JOURNAL OF BACTERIOLOGY, Feb. 1995, p. 1053–1058 Vol. 177, No. 4
0021-9193/95/$04.00+0

Detection and Quantification of *Vibrio fischeri* Autoinducer from Symbiotic Squid Light Organs

KATHERINE J. BOETTCHER† AND EDWARD G. RUBY*

Department of Biological Sciences, University of Southern California, Los Angeles, California 90089-0371

Received 6 October 1994/Accepted 12 December 1994

Vibrio fischeri **is the specific light organ symbiont of the sepiolid squid species *Euprymna scolopes* and *Euprymna morsei*. Both species of squid are luminescent by virtue of their bacterial symbionts, but the natural symbionts of *E. scolopes* do not produce visible luminescence in laboratory culture. The primary cause of this depressed luminescence by *E. scolopes* symbionts in culture was found to be the production of relatively low levels of *V. fischeri* autoinducer, a positive transcriptional coregulator of the *lux* regulon, identified as *N*-(3-oxohexanoyl) homoserine lactone. Concentrations of autoinducer activity produced by these symbionts in culture were quantified and found to be at least 10-fold lower than those produced by *E. morsei* isolates (which are visibly luminous outside the association) and perhaps 10,000-fold lower than those of the brightest *V. fischeri* strains. Despite the differences in their symbiont strains, the intact light organs of the two species of squid contained comparable amounts of extractable autoinducer activity (between 100 and 200 pg per adult animal). The chromatographic behavior of this autoinducer activity on reverse-phase high-performance liquid chromatography was consistent with its presumptive identification as *V. fischeri* autoinducer. Within the 5-μl volume of the epithelial core of the light organ in which the symbiotic *V. fischeri* strains are housed, these amounts would result in an effective autoinducer concentration of at least 100 nM. Because these levels are over 40-fold higher than the concentration needed for the induction of luminescence of bacteria in culture, we conclude that the inherent degree of autoinducer production by strains of *V. fischeri* may not influence their effectiveness as light organ symbionts. Furthermore, this study provides the first direct evidence that the phenomenon of cell density-dependent autoinduction, discovered and described first for laboratory cultures of *V. fischeri* but believed to be a general phenomenon in many species of host-associated symbionts and pathogens, is in fact a consequence of bacterial colonizations of host tissue.**

Isolates of the bioluminescent bacterium *Vibrio fischeri* are easily obtained both from seawater, where they exist at a concentration of only a few cells per milliliter, and from the light-emitting organs of certain marine animals, in which they are found in epithelium-lined crypts at densities of over 10^{10} cells per ml (26, 35, 39). Studies of *V. fischeri* in laboratory culture have revealed a complex system of physiological and genetic regulatory mechanisms that control the expression of the genes of the *lux* regulon, which is responsible for the synthesis of bacterial luciferase and the substrates of the luminescence reaction (28). These mechanisms allow *V. fischeri* to sense environmental cues and thereby modulate the amount of light produced per cell by a factor of over 10,000 (8, 35).

In laboratory culture, the principal level of control responds to cell density, which is signaled by the ambient concentration of a small molecule, *N*-(3-oxohexanoyl) homoserine lactone (13, 23), which is normally produced by *V. fischeri* cells at a low constitutive level (31). This molecule, termed *V. fischeri* autoinducer (VAI), freely diffuses across the cellular membrane (23) and thus will accumulate in the cytoplasm only when the cells are contained within an enclosed space and/or are at a high population density ($>10^7$/ml [38]). In conjunction with LuxR, a DNA-binding regulatory protein, the accumulation of cytoplasmic VAI induces transcription of the *lux* regulon. This regulon contains the putative gene for VAI synthase (*luxI* [14, 15, 27]), and when a critical external concentration of VAI is reached, the specific activity of bacterial light production increases exponentially (2, 31).

In recent years, other genera, including nonluminous, host-associated gram-negative bacteria, have been shown to exhibit a cell density-dependent regulatory mechanism homologous to that of *V. fischeri* (17) but for which the autoinducer molecule is usually a structural analog of VAI. It has been proposed that regulation of luciferase synthesis by such "quorum sensing" has evolved in luminous bacteria because light emission is an energetically expensive process (21) and that luminescence provides a selective advantage to the bacteria (by enhancing survival and propagation) only under conditions where high densities can be achieved (i.e., in a host light organ [31] or as a colony growing on a piece of marine detritus [30]). By analogy, it has been proposed that other organisms use their quorum-sensing gene regulation to assay their cell density in the host, allowing the expression of particular phenotypes that are more, or perhaps exclusively, beneficial within the confined environments of their hosts' tissue (17). Although this model for the function of autoinduction has been frequently cited over the past 20 years as an explanation for symbiotic light emission, it has never been tested; that is, the accumulation of autoinducer in any light organ or other colonized tissue has never been demonstrated (20).

The discovery that symbiotic *V. fischeri* strains isolated from the light organ of the sepiolid squid *Euprymna scolopes* are not visibly luminous when grown in culture refocused an interest on the regulation of luminescence in such symbioses (2). Subsequently, it was found that these symbionts produce relatively low levels of VAI, although they do possess a completely functional *luxI* gene (18, 19) and respond normally to addition of exogenous VAI (2). It was concluded that some condition(s) or

* Corresponding author. Phone: (213) 740-5803. Fax: (213) 740-8123. Electronic mail address: eruby@molbio.usc.edu.

† Present address: Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine, Orono, ME 04469.

compound(s) unique to the light organ environment might be responsible for the enhanced luminescence of these strains in the symbiotic association.

Besides autoinducer, a number of other factors (e.g., iron and oxygen availability and osmolarity, etc.) can significantly influence the induction of light production by *V. fischeri*. In culture, these factors typically play only a secondary role, but it has been proposed that such effectors might be of even greater importance than autoinduction in the regulation of luminescence within light organ symbioses (7, 9, 11, 16, 22, 33, 41). To date, however, no direct evidence exists regarding the biochemistry of the light organ environments of any luminous bacterium-host symbiosis to give credence to any of these hypotheses.

In this study, we demonstrate the presence of inducing levels of VAI in the light organs of two species of sepiolid squid (*E. scolopes* and *Euprymna morsei*) whose natural symbionts differ dramatically in their respective abilities to induce luminescence in culture (2, 39). Furthermore, we investigated a possible mechanism by which VAI may be able to accumulate within the crypts of the symbiotic host's light organ. These data suggest further implications concerning the inherent differences in VAI production among *V. fischeri* symbionts.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study were *V. fischeri* ES114, a light organ isolate from *E. scolopes* (2), and EM1, a light organ isolate from *E. morsei* (39); *Vibrio harveyi* D1 (5) and B392 (37); and *Escherichia coli* VJS533 (43) containing pHV200I$^-$ (36). The *Vibrio* strains were grown at 24°C in a 70% seawater nutrient medium (SWT) containing (per liter): 5 g of tryptone, 3 g of yeast extract, and 3 ml of glycerol. Cultures of *E. coli* were grown at 28°C in Luria-Bertani medium (42) containing ampicillin (100 μg/ml) to ensure plasmid maintenance. Specimens of *E. scolopes* and *E. morsei* were obtained as previously described (2, 3, 39).

Preparation of extracts. In a previous study, we demonstrated that addition of an ethyl acetate extract of a homogenized *E. scolopes* light organ stimulated luminescence of *V. fischeri* ES114 (2). We have subsequently found that autoinducing activity is more consistently extracted directly from intact light organs; thus, this was the method used in the present study. Freshly dissected whole light organs from adult specimens of *E. scolopes* or *E. morsei* were extracted overnight at room temperature with two sequential 500-μl portions of anhydrous ethyl acetate (Aldrich Chemical Co., Milwaukee, Wis.). The combined extracts were evaporated to dryness under N_2 and used immediately either for autoinducer bioassays (31) or for high-performance liquid chromatography (HPLC) analyses. In all instances, the light organ extracts were used within 24 h because longer storage, even at −20°C, resulted in a detectable decrease in activity. In addition, a volume-dependent inhibitory effect was observed when bioassays were performed with extracts produced by evaporation of >1 ml of ethyl acetate. Investigation of the source of this effect revealed that a trace residue from the ethyl acetate solution itself contained the inhibitory activity. Therefore, to serve as internal controls, known amounts of synthetic VAI were added to volumes of ethyl acetate equal to those of the experimental samples before they were evaporated and used in bioassays.

Autoinducer activity was extracted from the spent culture media of both an *E. scolopes* symbiont (*V. fischeri* ES114) and an *E. morsei* symbiont (*V. fischeri* EM1) according to the method of Eberhard et al. (13). Batch cultures that had been grown in SWT broth to maximal luminescence (A_{600} of about 1.5) were harvested at 16,000 × *g* for 10 min, and the resulting supernatant was recentrifuged and passed through a 0.2-μm-pore-size membrane filter (Millipore Inc., Bedford, Mass.). Autoinducer activity from these cell-free supernatants was extracted and partially purified (to the point of silica gel filtration as described in reference 13). The resulting material was solubilized in 200 μl of ethyl acetate and stored at −20°C. Interestingly, unlike extracts prepared from animal tissue, these extracts of culture media did not show a significant decrease in activity when stored this way indefinitely.

Autoinducer bioassays. To assay VAI, cells of either *V. fischeri* ES114 or *E. coli* VJS533(pHV200I$^-$) were grown to an A_{600} of 1.0 (equivalent to about 5×10^8 CFU/ml) and diluted 100-fold in either *V. harveyi*-conditioned medium (31) or *E. coli* bioassay medium (36), respectively. To initiate each assay, 200 μl of indicator cell suspension was added to 4-ml glass vials containing an ethyl acetate extract that had been evaporated to dryness under a stream of N_2. The vials were shaken continuously at between 23 and 25°C, and the development of luminescence was monitored at regular intervals by using a sensitive photometer (2). Vials containing known amounts of synthetic VAI were assayed in parallel to allow the quantification of autoinducer activity in experimental samples.

V. harveyi autoinducer (HAI) was bioassayed by using *V. harveyi* D1 as the indicator strain (5). Cells of this mutant were grown to an A_{600} of 1.0 and diluted 20-fold in medium conditioned by *V. fischeri* ES114 (35). Samples containing HAI in chloroform were evaporated in glass vials before the addition of 200 μl of the indicator cell suspensions. The subsequent development of luminescence was monitored as described above.

HPLC analysis. Known amounts of synthetic autoinducer (either VAI or HAI) were resuspended in 200 μl of 15% methanol (in water). These samples were injected through a 100-μl loop onto a reverse-phase Vydac C_{18} column (The Sep/a/ra/tions Group, Hesparia, Calif.) and run in an isocratic gradient of 15% methanol with a flow rate of 1 ml/min (modified from the procedure given in reference 24). Fractions were collected every minute for 20 min, and 500 μl of each fraction was placed in a microcentrifuge tube and evaporated to dryness in vacuo. For detection of VAI activity, 50 μl of ethyl acetate was added to each microcentrifuge tube and the resulting solutions were transferred to glass vials. After the ethyl acetate was evaporated, the VAI bioassay was performed as described above. For detection of HAI, 200 μl of indicator cell suspension was added to each of the dried HPLC fractions in their microcentrifuge tubes and the mixtures were immediately transferred to glass vials to initiate the bioassay.

Light organs dissected from nine adult *E. scolopes* specimens were extracted with ethyl acetate as described above. The combined extracts were then completely evaporated in a 4-ml glass vial before the addition of 200 μl of 15% methanol. The resulting suspension was centrifuged twice at 14,000 × *g* for 5 min to yield a clarified supernatant. Fifty microliters of this supernatant was put aside for subsequent autoinducer bioassay, while another 100 μl was applied to the Vydac C_{18} HPLC column in 15% methanol. Fractions were collected every minute for 20 min, after which the entire 1 ml of each of the fractions was evaporated to dryness in vacuo. Fifty microliters of ethyl acetate was subsequently added to each of the fractions, which were then transferred to glass vials and assayed for autoinducer activity.

Diffusion experiments. The ability of externally added VAI to diffuse into either bacterial cells or animal host tissue was determined by comparing the partitioning of radioactive tracers across cell compartments. Overnight cultures of *V. fischeri* ES114 were harvested by centrifugation to yield pellets containing about 5×10^{10} cells. Each pellet (approximately 150 mg [wet weight]) was resuspended in 150 μl of 1.25× artificial seawater (32) containing 10 mM sucrose (ASW-S) to which was added 0.15 μCi of one of the following isotopes: [^{3}H]H_2O, ^{3}H-VAI, and [^{14}C]sucrose. The cell suspensions were mixed well and incubated for 10 min, after which the cells were pelleted by centrifugation at 12,000 × *g* for 10 min. The resulting cell-free supernatants were removed and transferred to vials containing 20 ml of Cytoscint ES scintillation cocktail (ICN Pharmaceuticals, Inc.). The cell pellets were then resuspended in 75 μl of ASW-S and solubilized in 20 ml of scintillation cocktail. Control vials containing 150-μl aliquots of the initial ASW-S label solutions were similarly prepared, and the radioactivity in all the samples was determined by standard liquid scintillation counting procedures.

The bacterium-containing epithelial core tissues from the light organs of six adult *E. scolopes* specimens were removed, individually weighed, and placed in microcentrifuge tubes. Each organ was immersed in 10 μl of ASW-S and allowed to equilibrate for 10 min, after which the surrounding fluid was removed. This procedure was repeated once, and an equal amount (volume per quantity of organ [wet weight]) of ASW-S (between 4 and 7 μl), containing one of each of the isotopes described above, was added to the tube. After a 10-min incubation, the tubes containing the light organ cores were centrifuged at 3,000 × *g*. The resulting supernatants and core tissues were each separately transferred to 5 ml of scintillation cocktail and allowed to solubilize overnight, after which their radioactivity was counted by liquid scintillation.

RESULTS

Autoinducer activity in light organ extracts. In our initial studies, cells of *V. fischeri* ES114 were used to assay VAI activity because of their relatively high sensitivity to low concentrations of this molecule (2). However, enhancement of this strain's growth by compounds present in animal extracts occasionally indirectly resulted in premature induction and thus an inaccurate estimation of the added autoinducer activity. Therefore, *E. coli* VJS533(pHV200I$^-$) was used for the majority of assays. This strain carries a plasmid containing a modified *V. fischeri* ES114 *lux* regulon that lacks a functional gene for autoinducer synthase (*luxI*). As a result, even at high cell densities this strain will not induce luminescence in the absence of added VAI. This *E. coli* strain was as sensitive to synthetic VAI as was strain ES114, producing detectable luminescence at VAI concentrations at or above 0.5 ng/ml. Similarly, comparable amounts of autoinducing activity were detected in bio-

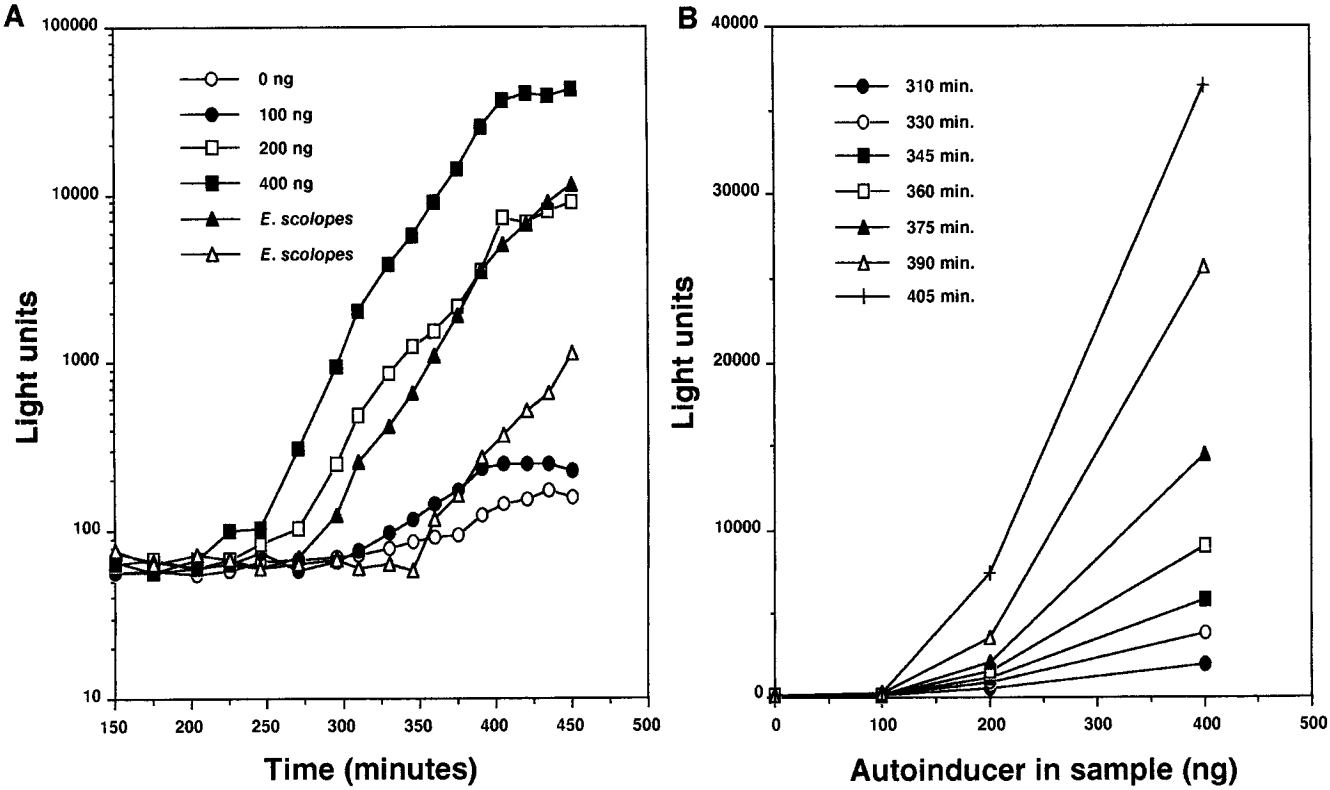

FIG. 1. Bioassay of *E. scolopes* light organ extracts. (A) Ethyl acetate extracts of two light organs were added to separate glass vials and evaporated to dryness. The bioassay was initiated by the addition to the vials of 5×10^6 cells of *E. coli* VJS533(pHV200I$^-$) in conditioned medium. Bioluminescence was monitored at regular intervals as previously described (2). Vials containing known amounts of synthetic autoinducer were assayed simultaneously as controls. (B) Data from selected time points shown in panel A were replotted as the luminescence elicited by known amounts of autoinducer. This method has been used to graphically estimate autoinducer activity in experimental samples (31).

logical samples regardless of the indicator strain used in the bioassay.

Autoinducing activity extracted from the light organs of both *E. scolopes* (Fig. 1A) and *E. morsei* (1, 3) squids was quantitatively estimated by comparison with synthetic VAI standards that were simultaneously assayed with the biological samples. Both the pattern of onset of induction and the extent of light production that resulted from exposure to these organ extracts were identical to those elicited by either natural (1, 3) or synthetic VAI. The linearity of the response is evident when the data are plotted as the amount of luminescence produced at different times during the assay as a result of the addition of known amounts of synthetic VAI (Fig. 1B). A total of 20 specimens of adult squid were extracted and assayed by using this procedure, each specimen yielding between 100 and 200 pg of autoinducer activity per light organ. This translated into an average estimated concentration of at least 100 nM in the epithelial core tissue of the light organs of both *E. scolopes* and *E. morsei* squids (Table 1).

HPLC analysis of light organ extracts. To date, VAI is the only known compound that induces a logarithmic increase in *V. fischeri* light production. However, it was possible that some substance and/or factor in light organ extracts other than VAI was responsible for the induction illustrated in Fig. 1A. Therefore, the various components present in light organ extracts were subjected to reverse-phase HPLC separation to compare the relative retention (i.e., rate of migration) of extract autoinducer activity with that of synthetic VAI. Synthetic VAI eluted primarily in HPLC fraction 8, with a small but detectable amount present in fraction 9 (Fig. 2). When the combined material from nine adult specimens of *E. scolopes* was subjected to HPLC separation, the autoinducer activity exhibited an identical elution pattern (Fig. 2), consistent with the hypothesis that the activity present in *E. scolopes* light organ extracts is, in fact, VAI. Synthetic HAI, which is structurally identical to VAI except that its fatty acid moiety chain length is shorter by two carbon atoms and contains a β-hydroxyl group instead of a β-keto group, migrated in a fashion distinct from that of VAI. When fractionated under the same conditions, the majority of HAI activity eluted in HPLC fraction 5 (Fig. 2), confirming that the column was able to efficiently separate structurally closely related compounds.

TABLE 1. Autoinducer concentrations produced by symbiotic *V. fischeri*

Environment	Concn (nM) produced by symbiont of:		
	E. scolopes	*E. morsei*	*Monocentris japonicus*
Light organ[a]	118 ± 31	140 ± 42	ND[b]
Laboratory culture[c]	0.4	5.0	4,700[d]

[a] The mean values and standard errors are shown for *E. scolopes* ($n = 6$; one value is derived from the pooled light organs of nine adult specimens) and *E. morsei* ($n = 6$).
[b] ND, not determined.
[c] All strains induce at 2.3 nM.
[d] Data are from reference 13.

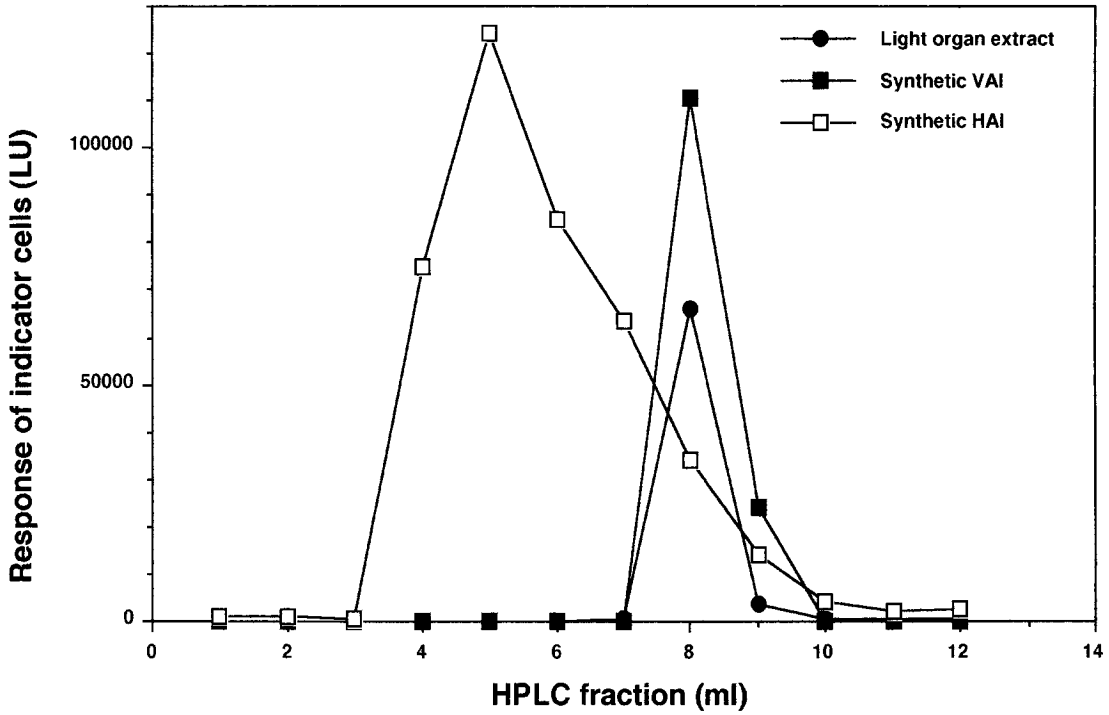

FIG. 2. Relative chromatographic mobilities of HAI [*N*-(3-hydroxybutanoyl) homoserine lactone], VAI [*N*-(3-oxohexanoyl) homoserine lactone], and autoinducing activity from *E. scolopes* light organ extracts were compared by reverse-phase HPLC. Samples were evaporated to dryness, resuspended in 15% methanol, and injected onto a Vydac C_{18} column. Elution was performed with 15% methanol at a rate of 1 ml/min, and fractions were collected every minute. The fractions were evaporated to dryness before bioassay for autoinducer activity (see Materials and Methods). The maximum light emission elicited in the bioassay of each fraction is presented. LU, light units.

As mentioned in Materials and Methods, we found that the residue produced from the evaporation of >1 ml of ethyl acetate in the bioassay vials produced a delayed response in the induction of luminescence by indicator cells. The identity of the responsible inhibitory compound(s) is unknown, and it is not clear whether it is related to the inhibitor of bioluminescence known to be present in complex nutrient media (12). However, we found that the inhibitor(s) present in the ethyl acetate extracts was apparently separated from VAI during reverse-phase HPLC, although the point of elution of this inhibitor was not determined (1). In a related study, Cao and Meighen (4) discovered an inhibitor present in chloroform extracts of HAI and demonstrated that it eluted on reverse-phase HPLC immediately prior to HAI.

Bioassays of culture extracts. Unlike *V. fischeri* strains isolated from the light organs of *E. scolopes*, which are not visibly luminous in culture without the addition of exogenous VAI, the natural symbionts of *E. morsei* typically produce visibly luminous colonies on agar medium (39). Because the actual amount of autoinducer produced by *E. scolopes* symbionts in culture has not been quantified, and to confirm that the 1,000-fold difference in light production of *E. scolopes* and *E. morsei* symbionts in culture is a direct result of their relative ability to produce VAI, ethyl acetate extracts were prepared from the spent media of a strain isolated from each of these two squid host species. Using the VAI bioassay, we found that the concentrations of autoinducer activity produced by the *E. scolopes* symbiont in culture were at least 10-fold lower than those produced by the *E. morsei* symbiont (Table 1). Strain MJ1, one of the brightest *V. fischeri* isolates identified, produces levels of autoinducer activity almost 1,000-fold higher than those produced by even the *E. morsei* isolate (Table 1). Thus, as previously demonstrated (31), luminescence of *V. fischeri* strains in laboratory culture is a direct function of their relative ability to produce VAI.

Diffusion experiments with light organ core epithelium. The results presented in Table 1 indicated that the concentrations of VAI within the light organs of *Euprymna* spp. are much higher than those that can be achieved in laboratory cultures of their symbionts. One explanation for this would be that the epithelium lining the light organ crypts creates an impermeable barrier to autoinducer diffusion, thereby allowing its accumulation in the space occupied by the symbiotic bacteria. When isolated light organ cores from adult *E. scolopes* were incubated with $[^3H]H_2O$, it equilibrated across the core epithelial cell membranes, although apparently a portion of the dissected light organ core (perhaps the acellular portion of the reflector tissue [26]) remained impermeable to water. In contrast, [^{14}C]sucrose did not equilibrate with the majority of the light organ core volume, consistent with the exclusion of this molecule from the interior of the core epithelial cells (Table 2). Labelled VAI fractionated between the core tissue and the supernatant in a manner similar to that of water, suggesting that it too is freely diffusible across the squid core epithelium cell membranes.

Diffusion experiments with bacterial cells. VAI diffuses easily across the cytoplasmic membranes of both *V. fischeri* MJ1 and *E. coli* grown in culture (24). However, because of possible strain differences between MJ1 and *V. fischeri* ES114, we determined whether there was a difference in the cytoplasmic membrane's permeability to VAI between these two bacterial strains. The cytoplasmic membrane of strain ES114 was freely permeable to both water and VAI, while only 40% of the sucrose was retained in the cell pellet after centrifugation (Ta-

TABLE 2. Diffusion of labeled compounds[a] into squid light organ epithelium and *V. fischeri* cells

Compound	Amt (%) present in:			
	E. scolopes light organ[b]		*V. fischeri* cells[c]	
	Supernatant	Tissue	Supernatant	Pellet
[^{3}H]H_2O	58.1	41.9	50.8	49.2
^{3}H-VAI	52.6	47.4	48.7	51.3
[^{14}C]sucrose	78.6	21.4	61.3	38.7

[a] A total of 0.15 μCi was used for each treatment. Total recovery was between 85 and 99% (>250,000 dpm).

[b] One dissected light organ was used per treatment. Numbers represent averages for two treatments per isotope. Small, variable amounts of insoluble material in the dissected light organ cores may have reduced the equilibration of water and VAI to a value of less than 50%.

[c] Two pellets of 5×10^{10} cells were used per treatment. Numbers represent averages for two treatments per isotope.

ble 2). These numbers are consistent with the prediction that sucrose would diffuse only into the periplasm of the cells (roughly 30% of the cell volume [44]) and suggest that there are no significant differences in membrane permeability between strains ES114 and MJ1.

DISCUSSION

Previously, it had not been demonstrated that VAI is actually present at inducing levels in the light organ of any host involved in symbiosis with *V. fischeri*. Therefore, the influence of VAI, relative to that of any physiological factor, on the luminescence of light organ symbionts has been in question. Similarly, in spite of the recent discovery of additional cell density-responsive induction systems (17), there have been no reports that any autoinducer homolog accumulates in its natural target tissue environment. Our interest in determining the relevance of such autoinduction systems in a natural symbiotic environment, as well as the increasing recognition of the importance of biochemical communication between animal or plant hosts and their bacterial symbionts (6, 10, 40), provided the impetus for this study.

Ethyl acetate extracts of the light organs from two species of *Euprymna* contained extractable autoinducer activity that corresponded to between 100 and 200 pg of VAI per animal (Table 1). Although these quantities were too minute to be purified and analyzed by either nuclear magnetic resonance spectrometry or mass spectroscopy, the inducing compound is believed to be bona fide VAI on the basis of the following evidence: (i) luminescence is induced in both *V. fischeri* ES114 (a strain that underproduces autoinducer) and *E. coli* VJS533 (a strain that contains the *V. fischeri lux* genes but is completely deficient in autoinducer production), (ii) the pattern of induction and extent of light production in these strains are indistinguishable from those produced by both natural and synthetic autoinducers, and (iii) the chromatographic behavior of this inducing activity on reverse-phase HPLC is identical to that of synthetic VAI and distinct from that of the structurally related HAI.

Approximately 10^8 bacteria are present within the core tissue of an adult squid light organ (39) and are localized within epithelium-lined crypts that have an estimated volume of about 15 nl (29). If all of the VAI produced by the bacteria was contained within the crypts, its effective concentration would be at least 30 μM (a level over 1,000 times that needed for induction of *V. fischeri* luminescence in culture). However, we have shown that autoinducer appears to freely diffuse across the cell membranes of the symbionts (like *V. fischeri* MJ1 [24]) and the epithelium of the animal cells that line the bacterium-containing crypts. Therefore, VAI may be spread more or less uniformly throughout the entire 5 μl of central core tissue (i.e., including the epithelial cell volume). If this assumption is correct, the ambient concentration present in the crypts (approximately 100 nM) would still result in a fully induced symbiont population (24). It is not clear how these levels are reached and maintained; however, it is possible that the production rate of VAI exceeds the rate at which it exits the light organ, thereby allowing a rapid accumulation of autoinducer around the tightly packed symbionts (39).

Bacteriological (2) and electron microscopic (26) analyses suggest that >95% of the bacterial symbionts are healthy, growing cells; thus, it is not unreasonable to assume that they are all producing VAI. To date, the maintenance of metabolically active *V. fischeri* cells at a concentration of $>5 \times 10^9$/ml has not been achieved in laboratory culture (2, 38), although advances in immobilized cell matrices (45) may allow the creation, in vitro, of an artificial light organ containing concentrations of healthy cells approaching the $>10^{10}$/ml found in tissue of the natural symbiotic host.

The results reported here can be explained partially by a hypothesis put forward by Gray and Greenberg (18, 19) that the luminescence induction system in *V. fischeri* evolved to allow the bacteria to eliminate light production at cell densities lower than those characteristic of light organ populations. The absence of an important role for luminescence at cell densities less than those achieved in the light organ might explain the apparent selection for diminished autoinducer synthesis characteristic of the *E. scolopes* symbionts.

Because *E. scolopes* squids are colonized by bacteria from the surrounding seawater (46) and periodically release a significant portion of their symbiont population into the environment (1, 25, 39), symbiotic *V. fischeri* strains continually alternate between the free-living lifestyle and their associations with the host. The fact that *E. morsei* symbionts produce sufficient VAI activity to induce light production at cell densities less than those achieved in the light organ of their host while the symbionts of *E. scolopes* do not suggests that *E. morsei* symbionts may have an additional important natural niche in which they exist at intermediate densities (e.g., 10^8 to 10^9 cells per ml) and where luminescence also exerts a positive selection pressure (30). This hypothesis is supported both by the observation that *V. fischeri* strains are an important component of animal enteric tracts in temperate coastal waters such as those in which *E. morsei* occurs, but not in the tropical environment inhabited by *E. scolopes* (34), and by evidence that the *E. scolopes* symbiosis may be the only major factor responsible for the abundance and distribution of *V. fischeri* in the habitats of these squids (25). The fact that variations in luminescence of *V. fischeri* are common and usually directly attributable to relative levels of autoinducer production (31) suggests that not all strains are experiencing the same temporal and spatial environments or the same selective pressures.

This study provides the first direct evidence that VAI accumulates in the host light organs colonized by *V. fischeri* and that the relative degrees of autoinducer production by symbiotic strains do not appear to be a significant factor in their bioluminescence effectiveness with sepiolid squid species. The contributions of other physiological effectors of luminescence (i.e., iron and oxygen availability) in this and other light organ symbioses remain to be addressed and may well be important to our understanding of bacterium-host associations in general. Furthermore, it is likely that the accumulation of related autoinducers by other gram-negative bacteria in their respective

host tissues will soon be demonstrated. It has already been suggested that such autoinducers may be regarded as virulence determinants in some of these species (17). However, in light of the results presented here, we suggest that differences among these other host-associated bacteria in their respective abilities to produce their specific autoinducers should be interpreted with caution when considering the expression of high-density-related phenotypes.

ACKNOWLEDGMENTS

We thank M. McFall-Ngai for valuable discussions and technical assistance with the HPLC analysis. Synthetic VAI was a gift from E. P. Greenberg, *E. coli* VJS533(pHV200I$^-$) was provided to us by Kendall Gray, and E. A. Meighen generously supplied *V. harveyi* D1 and synthetic HAI.

This work was supported by grants to E.G.R. from the Office of Naval Research (N0014-93-1-0846), to M. McFall-Ngai and E.G.R. from the National Science Foundation (IBN 9220482), and to K.J.B. from the Los Angeles chapter of the Achievement Rewards for College Scientists (ARCS) foundation.

REFERENCES

1. **Boettcher, K. J.** 1994. Unpublished data.
2. **Boettcher, K. J., and E. G. Ruby.** 1990. Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes*. J. Bacteriol. **172:** 3701–3706.
3. **Boettcher, K. J., and E. G. Ruby.** 1992. Detection of *Vibrio fischeri* autoinducer in symbiotic squid light organs, abstr. I-74, p. 249. *In* Abstracts of the 92nd General Meeting of the American Society for Microbiology. American Society for Microbiology, Washington, D.C.
4. **Cao, J.-G., 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.
5. **Cao, J.-G., and E. A. Meighen.** 1993. Biosynthesis and stereochemistry of the autoinducer controlling luminescence in *Vibrio harveyi*. J. Bacteriol. **175:** 3856–3862.
6. **Clarke, H. R. G., J. A. Leigh, and C. J. Douglas.** 1992. Molecular signals in the interactions between plants and microbes. Cell **71:**191–199.
7. **Dolan, K. M., and E. P. Greenberg.** 1992. Evidence that GroEL, not σ^{32}, is involved in transcriptional regulation of the *Vibrio fischeri* luminescence genes in *Escherichia coli*. J. Bacteriol. **174:**5132–5135.
8. **Dunlap, P. V.** 1991. Organization and regulation of bacterial luminescence genes. Photochem. Photobiol. **54:**1157–1170.
9. **Dunlap, P. V., and E. P. Greenberg.** 1985. Control of *Vibrio fischeri* luminescence gene expression in *Escherichia coli* by cyclic AMP and cyclic AMP receptor protein. J. Bacteriol. **164:**45–50.
10. **Dunlap, P. V., and E. P. Greenberg.** 1991. Role of intercellular chemical communication in the *Vibrio fischeri*-monocentrid fish symbiosis, p. 219–253. *In* M. Dworkin (ed.), Microbial cell-cell interactions. American Society for Microbiology, Washington, D.C.
11. **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.
12. **Eberhard, A.** 1972. Inhibition and activation of bacterial luciferase synthesis. J. Bacteriol. **109:**1101–1105.
13. **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.
14. **Engebrecht, J., K. H. Nealson, and M. Silverman.** 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. Cell **32:**773–781.
15. **Engebrecht, J., and M. Silverman.** 1984. Identification of genes and gene products necessary for bacterial bioluminescence. Proc. Natl. Acad. Sci. USA **81:**4154–4158.
16. **Friedrich, W. F., and E. P. Greenberg.** 1985. Glucose repression of luminescence and luciferase in *Vibrio fischeri*. Arch. Microbiol. **134:**87–91.
17. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. **176:**269–275.
18. **Gray, K. M., and E. P. Greenberg.** 1992. Sequencing and analysis of *luxR* and *luxI*, the luminescence regulatory genes from the squid light organ symbiont *Vibrio fischeri* ES114. Mol. Mar. Biol. Biotechnol. **1:**414–419.
19. **Gray, K. M., and E. P. Greenberg.** 1992. Physical and functional maps of the luminescence gene cluster in an autoinducer-deficient *Vibrio fischeri* strain isolated from a squid light organ. J. Bacteriol. **174:**4384–4390.
20. **Hastings, J. W., J. Makemson, and P. V. Dunlap.** 1987. How are growth and luminescence regulated independently in light organ symbionts? Symbiosis **4:**3–24.
21. **Hastings, J. W., W. H. Riley, and J. Massa.** 1965. The purification properties and chemiluminescent quantum yield of bacterial luciferase. J. Biol. Chem. **240:**1473–1481.
22. **Haygood, M. G., and K. H. Nealson.** 1985. Mechanisms of iron regulation of luminescence in *Vibrio fischeri*. J. Bacteriol. **162:**209–216.
23. **Kaplan, H. B., A. Eberhard, C. Widrig, and E. P. Greenberg.** 1985. Synthesis of N-[3-oxo-(4,5-$^3H_2$)-hexanoyl] homoserine lactone: biologically active tritium-labelled *Vibrio fischeri* autoinducer. J. Labelled Compd. Radiopharm. **22:**387–395.
24. **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.
25. **Lee, K.-H., and E. G. Ruby.** 1994. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl. Environ. Microbiol. **60:**1565–1571.
26. **McFall-Ngai, M., and M. K. Montgomery.** 1990. The anatomy and morphology of the adult bacterial light organ of *Euprymna scolopes* Berry (Cephalopoda:Sepiolidae). Biol. Bull. **179:**332–339.
27. **Meighen, E. A.** 1991. Molecular biology of bacterial bioluminescence. Microbiol. Rev. **55:**123–142.
28. **Meighen, E. A., and P. V. Dunlap.** 1993. Physiological, biochemical and genetic control of bacterial bioluminescence. Adv. Microb. Physiol. **34:**1–67.
29. **Montgomery, M. K.** 1993. Development of the symbiotic light organ of *Euprymna scolopes* (Cephalopoda:Sepiolidae). Ph.D. dissertation. University of Southern California, Los Angeles.
30. **Morin, J. G.** 1983. Coastal bioluminescence: patterns and functions. Bull. Mar. Sci. **33:**787–817.
31. **Nealson, K. H.** 1977. Autoinduction of bacterial luciferase. Occurrence, mechanism and significance. Arch. Microbiol. **112:**73–79.
32. **Nealson, K. H.** 1978. Isolation, identification and manipulation of luminous bacteria. Methods Enzymol. **57:**153–166.
33. **Nealson, K. H., and J. W. Hastings.** 1977. Low oxygen is optimal for luciferase synthesis in some bacteria: ecological implications. Arch. Microbiol. **112:**9–16.
34. **Nealson, K. H., and J. W. Hastings.** 1979. Bacterial bioluminescence: its control and ecological significance. Microbiol. Rev. **43:**469–518.
35. **Nealson, K. H., and J. W. Hastings.** 1991. The luminous bacteria, p. 625–639. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, 2nd ed. Springer-Verlag, New York.
36. **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.
37. **Reichelt, J. L., and P. Baumann.** 1973. Taxonomy of the marine, luminous bacteria. Arch. Mikrobiol. **94:**283–330.
38. **Rosson, R., and K. H. Nealson.** 1981. Autoinduction of bacterial bioluminescence in a carbon limited chemostat. Arch. Microbiol. **129:**299–304.
39. **Ruby, E. G., and L. M. Asato.** 1993. Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. Arch. Microbiol. **159:**160–167.
40. **Ruby, E. G., and M. J. McFall-Ngai.** 1992. A squid that glows in the night: development of an animal-bacterial mutualism. J. Bacteriol. **174:**4865–4870.
41. **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. **151:**574–586.
42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **Stewart, V. J., and J. V. Parles.** 1988. Identification and expression of genes *narL* and *narX* of the *nar* (nitrate reductase) locus in *Escherichia coli* K-12. J. Bacteriol. **170:**1589–1597.
44. **Stock, J. B., B. Rauch, and S. Roseman.** 1977. Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. J. Biol. Chem. **252:**7850–7861.
45. **Thiagarajan, V. S., K. L. Swope, and M. C. Flickinger.** 1994. A novel plug reactor for the study of bacteria immobilized in thin films, abstr. O-18, p. 358. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology. American Society for Microbiology, Washington, D.C.
46. **Wei, S. L., and R. E. Young.** 1989. Development of symbiotic bacterial bioluminescence in a nearshore cephalopod, *Euprymna scolopes*. Mar. Biol. **103:**541–546.