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Effect of Transposon-Induced Motility Mutations on Colonization of the Host Light Organ by *Vibrio fischeri*†

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***Vibrio fischeri* is found both as a free-living bacterium in seawater and as the specific, mutualistic light organ symbiont of several fish and squid species. To identify those characteristics of symbiosis-competent strains that are required for successful colonization of the nascent light organ of juvenile *Euprymna scolopes* squids, we generated a mutant pool by using the transposon Mu dI 1681 and screened this pool for strains that were no longer motile. Eighteen independently isolated nonmotile mutants that were either flagellated or nonflagellated were obtained. In contrast to the parent strain, none of these nonmotile mutants was able to colonize the juvenile squid light organ. The flagellated nonmotile mutant strain NM200 possessed a bundle of sheathed polar flagella indistinguishable from that of the wild-type strain, indicating that the presence of flagella alone is not sufficient for colonization and that it is motility itself that is required for successful light organ colonization. This study identifies motility as the first required symbiotic phenotype of *V. fischeri*.**

A number of species in the genus *Vibrio* are found living in intimate association with specific animal hosts. The associations are often pathogenic, for example, those between *Vibrio cholerae*, *V. vulnificus*, or *V. anguillarum* and various vertebrate and invertebrate species (13); however, mutualistic symbioses also exist, such as that between *V. fischeri* and the luminous squid *Euprymna scolopes* (6, 34). By examining genetic determinants that have evolved to play a role in mutualistic as well as pathogenic associations, it may be possible to uncover unifying principles that govern the establishment and development of bacterial colonizations of animal host tissues. In this study, we have used transposon mutagenesis to identify for the first time a required symbiotic determinant of a nonpathogenic, animal-associated bacterium.

Upon hatching, the juvenile *E. scolopes* squid is aposymbiotic (i.e., its nascent light organ is devoid of bacteria); thus, the symbiosis needs to be reestablished with each successive generation (24, 41). To initiate this benign infection, symbiosis-competent *V. fischeri* from the ambient seawater must enter the juvenile light organ through superficial pores and travel down narrow, ciliated ducts that lead into several epithelium-lined crypts (26). An inoculum of fewer than 10 bacterial cells enters the light organ and proliferates so rapidly that within 10 to 12 h the crypts are filled with an extracellular, monospecific culture of about 10^5 *V. fischeri* cells whose luminescence can be easily detected (24, 33).

The natural occurrence of aposymbiotic juveniles, combined with the rapid and readily initiated colonization process, provides the opportunity to test mutant strains of *V. fischeri* for the loss of symbiotic infectivity, thereby identifying genes required for the colonization of the juvenile *E. scolopes* light organ. Motility- and flagellum-associated structures have been shown to be important colonization or virulence determinants in a number of bacterial pathogens of animals (27, 29, 30), and thus motility might be presumed to play a critical role in allowing *V. fischeri* to enter the narrow pores and move into the internal location of the light organ crypts. For these reasons, we hypothesized that nonmotile *V. fischeri* would be unable to successfully colonize juvenile *E. scolopes*.

Numerous examples of virulence mutants have been generated by transposon mutagenesis (5, 18). In particular, genetic studies have successfully used bacteriophage Mu-derived transposons to produce mutants of *V. parahaemolyticus* and *V. harveyi* (3, 4, 21, 38). We thus chose the Mu derivative Mu dI 1681 (7) to mutagenize a symbiosis-competent strain of *V. fischeri*, isolate and identify several classes of motility mutants, and test their ability to colonize juvenile squids. The results demonstrate the importance of motility in the establishment of the mutualistic light organ symbiosis between *V. fischeri* and its host *E. scolopes*.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. *V. fischeri* ES114 is a symbiosis-competent strain isolated from the light organ of an adult *E. scolopes* (6). ESR1, a spontaneous rifampicin-resistant (Rif^r) derivative of ES114, was generated by the method of Miller (25). Like its parent, ESR1 is motile, inducibly luminescent, and capable of colonizing the nascent light organ of juvenile *E. scolopes* (16).

Media and growth conditions. Unless otherwise stated, *V. fischeri* strains were grown at 23°C in LBS medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 2% [wt/vol] NaCl, 0.3% [vol/vol] glycerol, 50 mM Tris-HCl [pH 7.5]) as described by Dunlap (9). When appropriate, media were solidified with 1.5% Bacto Agar (Difco Laboratories, Detroit, Mich.). Transposon mutants were generally cultured at 15°C. Cells were prepared for light organ colonization assays by overnight growth at 23°C in SWT, a complex seawater-based medium (6, 28), with the addition of appropriate antibiotics. One milliliter of this overnight culture was then inoculated into 4 ml of an artificial-seawater-based basal medium containing glycerol without antibiotics (6), and the culture was incubated without shaking. After 8 h of incubation, the culture was diluted again in the same medium and incubated for an additional 24 h.

Escherichia coli strains used in the construction of pPD104

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TABLE 1. Bacterial strains used in this study

Strain	Characteristics[a]	Reference
E. coli		
DH1 P1*cm1 ts*	P1 phage	37
JR234	*recA56*	8
PD101	*zah-735*::Tn*10*Δ(*argF-lac*) *U169*, Mu *cts*	11
PD1681	POI1681, *recA56*	This study
POI1681	::Mu dI 1681	7
S17-1	RP4 *mob* site	39
V. fischeri		
ES114[b]	Wild type, Sm^r	6
ESR1	ES114, Rf^r	16
IL200	ESR1::Mu dI 1681, Mot^+ Fla^+	This study
N209–N217[c]	ESR1::Mu dI 1681, Mot^-	This study
NF201, NF202, NF204, NF205, NF207, NF208	ESR1::Mu dI 1681, Mot^- Fla^-	This study
NM200	ESR1::Mu dI 1681, Mot^- Fla^+	This study
NM203, NM206	ESR1::Mu dI 1681, Mot^- Fla^s	This study

[a] The Fla^s phenotype is an abnormally straight and thin flagellum observed on a small proportion of otherwise nonflagellated cells.
[b] Isolated from the light organ of an adult *E. scolopes* (6).
[c] Not analyzed by electron microscopy for the presence of flagella.

were grown at 37°C in LB medium (35). When appropriate, antibiotics (Sigma Chemical Co., St. Louis, Mo.) were added at the following concentrations (in micrograms per milliliter): chloramphenicol, 30 for *E. coli* and 5 for *V. fischeri*; kanamycin, 100; tetracycline, 15; and rifampicin, 100.

Construction of a transposon delivery plasmid, pPD104. Earlier studies (10, 12) had demonstrated that the vector pSUP102 (Cm^r Tc^r), a pACYC184 derivative that contains the approximately 2-kb RP4 broad-host-range mobilization (*mob*) recognition region, could be conjugatively tranferred into *V. fischeri* from *E. coli* S17-1, a strain engineered to contain the RP4 transfer (*tra*) function genes in its chromosome (39). We therefore chose to use this vector to deliver Mu dI 1681 (Km^r *lacZYA*) into derivatives of *V. fischeri* ES114 as a means of constructing chromosomal insertion mutants. The Mu dI 1618 delivery vector, pPD104, was constructed essentially by the method of Castillho et al. (7) as follows. First, a *recA* derivative of *E. coli* POI1681 (Mu dI 1681, Mu *cts*) (7), designated PD1681, was constructed by P1::Tn*9 clr-100*-mediated transduction from strain DH1 P1*cml ts* (37) of the *recA56* allele from *E. coli* JR234, using selection for resistance to tetracycline provided by the closely linked *srl300*::Tn*10* insertion. PD1681 was then transformed with pSUP102 by the method of Hanahan (19), with selection on LB agar containing chloramphenicol, except that the heat shock step was performed at 30°C. Following heat-induced Mu transposition and cell lysis of PD1681(pSUP102), the lysate was used to transduce *E. coli* PD101 (11), with selection on kanamycin and chloramphenicol. Transductants, which expressed β-galactosidase on LB agar containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; 25 μg/ml) and were sensitive to tetracycline, were indicative of a Mu dI 1681 insertion in the Tc^r gene of pSUP 102; one of these was designated pPD104. This plasmid was isolated from PD101(pPD104) and used to transform S17-1 with selection on chloramphenicol and kanamycin. The presence of Mu dI 1681 in pPD104, and its position in the Tc^r gene, were confirmed by restriction mapping (15). Standard molecular biological methods were followed as specified by Sambrook et al. (35) and Rodriguez and Tait (32).

Transposon mutagenesis of *V. fischeri*. For the isolation of *V. fischeri* transposon mutants, the conjugation procedure was modified from those of Simon et al. (39) and Graf et al. (16) as follows. The donor, *E. coli* S17-1 (pPD104), and recipient, *V. fischeri* ESR1, were grown at 23°C to mid-exponential phase in LBS without antibiotics. The cells were concentrated by centrifugation, and a conjugation mix of approximately 2×10^8 recipient cells and 5×10^7 donor cells was spotted onto LBS agar plates. After incubation for 8 h at 23°C, the entire mix was resuspended in 1 ml of LBS, and 100-μl portions were spread onto the primary selection plates (LBS containing rifampicin and kanamycin) and incubated at 15°C for 72 to 96 h. In initial experiments, these conjugation conditions were varied to study the effects of both the temperature and the length of conjugation time on the number of genomic transposon insertions per recipient.

Isolation of nonmotile mutants. Transconjugants from primary selection plates were individually stabbed into semisoft LBS medium (containing rifampicin, kanamycin, and 0.45% agar) in 96-well microtiter dishes and were incubated at 15°C for between 24 and 48 h. Transconjugants that grew but did not form halo-shaped colonies were presumed to be nonmotile. Such strains were isolated, grown to mid-exponential phase at 15°C in LBS broth containing rifampicin and kanamycin and observed by phase-contrast microscopy for motility. Transconjugants that neither formed halos in soft agar nor were visibly motile were designated nonmotile mutants.

Characterization of nonmotile mutants. All of the nonmotile strains were analyzed for the number of transposon insertions and the retention of pPD104. The presence and number of Mu dI 1681 insertions were determined, both before and after the curing of the delivery vector, by agarose gel electrophoresis followed by Southern blot analysis of total cellular DNA, using a ^{32}P-labeled 9-kb *SalI* fragment of pPD104 containing the *lacZYA* region of Mu dI 1681 as the probe (2, 40). Plasmid retention (indicated by chloramphenicol resistance) was confirmed by Southern blot analysis using the same probe.

Flagellar arrangement and structure of nine of the motility mutants were determined with a Philips 300 transmission electron microscope. *V. fischeri* cells in mid-exponential growth phase were gently pelleted and resuspended in a 2% (wt/vol) filter-sterilized NaCl solution. The cells were then negatively stained with 1% aqueous uranyl acetate on Formvar-coated grids. In addition, the growth rates of nonmotile strains in SWT at 23°C were determined spectrophotometrically, and the luminescence of each of these cultures was quantified with a sensitive photometer (6).

Mutant infectivity assay. A previously described (24) colonization assay was modified to determine whether the nonmotile strains had lost the ability to colonize the nascent light organ of aposymbiotic *E. scolopes*. Briefly, newly hatched juvenile squids were individually placed in vials containing 5 ml of seawater to which had been added a cell suspension of either the wild-type or the mutant *V. fischeri* strain to be tested. The squid was exposed to the bacteria for a period of either 3 or 12 h (the concentration of the bacteria tested was 10^5 or 10^3 CFU/ml, respectively), after which the animal was placed in symbiont-free seawater and maintained for another 2 days at 23°C. Successful colonization was determined both by the appearance of light organ luminescence (determined photometrically) and by the presence of *V. fischeri* CFU in a homogenate of the juvenile light organ (24).

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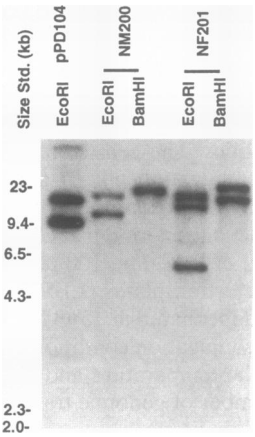

FIG. 1. Southern blot analysis of two nonmotile *V. fischeri* mutant strains indicating (i) a single genomic transposon insertion in NM200, (ii) two insertions in NM201, and (iii) the absence of the donor plasmid in both strains. The ^{32}P-labeled probe was a 9-kb fragment of Mu dI 1681 containing one internal *Eco*RI restriction site and no internal *Bam*HI site. Each transposon insertion in the chromosome produced two fragments that hybridized with *Eco*RI-digested DNA and one fragment that hybridized with *Bam*HI-digested DNA. The *Eco*RI-digested donor plasmid, pPD104, produced one 9-kb and one 15-kb fragment that hybridized with the probe.

RESULTS

Transposon mutagenesis. *V. fischeri* ESR1 was mutagenized by using the transposon Mu dI 1681, which was mobilized on the conjugative plasmid vector pPD104 carried in *E. coli* S17-1. The standard conjugation procedure had an efficiency of about 4×10^{-5} transconjugants per recipient, and 75% of these transconjugants contained single genomic transposon insertions. Because no preferential insertion sites were detected by Southern blot analysis of 80 transconjugants (Fig. 1) (15), the Mu dI 1681 appeared to insert randomly into the genome of *V. fischeri*. This conclusion was further supported by the fact that nonmotile mutants were isolated at a frequency of about 1% among the transconjugants, a proportion similar to the 0.5% obtained in a random mutagenesis of *V. cholerae* (31). A complicating factor in these analyses was that at the selection temperature of 15°C, the donor plasmid was maintained in 94% of the *V. fischeri* recipients (15). Raising the growth temperature to 23°C in the absence of antibiotic selection resulted in the loss of pPD104 from all of the strains tested. While in some of the mutants one or two additional insertions occurred during the curing, NM200 and NM203 possessed only the original single transposon insertion (Fig. 1) (15).

An examination of the effects of conjugation time and temperature on the transposition of Mu dI 1681 in *V. fischeri* ESR1 revealed that when both mating and selection were performed at 28°C (the optimal growth temperature for ESR1), the average number of genomic insertions per transconjugant ranged between 2.5 and 3.4 (Table 2). Either lowering the temperature or shortening the conjugation time resulted in a significant decrease (*t*-test values below 0.045) in the number of genomic insertions per cell. For example, lowering the temperature of mating to 23°C and that of selection to 15°C, as well as limiting the mating time to 12 h or less, resulted in only 1.3 to 1.5 genomic insertions per transconjugant (Table 2). Matings performed either at 15°C or for a period of less than 6 h at 23°C produced no evidence of successful conjugation between *E. coli* and *V. fischeri*.

TABLE 2. Effects of mating and selection conditions on the resulting number of transposon insertions in *V. fischeri*[a]

Mating period (h)	No. of insertions[b]	
	Mate at 23°C and select at 15°C	Mate at 28°C and select at 28°C
6	1.5 (0.8) (n = 12)	2.7 (1.0) (n = 12)
8	1.3 (0.9) (n = 21)	2.5 (0.8) (n = 11)
12	1.5 (0.6) (n = 6)	ND
19	ND	3.4 (0.9) (n = 14)
24	2.5 (0.7) (n = 2)	ND

[a] Conjugations were performed with the indicated combination of mating and selection temperatures. The matings were conducted for a period of between 6 and 24 h.

[b] The mean number of genomic insertions (with standard deviation in parentheses) was determined by Southern blot analysis of n strains. ND, not determined.

Characterization of motility mutants. Of the 2,462 mutants screened, 41 did not form distinct halos when stabbed into soft agar. To eliminate from these presumptive nonmotile mutants those that failed to form halos because of either the loss of chemotaxis or a severe restriction in growth rate, cells of these 41 strains were observed directly for motility. Twenty-four of the forty-one exhibited no sign of motility under phase-contrast microscopy. All of the nonmotile mutants tested, as well as the motile mutant IL200, were luminous and grew in liquid medium only slightly (between 3.5 and 20%) more slowly than the wild-type strain ES114 at 23°C. This finding suggested that the lack of halo formation by the nonmotile mutants did not result simply from a severely depressed growth rate.

Examination by electron microscopy of nine strains revealed that these nonmotile mutants of *V. fischeri* fell into one of three classes: (i) nonmotile yet retaining the characteristic tuft of sheathed, polar flagella (1); (ii) nonmotile and having morphologically abnormal flagella; and (iii) nonmotile and nonflagellated (Fig. 2). Strains observed to be missing flagella were subsequently renamed with the designation NF (not flagellated), and those with nonfunctional flagella were designated NM (not motile) (Table 3).

Of the group of 24 nonmotile mutants, 18 strains (N209 to N217, NF201, NF202, NF204, NF205, NF207, NF208, NM200, NM203, and NM206) contained single genomic insertions. Surprisingly, the donor plasmid was retained in all but one (NM200) of these strains (Fig. 1). It was readily cured from these other strains by subculturing twice at 23°C on LBS without antibiotics, although during this procedure the Mu dI 1681 inserted at additional sites (Fig. 1) (15).

Infectivity of mutants. The capability of these nonmotile mutants to successfully colonize the symbiotic light organ of their animal host was determined by exposing aposymbiotic juvenile squids to suspensions of individual mutant strains under conditions that normally lead to colonization by the parent strain, ESR1. There was no evidence of colonization by any of either the nonflagellated or nonmotile mutants; i.e., host luminescence was not detected (Fig. 3), and CFU were not present in light organ homogenates (Table 3). Even when the mutants were presented in suspensions either at 100 times the concentration (10^5 CFU/ml), or for 4 times the length of time (12 h) typically sufficient for both the parent strain and a motile transposon-carrying strain (IL200) to colonize the juvenile light organ (24), no sign of symbiosis was detected.

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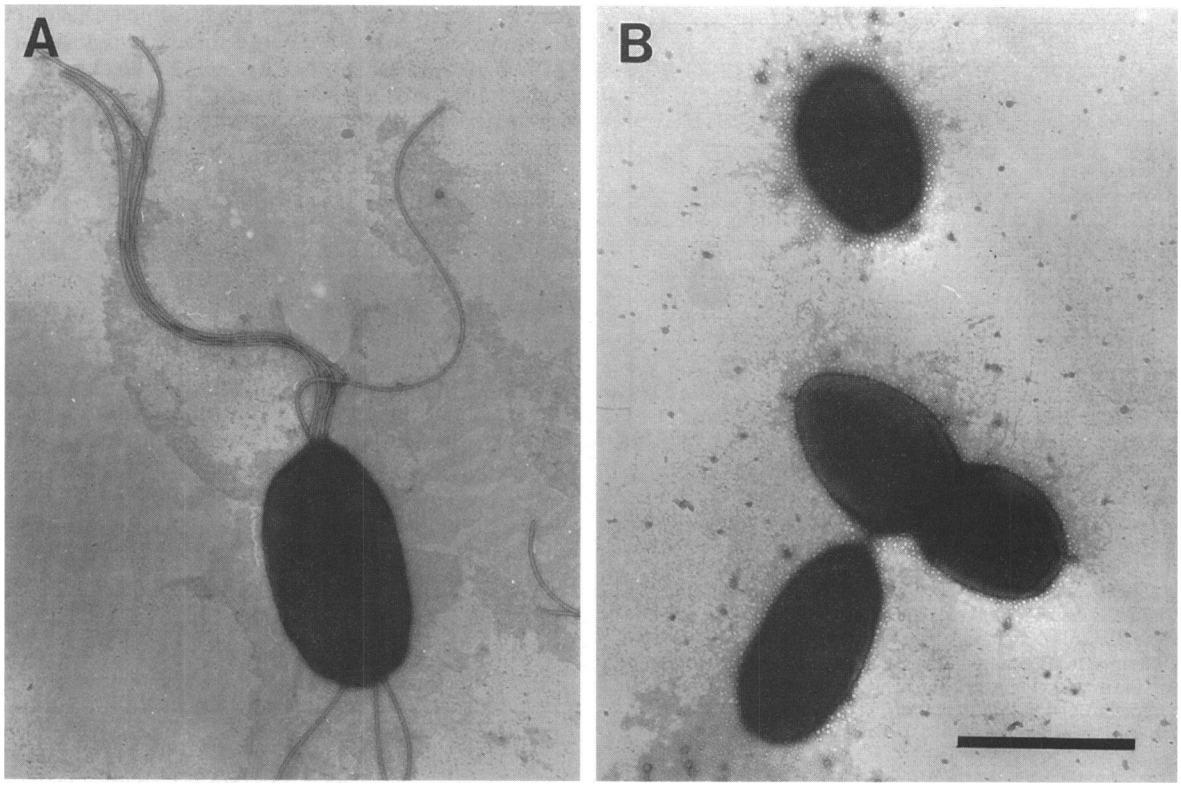

FIG. 2. Flagellar structure of nonmotile *V. fischeri* mutant strains. Transmission electron micrographs of negatively stained cells show the presence of a flagellar bundle similar to that of wild-type cells in strain NM200 (A) and the complete absence of flagella in strain NF201 (B). The size bar is equivalent to 1 μm.

DISCUSSION

Mu dI 1681 mutagenesis. We report here the first identification of a required symbiosis phenotype in an animal-bacterium mutualism. This discovery was made possible by developing the use of transposon mutagenesis in the squid light organ symbiont *V. fischeri*. Previously the RP4 transfer system has allowed the introduction of foreign DNA into *V. fischeri* by conjugation (10, 12). Because bacteriophage Mu-derived transposons have been successfully used to generate random mutational insertions in closely related *Vibrio* spp. (3, 4, 21, 22), we moved the Mu derivative Mu dI 1681 onto a vector containing the RP4 *mob* site (7), allowing transposon delivery into *V. fischeri* by conjugation.

This report demonstrates that Mu dI 1681 can be used successfully to produce mutants of *V. fischeri* with single, apparently random, transposon insertions, thus making in vivo mutagenesis possible. We have also shown that transposition is significantly temperature and time dependent in *V. fischeri*: only when matings were performed at 23°C for less than 12 h, and the recipient ESR1 cells were grown at 15°C, did 70% of the transconjugants contain a single genomic transposon insertion. In addition, 94% of these strains still retained the donor plasmid, although the plasmid was readily lost when the recipient was subsequently grown at temperatures above 15°C.

These results have revealed interesting differences in the process of Mu dI 1681 transposition in *V. fischeri* and *E. coli*. When it is carried in enteric bacteria, a growth temperature of 32°C is apparently sufficiently low to prevent the inactivation of Cts62, the temperature-sensitive repressor of transposition (18); however, Mu dI 1681 will proliferate in *V. fischeri* when the temperature is raised from 15 to 23°C (15). If this

TABLE 3. Characteristics of parent and mutant strains

Strain	Motility[a]	Light organ colonization[b]	
		Luminescence	CFU
ES114	+	+	+
ESR1	+	+	+
IL200	+	+	+
N209	−	−	−
N210	−	−	−
N211	−	−	−
N212	−	−	−
N213	−	−	−
N214	−	−	−
N215	−	−	−
N216	−	−	−
N217	−	−	−
NF201	−	−	−
NF202	−	−	−
NF204	−	−	−
NF205	−	−	−
NF207	−	−	−
NF208	−	−	−
NM200	−	−	−
NM203	−	−	−
NM206	−	−	−

[a] Determined both by the production of halos on soft agar medium and by observation under phase-contrast microscopy.

[b] Colonization of the light organ of juvenile squids was determined both by the production of light by the animal 24 h after exposure to the strain and by the presence of either more than 10^5 (+) or less than 15 (−) CFU per light organ homogenate.

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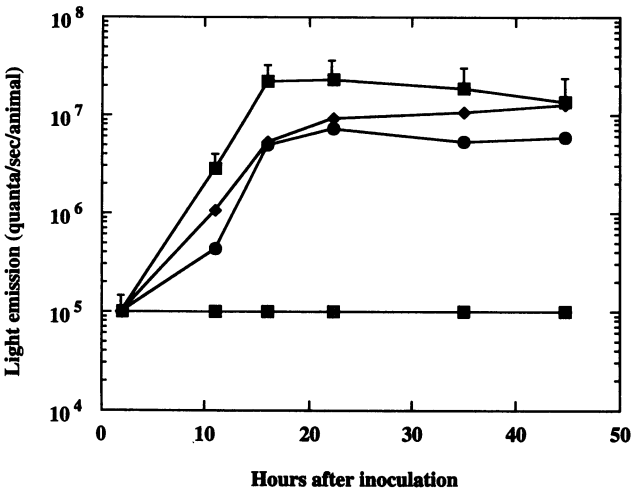

FIG. 3. Light emission by juvenile *E. scolopes* as an indication of light organ colonization by *V. fischeri*. Animals were exposed to suspensions of strains ES114 (■), ESR1 (●), IL200 (♦), NF201 (×), NM200 (+), or no added *V. fischeri* (□) as described in Materials and Methods, and the development of light emission by the animals was monitored periodically. The background electrical noise was equivalent to a reading of 10^5 quanta/s per animal. The datum points are averages of the light emission from five individual animals, and the error bars indicate 1 standard deviation.

subsequent transposition requires inactivation of the normally functional Cts62, then such inactivation appears to occur at a temperature about 9°C lower in *V. fischeri* than in *E. coli*, perhaps reflecting the approximately 10°C difference between the optimal growth temperatures of these two species. In any case, some of the difficulties encountered with the use of Mu dI 1681 in *V. fischeri* can be overcome either by growing *V. fischeri* at 15°C, by using mini-Mu constructions that do not carry the transposase gene, or by using another transposon system such as mTn*5* (17).

Nonmotile mutants. The ability to perform transposon mutagenesis in *V. fischeri*, and to subsequently test the capability of individual mutants to colonize the developing light organ of juvenile *E. scolopes*, has allowed us to begin to identify colonization determinants in a nonpathogenic *Vibrio*-animal host association. Both motility and flagellum structure have been implicated as virulence determinants in several pathogens. Motile, functional flagella are required for *Campylobacter jejuni* infection of 3-day-old chicks (27), and surface antigens associated with the flagellar sheath are needed for efficient host infection by *V. anguillarum* (29). These studies led us to address the importance of either motility or the flagellum structure in the infection of the juvenile light organ.

A total of 18 nonmotile mutants were isolated from the mutant bank, including 6 nonflagellated and 3 flagellated, nonmotile strains. The members of the more numerous nonflagellated mutant class may have had an insertion either (i) in the flagellar structural genes themselves, leading to a cessation of flagellar synthesis, as reported for *Salmonella typhimurium* (14), or (ii) in a gene encoding a positive regulator of the expression of these genes, such as a flagellar sigma factor (20). The less common flagellated, nonmotile mutants, when observed by electron microscopy, fell into two other classes. One class possessed thin and straightened flagella, a morphology probably due to a component of the flagellum or its sheath having been altered or inactivated by the mutagenesis. The other class possessed a tuft of sheathed polar flagella that was indistinguishable from that of the motile parent strain (Fig. 1). Although these mutants could elaborate the flagellar structure, some motor function was apparently interrupted. Taken as a whole, the nonmotile mutants ranged from having apparently wild-type flagella to having no flagellar structure.

When each of these nonmotile mutants was tested for the ability to colonize the juvenile squid light organ, none was able to initiate a symbiosis. Because the flagellar tuft of NM200 seemed identical to that of wild-type *V. fischeri*, the most parsimonious conclusion appears to be that motility itself is an essential symbiotic determinant; the question of the whether a flagellum is required not only for motility but also for adherence to host tissue, cannot be addressed. It is still formally possible that all of these different nonmotile mutants either (i) are regulatory mutants that coregulate both symbiotic determinants and motility (as in *S. typhimurium* [36]) or (ii) have symbiotic and motility genes in the same operon. This possibility will remain open until the genetics of *V. fischeri* flagella elaboration are better described. However, it seems unlikely that all of the nonmotile mutants described here can be explained in this manner.

These results lead us to hypothesize that during the initiation of the symbiotic infection, the bacteria are not simply swept along by epithelial cell ciliary movement of the host (24, 26) but are active participants in the colonization process, propelling themselves into the crypts of the nascent light organ. The bacteria are perhaps guided by a chemotactic response to a host-derived attractant(s) that directs them to proceed through the pore and into the crypts. Once there, the bacteria may be recognized by specific host tissue adhesins (23), in a process for which the flagella may yet be found to play another vital role. In any case, this study identifies only the first of what will likely be a panoply of classes of mutants with lesions in genes required by *V. fischeri* to colonize the light organ of juvenile *E. scolopes*.

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