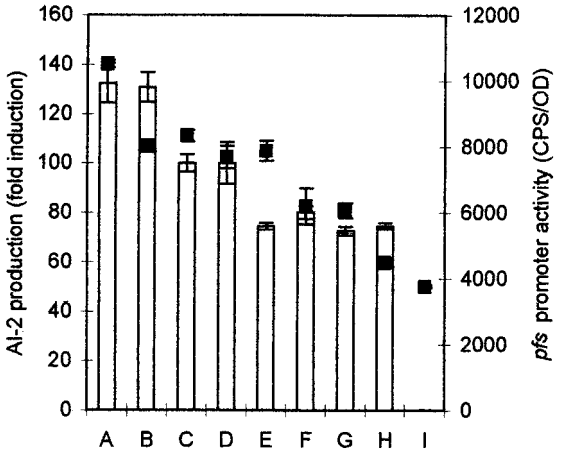

FIG. 2. Effects of different carbohydrate supplements on *pfs* expression and AI-2 production. *pfs* expression (using pAB13) during growth in LB medium with 0.5% of various carbohydrates was measured every hour. The AI-2 activity of these cultures was also assayed by using *V. harveyi* BB170 AI-2 reporter strain on cell-free culture supernatants. The growth conditions (in columns A to I) were LB medium plus a 0.5% concentration of one of the following carbohydrates: A, arabinose; B, galactose; C, glucose; D, mannose; E, raffinose; F, melibiose; G, maltose; H, glycerol; and I, LB. The maximal AI-2 activity (bars) is depicted as a percentage relative to glucose, and *pfs* expression levels (datum points, ■) are presented. In each case, they coincided at the same time point.

and similar expression profiles were observed (data not shown).

Effects of different carbohydrates on *pfs* transcription and AI-2 production. The AI-2 production profile by *Salmonella* serovar Typhimurium can be significantly different when carbohydrate supplements other than glucose are used in LB medium. Using pAB13 as a reporter construct for *pfs* transcription, we monitored *pfs* expression during growth in LB supplemented with 0.5% (nonlimiting) concentrations of various carbohydrates (Fig. 2). The following carbohydrates were tested: monosaccharides, including arabinose, galactose, and mannose; disaccharides, including maltose and melibiose; a trisaccharide (raffinose); and glycerol. Although the levels of AI-2 activity were dramatically affected by growth in alternative carbohydrate sources, these results show that, regardless of the carbohydrate supplement, *pfs* expression correlated to AI-2 production. For cultures supplemented with each of the carbohydrates, *pfs* expression and AI-2 production profiles were qualitatively similar to those of cultures supplemented with glucose; in each case, the maximum AI-2 production correlated with the peak in *pfs* expression (Fig. 2).

AI-2 does not regulate *pfs* and *luxS* transcription. *Salmonella* serovar Typhimurium CS132 was used to analyze AI-2 regulation of *luxS* expression. CS132 has a *luxS*::*lacZ* transcriptional fusion created by a *Mu*dJ insertion in *luxS*. *luxS* expression was examined in *Salmonella* serovar Typhimurium CS132 with or without *luxS* complementation in *trans* by pMS234 (*luxS* cloned in the arabinose-inducible vector, pBAD18). Strains were grown in LB with or without 0.5% arabinose, and β-galactosidase assays performed at 4 h postsubculture when the cultures were growing maximally at mid-log phase. The results of these experiments indicated that overexpression of *luxS* in *trans* does

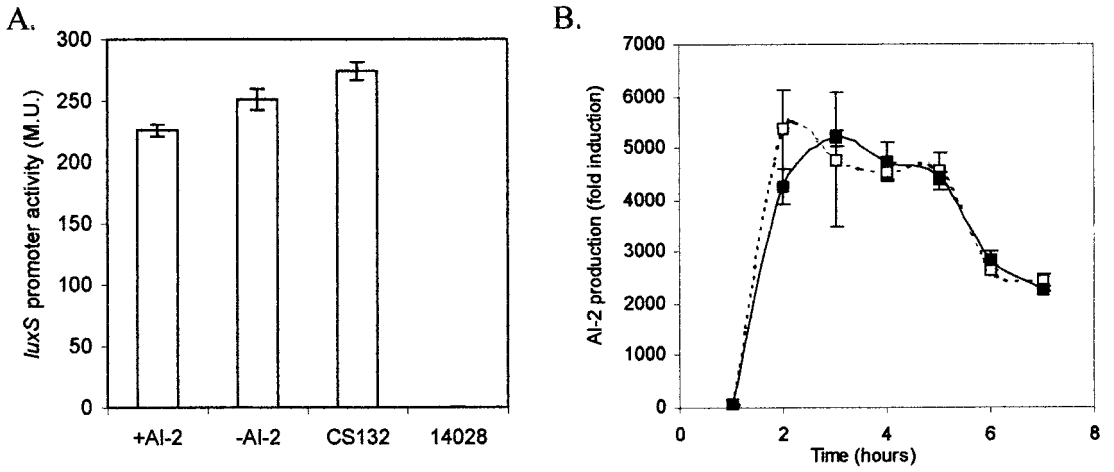

FIG. 3. AI-2 does not regulate transcription of *luxS*. (A) The *luxS*::*lacZ* transcriptional fusion in *Salmonella* serovar Typhimurium CS132 (*luxS*::*Mu*dJ) was used to monitor *luxS* expression (β-galactosidase activity measured in Miller units [M.U.]). Mid-log-phase (4 h) *luxS* expression in CS132/pMS234 (pBAD18 containing *luxS*) was measured when AI-2 was produced during growth in LB plus 0.5% arabinose. The expression of *luxS* in CS132/pMS234 was determined in the absence of AI-2 during growth in LB without arabinose induction. *luxS* expression in CS132 without pMS234 and 14028 (negative control) are also shown. (B) Overexpression of LuxS does not result in increased AI-2 production over wild-type levels. AI-2 production levels by *Salmonella* serovar Typhimurium 14028 (wild type) and *Salmonella* serovar Typhimurium CS132 (*luxS*)/pMS234 are indicated by solid and dashed lines, respectively.

not affect transcription of the native *luxS* promoter (Fig. 3A). In addition, we observed that LuxS overexpression from pMS234 in CS132 complements the AI-2 defect but does not result in increased AI-2 production from that generated by wild-type 14028, even though LuxS levels are much higher (Fig. 3B). This provides additional support for the hypothesis that AI-2 production by LuxS is regulated at the level of substrate (SRH) availability.

Possible regulatory effects on *pfs* transcription by AI-2 were also investigated. The *pfs* promoter fusion to *luxCDABE* (pAB13) was used to examine *pfs* transcription in the *luxS* mutant strain SS007 (*luxS*::T-POP) background and compared to that observed in wild-type *Salmonella* serovar Typhimurium 14028 (Fig. 4). These expression studies indicated that *pfs* transcription is not affected by the availability of AI-2.

In addition, no effect on *luxS* or *pfs* expression was observed when AI-2 was added exogenously. This finding provides additional support to our hypothesis that AI-2 does not affect expression of genes whose products are directly involved in its own production and regulation of *luxS* and *pfs* expression is at the level of substrate availability.

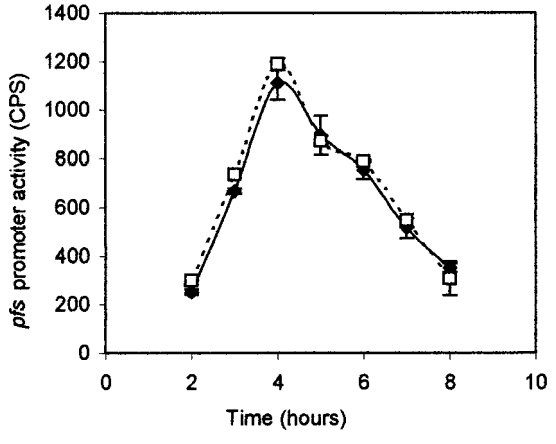

FIG. 4. AI-2 does not regulate the transcription of *pfs*. The *pfs-luxCDABE* transcriptional fusion was used to monitor *pfs* expression in the presence or absence of AI-2 in *Salmonella* serovar Typhimurium 14028 (*luxS*$^+$) or SS007 (Δ*luxS*) strain backgrounds. *pfs* expression (light production) was measured in the two strains during growth in LB with 0.5% glucose. The *pfs* expression profile in *Salmonella* serovar Typhimurium 14028/pAB13 is indicated by the solid line and in *Salmonella* serovar Typhimurium SS007 (*luxS*::T-POP)/pAB13 by the dashed line.

DISCUSSION

AI-2 is a unique bacterial signal since it is produced by both gram-negative and gram-positive species, and it has a novel structure. AI-2 is thought to be a universal signal and to mediate interspecies communication (34). The production of AI-2 by *Salmonella* serovar Typhimurium is tightly regulated. Maximal signaling activity is observed during rapid growth in exponential phase with the presence of a preferred carbon source, including glucose (40). AI-2 production by a variety of bacteria has also been shown to be regulated by growth phase and media conditions (9, 14; A. L. Beeston and M. G. Surette, unpublished observations).

LuxS utilizes SRH to generate homocysteine and the AI-2 signal (35). The enzyme Pfs acts directly upstream of LuxS in the AI-2 production pathway and is responsible for generation of adenine and the LuxS substrate SRH from SAH (35). Although the contribution of the *luxS* transcriptional profile on the regulation of AI-2 production appears to be minimal, the pattern of *pfs* transcription is correlated to the AI-2 production profile in *Salmonella* serovar Typhimurium (Fig. 1 and 2) in LB medium containing glucose or alternative carbohydrate supplements. This result indicates that the tight regulation of AI-2 production by *Salmonella* is largely at the level of *pfs* transcrip-

tion. The increase in AI-2 production due to osmotic or acid shock is not regulated at the level of *pfs* or *luxS* transcription, which suggests that these conditions may increase SAM utilization or alter Pfs and/or LuxS enzyme activity.

Although the maximal levels of *pfs* promoter activity and AI-2 production coincide temporally, the decrease in AI-2 levels after log-phase growth and *pfs* expression in LB without glucose supplements do not correlate as well. AI-2 degradation (40) or removal (42) at the onset of stationary phase is most likely responsible for this discrepancy. Alternatively, differences between AI-2 production and *pfs* transcription at this time or condition could be related to differences in the stability of Pfs or to various degrees of protein turnover in cells grown without glucose. The *lux* reporter is most likely not linked to this discrepancy since *pfs* expression profiles generated by *lux CDABE* and *lacZ* reporters were similar. The level of AI-2 is a consequence of both production and degradation rates, both of which may be independently subject to change during growth.

The decrease in *pfs* expression after maximum detectable levels of AI-2 production is consistent with a negative autoregulatory pattern. However, our results suggest that *pfs* is not transcriptionally regulated by the availability of AI-2 in the wild type or by the accumulation of the Pfs reaction product in the *luxS* null mutant (Fig. 4). In a *luxS* mutant, SRH is predicted to accumulate intracellularly since no enzymes other than LuxS are known to utilize SRH. The apparent accumulation of SRH does not result in toxicity, since we have observed that a *luxS* mutant shows no growth defects compared to wild-type *Salmonella* serovar Typhimurium or *E. coli*. If the decrease in *pfs* expression is due to accumulation of SRH, the observation that *pfs* expression profiles are the same in wild-type and isogenic *luxS* mutant strains would indicate that AI-2 production is not the major pathway for SRH catabolism (or removal from the cell). It is possible that mechanisms other than LuxS activity function to catabolize SRH in bacteria. AI-2 production may also be dependent on the availability of SAH due to the utilization of SAH as a substrate by Pfs. Intercellular SAH levels will be dependent on methionine and SAM synthesis, in addition to SAM-dependent methyl transfer reactions, in one-carbon-compound metabolism, including DNA (restriction-modification, phase variation, DNA replication), RNA, and protein methylation (such as in chemotaxis).

Similarly, *luxS* transcription also is not regulated by a negative autoregulatory feedback loop (Fig. 3A). The decrease in AI-2 production observed during the late log phase and the stationary phase is not due to a decrease in *luxS* expression. Regulation of AI-2 production was shown to occur largely through the activity of the *pfs* promoter and is seemingly under the control of a regulatory network that is tightly linked to methionine metabolism. AI-2-dependent signaling may be a reflection of metabolic state of the cell or metabolic potential of the environment rather than a consequence of cell density as reported for HSL-dependent signaling. Futhermore, overexpression of LuxS does not result in increased AI-2 production (Fig. 3B), supporting our hypothesis that AI-2 production is limited by SRH availability.

In addition to the role of Pfs in AI-2 production, this enzyme may indirectly influence the activity of the LuxI family of HSL synthases. The dual functions of Pfs prevent the accumulation of MTA and SAH, both of which have been shown to inhibit the activity of the HSL synthase family of enzymes (31). AI-2 and HSL signal generation pathways are dependent on central methionine metabolism, since both are derived from SAM, an essential metabolite, and both involve Pfs activity.

The environment of the intestinal lumen has been reported to be rich in nutrients (including carbohydrates) and of low pH and high osmolarity (20). AI-2 production by *Salmonella* serovar Typhimurium is also maximal under such conditions, suggesting that it may produce large amounts of AI-2 during infection (40). Interestingly, *pfs* is the first gene in a three-gene operon consisting of *pfs*, *btuF*, and *yadS*, where *pfs* and *btuF* have recently been shown to be cotranscribed (5). BtuF acts as a periplasmic vitamin B_{12} (cobalamin)-binding protein (5, 43), and the function of *yadS* has not been determined. Despite the genetic organization of these genes, the *pfs* and *yadS* gene products do not appear to be involved in cobalamin transport or utilization (5). Vitamin B_{12} synthesis is suppressed during aerobic growth and in media containing glucose (32), indicating that under these conditions *Salmonella* serovar Typhimurium requires BtuF-dependent cobalamin uptake from the environment. The coexpresssion of *pfs* and *btuF* (despite exhibiting different functions) indicates that *pfs* expression is high in conditions of excess vitamin B_{12}. We have observed increased *pfs* expression in LB media containing 0.5% glucose with 0.5 to 5.0 μM cyanocobalamin or hydroxycobalamin supplements (data not shown).

The vitamin B_{12} biosynthetic pathway is not required for virulence of *Salmonella* serovar Typhimurium (33), suggesting that significant amounts of vitamin B_{12} are present in the intestinal tract. The presence of *pfs* and *btuF* in the same operon may have evolved to facilitate coregulation in similar environments. Interestingly, *pfs* and *btuF* share an operon only in enteric species, suggesting that AI-2 production is tightly linked to cobalamin uptake in the intestinal tract. Understanding that the molecular basis of the regulation of AI-2 production by pathogenic bacteria is at the level of *pfs* expression may lead to the development of therapeutics that interfere with AI-2-dependent signaling.

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