

OpaR, a Homolog of *Vibrio harveyi* LuxR, Controls Opacity of *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus is an organism well adapted to communal life on surfaces. When grown on a surface or in a viscous layer, the bacterium induces a large gene system and differentiates to swarmer cells capable of movement over and colonization of surfaces. *V. parahaemolyticus* displays additional phenotypic versatility manifested as variable colony morphology, switching between translucent and opaque colony types. Although not itself luminescent, *V. parahaemolyticus* produces autoinducer molecules capable of inducing luminescence in *Vibrio harveyi*. To examine the role of quorum signaling in the lifestyles of *V. parahaemolyticus*, the functional homolog of the gene encoding the *V. harveyi* autoinducer-controlled transcriptional regulatory protein LuxR was cloned. Sequence analysis of the clone predicted an open reading frame with a deduced product 96% identical to LuxR. Introduction of the clone carrying the luxR-like locus into *V. parahaemolyticus* dramatically affected colony morphology, converting a translucent strain to an opaque one. When the coding sequence for the luxR homolog was placed under the control of the Ptac promoter, conversion to the opaque phenotype became inducible by isopropyl- β -D-thiogalactopyranoside. Allelic disruption of the luxR-like gene on the chromosome of an opaque strain produced a translucent strain proficient in swarming ability. Primer extension mapping demonstrated *opaR* transcription in opaque but not translucent cell types. It is postulated that this gene, which has been named *opaR*, encodes a transcription factor controlling cell type. The underlying genetic basis for opaque-translucent variation may be the consequence of a genomic alteration detected in the *opaR* locus of opaque and translucent strains.

In order to survive in changing environments, bacteria possess enormous adaptive capabilities that allow them to modulate their behavior and reprogram gene expression in response to environmental cues. *Vibrio parahaemolyticus*, a ubiquitous marine bacterium and human pathogen, seems particularly adapted to growth on surfaces or in biofilms. In response to its physical environment, *V. parahaemolyticus* induces the expression of a large gene system that allows differentiation to swarmer cells. This cell type is adapted for movement on surfaces or through highly viscous environments and thus for colonization of surfaces (20).

V. parahaemolyticus displays another adaptive mechanism. The organism switches between a translucent colony type and an opaque colony type. Opaque-translucent variation has been observed in other bacteria. For example, in *Neisseria gonorrhoeae*, phase-variable opacity is associated with the presence of specific outer membrane proteins (35). Furthermore, the expression of opacity proteins is correlated with adherence (17, 18). It has been postulated that cell surface components, e.g., the *N. gonorrhoeae* opacity proteins, lead to autoaggregation and differential cell packing. The organization presumably translates into differences in the transmission of light by the bacterial colony (34). Opacity has also been associated with the production of extracellular polysaccharide. The closely related bacterium *Vibrio vulnificus* undergoes opaque-translucent switching (26). For this bacterium, virulence and opacity have been correlated with the presence of capsular polysaccharide (38).

Density-dependent sensing has been postulated to be an important component of bacterial colonization and growth in

communities (6, 23). Small-molecule signaling and intercellular communication may provide the bacterial cell methods for discrimination between a free-living, low-cell-density state and an attached, high-density environment (13, 36). Intriguingly, *N*-acyl homoserine lactones (AHLs) have been demonstrated to be involved in the swarming behavior of the bacterium *Serratia liquefaciens* (10). This observation, i.e., that there is a cell-density-dependent component of the swarming of *S. liquefaciens*, coupled with the knowledge that *V. parahaemolyticus* produces autoinducer molecules (14), provoked examination of the role of density-dependent signaling in *V. parahaemolyticus*.

Nonluminescent *V. parahaemolyticus* produces autoinducer molecules that stimulate light production of luminous *Vibrio harveyi* (14, 30). The molecular scheme of density-dependent signaling in *V. harveyi* contrasts with the *luxI-luxR* system found in many bacteria, the best example being that of the regulation of luminescence in *Vibrio fischeri*. In *V. fischeri*, *luxI* encodes a protein responsible for the synthesis of an AHL autoinducer, and *luxR* encodes a protein that activates transcription of the luminescence operon in response to the level of autoinducer (13). When the level of autoinducer exceeds a threshold concentration, LuxR binds autoinducer (15) and activates the expression of luminescence genes. A variety of bacteria produce AHL signaling molecules, and a number of homologs of the *V. fischeri luxR* gene product have been demonstrated to be involved in intercellular communication controlling diverse gene systems (13, 36). These proteins contain signature motifs for DNA binding and autoinducer binding.

Luminescence in *V. harveyi* is also controlled in a cell-density-dependent manner; however, the model for quorum sensing in *V. harveyi* is more elaborate than the *luxI-luxR* paradigm of *V. fischeri* in that there are additional layers of gene control. *V. harveyi* LuxR is the transcriptional activator of the luminescence operon (29), although it is not homologous with mem-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
<i>V. parahaemolyticus</i>		
BB22TR	Wild type; translucent variant	R. Belas (7)
BB22OP	Wild type; opaque variant	BB22TR (this work)
LM1017	<i>lfgE313::lux</i> (formerly <i>lafX313::lux</i>)	RS313 (22)
LM4449	Opaque phenotype; BB22TR/pLM1950	BB22TR (this work)
LM4437	Translucent phenotype; <i>opaR::Tn5</i>	BB22TR (this work)
LM4462	Translucent phenotype; <i>opaR::Tn5</i>	BB22OP (this work)
LM4565	IPTG-inducible opaque phenotype; LM4462/pLM2035	LM4462 (this work)
LM4572	Translucent phenotype; LM4462/pLM1835	LM4462 (this work)
LM4582	Translucent phenotype; BB22TR/pLAFRII	BB22TR (this work)
<i>E. coli</i>		
DH5 α	F ⁻ <i>endA1 hsdR17</i> (r_K^-) <i>supE44 thi-1 λ^- recA1 deoR</i> <i>gyrA96 relA1 Δ(argF-lacZYA)U169 ϕ80dlacZΔM15</i>	Bethesda Research Laboratories
LLM1956	DH5 α /pRS205 (dark)	This work
LLM1957	DH5 α /pRS205/pLM1950 (bright)	This work
LLM1958	DH5 α /pRS205/pLM1952 (dark)	This work
Plasmids		
pLAFRII	Tet ^r broad-host-range cosmid vector	11
pRS205	Ap ^r ; <i>luxCDABE</i> ⁺ operon (<i>V. harveyi</i>)	R. Showalter (29)
pLM1835	Cam ^r Ap ^r broad-host-range expression vector carrying <i>lacI</i> ^q and Ptac	pMMB66EH (12, 33)
pLM1950	Tet ^r <i>opaR</i> ⁺	pLAFRII <i>V. parahaemolyticus</i> bank (21)
pLM1952	Tet ^r Kan ^r <i>opaR294::Tn5</i>	pLM1950 (this work)
pLM1980	Ap ^r ; pUC19 with 2.0-kb <i>PstI</i> fragment from pLM1952 containing part of Tn5 insertion in <i>opaR</i>	pUC19 and pLM1952
pLM2035	Cam ^r Ap ^r <i>opaR</i> ⁺ under Ptac control	pLM1835 (this work)

bers of the *V. fischeri* LuxR family. LuxR of *V. harveyi* does not interact with an autoinducer. *V. harveyi* produces two autoinducer molecules. It is postulated that the two autoinducers each interact with cognate regulatory proteins to control the transcription of *luxR*. In this cascade manner, the synthesis of LuxR and the ultimate output of light are controlled by the density of the culture and the autoinducer levels (5, 25).

V. parahaemolyticus BB22 produces the two autoinducer molecules which are capable of stimulating the dual systems found in *V. harveyi* (4). Since *luxR* transcription is modulated by multiple density-sensing inputs, a loss of function of one signaling system, for example, in the gene encoding one of the autoinducer synthases, does not eliminate luminescence or the regulation of luminescence in *V. harveyi* because the second system remains functional. So, in order to investigate the role of density-dependent signaling in *V. parahaemolyticus*, it seemed logical to begin by examining the ultimate transcriptional activator component, i.e., LuxR. A gene was cloned from *V. parahaemolyticus* which substituted for the *V. harveyi luxR* gene to activate the expression of the *V. harveyi* luminescence operon.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this work are described in Table 1. For propagation of *V. parahaemolyticus* strains, the following media were used: HI (25 g of heart infusion broth [Difco] and 20 g of NaCl per liter) and the minimal medium of Broach et al. (8) supplemented with 0.4% galactose, 20 mM NH₄Cl, and 2% NaCl (final concentration). Casamino Acids (Difco) were added to the minimal medium, as appropriate, at a final concentration of 0.25%. Solidified media were prepared by use of 2% Bacto Agar (Difco) for HI Swarm Minus, 1.5% Bacto Agar for HI Swarm Plus, and 1.4% Bacto Agar for minimal Swarm Plus plates. Medium that supports movement over the surface is designated Swarm Plus, while medium that does not allow swarming is designated Swarm Minus. *Vibrio* strains were grown at 30°C with overnight incubation on complex media, 5 to 6 days on minimal swarm

medium, and 2 to 3 days on minimal swarm medium with Casamino Acids. Minimal swarm plates were wrapped in Parafilm to prevent desiccation. Luria broth was prepared as described by Miller (24). Ampicillin, chloramphenicol, and tetracycline were used at final concentrations of 75, 10, and 10 μ g/ml, respectively. The final isopropyl- β -D-thiogalactopyranoside (IPTG; Gold Bio-Technology, Inc., St. Louis, Mo.) concentration was 0.5 mM.

Opaque-translucent variation is slow, so it is possible to obtain essentially "pure" cultures of opaque or translucent colony types having less than 1 alternate form per 1,000 colonies. Cultures for RNA and DNA preparations were checked for variation by diluting and plating on HI Swarm Minus medium to observe single colonies.

Bioluminescence measurements. Bioluminescence was quantified with a TD-20/22 luminometer (Turner Designs, Sunnyvale, Calif.) by measuring 0.1- and 0.2-ml samples of cultures appropriately diluted to give a linear response. Luminescence was reported as specific light units, which are relative light units per minute per milliliter per unit of optical density at 600 nm (OD₆₀₀). Bioluminescence was also monitored by exposing plates to Kodak XAR5 X-ray film.

Genetic and molecular techniques. Transposon mutagenesis with Tn5 has been described elsewhere (29). Procedures for the transfer of clones from *Escherichia coli* to *V. parahaemolyticus* via conjugation and gene replacement techniques have also been described elsewhere (31). General DNA manipulations were adapted from the methods of Sambrook et al. (27). Chromosomal DNA was prepared according to the protocol of Woo et al. (37). Southern blot analysis of restricted genomic DNA (19) was performed on Hybond-NX membranes (Amersham Life Science, Buckinghamshire, England). The cosmid bank was constructed by ligating 15 to 20 kb of size-fractionated *V. parahaemolyticus* DNA with vector pLAFRII (21). DNA for the bank, which was constructed in 1987, was prepared from a translucent strain; however, the culture from which the DNA was prepared was not monitored for opaque variants.

The primers for the PCRs that revealed differences between opaque and translucent colony types were HPT (also used for primer extension; see below) and LUXUR1 (5'-GAACTGACGGACAACATGGTTGAG-3'); they were used at an annealing temperature of 43°C. These primers should prime in the sequences beginning at bp 919 and bp 1539, respectively. Thirty cycles of amplification were performed by use of *Taq* polymerase (Perkin-Elmer, Foster City, Calif.) with extension times of 50 s. The molecular size standard on agarose gels was the 1-kb ladder obtained from GIBCO BRL (Gaithersburg, Md.).

For the construction of pLM2035, a gel-purified 660-bp PCR product was ligated into the *Bam*HI and *Sal*I restriction sites of pLM1835. The following primers were used at an annealing temperature of 49°C: LUXRF (5'-CGCGG ATCCATGGCAAGGAAAATGGATATGGAC-3') and LUXRR (5'-GGACC

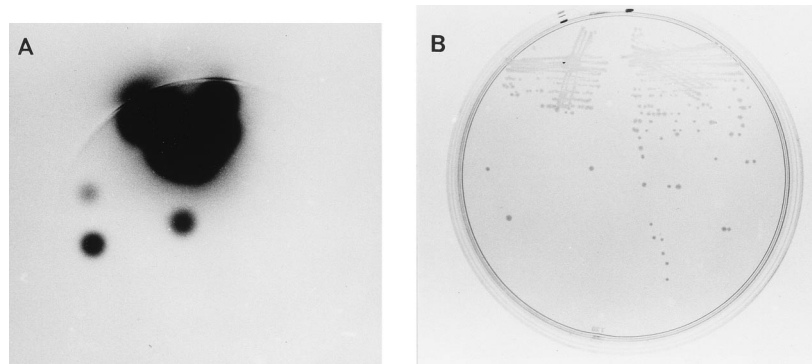


FIG. 1. Activation of luminescence by a *V. parahaemolyticus* clone. Strain LLM1957 (left side of each plate) is *E. coli* DH5 α containing two plasmids, pRS205 (*V. harveyi lux* operon) and pLM1950 (*V. parahaemolyticus luxR*-like locus). Strain LLM1958 (right side of each plate) is *E. coli* DH5 α containing two plasmids, pRS205 and pLM1952 (a mutated form of pLM1950 containing Tn5). Strains were grown overnight on a Luria broth-ampicillin-tetracycline plate and exposed to X-ray film for 15 min (A) or photographed directly in incident light (B).

TCGACGCTTTAGTGTTCGCGATTGTAGATGC-3'). Forty amplification cycles were performed with 1-min extension times. After ligation, clones were transformed into *E. coli* LLM1956 and screened for IPTG-inducible light production.

DNA sequencing analysis. The sequence of interest was obtained for both strands from cosmid clone pLM1950 by the DNA Core Facility of the University of Iowa by use of a fluorescence automated sequencer (model 373A; Applied Biosystems, Division of Perkin-Elmer). Sequence information precisely localizing the *opaR294*::Tn5 insertion was obtained from clone pLM1980 by use of the oligonucleotide TnR (5'-CCGACGATGAAGAGCAG-3'), which primed to the end of Tn5. Synthetic oligonucleotides were prepared by Integrated DNA Technologies, Inc. (Coralville, Iowa). Sequence assembly was performed by use of the Genetics Computer Group (GCG) software package. Searches for homology were performed at the National Center for Biotechnology Information (NCBI) with the BLAST network service (1, 2).

Primer extension analysis. RNA was prepared with Trizol reagent (GIBCO BRL Life Technologies, Grand Island, N.Y.) according to the manufacturer's protocol. Cells grown overnight on HI Swarm Plus plates were resuspended to an OD₆₀₀ of 2.0 units per ml, and 25 μ l was used to inoculate each HI Swarm Plus plate by spreading. Multiple plates were incubated for 5 to 6 h, and cells were harvested by resuspension in 0.3 M sucrose. Approximately 1 ml of Trizol reagent was used per 2.0 OD₆₀₀ units for RNA extraction. Primer extension analysis was performed by use of the avian myeloblastosis virus reverse transcriptase primer extension system (Promega, Madison, Wis.) according to the manufacturer's protocol. Reaction mixtures contained approximately 1 to 2 μ g of total RNA per final reaction volume of 13 μ l. Two oligonucleotide primers were used: 5'-GTTGCTTACGTTTAAGAGGAG-3' (OPAR) and 5'-CTTCACTGCCTTGTAAC-3' (HPT). Annealing reactions were performed at 48°C. Cosmid pLM1950 was primed with OPAR and HPT oligonucleotides for the sequencing reactions by the dideoxy chain termination procedure of Sanger et al. (28) with a Sequenase 2.0 kit (United States Biochemicals) and [α -³⁵S]dATP (Amersham).

Nucleotide sequence accession number. The nucleotide sequence studied here has been deposited in GenBank under accession no. AF035967.

RESULTS

The LuxR homolog of *V. parahaemolyticus*. The homolog of the *V. harveyi luxR* gene was retrieved from a *V. parahaemolyticus* cosmid gene bank by selecting in *E. coli* for clones encoding a transcriptional regulator that activated the expression of the *V. harveyi* luminescence operon *luxCDABE* carried on plasmid pRS205. The expression in *E. coli* of the *lux* operon from *V. harveyi* requires the product of the *luxR* gene; thus, *E. coli* LLM1956 carrying pRS205 is nonluminescent. The cosmid bank was conjugated into LLM1956, and exconjugants were screened for light production. Bright clones were obtained. One clone, pLM1950, was mutagenized with transposon Tn5 to obtain insertions inactivating and thus localizing the gene responsible for the induction of luminescence. Figure 1 contrasts the light produced by two *E. coli* strains harboring the *lux* plasmid and either the activating cosmid pLM1950 (on left side of each plate) or the mutated cosmid pLM1952 (on right side of each plate). Heterologous *trans* activation by the *V. para-*

haemolyticus clone of the *V. harveyi* promoter was approximately 25,000-fold (994,000.0 versus 3.0 specific light units for wild-type and mutated cosmids, respectively) and was of the same order of magnitude as that obtained for the homologous *V. harveyi luxR-luxCDABE* pair (29).

The Tn5 insertion targeted a 2-kb *Pst*I restriction fragment that was subcloned and used for initial sequencing and gene localization. The entire sequenced region, containing three complete open reading frames and one partial coding sequence, is depicted in Fig. 2. The four deduced protein sequences showed homology to *E. coli* YadF (and other members of a carbonic anhydrase family), a number of hypoxanthine ribosyltransferases (Hpt), *V. harveyi* LuxR, and *E. coli* dihydrolipoamide dehydrogenase (Lpd). The position of the transposon that eliminated the activation of luminescence is shown in Fig. 2. It mapped within the open reading frame encoding a deduced protein found 96% identical to *V. harveyi*

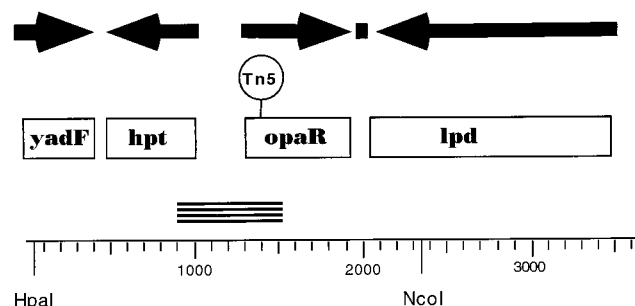


FIG. 2. Organization of the *luxR*-like locus of *V. parahaemolyticus*. The complete double-stranded nucleotide sequence of the region from bp 1 to 3605 was obtained from cosmid pLM1950. Boxes indicate open reading frames (ORFs). Arrows point in the direction of transcription of the deduced coding regions. The solid square shows the location of a potential rho-independent transcriptional terminator. The ORF for *yadF* (from bp 3 to 411) is incomplete; however, over 136 amino acids its product shows 72% similarity and 63% identity to *E. coli* YadF by GCG BestFit analysis. The predicted product of *hpt* (coding region from bp 488 to 1018) is 176 amino acids long and is 99% similar and 95% identical to *V. harveyi* Hpt. The ORF from bp 1338 to 1952 codes for a 204-amino-acid polypeptide showing 98% similarity and 96% identity to *V. harveyi* LuxR. The 475-amino-acid deduced product of the ORF from bp 2080 to 3507 exhibits 92% similarity and 87% identity to *E. coli* Lpd. Tn5 in pLM1952 is contained on a 2.3-kb *Hpa*I-*Nco*I restriction fragment, and DNA sequencing localized the insertion to bp 1392, which is within the coding region for the LuxR homolog. This gene is designated *opaR* in *V. parahaemolyticus*. The PCR product produced with the HPT and LUXUR1 primers is represented by the stacked short lines.

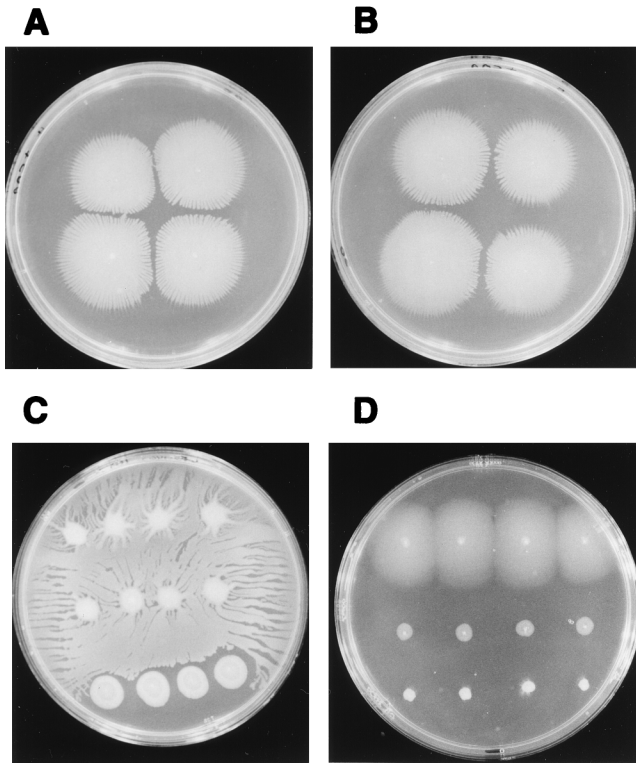


FIG. 3. Swarming patterns of wild-type and mutant strains with defects in the *luxR*-like gene *opaR*. (A and B) Wild-type BB22TR (A) and LM4437 (*opaR*::Tn5 in BB22TR) (B) on minimal Swarm Plus plates supplemented with Casamino Acids. (C) BB22TR, LM4462 (*opaR*::Tn5 in BB22OP), and LM1017 (a swarming-negative mutant) (top to bottom) on a minimal Swarm Plus plate with no Casamino Acids supplementation. (D) BB22TR, LM1017, and BB22OP (top to bottom) on a complex medium (HI) Swarm Plus plate. Four single colonies of each strain were inoculated into each row on each plate.

LuxR by GCG BestFit analysis. The predicted polypeptide also showed strong similarity (79% similarity and 72% identity by GCG BestFit analysis) to one other protein in the NCBI database, HapR, a positive regulator of the hemagglutinin/protease gene of *Vibrio cholerae* (16). The *hpt* genes are linked to the *luxR*-like genes in *V. parahaemolyticus*, *V. harveyi*, and *V. cholerae*.

Disruption of the *luxR*-like gene fails to affect swarming. Swarming motility of *S. liquefaciens* has been shown to be controlled by quorum sensing (10). An *S. liquefaciens* mutant strain unable to synthesize autoinducer failed to swarm on minimal medium supplemented with Casamino Acids. On rich medium, initiation of swarming by this mutant strain was severely delayed compared to that of the wild type. Swarming could be restored to wild-type patterns by the addition of exogenous autoinducer. To examine the role of quorum sensing in *V. parahaemolyticus*, the transposon insertion which abrogated *luxCDABE* transcriptional activation in *E. coli* and interrupted the coding region for the LuxR-like protein was introduced into the *V. parahaemolyticus* chromosome via allelic replacement. Construction of the correct gene replacement was confirmed by Southern blot analysis. The resulting strain, LM4437, was examined for swarming motility on a variety of media and showed no demonstrable defect in swarming. The swarming phenotype on minimal medium with Casamino Acids is shown in Fig. 3: the mutant strain (Fig. 3B) swarmed as well as the wild-type strain (Fig. 3A). It is important to note that in contrast to *S. liquefaciens*, *V. parahaemolyticus* swarmed

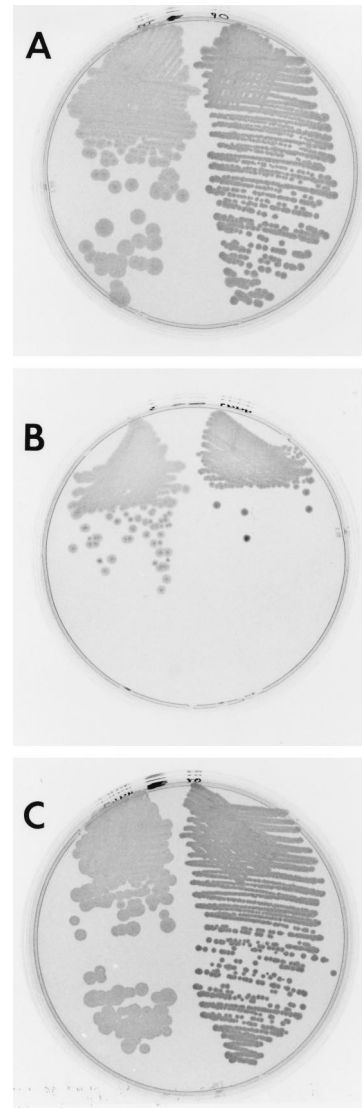


FIG. 4. Comparison of opaque and translucent phenotypes. In photographs, opaque strains appear darker than translucent strains. (A) BB22TR (left) and BB22OP (right). (B) LM4582 (BB22TR with pLAFRII; translucent phenotype) (left) and LM4449 (BB22TR with pLM1950; opaque phenotype) (right). (C) LM4462 (opaque strain with a transposon in *opaR*; translucent phenotype) (left) and BB22OP (right). Strains were grown overnight at 30°C on HI Swarm Minus plates with antibiotic as appropriate.

on unsupplemented minimal medium. There was no demonstrable difference in swarming on this type of medium between the wild-type strain and a strain with a defect in the *luxR*-like gene (Fig. 3C, rows 1 and 2, respectively). For comparison, strain LM1017, a nonswarming mutant, was also inoculated on this plate (Fig. 3C, row 3). Thus, the gene encoding the LuxR homolog is not required for swarming in *V. parahaemolyticus*.

The *V. parahaemolyticus* LuxR homolog affects the opaque and translucent phenotypes. Descendants of a single colony of *V. parahaemolyticus* can have multiple colony morphologies. The variants are described as opaque and translucent colony types as a result of differences in the transmission of light by the colonies (Fig. 4A). The properties of opaque and translucent colony types are distinct. For example, when an opaque colony is transferred from a plate with a toothpick, the entire colony adheres to the tip, sometimes lifting off the plate as a

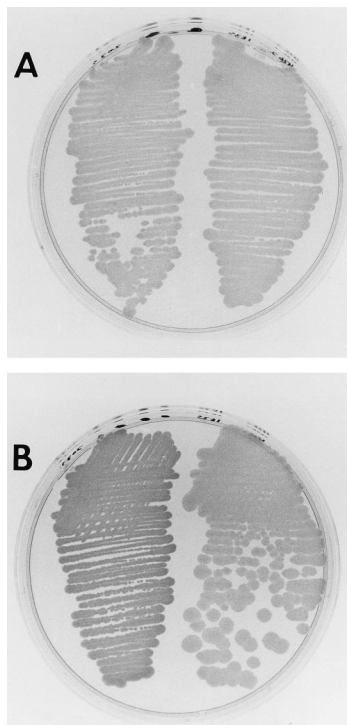


FIG. 5. IPTG-inducible opacity. On the right half of each plate is LM4572 (*opaR*::Tn5 with pLM1835), and on the left is LM4565 (*opaR*::Tn5 with pLM2035). Plasmid pLM2035 contains *opaR* under the control of the Ptac promoter in the vector pLM1835. The media used were HI Swarm Minus with chloramphenicol (A) and HI Swarm Minus with chloramphenicol and IPTG (B).

long string. In certain kinds of liquid medium, an opaque colony looks particulate due to small aggregates or clumps of cells, and on swarm plates an opaque colony performs very poorly, exhibiting little or no movement across the surface. A comparison of translucent (top row) and opaque (bottom row) swarming colonies is shown in Fig. 3D. Inoculated in the middle row of the plate is the nonswarming mutant strain LM1017.

Introduction of pLM1950, the cosmid carrying the *luxR*-like gene, into the translucent strain BB22TR converted the strain to an opaque colony type. Figure 4B shows the opaque phenotype of strain BB22TR carrying cosmid pLM1950 compared to the translucent phenotype of strain BB22TR carrying the control cosmid pLAFRII. To test whether the LuxR homolog was directly involved in the opaque-translucent switch, the transposon in the *luxR*-like gene was used for allelic replacement in an opaque strain. Introduction of the Tn5 mutation carried by pLM1952 into the chromosome of an opaque strain converted the opaque strain to a translucent colony type (strain LM4462; Fig. 4C). Furthermore, the introduction of the Tn5 mutation resulted in the concomitant gain of swarming ability. Strain LM4462 swarmed as well as strain BB22TR (Fig. 3C). Thus, the *V. parahaemolyticus* gene encoding the LuxR homolog seems to be implicated in the control of opacity and is designated *opaR*.

Expression of *opaR* controls opacity. In order to confirm the role of *opaR* in regulating opacity, a promoterless *opaR* coding region was cloned into the IPTG-inducible expression vector pLM1835. This vector also contains the *lacI^q* gene. Clone pLM2035 was transferred by conjugation to translucent strain LM4462, which contains the *opaR*::Tn5 insertion. The strain remained translucent when either pLM1835 or pLM2035 was introduced (Fig. 5A). The addition of IPTG to the

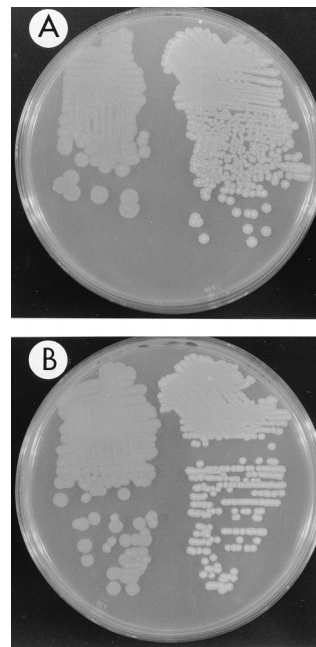


FIG. 6. Comparison of OpaR-induced opacity in opaque and translucent wild-type strains. (A) LM4572 (*opaR*::Tn5 with pLM1835) (left) and LM4565 (*opaR*::Tn5 with pLM2035) (right) were grown on HI Swarm Minus with chloramphenicol and IPTG. Plasmid pLM2035 contains *opaR* under the control of the Ptac promoter in the vector pLM1835. (B) Translucent (left) and opaque (right) wild-type strains were grown on HI Swarm Minus. Plates were photographed with illumination from above, in contrast to the plates in other figures, which were photographed with illumination from below.

plates induced the opaque morphology for the strain carrying pLM2035, but the control strain carrying pLM1835 remained translucent (Fig. 5B). A comparison of the IPTG-induced opaque phenotype with the wild-type translucent and opaque phenotypes is shown in Fig. 6. The plates were illuminated from above rather than below (as was done for the other figures) in order to show the contrast between the opaque and translucent colony types in another manner. The colony morphology of the wild-type opaque strain (Fig. 6B, right side) was identical to the IPTG-induced morphology (Fig. 6A, right side). Thus, induction of the expression of *opaR* resulted in transformation to the opaque phenotype. The introduction of pLM2035 into BB22TR also produced a translucent strain that could be converted to an opaque colony type by use of IPTG.

IPTG-induced expression of *opaR* induced the opaque phenotype. This finding suggests that the translucent phenotype is the result of the lack of expression of *opaR*. To test such an idea, as well as to establish the basis for examining the possibility of autoinducer-mediated regulation of *opaR* expression, primer extension mapping was used to define the promoter region and to examine the expression of *opaR* in opaque and translucent strains. RNA was prepared from opaque and translucent strains that had been grown on plates for 5 h. The labeled primer extension products for both the *hpt* and the *opaR* genes are indicated by the arrows next to the sequencing lanes in Fig. 7. The intergenic region between *hpt* and *opaR* is very similar to the equivalent *V. harveyi* region, and a comparison of the two sequences is shown below the autoradiograms of the primer extension gels in Fig. 7. The promoter regions for *opaR* and *luxR* seem well conserved with respect to potential σ^{70} promoter sequences and the transcription start sites. The expression of *opaR* correlated with the opaque phenotype. The

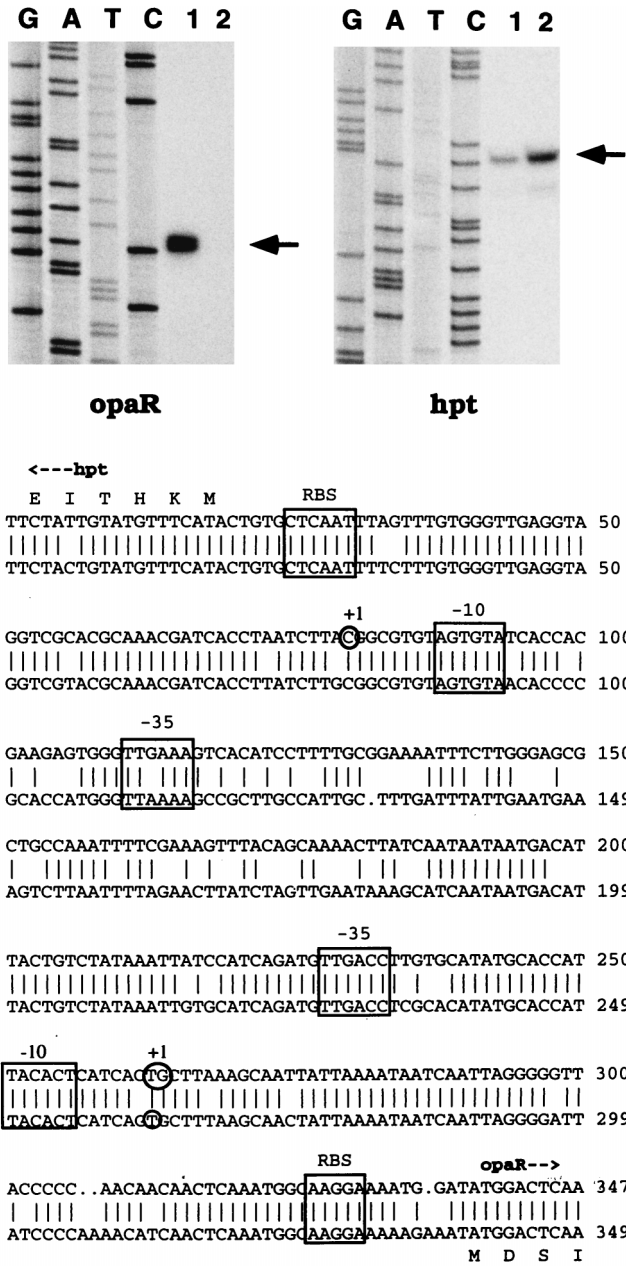


FIG. 7. Primer extension mapping of *opaR* and *hpt* transcripts. RNA was prepared from BB22OP (lane 1) and BB22TR (lane 2). Lanes G, A, T, and C correspond to the sequencing reactions generated with *opaR*- or *hpt*-specific primers for pLM1950 template DNA. The positions of the primer extension products are indicated by arrows. Below the autoradiogram is a GCG BestFit comparison of the *V. parahaemolyticus* (top) and *V. harveyi* (bottom) intergenic regions spanning the *hpt* and the *opaR* (or *luxR*) genes. Features of the sequence include potential ribosome binding sites identified by homology with the Shine-Dalgarno sequence (RBS), potential σ^{70} promoters (boxed -10 and -35 regions), and transcription start sites (circled nucleotides labeled +1). For *V. harveyi*, the *luxR* start site was determined by Miyamoto et al. (25), and the promoter boxes were proposed by Showalter et al. (29) and Miyamoto et al. (25). For *V. parahaemolyticus*, a strong doublet was observed for the *opaR* primer extension product (circle including two bases). The doublet was very clear in autoradiogram exposures shorter than the one shown here. It should be noted that there are multiple potential ATG start codons for *opaR*, and the translation depicted in this figure represents the particular N terminus having optimal spacing with respect to a potential Shine-Dalgarno sequence and corresponding to the predicted *V. harveyi* N terminus.

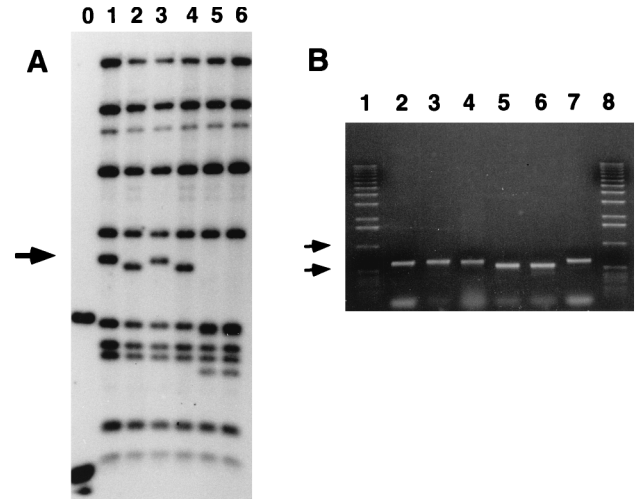


FIG. 8. Genomic organization of the *opaR* locus in opaque and translucent strains. (A) Southern blot of chromosomal DNAs prepared from the following strains: lanes 1 and 3, BB22OP; lanes 2 and 4, BB22TR; and lanes 5 and 6, LM4462 (*opaR*::Tn5). DNAs were digested with *HpaI* and *NcoI*, loaded in the indicated lanes, electrophoresed on an 0.8% agarose gel, Southern blotted, and probed with 32 P-labeled pLM1950. The 2.3-kb band marked with an arrow shows perturbations, while the other bands remained constant in size among the lanes. Molecular size markers were loaded in lane 0, and the two bands visualized correspond to 1.6 and 0.5 kb. (B) PCR amplification of *opaR*. Products were displayed on a 0.6% ethidium bromide-stained agarose gel. Lanes correspond to PCR products containing DNAs from the following sources: 2 and 7, cosmid pLM1950; 3 and 4, BB22OP; and 5 and 6, BB22TR. Molecular size markers were loaded in lanes 1 and 8. Arrows indicate 1- and 0.5-kb markers. The predicted size of the PCR product derived from the sequence of pLM1950 is 620 bp. The specific primers used were designed to amplify the sequence between bp 919 and 1539 depicted in Fig. 2.

production of *opaR* mRNA was detected only in the opaque strain and not in the translucent strain (Fig. 7, left panel, lane 1 versus lane 2). As a control, primer extension was performed with identical RNA preparations and conditions and with an *hpt*-derived primer. In this case, transcripts were identified for both opaque and translucent strain mRNAs (Fig. 7, right panel, lanes 1 and 2). In fact, slightly more total RNA was used in the translucent strain than in the opaque strain primer extension reactions, and this finding is reflected in more product in lane 2 than in lane 1 of Fig. 7 with the *hpt*-derived primer. The expression of *hpt* was observed in both cell types, while *opaR*-specific RNA could be detected only in the opaque strain and was not be observed in the translucent strain.

Opaque-translucent variation involves genomic rearrangements. Southern analysis of opaque and translucent variant strains revealed a difference in the chromosome structure in the *opaR* locus. Figure 8A shows the physical rearrangement observed on a Southern blot containing digested chromosomal DNA prepared from opaque and translucent strains. Also on this blot is digested DNA prepared from the *opaR*::Tn5 mutant strain LM4462. The blot was probed with cosmid pLM1950, which contains approximately 20 kb of recombinant *V. parahaemolyticus* DNA encompassing the *opaR* locus. Locations of transposon mutations can be mapped on a Southern blot because insertion of a transposon results in a large perturbation of the sequence. In Fig. 8A, lanes 5 and 6, which contained LM4462 DNA, a 2.3-kb fragment was missing. This finding is consistent with the sequence data, which precisely determined the insertion to be at bp 1392 (Fig. 2) within the coding region for *opaR* (from bp 1338 to bp 1952) and on a 2.3-kb *HpaI*-*NcoI* restriction fragment. Thus, the *opaR* gene can be mapped to

this fragment. This same fragment showed a size aberration in opaque and translucent variants, while the other restriction fragments remained relatively constant in size among the strains (except for the new bands appearing in LM4462, which were the consequence of the transposon insertion). When a blot identical to that shown in Fig. 8A was reprobed with only a structural coding sequence (with plasmid pLM2035), the fluctuating band in the 2.3-kb region was the only band detected (data not shown). The rearrangement in the chromosome structure observed for the opaque and translucent strains appeared to be closely linked to the *opaR* gene.

The rearrangement was analyzed in another manner (Fig. 8B). Two oligonucleotides were used to prime a PCR amplification between bp 919 and 1539 on the map shown in Fig. 2. This region contains the intergenic region between *hpt* and *opaR* and part of the N-terminal coding region for *opaR*. The sizes of the amplified products were different when template DNAs from opaque and translucent strains were used (two strains of each). The size of the product amplified from opaque strains (Fig. 8B, lanes 3 and 4) corresponded well with the size of the product produced when the template DNA was the sequenced clone pLM1950 (lanes 2 and 7), while the size of the translucent strain product was smaller by approximately 100 bp (lanes 5 and 6). Cosmid pLM1950 was cloned by selecting for the expression of *opaR* in *E. coli*, and the size of the PCR product amplified with pLM1950 corresponded to the size of the product produced with chromosomal DNA prepared from the opaque form, which is the state observed to express *opaR* mRNA. The size of the PCR product was different for translucent strains, and the translucent form failed to produce an *opaR* transcript. The PCR results in combination with the transcription data are consistent with the hypothesis that *opaR* is not expressed in translucent strains due to chromosomal organization.

DISCUSSION

V. parahaemolyticus is a bacterium with multiple personalities, each adapted for survival in a particular environment. The swimmer cell possesses a sheathed polar flagellum that is capable of rotating very fast and propelling the bacterium in liquid medium at speeds averaging 60 μm per s (3). However, in viscous medium or on surfaces, the polar flagellum fails to function, leading to the formation of the swarmer cell (20). The swarmer cell can be very long and multinucleate and can possess numerous lateral flagella in addition to the polar flagellum (7, 20). Induction of the lateral flagellar system endows the bacterium with the ability to move through viscous layers or over surfaces. The result of swarmer cell differentiation is colonization of surfaces. In addition to the swimmer-swarmer cell dimorphism, *V. parahaemolyticus* exhibits another kind of phenotypic switching, described as opaque-translucent variation in colony morphology. The molecular basis for the difference in colony morphology is not known. For other organisms it has been associated with differences in cell surface characteristics, e.g., outer membrane proteins and encapsulation (35, 38). It has been postulated that differences in colony structure or packing result in differential light transmission by opaque and translucent forms (34).

This work describes the identification of a gene, *opaR*, that encodes a transcriptional regulatory protein controlling opacity. The product of the *opaR* gene is 96% identical to and functionally exchangeable with the LuxR protein of *V. harveyi*. The gene was cloned by selecting for activation of the *V. harveyi luxCDABE* operon in *E. coli*. *V. parahaemolyticus* produces autoinducer molecules capable of inducing lumines-

cence in *V. harveyi* (4, 14). It has been a puzzle why nonluminescent *V. parahaemolyticus* makes signaling molecules, and one hypothesis has postulated a role in swarming behavior. Precedent for such an idea derives from *S. liquefaciens*. In this organism, loss of function of a gene encoding an autoinducer synthase abolishes swarming motility on certain types of media (10). To test this hypothesis in *V. parahaemolyticus*, the gene encoding the LuxR homolog was targeted for introduction of a knockout mutation. Strains carrying such a defect were demonstrated to display no swarming defect. In the course of these experiments, swarming was examined on minimal medium and, contrary to observations for other swarming organisms, such as *S. liquefaciens*, wild-type *V. parahaemolyticus* was observed to swarm on minimal medium without supplementation with Casamino Acids. Mutants with *opaR* defects also swarmed well on this medium.

Evidence suggests that *opaR* controls opacity. Introduction of a clone containing the *opaR* locus into a translucent strain transformed the colony morphology to opaque. Transposon-mediated disruption of *opaR* in an opaque genetic background converted the strain to a translucent phenotype. Expression of the gene from an exogenously controlled promoter determined opacity; i.e., opacity could be manipulated by use of an IPTG-inducible promoter controlling *opaR* expression.

Expression of *opaR* may be ultimately governed by the particular state of the DNA. The *opaR* gene is expressed in opaque strains but not in translucent strains. DNA rearrangements in the *opaR* locus were detected in opaque and translucent strains. The locus was cloned from a cosmid bank by use of positive selection for expression, i.e., activation of the *V. harveyi lux* operon in *E. coli*. Thus, both clone pLM1950 and opaque strains are expression competent for *opaR*. PCR analysis demonstrated that the physical state of the DNA on the clone appears to correspond to that found in opaque strains. Interestingly, the bank from which the clone was retrieved was constructed from a translucent strain (21). It is possible that the culture from which the chromosomal DNA was prepared contained some opaque variants. Current work involves analyzing other clones retrieved directly from the same bank by DNA hybridization as well as examining lineages of opaque-translucent variants in order to define the molecular basis for the rearrangement. It is possible that switching is the result of a gene or promoter inversion similar to *Salmonella typhimurium* flagellar phase variation (32) or some kind of phase-shifting event involving the loss of DNA. It is intriguing that the expression of luminescence in *V. harveyi* is also an unstable phenotype. Colonies show striking sectoring and segregate into bright and dim phenotypes with respect to light production (32).

What regulates opacity? Opaque-translucent switching may alternate between expression-competent and expression-incompetent states; however, the transcription of *opaR* may also be regulated by intercellular or environmental signaling mechanisms. Although *V. parahaemolyticus* and *V. harveyi* are phylogenetically closely related (9) and have functionally exchangeable components with respect to autoinducer and LuxR functions, it remains to be established whether *opaR* expression in *V. parahaemolyticus* is actually density dependent. It is provocative that the intergenic region and the transcription initiation sites are conserved between the organisms. Furthermore, the gene organization and the sequence conservation extend to the one other described homolog of *V. harveyi* LuxR, HapR, a positive regulator found in *V. cholerae* (16). HapR controls the expression of a soluble hemagglutinin. Interestingly, *hapR* mutants of El Tor strains have a rugose colony morphology.

What is the role of *opaR*? *opaR* is a master regulatory gene controlling cell type. Opaque and translucent strains form distinct colony structures. The colony organization is most probably determined by cell surface characteristics. Particular cell surface components, such as proteins or capsules, may aid survival under specific circumstances. For example, at times it may be beneficial to be adhesive. At other times, perhaps during the development of a biofilm, it may be critical to have mobility. Once colonization is established, the highly motile swarmer lifestyle may not always be advantageous. Also, an ability to detach, perhaps autoagglutinate, and take up a planktonic existence may play an important role in survival. These possibilities, plus the potential role of autoinducer signaling in the growth of communities, will be interesting to pursue. The alternating personalities of *V. parahaemolyticus* may have important significance for the development of ideas with respect to attachment, detachment, and how bacterial populations adapt to growth on surfaces, form structured communities, and develop in biofilms.

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REFERENCES

- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Atsumi, T., L. McCarter, and Y. Imae. 1992. Polar and lateral flagellar motors of marine *Vibrio* are driven by different ion membrane forces. *Nature (London)* **355**:182–184.
- Bassler, B. L., E. P. Greenberg, and A. M. Stevens. 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J. Bacteriol.* **179**:4043–4045.
- Bassler, B. L., M. Wright, and M. R. Silverman. 1994. Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. *Mol. Microbiol.* **13**:273–286.
- Batchelor, S. E., M. Cooper, S. R. Chhabra, L. A. Glover, G. S. A. B. Stewart, P. Williams, and J. I. Prossner. 1997. Cell density-regulated recovery of starved biofilm populations of ammonia-oxidizing bacteria. *Appl. Environ. Microbiol.* **63**:2281–2286.
- Belas, R., M. Simon, and M. Silverman. 1986. Regulation of lateral flagella gene transcription in *Vibrio parahaemolyticus*. *J. Bacteriol.* **167**:210–218.
- Broach, J., C. Neumann, and S. Kustu. 1976. Mutant strains (*nit*) of *Salmonella typhimurium* with a pleiotropic defect in nitrogen metabolism. *J. Bacteriol.* **128**:86–98.
- Dorsch, M., D. Lane, and E. Stackebrandt. 1992. Towards a phylogeny of the genus *Vibrio* based on 16S rRNA sequences. *Int. J. Syst. Bacteriol.* **42**:58–63.
- Eberl, L., M. K. Winson, C. Sternberg, G. S. A. B. Stewart, G. Christiansen, S. R. Chhabra, B. Bycroft, P. Williams, S. Molin, and M. Givskov. 1996. Involvement of N-acyl-L-homoserine lactone autoinducers in controlling the multicellular behavior of *Serratia liquefaciens*. *Mol. Microbiol.* **20**:127–136.
- Friedman, A., S. R. Long, S. E. Brown, W. J. Buikema, and F. Ausubel. 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene* **18**:289–296.
- Fuerste, J. P., W. Pansegrau, R. Frank, H. Blocker, P. Scholz, M. Bagdasarjan, and E. Lanka. 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene* **48**:119–131.
- Fuqua, C., S. C. Winans, and E. P. Greenberg. 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum sensing transcriptional regulators. *Annu. Rev. Microbiol.* **50**:727–751.
- Greenberg, E. P., J. W. Hastings, and S. Ulitzur. 1979. Induction of luciferase synthesis in *Beneckeia harveyi* by other marine bacteria. *Arch. Microbiol.* **120**:87–91.
- Hanselka, B. L., and E. P. Greenberg. 1995. Evidence that the N-terminal region of the *Vibrio fischeri* LuxR protein constitutes an autoinducer-binding domain. *J. Bacteriol.* **177**:815–817.
- Jobling, M. G., and R. K. Holmes. 1997. Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/protease gene *hap*, and its identification as a functional homologue of the *Vibrio harveyi* *luxR* gene. *Mol. Microbiol.* **26**:1023–1034.
- Kupsch, E. M., B. Knepper, T. Kuroki, I. Heuer, and T. F. Meyer. 1993. Variable opacity (Opa) outer membrane proteins account for the cell tropisms displayed by *Neisseria gonorrhoeae* for human leukocytes and epithelial cells. *EMBO J.* **12**:641–650.
- Lambden, P. R., J. E. Heckels, L. T. James, and P. J. Watt. 1979. Variations in surface protein composition associated with virulence properties in opacity types of *Neisseria gonorrhoeae*. *J. Gen. Microbiol.* **114**:305–312.
- Martin, M., R. Showalter, and M. Silverman. 1989. Identification of a locus controlling expression of luminescence genes in *Vibrio harveyi*. *J. Bacteriol.* **171**:2406–2414.
- McCarter, L., and M. Silverman. 1990. Surface-induced swarmer cell differentiation of *Vibrio parahaemolyticus*. *Mol. Microbiol.* **4**:1057–1062.
- McCarter, L. L., and M. Silverman. 1987. Phosphate regulation of gene expression in *Vibrio parahaemolyticus*. *J. Bacteriol.* **169**:3441–3449.
- McCarter, L. L., and M. E. Wright. 1993. Identification of genes encoding components of the swarmer cell flagellar motor and propeller and a sigma factor controlling differentiation of *Vibrio parahaemolyticus*. *J. Bacteriol.* **175**:3361–3371.
- McLean, R. J. C., M. Whitely, D. J. Stickler, and W. C. Fuqua. 1997. Evidence of autoinducer activity in naturally occurring biofilms. *FEMS Microbiol. Lett.* **154**:259–263.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Miyamoto, C. M., J. Chatterjee, E. Swartzman, R. Sztitner, and E. A. Meighen. 1996. The role of the *lux* autoinducer in regulating luminescence in *Vibrio harveyi*: control of *luxR* expression. *Mol. Microbiol.* **19**:767–775.
- Reddy, G. P., U. Hayat, C. Abeygunawardana, C. Fox, A. C. Wright, D. R. Maneval, Jr., C. A. Bush, and J. G. Morris, Jr. 1992. Purification and determination of the structure of capsular polysaccharide of *Vibrio vulnificus* M106-24. *J. Bacteriol.* **174**:2620–2630.
- 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.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
- Showalter, R., M. O. Martin, and M. R. Silverman. 1990. Cloning and nucleotide sequence of *luxR*, a regulatory gene controlling bioluminescence in *Vibrio harveyi*. *J. Bacteriol.* **172**:2946–2954.
- Showalter, R., M. Silverman, and L. McCarter. Unpublished observation.
- Silverman, M., R. Showalter, and L. McCarter. 1991. Genetic analysis in *Vibrio*. *Methods Enzymol.* **204**:515–536.
- Simon, M. I., and M. Silverman. 1983. Recombinational regulation of gene expression in bacteria, p. 211–227. *In* J. Beckwith, J. Davies, and J. A. Gallant (ed.), *Gene function in prokaryotes*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Stewart, B. J., J. L. Enos-Berlage, and L. L. McCarter. 1997. The *lonS* gene regulates swarmer cell differentiation of *Vibrio parahaemolyticus*. *J. Bacteriol.* **179**:107–114.
- Swanson, J. 1977. Studies on gonococcus infection. XII. Colony color and opacity variants of gonococci. *Infect. Immun.* **19**:320–331.
- Swanson, J. 1978. Studies on gonococcus infection. XIV. Cell wall protein differences among color/opacity colony variants of *Neisseria gonorrhoeae*. *Infect. Immun.* **21**:292–302.
- Swift, S., J. P. Throup, P. Williams, G. P. C. Salmond, and G. S. A. B. Stewart. 1996. Quorum sensing: a population-density component in the determination of bacterial phenotype. *Trends Biochem. Sci.* **21**:214–219.
- Woo, T. H. S., A. F. Cheng, and J. M. Ling. 1992. An application of a simple method for the preparation of bacterial DNA. *BioTechniques* **13**:696–697.
- Wright, L., L. M. Simpson, J. D. Oliver, and J. G. Morris, Jr. 1990. Relation of capsular materials and colony opacity to virulence of *Vibrio vulnificus*. *Infect. Immun.* **47**:446–451.