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Cross-Species Induction of Luminescence in the Quorum-Sensing Bacterium *Vibrio harveyi*

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Different species of bacteria were tested for production of extracellular autoinducer-like activities that could stimulate the expression of the luminescence genes in *Vibrio harveyi*. Several species of bacteria, including the pathogens *Vibrio cholerae* and *Vibrio parahaemolyticus*, were found to produce such activities. Possible physiological roles for the two *V. harveyi* detection-response systems and their joint regulation are discussed.

At least two species of marine bacteria, *Vibrio fischeri* and *Vibrio harveyi*, express bioluminescence in response to cell density. These two vibrios are found in different environments in the ocean. *V. harveyi* is found free-living in the sea as well as in the gut tracts of marine animals, where it exists at high population densities in association with other species of bacteria. *V. fischeri* is found in these habitats and also lives in pure culture as a light organ symbiont of various fish and squid (8). *V. fischeri* and *V. harveyi* accomplish density-dependent *lux* regulation by the synthesis, excretion, and detection of small signal molecules called autoinducers, which accumulate in the environment (9). The autoinducer that controls light production in *V. fischeri* is *N*-(3-oxohexanoyl)-L-homoserine lactone. Two autoinducers control density-dependent *lux* expression in *V. harveyi*. One of the *V. harveyi* autoinducers is *N*-(3-hydroxybutanoyl)-L-homoserine lactone (AI-1), and the second autoinducer (AI-2) remains to be identified. Recently, a number of other bacteria have been shown to control cell density-dependent functions through the excretion of and response to acyl-homoserine lactone autoinducers. Cell density-dependent regulation of *lux* expression is an example of a phenomenon called quorum sensing (5).

Genetic analysis of the density-sensing apparatus of *V. harveyi* has shown that two independent density-sensing systems exist, and each is composed of a sensor-autoinducer pair; system 1 is composed of sensor 1 and AI-1, and system 2 is composed of sensor 2 and AI-2 (1, 2). The two density-sensing systems are redundant, because a null mutation in either system alone results in Lux$^+$ strains, whereas null mutations in both systems render the cells dark and incapable of density sensing. In 1979 Greenberg et al. (6) reported that *V. harveyi* was stimulated to produce light following the addition of cell-free culture fluid from several species of nonluminous bacteria. At the time of that study it was not known that two autoinducer-detection systems existed in *V. harveyi*. Because *V. harveyi* mutants capable of responding to only AI-1 or AI-2 now exist, it is possible to determine through which pathway(s) the signals from these other organisms flow. Now that we also appreciate that many bacteria communicate intercellularly and control gene expression through the use of autoinducers, it is of interest to understand how they might accomplish cross-species communication and use it for survival in various niches. In this study we have used *V. harveyi* sensor mutants as reporters for specific autoinducers to begin to determine how cross-species communication might occur in marine systems.

Cell-free culture fluid from a number of bacterial species, of both marine and terrestrial origin, were prepared, and each culture fluid was tested for the ability to stimulate *V. harveyi* to produce light (Table 1). Three reporter strains of *V. harveyi* with different autoinducer response phenotypes were analyzed. Using this combination of *V. harveyi* strains allowed us to determine whether the stimulatory substances synthesized by other bacteria worked through *V. harveyi* signalling system 1, system 2, or both. The *V. harveyi* reporter strain BB120 is a wild-type strain, it is sensor 1$^+$ sensor 2$^+$, and it responds to both the *V. harveyi* autoinducers AI-1 and AI-2. Strain BB120 is the parent of the sensor mutants BB886 and BB170. *V. harveyi* strain BB886 is sensor 1$^+$ sensor 2$^-$ and responds only to AI-1, while strain BB170 is sensor 1$^-$ sensor 2$^+$ and responds only to *V. harveyi* AI-2. The construction and phenotypic analysis of these strains are described elsewhere (1, 2). We repeated the studies of Greenberg et al. using the *V. harveyi* reporter strain B392 and obtained essentially the same results as those reported (data not shown). Some of the strains assayed in 1979 were not available for the present study. We tested additional bacterial species, and in general, these are species that have recently become of interest in quorum-sensing investigations.

The bacterial strains tested for autoinducer production were grown for 16 h (to an approximate optical density at 600 nm of 1.0) at 30°C in autoinducer bioassay (AB) medium (6) unless otherwise noted. Cells were removed from the culture fluid by centrifugation at 5,000 × *g* for 10 min followed by passage of the culture fluids through 0.2-μm-pore-size membrane filters. Stimulation of light production in the *V. harveyi* reporter strains was assayed as reported elsewhere (1, 2). *V. harveyi* reporter strains were grown overnight at 30°C in AB medium. The cultures were diluted 1:5,000 in fresh medium, and cell-free culture fluids from the strains listed in Table 1 were added to a final concentration of 10%. The resulting light production was monitored at 30°C with a scintillation counter in the chemiluminescence mode. Maximal stimulation of light production in the *V. harveyi* reporter strains occurred between 3 and 4 h after dilution and addition of the cell-free culture fluids. All experiments were performed at least three times. The results are reported in Table 1. The stimulation of light production from addition of 1/10 volume *V. harveyi* BB120

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TABLE 1. Induction of luminescence in *V. harveyi* reporter strains by cell-free culture fluids from other bacteria

Species and strain	Induction of luminescence (%)[a] in *V. harveyi* reporter strain		
	BB120 (sensor 1^+ sensor 2^+)	BB886 (sensor 1^+ sensor 2^-)	BB170 (sensor 1^- sensor 2^+)
Vibrio harveyi BB120	**100**	**100**	**100**
V. harveyi BB152	**32**	1.0	**120**
V. harveyi B392	**90**	**100**	**125**
V. harveyi B72	**60**	**72**	**72**
V. harveyi D1	1.0	1.0	0.5
Vibrio fischeri MJ1	1.0	3.0	3.0
V. fischeri B61	0.5	1.0	2.0
V. fischeri ES114	1.0	3.0	0.3
Vibrio cholerae TSM301	0.5	0.3	**121**
V. cholerae C6706	1.5	0.2	**60**
V. cholerae 569B	1.5	10	**81**
Vibrio parahaemolyticus BB22	**69**	**98**	**89**
Vibrio anguillarum 19264[b]	**136**	1.0	**281**
Vibrio alginolyticus 118	**12**	0.5	**58**
Vibrio furnissii 1514	0.5	0.1	4.0
Vibrio proteolyticus 145	0.5	0	0
Vibrio natriegens 77	4.0	0	**24**
Vibrio angustum 70	0.2	10	0
Vibrio nereida B81	2.0	0	1.0
Vibrio logei SR6	0.3	0	10
Photobacterium phosphoreum NZ-11-D	**22**	0	**11**
Photobacterium leiognathi 721	0.3	3.0	0
Pseudomonas aeruginosa PAO1[c]	0.5	0	0.5
Yersinia enterocolitica JB580[d]	2.0	0	**82**
Escherichia coli CC118[c]	0	4.0	4.0
Salmonella typhimurium PS1[c]	10	0	0
Caulobacter crescentus CD15[e]	0.2	0	0
Bacillus subtilis 168[c]	0	0	0.1
Bacillus licheniformis 9445A[c]	0	0	0.5
Xenorhabditis luminescence Hm[c]	0	0	0
Xenorhabditis nematophilis Ang1°[c]	0	0	0.5

[a] The level of *V. harveyi* stimulation was normalized to 100%.

[b] A different strain of this species was used in the Greenberg et al. study.

[c] Grown at 30°C in Luria-Bertani medium (7).

[d] Grown at room temperature in 1% Bacto-tryptone, 0.5% Bacto-yeast extract, and 1.8% glucose.

[e] Grown at 30°C in M2 minimal salts medium containing 0.2% glucose (10).

culture fluid to *V. harveyi* reporter strains BB120, BB886, and BB170 is defined as 100% activity. Results of triplicate experiments from strains that produced an autoinducer-like activity (which we consider to be more than 10% of the *V. harveyi* BB120 stimulation) were within 15% of the average values reported in Table 1. Results of triplicate experiments from strains that showed no significant stimulation of light production in the *V. harveyi* tester strains (which we consider to be less than 10% of the *V. harveyi* BB120 stimulation) were within 50% of the reported average values. The media used for growth of each species were also tested in this assay, and none of them caused an increase in light production over the background in *V. harveyi.*

Our experiments confirm that *V. fischeri* does not produce an autoinducer that has an effect on *lux* expression in *V. harveyi*. Three strains of *V. fischeri*, MJ1, B61, and ES114, were tested for cross-stimulation of *V. harveyi*, and none showed detectable activity. Each of the wild-type *V. harveyi* strains tested (BB120, B392, and B72) apparently makes both AI-1 and AI-2. Culture fluids from these strains stimulated the wild-type BB120 strain (sensor 1^+ sensor 2^+), the sensor 1^+ strain BB886, and the sensor 2^+ strain BB170. *V. harveyi* BB152 is a mutant derived from BB120 that does not produce AI-1 (1). Culture fluid from BB152, as expected, had an effect on BB120 and BB170 but not on BB886. Cao and Meighen (4) isolated the dark mutant strain *V. harveyi* D1 following chemical mutagenesis of *V. harveyi* B392. They demonstrated that this mutant produces light in response to the addition of exogenous AI-1, and they also showed that culture fluids from *V. harveyi* D1 did not stimulate light production in the parent strain. We show that culture fluid from D1 did not induce luminescence in any of our *V. harveyi* reporter strains, BB120, BB886, and BB170. Taken together, the results suggest that the parent strain, *V. harveyi* B392, possesses both autoinducer production-detection systems and that the mutant strain D1 does not produce either AI-1 or AI-2. These findings indicate that strain D1 either contains defects in the genes for synthesis of both autoinducers or contains a defect in a regulatory function responsible for controlling synthesis of both AI-1 and AI-2. The location of the mutation(s) conferring the dark phenotype in *V. harveyi* D1 is unknown.

As described earlier (6), a number of bacterial species make substances that induce light production in *V. harveyi*. Greenberg et al. showed that culture fluid from *Vibrio anguillarum*, *Vibrio alginolyticus*, *Vibrio natriegens*, and *Photobacterium phosphoreum* had activity on *V. harveyi*. We show here that these species make substances that mimic the action of AI-2 but not AI-1. In each case, the sensor 2^+ strain BB170 was stimulated to produce light, whereas the sensor 1^+ strain BB886 was not. Among the other species of bacteria that we tested, we also found that the pathogens *Vibrio cholerae* and *Yersinia enterocolitica* produced an AI-2-like but not an AI-1-like activity.

Surprisingly, fluid from *V. cholerae*, *Y. enterocolitica*, and *V. natriegens* cultures did not stimulate the sensor 1^+ sensor 2^+ strain BB120 to produce light. Apparently the AI-2-like substance(s) made by these strains fails to induce system 2 when system 1 is present. Bassler et al. showed that the two *V. harveyi* sensory pathways are jointly regulated and that the system 1 and system 2 signals are both channeled to a common regulator protein (3). Our results suggest that when system 1 is present, system 2 is less sensitive to induction. Possibly, the AI-2-like activities produced by *V. cholerae*, *Y. enterocolitica*, and *V. natriegens* cannot stimulate the system 2 sensor under these conditions. However, in the absence of system 1 and its regulatory influence on system 2, the system 2 sensor is more inducible by AI-2 and AI-2-like activities. In contrast, culture fluid from the AI-1^- strain *V. harveyi* BB152 and culture fluids from *V. anguillarum*, *V. alginolyticus*, and *P. phosphoreum* stimulated both the sensor 2^+ strain, BB170, and the wild-type sensor 1^+ sensor 2^+ strain BB120. Presumably, the AI-2-like activities produced by these species can overcome the lowered sensitivity of system 2 in BB120.

Greenberg et al. found that fluid from *V. natriegens* stimulated wild-type *V. harveyi*, but we observed that the *V. natriegens* fluids had an effect only on BB170, not on the wild-type strain BB120. In order to understand this discrepancy, we tested *V. natriegens* fluid on *V. harveyi* B392. We also found that B392 was stimulated by *V. natriegens* culture fluids (19%). Apparently, the *V. harveyi* BB120 and *V. harveyi* B392 sensory systems have differing abilities to discern the presence of the *V. natriegens* AI-2-like activity. Our results suggest that *V. harveyi* B392 is more highly sensitive to the AI-2-like activity of *V. natriegens* than is *V. harveyi* BB120. Unfortunately, sensor mutants of *V. harveyi* B392 do not yet exist, so at present we are unable to further test this hypothesis.

Greenberg et al. also reported that *Vibrio parahaemolyticus* culture fluid induced *lux* expression in *V. harveyi*. We found that *V. parahaemolyticus* culture fluids induced all three *V. harveyi* reporter strains, BB120, BB886, and BB170. The level of activity in *V. parahaemolyticus* culture fluid was nearly that observed in *V. harveyi*. Apparently, this species makes distinct substances that can act through *V. harveyi* signalling system 1 and system 2. No other bacterial species that stimulated system 1 in *V. harveyi* was found. Finally, many strains that we tested did not produce any activity capable of inducing light production in *V. harveyi* under these conditions.

Purification of AI-2 from *V. harveyi* BB152 culture fluids is under way; however, standard autoinducer purification techniques which include organic extractions with either ethyl acetate or chloroform have not been successful (3a). Unlike the *V. harveyi* AI-1 [*N*-(3-hydroxybutanoyl)-L-homoserine lactone], the *V. harveyi* AI-2 activity is recovered in the aqueous phase following organic extraction. Similarly, the AI-2 activities produced by the bacteria examined in this study (*V. harveyi* BB120, *V. harveyi* BB152, *V. harveyi* B392, *V. harveyi* B72, *V. cholerae* TSM301, *V. cholerae* C6706, *V. cholerae* 569B, *V. parahaemolyticus* BB22, *V. anguillarum* 19264, *V. alginolyticus* 118, *P. phosphoreum* NZ-11-D, and *Y. enterocolitica* JB580) remained in the aqueous phase following ethyl acetate extraction of cell free culture fluids. In contrast, the AI-1-like activities from *V. harveyi* BB120, *V. harveyi* B392, *V. harveyi* B72, and *V. parahaemolyticus* BB22 fractionated to the organic phase.

Our studies show that *V. harveyi* is capable of responding through both of its independent autoinducer detection systems to substances produced by other species of bacteria. Because *V. harveyi* is found in marine environments inhabited by many other species of bacteria, it is possible that *V. harveyi* monitors its environment for signals produced by other species of bacteria and produces light in response to these interspecies stimuli (6). We found that besides *V. harveyi*, only one species, *V. parahaemolyticus*, produced an AI-1-like activity, indicating that *V. harveyi* system 1 is highly specific. However, several other species of bacteria produced an AI-2-like activity, indicating that *V. harveyi* system 2 is less specific. Additionally, system 2 appears to be less sensitive than system 1. Possibly, the function of the higher-sensitivity, higher-specificity system 1 is to monitor the environment for other *V. harveyi* organisms, while the function of the lower-sensitivity, lower-specificity system 2 is to monitor the environment for other species of bacteria. Coordination of the inputs from both of these detection systems could enable *V. harveyi* to express density-controlled functions, such as bioluminescence, in response to the cumulative, multispecies cell density.

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