

Multiple *N*-Acyl-L-Homoserine Lactone Autoinducers of Luminescence in the Marine Symbiotic Bacterium *Vibrio fischeri*†

ALAN KUO,¹ NEIL V. BLOUGH,^{2‡} AND PAUL V. DUNLAP^{1*}

Biology Department¹ and Chemistry Department,² Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

Received 24 August 1994/Accepted 14 October 1994

In *Vibrio fischeri*, the synthesis of *N*-3-oxohexanoyl-L-homoserine lactone, the autoinducer for population density-responsive induction of the luminescence operon (the *lux* operon, *luxICDABEG*), is dependent on the autoinducer synthase gene *luxI*. Gene replacement mutants of *V. fischeri* defective in *luxI*, which had been expected to produce no autoinducer, nonetheless exhibited *lux* operon transcriptional activation. Mutants released into the medium a compound that, like *N*-3-oxohexanoyl-L-homoserine lactone, activated expression of the *lux* system in a dose-dependent manner and was both extractable with ethyl acetate and labile to base. The *luxI*-independent compound, also like *N*-3-oxohexanoyl-L-homoserine lactone, was produced by *V. fischeri* cells in a regulated, population density-responsive manner and required the transcriptional activator LuxR for activity in the *lux* system. The *luxI*-independent compound was identified as *N*-octanoyl-L-homoserine lactone by coelution with the synthetic compound in reversed-phase high-pressure liquid chromatography, by derivatization treatment with 2,4-dinitrophenylhydrazine, by mass spectrometry, and by nuclear magnetic resonance spectroscopy. A locus, *ain*, necessary and sufficient for *Escherichia coli* to synthesize *N*-octanoyl-L-homoserine lactone was cloned from the *V. fischeri* genome and found to be distinct from *luxI* by restriction mapping and Southern hybridization. *N*-Octanoyl-L-homoserine lactone and *ain* constitute a second, novel autoinduction system for population density-responsive signalling and regulation of *lux* gene expression, and possibly other genes, in *V. fischeri*. A third *V. fischeri* autoinducer, *N*-hexanoyl-L-homoserine lactone, dependent on *luxI* for its synthesis, was also identified. The presence of multiple chemically and genetically distinct but cross-acting autoinduction systems in *V. fischeri* indicates unexpected complexity for autoinduction as a regulatory mechanism in this bacterium.

Autoinduction is an intercellular signalling and gene regulatory mechanism for population density-responsive control of luminescence in *Vibrio fischeri*, a bacterium that occurs at high population density in light-organ symbiosis (e.g., 10^{10} to 10^{11} cells ml⁻¹) and other habitats (23). Autoinducer (*N*-3-oxohexanoyl-L-homoserine lactone [*N*-3-oxohexanoyl-L-HSL]) is a self-produced, membrane-permeable compound that accumulates as *V. fischeri* population density increases (10, 20). At threshold concentrations, autoinducer, via the autoinducer receptor and transcriptional activator LuxR, triggers transcription of the luminescence (*lux*) operon, *luxICDABEG*, which contains genes for autoinducer synthase (*luxI*) and luminescence enzymes (13, 14, 30).

Besides *V. fischeri*, other species of proteobacteria that are symbionts or pathogens of higher organisms have recently been found to produce *N*-acyl-L-HSL autoinducers structurally similar or identical to the *V. fischeri* autoinducer. For example, *N*-3-hydroxybutanoyl-L-HSL mediates autoinduction of luminescence in *Vibrio harveyi*, *N*-3-oxooctanoyl-L-HSL mediates autoinduction of conjugation in *Agrobacterium tumefaciens*, and *N*-3-oxohexanoyl-L-HSL and *N*-3-oxododecanoyl-L-HSL mediate autoinduction of virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*, respectively (2, 4, 25, 32). In many cases, the genes for the autoinducer synthase and transcriptional activator exhibit substantial sequence similarity

to *luxI* and *luxR*, respectively, of *V. fischeri* (16, 17, 24, 26, 27). Recently, evidence has been obtained for an HSL-based signal of starvation in *Escherichia coli* (19). The diversity of species that use autoinduction and the chemical and genetic similarities of their autoinduction systems indicate that autoinduction is an evolutionarily conserved signalling and regulatory mechanism of general importance in proteobacteria.

In this report, we demonstrate that *V. fischeri* produces a second, novel autoinducer, *N*-octanoyl-L-HSL, that activates *lux* operon transcription via LuxR. The synthesis of *N*-octanoyl-L-HSL is directed by a novel autoinducer synthase locus, *ain*, that is distinct from *luxI*. A third *V. fischeri* autoinducer, *N*-hexanoyl-L-HSL, dependent on *luxI* for its synthesis, was also identified. The presence of multiple chemically and genetically distinct autoinduction systems in *V. fischeri* indicates unexpected complexity for autoinduction as a signalling and gene regulatory mechanism within a single bacterium.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions, and physiological assays. The strains used in this study are derivatives of *E. coli* K-12 and *V. fischeri* MJ-1 and are listed in Table 1. *V. fischeri* MJ-100, a spontaneously nalidixic acid-resistant (Nx^r) derivative of MJ-1 (8), and its derivatives were maintained on solid LBS medium (8) with 20 µg of nalidixic acid ml⁻¹. *E. coli* S17-1 is a strain capable of conjugatively transferring the mobilizable chloramphenicol resistance (Cm^r) plasmid pSUP102 to a wide range of recipients (29), including *V. fischeri* (6, 8). *E. coli* strains were maintained on solid LB medium (1) with appropriate antibiotics to ensure plasmid maintenance.

Growth conditions, cell density and luminescence assays, and the light-measuring equipment and standard were as

* Corresponding author. Mailing address: Biology Department, Redfield Laboratory, 86 Water St., Woods Hole Oceanographic Institution, Woods Hole, MA 02543. Phone: (508) 457-2000, ext. 3209. Fax: (508) 457-2195. Electronic mail address: pdunlap@whoi.edu.

† Contribution 8833 from Woods Hole Oceanographic Institution.

‡ Present address: Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>E. coli</i> K-12		
JM83	$\Delta(lac-proAB)$	31
PD100	<i>zah-735::Tn10</i> $\Delta(argF-lac)U169$, Tc ^r	7
S17-1	RP4 tra ⁺	29
<i>V. fischeri</i>		
MJ-1	Lux ⁺	28
MJ-100	MJ-1, Nx ^r	8
MJ-203	MJ-100, $\Delta luxA$ (168-bp <i>XhoI-NheI</i> in-frame deletion)	This study
MJ-207	MJ-100, $\Delta luxRICD$ (~3.5-kbp <i>PstI-BglII</i> deletion)	This study
MJ-208	MJ-100, $\Delta luxR$ (450-bp <i>PstI-XbaI</i> in-frame deletion)	This study
MJ-211	MJ-100, $\Delta luxI$ (~250-bp nonpolar deletion)	This study
Plasmids		
pSUP102	pACYC184, RP4 <i>mob</i> ⁺ , Cm ^r Tc ^r	29
pJE202	pBR322 with ~8.8-kbp <i>SalI</i> fragment from <i>V. fischeri</i> DNA (<i>luxR luxICDABEG</i>), Ap ^r	13
pNL121	pSUP102 with ~8.8-kbp <i>SalI lux</i> fragment of pJE202, Cm ^r	8
pPD749	<i>Ptac-luxR</i> , <i>lacI</i> ^s , Ap ^r	7
pJR551	pACYC184 with <i>luxR::Mu</i> Δ (<i>c nerAB</i>) dII681 (<i>lacZYA</i> Km ^s) and <i>luxICDABEG</i> with a nonpolar point mutation in <i>luxI</i> , Cm ^r	9
pAI002	pSUP102 with ~10.5-kbp fragment from <i>V. fischeri</i> , <i>ain</i> ⁺ , Cm ^r	This study
pAI004	pBR322 with ~2.7-kbp <i>HindIII V. fischeri</i> DNA fragment, <i>ain</i> ⁺ , Ap ^r	This study
pAK211	pNL121 with ~250-bp nonpolar deletion in <i>luxI</i>	This study
pAK411	pBR322 with ~8.5-kbp <i>lux</i> fragment of pAK211	This study
pAK208	pNL121 with 450-bp in-frame <i>PstI-XbaI</i> deletion in <i>luxR</i>	This study
pAK408	pBR322 with ~8.3-kbp <i>SalI</i> fragment of pAK208	This study

^a Ap^r, ampicillin resistant; Km^s, kanamycin sensitive; Tc^r, tetracycline resistant.

previously described (8). *V. fischeri* was inoculated into liquid LBS or ASH medium (8) to an $A_{660} < 0.01$. Liquid cultures of *E. coli* were handled similarly except that LB medium (1) with 50 mM Tris (pH 7.5) and an appropriate antibiotic was used.

For the measurement of autoinducer activity, filter-sterilized supernatants of high-density cultures (A_{660} of 1.5 in LBS for *V. fischeri* and 2.0 for *E. coli*) (i.e., conditioned media) were mixed 1:1 with autoinducer assay medium (an LB-Tris medium) (9). Alternatively, supernatants were extracted with acidified ethyl acetate (100 μ l of glacial acetic acid in 1 liter of ethyl acetate) and dried as described previously (10) and then redissolved in assay medium. Next, an exponential-phase culture ($A_{660} = 0.3$) of the autoinducer assay strain, *E. coli* PD100(pPD749, pJR551) (9), was added to a final A_{660} of 0.01, and subsequent luminescence and growth were monitored. The growth rates of the autoinducer assay strain within experiments were essentially similar. The responses of *V. fischeri* strains to an autoinducer were determined by inoculating the strain into a 1:1 mixture of unconditioned medium and medium conditioned by an appropriate autoinducer-producing strain.

Construction of *V. fischeri lux* mutants. Most of the deletions used in this study to construct *V. fischeri lux* mutants were generated in the subcloned *lux* genes (*luxR* and *luxICDABEG*) of pNL121 (8) by digestion with appropriate restriction enzymes (Fig. 1), blunt ending with Klenow fragment, ligating, transforming *E. coli* JM83, and selecting Cm^r transformants. An exception was the nonpolar *luxI* deletion of pAK211, which was constructed by partial digestion of pNL121 with *SfcI*, removal of approximately 250 bp of *luxI* DNA with the Promega (Madison, Wis.) Erase-a-Base System, ligation, and screening for JM83 transformants that luminesced only in the proximity of MJ-203, an autoinducer-producing derivative of MJ-100 that contains a nonpolar deletion in *luxA*, the gene specifying the α -subunit of the light-emitting enzyme luciferase (Fig. 1). Plasmids and *E. coli* strains were manipulated by using standard procedures (1).

V. fischeri lux mutants (Fig. 1) were then constructed by conjugating modified pNL121 from S17-1 to MJ-100 by a previously described mating and transconjugant selection procedure (8) and screening the resulting Nx^r Cm^s recombinant *V. fischeri* strains to confirm the mutant phenotype. For example, the nonpolar *luxI* deletion mutant MJ-211 was constructed by conjugating pAK211 into MJ-100 and screening for colonies that were Lux⁻ except in proximity to MJ-203. Mutant genotypes and phenotypes were verified by physiological measurements, complementation tests, and Southern hybridizations.

Southern hybridizations. DNA was transferred from 1% agarose gels to Zeta-Probe blotting membrane by standard procedures (1) and probed and developed with the Boehringer Mannheim (Indianapolis, Ind.) nonradioactive DNA labeling and detection kit. Probes were prepared by labeling purified fragments by random priming. Hybridizations and high-stringency washes were performed at 65°C.

RP HPLC of autoinducers. Synthetic *N*-acyl-L-HSLs, kindly provided by D. Lynn (University of Chicago), were dissolved in

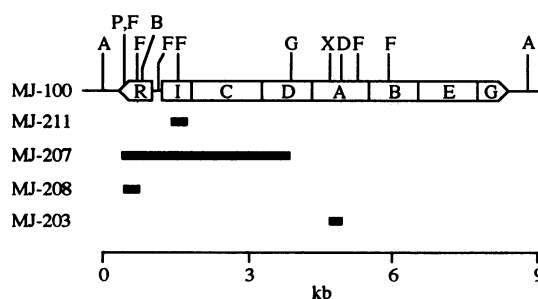


FIG. 1. The *lux* genes of *V. fischeri* and *V. fischeri lux* mutants constructed in this study. Solid bars indicate deletions. Restriction sites are denoted as follows: A, *SalI*; P, *PstI*; F, *SfcI*; B, *XbaI*; G, *BglII*; X, *XhoI*; D, *NheI*. Letters inside boxes indicate *lux* genes.

ethanol and diluted into fresh medium. Samples were concentrated on an in-line C_{18} enrichment column and separated with a previously described reversed-phase high-pressure liquid chromatography (RP HPLC) system (21). Flow rate was 1 ml min^{-1} . Fractions were dried in a VR-I HetoVac with vacuum but without heat, dissolved in 10 μl of ethanol, mixed with 3 ml of autoinducer assay medium, and assayed for autoinducer activity with PD100(pPD749, pJR551) as described above. For gradient elutions, either 750 μl of *luxI*-conditioned medium, 3 ml of *ain*-conditioned medium, or an appropriate amount (1 ng to 2 μg) of a synthetic autoinducer compound (*N*-hexanoyl-L-HSL, *N*-3-oxohexanoyl-L-HSL, *N*-octanoyl-L-HSL, *N*-3-oxooctanoyl-L-HSL, *N*-3-oxodecanoyl-L-HSL, or *N*-3-oxododecanoyl-L-HSL) was applied to the column. Mobile phase was composed of sodium phosphate (10 mM, pH 7) and methanol, and the gradient was 0 to 100% (vol/vol) methanol over 8 min. Fractions (500 μl) were collected, and the luminescence of assay cultures was measured after a 2-h incubation. For isocratic separations, either 2 ml of *luxI*-conditioned medium, 500 μl of *ain*-conditioned medium, or 5 ng of synthetic compound was applied. Mobile phase was composed of sodium phosphate and methanol. Fractions (250 μl) were collected and assayed.

Purification of *V. fischeri* autoinducer-2 (AI-2). JM83(pAI004) (*ain*⁺; Table 1) was cultured at 28°C in M9 medium (1) supplemented with 0.8% glycerol, 1 mM MgSO_4 , 230 mg of L-proline liter⁻¹, and 150 mg of ampicillin liter⁻¹ to an $A_{660} > 3$, after which cells were removed from the medium by centrifugation in a Sorvall GS-3 rotor at 4°C and 16,000 $\times g$ for 30 min. Then a peristaltic pump was used to pass 4 liters of culture supernatant through two in-series 60-ml Varian Mega Bond Elut C_{18} columns at a flow rate of 2 ml min^{-1} at 4°C. Columns were eluted with the following series of mixtures of water and increasing amounts (vol/vol) of methanol: fraction 1, 100 ml of 0% methanol; fraction 2, 30 ml of 25% methanol; fraction 3, 20 ml of 50% methanol; fraction 4, 10 ml of 75% methanol; fraction 5, 10 ml of 75% methanol; fraction 6, 10 ml of 100% methanol; fraction 7, 40 ml of 100% methanol. Respective fractions from the two columns were pooled, and 10 μl of each pooled fraction was assayed for autoinducer activity, which was found to be present in fractions 5 and 6. These two fractions were pooled, rotoevaporated to dryness at 42°C, redissolved in 10% methanol, filtered, and eluted isocratically with 40% phosphate buffer–60% methanol by the HPLC system described above. Autoinducer-containing fractions were pooled, dried, dissolved in water, and eluted isocratically by RP HPLC with 50% phosphate buffer–50% methanol. The resulting fractions were assayed for autoinducer activity, which occurred as a single peak at 10 to 13 min. Active fractions were dried, dissolved in 200 μl of water, and extracted twice with 200 μl of acidified ethyl acetate. The resulting highly purified material was pooled, dried again, and stored at –20°C.

Mass spectrometry and NMR spectroscopy. For mass spectrometry, samples were analyzed with a VG AutoSpec-Q mass spectrometer by utilizing a desorption chemical ionization probe and electron impact ionization. Magnet scanning was at 3.4 s decade⁻¹ from 310 to 41 Da. Low-resolution spectra ($M/\Delta M = 4,000$) were acquired in the centroid mode, whereas high-resolution spectra ($M/\Delta M > 10,000$) were acquired in the continuum mode. For nuclear magnetic resonance (NMR) spectroscopy, ¹H spectra of samples dissolved in a deuterated solvent (methanol or chloroform) were obtained with a Bruker AC-300 NMR spectrometer.

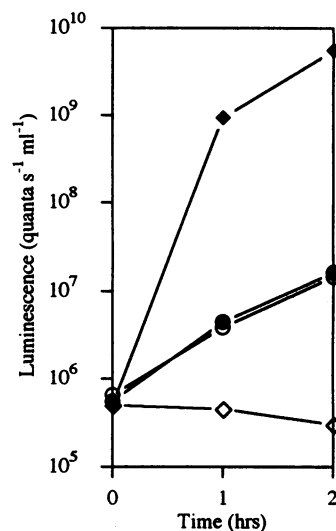


FIG. 2. Synthesis of autoinducer activity by *luxI* mutants of *V. fischeri*. Cell-free culture supernatants of MJ-100 (*lux*⁺) and two *luxI* deletion mutants, MJ-211 and MJ-207, were assayed for autoinducer. The medium was not conditioned (○) or was conditioned by MJ-100 (◆), MJ-207 (○), or MJ-211 (●).

RESULTS

Production of autoinducer activity by *luxI* mutants of *V. fischeri*. Previously, in a study of *lux* gene regulation with *lux::lacZ* (*Mu* dI) gene replacement mutants of *V. fischeri*, we observed that a mutant defective in *luxI*, expected to produce no autoinducer, nonetheless exhibited *lux* operon transcriptional activation (8). To examine the basis for that activation, we constructed a *V. fischeri luxI* deletion mutant, MJ-211, containing an approximately 250-bp nonpolar internal deletion in *luxI* (Fig. 1). The nonpolar nature of the deletion permitted transcription initiated from the *luxICDABEG* promoter to be monitored by luminescence, and the absence of foreign DNA (i.e., *E. coli lacZYA* and other genes of *Mu* dI present in *lux::lacZ* fusion mutants) avoided possible complications in interpreting results. Consistent with earlier results, luminescence in MJ-211 exhibited substantial *lux* operon transcriptional activation in the absence of added autoinducer. To determine if the *luxI* mutant actually produced autoinducer activity, cell-free supernatants of medium conditioned by the growth of MJ-211 were examined by a sensitive assay for autoinducers that activate expression of the *V. fischeri* luminescence system (9). Medium conditioned by MJ-211 activated *lux* operon expression (Fig. 2), suggesting that *V. fischeri* produced a second, *luxI*-independent autoinducer activity. To exclude the unlikely possibility that the remaining approximately 300 bp of *luxI* DNA in MJ-211 retained autoinducer synthase function, we constructed another mutant, MJ-207, in which the entire *luxI* gene and flanking sequences were removed (Fig. 1). Medium conditioned by MJ-207 gave results identical to those of MJ-211 (Fig. 2), demonstrating that the second autoinducer activity was independent of *luxI*. The autoinducer assay system did not respond to media conditioned by luminous bacteria closely related to *V. fischeri* (*Photobacterium leiognathi* LN-1a and *V. harveyi* B-392) or medium conditioned by *E. coli* JM83, indicating that the putative second autoinducer activity was due specifically to *V. fischeri* and not to a general conditioning effect.

The possibility, however, that the putative *luxI*-independent

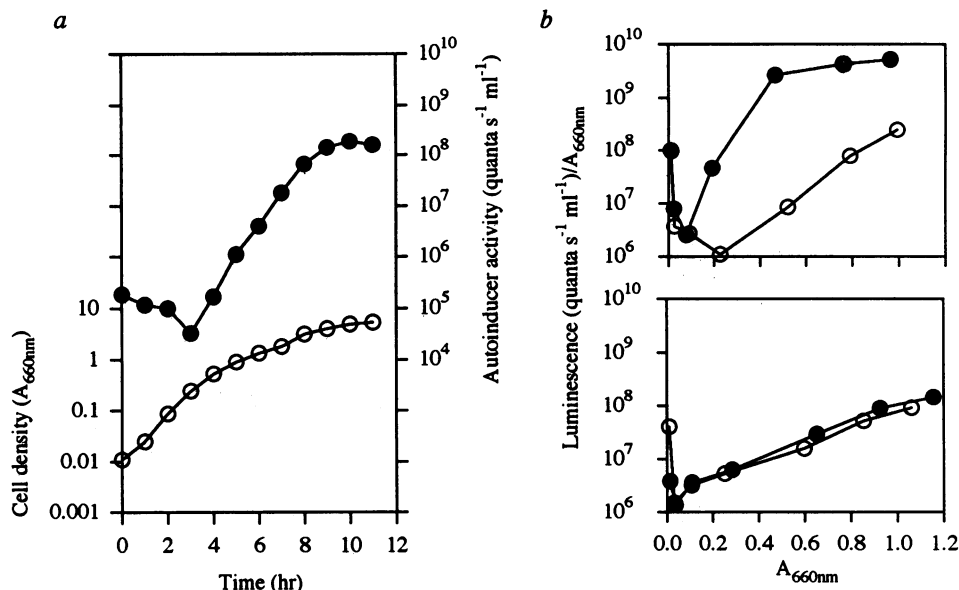


FIG. 3. Regulated synthesis of the *luxI*-independent autoinducer by *V. fischeri* and requirement of LuxR for *lux* operon transcriptional activation by the *luxI*-independent autoinducer. (a) Cells of *V. fischeri* MJ-211 ($\Delta luxI$) were inoculated into 50 ml of ASH broth, and cell density (○) and autoinducer activity (●) were monitored during culture growth. Autoinducer levels were measured as the luminescence response of autoinducer assay strain PD100 (pPD749, pJR551) by using 2-ml samples of cell-free growth medium of the MJ-211 culture at each time point (see Materials and Methods). (b) Responses of *luxI* deletion mutant MJ-211 (upper panel) and *luxR* deletion mutant MJ-208 (lower panel) to the *luxI*-independent autoinducer activity. Open symbols denote responses to unconditioned medium, and solid symbols denote responses to medium conditioned by MJ-207.

autoinducer activity did not result from the synthesis of a second autoinducer but from a spurious conditioning effect on the autoinducer assay system specific to *V. fischeri* existed. For example, *V. fischeri* cells might have released into the medium a compound, such as an iron chelator, that led to a change in the copy number of the *lux* gene-containing plasmids (5) in the *E. coli* autoinducer assay strain or a compound that activated transcription from plasmid-borne promoter sequences which continued on into the *lux* operon. To rigorously exclude this possibility, we used an approach modeled after that employed to extract and chemically characterize the first *V. fischeri* autoinducer, *N*-3-oxohexanoyl-L-HSL (*V. fischeri* AI-1) (10). We found the *luxI*-independent autoinducer activity, like AI-1, to be extractable from conditioned medium with acidified ethyl acetate. Furthermore, the *luxI*-independent activity, extracted and concentrated by rotoevaporation, was active in a dose-dependent manner and was destroyed by incubation at pH 12 for 10 min, like AI-1 (data not shown). Sensitivity to treatment with base suggested that the compound contained a lactone ring (10). Chemical similarities with AI-1 indicated that the *luxI*-independent activity was likely to be an autoinducer and not a spurious conditioning effect. This conclusion was supported by two additional similarities with AI-1, regulated synthesis of the *luxI*-independent activity and its requirement of LuxR for activity in the *lux* system, as described below.

Regulated synthesis of the *luxI*-independent autoinducer activity. In *V. fischeri*, the synthesis of AI-1 is regulated. The presence of *luxI* as part of the autoinducer-controlled *lux* operon results in an autocatalytic, positive-feedback loop for AI-1 synthesis, which leads to a rapid increase in the level of AI-1 once *lux* operon induction is triggered (5, 11, 13, 15). To determine if synthesis of the *luxI*-independent autoinducer activity also might be regulated, we assayed the cell-free supernatant of a culture MJ-211 for *luxI*-independent autoinducer activity during growth. The level of activity was low

initially and remained constant during the first few hours of culture growth. After the culture attained an A_{660} of approximately 0.3 to 0.5, however, the level of activity increased rapidly, at a rate faster than the rate of increase in cell density (Fig. 3a). These results suggest that the production of the *luxI*-independent autoinducer activity, like the production of AI-1, is inducible.

Requirement of LuxR for activity of the *luxI*-independent autoinducer. The experiments described above were conducted with *V. fischeri* and *E. coli* strains that contained an intact *luxR* gene, leaving open the possibility that the activation of *lux* operon transcription by the *luxI*-independent autoinducer, like activation of *lux* operon transcription by AI-1, required LuxR. To test this possibility, we examined the effect of medium conditioned by MJ-207 on luminescence in MJ-208 (Fig. 1), a *V. fischeri* strain with a deletion in *luxR*. Medium conditioned by MJ-207 stimulated luminescence in MJ-211 ($\Delta luxI$) but had no effect on luminescence in MJ-208 (Fig. 3b), which demonstrated that the *luxI*-independent autoinducer activated *lux* operon transcription via LuxR. The above results, as well as the known limited tolerance of LuxR for autoinducer compounds structurally different from AI-1 (12), led us to conclude that the *luxI*-independent compound was likely to be an *N*-acyl-L-HSL chemically similar to AI-1. For these reasons, we termed the compound *V. fischeri* AI-2.

A genetic locus that directs the synthesis of AI-2. The production of AI-2 by *luxI* deletion mutants indicated that another autoinducer synthase gene was present in the *V. fischeri* chromosome. To isolate the locus containing that gene, we transformed a plasmid-borne MJ-1 chromosomal library (6) into JM83(pAK411), which bears the *lux* genes with the same 250-bp nonpolar deletion in *luxI* as MJ-211 (Table 1). By conferring luminescence on cells in the presence of an autoinducer, pAK411 served as a reporter for DNA fragments that directed the synthesis of autoinducers active in the *V. fischeri*

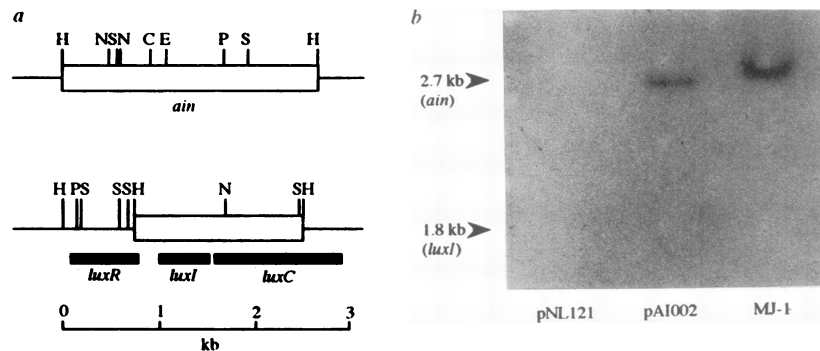


FIG. 4. Restriction map and Southern hybridization analysis of the novel *V. fischeri* autoinducer synthesis locus *ain*. The 2.7-kbp *Hind*III fragment containing *ain* was subcloned from the 10.5-kbp insert of pAI002, isolated from a genomic library of MJ-1 DNA that had been inserted into the *Bam*HI site of pSUP102 (6). (a) Physical maps of *ain* and *luxI*. Restriction sites are denoted as follows: H, *Hind*III; N, *Nsi*I; S, *Ssp*I; C, *Cla*I; E, *Eco*RV; P, *Pst*I. Black bars indicate *lux* genes. Open boxes indicate the 2.7- and 1.8-kbp *Hind*III fragments used as *ain* and *luxI* probes, respectively, in hybridization experiments. (b) Blot of *Hind*III digests of pNL121, pAI002, and MJ-1 genomic DNA, all probed with the 2.7-kbp *ain* fragment. In the reciprocal experiment, the 1.8-kbp *luxI* probe hybridized to same-sized *Hind*III fragments of pNL121 and MJ-1 DNA but did not hybridize to pAI002 (data not shown).

luminescence system. Two such fragments were found. Of approximately 50,000 transformant colonies screened for luminescence, 12 emitted high levels of light, and each of these was found by restriction analysis to have received a fragment containing *luxI*. Three others, however, emitted a low level of light that was consistent with the lower activation of luminescence by AI-2 in the autoinducer assay (Fig. 2); each had received a 10.5-kbp DNA fragment that by preliminary restriction analysis was distinct from *luxI*. This fragment contained a locus, designated *ain* (autoinducer), that was necessary and sufficient for JM83 to produce AI-2 (see below).

By subcloning, the *ain* locus was resolved to a 2.7-kbp *Hind*III fragment of the original 10.5-kbp clone. The detailed restriction map of the 2.7-kbp fragment differed unambiguously from that of the *luxI* region (Fig. 4a), and *ain* and *luxI* did not cross-hybridize (Fig. 4b), confirming that the two loci were distinct. The original 10.5-kbp *ain* clone did not, however, restore luminescence to JM83(pAK408), with a *lux* gene plasmid with a deletion in *luxR*. Therefore, the 10.5-kbp fragment did not express a protein that could substitute for LuxR in the *V. fischeri* luminescence system.

Chemical identity of AI-2. To chemically characterize AI-2, we fractionated media conditioned by *E. coli* and *V. fischeri* by RP HPLC and assayed fractions for autoinducer activity. From medium conditioned by JM83 containing *ain*, AI-2 was recovered in a fraction that eluted later (i.e., was more hydrophobic) than AI-1 from medium conditioned by JM83 containing *luxI* (Fig. 5a), indicating that AI-2 and AI-1 were chemically distinct compounds. Medium conditioned by MJ-207 yielded only AI-2, whereas medium conditioned by MJ-100 yielded AI-1, AI-2, and a second *luxI*-dependent autoinducer compound, AI-3 (data not shown). Furthermore, a *V. fischeri ain* gene replacement mutant failed to produce AI-2 but produced AI-1 and AI-3 (18). Thus, in *E. coli* as well as *V. fischeri*, the synthesis of AI-2 was dependent on the *ain* locus.

To chemically identify AI-2, we compared AI-2 chromatographically with several synthetic *N*-acyl-L-HSLs. Under RP HPLC gradient elution conditions, AI-2 separated unambiguously from most of the synthetic *N*-acyl-L-HSLs examined, including *N*-3-oxohexanoyl-L-HSL (AI-1), *N*-3-oxooctanoyl-L-HSL (the *A. tumefaciens* autoinducer), and *N*-3-oxododecanoyl-L-HSL (the *P. aeruginosa* autoinducer) (Fig. 5a). A fourth previously identified autoinducer, *N*-3-hydroxybutanoyl-

L-HSL (the *V. harveyi* autoinducer), is a more hydrophilic compound that does not bind to the RP HPLC C_{18} columns (4) used here. Fractions of medium conditioned by *E. coli* containing *ain* in which the *V. harveyi* compound eluted if present (i.e., 0% methanol) exhibited no activity in the autoinducer assay. However, AI-2 did coelute under the gradient conditions employed with two synthetic compounds, *N*-octanoyl-L-HSL, which was shown previously to activate expression of the *V. fischeri* luminescence system and to interfere with the activity of AI-1 (12), and *N*-3-oxododecanoyl-L-HSL (Fig. 5a).

To differentiate between these two candidate compounds, a higher-resolution isocratic elution system that unambiguously separated them was used. Under these conditions, AI-2 was indistinguishable from *N*-octanoyl-L-HSL but distinct from *N*-3-oxododecanoyl-L-HSL (Fig. 5b). These results suggested that AI-2 was *N*-octanoyl-L-HSL.

As *N*-octanoyl-L-HSL, AI-2 presumably lacked a 3-oxo group. Since most other known autoinducers contain a 3-oxo group, we sought to directly confirm the absence of this group in AI-2. We used a keto-group detection procedure that involved derivatization with 2,4-dinitrophenylhydrazine and separation and detection of the resulting hydrazones by RP HPLC and UV absorbance (22). To our knowledge, this is the first application of this method to studies of autoinducer chemical structure. Each of these *N*-3-oxoacyl-L-HSLs was detected as its corresponding hydrazone by this analysis, but AI-2 was not detected, confirming its lack of a 3-oxo group (data not shown). We concluded that AI-2 is *N*-octanoyl-L-HSL, an *N*-acyl-L-HSL compound that is structurally related to but distinct from other known autoinducers.

To confirm the identification of AI-2 as *N*-octanoyl-L-HSL, we used mass spectrometry to compare AI-2 purified from medium conditioned by the growth of JM83(pAI004) (see Materials and Methods) with synthetic *N*-octanoyl-L-HSL. The low-resolution mass spectrum of AI-2, with a molecular ion at $m/z = 227.2$, was essentially identical to that of synthetic *N*-octanoyl-L-HSL, with minor contaminants (Fig. 6). The monotonically decreasing peaks at 156.1, 170.1, 184.1, 198.1, and 212.1 were consistent with a simple unbranched alkyl chain, confirming the absence of a 3-oxo or 3-hydroxy group. High-resolution mass spectrometry revealed that the m/z of the molecular ion was 227.1513, which corresponded to the elemental composition of *N*-octanoyl-L-HSL, $C_{12}H_{21}NO_3$ (calcu-

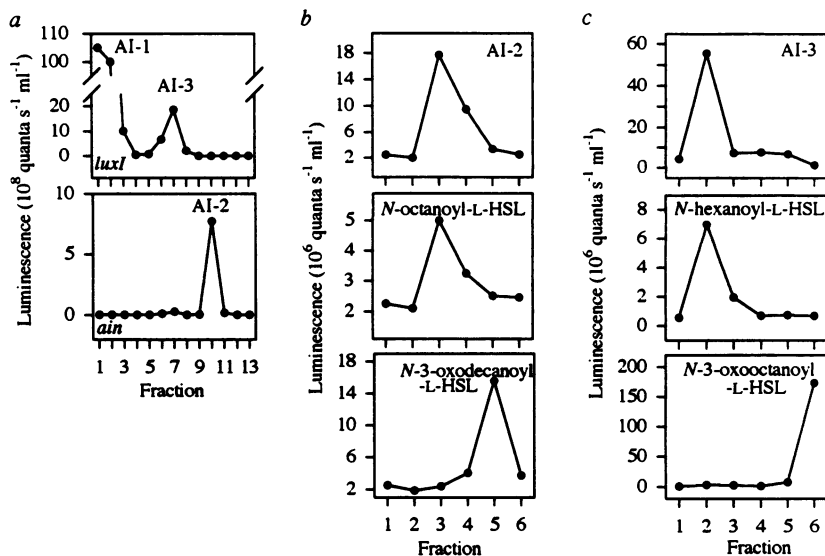


FIG. 5. Chromatographic identification of the novel *ain*-dependent autoinducer, AI-2, and a second *luxI*-dependent autoinducer, AI-3. (a) Gradient elution profiles of media conditioned by either *luxI*-expressing strain JM83(pJE202) (upper panel) or *ain*-expressing strain JM83(pAI004) (lower panel) and subjected to a methanol gradient. Synthetic *N*-acyl-L-HSLs eluted as follows: *N*-3-oxohexanoyl-L-HSL, fraction 2; *N*-hexanoyl-L-HSL and *N*-3-oxooctanoyl-L-HSL, fraction 7; *N*-octanoyl-L-HSL and *N*-3-oxodecanoyl-L-HSL, fraction 10; *N*-3-oxododecanoyl-L-HSL, fraction 12. (b) Isocratic separation (30% phosphate buffer-70% methanol) of JM83(pAI004)-conditioned medium and synthetic *N*-octanoyl-L-HSL and *N*-3-oxodecanoyl-L-HSL. (c) Isocratic elution (47% phosphate buffer-53% methanol) of JM83(pJE202)-conditioned medium and synthetic *N*-hexanoyl-L-HSL and *N*-3-oxooctanoyl-L-HSL.

lated $m/z = 227.1521$). The m/z of the base peak, 143.0576, also observed in mass spectra of AI-1 and the *P. aeruginosa* autoinducer (2, 10, 25), corresponded to the rearrangement product, $C_6H_9NO_3$ (calculated $m/z = 143.0582$).

To further confirm the structure of AI-2, we subjected the purified compound to NMR spectroscopy. The 1H NMR spectrum of AI-2 in deuterated methanol was consistent with an unbranched moiety with the following proton assignments (Fig. 7): a, $\delta_H = 2.20$, t, 2H; b, $\delta_H = 1.59$, t, 2H; c, $\delta_H = 1.29$, broad s, 8H; d, $\delta_H = 0.87$, t, 3H. Observed peaks diagnostic of HSL were as follows: α , $\delta_H = 4.55$, m, 1H; β_1 , $\delta_H = 2.50$, m, 1H; β_2 , $\delta_H = 2.25$, m, 1H; γ_1 , $\delta_H = 4.42$, m, 1H; γ_2 , $\delta_H = 4.26$, m, 1H.

The biological activity of AI-2 also matched that of *N*-octanoyl-L-HSL. Over the concentration range tested, 7 to 470 nM, the autoinducer assay system responded identically to purified AI-2 and synthetic *N*-octanoyl-L-HSL (data not shown).

Chemical identity of a third *V. fischeri* autoinducer. Conducting RP HPLC analyses of conditioned media, we found that *V. fischeri* produced a third autoinducer activity, AI-3. AI-3 exhibited by RP HPLC a hydrophobicity that was intermediate between those of *N*-3-oxohexanoyl-L-HSL and *N*-octanoyl-L-HSL, indicating it was chemically distinct from AI-1 and AI-2. JM83 containing *luxI* produced this activity (Fig. 5a), but JM83 containing the *lux* genes with a deletion in *luxI* did not. Furthermore, neither JM83 containing only *ain* nor MJ-207 produced AI-3. Thus, AI-3, like AI-1, was dependent on *luxI* for its synthesis. The RP HPLC gradient elution profile of AI-3 was consistent with it being either *N*-hexanoyl-L-HSL, which had been previously shown to activate expression of the *V. fischeri* luminescence system and to inhibit the activity of AI-1 (12), or *N*-3-oxooctanoyl-L-HSL, the *A. tumefaciens* autoinducer (Fig. 5a). Under a higher-resolution isocratic elution

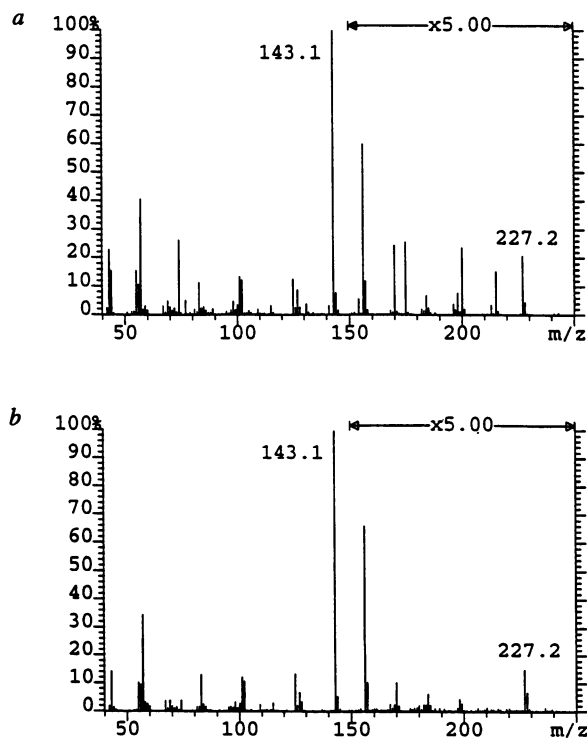


FIG. 6. Mass spectrometric analysis of *V. fischeri* AI-2. Low-resolution mass spectra of AI-2 purified from cell-free supernatant of *E. coli* JM83(pAI004) (a) and synthetic *N*-octanoyl-L-HSL (b). Peaks corresponding to the molecular ion ($m/z = 227.2$) and rearrangement product $C_6H_9NO_3$ ($m/z = 143.1$) are indicated. AI-2 was purified as described in Materials and Methods.

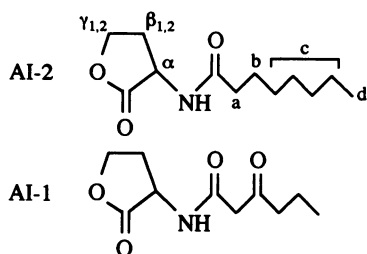


FIG. 7. Structural comparison of *V. fischeri* AI-2 (*N*-octanoyl-L-HSL) and AI-1 (*N*-3-oxohexanoyl-L-HSL). Carbon designations refer to proton assignments from NMR spectra described in the text.

regimen, the coelution of AI-3 with the synthetic compound identified it as *N*-hexanoyl-L-HSL (Fig. 5c). Thus, *V. fischeri* cells produced three chemically distinct autoinducers with activity in the *lux* system, AI-1 (*N*-3-oxohexanoyl-L-HSL) and AI-3 (*N*-hexanoyl-L-HSL), both of which are dependent on *luxI* for synthesis, and AI-2 (*N*-octanoyl-L-HSL), whose synthesis is dependent on the novel locus *ain*. The relative activities of autoinducer-containing RP HPLC fractions of medium conditioned by MJ-100 were 1.000, 0.033, and 0.167 for AI-1, AI-2, and AI-3, respectively.

DISCUSSION

In this study, we have identified a novel autoinducer of luminescence, *N*-octanoyl-L-HSL (AI-2), produced by the marine symbiotic bacterium *V. fischeri* and have isolated from the *V. fischeri* genome a novel locus, *ain*, involved in the synthesis of AI-2. This is the first report in which a second autoinducer from a bacterial species has been chemically identified and the gene directing its synthesis has been isolated. The *ain* locus and AI-2, acting via LuxR, constitute a third level of population density-responsive control of *lux* gene expression in *V. fischeri*, one that could supplement or inhibit transcriptional control by *N*-3-oxohexanoyl-L-HSL (AI-1) and enhance the mechanistically undefined effect of autoinducer-LuxR-independent modulation (8). We also have identified a second previously unrecognized autoinducer of luminescence in *V. fischeri*, *N*-hexanoyl-L-HSL (AI-3), whose synthesis, like that of AI-1, is dependent on *luxI*. Therefore, population density-responsive control of *lux* gene expression in *V. fischeri* is substantially more complex than previously envisioned.

The construction of *V. fischeri* mutants with defects in *luxI*, which eliminated the synthesis of AI-1 by these strains, led to the detection of AI-2, and isolation of the *ain* locus was facilitated by the use of a *lux* gene-containing plasmid with a nonpolar deletion in *luxI*, which served as a reporter for DNA fragments that directed the synthesis of autoinducers active in the *V. fischeri* luminescence system. These approaches may find applications with other autoinducer-utilizing bacteria to detect the presence of multiple autoinducers and to isolate the genes involved in the synthesis of them.

In this regard, other bacteria are likely to contain more than one autoinducer synthase gene and to synthesize more than one autoinducer. In *V. harveyi*, genetic evidence for an activity in addition to *N*-3-hydroxybutanoyl-L-HSL controlling luminescence has been obtained, although neither the chemical structure of the second activity nor the gene responsible for its synthesis has been identified (3). Furthermore, *A. tumefaciens* and *P. aeruginosa* apparently synthesize more than one autoinducer activity (25, 32).

Like autoinducers identified from other species of bacteria

(2, 4, 25, 32), *V. fischeri* AI-2 and AI-3, as *N*-acyl-L-HSLs, are structurally similar to *V. fischeri* AI-1 (Fig. 7). Besides the chemical similarity of autoinducers, in many cases, the genes encoding the autoinducer synthases and autoinducer receptor proteins from these other bacteria exhibit sequence similarity to *V. fischeri luxI* and *luxR*, respectively (16, 17, 24, 26, 27). The *ain* locus, however, exhibits no obvious sequence similarity to *luxI* (18), suggesting that it arose independently. With respect to a possible AI-2-specific transcriptional activator analogous or homologous to *luxR*, we know at this point only that the original 10.5-kbp *ain* fragment did not restore luminescence to *E. coli* carrying a *lux* plasmid with a deletion in *luxR*, indicating that this fragment does not express a protein that can substitute for LuxR in the *V. fischeri* luminescence system. The putative protein might not be expressed by *E. coli* or might not recognize the *lux* operator, or its gene might be incomplete on the 10.5-kbp fragment or occur elsewhere in the *V. fischeri* chromosome.

The total amounts of each of the three autoinducers produced by *V. fischeri* were such that they exhibited activity in the luminescence system as AI-1 > AI-3 > AI-2, which is consistent with AI-1 as the primary autoinducer of luminescence. Besides controlling luminescence in *V. fischeri*, the different autoinducers encoded by the two autoinducer synthase genes also might function either separately or cooperatively to control various other cellular responses to the high population densities this bacterium encounters in light-organ symbiosis and other habitats (23). On the basis of the smaller effect of AI-2 on luminescence compared with those of AI-1 and AI-3, one possibility is that the activation of luminescence by AI-2 is incidental to its principal function, that of controlling a set(s) of genes distinct from those involved in light production. Identification of these genes and a transcriptional activator specific to AI-2 would add substantial insight into cellular responses to high population density in *V. fischeri*.

A striking feature of the *V. fischeri* luminescence system is the versatility of LuxR and LuxI seen in this study. LuxR recognized a wide range of chemically distinct *N*-acyl-L-HSLs, including the three different compounds produced by *V. fischeri* (AI-1, AI-2, and AI-3) (Fig. 5), the chemically distinct autoinducers produced by *A. tumefaciens* and *P. aeruginosa*, and three other compounds (*N*-3-oxodecanoyl-L-HSL, *N*-decenoyl-L-HSL, and *N*-nonanoyl-L-HSL) (data not shown). A reasonable explanation for this versatility is that LuxR recognizes both of the main structural elements of autoinducers, the HSL and hydrophobic acyl moiety, but its level of activity is strongly influenced by the length and composition of the acyl group (12). Similarly, LuxI catalyzed a critical step in the synthesis of two distinct autoinducers that differed only in the structure of the acyl group. This versatility presumably reflects the ability of LuxI to recognize two alternative fatty acyl substrates, as well as reflecting the presence of those substrates in *V. fischeri* and *E. coli*. The versatility of LuxR and LuxI, together with our demonstration of a second autoinducer system in *V. fischeri*, implies that autoinduction of luminescence and other bacterial population density-responsive activities may be the summation of an unexpectedly complex network of multiple cross-acting regulatory elements.

ACKNOWLEDGMENTS

We thank D. Lynn for the generous gift of synthetic autoinducers, S. Caron for technical assistance with RP HPLC, C. Johnson for conducting the mass spectrometry and NMR spectroscopy experiments, and L. Gilson for comments on the manuscript.

This work was supported by the Woods Hole Oceanographic Institution Ocean Ventures Fund (A.K.), Office of Naval Research

grant N00014-89-J-1260 (N.V.B.), and National Science Foundation grants MCB 91-04653 and MCB 94-08266 (P.V.D.).

REFERENCES

- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl. 1987. Current protocols in molecular biology. John Wiley, New York.
- Bainton, N. J., P. Stead, S. R. Chhabra, B. W. Bycroft, G. P. C. Salmund, G. S. A. B. Stewart, and P. Williams. 1992. *N*-(3-Oxohehexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. *Biochem. J.* **288**:997-1004.
- Bassler, B. L., M. Wright, R. E. Showalter, and M. R. Silverman. 1993. Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Mol. Microbiol.* **9**:773-786.
- Cao, J., and E. A. Meighen. 1989. Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. *J. Biol. Chem.* **264**:21670-21676.
- Dunlap, P. V. 1992. Mechanism for iron control of the *Vibrio fischeri* luminescence system: involvement of cyclic AMP and cyclic AMP receptor protein and modulation of DNA level. *J. Biolumin. Chemilumin.* **7**:203-214.
- Dunlap, P. V., and S. M. Callahan. 1993. Characterization of a periplasmic 3':5'-cyclic nucleotide phosphodiesterase gene, *cpdP*, from the marine symbiotic bacterium *Vibrio fischeri*. *J. Bacteriol.* **175**:4615-4624.
- Dunlap, P. V., and E. P. Greenberg. 1988. Control of *Vibrio fischeri lux* gene transcription by a cyclic AMP receptor protein-LuxR protein regulatory circuit. *J. Bacteriol.* **170**:4040-4046.
- Dunlap, P. V., and A. Kuo. 1992. Cell density-dependent modulation of the *Vibrio fischeri* luminescence system in the absence of autoinducer and LuxR protein. *J. Bacteriol.* **174**:2440-2448.
- Dunlap, P. V., and J. M. Ray. 1989. Requirement for autoinducer in transcriptional negative autoregulation of the *Vibrio fischeri luxR* gene in *Escherichia coli*. *J. Bacteriol.* **171**:3549-3552.
- Eberhard, A., A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson, and N. J. Oppenheimer. 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry* **20**:2444-2449.
- Eberhard, A., T. Longin, C. A. Widrig, and S. J. Stranick. 1991. Synthesis of the *lux* gene autoinducer in *Vibrio fischeri* is positively autoregulated. *Arch. Microbiol.* **155**:294-297.
- Eberhard, A., C. A. Widrig, P. McBath, and J. B. Schineller. 1986. Analogs of the autoinducer of bioluminescence in *Vibrio fischeri*. *Arch. Microbiol.* **146**:35-40.
- Engebrecht, J., K. Nealson, and M. Silverman. 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell* **32**:773-781.
- Engebrecht, J., and M. Silverman. 1984. Identification of genes and gene products necessary for bacterial bioluminescence. *Proc. Natl. Acad. Sci. USA* **81**:4154-4158.
- Friedrich, W. F., and E. P. Greenberg. 1983. Glucose repression of luminescence and luciferase in *Vibrio fischeri*. *Arch. Microbiol.* **134**:87-91.
- Fuqua, W. C., and S. C. Winans. 1994. A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *J. Bacteriol.* **176**:2796-2806.
- Gambello, M. J., and B. H. Iglewski. 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. *J. Bacteriol.* **173**:3000-3009.
- Gilson, L., A. Kuo, and P. V. Dunlap. Unpublished data.
- Huisman, G. W., and R. Kolter. 1994. Sensing starvation: a homoserine lactone-dependent signalling pathway in *Escherichia coli*. *Science* **265**:537-539.
- Kaplan, H. B., and E. P. Greenberg. 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. *J. Bacteriol.* **163**:1210-1214.
- Kieber, D. J., and N. V. Blough. 1990. Determination of carbon-centered radicals in aqueous solution by liquid chromatography with fluorescence detection. *Anal. Chem.* **62**:2275-2283.
- Kieber, D. J., and K. Mopper. 1986. Trace determination of α -keto acids in natural waters. *Anal. Chim. Acta* **183**:129-140.
- Nealson, K. H., and J. W. Hastings. 1992. The luminous bacteria, p. 625-639. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), *The prokaryotes*, 2nd ed. Springer-Verlag, Berlin.
- Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski. 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* **260**:1127-1130.
- Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg. 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc. Natl. Acad. Sci. USA* **91**:197-201.
- Piper, K. R., S. B. von Bodman, and S. K. Farrand. 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature (London)* **362**:448-450.
- Pirhonen, M., D. Flego, R. Heikinheimo, and E. T. Palva. 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *EMBO J.* **12**:2467-2476.
- Ruby, E. G., and K. H. Nealson. 1976. Symbiotic association of *Photobacterium fischeri* with the marine luminous fish *Monocentris japonica*: a model of symbiosis based on bacterial studies. *Biol. Bull. (Woods Hole)* **141**:574-586.
- Simon, R., M. O'Connell, M. Labes, and A. Pühler. 1986. Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria. *Methods Enzymol.* **118**:640-659.
- Swartzman, E., S. Kapoor, A. F. Graham, and E. A. Meighen. 1990. A new *Vibrio fischeri lux* gene precedes a bidirectional termination site for the *lux* operon. *J. Bacteriol.* **172**:6797-6802.
- Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103-119.
- Zhang, L., P. J. Murphy, A. Kerr, and M. E. Tate. 1993. *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactones. *Nature (London)* **362**:446-448.