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Generation of cell-to-cell signals in quorum sensing: Acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein

(autoinduction/*lux* genes/cell density-dependent gene expression)

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ABSTRACT **Many bacteria use acyl homoserine lactone signals to monitor cell density in a type of gene regulation termed quorum sensing and response. Synthesis of these signals is directed by homologs of the *luxI* gene of *Vibrio fischeri*. This communication resolves two critical issues concerning the synthesis of the *V. fischeri* signal. (*i*) The *luxI* product is directly involved in signal synthesis—the protein is an acyl homoserine lactone synthase; and (*ii*) the substrates for acyl homoserine lactone synthesis are not amino acids from biosynthetic pathways or fatty acid degradation products, but rather they are *S*-adenosylmethionine (SAM) and an acylated acyl carrier protein (ACP) from the fatty acid biosynthesis pathway. We purified a maltose binding protein–LuxI fusion polypeptide and showed that, when provided with the appropriate substrates, it catalyzes the synthesis of an acyl homoserine lactone. In *V. fischeri*, *luxI* directs the synthesis of *N*-(3-oxohexanoyl)homoserine lactone and hexanoyl homoserine lactone. The purified maltose binding protein–LuxI fusion protein catalyzes the synthesis of hexanoyl homoserine lactone from hexanoyl-ACP and SAM. There is a high level of specificity for hexanoyl-ACP over ACPs with differing acyl group lengths, and hexanoyl homoserine lactone was not synthesized when SAM was replaced with other amino acids, such as methionine, *S*-adenosylhomocysteine, homoserine, or homoserine lactone, or when hexanoyl-SAM was provided as the substrate. This provides direct evidence that the LuxI protein is an autoinducer synthase that catalyzes the formation of an amide bond between SAM and a fatty acyl-ACP and then catalyzes the formation of the acyl homoserine lactone from the acyl-SAM intermediate.**

Many Gram-negative bacteria synthesize diffusible acyl homoserine lactone molecules. These molecules serve as signals in quorum sensing, a system for cell density-dependent expression of specific sets of genes. As such, they have been termed autoinducers (for recent reviews of quorum sensing, see refs. 1–4). Acyl homoserine lactone signaling was first described in the luminous marine bacterium, *Vibrio fischeri* (5, 6), and *V. fischeri* has since become a model for studies of quorum sensing (1, 3, 4). The *luxI* gene has been shown to direct *V. fischeri* to synthesize *N*-(3-oxohexanoyl)homoserine lactone (5), and, more recently, it also has been shown to direct the synthesis of hexanoyl homoserine lactone (7). These acyl homoserine lactones bind to the product of *luxR* (8), which then serves as a transcriptional activator of the luminescence genes (1–4, 9). *N*-(3-oxohexanoyl)homoserine lactone shows more activity as an autoinducer than does hexanoyl homoserine lactone (10, 11). A variety of plant and animal pathogens use quorum sensing systems homologous to the *luxR-luxI* system to control expression of extracellular virulence factors (1–4).

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There is very little known about how *luxI* or any of its homologs direct the synthesis of acyl homoserine lactones. Crude cell extracts of *V. fischeri* catalyze the synthesis of *N*-(3-oxohexanoyl)homoserine lactone in the presence of added *S*-adenosylmethionine (SAM) and 3-oxohexanoyl-coenzyme A (CoA; ref. 12). This suggests that SAM and 3-oxohexanoyl-CoA are substrates for synthesis of *N*-(3-oxohexanoyl)homoserine lactone. Alternatively, it is possible that either of these substrates is converted to unknown compounds in the *V. fischeri* cell extract. Conflicting results have been reported with *Escherichia coli* amino acid auxotrophic mutants containing *luxI*. One report suggests that homoserine lactone or homoserine may serve as the amino acid substrate for acyl homoserine lactone synthesis (13), and another supports the earlier suggestion that SAM is the amino acid substrate (14). In fact, while it is clear that the product of *luxI* is required for *N*-(3-oxohexanoyl)homoserine lactone and hexanoyl homoserine lactone synthesis in bacteria, it has been suggested and remains a possibility that the LuxI protein does not directly catalyze the synthesis of these molecules but that it directs other common bacterial enzymes to carry out this synthesis (3).

To determine whether LuxI itself can produce an acyl homoserine lactone and to determine the substrates required for acyl homoserine lactone synthesis, we have constructed a plasmid that directs *E. coli* to synthesize a soluble form of the LuxI protein, purified that protein, and shown that it can synthesize hexanoyl homoserine lactone in the presence of SAM and hexanoyl-acyl carrier protein (ACP).

MATERIALS AND METHODS

Construction of the Maltose Binding Protein (MBP)–LuxI Expression Vector, pBLH205. A 0.6-kb, *luxI* fragment was excised from pBLH105 (14) with *Eco*RI and *Bam*HI and ligated to pMal-c2 (New England Biolabs) that had been digested with *Eco*RI and *Bam*HI. The ligation mixture was used to transform *E. coli* XL1-Blue (15), and transformants were selected by plating on Luria agar (16) plus ampicillin (80 μg/ml). Several transformants were then screened by coculturing in Luria broth (16) with *E. coli* VJS533 (17) containing pHV200I$^-$. The pHV200I$^-$ vector contains functional copies of all of the *V. fischeri* genes required for luminescence, except *luxI* (18). Several cocultures were luminescent, and the *luxI* plasmid from one was selected for further study. This plasmid, pBLH205, directed *E. coli* to overexpress an MBP–LuxI fusion protein of the predicted molecular weight, ≈66,000, as judged by Western immunoblot analyses with anti-MBP antibodies (New England Biolabs). Further confirmation of the construc-

Abbreviations: SAM, *S*-adenosylmethionine; CoA, coenzyme-A; ACP, acyl carrier protein; MBP, maltose binding protein; IPTG, isopropyl β-D-thiogalactopyranoside.

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tion was obtained by sequencing *luxI* and the junction between *luxI* and pMal-c2 by using the chain-termination method (19) and a set of *luxI* primers (5′ → 3′: −6 to +24, +424 to +444, +189 to +172, and +579 to +568, with +1 corresponding to the first nucleotide of the *luxI* open reading frame).

Purification of the MBP–LuxI Fusion Protein from *E. coli* Containing pBLH205. *E. coli* XL1-Blue (pBLH205) was grown at 30°C, with shaking in 1-liter volumes of L-Broth plus D-glucose (20 mg/ml), ampicillin (100 μg/ml), and tetracycline (10 μg/ml). Isopropyl β-D-thiogalactopyranoside (IPTG; 1 mM) was added when the culture density reached 0.5 (optical density at 600 nm), and cells were harvested by centrifugation after 2 h in the presence of IPTG. The cells were resuspended (1 g of wet cell paste per 5 ml) in a buffer containing sodium phosphate (20 mM), EDTA (0.1 mM), sodium chloride (100 mM), dithiothreitol (1 μM), glycerol [10% (vol/vol)], phenylmethylsulfonyl fluoride (100 μg/ml), and leupeptin (0.5 μg/ml), with an overall pH of 7.0. The cells were then broken with a French pressure cell (at 6.9 kPa), and the cell extract was clarified by centrifugation at 9000 × *g* at 4°C for 30 min. The MBP–LuxI fusion protein was purified from the clarified cell extract by amylose column chromatography according to the manufacturer's instructions (New England Biolabs) and stored at −70°C.

Hexanoyl Homoserine Lactone Synthase Assays. The reactions were carried out in a 0.5-ml volume of sodium phosphate buffer (20 mM, pH 7.5) containing 4 μg of MBP–LuxI plus 300 μM SAM and 10 μM hexanoyl-ACP at 23°C for 60 min, unless otherwise specified. The reactions were stopped by addition of 1 ml of ethyl acetate. The material obtained from three sequential 1-ml ethyl acetate extractions was pooled and dried by rotary evaporation, and the autoinducer activity in the ethyl acetate extract was measured by an autoinducer bioassay (see below).

HPLC Analysis of the Reaction Product. The ethyl acetate extract was fractionated by C_{18} reverse-phase HPLC in a 20–100% (vol/vol) methanol-in-water gradient as described elsewhere (18). The fractions were analyzed with an autoinducer bioassay, and the elution profile of the autoinducer activity was compared with the elution profiles of synthetic hexanoyl homoserine lactone and *N*-(3-oxohexanoyl)homoserine lactone, prepared as described elsewhere (10).

Measurement of Acyl Homoserine Lactone Synthesis. Acyl homoserine lactone formation was measured with previously described autoinducer bioassays. Hexanoyl homoserine lactone in ethyl acetate extracts was measured with the *V. fischeri* autoinducer bioassay described previously (8, 18), except that the standard curve was generated with synthetic hexanoyl homoserine lactone. Butyryl homoserine lactone was measured with the factor 2 bioassay described by Pearson *et al.* (20), octanoyl homoserine lactone was measured by using the *Agrobacterium tumefaciens* autoinducer bioassay described by Piper *et al.* (21) with synthetic octanoyl homoserine as the standard, and decanoyl homoserine lactone was measured by using the *Pseudomonas aeruginosa* LasR autoinducer bioassay (18) with synthetic decanoyl homoserine lactone as the standard.

To determine the concentrations of *N*-(3-oxohexanoyl)homoserine lactone and hexanoyl homoserine lactone produced by *E. coli*, a culture of *E. coli* XL1-Blue (pBLH105) in the late logarithmic phase of growth was centrifuged at 9000 × *g* for 20 min, the supernatant fluid was extracted with ethyl acetate (18), and the autoinducer activities were separated by HPLC. The *V. fischeri* autoinducer activity in the *N*-(3-oxohexanoyl)-homoserine lactone peak was quantitated by comparison with *N*-(3-oxohexanoyl)homoserine lactone standards, and the activity in the hexanoyl homoserine lactone peak was quantitated by using the factor 2 bioassay with hexanoyl homoserine lactone as the standard. The culture was grown in Luria–Bertani medium containing ampicillin and IPTG at 30°C with shaking (20).

Chemicals. With the exception of acyl-ACPs and hexanoyl-SAM, all chemicals were purchased from Sigma. Acyl-ACPs were synthesized biochemically from fatty acids and holo-ACP by using purified *Vibrio harveyi* acyl-ACP synthetase as described previously (22). Holo-ACP was purified from an *E. coli* strain that overproduces the protein from a synthetic gene construct as described (23). Hexanoyl-SAM was purified by acylation of SAM with the *N*-hydroxysuccinimide of hexanoic acid as described for the synthesis of *N*-acyl-serine (24). The reaction gave a new UV-absorbing compound that was separated from SAM by thin layer chromatography. The product was ninhydrin-negative and, upon heating at pH 4, gave a UV-absorbing product that comigrated with thiomethyladenosine. The hexanoyl-SAM was purified by ion exchange chromatography as described for SAM (25) except that Dowex-50 (Na^+ form) resin was used. As shown by positive ion electrospray quadrapole mass spectrometry the molecular mass of the purified material was consistent with that of hexanoyl-SAM.

RESULTS

Purification of the MBP–LuxI Fusion Protein. The recent discovery that in *V. fischeri, luxI* directs the synthesis of hexanoyl homoserine lactone in addition to *N*-(3-oxohexanoyl)-homoserine lactone (7) led us to attempt to purify LuxI and study its activity *in vitro*. The probable fatty acyl substrates for synthesis of *N*-(3-oxohexanoyl)homoserine lactone, 3-oxohexanoyl-CoA, or 3-oxohexanoyl-ACP are not available commercially and are not easily synthesized, whereas the probable substrates for hexanoyl homoserine lactone synthesis, hexanoyl-CoA, and hexanoyl-ACP are readily available. Hexanoyl-CoA can be purchased commercially, and pure hexanoyl-ACP can be produced in high yield (22). To gain information on the relative amounts of *N*-(3-oxohexanoyl)homoserine lactone and hexanoyl homoserine lactone produced by LuxI-containing bacteria, we compared the amounts of the two compounds in *E. coli* (pBLH105) culture fluid and found them to be at roughly equimolar concentrations. Although the physiological significance of this production of relatively high levels of hexanoyl homoserine lactone is unknown, our results indicate that it is reasonable to substitute hexanoyl compounds for 3-oxo-hexanoyl compounds in our experiments.

We first overexpressed LuxI in *E. coli* XL1-Blue (pBLH105) and found that the overexpressed protein was primarily in the insoluble fraction of cell extracts after centrifugation at 9000 × *g* for 30 min (data not shown). The pBLH105 construct contains the *luxI* open reading frame cloned downstream of the *tac* promoter and a Shine–Dalgarno sequence in pKK223-3. There are 10 bp between the Shine–Dalgarno sequence and the start of *luxI*. To express a soluble form of LuxI, we constructed pBLH205 (see *Materials and Methods*), which directs *E. coli* XL1-Blue to overexpress an MBP–LuxI fusion protein. This fusion protein remained in the supernatant fluid after centrifugation of cell extracts. The fusion protein was purified from the cell extracts by amylose affinity chromatography (Fig. 1), with a yield of 2–3 mg of protein per liter of culture. The apparent molecular weight of the fusion protein, 66,000, estimated from the SDS/PAGE analysis (Fig. 1) is consistent with the predicted molecular weight of the fusion protein, 64,609. Although the fusion protein contains a factor X_A cleavage site, we were unable to cleave MBP and LuxI in repeated attempts with the factor X_A protease.

Autoinducer Synthase Activity of the Purified MBP–LuxI Fusion Protein. Incubation of the purified MBP–LuxI protein with hexanoyl-ACP and SAM resulted in synthesis of a molecule that activated the *V. fischeri lux* genes. The product showed an HPLC elution profile identical to that of hexanoyl homoserine lactone (Fig. 2). Thus we believe the autoinducer synthesized by the fusion protein in the presence of SAM and

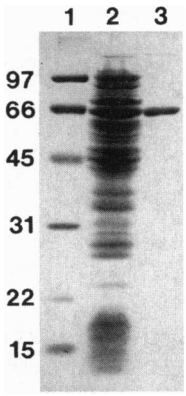

FIG. 1. Purification of the MBP–LuxI fusion protein from extracts of *E. coli* (pBLH205). Coomassie brilliant blue-stained SDS/polyacrylamide gel. Lane 1, low molecular mass (in kDa) standard proteins; lane 2, cell extract (40 μg of protein); and lane 3, purified MBP–LuxI following amylose affinity chromatography (4 μg of protein).

hexanoyl-ACP is hexanoyl homoserine lactone. The rate of hexanoyl homoserine lactone synthesis was linear for at least 90 min (data not shown).

An autoinducer activity was not produced when hexanoyl-CoA was used in place of hexanoyl-ACP. The presence of hexanoyl-CoA in the reaction mixture did not inhibit enzyme activity with hexanoyl-ACP and SAM (Table 1), indicating that the lack of activity with hexanoyl-CoA and SAM was not due to the detergent properties of the hexanoyl-CoA. These results demonstrate that the MBP–LuxI fusion protein has a hexanoyl homoserine lactone synthase activity and indicate that the fatty acyl substrate is derived from fatty acid biosynthesis through the acyl-ACP rather than from fatty acid degradation through the acyl-CoA.

In *V. fischeri*, LuxI directs the synthesis of *N*-(3-oxohexanoyl)homoserine lactone and hexanoyl homoserine lactone. We replaced hexanoyl ACP with fatty acyl-ACPs of differing acyl chain lengths to assess whether a specificity for the six-carbon fatty acyl-ACP was exhibited by the purified synthase. Small amounts of butyryl homoserine lactone and octanoyl homoserine lactone were produced when butanoyl-ACP and octanoyl-ACP were substituted for hexanoyl-ACP,

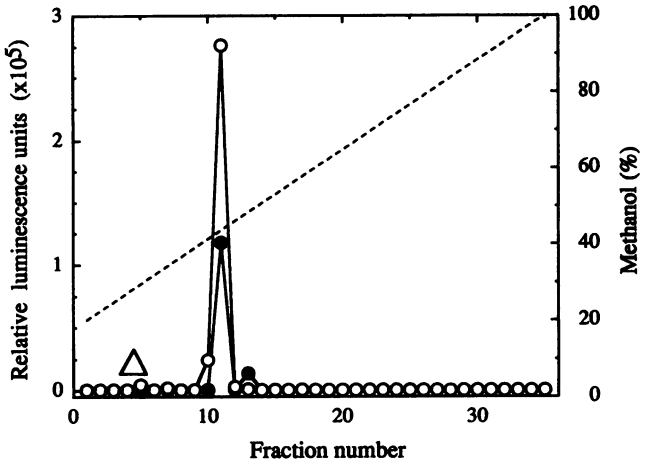

FIG. 2. HPLC analysis of autoinducer activity produced by the MBP–LuxI protein in the presence of hexanoyl ACP and SAM. Autoinducer activity synthesized by the MBP–LuxI protein (○) and synthetic hexanoyl homoserine lactone (●) are shown. The region in which *N*-(3-oxohexanoyl) homoserine lactone is eluted is indicated by the large triangle, and the dashed line shows the methanol concentration.

Table 1. Substrate requirements for synthesis of acyl homoserine lactones by purified MBP–LuxI

Substrates added	Acyl homoserine lactone produced,* pmol/min per mg of protein
None	<1
SAM plus hexanoyl-ACP	967
SAM	<1
Hexanoyl-ACP	<1
SAM plus hexanoyl-CoA†	<1
SAM plus butanoyl-ACP	33‡
SAM plus octanoyl-ACP	17‡
SAM plus decanoyl-ACP	<1‡
SAM plus hexanoic acid	<1
Methionine plus hexanoyl-ACP	<1
Homoserine plus hexanoyl-ACP	<1
Homoserine lactone plus hexanoyl-ACP	<1
Homocysteine plus hexanoyl-ACP	<1
S-adenosylhomocysteine plus hexanoyl-ACP	<1
S-adenosylcysteine plus hexanoyl-ACP	<1
Hexanoyl-SAM§	<1

*Except for reactions in the presence of hexanoyl-SAM, fatty acyl substrates were at a concentration of 10 μM and amino acid substrates were at 300 μM. Reactions were as described.

†The presence of hexanoyl-CoA at concentrations as high as 300 μM did not inhibit hexanoyl homoserine lactone synthesis from SAM and hexanoyl-ACP.

‡For reactions with butyryl, octanoyl, and decanoyl ACP, the product formed was determined by using synthetic butyryl, octanoyl, and decanoyl homoserine lactone as standards and bioassays as indicated.

§Hexanoyl-SAM was tested at a concentration of 10 μM. Hexanoyl-SAM (10 μM) did not inhibit hexanoyl homoserine lactone synthesis from hexanoyl ACP and SAM.

but enzyme activity with these alternate substrates was <5% of the activity with hexanoyl-ACP. Decanoyl homoserine lactone was not detected when the reaction mixtures contained decanoyl-ACP in place of hexanoyl-ACP (Table 1).

When SAM was replaced with methionine, homoserine, homoserine lactone, homocysteine, *S*-adenosyl homocysteine, *S*-adenosylcysteine, or hexanoyl-SAM, production of hexanoyl homoserine lactone or any compound that could function as a *V. fischeri lux* autoinducer was not detected (Table 1). We note that some preparations of hexanoyl-SAM contained low levels of autoinducer activity before incubation with MBP–LuxI. However, incubation with or without the MBP–LuxI protein did not result in increased levels of autoiducer activity (data not shown).

Kinetics of Hexanoyl Homoserine Lactone Synthase Activity. We determined the K_m for both hexanoyl-ACP and SAM. From experiments in which hexanoyl-ACP concentrations ranged from 0.5–50 μM at a SAM concentration of 300 μM, we calculated a K_m for hexanoyl-ACP of 9.6 μM. From experiments in which the SAM concentration ranged from 0.5–500 μM at a hexanoyl-ACP concentration of 20 μM, we calculated a K_m for SAM of 130 μM. With either hexanoyl-ACP or SAM, the calculated V_{max} was 1.1 mol of hexanoyl homoserine lactone per min per mol of MBP–LuxI.

Temperature and pH Optima. The autoinducer synthase activity occurred over a limited range of temperatures and pH values. The MBP–LuxI protein showed good activity at temperatures between 20 and 30°C. At 18°C, activity was 57% of maximal, and at 37°C it was 10% of maximal. The MBP–LuxI protein was reasonably active between pH 7.1 and 8.0. Maximum activity was at 7.8. At a pH of 8, activity was reduced to 50% of maximum, and at 7.1 activity was 65% of maximum. There was a sharp loss of activity below pH 7.1, and at pH 7.0 activity was 10% of the maximum.

DISCUSSION

We have purified an MBP–LuxI fusion protein and shown that it can catalyze the synthesis of hexanoyl homoserine lactone from hexanoyl-ACP and SAM. Additional cofactors or energy sources were not required for activity. This shows that the LuxI protein functioned as a synthase and argues against the hypothesis that LuxI homologs direct bacteria to synthesize autoinducers by regulating or modifying other cellular enzymes (3).

The LuxI activity requires SAM, no activity was detected when any of a number of other amino acid substrates were used in place of SAM (Table 1). This supports the earlier studies of Eberhard *et al.* (12) with crude cell extracts of *V. fischeri*, and our more recent analysis of *luxI*-directed *V. fischeri* autoinducer synthesis in *E. coli* amino acid auxotrophs (14). Thus there is a growing body of evidence against the suggestion that homoserine or homoserine lactone serve as an amino acid substrate for acyl homoserine lactone synthesis (13). Because our assay for enzyme activity was dependent on formation of a product with autoinducer activity, we cannot rule out the possibility that the MBP–LuxI catalyzed amide bond formation between some of the amino acid substrates we tested and hexanoyl-ACP.

We also showed that hexanoyl-ACP rather than hexanoyl-CoA serves as a fatty acid substrate for the hexanoyl homoserine lactone synthase activity. Eberhard *et al.* (12) showed that, in crude cell extracts of *V. fischeri*, an acyl-CoA was required together with SAM for autoinducer biosynthesis. Because high concentrations of the acyl-CoA were required for activity, it was suggested that the true substrate for the synthase enzyme might be the acyl-ACP. Our studies provide evidence for this suggestion. Apparently the fatty acid substrate for LuxI is acquired from the pool of acyl-ACPs generated during fatty acid biosynthesis rather than from products of fatty acid degradation. This would allow for a continuous supply of the fatty acid substrate regardless of the growth conditions. Furthermore, the MBP–LuxI protein showed a high degree of specificity for the six-carbon fatty acyl-ACP (Table 1). This provides an explanation for why *luxI* directs bacteria to produce *N*-(3-oxohexanoyl)homoserine lactone and hexanoyl homoserine in a rather specific fashion (7).

An interesting result from the kinetic analysis of hexanoyl homoserine lactone synthesis by the MBP–LuxI protein was the low V_{max}. Our results indicated that the turnover rate for the enzyme was one molecule per minute. It could be argued that this low rate of synthesis is a consequence of studying LuxI with a large polypeptide fused to its N terminus. However, recently the *A. tumefaciens* LuxI homolog, TraI, has been purified as a His-tagged polypeptide and appears to show kinetics similar to LuxI when provided with SAM and a chemically synthesized acyl-ACP (26). It is possible that these autoinducer synthases, which are required to produce low levels of cell-to-cell signal compounds, are designed to do so economically by means of an extraordinarily low V_{max}. These results should be considered with caution because the purifications of both autoinducer synthases were based on affinity to fused polypeptides rather than by following activity. Therefore a large fraction of both purified proteins could be inactive.

Our data support models for autoinducer synthesis such as that depicted by Sitnikov *et al.* (4). According to such models (Fig. 3), the acyl group is transferred from the ACP to an active site cysteine in LuxI, SAM binds to the enzyme and an amide bond is formed between carbon 1 of the fatty acid and the amino group of SAM to form an acyl-SAM intermediate. The formation of acyl-SAM is then followed by lactone ring formation to yield the acyl homoserine lactone and 5′-methylthioadenosine. Of interest, hexanoyl-SAM did not serve as a substrate for activity of the MBP–LuxI protein, at least under the conditions of our experiments. This suggests that perhaps hexanoyl-SAM is an enzyme-bound intermediate and

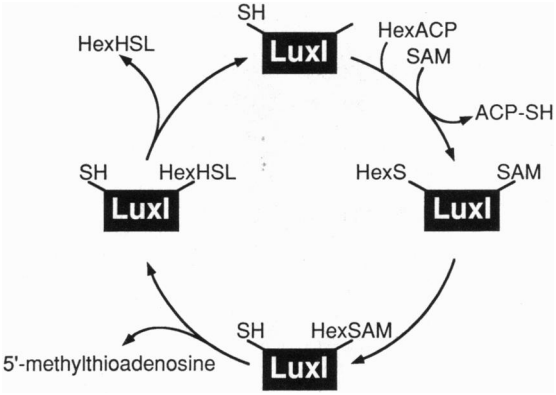

FIG. 3. Hypothetical scheme for hexanoyl homoserine lactone synthesis catalyzed by LuxI. HexACP indicates hexanoyl-ACP, HexSAM, hexanoyl-SAM, and HexHSL, hexanoyl homoserine lactone. The hexanoyl group is transferred from ACP to an active site cysteine on LuxI, and SAM binds to the active site. The hexanoyl group is released from the cysteine to form an amide bond with the amino group of SAM. 5′-Methylthioadenosine is released, and a lactonization reaction results in the synthesis of hexanoyl homoserine lactone.

that free hexanoyl-SAM cannot interact appropriately with the catalytic site of LuxI. It also indicates that the lactonization step does not readily occur spontaneously and that it requires catalysis by the enzyme as depicted in Fig. 3. The LuxI polypeptide is only 193-aa residues long, yet it serves as the sole enzyme for the multistep synthesis of hexanoyl homoserine lactone. Only the acylation of SAM should require extensive protein machinery. In fact, some of our hexanoyl-SAM preparations showed low levels of autoinducer activity, indicating that the lactonization of hexanoyl-SAM can occur spontaneously but not rapidly. Sulfonium compounds such as SAM and acyl-SAM are intrinsically unstable. For example, the formation of homoserine lactone in neutral and mildly acid solutions of SAM is well-known (27, 28). Thus the lactonization step may require only a minimal contribution from LuxI. It is clear that understanding the details of the autoinducer synthase reaction awaits further investigation, but the ability to study the synthesis of acyl homoserine lactones by a purified LuxI polypeptide should allow more rapid progress in elucidating the mechanism of autoinducer signal formation in cell density-dependent control of gene expression by Gram-negative bacteria.

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