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Chemoreceptor VfcA Mediates Amino Acid Chemotaxis in *Vibrio fischeri*

Caitlin A. Brennan,[a] Cindy R. DeLoney-Marino,[b] Mark J. Mandel[c]

Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, Wisconsin, USA[a]; Department of Biology, University of Southern Indiana, Evansville, Indiana, USA[b]; Department of Microbiology-Immunology, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA[c]

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Flagellar motility and chemotaxis by *Vibrio fischeri* are important behaviors mediating the colonization of its mutualistic host, the Hawaiian bobtail squid. However, none of the 43 putative methyl-accepting chemotaxis proteins (MCPs) encoded in the *V. fischeri* genome has been previously characterized. Using both an available transposon mutant collection and directed mutagenesis, we isolated mutants for 19 of these genes, and screened them for altered chemotaxis to six previously identified chemoattractants. Only one mutant was defective in responding to any of the tested compounds; the disrupted gene was thus named *vfcA* (*Vibrio fischeri* chemoreceptor A; locus tag VF_0777). In soft-agar plates, mutants disrupted in *vfcA* did not exhibit the serine-sensing chemotactic ring, and the pattern of migration in the mutant was not affected by the addition of exogenous serine. Using a capillary chemotaxis assay, we showed that, unlike wild-type *V. fischeri*, the *vfcA* mutant did not undergo chemotaxis toward serine and that expression of *vfcA* on a plasmid in the mutant was sufficient to restore the behavior. In addition to serine, we demonstrated that alanine, cysteine, and threonine are strong attractants for wild-type *V. fischeri* and that the attraction is also mediated by VfcA. This study thus provides the first insights into how *V. fischeri* integrates information from one of its 43 MCPs to respond to environmental stimuli.

Flagellar motility is one of several behaviors used by bacteria to migrate through their surroundings (1). Migration to preferred environmental conditions is mediated by a behavior known as chemotaxis (2), which allows the bacterium to sense gradients of attractants and respond by controlling the directionality of the flagellar motor (3). Methyl-accepting chemotaxis proteins (MCPs) function as receptors that bind attractants or repellants, usually in the periplasm. Upon ligand binding, MCPs transduce a signal through the CheAY two-component system to affect a change in the tumbling frequency of the flagella (4). By altering the tumbling frequency, MCPs direct an average change in the direction of travel for the bacteria. In *Escherichia coli* K-12, a total of five MCPs enable sensing of numerous attractants, including amino acids, peptides, galactose, ribose, and oxygen (5–9). However, as more diverse bacterial species have been studied, we have learned that bacterial chemotaxis is frequently more complex than the *E. coli* paradigm (10, 11). Bioinformatics and increased genome sequence availability have revealed that both the number and the domain structure of predicted MCPs vary greatly between species (12–14). Work in *Pseudomonas aeruginosa*, a species that encodes 26 MCPs in strain PAO1, has characterized 9 MCPs that mediate chemotaxis toward ligands such as amino acids, trichloroethylene, and malate (15–18). However, even in this relatively well-studied organism, high-throughput attempts to identify MCP ligands have been largely inconclusive and, in one case, only successful when energy taxis, mediated by Aer, is also disrupted (15).

Within the *Vibrionaceae*, the genetic basis of chemotaxis has not been well characterized. Even in the human pathogen *Vibrio cholerae*, only 3 of up to 45 putative MCPs, contributing to aerotaxis, amino acid chemotaxis, and chemotaxis toward the chitin-derived sugars *N*-acetylglucosamine (GlcNAc) and *N*,*N*′-diacetylchitobiose [$(GlcNAc)_2$] have been described (19–21). The genome of the squid symbiont *Vibrio fischeri* (strain ES114) encodes 43 predicted MCPs (22, 23), none of which have been studied, despite the importance of chemotaxis and flagellar motility in the symbiotic lifestyle of this organism (24–28). Because some of the nutrients provided by its squid host have been shown to be chemoattractants for *V. fischeri* (28, 29), we hypothesized that it would be possible to delimit the chemotactic signaling pathways in the bacterium—and during host colonization—by assigning ligands to the MCPs encoded by the bacterium. In the present study, we screened 19 MCPs in *V. fischeri* ES114 and identified a single gene product that mediates chemotaxis to multiple amino acids.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in the present study are listed in Table 1. *V. fischeri* strains are derived from strain ES114 (30) (isolate MJM1100) and were grown at 28°C in either Luria-Bertani salt (LBS) medium (10 g of Bacto tryptone/liter, 5 g of yeast extract/liter, 20 g NaCl/liter, and 50 ml of 1 M Tris buffer [pH 7.5] in distilled water) or seawater-based tryptone (SWT) medium (5 g of Bacto tryptone/liter, 3 g of yeast extract/liter, 3 ml of glycerol, 700 ml of Instant Ocean [Aquarium Systems, Inc., Mentor, OH] at a salinity of 33 to 35 ppt, and 300 ml of distilled water). *E. coli* strains, as used for cloning, were grown at 37°C in Luria-Bertani medium or brain heart infusion medium (BD, Sparks, MD). When appropriate, antibiotics were added to media at the following concentrations: erythromycin at 5 μg/ml for *V. fischeri* and 150 μg/ml for *E. coli* and chloramphenicol at 2.5 μg/ml for *V. fischeri* and 25 μg/ml for *E. coli*. Growth media were solidified with 1.5% agar as needed.

Construction and isolation of MCP mutants. To isolate mutants disrupted in one of the *V. fischeri* MCP genes, we utilized the MB transposon

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Address correspondence to Mark J. Mandel, m-mandel@northwestern.edu.
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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
V. fischeri		
MJM1100	ES114, sequenced wild-type *E. scolopes* light organ isolate	30
CAB1500	*vfcA*::pCAB15 Campbell mutant	This study
CAB1501	*VF_1133*::pCAB16 Campbell mutant	This study
CAB1502	*VF_1789*::pCAB17 Campbell mutant	This study
CAB1503	*VF_2161*::pCAB18 Campbell mutant	This study
CAB1504	*VF_A0325*::pCAB19 Campbell mutant	This study
CAB1505	*VF_A0448*::pCAB20 Campbell mutant	This study
CAB1506	*VF_A0677*::pCAB21 Campbell mutant	This study
MB06594	*VF_1503*::Tn*erm*	This study
MB08238	*VF_A0528*::Tn*erm*	This study
MB08251	*VF_A0481*::Tn*erm*	This study
MB08831	*VF_A0527*::Tn*erm*	This study
MB20125	*VF_1138*::Tn*erm*	This study
MB20164	*VF_A0170*::Tn*erm*	This study
MB21095	*VF_1652*::Tn*erm*	This study
MB20465	*VF_A1072*::Tn*erm*	This study
MB20467	*VF_1117*::Tn*erm*	This study
MB09616	*VF_A1084*::Tn*erm*	This study
MB09969	*VF_0987*::Tn*erm*	This study
MB21134	*VF_1618*::Tn*erm*	This study
MB09076	*vfcA*::Tn*erm*	This study
MB08701	*cheA*::Tn*erm*	Brennan et al., unpublished
CAB1516	MJM1100 harboring pVSV105	This study
CAB1517	MJM1100 harboring pCAB26	This study
CAB1523	MB09076 harboring pVSV105	This study
CAB1524	MB09076 harboring pCAB26	This study
E. coli		
DH5α-λpir	Cloning vector	55
Plasmids		
pEVS122	*oriR6K*-based suicide vector, Erm^r	32
pEVS104	Conjugative helper plasmid, Kan^r	33
pCAB15	pEVS122::′*vfcA*′	This study
pCAB16	pEVS122::′*VF_1133*′	This study
pCAB17	pEVS122::′*VF_1789*′	This study
pCAB18	pEVS122::′*VF_2161*′	This study
pCAB19	pEVS122::′*VF_A0325*′	This study
pCAB20	pEVS122::′*VF_A0448*′	This study
pCAB21	pEVS122::′*VF_A0677*′	This study
pVSV105	pES213-based plasmid used for complementation, Cm^r	37
pCAB26	pVSV105 containing *vfcA* ORF and 350 bp upstream	This study

[a] Cm^r, chloramphenicol resistance; Erm^r, erythromycin resistance; Kan^r, kanamycin resistance.

TABLE 2 Primers used in this study

Primer	Sequence (5′–3′)
MJM-313F	GCCCCGGGGCTCTTACTTGGTTGGCTTCTAAC
MJM-314R	GCGCATGCATCGTAATGGTATCTTTACCTGCAT
MJM-315F	GCCCCGGGATCCATGCCTTTAGTGAGCTTATC
MJM-316R	GCGCATGCTTTGATTAACGGCTTTATTAACCA
MJM-317F	GCCCCGGGTCAACTTGCTGCAAATATTGACTA
MJM-318R	GCGCATGCAAAGTAATGGTAAGCTGATGGTTG
MJM-319F	GCCCCGGGCGAGTTGCAAGTACAATAGACAACA
MJM-320R	GCGCATGCGCAATGATAAAAGCTAACCCGAAT
MJM-321F	GCCCCGGGACACAAATGATGGAGATGAAACG
MJM-322R	GCGCATGCGCCACAATAACTAATGTGAACAACA
MJM-323F	GCCCCGGGGCCTCTACACTTGAGGAAGAACA
MJM-324R	GCGCATGCGCTAAAATAGTTAGACCTGCTGTGG
MJM-325F	GCCCCGGGCACGCTTGAGCTTCCTACTATTCT
MJM-326R	GCGCATGCTGATATTACCGTCACCATCCATTA
MJM-127	ACAAGCATAAAGCTTGCTCAATCAATCACC
MJM-179	GCCACGGCCTTGTTCGCCGGC
MJM-173	ACCACGGCCACTTTCACCGGC
MJM-175	ACCACGCCCCATTTCACCAGC
MJM-176	GCCTCGACCATATTCACCAGC
MJM-181	TCCTCGACCTTGTTCGCCAGC
MJM-184	ACCTCGCCCTTGTTCACCCGC
MJM-186	TCCTCTTCCTTGTTCACCAGC
MJM-187	ACCACGACCATATTCACCTGC
MJM-192	ACCTCGGCCTTGCTCTCCAGC
MJM-193	ACCACGACCTTGTTCGCCAGC
MJM-195	CCCTCGCCCACTCTCACCAGC
MJM-201	ACCTCGACCAGATTCACCAGC
MJM-205	ACCACGACCATGATCACCAGC
MJM-206	ACCTCTTCCCGCTTCACCGGC
170Int2	AGCTTGCTCAATCAATCACC
ARB1	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT
170Ext3	GCAGTTCAACCTGTTGATAGTACG
ARB2	GGCCACGCGTCGACTAGTAC
170Int3	CAAAGCAATTTTGAGTGACACAGG
170Seq1	AACACTTAACGGCTGACA
0777compF	GCGCATGCCCAAGTTTAAAAGAATTACG
0777compR	GCGGTACCTTACAGTTTAAATCGGTCGA

mutant collection (C. A. Brennan et al., unpublished data) and modified an approach we have successfully used previously to identify mutants carrying a transposon insertion in a specific gene (31). Briefly, pooled templates were amplified by PCR with one primer anchored in the transposon (MJM-127; ~100 bp from either end) and a second primer or primer mixture that anneals to a conserved region within MCP genes (either MJM-179 or a mixture of 13 primers [MJM-173, MJM-175, MJM-176, MJM-181, MJM-184, MJM-186, MJM-187, MJM-192, MJM-193, MJM-195, MJM-201, MJM-205, and MJM-206]; Table 2). We predicted that a band of approximately 500 to 1,500 bp would appear only if there were a transposon insertion in or near an MCP. Since MCPs are often encoded in tandem in the genome, it was also possible to identify an insertion in the 3′ end of an MCP encoded upstream of the one in which the MCP-specific primer bound. Bands identified in this manner were purified and sequenced with primer 170Int2. The strain containing the mutation of interest was subsequently isolated. Using this approach, we screened 3,168 members of the MB collection and identified mutants separately disrupted in 12 of the 43 MCP-encoding genes.

Campbell-type mutagenesis using the suicide vector pEVS122 (32) was performed to generate disruptions in an additional seven MCP genes: *vfcA* (*VF_0777*), *VF_1133*, *VF_1789*, *VF_A0325*, *VF_A0448*, *VF_A0677*, and *VF_2161*. Using the following primer pairs, 500 bp of homology near the 5′ end of each open reading frame (ORF) was amplified for each gene by PCR: *VF_0777*, MJM-313F and MJM-314R; *VF_1133*, MJM-315F and MJM-316R; *VF_1789*, MJM-317F and MJM-318R; *VF_2161*, MJM-319F and MJM-320R; *VF_A0325*, MJM-321F and MJM-322R; *VF_A0448*, MJM-323F and MJM-324R; and *VF_A0677*, MJM-325F and MJM-326R. The primer pairs also added XmaI and SphI restriction enzyme sites, which were used to clone the amplified products into the XmaI/SphI-digested pEVS122 using standard techniques. The resulting constructs were conjugated into *V. fischeri* ES114 (MJM1100 isolate) as previously described (33).

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Plate-based chemotaxis assays. For assessment of chemotaxis in soft agar, the strains were grown overnight and then spotted onto modified SWT that lacks glycerol (5 g of Bacto tryptone, 3 g of yeast extract, 8.8 g of NaCl, 6.2 g of $MgSO_4$, 0.72 g of $CaCl_2$, and 0.38 g of KCl/liter) supplemented with 1.5 mM serine and 0.35% agar as previously described (29). Plates were incubated at 28°C for 4 to 6 h, at which point 10 μl of each chemoattractant was spotted outside of the migrating rings. The chemoattractants were spotted at the following concentrations: 2 M serine, 1.1 M GlcNAc, 0.24 M $(GlcNAc)_2$, 0.17 M *N*-acetylneuraminic acid (NANA), 4.8 M thymidine, and 1 M glucose.

Screening for inner-ring migration defects and transposon insertion site identification. We selected five 96-well plates from the MB collection of *V. fischeri* transposon mutants (Brennan et al., unpublished). These strains were inoculated into 100 μl of SWT buffered with 50 mM HEPES (pH 7.5), directly from frozen stocks, and grown overnight. Omnitrays (Nunc, Rochester, NY) containing SWT 0.3% agar supplemented with 1.5 mM serine were inoculated, in duplicate, with 1 μl of each overnight culture using a 96-pin replicator (V&P Scientific, San Diego, CA). Soft-agar plates were then incubated at 28°C for 4 to 6 h and examined for altered inner-ring migration. Candidate strains were further examined by inoculation into individual SWT-serine soft-agar plates, which were subsequently incubated at room temperature (23 to 24°C) for ~12 h.

The insertion site of the validated candidate *vfcA*::Tn*erm* mutant, MB09076, was identified using arbitrarily primed PCR as previously described (Brennan et al., unpublished) (34, 35). Briefly, the transposon junction site was amplified from a diluted overnight culture in two successive rounds of PCR using primer sets ARB1/170Ext3, followed by ARB2/170Int3 (Table 2). The sample was submitted for sequencing to the DNA Sequencing Center at the University of Wisconsin Biotechnology Center (Madison, WI) with primer 170Seq1. Analysis of the sequencing results was performed with DNASTAR (Madison, WI) Lasergene Seqman software.

Capillary chemotaxis assay. Strains were grown in SWT liquid medium to an optical density at 600 nm of ~0.3, pelleted gently for 5 min at 800 × *g*, and resuspended in buffered artificial seawater (H-ASW: 100 mM $MgSO_4$, 20 mM $CaCl_2$, 20 mM KCl, 400 mM NaCl, and 50 mM HEPES [pH 7.5]) (36). One-microliter capillary tubes (Drummond Scientific, Broomall, PA) were sealed at one end, filled with either H-ASW alone or H-ASW containing the indicated attractant, and inserted into microcentrifuge tubes containing the cell suspension. The tubes were incubated on their side for 5 min at room temperature (23 to 24°C), after which the capillary tubes were removed from the cell suspension and washed. The contents were expelled into 150 μl of buffer (either H-ASW or 70% Instant Ocean), and dilutions were plated for colony counts on LBS plates.

Construction of *vfcA* complementation plasmid. The *vfcA* ORF and 350 bp upstream were amplified by PCR using the primer pair 0777compF and 0777compR (Table 2) and cloned into the SphI/KpnI-digested fragment of pVSV105 (37) according to standard molecular techniques. Both the complementation construct and the vector control were conjugated into wild-type *V. fischeri* and into the *vfcA*::Tn*erm* mutant using the standard technique (33).

Squid colonization assay. Newly hatched squid were colonized by exposure to ~3,000 CFU of the indicated strain(s)/ml in 100 ml of filter-sterilized Instant Ocean (FSIO) for 3 h. Squid were then transferred to vials containing 4 ml of uninoculated FSIO for an additional 18 to 21 h, at which point they were euthanized and surface sterilized by storage at −80°C. Individual squid were then homogenized, and each homogenate was diluted and plated for colony counts on LBS agar according to standard methods (38). In competitive colonization experiments, 100 colonies were then patched onto LBS plates supplemented with erythromycin to determine the ratio of the *vfcA*::Tn*erm* mutant to wild type. The competitive index (CI) for each individual squid is calculated as the $\log_{10}$[(homogenate mutant CFU/wild-type CFU)/(inoculum mutant CFU/wild-type CFU)].

RESULTS

Generation and initial characterization of MCP mutants. The *V. fischeri* ES114 genome (22, 23) encodes 43 predicted MCPs. Our preliminary analyses included BLAST (39) queries with full-length proteins and with the N-terminal sensing domains against characterized chemoreceptors in other organisms; however, this approach did not yield ligand-specific signatures. Categorization by domain structure (Fig. 1) revealed four domain architectures that were common to at least five *V. fischeri* MCPs, and an additional 11 architectures represented in the genome. Because it appeared that many of the MCPs could be functional for chemotaxis signal transduction, we took an empirical approach to identifying whether mutants in MCP genes exhibited chemotaxis defects during an initial screen in a rich-medium soft-agar assay. We identified 12 mutants disrupted in MCP-encoding genes by PCR analysis of a transposon mutant collection as described in Materials and Methods (Table 1, Fig. 1). We also constructed plasmid-integration (Campbell-type) mutants in seven other MCP genes that were not identified by this method (Table 1, Fig. 1). Six of these seven specific target genes (*VF_0777* [*vfcA*], *VF_1133*, *VF_1789*, *VF_A0325*, *VF_A0448*, and *VF_A0677*) were selected as candidates because of evidence they might be regulated under conditions relevant to symbiosis: *vfcA*, *VF_1789*, *VF_A0325*, and *VF_A0677* were regulated by the flagellar master regulator FlrA in a transcriptomic study (Brennan et al., unpublished), *vfcA* is regulated by the AinS C8-homoserine lactone autoinduction pathway that exhibits squid initiation and maintenance phenotypes (40), and *VF_A0448* and *VF_1133* were activated when grown in either GlcNAc or $(GlcNAc)_2$, respectively (A. Schaefer and E. Ruby, unpublished data). The final directed mutant we analyzed, *VF_2161*::pCAB18, was constructed with an insertion in a gene whose N-terminal sensing domain was highly similar to the *V. cholerae* gene encoded by *VC_0449*, which, when mutated, has been reported to reduce chemotaxis toward GlcNAc and $(GlcNAc)_2$ (20).

All 19 of these mutant strains, as well as wild-type *V. fischeri*, were assayed for responses to glucose, serine, GlcNAc, $(GlcNAc)_2$, NANA, and thymidine in plate-based assays, as previously described (28, 29). Only one strain exhibited an altered chemotactic response to any of the assayed chemoattractants. The mutant disrupted in *VF_0777* (henceforth referred to as *vfcA* for *V. fischeri* chemoreceptor A) did not respond to exogenous serine but exhibited wild-type responses to the other chemoattractants (Fig. 2 and data not shown). It has been shown previously that, on SWT soft-agar plates, the outer ring of motile *V. fischeri* cells swim toward thymidine and the inner ring of cells swim toward serine (29). As shown in Fig. 2B, the *vfcA*::pCAB15 mutant's inner ring did not exhibit the robust response to the addition of serine that is observed in wild-type cells (Fig. 2A). Together, the mutant's lack of response to exogenous serine and its lack of a robust serine ring suggested that VfcA plays a role in mediating serine chemotaxis in *V. fischeri*.

We next conducted a screen for transposon insertion mutants that lacked an inner migration ring, since we reasoned that such a screen would yield insertions in *vfcA* or in genes that modulated other aspects of serine chemotaxis. We screened 480 transposon mutants in serine-supplemented SWT soft agar and isolated a single candidate, MB09076, which did not have an obvious inner ring (Fig. 3). The transposon insertion site was identified by arbitrarily

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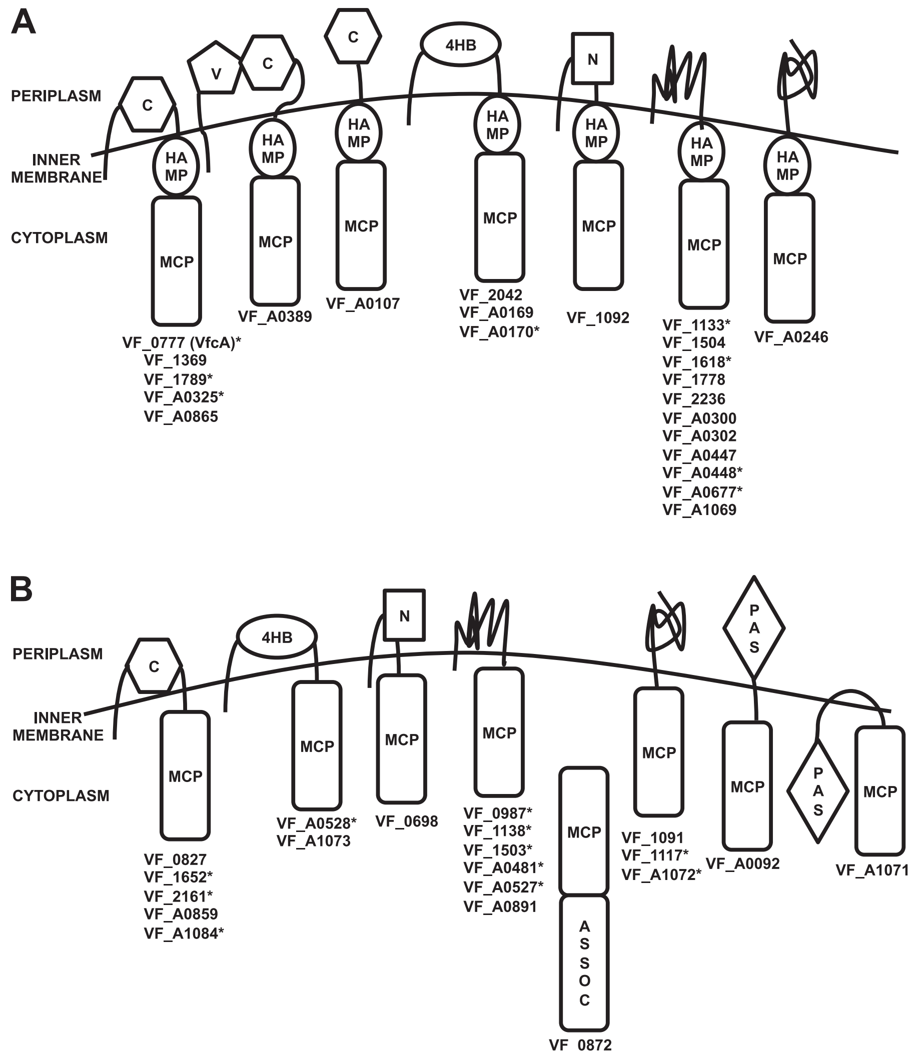

FIG 1 Representation of MCP domain-containing proteins in *V. fischeri* with (A) or without (B) predicted HAMP domains. The predicted domain structures of the 43 MCP domain-containing proteins were determined by Pfam and TmHMM analyses (47, 48). In each model, the N terminus is oriented to the upper left, and the C terminus is marked by the MCP domain, except in the case of VF_0872, in which the ASSOC domain is C terminal. Asterisks indicate loci in which a mutant in the corresponding gene has been identified and analyzed in the present study. Abbreviations: MCP, MCP signal domain; HAMP, HAMP domain; C, CACHE_1 or CACHE_2 domain; V, MCP_N domain; 4HB, 4HB_MCP_1 domain; N, NIT domain; PAS, PAS_3 or PAS_9 domain; ASSOC, MCP-signal associated domain (49–54).

primed PCR and determined to be within the *vfcA* ORF. Identification of an independent allele of *vfcA* that exhibited the same phenotypes as the Campbell allele supported our hypothesis that VfcA was linked to the formation of the inner migration ring. Future experiments were conducted with the *vfcA*::Tn*erm* mutant to avoid the possibility of reversion from the plasmid integration mutation.

Examination of chemotaxis behavior by capillary assay. Plate-based chemotaxis assays are indirect and require not only flagellar motility and chemotaxis but also utilization of putative attractants to generate a gradient (41, 42). Therefore, we performed a capillary chemotaxis assay to directly test whether VfcA plays a role in serine chemotaxis. This assay has not previously been used to examine chemotaxis behavior in *V. fischeri*. In this assay, wild-type *V. fischeri* responded strongly to serine and GlcNAc, a structurally distinct chemoattractant used as a positive control (Fig. 4). The *vfcA*::Tn*erm* mutant responded only to 1 mM GlcNAc and did not respond to any of the tested serine concentrations. As expected, a nonchemotactic *cheA*::Tn*erm* mutant, isolated from another study (Brennan et al., unpublished) also did not migrate toward any of the chemoattractants.

We then examined whether the phenotype of the *vfcA*::Tn*erm* mutant could be complemented by expression of a wild-type copy of *vfcA in trans*. As shown in Fig. 5, the chemotaxis response toward serine in the *vfcA*::Tn*erm* strain was restored upon heterologous expression of *vfcA*, supporting that it is the VfcA gene product that mediates the serine attraction. Expression of *vfcA* in wild-type *V. fischeri* conferred an enhanced chemotaxis response toward serine but not GlcNAc (Fig. 5), further supporting a role for VfcA in mediating chemotaxis toward serine (Fig. 2 and 4). Together, these data suggested that *vfcA* encodes the serine

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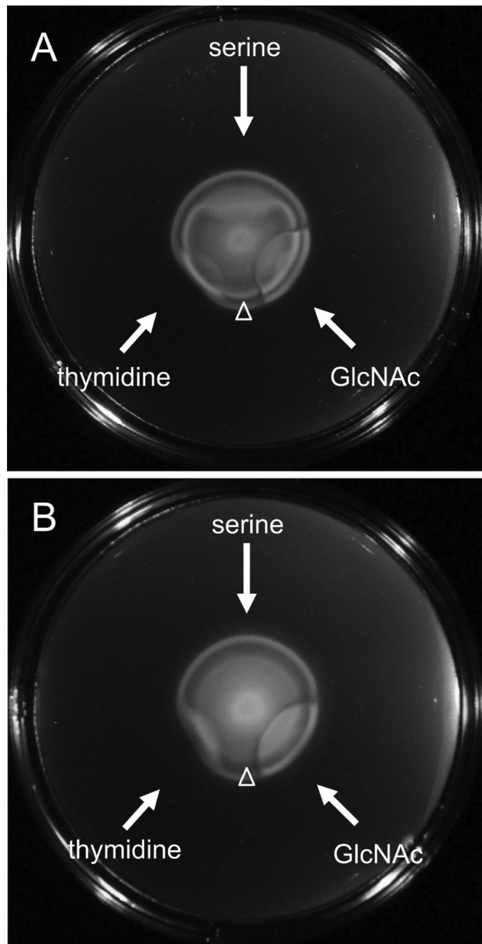

FIG 2 Responses of the wild type and the *vfcA*::pCAB15 mutant to exogenous chemoattractants. Wild-type (MJM1100) (A) and *vfcA*::pCAB15 mutant (CAB1500) (B) cells were inoculated into SWT-serine soft-agar plates and incubated at 28°C for 5 h, at which time chemoattractants (serine, thymidine, or GlcNAc) were spotted (as indicated by arrows) and allowed to incubate an additional 2 h. Open arrowheads mark migration of the inner ring. Chemoattraction to the provided compound manifests as a disruption of the migrating cells.

chemoreceptor in *V. fischeri*, which is functionally analogous to *tsr* in *E. coli*.

Because MCPs can sense multiple chemoattractants, we then investigated whether VfcA mediates chemotaxis toward other amino acids in *V. fischeri*. Previous work using plate-based assays showed that wild-type *V. fischeri* responded most strongly to serine and, to a lesser extent, alanine, arginine, asparagine, histidine, and threonine (29). Again, soft-agar assays report on a combination of growth and chemotaxis phenotypes. We therefore applied our capillary assay to quantify the wild-type chemotaxis repertoire across all 20 amino acids (Table 3). We observed strong attraction ($>5 \times 10^3$ CFU/capillary) to 1 mM concentrations of serine, cysteine, threonine, and alanine (Table 3). These responses were not observed in the *vfcA* mutant, suggesting that VfcA mediates chemotaxis toward each of these four amino acids. We also noticed that the *vfcA* mutant showed increased chemotaxis toward the aromatic hydrophobic amino acids (phenylalanine, tyrosine, and tryptophan) compared to wild-type *V. fischeri*.

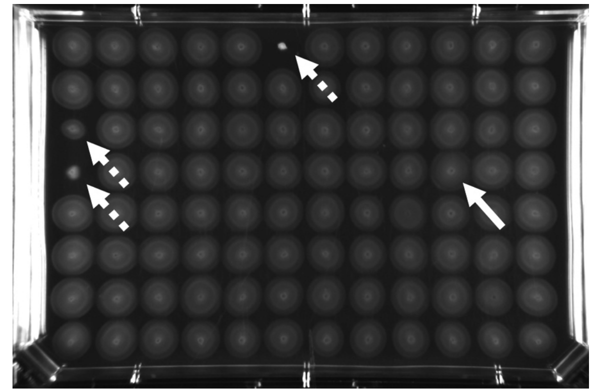

FIG 3 Screening for altered inner-ring migration in soft-agar motility plates. A representative SWT-serine soft-agar plate inoculated with 96 transposon mutants from the MB collection, as described in the Materials and Methods, and incubated for 4 to 6 h at 28°C. The *vfcA*::Tn*erm* mutant (MB09076; white arrow) was identified since its migration pattern lacked a well-organized inner ring. This was straightforward to identify visually compared to the surrounding cells, all of which displayed the ring. Also evident on the plate are strains with reduced or no soft-agar motility (dashed arrows), which were not examined further for the present study.

Squid colonization. Chemotaxis is important during squid colonization (24, 25) and host amino acids are critical to successful colonization by *V. fischeri* (43). Strains that are nonmotile are unable to colonize (25, 27), and nonchemotactic strains colonize inefficiently (24, 25). Because VfcA mediates essentially all of the significant amino acid chemotaxis in *V. fischeri* (Table 3), the *vfcA* mutant permitted us to test whether host-derived amino acids served as a signal for colonizing *V. fischeri* to locate their symbiotic niche. Our prediction was that, if amino acid chemoattraction is developmentally relevant, then the *vfcA* strain would exhibit a squid colonization defect. We colonized juvenile *Euprymna scol-*

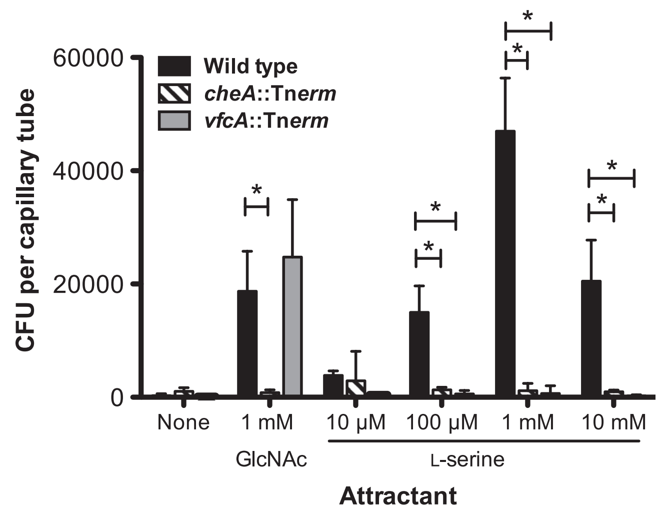

FIG 4 Responses to serine and GlcNAc in a capillary chemotaxis assay. The chemotactic responses of the wild type, the *cheA*::Tn*erm* mutant (MB08701), and the *vfcA*::Tn*erm* (MB09076) mutant to various concentrations of serine and 1 mM GlcNAc were measured by a capillary chemotaxis assay. The data represent the means and standard errors of the mean (SEM) of three independent assays performed in duplicate. Asterisks indicate significance at a *P* value of <0.01, as determined by two-way analysis of variance (ANOVA) with a Bonferroni correction.

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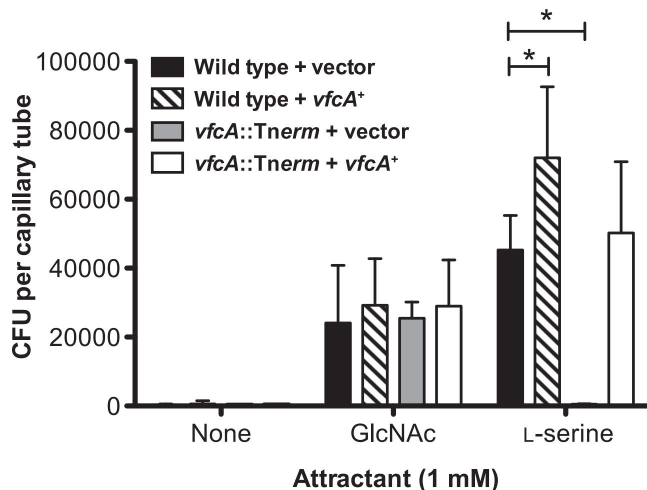

FIG 5 VfcA complementation as assayed by the capillary chemotaxis assay. The chemotactic responses of wild type harboring the vector control (CAB1516), wild type harboring the *vfcA* complementation construct (CAB1517), the *vfcA*::Tn*erm* mutant harboring the vector control (CAB1523), and the *vfcA*::Tn*erm* mutant harboring the *vfcA* complementation construct (CAB1524) to 1 mM GlcNAc or 1 mM L-serine were measured by a capillary chemotaxis assay. The data represent the means and SEM of three independent assays performed in duplicate. A single asterisk indicates a P of <0.01, as determined by two-way ANOVA with a Bonferroni correction.

opes with culture-grown wild-type and *vfcA*::Tn*erm* strains, either individually (single-strain colonization) or in a competitive colonization assay. In the single-strain assays, wild-type and mutant strains each colonized to comparable levels (Fig. 6A). Similarly, in competition, we observed a neutral competition phenotype, so this result did not indicate any competitive advantage or disadvantage for the mutant (Fig. 6B).

DISCUSSION

The main contributions from this study are as follows. (i) Using a capillary assay for the quantification of chemoattraction for the first time in *V. fischeri*, we probed the amino acid chemotaxis response of wild-type *V. fischeri* and revealed strong attraction to cysteine, serine, threonine, and alanine. (ii) Analyzing mutants disrupted in individual predicted MCP-encoding genes, we identified one MCP, VfcA, that mediates chemotaxis toward a wide range of amino acids. (iii) The *vfcA* mutant colonizes squid as effectively as wild-type *V. fischeri*, both alone and in competition.

A number of studies have examined flagellar motility and chemotaxis in *V. fischeri* (24–26, 28, 29). Chemotaxis experiments in these works have relied on soft-agar motility assays. Our work has affirmed conclusions in those studies (e.g., chemotaxis toward serine), and the capillary chemotaxis assay provides the ability to assay chemotaxis in liquid medium without confounding factors such as growth (42). This technique allowed us to examine chemotaxis toward all 20 standard amino acids. In the wild-type strain, we measured significant chemotaxis toward nine of these amino acids, and this technique can now be applied to other relevant macromolecules in *V. fischeri*.

We mutated 19 of the 43 putative *V. fischeri* MCPs and found that only the *vfcA* mutant exhibited detectable chemotactic defects to any of the six *V. fischeri* chemoattractants tested. Using the capillary chemotaxis assay, we showed that the *vfcA* mutant does not exhibit strong chemotaxis toward serine, cysteine, threonine, and alanine. Since we observed no alteration in the *vfcA* mutant's ability to respond toward the structurally distinct chemoattractant GlcNAc, we attribute the amino acid phenotypes to specific signal transduction through VfcA rather than to pleiotropic effects on chemotaxis overall. Furthermore, the mutant displays 5- to 10-fold-enhanced attraction toward aromatic amino acids phenylalanine, tyrosine, and tryptophan. These data may indicate either that such aromatic amino acids act directly as VfcA-mediated chemorepellents or that the loss of VfcA enhances recognition of this class of amino acids, perhaps by biasing the highly organized structure of MCP arrays (44).

Amino acids, including alanine and serine, are found in the squid light organ environment at micromolar concentrations (43), and we hypothesized that these amino acids could serve as a chemotactic signal during symbiotic initiation. However, the *vfcA* mutant did not exhibit a colonization defect. Since *vfcA* mutants are defective in essentially all of the major amino acid chemotaxis responses observed in wild type, we conclude that amino acid chemotaxis is not relevant for the colonizing symbiont to be directed into the its symbiotic niche. An alternate interpretation of the colonization data is that the mutant's enhanced chemoattraction toward aromatic amino acids mediates symbiotic colonization through a distinct pathway from wild-type *V. fischeri*; how-

TABLE 3 Normalized chemotactic responses of wild-type and *vfcA*::Tn*erm* mutant strains to 1 mM L-amino acids

		Mean CFU ($\times 10^3$)/capillary tube ± SEM[a]	
Amino acid classification	Attractant	Wild type	*vfcA*::Tn*erm* mutant
Polar (basic)	Arginine	BD	0.8 ± 0.5
	Histidine	BD	BD
	Lysine	BD	BD
Polar (acidic)	Aspartic acid	0.3 ± 0.2	0.5 ± 0.8
	Glutamic acid	**0.6 ± 0.2**	0.2 ± 0.2
Polar (neutral)	Serine	**55.6 ± 2.8**	BD
	Cysteine	**78.3 ± 12.3**	BD
	Threonine	**22.0 ± 6.2**	0.1 ± 0.2
	Asparagine	BD	3.0 ± 3.2
	Glutamine	0.2 ± 0.2	BD
	Methionine	0.4 ± 0.2	BD
Hydrophobic (aliphatic)	Alanine	**8.0 ± 2.1**	BD
	Valine	BD	1.4 ± 0.8
	Isoleucine	BD	BD
	Leucine	BD	BD
Hydrophobic (aromatic)	Phenylalanine	**0.3 ± 0.1**	**1.6 ± 0.6**
	Tyrosine	**0.6 ± 0.2**	**11.5 ± 5.1**
	Tryptophan	**1.6 ± 0.5**	**9.3 ± 2.3**
Unique	Glycine	**0.3 ± 0.1**	BD
	Proline	0.4 ± 0.3	BD

[a] Values represent the means of four independent assays performed in duplicate and normalized by the subtraction of no-attractant controls. BD, below detection (0.1×10^3 adjusted CFU/capillary tube). Bold indicates significance compared to no-attractant controls (0 adjusted CFU/capillary tube) at $P < 0.05$, as determined by one-tailed Student t test.

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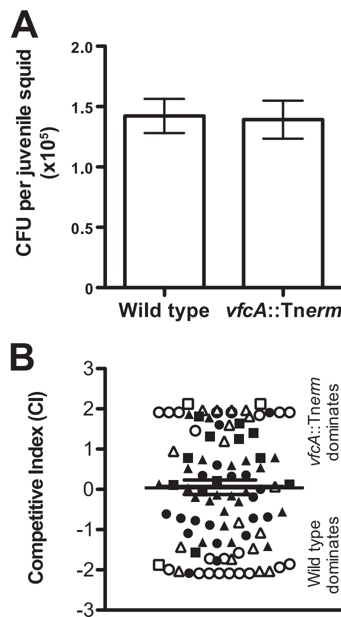

FIG 6 Single-strain and competitive colonization of juvenile squid. (A) Bacterial levels after 24 h in squid exposed to either wild type (MJM1100) or *vfcA*::Tn*erm* (MB09076) from three independent experiments, representing a total of 80 squid per condition. Starting inoculum levels ranged from 2,300 to 4,200 CFU/ml. (B) Competitive index (CI) values of squid exposed to both wild type (MJM1100) and *vfcA*::Tn*erm* (MB09076). Different symbols (circles, triangles, and squares) represent animals from different experiments (inocula, 2,400 to 4,400 CFU/ml). Open markers indicate squid in which only one strain was detected. Bars indicate means ± the SEM, as determined for all squid in which both strains were observed.

ever, it is more parsimonious that amino acid chemotaxis does not play a significant role in bacterial migration into the light organ.

We characterized here the first identification of MCP-chemoattractant pairing in *V. fischeri*. This could not have been predicted by sequence alone, since BLASTP comparison of the ligand-binding domain from the *E. coli* serine-sensing chemoreceptor, Tsr, to the ES114 proteome does not yield a single MCP within the significant hits. In addition, recent work identified a distinct MCP in *V. cholerae*, named Mlp24/McpX, that mediates chemotaxis toward multiple amino acids (21). It is not yet known whether amino acid chemotaxis appears elsewhere among the other 42 MCP-domain encoding proteins of *V. fischeri* such as the McpX ortholog.

Although we identified an amino acid chemoreceptor in *V. fischeri*, the genetic basis for chemotaxis by the bacterium toward the other tested chemoattractants remains elusive. Since we identified mutants in only 44% of the MCPs, it is possible that these behaviors are mediated by the remaining chemoreceptors and could be clarified by continuing to construct single MCP mutants. However, functional redundancy or masking by other behaviors (i.e., energy taxis) could similarly prevent chemoreceptor-ligand identification (15). Alternative approaches may be needed to uncover these relationships. Interestingly, *vfcA* is one of only four MCP genes that are regulated by the flagellar master activator FlrA in *V. fischeri* (Brennan et al., unpublished). As such, it resembles the five *E. coli* MCPs that are all part of the flagellar regulon (45). Identifying such characteristics that show similarity to the canonical MCPs in *E. coli* may suggest an initial filter for chemoreceptors that mediate general (e.g., amino acid) chemoattraction, rather than lifestyle-specific chemotactic responses that might require specific induction (46).

The present study and other recent works (24, 28; Brennan et al., unpublished) have expanded the tools available for studying chemotaxis and motility in *V. fischeri*, including large-scale mutant availability and chemoattractant disruption in the host. Since there is evidence that chemoattraction to the carbohydrate *N*,*N*′-diacetylchitobiose is relevant during squid colonization (28), it will be of interest to apply these methods to examine the genetic basis for *V. fischeri* sensing of other chemoattractants. Although the chemoattractant-chemoreceptor networks in bacterial species that encode high numbers of MCPs are still poorly understood, our work suggests that *V. fischeri* may serve as a valuable model for characterizing the relevant roles of MCPs in mediating both planktonic and symbiotic chemotactic signals.

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