Published in final edited form as:
Can J Microbiol. 2012 January ; 58(1): 29–38. doi:10.1139/w11-107.

Role for *cheR* of *Vibrio fischeri* in the *Vibrio*-Squid Symbiosis

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Abstract

Upon hatching, the Hawaiian squid *Euprymna scolopes* is rapidly colonized by its symbiotic partner, the bioluminescent marine bacterium *Vibrio fischeri. V. fischeri* cells present in the seawater enter the light organ of juvenile squid in a process that requires bacterial motility. In this study, we investigated the role chemotaxis may play in establishing this symbiotic colonization. Previously we reported that *V. fischeri* migrates toward numerous attractants, including *N*-acetylneuraminic acid (NANA), a component of squid mucus. However, whether or not migration toward an attractant such as squid-derived NANA helps the bacterium to localize toward the light organ is unknown. When tested for the ability to colonize juvenile squid, a *V. fischeri* chemotaxis mutant defective for the methyltransferase CheR was outcompeted by the wild-type strain in co-inoculation experiments, even when the mutant was present in 4-fold excess. Our results suggest that the ability to perform chemotaxis is an advantage during colonization, but not essential.

Keywords

Vibrio fischeri; chemotaxis; colonization; CheR; symbiosis

Introduction

Over the past few decades, the study of long-term associations, or symbioses, between animals and microorganisms has become an important area of research in both the fields of microbial ecology and pathogenic microbiology (e.g., (Dethlefsen *et al.*, 2007; Nyholm and Nishiguchi, 2008; Ruby *et al.*, 2004)). The *Vibrio*-squid symbiosis has emerged as a prominent model for studying the establishment of a symbiotic relationship between animal host and microbial symbiont. The bobtail squid host, *Euprymna scolopes*, is found in the shallow waters off of the island of Oahu, and rapidly becomes colonized by the bioluminescent bacterium, *Vibrio fischeri*, shortly after hatching (for reviews, see (McFall-Ngai, 1999; Nyholm and McFall-Ngai, 2004; Stabb, 2006; Visick and McFall-Ngai, 2000). The juvenile squid's symbiotic light organ has a physiology that permits entrance into, and permanent colonization by *V. fischeri*, while excluding all other species of bacteria found in the seawater.

Newly-hatched, non-colonized squid encounter *V. fischeri* cells in seawater being vented through the mantle cavity of the animal. Cilia present on specialized tissue appendages of the light organ help direct the bacteria towards the organ's surface (Nyholm *et al.*, 2000; Nyholm *et al.*, 2002). The bacteria are thus swept into the vicinity of the light organ where

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they accumulate in mucus secreted on the surface of the light organ in response to bacterial exposure (Nyholm *et al.*, 2000; Nyholm *et al.*, 2002). To colonize their host, bacteria attached to the surface of the light organ must enter the pores, traverse through ducts and antechambers, both of which are non-permissive for colonization, before reaching the sites of colonization, or crypts, where they grow to a high density (Sycuro *et al.*, 2006). It is in these crypts that *V. fischeri* begins to produce bioluminescence, which is used by the squid to avoid detection by predators (Jones and Nishiguchi, 2004).

Motility is critical for colonization, as non-motile mutants fail to colonize the cypts (Graf *et al.*, 1994; Millikan and Ruby, 2003; Wolfe *et al.*, 2004). Given the complex path to colonization, it seems likely that the ability to sense and move toward a chemoattractant, known as chemotaxis, may also play a role. For example, chemotaxis may be important in the ability of *V. fischeri* to migrate to the pores of the light organ and/or to traverse through the ducts and antechambers and ultimately reach the internal crypts. Other marine vibrio species use chemotactic motility to colonize their hosts: *V. anguillarum* and *V. alginolyticus* migrate toward mucus of their respective fish hosts (Bordas *et al.*, 1998; Larsen *et al.*, 2001; O'Toole *et al.*, 1996; O'Toole *et al.*, 1999). We have previously identified a number of attractants for *V. fischeri* (DeLoney-Marino *et al.*, 2003), one of which (*N*-acetylneuraminic acid, NANA) is a component of the squid-derived mucus secreted on the symbiotic light organ (Nyholm *et al.*, 2000). In addition, analysis of gene expression profiles of both free-living and symbiotic *V. fischeri* identified a number of transcripts that were solely expressed by planktonic cells, including two genes for methyl-accepting chemotaxis proteins (Guerrero-Ferreira and Nishiguchi, 2010), suggesting that a chemotactic attraction may help to guide the bacterium into the squid light organ, but may no longer be necessary once the microbe has established symbiotic colonization.

Chemotaxis, best-studied in *Escherichia coli* and *Salmonella typhimurium*, enables a bacterium to migrate toward an attractant by extending the time the bacterium rotates its flagella counter-clockwise (CCW), resulting in a "run" (for reviews, see (Eisenbach, 2007; Wadhams and Armitage, 2004)). These runs are interrupted continuously by short intervals of clockwise rotation (which decrease in frequency in the presence of attractant), resulting in tumbling and nearly random changes in direction. In *E. coli* and *S. typhimurium*, the signal transduction pathway that permits cells to make these comparisons consists of a group of cytoplasmic proteins which enable transmembrane chemoreceptors, known as methyl-accepting chemotaxis proteins (MCPs), to communicate with switch components of flagellar motors (reviewed in (Clausznitzer *et al.*, 2010)). One of these signaling proteins is CheY, which interacts directly with the flagellum to cause a reversal in rotation, resulting in a tumble and permitting cells to re-orient. In *V. fischeri*, *cheY* is important for colonization: a *cheY* mutant was outcompeted by wild-type cells for colonization of juvenile squid (Hussa *et al.*, 2007). These data suggested that chemotaxis may be important for symbiotic colonization, although little else is known about the role chemotaxis may play in the establishment of the *V. fischeri*-squid symbiosis.

Once cells such as *E. coli* and *Salmonella* recognize and respond to a stimulus, the bacteria must reset their response to ligand binding (monitored as receptor methylation) so that they may either increase or decrease the frequency of tumbling, depending on where they find themselves in the gradient of attractant (Macnab and Koshland, 1972). The methyltransferase CheR and the methylesterase CheB attenuate the signaling states by adding and removing methyl residues from MCPs, allowing the cells to continuously adapt to changes in a gradient of attractant (reviewed in (Lux and Shi, 2004)). *E. coli cheR* mutants cannot methylate the MCPs, and as a consequence cannot adapt: they cannot reorient their direction to detect gradients of attractants (Manson, 1992). Such chemotaxis

mutants do not swim toward attractants and therefore can be used to study the role of chemotaxis in certain processes such as colonization.

The colonization defect of the *cheY* mutant suggests that the ability to control the chemotactic excitation response is important to symbiotic colonization by *V. fischeri*; however, whether or not the ability to adapt to changes in gradient of an attractant (such as encountering squid secretions) via a CheR-based attenuation of an excitation signal is also needed to establish colonization is unknown (for a review of the chemotactic signal transduction pathway, see (Szurmant and Ordal, 2004). To further explore the role of chemotaxis in the symbiotic colonization of *V. fischeri* with *E. scolopes*, we identified a gene that encodes a homolog to the CheR protein of *E. coli*. We subsequently constructed a *cheR* mutant of *V. fischeri* and examined the role of CheR in chemotaxis in laboratory media. Finally, we evaluated the ability of the *cheR* mutant to form a symbiotic colonization with juvenile *E. scolopes*. We conclude from these studies that *V. fischeri* uses a CheR protein to promote chemotaxis, likely by functioning in a manner similar to the homologous *E. coli* protein, and that disabling this activity significantly decreases the bacterium's ability to compete against wild-type cells for colonization of juvenile squid.

Materials and Methods

Strains and media

Strains used in this study are listed in Table 1. *V. fischeri* strains were grown in seawater-tryptone (SWT) (Yip *et al.*, 2005) or LB-Salt (LBS) (Dunlap, 1989; Graf *et al.*, 1994) media for routine culturing. For experiments in which motility was important, we used SWT, while LBS was the medium of choice for genetic manipulation of *V. fischeri*. In addition, for motility experiments, we used tryptone broth seawater (TB-SW) (DeLoney-Marino *et al.*, 2003). *E. coli* strains were grown in Luria Bertani (LB) medium (Davis *et al.*, 1980). Agar was added to a final concentration of 0.25% for soft agar and 1.5% for solid agar. Where appropriate, antibiotics were added to the following final concentrations: Chloramphenicol (Cm), 1-5 μg ml^{-1} for *V. fischeri* and 30 μg ml^{-1} for *E. coli*; Erythromycin (Erm), 5 μg ml^{-1} for *V. fischeri* and 150 μg ml^{-1} for *E. coli*; Kanamycin (Kn), 100 μg ml^{-1} for *V. fischeri* and 50 μg ml^{-1} for *E. coli*.

Soft agar and motility assays

Cultures of *V. fischeri* were grown at 28°C to mid-exponential phase (OD_{600} ≈ 0.3-0.7) in SWT. For chemotaxis studies, aliquots of approximately equal numbers of bacterial cells were inoculated near the center of soft agar motility plates and incubated at 28°C for 5 to 22 h. Soft agar motility plates were handled as described previously (DeLoney-Marino *et al.*, 2003). Images were taken with a digital camera.

Molecular and genetic techniques

All plasmid constructions were carried out using standard molecular biology techniques with restriction and modifying enzymes obtained from New England Biolabs (Beverly, MA) or Promega (Madison, WI). Plasmids used or constructed in this study are shown in Table 1. Conjugations were performed as described previously (DeLoney *et al.*, 2002; Visick and Skoufos, 2001).

Flagella and chemotaxis genes were cloned by complementing a non-motile transposon-insertion mutant (KV661) with a plasmid library of *Bgl*II-digested ES114 chromosomal DNA. The library was conjugated into KV661, and the resultant conjugation mixture was inoculated onto a soft agar motility plate. The plates were incubated at 28°C until motile transconjugants swam away from the inoculation site. From the motile bacteria, a plasmid

(pKV119) was isolated that contained a 5.5 kb chromosomal fragment. A portion of this plasmid was subcloned into the conjugal vector pEVS79, generating pAM1. To obtain additional flanking DNA, pAM1 was recombined into the ESR1 chromosome; the chromosomal DNA was then digested with enzymes that cut outside the vector, and self-ligated. The resulting plasmid, pAM4, contained an additional 3 kb of upstream DNA.

Strain construction

The *cheR* frameshift mutant KV1172 was constructed with strains and plasmids as described in Fig. 2. KV1172 was complemented with a wild-type copy of the *cheV-cheR* region inserted in single copy at the Tn*7* insertion site (*att* Tn*7*) (Bao *et al.*, 1991) in the *V. fischeri* chromosome as follows. *E. coli* cells carrying the Tn*7*::*cheV-cheR*-containing plasmid pCD5 served as a donor in a tetra-parental mating, which also included *E. coli* cells carrying the transposase-encoding plasmid pUX-BF13, *E. coli* cells carrying the self-transmissible plasmid pEVS104, and the *V. fischeri* recipient (KV1172). Similarly, antibiotic-resistant *cheR* mutant strain KV1424 and wild-type strain KV1421 were constructed by introducing pNDM50 (Tn*7*::*cm*) and pEVS107 (Tn*7*::*erm*) respectively into KV1172 and ES114 by conjugation. The resulting transconjugants were screened for loss of Kn resistance (resulting from loss of the delivery plasmid) and stable antibiotic resistance (Cm or Erm, resulting from the integration of the appropriate Tn*7* cassette at the Tn*7att* site).

Southern blotting

KV1176 (derived from KV1172) was confirmed by Southern blot analysis as follows. Chromosomal DNA isolated from *V. fischeri* strains ES114 and KV1172 was digested with either *Hpa*I (which cuts once within *cheR*), or *Hpa*I/*Xba*I (which cuts twice within and twice outside *cheR*). DNA fragments were separated by electrophoresis, transferred onto a nylon membrane (Hybond XL, Amersham-Pharmacia Biotech, Piscataway, NJ), hybridized and detected as described previously (DeLoney *et al.*, 2002). Purified pAM1 plasmid DNA was used to generate digoxygenin-labeled DNA complementary to the *cheR* gene, which served as the probe.

Colonization assays

To determine whether the *cheR* mutant was able to form a symbiotic association with *E. scolopes*, cells were inoculated into artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH) as a 1:1, 1:2, or 1:4 mixture of wild-type and *cheR* mutant cells to a final concentration of 2000 to 6000 cells per ml. Juvenile squid were then placed into this inoculum and analyzed for presence of bacteria in the light organ as described previously (Ruby, 1996). The actual ratio of wild-type to mutant cells was determined by plating a dilution of the homogenate onto SWT and selective media containing either Erm or Cm. The limit of detection is 14 *V. fischeri* cells per squid.

Animal use

Squid were handled according to the protocols approved by the Institutional Animal Care and Use Committee at Loyola University Chicago.

Results

Identification of the *cheR* gene of *V. fischeri*

To further understand the role of chemotaxis in symbiotic colonization by *V. fischeri*, we chose to generate and characterize a *cheR* mutant. We began with a search for flagella and chemotaxis mutants of *V. fischeri* from a library of Tn*10*::*luxAB* transposon mutants. We further characterized one of the non-motile mutants, KV661, by complementing the motility

defect with a chromosomal DNA library. The complementing construct contained, along with several other genes, a putative *cheR* gene, VF_1878 (Fig. 1A). We used comparative BLAST analyses to evaluate the putative CheR protein (Marchler-Bauer and Bryant, 2004; Marchler-Bauer *et al.*, 2009; Marchler-Bauer *et al.*, 2011). These analyses revealed that, in contrast to some other bacteria, the *V. fischeri* genome contained a single copy of the *cheR* gene. They also revealed that the predicted protein was homologous to numerous characterized and putative CheR proteins. The most closely related, with E-values of zero, were six putative CheR homologs found in related vibrio species. The alignment of the *V. fischeri* protein with two of these related CheR proteins, from *Aliivibrio salmonicida*, (95% identity, 99% positives, 0% gaps; (Hjerde *et al.*, 2008) and *Vibrio harveyi* (87% identity, 93% positives, 0% gaps; Naval Research Laboratory sequencing effort GenBank CP001223-5), is shown in figure 1B. For comparison, we also analyzed a characterized CheR protein from *E. coli*, which yielded a much lower sequence homology with only 31% identity, 54% positives and 7% sequence gaps (Fig. 1B) (*E*-value of 5e-38; (Mutoh and Simon, 1986)).

Importantly, our analysis revealed the presence of essential conserved domains. The protein encoded by VF_1878 contained the catalytic S-adenosylmethionine-dependent methyltransferase (SAM or AdoMet-MTase) domain common to the methyltansferase superfamily, as well as both the N-terminal all-alpha domain and beta-subdomains of the catalytic domain found exclusively in chemotaxis methyltransferases (Shiomi *et al.*, 2002). Within those domains, a number of important residues known to be important for function in the well-studied microbe *E. coli* were conserved (Fig. 1B). For example, residue Gly-23 corresponds to Gly-39 of CheR of *E coli*, which determines a crucial structural component in the protein and is known to be strictly conserved in all CheR homologues (arrow in Fig. 1B). In addition, the (G/A)X(G/A/S)XG signature sequence, which is found in most MTase catalytic domains, was conserved (asterisks in Fig. 1B) (Shiomi *et al.*, 2002). We conclude from these analyses that VF_1878 likely encodes a protein with a function similar to the CheR protein of *E. coli*.

Genetic approaches to generate a *cheR* mutant

We used these cloned sequences to generate *cheR* mutants as follows. First, we subcloned *cheR* and flanking DNA into a plasmid that could be used for recombination in *V. fischeri*, thus constructing pAM1 (Table 1). However, the *cheR* gene was located at one end of the cloned chromosomal DNA (in both pAM1 and its precursor), leaving no room for homologous recombination upstream of the *cheR* gene (Fig. 2A). We therefore devised an approach to overcome this problem. Specifically, we introduced this plasmid into *V. fischeri*, and sought and obtained a recombinant that integrated the plasmid into the chromosome (Fig. 2A). We then isolated chromosomal DNA from that strain, digested it with *Hind*III, ligated the DNA fragments and used the ligation to transform *E. coli* cells, selecting for chloramphenicol resistance. With this approach, we obtained plasmid pAM4, which contained *cheR* gene and flanking DNA, including an additional ~3 kb of DNA upstream of *cheR*, sufficient for subsequent gene replacement approaches.

Using pAM4, we generated two additional constructs that contained disruptions of the *cheR* gene. The *cheR* gene in pAM9 had an insertion of the erythromycin gene, while the *cheR* gene in pAM7 contained an unmarked frame-shift mutation. To obtain *cheR* mutants, we first used pAM9 to introduce the marked mutation into *V. fischeri* to construct strain KV1007 (Fig. 2B). Then, we used KV1007 as a recipient and pAM7 as donor DNA to replace the erythromycin cassette with the unmarked frame-shift mutation to generate KV1172 (Fig. 2C). We used this latter unmarked mutant, KV1172, in our subsequent studies as we previously determined that cells carrying the erythromycin resistance cassette exhibited slight defects in growth and motility in soft agar assays (O'Shea *et al.*, 2006).

Finally, to ensure that any phenotype we observe was due to the loss of *cheR* and not to a spontaneous mutation elsewhere, we introduced the *cheR* operon (including the upstream gene *cheV*) into the chromosome of KV1172 at the Tn*7* site, a neutral location that permits single copy complementation, thus generating KV1176.

Migration of chemotaxis mutants in soft agar assays

To evaluate the role of *cheR* in chemotaxis, we used soft agar assays as described previously (DeLoney-Marino *et al.*, 2003). Briefly, when inoculated near the center of a petri plate containing semisolid (soft) nutrient agar, motile bacteria migrate outward in concentric bands formed by chemotactic responses of the bacteria to spatial gradients of attractants resulting from metabolism and diffusion of the attractants (Wolfe and Berg, 1989). To observe the chemotactic behavior of the *V. fischeri cheR* mutant in soft agar, we inoculated log-phase cells of the unmarked *cheR* mutant along with control strains onto the surface of TB-SW soft agar. Within 3 h of incubation at 28°C, wild-type strain ES114 began to migrate beyond the point of inoculation, and within 5 to 6 h, this strain had formed the two distinct chemotaxis rings typical for *V. fischeri* (Fig. 3A and data not shown) (DeLoney-Marino *et al.*, 2003). In contrast, no migration was observed for the *cheR* mutant after 5 to 6 hours incubation at 28°C, a phenotype that was indistinguishable from that of the *cheY* mutant KV2510 and the non-motile mutant KV661 (Fig. 3A). By 22 hours, a fuzzy haze of growth characteristic of diffusion in soft agar had appeared around the *cheR* mutants (KV1007 and KV1172) but not around the non-motile mutant KV661 (Fig. 3B). Furthermore, despite this migration, the *cheR* mutant failed to form concentric migrating bands. This phenotype is indicative of a chemotaxis defect (Fig. 3B). Together, these data indicated that the *cheR* mutants were not non-motile, but exhibited a severe defect in directed migration through soft agar.

Our data were consistent with what is known for the well-characterized *cheR* mutants of *E. coli*. Specifically, due to an inability to methylate the MCPs, such *cheR* mutants have extended periods of runs and cannot bias their movement in a specific direction (Springer and Koshland, 1977; Stock *et al.*, 1981). This same smooth swimming phenotype has been seen in *cheR* mutants of other bacterial species as well (Jones *et al.*, 1992; Stephens *et al.*, 2006), including in *V. anguillarum* (O'Toole *et al.*, 1996), although in *Bacillus subtilis*, *cheR* mutants exhibit a "tumbly" phenotype (Kirsch *et al.*, 1993). To determine if the *V. fischeri cheR* frameshift mutant exhibited a similar swimming behavior to that of *E. coli cheR* mutants, we examined the strains microscopically. When visualized for motility microscopically, the *cheR* mutant swam predominantly in extended runs, while the wild-type strain exhibited runs interspersed with frequent tumbles and the non-motile mutant did not display any apparent motility other than Brownian motion (data not shown). These results suggested that the inability of the mutant to migrate in TB-SW soft agar did not result from an inability to swim, but rather from a decreased ability to change the direction of its flagellar rotation and thus navigate the channels in the agar (Wolfe and Berg, 1989). When complemented with a wild-type copy of the *cheVR* genes, the *cheR* frameshift mutant migrated at a rate similar to that of the wild-type strain (Fig 3A). Thus, the *cheR* mutant KV1172 displayed motility and chemotactic behaviors characteristic of *E. coli cheR* mutant strains, and these phenotypes could be rescued with a wild-type copy of *cheR*. We conclude that VF_1878 encodes a CheR homolog.

Colonization by the *cheR* mutant

Because our data supported the identification of *cheR* as a chemotaxis gene with the same function that has been demonstrated for other organisms (Springer and Koshland, 1977; Stock *et al.*, 1985), we proceeded to determine whether CheR plays an important role in colonization by *V. fischeri* of its symbiotic host *E. scolopes*. To evaluate the role of *cheR*,

we assayed the ability of the frame-shift *cheR* mutant to form a symbiotic association with juveniles of the squid *E. scolopes* when presented in competition with a chemotaxis-proficient (wild-type) strain. Newly hatched juvenile *E. scolopes* were inoculated into seawater containing various ratios of parent strain to mutant. When co-inoculated with a ratio of 1:2 wild-type to mutant, wild-type bacteria represented 100% of the recovered cells from 8 of 9 animals (Fig. 4). Even when inoculated with a wild-type to mutant ratio of 1:4, 8 of 10 animals were predominately colonized by the parent strain (Fig. 4), indicating that the *cheR* mutant has a colonization disadvantage compared to the wild-type strain. These data demonstrate that CheR plays a critical role in symbiotic colonization and suggest that the ability to perform chemotaxis is an advantage during colonization.

Discussion

A number of studies have investigated the role chemotactic motility plays in colonization of animal hosts, both in pathogenic (e.g., (Bordas *et al.*, 1998; Foynes *et al.*, 2000; Hawes and Smith, 1989; Larsen *et al.*, 2001; O'Toole *et al.*, 1996; O'Toole *et al.*, 1999; Terry *et al.*, 2005; Yao and Allen, 2006) and symbiotic associations (e.g., (Bashan and Holguin, 1994; Miller *et al.*, 2007; Yost *et al.*, 1998)). While the requirement of normal motility for symbiotic colonization of juvenile squid by *V. fischeri* is well established (Graf *et al.*, 1994; Millikan and Ruby, 2002; Nyholm *et al.*, 2000), only a few studies have investigated the role chemotaxis may play in establishing the *Vibrio*-squid symbiosis (Guerrero-Ferreira and Nishiguchi, 2010; Hussa *et al.*, 2007). A *cheY* chemotaxis mutant of *V. fischeri* was found to exhibit a competitive colonization defect compared to wild type (Hussa *et al.*, 2007), suggesting that the ability to control the excitation response to a chemical gradient is important to symbiotic colonization by *V. fischeri*. In this work, we characterized the ability of a *cheR* chemotactic adaptation mutant of the marine bacterium *V. fischeri* to perform chemotaxis in laboratory culture and investigated the role of this behavior during symbiotic association with the Hawaiian squid *E. scolopes*.

We identified the *cheR* gene during our sequence analysis of a flagella gene cluster. The predicted protein exhibited extensive sequence identity with CheR proteins of other vibrio species and contained the three domains commonly found in CheR homologues. We subsequently mutated *cheR* by frame-shift mutation. The mutant exhibited defects similar to that of *E. coli cheR* mutants: smooth swimming in liquid culture and an inability to migrate proficiently through soft agar. Finally, we determined that a *V. fischeri cheR* chemotaxis mutant exhibits a competitive disadvantage compared to the parental wild-type strain to colonize juvenile squid even when present in 4-fold excess over the wild-type strain.

Previously, two possibilities were suggested to account for the defect of the *cheY* mutant in colonization (Hussa *et al.*, 2007). One was that the mutant failed to sense and respond to a gradient of attractant that would direct the cells to the crypts. The second was that the smooth swimming phenotype of the *cheY* mutant resulted in the cells getting "stuck" in the mucus, similar to their getting stuck in the soft agar medium. For the *cheR* mutant, both explanations are possible. We hypothesized that, if the *cheR* mutant were getting stuck in the mucus, it might colonize poorly even when presented to the squid alone (rather than in competition). To test this idea, we performed several single strain inoculation experiments, and found that the *cheR* mutant could colonize when presented alone. However, the ability of this mutant to colonize was highly variable: in one experiment, 4 of 11 animals inoculated with the *cheR* mutant became colonized to high levels within 24 hours while 7 of 7 wild-type-inoculated animals became colonized; in another experiment, we observed 7 of 10 animals became colonized with the *cheR* mutant while 9 of 10 wild-type-inoculated animals became colonized. These results do not permit us to conclusively distinguish between the two possibilities. However, because the *cheR* mutant could colonize within a reasonable

period of time, we favor the former hypothesis, that the *cheR* mutant colonizes poorly because it fails to respond appropriately to an attractant. Although the identification of a hypothetical squid-produced attractant for *V. fischeri* remains unknown, a number of possibilities exist, including an amino acid (e.g., serine), a peptide(s) present inside the light organ (Graf and Ruby, 1998) and a component of the squid-secreted mucus (e.g. NANA, (DeLoney-Marino *et al.*, 2003)). Marine pathogens such as *V. anguillarum* and *V. alginolyticus* migrate to fish mucus (Bordas *et al.*, 1998; Larsen *et al.*, 2001; O'Toole *et al.*, 1996; O'Toole *et al.*, 1999), although the glycan sugar components of the mucus do not serve as attractants for *V. anguillarum* (O'Toole *et al.*, 1999). Finally, it is not unprecedented that a mutant could simply be inefficient at reaching the crypt spaces: a poorly motile mutant defective for the *flaA* flagellin gene exhibited both a significant delay in colonization initiation and an attenuated ability to colonize the crypts, despite being able to aggregate on the light organ surface and migrate into the pores (Millikan and Ruby, 2004). Thus, the *cheR* mutant may be slightly less efficient in its ability to reach the deep crypts to colonize, and therefore gets outcompeted by parental strains of the bacterium in co-inoculation experiments.

In any case, the results presented here demonstrate a requirement for chemotactic motility--at the very least, the ability to perform the characteristic run and tumble behaviors necessary for migrating up a gradient of attractant during symbiotic colonization. These results, in combination with previous studies indicating the stringent requirement for normal motility, further support the notion that *V. fischeri* must actively participate to initiate symbiotic colonization. The identity of a hypothetical chemoattractant(s) during colonization of *E. scolopes* by *V. fischeri* remains to be determined.

Acknowledgments

We thank Alan Wolfe for guidance with respect to the chemotaxis assays, Ana Mrejeru, Line Skoufos, Nathan Montgomery, Therese O'Shea, and Andrew Morris for expert technical assistance, Landon Moore for assistance with the protein sequence alignment, members of our labs for critical reading of the manuscript, and Integrated Genomics for the use of ERGO for some of our bioinformatics analyses. This work was supported by NIH grant GM59690 awarded to K.L.V., and by both the National Science Foundation under a Research Fellowship in Microbial Biology awarded in 2001 and under a University of Southern Indiana Sponsored Research Office Summer Research Fellowship awarded in 2011 to C.R.D.

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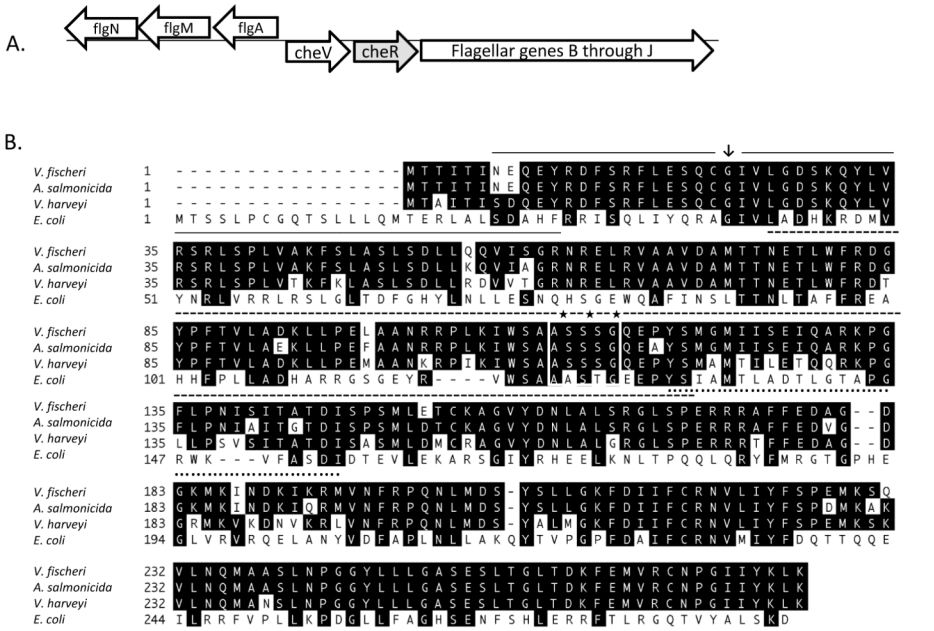

Figure. 1.

A. Chromosomal arrangement of flagellin genes *flgB-flgJ* and chemotaxis genes *cheV* and *cheR* of *V. fischeri*. B. Multiple sequence alignments of the *cheR* genes of *Vibrio fischeri* ES114, *Allivibrio salmonicida* LFI1238, *Vibrio harveyi* ATCC BAA-1116, and *E. coli* showing conserved domains and key residues above the alignments known to be important for protein function (Shiomi *et al.*, 2002). Solid line: CheR methyltransferase, all-alpha domain. Dashed line: AdoMet-MTase catalytic domain. Dotted line: beta-subdomain. Arrow: Residue Gly-23, which corresponds to Gly-39 of *E. coli* which is strictly conserved in all CheR homologs and corresponds to a crucial turn in the secondary structure of the protein. Three stars: signature sequence involved in binding to AdoMet (Malone *et al.*, 1995). NCBI gene identification numbers: *V. fischeri*, YP 205261.1; *A. salmonicida*, YP 002263696; *V. harveyi*, YP 001444487; *E. coli*, AAA23568.1.

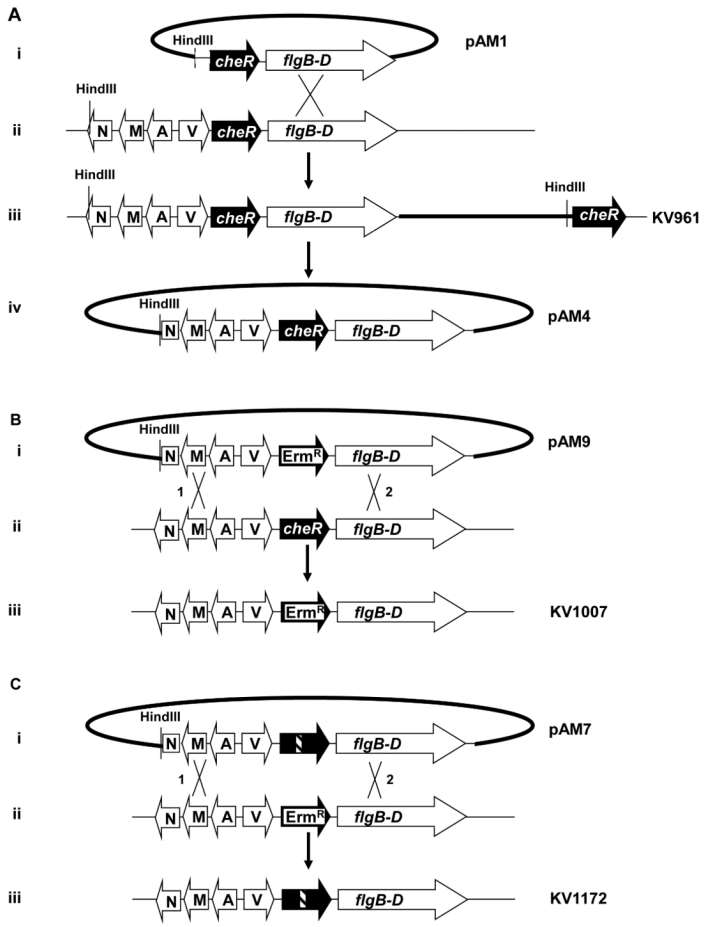

Figure 2.

Genetic manipulations involved in the construction of *cheR* mutants. (A) A Genetic approach to obtaining DNA flanking the *cheR* gene. The starting plasmid, pAM1 (i), was recombined into the chromosome of wild-type *V. fischeri* (ii) to generate KV961 (iii). Chromosomal DNA was isolated from KV961, digested with *Hind*III, ligated, and used to transform *E. coli* cells to chloramphenicol resistance. A plasmid, pAM4, containing cheR and about 3 kb of upstream flanking DNA, was isolated (iv). (B) Plasmid pAM9 (i), a derivative of pAM4 in which the erythromycin resistance cassette (Erm^R) was inserted into the *cheR* gene, was introduced into wild-type ES114 cells (ii). A mutant, KV1007 (iii), in which the *cheR* gene in the chromosome was exchanged with for Erm^R-disrupted gene, was isolated. (C) Plasmid pAM7 (i), a derivative of pAM4 in which *cheR* gene was disrupted by frame-shift mutation (indicated by the striped bar within the black arrow representing *cheR*), was introduced into *cheR*:: Erm^R mutant KV1007 (ii). A mutant, KV1172 (iii), in which the *cheR*:: Erm^R allele in the chromosome was exchanged with for *cheR*-frame-shift allele, was isolated. The crossed lines indicate possible recombinational cross-over events.

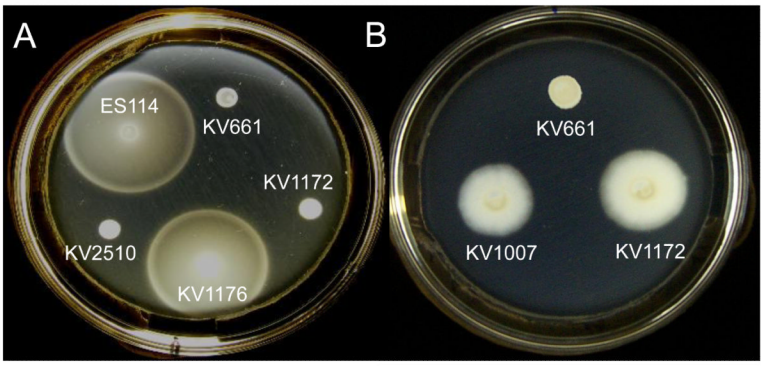

Figure 3.

Motility phenotype of the *cheR* mutant. (A-B) Migration of *V. fischeri* in soft agar. *V. fischeri* cells were inoculated onto TB-SW plates and incubated at 28°C for 5h (A) or 22h (B). ES114 = parental squid isolate; KV661 = nonmotile *flg*$^{-}$; KV1007 = *cheR*::*erm*; KV1172 = *cheR*-frame-shift; KV1176 = *cheR*-frame-shift *att*Tn*7*::*cheV-cheR*$^{+}$; KV2510 = *cheY*::pKV215.

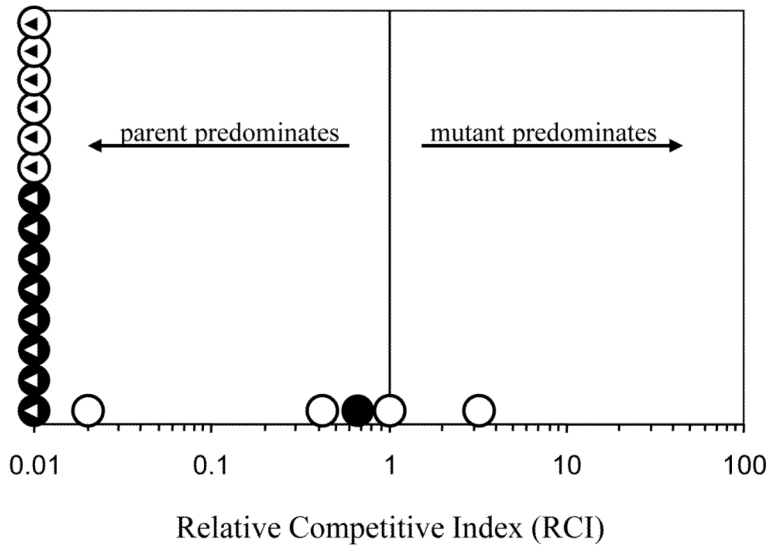

Figure 4.

Colonization defect of a *cheR* mutant. Competition between wild-type and *cheR* mutant *V. fischeri* strains for symbiotic colonization. 9 newly-hatched squid were incubated with a mixed inoculum containing an approximate 1:2 ratio of Erm-resistant wild-type strain KV1421 to Cm-resistant *cheR* mutant strain KV1424 (solid circles). In a separate experiment, 10 newly-hatched squid were incubated with a mixed inoculum containing an approximate 1:4 ratio of KV1421 to KV1424 (open circles). The level of colonization achieved by each strain was determined by homogenization and plating at 20 h after the organisms were placed together. The ratio of mutant to wild-type cells extracted from individual squid is expressed as the relative competitive index (RCI) and is indicated by a circle. An RCI less than 1 indicates animals colonized predominately by the wild-type strain. Circles with an arrow indicate an RCI of <0.01. The data reported are representative of 4 different experiments.

TABLE 1

Bacterial strains and plasmids used in this study

Strain	Relevant genotype or characteristics	Reference or source
E. coli		
DH5α	*endA1 hsdR17* ($r_k^-m_k^+$) *glnV44 thi-1 recA1 gyrA* (Nal^R) *relA1* Δ*(lacIZYA-argF)U169 deoR* (ϕ80*dlac*Δ[*lacZ*]*M15*)	(Woodcock *et al.*, 1989)
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20*	(Herrero *et al.*, 1990)
S17-1λ*pir*	*thi, pro, hsdR*$^-$ *hsdM*$^+$ *recA* λ *pir*	(Simon *et al.*, 1983)
V. fischeri		
ES114	Wt	(Boettcher and Ruby, 1990)
ESR1	Rf^R	(Graf *et al.*, 1994)
KV661	Rf^R *flg*::Tn*10luxAB* Δ*luxA*::*erm*	This study
KV961	Rf^R, Cm^R (pAM1 integrated into chromosome)	This study
KV1007	*cheR* :: *erm*	This study
KV1172	*cheR*-frameshift	This study
KV1176	*cheR*-frameshift *att*Tn*7*::*cheV-cheR*$^+$	This study
KV1421	*att*Tn*7*::*erm*	(O'Shea *et al.*, 2006)
KV1424	*cheR*-frameshift attTn*7*::cm	This study
KV2510	VF1833 (*cheY*)::pKV215 (erm^R)	(Hussa *et al.*, 2007)

Plasmid	Characteristics/Derivation	Reference or source
pAM1	pEVS79 (Stabb and Ruby, 2002) (*SalI*) + 2.3-kb *Sal*I fragment from pKV119 (*cheR flgB-D*)	This study
pAM4	pEVS79 + *flgNMA, cheVR flgB-D*	This study
pAM7	pAM4 *XbaI* partial digest, filled-in (results in *cheR*-frameshift)	This study
pAM8	pAM4 *XbaI* partial digest, filled-in (results in loss of *XbaI* in the MCS)	This study
pAM9	pAM8 *XbaI* filled-in + *Cla*I/*Xba*I - filled-in erm^R from pLMS37 (results in *cheR*::*erm*)	This study
pCD5	pEVS107 *Ase*I, filled-in + 2.6-kb fragment from pAM4 *Ase*I, filled-in (results in Tn*7*::*cheV-cheR*)	This study
pEVS104	Conjugal helper plasmid (*tra, trb*), Kn^R	(Stabb and Ruby, 2002)
pEVS107	Tn*7* delivery plasmid, Kn^R, Erm^R	(McCann *et al.*, 2003)
pKV119	pVO8 (Visick and Ruby, 1997)(*BamHI*) + 5.5 kb *Bgl*II fragment from ES114 chromosome	This study
pLMS37	pBS (*Eco*RV) + 1.2-kb erm^R *Sma*I/*Eco*RV fragment from pKV25 (Visick and Ruby, 1998)	This study
pNDM50	Tn*7* delivery plasmid, Kn^R, Cm^R	This study
pRK2013	Conjugal helper plasmid (*tra, trb*), Kn^R	(Figurski and Helinski, 1979)
pUX-BF13	Encodes Tn*7* transposase (*tnsA, B, C, D, E*), ApR	(Bao *et al.*, 1991)