

## Quorum Sensing in *Vibrio fischeri*: Probing Autoinducer-LuxR Interactions with Autoinducer Analogs

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**The *Vibrio fischeri* luminescence genes are activated by the transcription factor LuxR in combination with a diffusible signal compound, *N*-(3-oxohexanoyl) homoserine lactone, termed the autoinducer. We have synthesized a set of autoinducer analogs. Many analogs with alterations in the acyl side chain showed evidence of binding to LuxR. Some appeared to bind with an affinity similar to that of the autoinducer, but none showed a higher affinity, and many did not bind as tightly as the autoinducer. For the most part, compounds with substitutions in the homoserine lactone ring did not show evidence of binding to LuxR. The exceptions were compounds with a homocysteine thiolactone ring in place of the homoserine lactone ring. Many but not all of the analogs showing evidence of LuxR binding had some ability to activate the luminescence genes. None were as active as the autoinducer. While most showed little ability to induce luminescence, a few analogs with rather conservative substitutions had appreciable activity. Under the conditions we employed, some of the analogs showing little or no ability to induce luminescence were inhibitors of the autoinducer.**

Quorum sensing is used by a number of gram-negative bacterial genera to regulate expression of specific sets of genes in a cell density-dependent fashion (10, 20, 21). Certain pathogenic bacteria use quorum sensing in the regulation of genes encoding extracellular virulence factors (17, 19). The cell density control of luminescence in the symbiotic marine bacterium *Vibrio fischeri* is the best-studied quorum sensing system, and although each of the known systems has unique features, the *V. fischeri* luminescence system is considered the model (10, 20, 21). There are two regulatory genes involved in quorum sensing, the *I* and *R* genes. The *I* gene directs the synthesis of an *N*-acyl homoserine lactone (HSL) signal molecule termed the autoinducer. The *R* gene codes for a transcription factor that is responsive to the *N*-acyl HSL signal. In *V. fischeri*, the *luxI* gene directs the synthesis of 3-oxohexanoyl HSL, the autoinducer signal required for luminescence gene activation (6, 8, 9). Cells are permeable to this signal, and thus high cell densities are required to achieve a critical concentration of the autoinducer required to bind the *luxR* product, which in turn activates transcription of the luminescence genes (1, 8, 13, 15).

Little is known about the interaction of the *V. fischeri* autoinducer, 3-oxohexanoyl HSL, and the LuxR protein. The LuxR polypeptide consists of two domains. The available evidence indicates that 3-oxohexanoyl HSL binds to the N-terminal domain and that this binding allows a productive interaction of the LuxR C-terminal domain with the transcription-initiation complex of the luminescence genes (4, 13). There is one previous study of the influence of autoinducer analogs on induction of luminescence in *V. fischeri* (7). The analogs showed a spectrum of activities: some were capable of inducing luminescence, some inhibited activation by 3-oxohexanoyl HSL, and others showed little or no effect on induction of luminescence. In a similar type of analysis, Chhabra et al. (3) found that a number of autoinducer analogs had appreciable activity when substituted for 3-oxohexanoyl HSL as an inducer of carbap-

enem synthesis in *Erwinia carotovora*. These studies were limited by an inability to measure the binding of 3-oxohexanoyl HSL to cells containing the R protein or to measure the influence of autoinducer analogs on that binding. It is now possible to measure the binding of 3-oxohexanoyl HSL to recombinant *Escherichia coli* containing *luxR* (1, 13). It is also possible to use strains without a functional *luxI* so that autoinducer concentrations can be controlled precisely by the amount added exogenously.

We have studied the ability of a variety of *V. fischeri* autoinducer analogs (i) to competitively inhibit binding of <sup>3</sup>H-labeled 3-oxohexanoyl HSL to LuxR-containing *E. coli*, (ii) to activate the *V. fischeri* luminescence genes in *E. coli*, and (iii) to competitively inhibit the activity of 3-oxohexanoyl HSL as an inducer of luminescence in recombinant *E. coli*. This analysis provides some insight into how 3-oxohexanoyl HSL might interact with LuxR, and it provides information relevant to the development of therapeutic quorum sensing inhibitors of virulence gene expression in pathogenic bacteria.

### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** For autoinducer binding studies, we used *E. coli* XL1-Blue (2) containing pHK724 (16) and pGroESL (11). Induction of luminescence was measured in *E. coli* VJS533 (22) containing pHV200I<sup>-</sup> (18). The plasmid pHK724 contains a *ptac*-controlled *luxR*, pGroESL contains a *plac*-controlled *groE* operon, and pHV200I<sup>-</sup> contains a *V. fischeri* luminescence gene cluster in which *luxI* has been inactivated. Cultures were grown in Luria broth plus isopropyl-β-D-thiogalactoside and the appropriate antibiotics for plasmid maintenance at 30°C as described elsewhere (4, 13).

**Autoinducer binding experiments.** Binding of tritium-labeled 3-oxohexanoyl HSL to *E. coli* containing pHK724 and pGroESL (5) was measured as described previously (13). Competition studies with unlabeled autoinducer analogs were carried out by incubating cells with both tritiated 3-oxohexanoyl HSL (230 nM) and the indicated analog at a concentration of 230 nM, 1.2 μM, or 2.3 μM for 10 min at 25°C. The amount of tritium bound to the cells was then determined. Results of the competition experiments are given as a percentage of inhibition of <sup>3</sup>H-labeled 3-oxohexanoyl HSL binding in the absence of an added analog.

**Effects of 3-oxohexanoyl HSL analogs on the induction of luminescence.** Induction was assessed by measuring the luminescence of *E. coli* containing pHV200I<sup>-</sup>. This recombinant *E. coli* produces light in response to autoinducer in a dose-dependent manner (18). The influence of the analogs on autoinduction of luminescence was investigated in two ways. First, we determined the ability of each analog that inhibited autoinducer binding to serve in place of 3-oxohexanoyl HSL to activate luminescence. Then we tested those analogs which inhibited

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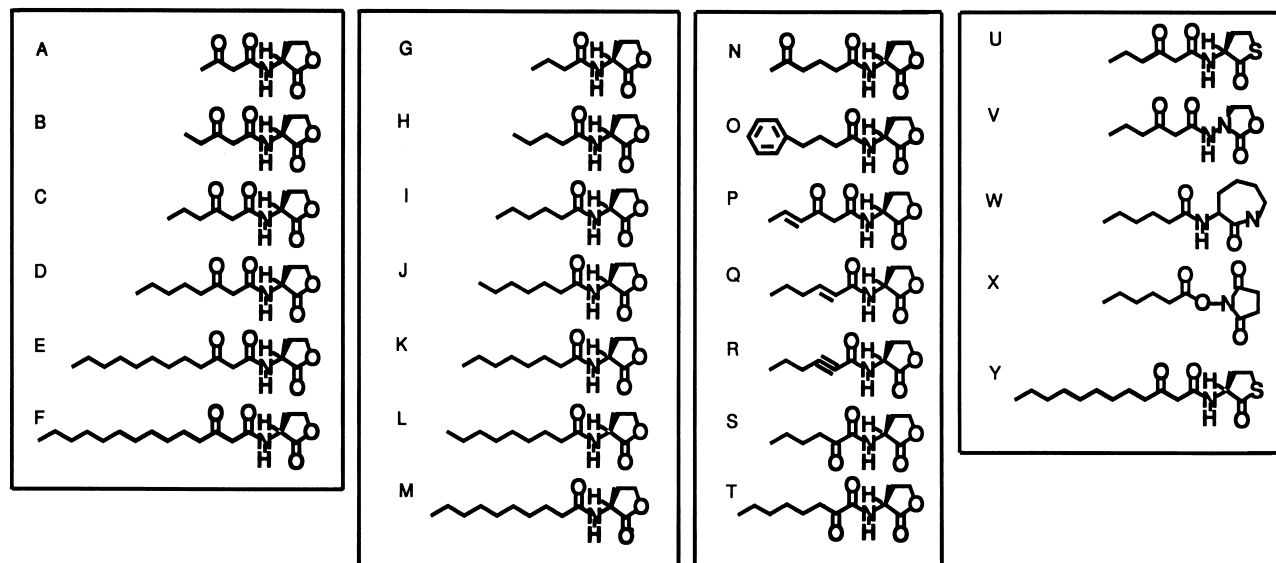


FIG. 1. Structures of *N*-acyl HSL compounds used in this study. A, 3-oxobutanoyl HSL; B, 3-oxopentanoyl HSL; C, 3-oxohexanoyl HSL; D, 3-oxooctanoyl HSL; E, 3-oxododecanoyl HSL; F, 3-oxotetradecanoyl HSL; G, butanoyl HSL; H, pentanoyl HSL; I, hexanoyl HSL; J, heptanoyl HSL; K, octanoyl HSL; L, nonanoyl HSL; M, decanoyl HSL; N, 5-oxohexanoyl HSL; O, 4-phenylbutanoyl HSL; P, 3-oxo-4-hexenoyl HSL; Q, 2-hexenoyl HSL; R, 2-hexynoyl HSL; S, 2-oxohexanoyl HSL; T, 2-oxooctanoyl HSL; U, 3-oxohexanoyl homocysteine thiolactone; V, 3-oxohexanoyl-aza-HSL; W, 3-oxohexanoyl caprolactam; X, succinimidyl hexanoate; Y, 3-oxodecanoyl homocysteine thiolactone.

autoinducer binding to LuxR but were not strong activators of luminescence for their ability to inhibit the activity of 3-oxohexanoyl HSL. Except where indicated, the induction experiments were performed by measuring the luminescence of cells incubated for 3 h in the low-nutrient autoinducer bioassay medium as described previously (18). In these experiments, we tested each analog at concentrations of 20, 100, and 200 nM. To test for inhibitory effects of analogs, we included 20 nM 3-oxohexanoyl HSL in the cell suspensions. This concentration of 3-oxohexanoyl HSL is approximately one-half of that required for maximal induction of luminescence in our experiments.

**Chemical synthesis of autoinducer analogs.** The synthesis of  $^3\text{H}$ -labeled 3-oxohexanoyl HSL has been described previously (14), as has the synthesis of compounds A, C, D, G through N, S, T, U, and W (Fig. 1) (7) and compounds E (18) and P (14). All compounds were purified by high-performance liquid chromatography (HPLC), and the structure of each compound is shown in Fig. 1.

Compound B was prepared from propionyl chloride and Meldrum's acid (2,2-dimethyl-1,3-dioxane-4,6-dione; Aldrich Chemical Co.) in methylene chloride with two equivalents of pyridine. After reaction at 0°C for 1 h and at room temperature for 1 h, the methylene chloride was removed and the residue was heated under reflux for 1 h with an excess of pyridine and one equivalent of HSL hydrobromide. After evaporation of the pyridine, the residue was taken up in ethyl acetate and the product was purified by preparative reverse-phase HPLC.

Compound O was prepared from sodium 4-phenyl butanoate and HSL hydrobromide with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in water and then extracted with ethyl acetate and purified by preparative reverse-phase HPLC. Compound Q was prepared similarly from sodium *trans*-2-hexenoate, and compound R was prepared from sodium 2-hexenoate (Farchan Laboratories, Gainesville, Fla.).

Compound V was prepared from the lithium salt of the ethylene glycol ketal of 3-oxohexanoic acid (7) and 3-amino-2-oxazolidinone sulfate (Aldrich Chemical Co.) with dicyclohexyl carbodiimide in pyridine. After reaction at room temperature for several days, the mixture was treated with water and filtered. The filtrate was washed with ether and then evaporated to give a syrup which was taken up in ethyl acetate. Evaporation of the ethyl acetate gave a syrup which was refluxed with dilute HCl. The product was purified by preparative reverse-phase HPLC.

Compound X was prepared from hexanoic acid and *N*-hydroxysuccinimide with dicyclohexyl carbodiimide in dioxane. After reaction overnight, the mixture was filtered and the filtrate was evaporated. The residue was taken up in ethyl acetate, and the product was purified by preparative reverse-phase HPLC. Compound F was a gift from Luciano Passador, and compound Y was a gift from Paul Williams.

## RESULTS AND DISCUSSION

**Inhibition of autoinducer binding by analogs.** One way to assess whether an analog can bind to LuxR is to determine if

it inhibits the binding of 3-oxohexanoyl HSL. Thus we examined the influence of the analogs on binding of tritium-labeled 3-oxohexanoyl HSL (compound C) to *E. coli* containing *luxR*. As a control, we used unlabeled 3-oxohexanoyl HSL as the competitor. The degree of inhibition observed over the range of 3-oxohexanoyl HSL concentrations tested (Fig. 2A) was close to that predicted on the basis of the assumption that the tritium-labeled and unlabeled compounds bind to the same site with the same affinity. Similar results were obtained regardless of the order in which the labeled and unlabeled compounds were added.

A series of analogs differing from 3-oxohexanoyl HSL only in the length of the acyl side chain was tested (Fig. 2A). The C-4 analog (compound A) showed no inhibition of autoinducer binding, but compounds with side chain lengths of C-5 to C-14 (compounds B to F) inhibited autoinducer binding. The C-8 analog (compound D) was as effective an inhibitor as the C-6 autoinducer (compound C). This indicates that there is a minimum side chain length of five carbons required for binding to LuxR and that, although LuxR can accommodate analogs with acyl side chains of 14 carbons, it most efficiently binds to compounds with acyl side chain lengths of six or eight carbons.

We also tested a series of compounds that did not possess a carbonyl at position 3 (Fig. 2B). Again, the C-4 derivative (compound G) showed no inhibition of autoinducer binding, but all of the compounds with longer acyl side chains served as inhibitors (compounds H to M). In each case in which a comparison can be made, the 3-oxo derivative was a better inhibitor than its 3-methylene counterpart. This indicates that the carbonyl at position 3 improves the binding efficiency but is not imperative for binding. A compound with the carbonyl at position 5 (compound N) rather than position 3 (compound C) showed a reduced but measurable inhibition of autoinducer binding whereas neither a C-6 nor a C-8 analog with a carbonyl in position 2 (compounds S and T; data for compound T not shown) rather than position 3 showed any activity as an inhibitor of autoinducer binding (Fig. 2C).

Introduction of a double bond in the side chain resulted in a

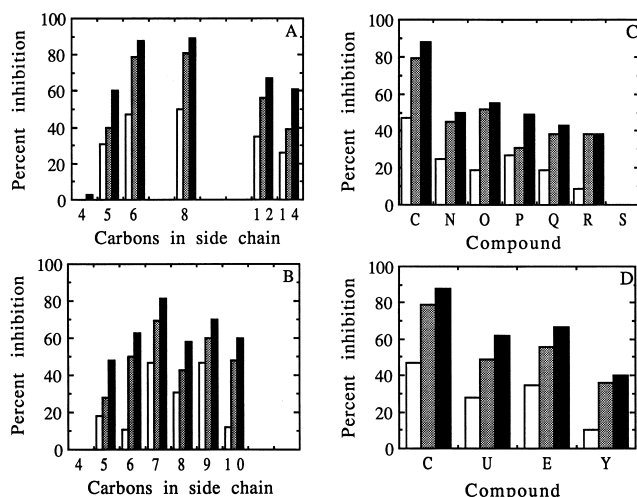


FIG. 2. Inhibition of  $^3\text{H}$ -labeled 3-oxohexanoyl HSL binding to *E. coli* containing LuxR by autoinducer analogs. Each analog was tested at 230 nM (open bars), 1.2  $\mu\text{M}$  (stippled bars), and 2.3  $\mu\text{M}$  (solid bars) concentrations. In all cases, the  $^3\text{H}$ -labeled 3-oxohexanoyl HSL concentration was 230 nM. The y axis is the percent inhibition of  $^3\text{H}$ -labeled 3-oxohexanoyl HSL binding. (A) Inhibition of binding by analogs containing a carbonyl group at the C-3 position. The x axis represents the number of carbons in the acyl side chain of each analog tested. (B) Inhibition by analogs with a methylene group at position 3 of the acyl side chain. The x axis represents the number of carbons in the acyl side chain. (C) Inhibition by analogs with a substituted six-carbon acyl side chain. The letters on the x axis correspond to the analogs shown in Fig. 1. Inhibition by unlabeled 3-oxohexanoyl HSL (compound C) is shown for reference. (D) Inhibition of binding by analogs with substitutions in the HSL ring. U and Y. The corresponding compounds with unsubstituted HSL rings, C and E, are shown for reference. Compounds V, W, and X did not inhibit binding (data not shown). Each value represents the mean of four experiments. In all cases, the range was  $\pm 10\%$  of the average.

reduced capacity to inhibit binding, and the extent of inhibition was about the same regardless of the location of the double bond (Fig. 2C, compounds P and Q). Introduction of a 2,3-triple bond (compound R) caused a more dramatic decrease in inhibition of 3-oxohexanoyl HSL binding. Interestingly, an analog with a bulky phenyl group at the end of the side chain (compound O) showed activity as an inhibitor of 3-oxohexanoyl HSL binding to LuxR (Fig. 2C).

These data lead us to speculate that analogs with a sufficiently long acyl side chain can bind to LuxR and interfere with autoinducer binding. Compounds with decreased side chain flexibility have decreased activity, and the compounds with decreased flexibility in position 2, near the amide bond, have no measurable activity.

Of several analogs with substitutions in the ring, only those with the conservative substitution of a thiolactone ring for the HSL ring showed an appreciable activity (Fig. 2D). The binding studies taken as a whole suggest that the acyl side chain is involved in a somewhat nonspecific interaction with a hydrophobic surface of LuxR and that this brings the HSL group in the vicinity of specific amino acid residues with which it must interact. A certain flexibility in the side chain is required so that an appropriate interaction between the HSL and the specific LuxR residues can occur.

**Which analogs can activate transcription of the luminescence genes?** To determine which of the analogs showing evidence of binding to LuxR (Fig. 2) could activate the luminescence genes, we carried out autoinducer bioassays in which 3-oxohexanoyl HSL was replaced with each analog (Table 1). None of the analogs tested were as active in the bioassay as was 3-oxohexanoyl HSL, but several showed considerable induc-

tion of luminescence. Considering the results with the 3-oxo analogs and the analogs missing the carbonyl on carbon 3, it appears that compounds with side chain lengths of five to nine carbons have at least slight activity. Compounds with side chains of 10 or more carbons showed little or no activity (Table 1). While not critical for binding to LuxR (Fig. 2A and B), the carbonyl in position 3 greatly increased the potency of a compound as an inducer of luminescence (compare compounds B to D with compounds H to K in Table 1). With the exception of 5-oxohexanoyl HSL (compound N) and 3-oxohexanoyl homocysteine thiolactone (compound U), none of the other analogs showed much autoinducer activity (Table 1). The general trend showed that those analogs that were the most effective inhibitors of autoinducer binding also were the most effective inducers of luminescence. However, a few compounds such as 3-oxododecanoyl HSL (compound E) showed inhibition of  $^3\text{H}$ -labeled 3-oxohexanoyl HSL binding but did not activate luminescence.

**Analog inhibition of 3-oxohexanoyl HSL activity.** Each analog that showed any evidence of binding to LuxR (Fig. 2) was tested for its ability to interfere with induction of luminescence by 3-oxohexanoyl HSL as measured with the autoinducer bioassay. Five of the analogs inhibited 3-oxohexanoyl HSL activity by 80% or more, at least at the highest analog concentration tested (Fig. 3). These five compounds were those which bound most tightly to LuxR without showing much activation of luminescence.

We tested the influence of autoinducer analogs on inhibition of the *V. fischeri* autoinducer (compound C) only under a limited set of conditions in recombinant *E. coli*. Eberhard et al. (7) reported previously that decanoyl HSL (compound M) was a more potent inhibitor of autoinducer in *V. fischeri* than we have found in *E. coli*. We have confirmed this and shown that the *Pseudomonas aeruginosa* autoinducer (compound E) is a weaker inhibitor in *V. fischeri* than in *E. coli* (5a). Eberhard et al. (7) also found that 3-oxooctanoyl HSL (compound D) was nearly as effective an inducer in *V. fischeri* as 3-oxohexanoyl HSL, while we found it to be a much weaker inducer in *E. coli* (Table 1) but a strong competitive inhibitor of 3-oxohexanoyl HSL binding (Fig. 2). Thus, while we find similar trends in *V. fischeri* and recombinant *E. coli*, there are subtle differences. In a recent review, Sitnikov et al. (21) indicated that they found decanoyl HSL to be an inhibitor of *lux* gene induction in *V. fischeri* and an activator in recombinant *E. coli*. On the basis of this sort of discrepancy, they postulated that the autoinducer binds not to LuxR but to an accessory protein, which relays information to LuxR. The postulated accessory protein in *V. fischeri* would have a different specificity than its *E. coli* counterpart. Our results with decanoyl HSL are not consistent with those discussed by Sitnikov et al. (21); we could not detect any induction of luminescence in *E. coli* by as much as 200 nM decanoyl HSL, but this concentration of decanoyl HSL inhibited 3-oxohexanoyl HSL induction of luminescence by about 80% (Fig. 3). On the basis of our observations, we favor the more generally accepted model of direct autoinducer binding to LuxR (1, 13) over the recently proposed signal transduction model (21).

This study confirms and extends previous studies of the influence of autoinducer analogs on luminescence in *V. fischeri* and carbapenem synthesis in *E. carotovora* (3, 7). Here we show that a variety of analogs can inhibit autoinducer binding to *E. coli* containing LuxR, presumably by binding to the autoinducer-binding site. Some of the analogs such as 3-oxooctanoyl HSL (compound D) appeared to have a binding affinity approaching that of the authentic autoinducer, 3-oxohexanoyl HSL (Fig. 2A). Other analogs were much less effective as

TABLE 1. Activation of the *V. fischeri* luminescence system in *E. coli* by autoinducer analogs

Analog <sup>a</sup>	Relative luminescence at indicated analog concn <sup>b</sup> (nM)			
	0	20	100	200
3-Oxopentanoyl HSL (B)	1	350	5,000	9,000
3-Oxohexanoyl HSL (C, <i>V. fischeri</i> inducer)	1	3,000	15,000	20,000
3-Oxooctanoyl HSL (D)	2	35	450	350
3-Oxododecanoyl HSL (E)	1	1.5	1.5	1.5
3-Oxotetradecanoyl HSL (F)	1	1.5	1.5	1.5
Pentanoyl HSL (H)	1	2	3	4
Hexanoyl HSL (I)	1	10	50	100
Heptanoyl HSL (J)	1	4	10	15
Octanoyl HSL (K)	2	2.5	5	10
Nonanoyl HSL (L)	1	4.5	5	15
Decanoyl HSL (M)	1	1.5	1.5	1.5
5-Oxohexanoyl HSL (N)	1	200	1,000	2,000
2-Hexynoyl HSL (R)	1	1	1	1
3-Oxo-4-hexenoyl HSL (P)	1	2	10	10
2-Hexenoyl HSL (Q)	1.5	1.5	2.5	3.5
4-Phenylbutanoyl HSL (O)	1	1.5	1.5	1.5
3-Oxohexanoyl homocysteine thiolactone (U)	1	10	100	150
3-Oxododecanoyl homocysteine thiolactone (Y)	1	1.5	1.5	1.5

<sup>a</sup> The structure of each analog is shown in Fig. 1, and the letters in parentheses correspond to the designations in the figure.

<sup>b</sup> Experiments were performed under the standard bioassay conditions (see Materials and Methods). One relative unit of luminescence is  $2 \times 10^4$  quanta/s/ml.

inhibitors of 3-oxohexanoyl HSL binding. Most of those analogs that showed relatively strong binding to *E. coli* containing *luxR* could activate the luminescence genes. Although none of the analogs activated luminescence as well as the authentic autoinducer, several showed considerable activity (Table 1). Some showed little or no ability to activate the luminescence genes (Table 1). Among this group, five compounds showed considerable activity as inhibitors of 3-oxohexanoyl HSL activity (Fig. 3). These data suggest that it might be possible to identify compounds that effectively inhibit the production of autoinducible virulence factors in pathogens such as *P. aeruginosa*, but an effective inhibitor must be designed such that it binds to LuxR irreversibly.

Finally, the evidence indicating that a wide variety of autoinducer analogs can bind to LuxR and either activate the luminescence genes or inhibit the activity of the natural autoinducer focuses attention on the possibility of interspecies communication. This idea was introduced 15 years ago when

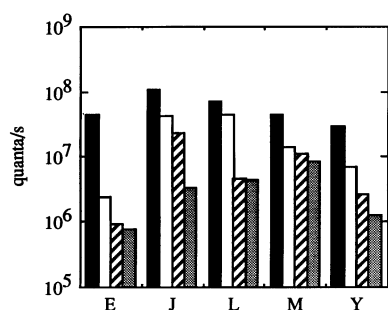


FIG. 3. Inhibition of autoinducer activity by compounds E, J, L, M, and Y. Each compound was tested against 20 nM autoinducer at three concentrations. The ratio of analog to autoinducer was no analog (solid bars), 1:1 (open bars), 5:1 (hatched bars), and 10:1 (stippled bars). The background luminescence in the absence of added autoinducer or analog was  $2 \times 10^4$  quanta/s/ml. The values are the averages of two or three independent experiments. Ranges were within  $\pm 10\%$  of the mean.

the term alloinduction (12) was used to describe the induction of the *Vibrio harveyi* luminescence genes by extracellular signals (alloinducers) from other marine bacteria that coexist with this species. That under some conditions the *P. aeruginosa* autoinducer is a strong inhibitor of the *V. fischeri* luminescence gene autoinduction suggests that we must consider the idea of alloinhibition: that a given species at a high cell density can produce an extracellular signal that interferes with the ability of other species to successfully colonize a particular habitat.

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#### ADDENDUM

A recent report showed that, in *E. coli* or *V. fischeri*, 3-oxooctanoyl HSL (compound D) can inhibit the activity of 3-oxohexanoyl HSL (compound C) and display a modest autoinducer activity of its own (16a). This is consistent with our autoinducer binding experiment (Fig. 2), our induction studies (Table 1), and a previous study of *V. fischeri* (7).

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