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Regulation of Lateral Flagella Gene Transcription in *Vibrio parahaemolyticus*

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Two distinctly different organelles of locomotion are produced by *Vibrio parahaemolyticus*. The polar flagellum is responsible for motility in a liquid environment (swimming), and the lateral flagella enable the bacteria to move over surfaces (swarming). Synthesis of lateral flagella occurs when *V. parahaemolyticus* is grown on agar media but not when it is grown in liquid media. We used *lux* (luminescence gene) fusions to conveniently and sensitively analyze the factors which influence transcription of lateral flagella genes (*laf*). Transposon mini-Mu *lux* was used to mutagenize *V. parahaemolyticus* and to generate *laf*::*lux* transcriptional fusions. Mutants with insertions of mini-Mu *lux* in *laf* genes were defective in the swarming phenotype and produced light when the bacteria were propagated on agar media, but not when cells were grown in liquid media. Thus, surface-dependent expression of lateral flagella synthesis is controlled by regulation of transcription. Such fusion strains were also used to further define the environmental conditions which induce *laf* gene expression. Cultivation on media solidified by gelling agents other than agar also induced light production in fusion strains, as did growth on a variety of hydrophilic membrane filters suspended over liquid media. Growth at an air-surface interface was not necessary for expression since embedding the fusion strains in agar was also effective. Furthermore, induction of *laf* gene transcription could also be accomplished by increasing the viscosity of the liquid medium by the addition of a high-molecular-weight polymer such as polyvinylpyrrolidone. Increase in luminescence of the fusion strains was detected within 30 min of initiation of the inducing circumstance, and reversal of induction, e.g., by dilution of the viscous medium, resulted in a rapid decline in the rate of increase in luminescence. Conditions that induced luminescence in the fusion strains also induced the synthesis of lateral flagella in wild-type *V. parahaemolyticus*. The growth environment of the fusion strains was manipulated extensively to identify those conditions which resulted in transcription of *laf* genes, and it appears that the signal that triggers *laf* expression is physical rather than chemical in nature. Possibilities for a sensing mechanism are discussed.

Bacteria in the ocean are capable of colonizing a myriad of habitats. They can be found free-swimming in the water column, in the gut tracts of marine animals, or attached to a variety of animate and inanimate surfaces (1, 8, 9). Collectively, a great diversity of bacterial species has evolved to survive in the marine ecosystem. Individual marine bacteria also encounter a varied and changing environment, and survival of a single cell would be expected to depend on the ability to express different phenotypes each appropriate to a particular circumstance. For example, *Vibrio parahaemolyticus* occupies a variety of habitats in marine and estuarine environments, where it can be isolated as a free-living form or attached to submerged surfaces (16). From laboratory studies, it is apparent that the same strain of *V. parahaemolyticus* can express one phenotype appropriate for growth in liquid media and another phenotype appropriate for growth on surfaces (10, 11).

When grown in liquid media, *V. parahaemolyticus* produces a single, polar flagellum which is covered by a membranous sheath that is contiguous with the outer membrane of the bacterium. The polar flagellum propels the cells through the liquid environment. When these bacteria are propagated on media solidified with agar, the cells undergo a morphological change. Shortly after being plated on agar media the cells cease septation and begin to elongate. Elongation can eventually result in cells 30 to 40 μm in length and occurs concomitantly with the synthesis of distinctly different flagellar structures. Hundreds of these unsheathed flagella, called lateral flagella, are assembled in a peritrichous arrangement around the cell and function to translocate the bacteria over the surface, an event termed swarming (17). If the swarmer cells are transferred from agar medium to a liquid medium, the cells septate and fragment into short cells (0.5 by 1.5 to 2.0 μm). Synthesis of lateral flagella also ceases, and remaining lateral flagella appear to be lost or shed into the surrounding environment (3, 10, 11).

The laterally flagellated swarmer cell has a specialized function appropriate to life on a surface. Cells with lateral flagella adhere more firmly to surfaces (2) and move over the surface, resulting in expansion of the area of colonization. In cross sections of a large swarming colony the swarmer cells are found mostly at the periphery, while interior regions are inhabited by short cells possessing only the polar flagellum (21). These shorter cells apparently are the descendants of swarmer cells in which the differentiation process has been reversed, making them better suited for dispersal into the liquid environment. Thus, *V. parahaemolyticus* is capable of producing different cell types adapted to different modes of existence. How does this organism regulate the expression of these phenotypes so that a form appropriate to a given habitat is produced?

Because the ability of *V. parahaemolyticus* to adapt its form to particular habitats must ultimately be controlled by a genetic program, we chose to study this differentiation process by analyzing the regulation of lateral flagella gene (*laf*) expression. Specifically, regulation of *laf* gene expression was analyzed by constructing fusions between *laf* genes and indicator luminescence genes (*lux*). The fusions were

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constructed in vivo with the use of a specialized transposon, mini-Mu *lux*, which in the process of transposition can insert *lux* genes into target genes in the genome of *V. parahaemolyticus* (14). In strains with *laf*::*lux* fusions, transcription of *lux* genes is coupled with that of the *laf* genes, and as a consequence, light production, the phenotype of the *lux* genes, is governed by factors which regulate the transcription of *laf* genes (see Fig. 1). By constructing such fusion strains it is now possible to measure light production instead of quantitating a complex morphological differentiation. Furthermore, very sensitive measurements of *laf* gene expression can be made over a wide range of experimental configurations without disturbing the cells or the environment surrounding them.

We used *laf*::*lux* fusion strains to answer a number of questions about regulation of the swarming phenotype. Is expression of swarming regulated at the level of gene transcription? What other surfaces or environmental conditions induce formation of lateral flagella? Does induction require cell-to-cell communication? What are the kinetics of *laf* gene turnon and turnoff? The results reported here were then used to formulate more specific hypotheses to explain what *V. parahaemolyticus* senses and how this signal actuates *laf* gene expression.

MATERIALS AND METHODS

Bacteria, media, and culture conditions. Wild-type *V. parahaemolyticus* BB22 (from R. Belas, Agouron Institute) was routinely grown at 30°C in Difco marine broth at 75% the recommended concentration (28 g/1,000 ml of H_2O and herein referred to as 2216 medium) or in heart infusion broth (Difco heart infusion broth supplemented with 20 g of NaCl per liter). *Escherichia coli* MC4100 harboring mini-Mu *lux* (Tet^r) was grown in L broth (10 g of tryptone [Difco], 5 g of yeast extract [Difco], 10 g of NaCl per liter). For solid media, 15 g of agar was added per liter of liquid medium. Tetracycline (Calbiochem-Behring, La Jolla, Calif.) was added to a final concentration of 20 μg/ml for selection in *E. coli* and was used at 10 μg/ml for selection of tetracycline-resistant *V. parahaemolyticus*.

The following compounds were used to increase the viscosity of aqueous solutions: polyvinylpyrrolidone, average molecular weight (MW), 40,000 (PVP-40) and 360,000 (PVP-360); polyethylene glycol, average MW, 1,000, 6,000, and 20,000 (PEG 1000, PEG 6000, and PEG 20,000, respectively); Ficoll70 (70,000 MW) and Ficoll400 (400,000 MW); gums guar, karaya, locust bean, tragacanth, and xanthan. All viscosity-increasing compounds were purchased from Sigma Chemical Co., St. Louis, Mo. They were dissolved in distilled H_2O and dialyzed overnight against 10 changes of 250 volumes of distilled H_2O and sterilized. Final stock solutions were made by the addition of equal volumes of 2× viscosity-increasing agent and 2× 2216 broth. A 10% (wt/vol) solution of PVP-360 was used as a standard reference of viscosity. The viscosity at 22°C of a 10% (wt/vol) solution of PVP-360 in 2216 broth was 40 cP as measured with a Cannon-Fenske calibrated viscometer (Thomas Scientific). The viscosity of H_2O equals 1 cP or 10^{-3} kg m^{-1} s^{-1}. Solutions of guar, karaya, locust bean, tragacanth, and xanthan gums were adjusted to this viscosity (40 cP) with the same apparatus.

Mutagenesis with mini-Mu *lux* (Tet^r). Transduction of mini-Mu *lux* (Tet^r) (14) into *V. parahaemolyticus* was performed as described previously (4). *E. coli* MC4100 (mini-Mu *lux* [Tet^r], P1 *clr-100* CM) was temperature induced at 42°C to prepare transducing lysates. Infected cells were spread onto heart infusion agar plates (heart infusion broth supplemented with 15 g of NaCl per liter and 20 g of agar per liter) containing 10 μg of tetracycline per ml. This medium does not affect the induction of swarming, but it blocks the spreading of the swarming colony, thus allowing the recognition of single clones. Tetracycline-resistant transductants were picked onto a master array after overnight growth at 30°C. To test for nonswarming mutants, arrays of colonies were replica plated onto heart infusion agar containing 20 g of NaCl and 15 g of agar per liter. Swarming was measured after 8 h of incubation at 30°C, and nonswarming mutants were saved for further analysis. *lux*::*laf* fusion mutants of *V. parahaemolyticus*, which is naturally nonluminescent, were detected by growing colonies on an agar medium and: (i) visually inspecting colonies for luminescence in a darkroom, (ii) allowing luminescent colonies to expose an undeveloped sheet of Kodak XAR X-ray film in a darkroom, or (iii) measuring light units with an LKB 1211 scintillation counter in the chemiluminescence mode. Nonswarming mutants that produced light when grown on an agar surface, but not in liquid, were collected for analysis.

Measurement of luminescence in *laf*::*lux* fusions. Conditions used for measuring the levels of gene expression in *laf*::*lux* transcriptional fusions were as follows. *V. parahaemolyticus laf*::*lux* fusion strains were grown overnight at 30°C in 2216 broth supplemented with 10 g of tetracycline per ml. Cells were diluted 1 to 2,000 in fresh 2216 broth (without tetracycline) and incubated with shaking at 30°C until the optical density was 0.05 at a wavelength of 600 nm (OD_{600}). The time required for the bacterial culture to reach this OD was approximately 2 h. A 5-μl sample was used to inoculate 500 μl of liquid medium contained within a plastic 1.5-ml centrifuge tube, or the inoculum was added to the surface of a cylindrical core of agar-solidified medium approximately 1 cm in diameter and 1 cm in depth. Although agar was routinely used as a solid substrate when measuring luminescence in *laf*::*lux* fusions, carrageenan (Sigma) and Gelrite (Kelco, San Diego, Calif.) were also used. Carrageenan (2%, wt/vol) was added to liquid media and used like agar. Gelrite (1%, wt/vol) in H_2O was sterilized separately and added to sterile 2× liquid media, poured in petri plates, and solidified. The final concentration of Gelrite was 0.5% (wt/vol).

Membrane filter disks were also used as substrates to test surface specificity of *laf*::*lux* fusions. Membrane circles of 1-cm diameter were cut from nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.), cellulose acetate (Celotate; Millipore Corp., Bedford, Mass.), and polyvinylidene difluoride (hydrophilic Durapore; Millipore). The filters were placed in scintillation vials containing pads of absorbent paper (3MM filter paper; Whatman, Inc., Clifton, N.J.) saturated with liquid medium and inoculated with a 5-μl sample of the *laf*::*lux* fusion. The *laf*::*lux* fusion strains were then placed in an LKB 1211 scintillation counter programmed so that each sample would be repetitively counted at 30-min intervals. Luminescence was measured as the output of the chemiluminescence channel of the scintillation counter and is referred to as light units. To obtain values for light units per cell (relative light units), replicate samples were arranged in the scintillation counter. At 30-min intervals, a sample was removed, diluted in 2216 broth, spread on 2216 agar containing 10 μg of tetracycline per ml, and incubated overnight at 30°C. Cell number was expressed as CFU.

Photomicroscopy. Wild-type *V. parahaemolyticus* and nonswarming *laf*::*lux* transposon fusion strains were exam-

ined for morphological changes induced by incubation in viscous medium with a Nikon Optiphot microscope equipped with an AFX-II automatic exposure meter. Photographic exposures were recorded on Ilford HP5 film.

RESULTS

Mutagenesis with mini-Mu *lux*. Transposon mini-Mu *lux*, like transposon mini-Mu from which it was derived, mutagenizes by transposing into DNA sequences in the bacterial genome (7, 14). Insertion of the transposon, which is approximately 18 kilobase pairs in length, interrupts the continuity of the target gene and results in a null mutant phenotype. Since the transposon encodes a drug resistance marker, a collection of mutants can readily be isolated by plating a mutagenized culture on selective antibiotic media. The region flanking the site of insertion can also be cloned by selecting recombinants which express the drug resistance phenotype. The introduction of a large amount of extraneous DNA into the target gene also facilitates mapping of transposon-generated mutations. In addition, mini-Mu *lux* contains all the luminescence genes required for light production in a wide variety of bacteria. The *lux* genes (*luxCDABE* from *Vibrio fischeri*; 13) are positioned adjacent to one terminus of the transposon (Fig. 1) such that insertion of the transposon in one of the two possible orientations aligns transcription of the target gene with that of the *lux* genes. Since the native promoter element of the *lux* operon has been removed, transcription of the *lux* gene is dependent on the target gene promoter, and activation of transcription of the target gene results in transcription of the *lux* genes and the subsequent production of light (14).

Mutagenesis was accomplished by using coliphage P1 (specifically P1 *clr-100 CM*) to transduce transposon mini-Mu *lux* from *E. coli* into *V. parahaemolyticus*. Transducing phage P1 is capable of packaging mini-Mu *lux* DNA and infects, but does not replicate in, *V. parahaemolyticus* (4). Both the P1 repressor and the repressor of Mu transposase of mini-Mu *lux* are inactivated by temperature shift to 42°C, so the lytic cycle of phage P1 and mini-Mu *lux* replication can be coinduced. The resultant transducing lysate contains a relatively high proportion of transducing particles containing mini-Mu *lux*. Transduction conditions were developed originally for use with another transposon, Tn*5-132* (4) and are also effective for mini-Mu *lux* (4, 14). Briefly, *V. parahaemolyticus* was infected with a transducing lysate prepared from an *E. coli* strain lysogenized with P1 *clr-100 CM* and containing a mini-Mu *lux* transposon residing in the phage genome. Infected cells were then plated on medium containing tetracycline to select for those transductants which had a mini-Mu *lux* insertion. Initial selection and propagation was done on a modified heart infusion medium (see above) which inhibited swarming so individual colonies could be maintained. A mutant bank of approximately 10,000 tetracycline-resistant colonies was assembled on master plates in a grid pattern with 49 colonies. Colonies were then transferred with a replicator device to a heart infusion medium formulated to support swarming, and those mutants which did not swarm were saved for further analysis. Approximately 1% (106 of 9,800) of the colonies in the mutant bank were incapable of swarming.

Transcriptional control of *laf* genes. Light production by the mutants defective in swarming (Laf$^-$) was measured by placing agar plates containing the mutants in close proximity to X-ray film in a darkroom. About 40% of the Laf$^-$ mutants

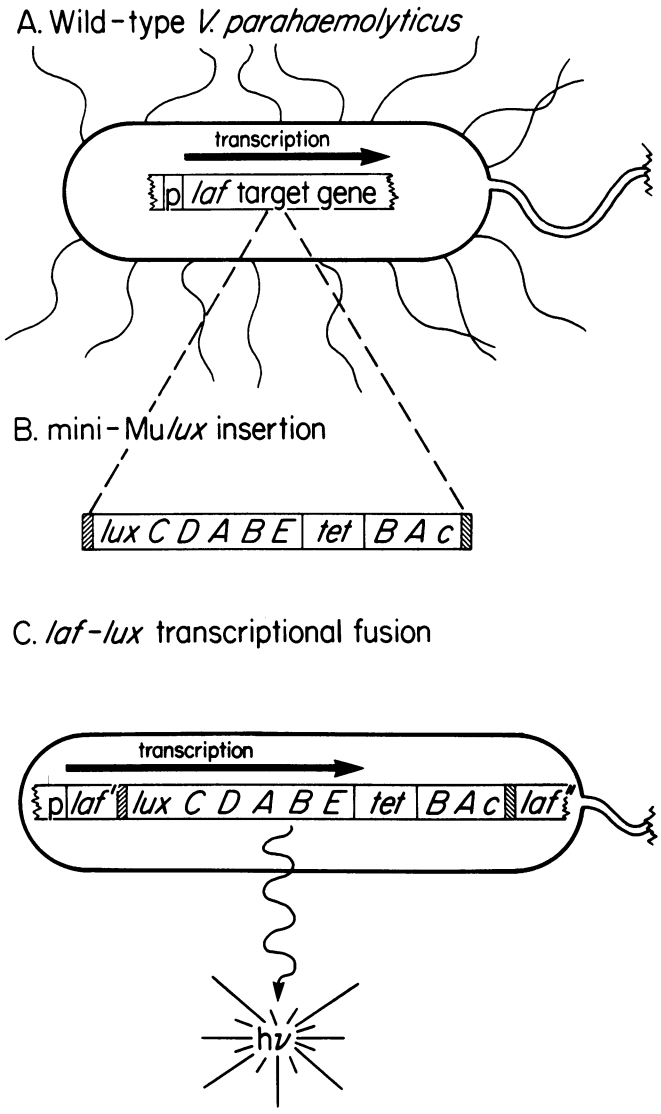

FIG. 1. Mutagenesis with transposon mini-Mu *lux*. (A) Wild-type *V. parahaemolyticus* possesses a single polar flagellum which is sheathed by a continuation of the outer membrane, represented at the right of the cell, and numerous, unsheathed lateral flagella, peritrichously arranged over the bacterium. In this example, a target *laf* (lateral flagella) gene, encoding an essential element involved with the swarming phenomenon, is represented. Surface-dependent transcription commences after the promoter (p) and proceeds left to right. (B) Structure of mini-Mu *lux*. *luxC*, *-D*, and *-E* encode aldehyde substrate synthesis or recycling functions, and *luxA* and *-B* encode the α and β subunits of luciferase. Tetracycline resistance is encoded by *tet*, and Mu-specific transposase and repressor activity is encoded by *B*, *A*, and *c*. The *lux* operon promoter was removed during construction of this transposon; therefore light is produced only if the transposon inserts downstream from an actively transcribed gene and aligns in the correct orientation. (C) Capacity of mini-Mu *lux* to form transcriptional fusions between the *laf* target gene and *lux* genes on the transposon. The insertion of mini-Mu *lux* into the target *laf* gene results in the null phenotype: nonswarming colonies lacking lateral flagella. Transcription of the *lux* genes requires active transcription from a *laf* promoter, and light (hν) is produced when bacteria are grown on an agar surface, but not when cells are liquid grown.

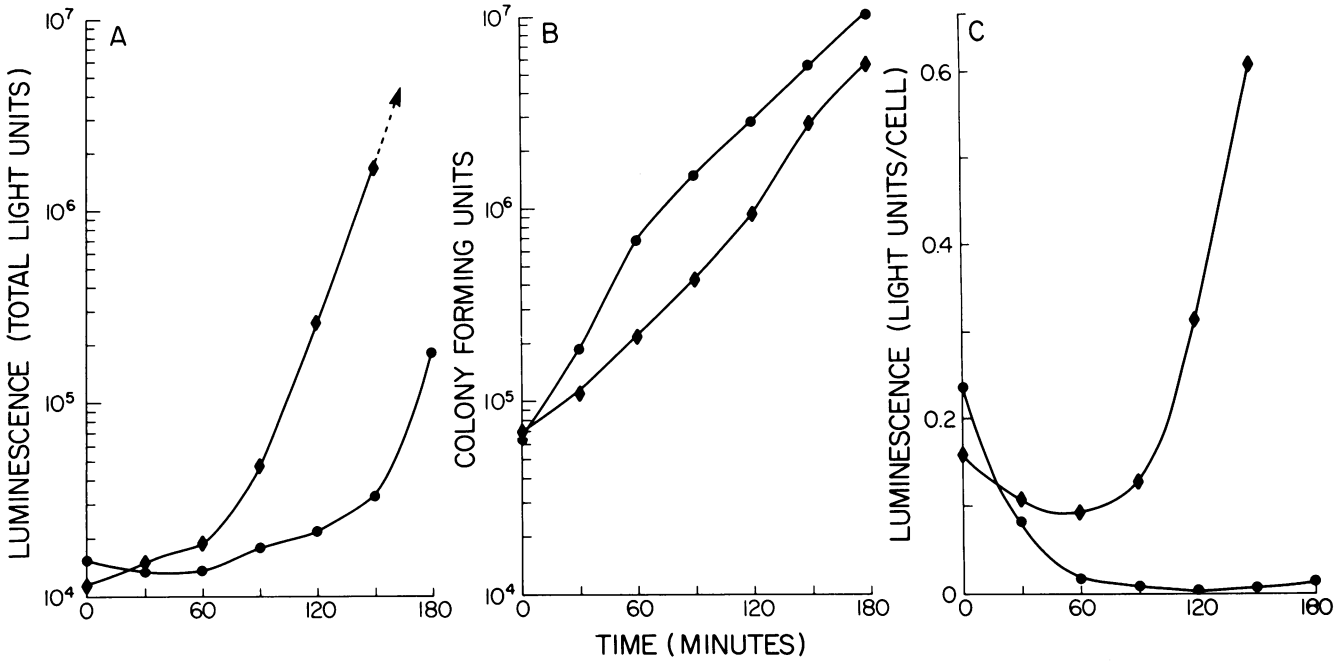

FIG. 2. Kinetics of light production in *laf*::*lux* fusion strain RS3639. (A) Total light production from cells grown in liquid medium (●) and on agar medium (◆). Cells were grown to an OD_{600} of 0.05, and 5 μl was inoculated in either 500 μl of liquid medium or on an agar core. Cells were placed in a scintillation counter, and light was measured at 30-min intervals, using single photon event or chemiluminescence mode. Dashed line and arrow represent increase in luminescence beyond the upper counting limit of the liquid scintillation counter. (B) Growth of cells in liquid and on agar medium. Replicate samples were set up in parallel to those used to record luminescence. These samples were sacrificed at 30-min intervals, diluted, spread on agar medium, and incubated overnight at 30°C to obtain CFU. (C) Luminescence per individual cell for liquid-grown and agar-grown cells. Graph is derived from data represented in panels A and B.

produced light. Since only one of the two possible orientations of transposon insertion can align *lux* gene transcription with that of the target genes, no more than 50% of the fusion strains would be expected to produce light. Insertion into weakly transcribed genes or into untranscribed control regions would also result in fusions which produce no light. Of the nonswarming fusion strains which produced light on agar medium, approximately 70% produced significantly less light (by a factor of about 100) when propagated in liquid medium. The remainder (approximately 30%) produced a comparable amount of light per cell on agar and in liquid medium. Thus, light production in the latter strains appears to be constitutively expressed. By inference, most of the strains which produced light are transposon fusions with *laf* genes whose expression is regulated by growth on surfaces. Characterization of the swarming defects in the Laf^- mutants will be reported elsewhere, but generally speaking, most of the Laf^- mutants which manifested surface-dependent expression of light synthesized no detectable lateral flagellar structures. Analysis of the genetic linkage of *laf* genes is also in progress, and the target *laf* genes in the fusion strains which show surface-dependent expression of light are located in several gene clusters. The *laf*::*lux* fusion strains produced a fused mRNA transcript, but the *laf* and *lux* gene products were synthesized as individual rather than fused polypeptides. Expression of the *lux* genes in the fusion strains depended primarily upon factors which influenced transcription but not translation of the *laf* mRNA. Therefore, we conclude from the properties of the fusion strains that control of expression of most of the *laf* genes operates at the level of gene transcription. A more limited study of *laf* regulation has been performed with *laf*::*lacZ* fusion mutants, and as observed with *laf*::*lux* fusions, production of β-galactosidase with most Lac^+ fusion strains was dependent on growth on the surface of an agar medium (18).

Since sensitive measurement of light production can be made without disturbing the bacterial culture, further experimentation with *laf*::*lux* fusion strains was performed in vials with a scintillation counter (see above). Fusion strains were inoculated on agar medium cores, in liquid medium in microcentrifuge tubes, or in a variety of other configurations inside vials cycled continuously in a scintillation counter monitoring single photon events (chemiluminescence mode). Six *laf*::*lux* fusion strains, RS313 (*laf-313*::mini-Mu *lux*), RS632 (*laf-632*::mini-Mu *lux*), RS1483 (*laf-1483*::mini-Mu *lux*), RS1522 (*laf-1522*::mini-Mu *lux*), RS3639 (*laf-3639*::mini-Mu *lux*), and RS5712 (*laf-5712*::mini-Mu *lux*) were used for detailed study. Data obtained with RS3639 are shown in this report, although similar results were obtained with the other fusion strains. For comparison, measurements were also made with two luminescent Laf^+ fusion strains, RS1495 and RS1581. The identity of the target genes in the latter strains was not known.

As a starting point in our effort to identify the particular physical or chemical factors which induce *laf* gene expression, we examined the influence of medium composition and the stage of growth of the inoculum on induction. For example, induction by growth on an agar medium was judged to have occurred if light production per cell was at least 10-fold higher than that observed for the fusion strain grown in liquid medium. This comparison was made 90 to 120 min after initiation of the experiment. Light production was influenced little by the composition of the medium used. A modified Luria medium (4), marine medium 2216, and heart infusion medium (see above) were used. The stage of growth of the inoculum, however, did affect the response of

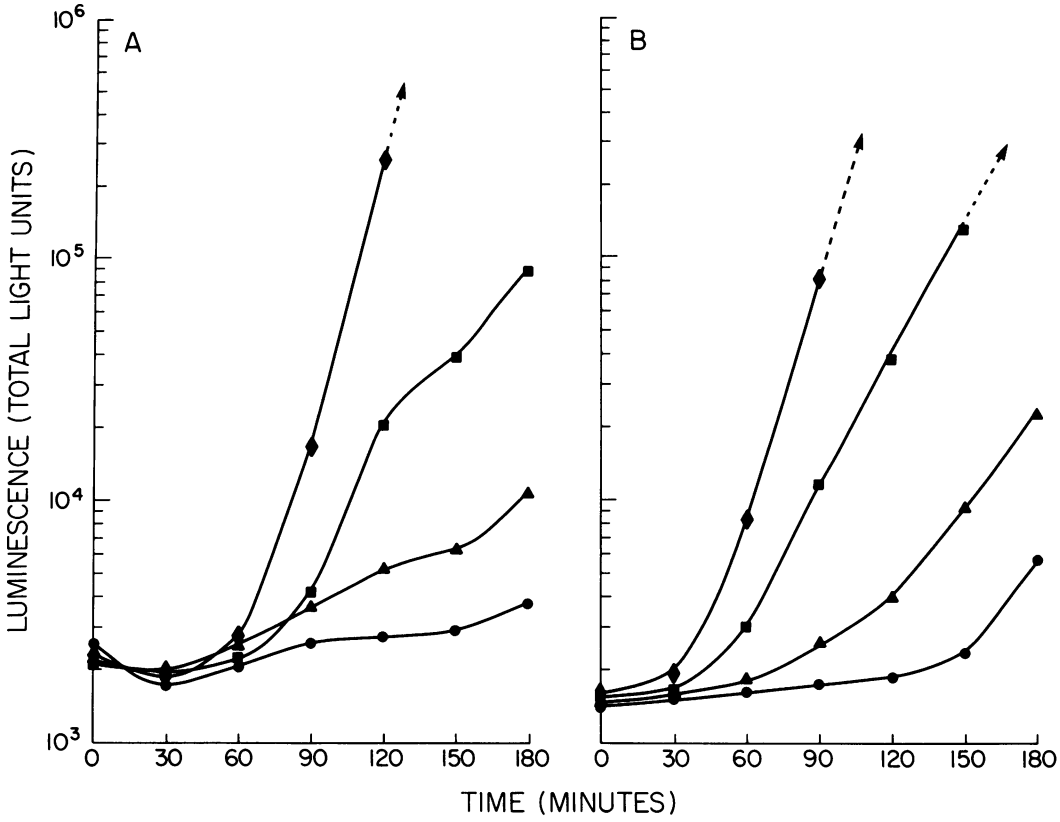

FIG. 3. Induction of luminescence in *laf*::*lux* fusion RS3639 when embedded in agar or when grown in viscous medium. (A) Light production of embedded bacteria as a function of increasing agar concentration. Cells were grown overnight in 2216 medium, diluted 1 to 2,000, and grown at 30°C until the culture reached an OD_{600} of 0.05. A 5-μl sample was added to 500 μl of molten agar at 0% (●), 0.2% (▲), 0.4% (■), and 0.7% (◆) (wt/vol) agar, and the agar was allowed to gel. Embedded bacteria were placed in a scintillation counter set to record single photon events, and luminescence was recorded at 30-min intervals. (B) Luminescence of *laf*::*lux* fusion when grown in 2216 broth amended with 0% (●), 2% (▲), 4% (■), and 10% (◆) (wt/vol) PVP-360. Dashed line and arrow indicate continued increase in luminescence beyond counting range of equipment.

fusion strains to growth on an agar core. Cells were diluted 1 to 2,000 from an overnight culture into fresh liquid medium and grown to a variety of cell densities. Cells from these different suspensions were tested for light production on cores and in liquid media. Inocula from very early logarithmic-phase cultures (OD_{600} of about 0.05 or about 5×10^7 cells per ml) gave very substantial induction of the *laf*::*lux* fusion strains, while cells in the mid-logarithmic phase (OD_{600} of 0.4) or late logarithmic phase (OD_{600} of 0.8) induced poorly. Therefore, cells in the early logarithmic phase were used for subsequent experimentation. The apparent lack of responsiveness of cells from relatively dense cultures will be considered later (see below).

Induction of bioluminescence in strain RS3639 by growth on an agar medium is shown in Fig. 2. At 60 min after initiation of the experiment, total light production from the fusion strain on agar medium increased rapidly relative to that observed for broth-grown cells (Fig. 2A). Cell division must be considered to obtain a measure of light production per cell, so extra experimental samples were grown in parallel and harvested for determination of CFU. Growth was similar and proceeded without a lag for both cultures, although the initial rate of growth on the agar medium was less than for the liquid medium (Fig. 2B). Light production per cell was calculated by dividing total light units by CFU. Light production per cell in the agar medium culture increased most rapidly after 60 min of incubation, but it is apparent that even in the first 30 min of incubation light production by agar-grown cells was increasing relative to that of broth-grown cells. Growth on agar medium of control strains with *lux* fusions in other genes, strains RS1495 and RS1581, did not induce light production (data not shown).

Factors which influence induction. What specific property of growth on an agar surface induced expression of the *laf*::*lux* fusion strains? Experimentation was guided by three general hypotheses. (i) *V. parahaemolyticus* senses the depletion of a component of the nutrient medium. Depletion might occur when cells are grown on a surface because consumption would not be balanced by replenishment owing to diffusion (5). (ii) The cells sense the accumulation of a particular metabolite or substance produced by the cells which accumulates to a relatively high concentration because dispersal is limited to diffusion (5). (iii) The cells sense a physical property of the growth medium. The composition of the growth medium was modified by adding a variety of cations and anions to a concentration of 10 mM. K^+, NH_4^+, Ca^{2+}, Mg^{2+}, Fe^{3+} (as ferric ammonium citrate), NO_3^-, CO_3^{2-}, SO_4^{2-}, and PO_4^{3-} were added to both broth and agar media, but were without effect on the production of light by the fusion strains. Although the concentration of NaCl in the medium affects the motility of *V. parahaemolyticus* (19), adjustment of [NaCl] from 0.5 to 4% (wt/vol) did not affect *laf* induction. Expression of *laf* is apparently not controlled by sensing the osmolarity of the medium. Defined medium

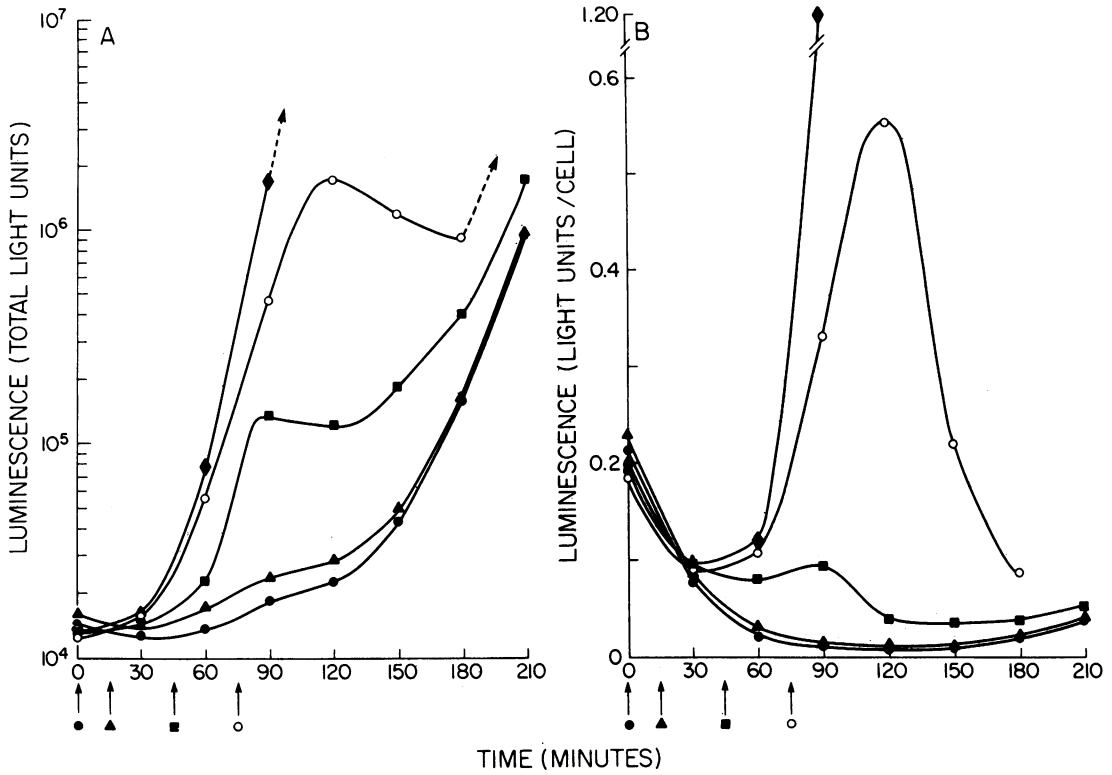

FIG. 4. Kinetics of light production from *laf*::*lux* fusion RS3639 as a function of removal of viscous environment. (A) Total luminescence of RS3639. Cells were grown overnight at 30°C, diluted 1 to 2,000 in 2216 broth, and grown to an OD_{600} of 0.05. A 5-μl sample was inoculated into 50 μl of 10% (wt/vol) PVP-360 in 2216 broth, and at 0 (●), 15 (▲), 45 (■), and 75 (○) min, 1 ml of 2216 broth was added to separate cultures, reducing the viscosity to <0.5% (wt/vol) PVP-360, a concentration of PVP-360 which does not induce transcription of the *laf*::*lux* fusion. The control culture (◆) was not diluted with 2216 broth. Light was measured as described in the Materials and Methods. (B) Light production per individual cell during noninducing conditions. Replicate samples were prepared and sacrificed at 30-min intervals, and the number of bacteria was quantified as CFU. Total light production was divided by the number of CFU and plotted. Symbols are the same as in panel A. Dashed line and arrow represent extrapolation of curves beyond upper limit of detection by liquid scintillation counter.

with reduced PO_4^{3-} (<0.1 mM) and media with Fe^{3+} chelated by EDDA [ethylenediamine-di(*o*-hydroxyphenyl acetic acid)] or with Mg^{2+} chelated by EDTA were also prepared, but these changes did not specifically influence *laf* induction. Depletion of Fe^{3+} actually increased light production substantially with both *laf*::*lux* fusions and the control fusion strains. This effect was probably due to reduction in the synthesis of functional cytochromes which makes more reductant available for the luminescence reaction. Exudates from spent agar plates were collected by repeated freeze-thaw treatments. These exudates, when added to broth cultures, did not stimulate induction of the *laf*::*lux* fusion strains. Inducers of other biological systems such as cyclic AMP and cyclic GMP (at 10 mM); the inducer of bioluminescence, *N*-(β-ketocaproyl) homoserine lactone, in *V. fischeri* (12); and a mixture of compounds (*N*-acetylglucosamine, *N*-acetylmuramic acid, diaminopimelic acid, and D-alanine at 10 mM) which induce developmentally programmed lysis in *Myxococcus* species (15) also did not induce light production of broth-grown fusion strains. Several volatile compounds (propionic acid, isovaleric acid, diethylmalonic ester) reported (20) to induce swarming of *Vibrio alginolyticus* were also ineffective. Attempts to identify a component of the growth medium or a substance produced by the bacteria which caused induction of *laf* were not informative, although an exhaustive search was not made.

The particular gelling agent used did not influence induction since agarose, carrageenan, or Gelrite could be substituted for agar. Furthermore, the surface could be a porous hydrophilic membrane filter since membranes of nitrocellulose, cellulose acetate, or hydrophilic polyvinylidene difluoride positioned over a liquid medium also elicited the induction response for the fusion strains. Growth at an air-water (agar) interface was a common feature of these culture configurations. However, the air-water interface could be eliminated by growing the fusion strain between two agar surfaces (agar core sandwich), and this growth configuration also induced *laf* expression. Since confining the bacteria between two layers of agar medium was effective, the outcome of embedding the fusion strains directly in the agar medium was examined. Strain RS3639 was added to molten agar medium which was then allowed to gel, and confinement in an agar matrix did induce *laf* expression (Fig. 3A). The magnitude of the response was proportional to the concentration of agar used to form the gel, with 0.7% agar giving maximal induction. Light production from agar-embedded cells was similar to that obtained by propagating the fusion strain on the surface of an agar core (1.5% agar).

All of the inducing configurations examined so far impair the ability of the bacteria to move relative to their surroundings. *V. parahaemolyticus* is stationary on an agar surface until lateral flagella are assembled. Chemotaxis studies with *E. coli* commonly employ motility medium which contains 0.3% agar. Higher concentrations of agar impaired the motility of polarly flagellated *V. parahaemolyticus*, but these

higher concentrations were also the most effective at inducing *laf* gene expression. Thus, there appears to be a correlation between the confining nature of the medium and its effectiveness in inducing *laf* expression. Other means to confine the fusion strains in their nutritive environment were sought, and since viscous solutions inhibit the motility of many bacteria including *V. parahaemolyticus*, we examined the influence of viscous media on *laf* induction. Fusion strain RS3639 was added to media containing differing amounts of PVP-360. Media with 7 to 10% (wt/vol) PVP-360, which affects the macroviscosity of the solution (6), were very effective at inducing *laf* expression (Fig. 3B). Maximal induction, achieved at 10% PVP-360, was comparable to or even of higher magnitude than that obtained by any other inducing condition. A 10% (wt/vol) solution of PVP-360 has a viscosity at 22°C of 40 cP. A solution containing 10% PVP-40 did not cause *laf* induction. Solutions with 10% (wt/vol) PEG (PEG 1000, PEG 6000, or PEG 20,000) also did not elicit *laf* expression. Solutions of Ficoll70 and Ficoll400 (up to 30%, wt/vol), which increase the microviscosity of the medium (6) but not the macroviscosity, did not induce expression of the *laf*::*lux* fusion. On the other hand, media with other high-MW polymers used at concentrations which gave the same macroviscosity as PVP-360 also activated the production of light by the fusion strains. These polymers included xanthan gum (0.4%), tragacanth gum (0.7%), guar gum (0.7%), locust bean gum (0.9%), and karaya gum (1.0%). Induction of *laf* was influenced by the macroviscosity of the medium and not by the chemical composition of the particular polymer.

Expression of *laf* in viscous media. Compared with other experimental configurations which induced *laf* expression, it was more convenient to use viscous media for a detailed analysis of *laf* control. The degree of induction could be manipulated by adjusting the concentration of the high-MW polymer. The bacteria could be rapidly shifted to a noninducing environment by simply diluting the viscous media, and samples could be conveniently removed for determination of CFU. It was more difficult to accomplish these operations with embedded or plate-grown bacteria. Measurement of the kinetics of *laf* gene turnon and turnoff in strain RS369 is shown in Fig. 4. The experiment was initiated by inoculating medium containing 10% PVP-360 with strain RS3639, and the shift to the noninducing condition was done by diluting the viscous culture 20-fold with unamended broth medium. Multiple cultures were started in parallel so that single cultures could be diluted at different times to terminate the induction process. Light production per cell in the viscous medium matched that observed for the broth culture control until 30 min of incubation. After 30 min, light production per cell in the viscous medium increased rapidly with respect to the broth culture. The induced culture was producing approximately 100-fold more light than the broth culture after 90 min of incubation. The lag in the onset of induction and the rapid increase of light production were also characteristic of other inducing conditions (Fig. 2 and 3).

When a viscous culture was shifted to the noninducing condition immediately after initiation of the experiment, light production was the same as that observed for a broth culture. No measurable response was evident after 15 min of exposure to the viscous medium, but 45 min of exposure resulted in a significant, albeit transient, induction of luminescence. After 75 min of growth in the viscous medium, induction was even more pronounced. Shifting the fusion strain to the low-viscosity medium did reverse the induction process, but light production continued to increase, at a reduced rate, for about 45 min after the shift. This overshoot will be discussed later.

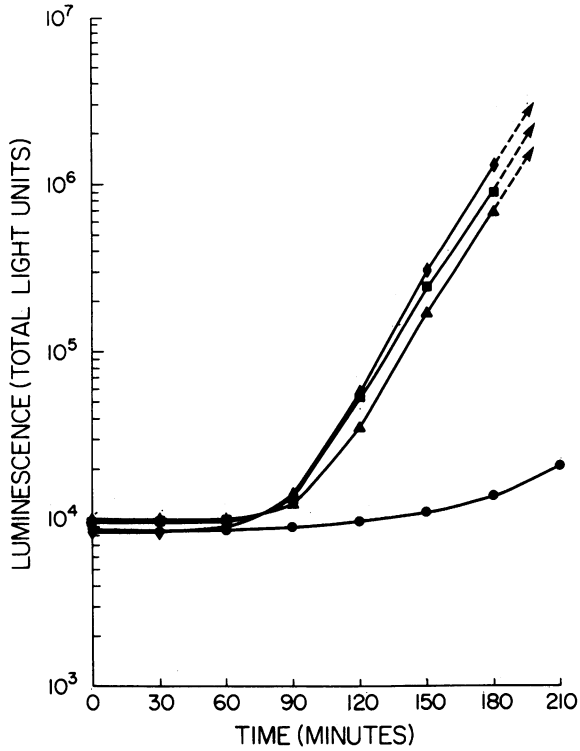

FIG. 5. Effect of bacterial density on induction of *laf*::*lux* fusion grown in 10% (wt/vol) PVP-360. *laf*::*lux* fusion RS3639 was grown overnight at 30°C, diluted 1 to 2,000 in 2216 broth, and incubated to an OD_{600} of 0.05. A 5-μl sample of this culture was inoculated into 10% (wt/vol) PVP-360 to yield a final bacterial concentration of 6.5×10^4 cells per ml (▲), 6.5×10^5 cells per ml (■), or 6.5×10^6 cells per ml (◆). A 5-μl sample of cells was also inoculated into unamended 2216 broth at a final cell concentration of 6.5×10^4 cells per ml (●) and served as a control. Dashed line and arrow indicate light production beyond upper limit of counting.

If *laf* induction was to require communication or an interaction between cells, we would expect induction to be influenced by the density of the cell suspension. The effect of cell density on the induction of *laf* could be measured conveniently by suspending a constant amount of bacteria in different volumes of viscous medium. Cell suspensions with bacteria at densities ranging from 6.5×10^4 to 6.5×10^6 cells per ml were tested for the induction of luminescence. Light production from samples representing a 100-fold range in cell density was very similar (Fig. 5).

The expression of luminescence in *V. fischeri* is regulated by a substance, *N*-(β-ketocaproyl) homoserine lactone (autoinducer), produced by the bacteria (12). Gene induction in *V. fischeri* is strongly influenced by density because an effective concentration of autoinducer is not achieved until the bacterial culture reaches a high cell density. Increasing the viscosity of the medium in which *V. fischeri* was grown did not accelerate induction of the genes controlled by autoinducer (data not shown). Since viscous media did not affect the regulation of a gene system known to be controlled by an effector excreted into the growth environment, it appears unlikely that *laf* expression is controlled by a similar mechanism. Furthermore, the experiment with *V. fischeri*

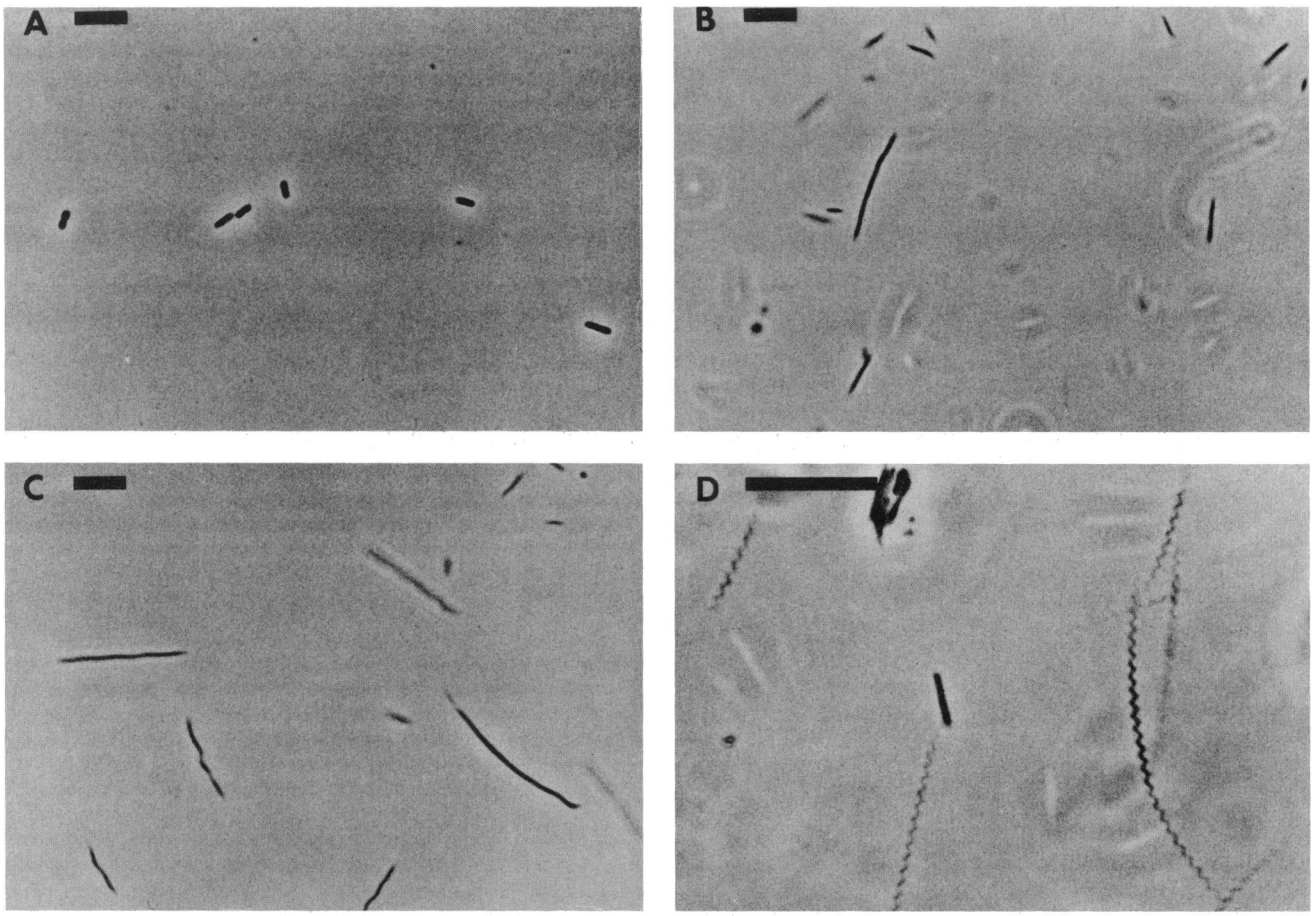

FIG. 6. Induction of Laf$^+$ phenotype in wild-type *V. parahaemolyticus*. Cells were grown overnight in 2216 broth, diluted 1 to 2,000 in fresh 2216 broth, and incubated at 30°C until the density reached an OD_{600} of 0.05. A 5-μl sample was inoculated into 500 μl of 10% (wt/vol) PVP-360 and observed at 0 h (A), 4 h (B), 7 h (C), and 18 h (D). Bar represents 10 μm.

confirms our expectation that the viscous medium used for induction of *laf* does not significantly restrict the diffusion of small molecules. If diffusion was significantly reduced in the viscous medium, autoinducer would accumulate more rapidly around the cells which synthesized it, and induction of the *V. fischeri lux* genes would occur at a lower cell density.

Induction of the Laf$^+$ phenotype. When wild-type *V. parahaemolyticus* is propagated on agar media, the cells produce 100 to 1,000 lateral flagella each (10, 11). An integral part of lateral flagella synthesis is the formation of long, nonseptated cells. This adaption or differentiation to life on an agar surface is pronounced. The cellular dimensions of broth-grown *V. parahaemolyticus* are approximately 0.5 by 1.5 to 2.0 μm. In contrast, agar-grown cells taken from the periphery of an actively swarming colony average ca. 20 μm in length and are often twice as long. The data shown above suggest that viscous media induced transcription of the *laf*::*lux* fusions. Can we extrapolate from a condition which affects *laf* gene transcription to induction of a complex phenotype? Does a viscous medium cause differentiation of the wild-type strain into swarmer cells? Photomicrographs of *V. parahaemolyticus* after 0, 4, 7, and 18 h of incubation are shown in Fig. 6. As was observed when cells were grown on agar media, differentiation to elongated cells did occur when *V. parahaemolyticus* was grown in 10% (wt/vol) PVP-360. Giant flagellar bundles (21), aggregates of sheared lateral flagella seen after overnight incubation on agar media, were prominent in overnight cultures grown in viscous media. Lateral flagellar bundles were not observed when nonswarming *laf*::*lux* fusion strain RS3639, which does not produce lateral flagella, was grown overnight in media containing 10% (wt/vol) PVP-360.

DISCUSSION

Swarmer cells are formed when *V. parahaemolyticus* is propagated on the surface of agar media. These laterally flagellated, elongated cells translocate over an agar surface and give rise to radially expanding colonies which appear similar to those observed with *Proteus* species (22). Since the swarmer cell phenotype is not expressed when the bacteria are grown in liquid media, this differentiation process must be induced by some circumstance peculiar to life on the surface of an agar medium. To simplify analysis of this process, we constructed gene fusion strains in which indicator genes (*lux*) were linked to the genes determining the swarming phenotype (*laf*). This made it possible to study expression of the *laf* genes by measuring the production of light. Construction of the fusion strains was accomplished in vivo with a specialized transposon, mini-Mu *lux*, which upon insertion can align *lux* genes on the transposon with the transcriptional control element of the target gene.

Approximately 70% of the luminescent (Lux$^+$) strains defective in swarming (Laf$^-$) produced significantly more

light per cell (>100-fold) when grown on an agar medium than when grown in a liquid medium. We conclude that most (about 70%) of the *laf* genes are induced by growth on an agar medium and that control of *laf* induction operates at the level of gene transcription since mini-Mu *lux* generates transcriptional fusions. Closer examination of induction on an agar medium revealed that early-logarithmic-phase cells were the most responsive to induction. Bacteria in the interior of a swarming colony do not have the swarmer cell phenotype (21), so a mechanism probably exists to reverse or prevent initiation of the differentiation process. Possibly cells in dense regions of the colony are not differentiated because they have become refractile or unresponsive to the inducing condition.

On the basis of a variety of experiments, we conclude that changes in the viscosity of the medium are sufficient to induce all the phenotypic changes associated with swarmer cell differentiation. Viscous media prepared with a large variety of high-MW polymers were even more effective than agar media in inducing *laf*::*lux* expression in the fusion strains. This was not due to the chemical nature of the polymer since 10% (wt/vol) PVP-360 induced the fusion strains, but a 10% (wt/vol) solution of PVP-40 did not.

Induction in a viscous medium (or on the surface of an agar medium) was not immediate but lagged, becoming pronounced after about 30 min in the inducing environment. We do not know what events are occurring during the lag period, but activation of *laf* gene transcription could be the consequence of a series of information-processing steps. It is clear that transcription of *laf*::*lux* requires the continuous presence of the inducing stimulus. Thus, if the cells are removed from an agar medium or diluted from a viscous medium, transcription rapidly declines. When induction was terminated (Fig. 4), light production continued to increase, but the rate of increase declined rapidly. The bacteria may have a "memory" of the inducing stimulus, but this overshoot in light production could also result from persistence of the mRNA for the *laf*::*lux* genes.

The same conditions which induced light production in the fusion strains also triggered the formation of swarmer cells in the wild-type strain, so we are confident that the results with the *laf*::*lux* fusion strains are relevant to understanding the swarming process. But, what specific signal induces swarming? Can *V. parahaemolyticus* sense viscosity? Motility in liquid media is the result of the polar flagellum; however, this motility is markedly reduced in viscous media. Can the organism actually sense and respond to forces which restrict its movement? Viscometers measure the energy required to move an object relative to its fluid surroundings, and the polar flagellum moves relative to the surrounding fluid and to the cell body. Therefore, this bacterium may have some of the elements of a viscometer. This hypothesis is in accord with our data and suggests that conditions which induce *laf* expression also restrict the movement of the polar flagellum. We are currently analyzing mutants defective in polar flagellum function to appraise the role of this organelle in the regulation of lateral flagella synthesis.

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