JOURNAL OF BACTERIOLOGY, Feb. 1996, p. 971–976
0021-9193/96/$04.00+0

Vol. 178, No. 4

Modulation of Luminescence Operon Expression by *N*-Octanoyl-L-Homoserine Lactone in *ainS* Mutants of *Vibrio fischeri*†

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Population density-dependent expression of luminescence in *Vibrio fischeri* is controlled by the autoinducer *N*-3-oxohexanoyl-L-homoserine lactone (autoinducer 1 [AI-1]), which via LuxR activates transcription of the *lux* operon (*luxICDABEG*, encoding the putative autoinducer synthase [LuxI] and the luminescence enzymes). We recently identified a novel *V. fischeri* locus, *ainS*, necessary for the synthesis of a second autoinducer, *N*-octanoyl-L-homoserine lactone (AI-2), which via LuxR can activate *lux* operon transcription in the absence of AI-1. To define the regulatory role of AI-2, a *luxI ainS* double mutant was constructed; in contrast to the parental strain and a *luxI* mutant, the *luxI ainS* mutant exhibited no induction of luminescence and produced no detectable luminescence autoinducer, demonstrating that *V. fischeri* makes no luminescence autoinducers other than those whose synthesis is directed by *luxI* and *ainS*. A mutant defective only in *ainS* exhibited accelerated luminescence induction compared with that of the parental strain, indicating that AI-2 functions in *V. fischeri* to delay luminescence induction. Consistent with that observation, the exogenous addition of AI-2 inhibited induction in a dose-dependent manner in *V. fischeri* and *Escherichia coli* carrying the *lux* genes. AI-2 did not mediate *luxR* negative autoregulation, alone or in the presence of AI-1, and inhibited luminescence induction in *E. coli* regardless of whether *luxR* was under the control of its native promoter or a foreign one. Increasing amounts of AI-1 overcame the inhibitory effect of AI-2, and equal activation of luminescence required 25- to 45-fold-more AI-2 than AI-1. We conclude that AI-2 inhibits *lux* operon transcription. The data are consistent with a model in which AI-2 competitively inhibits the association of AI-1 with LuxR, forming a complex with LuxR which has a markedly lower *lux* operon-inducing specific activity than that of AI-1–LuxR. AI-2 apparently functions in *V. fischeri* to suppress or delay induction at low and intermediate population densities.

Autoinduction of light production by the marine bacterium *Vibrio fischeri* provides a model for understanding population density-responsive gene expression in bacteria. Luminescence in *V. fischeri* is induced at a high population density through the activity of a self-produced, membrane-permeant autoinducer, *N*-3-oxohexanoyl-L-homoserine lactone (*N*-3-oxohexanoyl-L-HSL; autoinducer 1 [AI-1]), which accumulates in a population density-dependent manner during growth. At a threshold concentration, AI-1 activates via LuxR transcription of the *luxICDABEG* operon, encoding the putative autoinducer synthase (LuxI) and the luminescence enzymes (14, 17, 18, 25, 35, 37–39). The autoinduction mechanism in *V. fischeri* also involves, among other regulatory aspects, AI-1-mediated *luxR* negative autoregulation (9, 12, 19, 33, 34, 40). For many years, autoinduction was thought to be unique to the luminescence system of *V. fischeri* and certain closely related marine bacteria. However, several other species of bacteria have recently been found to produce *N*-acyl-L-HSLs chemically similar or identical to that of *V. fischeri*. Autoinducers regulate activities as diverse as the conjugative transfer of plasmids, synthesis of antibiotics, and production of extracellular enzymes. In many cases, proteins homologous to *V. fischeri* LuxI and LuxR have been identified in these other bacteria (8, 23). The diverse species using autoinduction and the chemical and genetic similarities of autoinduction systems indicate that autoinduction is an evolutionarily conserved regulatory mechanism for sensing and responding to population density which is common among bacteria. Detailed knowledge of luminescence autoinduction in *V. fischeri* provides a foundation for understanding these newly discovered autoinduction systems.

We recently demonstrated that *V. fischeri* produces three *N*-acyl-L-HSL autoinducers which can independently activate *lux* operon expression via LuxR (29). The synthesis of two of these compounds, the previously identified AI-1 and the newly identified *N*-hexanoyl-L-HSL (AI-3), is dependent on the previously characterized putative autoinducer synthase gene *luxI*. The synthesis of the third compound, *N*-octanoyl-L-HSL (AI-2), however, is dependent on a newly identified putative autoinducer synthase gene, *ainS* (24, 29). Remarkably, AinS does not exhibit significant amino acid sequence similarity to members of the LuxI family of proteins, indicating that AinS is a representative of a new family of putative autoinducer synthases (29). In an earlier study with synthetic AI-1 analogs, AI-2 was shown to stimulate luminescence by a small amount and to interfere with the luminescence-stimulating activity of AI-1 (16). The production of AI-2 by *V. fischeri* and its apparent contribution to *lux* gene regulation suggest that the luminescence autoinduction mechanism is substantially more complex than previously understood.

In the present study, we sought to determine whether AI-2

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli K-12		
JM83	Δ(*lac-proAB*)	41
PD100	*zah-735*::Tn*10*Δ(*argF-lac*)*U169*, Tc^r	9
S17-1	RP4 *tra*$^+$	36
V. fischeri		
MJ-1	Lux^+	32
MJ-100	MJ-1, Nx^r	11
MJ-211	MJ-100, Δ*luxI* (~250-bp nonpolar deletion)	29
MJ-215	MJ-211, *ainS* (2-bp insertion)	This study
MJ-216	MJ-100, *ainS*::*neo* (1.8-kb Nm^r cassette)	24
Plasmids		
pSUP102	pACYC184, RP4 *mob*$^+$, Cm^r, Tc^r	36
pNL121	pSUP102 with *V. fischeri luxR luxICDABEG*, Cm^r	11
pPD749	p*tac-luxR*, *lacI*^{q}, Ap^r	9
pJR551	pACYC184 with *luxR*::MuΔ(*c*, *nerAB*) dI1681 (*lacZYA*, Km^s) and *luxICDABEG* with a nonpolar point mutation in *luxI*, Cm^r	12
pAK011	pSUP102 with 11.8-kb *Bgl*II fragment from pJR551, *luxR*::*lacZ luxI luxC*$^+$	This study
pAK411	pBR322, *luxR luxICDABEG* with ~250-bp nonpolar deletion in *luxI*, Ap^r	29
pAI009	pSUP102 with 2.7-kb *ain* fragment	24
pAI015	pAI009 with 2-bp insertion in *ainS*	This study

[a] Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Nm^r, neomycin resistant; Tc^r, tetracycline resistant.

actually contributes to luminescence autoinduction in *V. fischeri* and if so, how it operates. The results demonstrate that AI-2 negatively modulates induction, operating by directly interfering with AI-1-mediated *lux* operon expression and not by enhancing *luxR* negative autoregulation. A model for competitive interaction between AI-1 and AI-2 is presented.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used in this study, derivatives of *E. coli* K-12 and *V. fischeri* MJ-1, are listed in Table 1. *E. coli* JM83 (41) was used for most cloning manipulations, which were done by standard procedures (2). *E. coli* S17-1 is a strain capable of conjugatively transferring IncP plasmids, such as the mobilizable chloramphenicol resistance (Cm^r) plasmid pSUP102, to a wide range of recipients (36), including *V. fischeri* (11, 29). *V. fischeri* MJ-100 is a spontaneously nalidixic acid-resistant (Nx^r) derivative of the wild-type strain *V. fischeri* MJ-1 (11, 32).

pAK411 contains the *V. fischeri* MJ-1 *lux* genes (17) cloned into pBR322, with a nonpolar deletion in *luxI* (29). JM83 containing pAK411 produces visible luminescence only in the presence of exogenous autoinducer. pAK011 is an 11.8-kb *Bgl*II fragment from pJR551, which contains the *lux* genes with a *lacZ* fusion in *luxR* and a point mutation in *luxI* (12), cloned into the *Bam*HI site of pSUP102. pAI009, which directs the synthesis of AI-2 in JM83, is pSUP102 containing the 2.7-kb *Hin*dIII *ain* fragment from MJ-1 (24). Other plasmids were constructed by standard procedures, as indicated below and in Table 1.

Culture conditions and physiological assays. *E. coli* strains were maintained on LB medium (2) with appropriate antibiotics to ensure plasmid maintenance. *V. fischeri* strains were maintained on solid LBS medium (11), with nalidixic acid for MJ-100 and its derivatives. *E. coli* liquid cultures were grown in LB with 50 mM Tris (pH 7.5) and an appropriate antibiotic, whereas liquid cultures of *V. fischeri* were grown in ASH medium (11) without antibiotics, except as indicated. Antibiotics were used at the following concentrations (per liter): nalidixic acid, 20 mg; ampicillin, 150 mg; chloramphenicol, 34 mg; kanamycin, 20 mg; and neomycin, 200 mg, unless indicated otherwise. The growth conditions and measurement (A_{660}), luminescence assays, and light-measuring equipment and standard were previously described (11). β-Galactosidase activity was assayed by standard procedures (30), and the assay values reported are the averages of four replicates (range, <10%).

The autoinducer activities in media conditioned by the growth of bacteria were assayed by measuring the luminescence response of *E. coli* PD100(pJR551, pPD749), as described previously (12, 29). In certain experiments, as indicated, isopropyl-β-D-thiogalactopyranoside (IPTG) was used at 25 μM instead of 1 mM. When distinctions among the different *V. fischeri* autoinducers were required, samples were fractionated by reversed-phase high-pressure liquid chromatography (HPLC) and the fractions were assayed for autoinducer activity (29). Synthetic autoinducers were added to culture tubes as solutions in chloroform, and the solvent was removed by drying with a stream of air before the culture medium and bacteria were added.

Construction of *V. fischeri ainS* mutants. The *ainS* plasmid pAI015 was constructed by partially digesting pAI009 (24) with *Cla*I (one site in *ainS* and one site in vector), blunt ending, gel purifying the singly cut plasmid, and ligating. The resulting plasmid theoretically has a frameshift 2-bp insertion in *ainS* that eliminates the *Cla*I site. JM83(pAI015) is Cm^r and does not produce AI-2. The *V. fischeri* Δ*luxI ainS* double mutant MJ-215 was constructed by conjugatively transferring pAI015 from *E. coli* S17-1 to MJ-211 and screening the transconjugant *V. fischeri* for Nx^r Cm^s recombinants that emitted no light detectable by the eye. Southern analysis confirmed that MJ-215 lacks the *Cla*I site in *ainS*. MJ-215 synthesized no autoinducer activity detectable by the standard PD100(pJR551, pPD749) assay. The introduction of pAI009, but not pSUP102 or pAI015, restored luminescence in MJ-215. The *V. fischeri ainS*::*neo* mutant MJ-216, which contains the 1.8-kb *Bam*HI-*Hin*dIII *neo* fragment from Mu dI1734 (5), was constructed and confirmed by similar procedures (24). MJ-216 produces AI-1 and AI-3 but does not produce AI-2 (24).

RESULTS

Absence of luminescence induction and autoinducers in a *luxI ainS* double mutant. Initially, we determined that *V. fischeri* produces no *N*-acyl-L-HSLs that activate *lux* operon transcription via LuxR other than those whose synthesis is dependent on *luxI* and *ainS*. A *V. fischeri* mutant defective in both *luxI* and *ainS*, MJ-215, was constructed by gene replacement procedures (see Materials and Methods), and its growth and luminescence behavior were examined. MJ-215 grew at a rate essentially identical to those of the parent strain, MJ-100, and direct progenitor strain, MJ-211 (Δ*luxI ain*$^+$). However, in contrast to MJ-100 and MJ-211, MJ-215 did not induce luminescence (Fig. 1). Consistent with the lack of induction, culture medium conditioned by the growth of MJ-215 contained no autoinducer activity detectable with a sensitive assay for *N*-acyl-L-HSLs that activate *lux* operon transcription via LuxR (see Materials and Methods). Nonetheless, the addition of AI-1 or AI-2 strongly stimulated luminescence in MJ-215, indicating that this mutant is capable of responding to exogenous autoinducers. For example, MJ-215 at an A_{660} of 0.5 produced approximately 8×10^8 quanta s^{-1} ml^{-1} in the presence of 750 nM AI-2, compared with approximately 2×10^6 quanta s^{-1} ml^{-1} with no addition. The response of this mutant confirms

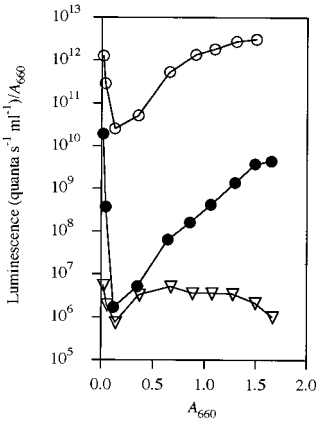

FIG. 1. Luminescence induction behavior of *luxI* and *luxI ainS* mutants of *V. fischeri*. Luminescence (in quanta per second per milliliter/A_{660}) during growth (A_{660}) of MJ-100 (*luxI*$^+$ *ain*$^+$) (○), MJ-211 (*luxI*) (●), and MJ-215 (*luxI ainS*) (▽).

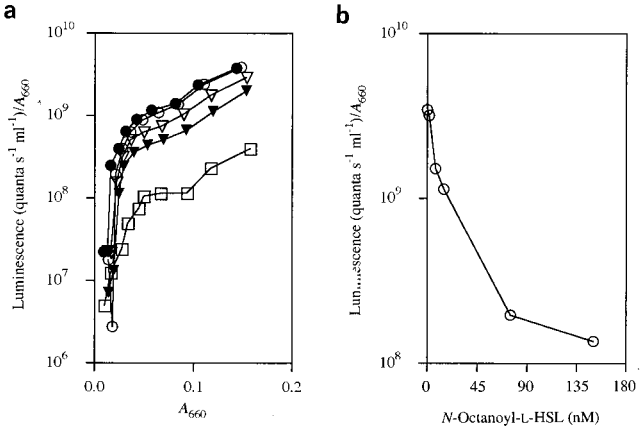

FIG. 3. Inhibition of AI-1-mediated activation of *lux* operon expression by AI-2. (a) Luminescence (in quanta per second per milliliter/A_{660}) of *E. coli* JM83(pAK411, pSUP102) during growth in the presence of *N*-3-oxohexanoyl-L-HSL (AI-1) added to 50 nM and of AI-2 either not added (○) or added to 1.5 (●), 15 (▽), or 150 (▼) nM or 1.5 μM (□). (b) Luminescence at an A_{660} of 0.1 of *V. fischeri* MJ-215 (*luxI ainS*) in the presence of AI-1 added to 50 nM and of increasing concentrations of AI-2.

that AI-2, at least in the absence of AI-1, activates *lux* operon transcription in *V. fischeri* (16, 29). The lack of induction in MJ-215 indicates that even if *V. fischeri* does produce autoinducers other than those whose synthesis is directed by *luxI* and *ainS*, they are not likely to contribute meaningfully to luminescence autoinduction.

Inhibition of luminescence induction by AI-2. The luminescence behavior of a mutant defective only in *ainS* indicated that AI-2 delays the induction of luminescence in *V. fischeri*. In mutant MJ-216, AI-2 synthesis had been eliminated by the insertion of a *neo* cassette into *ainS* (24). Colonies of MJ-216 growing on agar plates exhibited high levels of luminescence, similar to those of colonies of the parent strain, MJ-100. However, the induction of luminescence in MJ-216 grown in broth culture occurred at a lower population density than it did in MJ-100, and luminescence increased more rapidly once induction started (Fig. 2a). The accelerated induction of luminescence in *V. fischeri* in the absence of AinS is consistent with the inhibition of *lux* operon induction by AI-2. Alternatively, however, the accelerated induction in MJ-216 might have been due in some way to the presence of the *neo* cassette. For example,

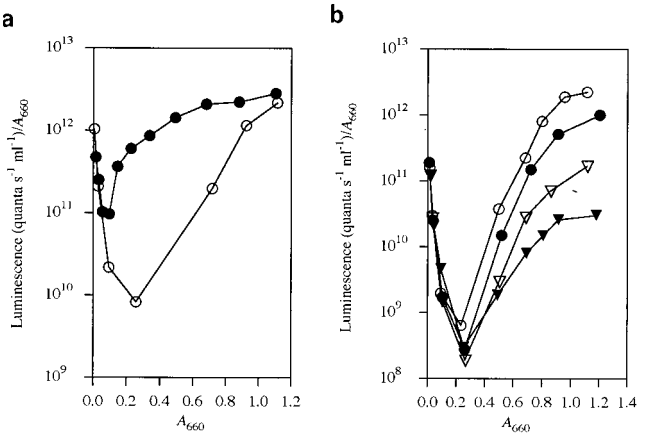

FIG. 2. Inhibition of luminescence induction by *ainS* and *N*-octanoyl-L-HSL (AI-2) in *V. fischeri*. (a) Luminescence (in quanta per second per milliliter/A_{660}) during growth (A_{660}) of MJ-100 (*luxI*$^+$ *ain*$^+$) (○) and MJ-216 (*ainS*) (●); (b) luminescence during growth of MJ-1 (*luxI*$^+$ *ain*$^+$) in the presence of 0 (○), 7.5 (●), 75 (▽), and 750 (▼) nM exogenous AI-2.

MJ-216 grew with a doubling time of 130 min, a rate much slower than the 40-min doubling time for MJ-100. In *V. fischeri*, the presence of a *neo* cassette in the genome can lead to slower growth (28), and slower growth under some conditions can lead to the induction of luminescence at a lower population density (10).

To resolve this issue, we examined the effect of exogenously added AI-2 on luminescence induction in *V. fischeri*. For the *lux*$^+$ *ain*$^+$ strains MJ-1 (Fig. 2b) and MJ-100 and the *ainS* mutant MJ-216, the addition of increasing amounts of AI-2 progressively delayed the induction of luminescence until cultures had attained proportionately higher population densities, and once induction started, the rate at which luminescence increased was progressively lower with increasing amounts of added AI-2. The addition of AI-2, however, had no effect on the growth rates of these strains at any of the concentrations tested. These results demonstrate that the presence of AI-2 inhibits luminescence induction in *V. fischeri* and does so without affecting growth.

Establishment of luminescence inhibition by AI-2 in *E. coli*. We next sought to establish the effect of AI-2 on luminescence induction in *E. coli* containing cloned *V. fischeri lux* genes. *E. coli* makes no autoinducer detected with the LuxR-dependent assay used in this study (29); therefore, it is a simpler system than *V. fischeri* for studying the mechanism by which AI-2 inhibits luminescence induction. *E. coli* JM83 containing pAK411, a *lux* plasmid with a 250-bp nonpolar deletion in *luxI* (29), was treated with a fixed amount of AI-1 (50 nM) and with increasing amounts of AI-2 (0 to 1.5 μM). The addition of AI-2 decreased the AI-1-activated luminescence of JM83(pAK411) in a dose-dependent manner (Fig. 3a). This result was confirmed with *V. fischeri* MJ-215 (*luxI ainS*) (Fig. 3b). The ability to establish luminescence inhibition by AI-2 in *E. coli* indicates that this inhibition requires no other regulatory elements besides AI-1 and LuxR and no regulatory sites other than those of the *lux* genes.

Inability of AI-2 to mediate *luxR* negative autoregulation. With the establishment of luminescence inhibition by AI-2 in both *E. coli* and *V. fischeri*, we were in a position to examine its mechanism of action. AI-1 via LuxR activates *lux* operon transcription and represses *luxR* expression (9, 12, 33). Conse-

TABLE 2. Luminescence and β-galactosidase activities of *E. coli* PD100(pJR551, pPD749) and *V. fischeri* MJ-215(pAK011)

Strain	Addition	Luminescence (quanta/s/ml)[a]	β-Galactosidase activity (Miller units)[a]
PD100(pJR551, pPD749)	None	2.2×10^8	310
	AI-1 (160 nM)	8.1×10^{11}	190
	AI-2 (7.1 μM)	7.4×10^{11}	320
MJ-215(pAK011)[b]	None	2.3×10^7	670
	AI-1 (300 nM)	7.8×10^{10}	500
	AI-2 (7.5 μM)	2.0×10^{10}	720

[a] At an A_{660} of 1.0 for *E. coli* and 1.1 for *V. fischeri.*
[b] Grown in the presence of 17 mg of chloramphenicol per liter to ensure plasmid maintenance.

TABLE 3. Luminescence and β-galactosidase activities of *E. coli* JM83(pAK011, pAK411)

Addition(s)	Luminescence (quanta/s/ml)[a]	β-Galactosidase activity (Miller units)[a]
None	8.0×10^6	53
AI-1 (50 nM)	4.3×10^8	52
AI-2 (1.5 μM)	1.0×10^7	53
AI-1 and AI-2	5.4×10^7	53

[a] At an A_{660} of 0.27.

quently, AI-2 could inhibit luminescence induction by interfering with AI-1-mediated activation of *lux* operon transcription or by increasing *luxR* negative autoregulation.

We found no evidence, however, to support the idea that AI-2 operates through *luxR* negative autoregulation. Initially, we used conditions similar to those used to define AI-1-dependent *luxR* transcriptional negative autoregulation (9, 12). Specifically, *E. coli* PD100 containing pJR551 (*luxR*::*lacZ luxICDABEG*, with a point mutation in *luxI*) and pPD749 (p*tac luxR*) was grown in the presence of IPTG and in the presence or absence of AI-1 and AI-2. β-Galactosidase activity and luminescence were measured as reporters of expression from the *luxR* and *lux* operon promoters, respectively. The addition of AI-1 activated luminescence approximately 1,000-fold but decreased β-galactosidase activity approximately 2-fold (Table 2). These results are similar to those of earlier reports for AI-1-mediated *luxR* negative autoregulation (9, 12). However, even when AI-2 was added at a concentration sufficiently high to activate luminescence to approximately the same extent as that of added AI-1, it had no effect on β-galactosidase activity (Table 2).

The *E. coli* results were confirmed by examination of *V. fischeri* MJ-215 (*luxI ainS*) into which was mobilized pAK011 (*luxR*::*lacZ*). The addition of AI-1 to MJ-215(pAK011) increased luminescence strongly but decreased β-galactosidase activity by a small amount (Table 2). To our knowledge, this is the first demonstration of *luxR* negative autoregulation in *V. fischeri*. The addition of AI-2 at a concentration sufficient to activate luminescence to approximately the same level as that of added AI-1, however, did not decrease β-galactosidase activity (Table 2). These results indicate that although AI-2 is able to activate *lux* operon transcription via LuxR, it does not mediate detectable *luxR* negative autoregulation, at least in the absence of AI-1.

Similar results were also obtained in the presence of AI-1. The addition of AI-1 alone to *E. coli* JM83(pAK411, pAK011) stimulated luminescence; this stimulation was strongly decreased in the presence of AI-2. Under these conditions, AI-2 alone weakly stimulated luminescence. However, the presence of AI-2, alone or with AI-1, had no effect on β-galactosidase activity (Table 3).

It should be noted that at low culture densities (i.e., A_{660} of 0.27) at which the inhibitory effect of AI-2 on AI-1-stimulated luminescence could be detected, AI-1-mediated *luxR* negative autoregulation was not observed (Table 3). Conversely, at the high culture density (A_{660} of 1.0) typically used to detect AI-1-mediated *luxR* negative autoregulation (9, 12), the inhibition of AI-1-stimulated luminescence by AI-2 was not observed (data not shown). We attribute this complexity to the need for high cellular levels of active LuxR, available at high population densities (1), to effect *luxR* negative autoregulation and to the ability of high levels of the putative AI-1–LuxR complex to overcome the inhibition of AI-1-stimulated luminescence by AI-2 (see below). Nonetheless, the results (Tables 2 and 3) demonstrate that AI-2 does not mediate detectable *luxR* negative autoregulation, even under conditions in which it inhibits AI-1-mediated activation of *lux* operon expression.

Direct inhibition of *lux* operon expression by AI-2. The results discussed above alternatively suggested that AI-2 inhibits luminescence by inhibiting *lux* operon transcription. Two sets of experimental results support this alternative. First, inhibition by AI-2 was apparent even when the expression of LuxR was not under the control of its native promoter. We examined the effects of increasing concentrations of AI-2 on the luminescence of *E. coli* PD100(pJR551, pPD749), with the addition of different amounts of AI-1 to activate *lux* operon expression via LuxR to different levels. A low concentration of IPTG (25 μM) was used to partially activate LuxR expression from the p*tac* promoter. Under these conditions, we found that at each concentration of AI-1 used, the addition of increasing amounts of AI-2 progressively inhibited luminescence (Fig. 4a).

Second, inhibition by AI-2 could be overcome by AI-1. We examined the effects of increasing concentrations of AI-1 on the AI-2-mediated inhibition of luminescence of PD100 (pJR551, pPD749) by using the low concentration of IPTG mentioned above. Regardless of the initial extent of inhibition,

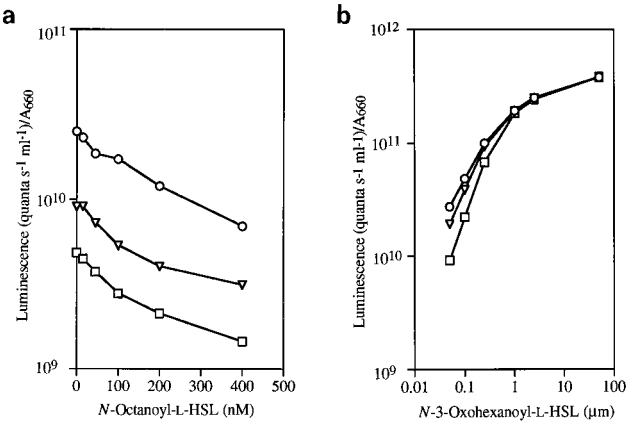

FIG. 4. Inhibition of AI-1-mediated activation of *lux* operon expression by AI-2 and reversal of AI-2-mediated inhibition by AI-1 in *E. coli*. (a) Luminescence (in quanta per second per milliliter/A_{660}) at an A_{660} of 0.2 of PD100(pJR551, pPD749) in the presence of AI-1 added to 25 (□), 50 (▽), or 100 (○) nM and in the presence of increasing concentrations of AI-2. (b) Luminescence at an A_{660} of 0.2 of PD100(pJR551, pPD749) in the presence of AI-2 added to 0 (○), 45 (▽), or 200 (□) nM and in the presence of increasing concentrations of AI-1. The expression of LuxR from p*tac-luxR* was activated by the addition of 25 μM IPTG.

high concentrations of AI-1 overcame the inhibition (Fig. 4b). These results are consistent with direct inhibition of AI-1-mediated *lux* operon expression by AI-2. Furthermore, they are strongly indicative of competitive interaction between AI-1 and AI-2.

DISCUSSION

The results of this study demonstrate that the newly identified *ainS*-dependent autoinducer *N*-octanoyl-L-HSL (AI-2) functions in *V. fischeri* to negatively modulate luminescence induction. Previously, using a genetically defined *luxI* mutant unable to produce *N*-3-oxohexanoyl-L-HSL (AI-1) and *N*-hexanoyl-L-HSL (AI-3), we demonstrated that AI-2 can activate transcription from the *lux* operon promoter via LuxR by a small amount (29). That observation was consistent with the results of an earlier study using a genetically uncharacterized autoinducer-deficient strain of *V. fischeri* in which it was shown that various synthetic *N*-acyl-L-HSLs structurally similar to AI-1, including AI-2, could stimulate luminescence (16). The earlier study also demonstrated that AI-2 and certain other compounds could inhibit the luminescence stimulated by AI-1 (16). In the present study, with the recognition that *V. fischeri* produces AI-2 (29), we sought to determine whether AI-2 actually contributes to luminescence autoinduction in *V. fischeri* and if so, how it operates. We found that in the absence of both *luxI* and *ainS*, *V. fischeri* did not induce luminescence and produced no detectable luminescence autoinducer, indicating that luminescence autoinducers other than those whose synthesis is dependent on *luxI* and *ainS* are not likely to be produced by *V. fischeri*. However, a mutant defective only in *ainS* exhibited accelerated luminescence induction compared with that of the parental strain. The behavior of the *ainS* mutant was surprising because it suggested that AI-2 operates in *V. fischeri* not to supplement the activity of AI-1, as could be expected from the ability of AI-2 to activate luminescence in the absence of AI-1, but to inhibit luminescence induction. This latter interpretation is consistent with the inhibitory activity of AI-2 demonstrated elsewhere (16). Indeed, the general concordance between the results for AI-2 in the present study and those in the earlier report suggests that the *luxI*-dependent autoinducer AI-3 also might function in *V. fischeri* to inhibit AI-1-mediated luminescence induction (16, 29). The inhibitory activity of AI-2 was confirmed through its exogenous addition, which inhibited luminescence induction in *V. fischeri* and *E. coli* containing the *lux* genes.

This is the first report of negative modulation of an autoinducible operon by a self-produced autoinducer. However, there is precedent for negative regulation of autoinducible systems in other species. The recently identified *Agrobacterium tumefaciens* protein TraM negatively modulates the response of TraR, a LuxR homolog, to *N*-3-oxooctanoyl-L-HSL (22, 26). In *Vibrio harveyi*, the repression of *lux* operon expression by LuxO apparently is relieved by two separate putative autoinducer-sensor protein complexes (4). The synthesis of two autoinducers by *Pseudomonas aeruginosa* (31), both of which are required for normal induction of the target genes, introduces the possibility of autoinducer-mediated negative modulation in this species as well, assuming both positive and negative roles for at least one of these autoinducers. Negative modulation by an autoinducer and other regulatory elements highlights the notion that autoinduction is a complex regulatory mechanism which may require fine-tuning in response to diverse and varying environmental conditions (4, 24).

With respect to the mechanism by which AI-2 operates, we found no evidence for AI-2-mediated *luxR* negative autoregulation, even under conditions in which AI-2 inhibits AI-1-dependent activation of *lux* operon expression. Conversely, AI-2 was found to inhibit luminescence induction in *E. coli* regardless of whether *luxR* was under the control of its native promoter or a foreign promoter, and the inhibitory effect of AI-2 was overcome by high levels of AI-1. These observations indicate that AI-2 operates by directly inhibiting *lux* operon expression rather than by increasing *luxR* negative autoregulation, and they are consistent with competitive interaction between AI-1 and AI-2. Nonetheless, we have not excluded the possibility that AI-2 operates by other means, such as the stimulation of the synthesis or activity of an unidentified repressor of *lux* operon transcription. However, postulating the existence of such a repressor seems unlikely, since it would have to be active in both *V. fischeri* and *E. coli* containing the *lux* genes.

On the basis of the data presented here, we propose a model in which AI-2 competitively inhibits the association of AI-1 with LuxR. This model is generally consistent with current models for the interaction of an autoinducer with LuxR (6, 16, 18, 23, 38, 40). According to this model, AI-2 associates with LuxR, forming a complex with markedly lower *lux* operon-inducing specific activity than that of the putative AI-1–LuxR complex. Consistent with the proposed lower activity, 25- to 45-fold-more AI-2 is needed to activate *lux* operon expression to the same extent as that of AI-1 (Table 2). This lower specific activity could result from a nonoptimal conformational change in LuxR, leading to a lower affinity of the AI-2–LuxR complex for the LuxR protein binding site in the *lux* regulatory region (the *lux* box) or to the weaker ability of this complex to facilitate the association of RNA polymerase with the *lux* operon promoter (3, 7, 20, 38). Since LuxR is thought to operate as a dimer (6, 16), lower activity presumably could result from one monomer binding AI-2 and one binding AI-1 or both monomers binding AI-2. Consistent with this model, the apparent inability of AI-2 to mediate *luxR* negative autoregulation, which even when mediated by AI-1 is a small effect, could result from the lower specific activity of the AI-2–LuxR complex, making its *luxR* negative autoregulation activity very minor and difficult to detect.

The inhibitory activity of AI-2 may help explain how induction at low population densities is prevented in *V. fischeri*. Some *lux* operon induction can occur in the presence of only one to two molecules of AI-1 per cell (27), and the positive feedback nature of *lux* operon regulation by AI-1 (15, 16, 21) suggests that under these conditions, induction should quickly increase to a high level, at least in the absence of inhibitory factors. Indeed, complete medium, used in most regulatory studies of *V. fischeri* luminescence, contains an unidentified factor, removed by cells during growth, that inhibits induction (13). The production of AI-2 apparently is a natural means by which *V. fischeri* prevents induction at low population densities. According to the competition model outlined above, the presence of AI-2 retards *lux* operon expression and temporarily short-circuits induction by delaying the accumulation of AI-1. Because of the AI-1-dependent feedback nature of AI-1 synthesis, with the putative AI-1 synthase gene *luxI* as part of the *lux* operon, even a small decrease in the synthesis of AI-1 by AI-2 could substantially delay the buildup of AI-1. However, AI-2 does not completely block the synthesis of AI-1, because it activates *lux* operon transcription via LuxR by a small amount. Relief from inhibition by AI-2 presumably develops from the accumulation at high population densities of sufficient amounts of AI-1 to outcompete AI-2 for the available LuxR, as well as from the increased availability of active LuxR in the late exponential phase of growth (1).

We have also shown that the production of AI-2 in *V. fischeri* increases rapidly at high population densities, similar to the production of AI-1 (29). Furthermore, the presence of a *lux* box-like palindrome in the proposed *ainS* promoter region (24) suggests that AI-2 production is under autoinducer control. It is possible therefore that basal levels of AI-2 prevent premature induction at low population densities and that induced AI-2 synthesis helps to restrain luminescence induction at intermediate population densities as AI-1 accumulates. These roles for AI-2 in negatively modulating *lux* gene expression may parallel roles in positively and negatively regulating non-*lux* genes in *V. fischeri* (24, 29).

ACKNOWLEDGMENTS

We thank N. Blough for assistance with reversed-phase HPLC and D. Lynn for the generous gift of synthetic autoinducers.

This work was supported by National Science Foundation grant MCB 94-08266.

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