Mutational Analysis of the *Vibrio fischeri* LuxI Polypeptide: Critical Regions of an Autoinducer Synthase

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Synthesis of the *Vibrio fischeri* autoinducer, a signal involved in the cell density-dependent activation of bioluminescence, is directed by the *luxI* gene product. The LuxI protein catalyzes the synthesis of *N*-acyl-homoserine lactones from *S*-adenosylmethionine and acylated-acyl carrier protein. We have gained an appreciation of the LuxI regions and amino acid residues involved in autoinducer synthesis by isolating and analyzing mutations generated by random and site-specific mutagenesis of *luxI*. By random mutagenesis we isolated 13 different single amino acid substitutions in the LuxI polypeptide. Eleven of these substitutions resulted in no detectable autoinducer synthase activity, while the remaining two amino acid substitutions resulted in reduced but detectable activity. The substitutions that resulted in no detectable autoinducer synthase activity mapped to two small regions of LuxI. In *Escherichia coli*, wild-type *luxI* showed dominance over all of the mutations. Because autoinducer synthesis has been proposed to involve formation of a covalent bond between an acyl group and an active-site cysteine, we constructed site-directed mutations that altered each of the three cysteine residues in LuxI. All of the cysteine mutants retained substantial activity as an autoinducer synthase in *E. coli*. Based on the analysis of random mutations we propose a model in which there are two critical regions of LuxI, at least one of which is an intimate part of an active site, and based on the analysis of site-directed mutations we conclude that an active-site cysteine is not essential for autoinducer synthase activity.

N-Acyl-homoserine lactones (commonly called autoinducers) are produced by many gram-negative bacteria and serve as intercellular signals that facilitate a phenomenon termed quorum sensing (for recent reviews see references 9, 10, 22, and 26). Quorum sensing allows a bacterial species to monitor its population density and activate specific sets of genes at a sufficiently high cell density. Regulation of luminescence in *Vibrio fischeri* was the first reported example of *N*-acyl-homoserine lactone-mediated quorum sensing, and it continues to serve as a model system (5, 9, 10, 18, 22, 26). In *V. fischeri*, the *luxR* gene encodes a transcriptional regulator that activates expression of the luminescence genes in the presence of a sufficient concentration of *N*-(3-oxohexanoyl)homoserine lactone (7, 9, 10, 22, 26). The *luxI* gene encodes an autoinducer synthase that catalyzes the production of *N*-(3-oxohexanoyl)homoserine lactone (5, 7). There is relatively little known about the mechanism of autoinducer synthesis.

Recently, LuxI from *V. fischeri* (24) and TraI from *Agrobacterium tumefaciens* (17) were shown to catalyze the synthesis of *N*-acyl-homoserine lactones from *S*-adenosylmethionine (SAM) and acylated-acyl carrier protein (ACP). These findings are consistent with proposed models for the mechanism of autoinducer synthesis (6, 17, 26). The models suggest that a covalent intermediate forms between an acyl group, transferred from ACP, and a cysteine residue on the enzyme. SAM binds to the enzyme, followed by amide bond formation between the carboxyl group of the fatty acid and the amino group of SAM, yielding an acyl-SAM intermediate. Finally, the acyl-

SAM intermediate cyclizes, resulting in release of the acyl-homoserine lactone and 5'-methylthioadenosine.

We have generated random and site-directed mutations in the *luxI* gene and studied the activity of the encoded LuxI polypeptides in vivo. Seven of 11 mutations that inactivated *luxI* mapped to a region spanning residues 25 to 70 of this 193-amino-acid polypeptide. The other four mutations mapped to a second region between residues 104 and 164. Two random mutations that resulted in reduced autoinducer synthase activity mapped between the two regions. We altered the three cysteine codons in *luxI* by site-directed mutagenesis and showed that none of the cysteines were essential for autoinducer synthase activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. The bacterial strains and plasmids used in this study are described in Tables 1 through 3. Unless otherwise specified, cultures were grown in Luria broth or Luria agar (25) containing the appropriate antibiotics for plasmid screening and maintenance (100 µg of ampicillin per ml, 30 µg of chloramphenicol per ml, and 10 µg of tetracycline per ml) at 30°C. Where indicated, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to cultures at a final concentration of 1 mM.

Random mutagenesis. Hydroxylamine mutagenesis of pBLH105 in vitro was performed as described by Slock et al. (27), except that the plasmid was treated with hydroxylamine for 18 to 24 hours. *Escherichia coli* DH5α was transformed with mutagenized plasmids, and the transformants were grown on Luria agar containing ampicillin. Transformants were screened for production of *N*-(3-oxohexanoyl)homoserine lactone by coculturing with the autoinducer bioassay strain *E. coli* VJS533(pHV2001[−]) as described previously (24). Transformants that did not induce light production in the reporter strain were further characterized.

Site-directed mutagenesis. A *Bam*HI fragment from pBLH105 containing the *luxI* gene and *tac* promoter was cloned into pALTER-1 (Promega, Madison, Wis.) to create the plasmid pLuxIMut-9. Mutagenesis was performed with the Altered Sites II in vitro mutagenesis system (Promega) as described by the manufacturer. Oligonucleotide primers containing the appropriate base substitutions for each mutation were obtained from Genosys Biotechnologies, Inc. (The Woodlands, Tex.). The plasmids pLIC68A, pLIC59S, and pLIC160S were constructed by cloning a *Bam*HI fragment from mutagenized pLuxIMut-9 into

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s) ^a	Source or reference ^b
<i>E. coli</i> strains		
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F' proAB lacI^qΔM15 Tn10(Tc^r)]</i>	2
DH5α	<i>recA1 Δ(lacZYA-argF) U169 hsdR17 thi-1 gyrA96 supE44 endA1 relA1 (φ80dlacZΔM15)</i>	12
VJS533	<i>ara Δ(lac-proAB) rpsL φ80lacZΔM15 recA56</i>	28
MG4	<i>Δ(argF-lac) U169 zah-735::Tn10 recA56 srl::Tn10</i>	21
Plasmids		
pHV2001 ⁻	8.8-kb <i>V. fischeri</i> ES114 <i>lux</i> regulon with inactivated <i>luxI</i> in pBR322; Ap ^r	19
pKDT17	<i>lasB::lacZ</i> translational fusion and <i>plac-lasR</i> in pTS400; Ap ^r	19
pALTER-1	Site-directed mutagenesis vector; Tc ^r	Promega
pLuxMut-9	0.8-kb <i>Bam</i> HI fragment from pBLH105 in the <i>Bam</i> HI site of pALTER-1; Tc ^r	This study
pECP61.5	<i>rhlA::lacZ</i> translational fusion and <i>ptac-rhlR</i> in pSW205; Ap ^r	Everett Pesci
pMAL-c2	Cytoplasmic MBP fusion expression vector; Ap ^r	NEB
pBLH105	0.6-kb fragment encoding <i>V. fischeri</i> MJ-1 <i>luxI</i> in the <i>Eco</i> RI- <i>Sma</i> I site of pKK223-3 with optimally spaced ribosomal binding site; Ap ^r	13
pBLH205	0.6-kb <i>luxI Eco</i> RI- <i>Bam</i> HI fragment from pBLH105 in pMAL-c2 encoding MBP-LuxI fusion protein; Ap ^r	24
pBLH305	0.8-kb PCR product containing the <i>ptac</i> and <i>luxI</i> regions of pBLH105 in the <i>Bam</i> HI site of pACYC184; Cm ^r	This study

^a Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Tc^r, tetracycline resistant.

^b NEB, New England Biolabs, Beverly, Mass.

pKK223-3 digested with *Bam*HI. This procedure removes the *tac* promoter and multiple cloning site from pKK223-3, but an analogous fragment is contained on the cloned *Bam*HI fragment. The plasmids from the resulting transformants were screened by restriction digestion to select clones with the *Bam*HI fragment in the same orientation as that of pBLH105. Because the mutation that produced the Cys-68-Ser substitution generated an internal *Bam*HI site, pLIC68S was constructed by cloning the *luxI* mutation on an *Eco*RI-*Sma*I fragment into pKK223-3. The resulting plasmids were identical to pBLH105 except for the site-directed mutations in the Cys codons of *luxI*.

DNA sequencing. The *luxI* mutations and *tac* promoter regions of all of the LuxI mutant plasmids were sequenced by the chain termination method, with previously described primers (24).

Preparation of anti-LuxI serum. A maltose-binding protein (MBP)-LuxI fusion protein was prepared from extracts of *E. coli* XL1-Blue(pBLH205) by affinity chromatography as described by Schaefer et al. (24). The MBP-LuxI antiserum was from New Zealand White rabbits (Berkeley Antibody Company, Richmond, Calif.). The antiserum was preadsorbed with *E. coli* XL1-Blue (pMAL-c2) cells (23), and immunoglobulin G was isolated by precipitation with 33% ammonium sulfate and stored in phosphate-buffered saline (4).

Gel electrophoresis and Western immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 12% acrylamide resolving gel as previously described (14). For Western immunoblotting we used a 1:500 dilution of preadsorbed MBP-LuxI antiserum with the procedure described previously (1).

Measurement of acyl-homoserine lactones in culture fluids. As a screen for production of acyl-homoserine lactones by mutant LuxI polypeptides in *E. coli* XL1-Blue, 250-ml cultures in 1-liter flasks were grown to an optical density (at 600 nm) of approximately 1.5 in Luria broth containing 0.8% glycerol, ampicillin, tetracycline, and IPTG. Cells were removed by centrifugation, the culture fluids were extracted with 2 equal volumes of acidified ethyl acetate, and the extracts were concentrated 100-fold as described previously (19). *N*-Acyl-homoserine lactones in the extracts were measured by using the *V. fischeri* autoinducer bioassay with strain VJS533(pHV2001⁻) (19), the *Pseudomonas aeruginosa las* bioassay with strain MG4(pKDT17) (19), and the *P. aeruginosa rhl* bioassay with strain DH5α(pECP61.5) (20). Standard curves were generated with synthetic *N*-(3-oxohexanoyl)homoserine lactone, *N*-(3-oxododecanoyl)homoserine lactone, and *N*-butyrylhomoserine lactone, respectively.

For all cultures in which *N*-(3-oxohexanoyl)homoserine lactone was detected, a more accurate measurement of the quantity of this substance was made by extraction of 1.5 ml of culture fluid (optical density at 600 nm of 1.0) twice with 3 ml of ethyl acetate. The concentration step described above was not necessary and was omitted to eliminate a possible source of error in measurement of *N*-(3-oxohexanoyl)homoserine lactone. The *V. fischeri* autoinducer bioassay was used to measure *N*-(3-oxohexanoyl)homoserine lactone in the extracts.

RESULTS

Isolation of pBLH105 derivatives with *luxI* mutations. Approximately 5,000 *E. coli* transformants, containing pBLH105

derivatives, were screened for the ability to induce luminescence in the LuxI⁻ mutant reporter strain, *E. coli* VJS533 (pHV2001⁻), by coculture in individual microtiter dish wells (24). Thirty-three transformants failed to induce luminescence by the reporter and were studied further. These transformants were screened for expression of LuxI polypeptides by Western immunoblotting. We observed, as previously reported by other investigators (8), that the LuxI polypeptide migrated anomalously in SDS-PAGE. Although the molecular weight of LuxI predicted from the DNA sequence is 21,909, SDS-PAGE indicated a mass of 25,000 to 28,000 daltons (8) (Fig. 1A). Twenty-three of the 33 transformants contained plasmids that directed the expression of LuxI polypeptides. Two of these LuxI polypeptides appeared significantly smaller than the wild-type LuxI (data not shown). DNA sequencing showed that the plasmids encoding these LuxI polypeptides contained nonsense mutations in *luxI*. Of the remaining 21 plasmids, pBLH107 directed the synthesis of LuxI at a level slightly lower than that of the wild type (Fig. 1B, lane 3), and pBLH108 directed expression of a LuxI polypeptide with a slightly faster electrophoretic migration than those of the other LuxI polypeptides

FIG. 1. Anti-LuxI Western immunoblot analysis of *E. coli* XL1-Blue containing *ptac luxI* plasmids. (A) The wild-type *ptac-luxI* plasmid pBLH105. Lanes: 1, negative control (*E. coli* without a *luxI* plasmid grown with IPTG); 2, *E. coli* (pBLH105) grown without IPTG; 3, *E. coli* (pBLH105) grown with IPTG. (B) *E. coli* containing mutant *ptac luxI* plasmids obtained by hydroxylamine treatment of pBLH105. All cultures were grown in the presence of IPTG. Lanes: 1, negative control (*E. coli* without a *luxI* plasmid); 2, *E. coli* (pBLH105); 3, *E. coli* (pBLH107); 4, *E. coli* (pBLH108); 5, *E. coli* (pBLH109); 6, *E. coli* (pBLH110); 7, *E. coli* (pBLH111); 8, *E. coli* (pBLH112); 9, *E. coli* (pBLH114); 10, *E. coli* (pBLH116); 11, *E. coli* (pBLH118); 12, *E. coli* (pBLH120); 13, *E. coli* (pBLH121); 14, *E. coli* (pBLH124); 15, *E. coli* (pBLH126). Molecular masses are given on the left of each panel, in kilodaltons.

TABLE 2. Analysis of random *luxI* mutations in pBLH105 derivatives

LuxI plasmid	Nucleic acid base and substitution ^a	Amino acid substitution	<i>V. fischeri</i> autoinducer concn (nM) ^b
pKK223-3	— ^c	—	<5
pBLH105	None (wild type)	None	15,000
pBLH112	G-74 to A	Arg-25 to His	<5
pBLH108	G-130 to A	Glu-44 to Lys	<5
pBLH116 ^d	G-136 to A	Asp-46 to Asn	<5
pBLH118	G-145 to A	Asp-49 to Asn	<5
pBLH120	G-178 to A	Asp-60 to Asn	<5
pBLH109	G-200 to A	Gly-67 to Glu	<5
pBLH124 ^d	G-209 to A	Arg-70 to His	<5
pBLH110	C-221 to T	Thr-74 to Ile	40
pBLH121	C-280 to T	Pro-94 to Ser	90
pBLH126	G-311 to A	Arg-104 to His	<5
pBLH111 ^d	G-397 to A	Ala-133 to Thr	<5
pBLH107	G-448 to A	Glu-150 to Lys	<5
pBLH114 ^d	G-491 to A	Gly-164 to Glu	<5

^a Base substitutions indicated are in the *luxI* coding strand.^b Measured in culture fluid as described in Materials and Methods. The limit of detection of the sampling and bioassay method was 5 nM.^c Dashes indicate the absence of a *luxI* gene or product.^d A duplicate, independently obtained plasmid with the same *luxI* mutation was isolated.

(Fig. 1B, lane 4). The remaining plasmids directed the synthesis of LuxI polypeptides that could not be distinguished from wild-type LuxI by Western immunoblotting (Fig. 1B).

DNA sequence analysis of *luxI* mutations. The specific base changes in the 21 plasmids that directed the synthesis of full-length or nearly full-length LuxI were determined by nucleotide sequencing. The nucleotide changes and resulting amino acid substitutions are shown in Table 2. Four pairs of plasmids had identical mutations and were obtained from the same transformation mixture; for this reason these pairs were assumed to be siblings. Four additional pairs of plasmids had identical mutations but were obtained from either different transformations or different hydroxylamine reaction mixtures; these were independent mutations. This suggests that we have approached saturation for this mutagenesis procedure. Only one plasmid from each of the identical pairs was selected for further characterization. Thus, we obtained 13 unique *luxI* mutations, 4 of which were obtained more than once in independent screens. Each of the plasmids encoding a full-length polypeptide had a single base pair substitution in *luxI* that resulted in a single amino acid codon change. All 13 of the mutations were GC to AT transitions, as expected (3) from hydroxylamine mutagenesis (Table 2).

Measurement of acyl-homoserine lactones produced by *E. coli* containing the mutant *luxI* plasmids. Extracts of culture fluid from *E. coli* with mutant LuxI polypeptides were screened to determine if small quantities of *N*-(3-oxohexanoyl)homoserine lactone, which could not be detected by the coculture screen, were produced (Table 2). The extracts were also tested with the *P. aeruginosa las* and *rhl* bioassays to determine if any of the mutant LuxI polypeptides synthesized an acylated-homoserine lactone other than *N*-(3-oxohexanoyl)homoserine lactone. The *las* bioassay can be used to detect 3-oxoacylhomoserine lactones with 8 to 14 carbons in the acyl chain (11, 19), and the *rhl* bioassay can be used to detect butyryl or hexanoylhomoserine lactone (16, 20). In addition to *N*-(3-oxohexanoyl)homoserine lactone, wild-type LuxI produces smaller quantities of hexanoylhomoserine lactone and *N*-(3-oxooctanoyl)homoserine lactone (reference 15 and unpublished data). Of the 13 mutants, 2, the Pro-94-Ser and Thr-74-Ile LuxI polypep-

tides, produced acyl-homoserine lactones detected by each of the bioassays. *E. coli* containing the Pro-94-Ser LuxI polypeptide produced 170-fold less *V. fischeri* autoinducer than wild-type LuxI, and *E. coli* containing the Thr-74-Ile LuxI polypeptide produced 400-fold less (Table 2). None of the other LuxI mutant strains produced autoinducers detected by any of the three bioassays. For the Pro-94-Ser and Thr-74-Ile LuxI mutants, the relative levels of activity in each of the three bioassays were similar to those found with *E. coli* strains containing wild-type *luxI*. Furthermore, high-performance liquid chromatography analysis showed that *N*-(3-oxohexanoyl)homoserine lactone remained as the major product of the Pro-94-Ser and Thr-74-Ile mutant LuxI polypeptides (data not shown). Our results indicate that the Pro-94-Ser and Thr-74-Ile mutants do not alter the relative abundance of acylated homoserine lactones produced by wild-type LuxI.

Of the 11 mutations that resulted in no detectable autoinducer synthase activity, 7 mapped to a region between residues 25 and 70 of LuxI and the other 4 mapped to a region between residues 104 and 164. The two mutations that resulted in low levels of activity mapped to an area between the two regions described above. Of interest, four of the mutations altered amino acid residues in four of the seven positions that are completely conserved among the 12 LuxI homologs available for our database search, and four mutations altered amino acids conserved in 80% or more of the homologs (9). The eight highly conserved residues (80% or better) are Arg-25, Glu-44, Asp-46, Asp-49, Arg-70, Arg-104, Ala-133, and Gly-164. We identified five residues that were less well conserved. The mutations in three of these less well conserved residues, Asp-60, Gly-67, and Glu-150, resulted in the loss of all detectable autoinducer synthesis (Table 2). Alignments of the LuxI homologs revealed little conservation at Asp-60; there are eight different amino acid residues at this position in the 12 LuxI homologs that were compared (9). The position corresponding to Gly-67 shows considerably more conservation. Five of the LuxI homologs have a Gly at this position, and the remaining seven have a cysteine at this position. The Glu at position 150 is conserved in five LuxI homologs. Another five have a Leu at this position, one has a Phe, and one has an Arg. Of the two residues in which substitutions resulted in a low but detectable activity of LuxI, Thr-74 is conserved in 7 of the 12 aligned sequences and all of the sequences have a hydrophobic residue at this position. Pro-94 is conserved in four of the sequences. Of the other eight sequences, four have a Leu, three have an Ile, and one has a Ser at this position.

Dominance of the wild type over *luxI* mutations in *E. coli*. We measured autoinducer levels produced by *E. coli* containing the wild-type *luxI* plasmid pBLH305 (which encodes expression of *luxI* at a level similar to that of pBLH105 [data not shown]) and mutant *luxI* plasmids. For these experiments IPTG was omitted from the medium because growth ceased when *luxI* overexpression from both plasmids was driven by IPTG. Culture fluid from *E. coli* containing both pBLH105 and

FIG. 2. Anti-LuxI Western immunoblot analysis of *E. coli* XL1-Blue containing plasmids with mutations in cysteine codons. All cultures were grown with IPTG. Lanes: 1, *E. coli*; 2, *E. coli*(pBLH105); 3, *E. coli*(pLIC59S); 4, *E. coli*(pLIC68S); 5, *E. coli*(pLIC68A); 6, *E. coli*(pLIC160S). Molecular masses are given on the left, in kilodaltons.

TABLE 3. Analysis of site-directed *luxI* mutations

LuxI plasmid	Nucleic acid base and substitution ^a	Amino acid substitution	<i>V. fischeri</i> autoinducer concn (nM) ^b
pKK223-3	— ^c	—	<5
pBLH105	None (wild type)	None	15,000
pLIC59S	G-176 to C	Cys-59 to Ser	4,500
pLIC68S	G-203 to C	Cys-68 to Ser	800
pLIC68A	T-202 to G, G-203 to C	Cys-68 to Ala	2,200
pLIC160S	G-479 to C	Cys-160 to Ser	5,600
pBLH110 ^d	C-221 to T	Thr-74 to Ile	40

^a Base substitutions indicated are in the *luxI* coding strand.
^b Measured in culture fluid as described in Materials and Methods.
^c Dashes indicate the absence of a *luxI* gene or product.
^d This plasmid, which was obtained by random mutagenesis and directs the synthesis of a low-activity autoinducer synthase, is shown for comparison to the cysteine mutants.

pBLH305 contained 7.1 μM ($\pm 1.5 \mu\text{M}$ in three separate experiments) *N*-(3-oxohexanoyl)homoserine lactone. Culture fluid from *E. coli* containing pBLH305 and pKK223-3 (a plasmid without *luxI*) produced 3.5 μM ($\pm 1.5 \mu\text{M}$) *N*-(3-oxohexanoyl)homoserine lactone (this served as a control). Culture fluids from *E. coli* containing pBLH305 and any *luxI* mutant plasmid produced between $1.7 \pm 0.5 \mu\text{M}$ and $3.3 \pm 1.5 \mu\text{M}$. Thus, under the conditions of our experiments, the mutant LuxI polypeptides had little or no influence on the activity of wild-type LuxI. From this we conclude that wild-type *luxI* is dominant over all of the *luxI* mutations. This is consistent with the report that TraI from *A. tumefaciens* functions as a monomer (17).

Characterization of site-directed cysteine substitution mutants. Because the proposed model for autoinducer synthesis involves transfer of the fatty acyl substrate from ACP to a cysteine residue on the enzyme, forming a covalent bond with the sulfhydryl group (6, 17, 26), we mutated each of the three cysteine codons in *luxI* (Cys-59, Cys-68, and Cys-160) and determined whether the encoded polypeptides retained autoinducer synthase activity. We were particularly interested in Cys-68 because it is conserved among the 12 LuxI homologs that have been described (9). Each of the site-directed *luxI* mutations were expressed by *E. coli* at levels similar to that of

the wild type (Fig. 2). Cocultures of *E. coli* containing each of the mutant *luxI* genes and the autoinducer bioassay strain were visibly luminous and indistinguishable from *E. coli* expressing wild-type *luxI*. Measurements of autoinducer produced by *E. coli* containing LuxI with a Cys-to-Ser substitution at position 59, position 68, or position 160 demonstrated that these mutant polypeptides produced relatively high levels of *V. fischeri* autoinducer, although the levels were lower than those produced by *E. coli* containing wild-type LuxI (Table 3). Because none of the cysteine substitutions abolished autoinducer synthase activity, we conclude that the formation of a covalent enzyme-fatty acyl intermediate with a cysteine residue is not required for enzymatic autoinducer synthesis.

Because serine is structurally and chemically similar to cysteine, the possibility remained that a serine hydroxyl group at the active site could form an alternative covalent intermediate with a fatty acyl group, allowing the enzyme to retain a high level of activity. However, an alanine residue would not provide a functional group for the formation of a covalent bond; therefore, we constructed a *luxI* gene encoding a polypeptide with a Cys-68-Ala substitution. Culture extracts of *E. coli* with this *luxI* mutation contained about fivefold less autoinducer than culture extracts of *E. coli* expressing wild-type *luxI* (Table 3). This is consistent with our hypothesis that covalent bond formation between the acyl substrate and an amino acid at position 68 is not required for autoinducer synthesis.

DISCUSSION

Our analysis of random point mutations in *luxI* has revealed two regions of the LuxI polypeptide that appear to be critical for its activity as an autoinducer synthase. Seven of 11 mutations that inactivate LuxI map to a region spanning amino acid residues 25 to 70. The other four span residues 104 to 164. In comparing the map positions of the mutations with a sequence alignment of LuxI homologs, it becomes apparent that the regions we have identified show a high density of conserved residues within the family (Fig. 3). The region between residues 25 and 70 represents less than 25% of the length of LuxI and contains six of the seven amino acids conserved throughout the LuxI family (Fig. 3). In fact, three of the mutations that we identified mapped to three of the six conserved residues in this region. We propose that this region constitutes an impor-

FIG. 3. Diagram of the LuxI polypeptide. Lines through the diagram indicate the positions of amino acid residues conserved in 10 or more of the 12 LuxI homologs (9). The letters above the LuxI diagram are the single-letter designations for amino acids conserved in 100% of the 12 LuxI homologs. The green C indicates the position of the proposed active-site cysteine, which, as described in the text, is conserved among all of the LuxI homologs. Letters below the diagram indicate the positions and nature of the random mutations described in this study. Uppercase letters denote substitutions that reduce activity to below detectable levels, and lowercase letters indicate substitutions that retain some detectable autoinducer synthase activity. The red region is the proposed active-site region, the orange region may define an area important for conformation of the active site, and the yellow region might be involved in acyl-ACP selection (see Discussion). The red, orange, and yellow regions encompass 25 of the 28 highly conserved residues in LuxI, and the remaining 3 highly conserved residues are in close proximity to these regions.

tant part of an active site in which an amide bond is formed between SAM and the acyl group transferred from ACP.

Two mutant polypeptides detected in our screen, the Thr-74-Ile and Pro-94-Ser LuxI mutants, retained low but detectable autoinducer synthase activity (Table 2). Furthermore, a *luxI* gene encoding a polypeptide with a Lev-88-Phe substitution was identified as a temperature-sensitive mutation by using a slightly different screening method than that described here (27a). In *E. coli* at the nonpermissive temperature (30°C) the Leu-88-Phe LuxI mutant retains some activity; it is approximately 75-fold less active than wild-type LuxI. Thus, the region between residues 74 and 94 may not participate in catalysis directly but rather may serve to optimize the conformation of the active site.

In the proposed active-site region, there are three residues conserved within the LuxI family that were not identified by random mutagenesis: Phe-29, Trp-35, and Cys-68. For the reasons described below we changed the Cys-68 codon of *luxI* to a Ser codon and an Ala codon by site-directed mutagenesis. Both of these Cys-68 mutant polypeptides retained autoinducer synthase activity (Table 3) and would not have been detected by our screening method. We did not mutagenize the Phe-29 and Trp-35 codons. The remaining conserved amino acid is Arg-104 (Fig. 3). A mutation of the Arg-104 codon to a His codon was obtained in our screen, and activity of the Arg-104-His LuxI mutant was below detectable levels (Table 2).

It is not clear whether Arg-104 should be considered part of a large catalytic domain of LuxI that includes the putative active site or whether it should be grouped with the three remaining mutations identified by random mutagenesis (Ala-133-Thr, Glu-150-Lys, and Gly-164-Glu) (Table 2). Regardless, it is clear that at least residues 133 through 164 represent a second region of LuxI that is important for activity. As indicated in Fig. 3, this region shows less conservation among LuxI homologs than the putative active-site region. LuxI homologs catalyze the synthesis of acyl-homoserine lactones, but the acyl side chains often differ between the different LuxI homologs (9), and side chain selectivity has been demonstrated for LuxI (5, 15, 24). We propose that the less conserved C-terminal region might be involved in binding of the appropriate acyl-ACP (3-oxohexanoyl-ACP for LuxI) and delivery of the acyl group to the active site. There are other equally plausible hypotheses, and understanding the function of the region between residues 133 and 164 requires further investigation.

One model for acyl-homoserine lactone synthesis by LuxI and its homologs suggests that the fatty acyl substrate is transferred from ACP to an active-site cysteine and forms a covalent bond with the sulfhydryl group of that cysteine (6, 17, 26). A cysteine residue at position 68 of the LuxI polypeptide aligns within one amino acid position with cysteine residues in all 12 of the LuxI homologs we examined (9). This residue falls in the proposed active site of LuxI, and several inactive *luxI* genes isolated in this study have alterations in amino acid residues near this cysteine (Fig. 3). Because neither of the two remaining cysteines in LuxI correspond to cysteine residues in the other LuxI homologs, Cys-68 is the best candidate for a cysteine directly involved in the enzymatic reaction. We made mutations in *luxI* in the Cys-68 codon and the two other Cys codons and measured the autoinducer produced by *E. coli* containing the mutant LuxI polypeptides. All made reduced but substantial amounts of autoinducer (Fig. 3). Thus, we believe that covalent bond formation between the fatty acyl substrate and a LuxI cysteine residue is not essential for enzymatic synthesis of the autoinducer. However, we cannot exclude the possibility that a nonessential covalent bond forms between a

cysteine residue and the fatty acyl substrate during the catalysis. It is also possible that a covalent bond forms with a residue other than a cysteine. Several well conserved charged residues in the hypothetical active site were identified by random mutagenesis (Table 2 and Fig. 3). We speculate that these charged residues are solvent exposed and participate directly in the enzymatic catalysis of *N*-(3-oxohexanoyl)homoserine lactone.

This mutational analysis provides a general view of LuxI structure that needs to be refined and validated by future biochemical and genetic studies. It appears that amide bond formation is catalyzed in an active site within the N-terminal region of LuxI and that, in opposition to previous proposals, formation of a covalent bond between a LuxI Cys residue and the acyl group from ACP is not essential for autoinducer synthase activity. We propose that the C-terminal region may play an important role in providing LuxI specificity in acyl-ACP substrate acquisition.

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