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Squid-Derived Chitin Oligosaccharides Are a Chemotactic Signal during Colonization by *Vibrio fischeri*


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Chitin, a polymer of N-acetylglucosamine (GlcNAc), is noted as the second most abundant biopolymer in nature. Chitin serves many functions for marine bacteria in the family *Vibrionaceae* ("vibrios"), in some instances providing a physical attachment site, inducing natural genetic competence, and serving as an attractant for chemotaxis. The marine luminous bacterium *Vibrio fischeri* is the specific symbiont in the light-emitting organ of the Hawaiian bobtail squid, *Euprymna scolopes*. The bacterium provides the squid with luminescence that the animal uses in an antipredatory defense, while the squid supports the symbiont’s nutritional requirements. *V. fischeri* cells are harvested from seawater during each host generation, and *V. fischeri* is the only species that can complete this process in nature. Furthermore, *chitin* is located in squid hemocytes and plays a nutritional role in the symbiosis. We demonstrate here that chitin oligosaccharides produced by the squid host serve as a chemotactic signal for colonizing bacteria. *V. fischeri* uses the gradient of host chitin to enter the squid light organ duct and colonize the animal. We provide evidence that chitin serves a novel function in an animal-bacterial mutualism, as an animal-produced bacterium-attracting synomone.

Horizontally transmitted microbial symbioses entail specificity challenges for both partners during each host generation (17). The juvenile host often enters the world lacking its partner microbe(s) and recruits them from a complex environmental assemblage of bacteria; for their part, the bacterial symbionts find the correct host niche to the exclusion of nonsymbiotic and pathogenic bacteria. Colonization specificity has been well studied in nitrogen-fixing plant symbionts, revealing detailed chemical communication that takes place between host plants and colonizing rhizobia to direct the symbionts to the correct host (15, 32). For example, plant flavonoids induce the production of bacterial nod factors, chitin derivatives decorated with oligosaccharides that are specific for their cognate host plant. In turn, these factors signal plant-specific receptor kinases that result in plant tissue development.

In animal associations—especially those in marine environments, which often contain >10⁶ bacterial cells per milliliter of seawater—the need for effective mechanisms to assure recruitment and host specificity is clear; however, the underlying molecular determinants are poorly understood. Colonization of the Hawaiian bobtail squid *Euprymna scolopes* by the bioluminescent bacterium *Vibrio fischeri* has proven to be an especially valuable platform for identifying and characterizing such determinants. A "winnowing" process has been described (30) during which first Gram-negative bacteria, then *V. fischeri*, and finally motile *V. fischeri* specifically are selected for their ability to colonize this model host. Within the mantle cavity of the squid, the ciliated surface epithelium of the nascent light-emitting organ plays an important role. In the presence of host-derived mucus released by this epithelium, *V. fischeri* cells aggregate and outcompete other congeneric bacteria (27). Aggregation of potential symbionts in this mucus is largely dependent on the production of exopolysaccharide (EPS) (41), and proper regulation of EPS production is required for squid-specific colonization (19).

In the present study we sought to determine how environmental V. *fischeri* cells are directed into the light-organ crypts of the hatching squid. Previous work has established that motility and chemotaxis are important bacterial behaviors during squid colonization (7, 12, 22–24, 33), but the chemical identity of the host-derived signals remain undescribed. Specifically, while *V. fischeri* have been shown to migrate toward multiple compounds in culture (8), the relevant compound(s) to which the bacteria swim during squid colonization have not been assessed, nor has a developmental stage been identified for which chemotaxis is required.

We examine here the role played by squid-derived chitin oligosaccharides in recruiting *V. fischeri* symbionts. We recently described the presence of chitin in the squid light-organ (10, 39). Previous work identified a robust chemotaxis response by *V. fischeri* to the chitin monosaccharide *N*-acetylglucosamine, GlcNAc (8), and by related vibrios to chitin oligosaccharides, (GlcNAc)₃₋⁵ (3, 4, 11), suggesting a role for chemotaxis toward chitin oligosaccharides during squid colonization. Here we evaluate a role for chitin oligosaccharides as a chemotactic signal that directs potential symbionts from the external environment of the mantle cavity into the light-organ crypts.

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MATERIALS AND METHODS

Strains, plasmids, culture conditions, and chemicals. The strains used in the present study are derivatives of wild-type *V. fischeri* ES14, originally isolated from *E. coli* (5). They were grown in either seawater-tryptone medium (SWT) (5) or Tris minimal medium (MM) (16) at 28°C. *Escherichia coli* DH5α λpir was maintained in LB medium (35). Plasmid pVSV102 encodes constitutive green fluorescent protein (GFP) on a plasmid that is stable in *V. fischeri* (9). When required, antibiotics were added to the media at the following concentrations: kanamycin at 100 μg/ml for *V. fischeri* and 50 μg/ml for *E. coli* and erythromycin at 5 μg/ml for *V. fischeri* and 150 μg/ml for *E. coli*. N-Acetylglucosamine was purchased from Sigma-Aldrich Chemical Co., and high-pressure liquid chromatography-purified N-N′-diacetyctibiobiose was purchased from Seikagaku America via Cape Cod Associates. The cheA::Tnerr (MB08701) and flrA::Tnerr (MB21407) mutant strains were isolated in a screen for random transposon insertions that led to chemotaxis and/or motility defects in swim plates (C. A. Brennan, M. J. Mandel, M. C. Gyllborg, K. A. Thom- asgard, and E. G. Ruby, unpublished data). Mutations were obtained in 19 putative MCPs in one of two ways. Transposon insertion mutants were identified by pooled PCR from an arrayed library as described earlier (37) to isolate strains with random mutations in the following open reading frames (ORFs): VF_0987, VF_1117, VF_1138, VF_1503, VF_1618, VF_1652, VF_A0170, VF_A0481, VF_A0527, VF_A0528, VF_A1072, and VF_A1084. Alternatively, genes encoding presumptive MCPs were inactivated by insertion-duplication mutagenesis (plasmid integration) in the 5′ portion of the ORF in VF_0777, VF_1133, VF_1789, VF_2161, VF_A0325, VF_A0448, and VF_A0677.

Light-organ colonization assays. To survey the capacity of several *V. fischeri* chemoeffectorants to diminish the efficiency of colonization, squid were inoculated into filter-sterilized Instant Ocean (FSIO; Aquarium Systems, Inc.) containing ~3,000 CFU of *V. fischeri/ml and 0.625% (wt/vol) of mannose, thymidine, GlcNAc, or (GlcNAc)2. The percentage of juvenile squid that had been colonized for 24 h after staging was determined at 17 h after inoculation (i.e., 1 h after spotting). If the bacteria swim toward a gradient of the provided compound, then the spot will disrupt the front of moving cells (8).

**RESULTS**

Chitin visualization. Juvenile squid that had been colonized for 24 h were anesthetized in 2% (vol/vol) ethanol in FSIO and then fixed over night in 4% (vol/vol) paraformaldehyde in marine phosphate-buffered saline (mPBS; 0.45 M NaCl in 50 mM sodium phosphate buffer [pH 7.4]) (13). Fixed animals were washed four times for 1 h each time in mPBS, and the light organs were removed by dissection. The dissected light organs were permeabilized with 1% Triton X-100 in mPBS before staining. Briefly, chitin-specific staining with fluorescein isothiocyanate (FITC)- conjugated chitin-binding protein (New England Biolabs) was performed at a 1:250 dilution in 1% Triton X-100 in mPBS for 3 days at 4°C in the dark. After three 1-h washes in mPBS, the samples were stained with the nucleic acid dye TOTO-3 (Invitrogen/Molecular Probes) at a concentration of 2 μM in 2× sodium chloride-sodium citrate buffer (38) at room temperature. Stained light organs were then visualized by confocal microscopy.

Aggregation assays. To visualize bacterial cells during the initial hours of colonization, newly hatched squid were exposed to an inoculum of ~2 × 104 CFU per ml of seawater. Included in the seawater was 0.625% (wt/vol) GlcNAc or (GlcNAc)2, as indicated. At various times postinoculation, animals were anesthetized (2% [vol/vol] ethanol in FSIO) and examined on a Carl Zeiss LSM 510 confocal microscope as described previously (27). The host tissues were visualized by staining with CellTracker Orange CMROra or MitoTracker Red CMXRsos (Invitrogen/Molecular Probes), and the positions of aggregates of *V. fischeri* cells relative to the pores of the light organ were determined based on the expression of GFP. Data were obtained from three independent experiments, in which the behavior of bacterial aggregates was observed in a total of more than 60 animals.

Soft agar chemotaxis assays. The ability of *V. fischeri* to exhibit chemotaxis toward 1 mM GlcNAc or 0.75 mM (GlcNAc)2 was assessed using soft-agar swim plates as described previously (8). The addition of 25 mM mannitol, which is not a chemoeffectorant, was included in all swim plates as a carbon source (8). After 14 h, 5 to 10 μl of the putative chemoeffector (between 0.24 and 1.1 M; an excess compared to that in the plate) was spotted onto the plate immediately ahead of the ring of swimming bacteria. Plates were imaged at 3 h after spotting (i.e., 17 h after inoculation) and in some cases at later time points. If the bacteria swim toward a gradient of the provided compound, then the spot will disrupt the front of moving cells (8).

Disruption of the squid-produced chitin oligosaccharide gradient diminishes colonization efficiency. In culture we observed robust chemoattraction toward GlcNAc and (GlcNAc)2 in a swim-plate assay (Fig. 2). For *V. fischeri*, mannitol serves as a nonattracting carbon source (8). The wild-type strain exhibited robust swim ring formation when either of these attractants was added alone to the mannitol base medium (Fig. 2A and B). We characterized additional strains as controls: a nonmotile strain, *flrA::Tnerr*, and a motile but not chemotactic strain, *cheA::Tnerr*. Using light microscopy, we confirmed that *flrA::Tnerr* was nonmotile and that *cheA::Tnerr* exhibited wild-type motility when grown in liquid culture (data not shown). The *flrA::Tnerr* mutant did not swim out on soft agar (Fig. 2G to I), whereas the nonchemotactic *cheA::Tnerr* exhibited a phenotype consistent with nondirected swimming: the bacterial mass expanded over time, slower than a wild-type strain that could follow a chemotactic gradient and faster than the *flrA::Tnerr* mutant that was amo- nile (Fig. 2G to I). In addition, the *cheA::Tnerr* strain did not
respond to spotted chemoattractants (asterisk in Fig. 2D and E), supporting the assignment of this mutant as motility positive and chemotaxis negative.

We next proceeded to examine the relevance of chemotaxis toward specific compounds during host colonization. If chemotraction toward GlcNAc and/or (GlcNAc)_2 is a necessary developmental step for squid colonization, then we predicted that exogenous addition of such a chemoattractant to the seawater would disrupt the endogenous gradient and diminish the efficiency of colonization. We tested several compounds in such an assay. As shown in Fig. 2J, addition of GlcNAc or two other compounds known to be chemoattractants in V. fischeri (8) had no effect on the number of animals colonized. Because compounds diffuse freely between the light organ and the seawater, these results exclude a requirement for swimming toward these compounds during the initiation of symbiotic development. In contrast, addition of (GlcNAc)_2 had a dramatic effect, since fewer than 10% of the animals were colonized (Fig. 2J). If chemogradient disruption were the mechanism by which (GlcNAc)_2 interferes with colonization, then we would predict that the reduction in colonization efficiency would subside at concentrations lower than that of the endogenous gradient encountered by the bacteria. Such an exper-

FIG 1 Chitin is present in the E. scolopes light-organ duct. (A) Surrounding the epithelial cells of the squid light-organ duct, chitin is detected by staining with an FITC-conjugated chitin-binding protein in green. (B) Host nuclei, stained with TOTO-3, are shown in blue. (C) Merged image of the staining. Scale bar, 20 μm for all panels.

FIG 2 Addition of the chemoattractant (GlcNAc)_2 inhibits the colonization of squid by V. fischeri. (A to I) Chemotaxis of V. fischeri in soft agar. Wild-type, flrA::Tn and cheA::Tn strains were inoculated into HEPES minimal medium-mannitol (MM-M) (8) containing GlcNAc (A, D, and G) or containing (GlcNAc)_2 (B, E, and H) or with no addition (C and F) or onto rich medium SWT (I). Chemoattractants were included in the plates and also spotted at 14 h postinoculation on the side noted by each asterisk (*). Disruption of the outer chemotaxis ring was observed in the wild type (A and B), confirming that it is the chitin monosaccharide (GlcNAc) and disaccharide [(GlcNAc)_2], respectively, that are responsible for the chemotaxis phenotype. No disruption was observed for the flrA::Tn or cheA::Tn strains. (J) Effect of the addition of chemoattractants at 0.625% (wt/vol) on the efficiency of squid colonization [this concentration is equivalent to 35 mM mannose, 26 mM thymidine, 28 mM GlcNAc, or 15 mM (GlcNAc)_2]. Shown are the averages of two separate experiments using 17 to 21 animals per treatment. The bars indicate the range. (K) Dose effect of (GlcNAc)_2 addition on squid colonization efficiency. Shown are the averages of two separate experiments, using 10 to 12 animals per (GlcNAc)_2 concentration. The bars indicate the range.
iment showed that at 10 mM there was an ~60% reduction in colonization efficiency, while at 1 mM there was no significant effect (Fig. 2K).

**Chemotaxis toward chitin oligosaccharides is required for duct entry.** To better understand the behavior of *V. fischeri* symbionts during the first few hours of the colonization process, we used confocal microscopy to observe GFP-labeled bacteria initiating the infection in the presence of exogenously added (GlcNAc)$_2$ or the control GlcNAc. At 2 h after inoculation, bacterial aggregates had formed in the mucus field of all of the animals examined and had migrated to a position just outside of the light-organ pores, regardless of whether GlcNAc or (GlcNAc)$_2$ was present in the seawater (Fig. 3C to E). The table lists the observed frequency of each behavior after treatment with either GlcNAc or (GlcNAc)$_2$. (D) GlcNAc treatment, 2 h postinoculation (hpi). (E) (GlcNAc)$_2$ treatment, 2 h. (F) GlcNAc treatment, 4 hpi. The bacteria have transited through the pore and entered the duct (outlined with the white dashed line) and are located ~40 μm into the pore. (G) (GlcNAc)$_2$ treatment, 4 hpi. (H) Colonization with a *cheA*::Tn strain with no compound addition, 4 hpi. Animals were counterstained with CellTracker Orange (B, D, E, G, and H) or with MitoTracker Red (F).

In each case where we observed colonization in the presence of (GlcNAc)$_2$, only one lobe of the bilaterally symmetrical organ was colonized; imaging of the opposite lobe from the same organ revealed no bacteria within the ducts. We conclude that the effect of (GlcNAc)$_2$ addition is perceived locally at the pore, rather than having widespread physiological effects on either the bacteria or the host. These data suggest that chemotaxis to chitin oligosaccharides emanating from the light organ plays an important role in directing *V. fischeri* cells to migrate from the pore entrance toward the deeper recesses of the ducts and crypts. When juvenile animals were exposed to the chemotaxis-defective strain of *V. fischeri* (*cheA*::Tnerm), even in the absence of added chitin oligosaccharides, this strain became arrested above the pores in a fashion similar to that of wild-type cells in the presence of (GlcNAc)$_2$ (Fig. 3H). The *cheA*::Tnerm strain arrested outside of the pore, and the cells did not enter the duct, even when followed to 6 h postinoculation. Taken together, the colonization data support the hypothesis that chitin-oligosaccharide addition interferes with a natural chemotaxis gradient.

In proteobacteria, chemotaxis is mediated by methyl-accepting chemotaxis proteins (MCPs), which transduce binding of the chemoeffectector ligand to the flagellar motor via the CheA-CheY two-component system (31). We investigated whether *V. fischeri* has a single MCP that mediates this chemotaxis to soluble chitin breakdown products. Work with *V. cholerae* has reported that one
MCP (VC0449) mediates chemotaxis responses toward both GlcNAc and (GlcNAc)₂, as evidenced by diminished swimming outgrowth in an MCP mutant (21). When we mutated the MCP in V. fischeri (VF_2161) that shares the greatest similarity in the N-terminal sensing domain to VC0449, no chemotaxis or colonization defect was detected. We mutated an additional 18 of the 43 total V. fischeri genes that encode predicted MCPs (18, 34), and none of these 19 MCP mutants exhibited chemotaxis defects toward either GlcNAc or (GlcNAc)₂.

**DISCUSSION**

Our data demonstrate that squid-derived chitin is present in the duct tissue just internal to the pores, and as chitinase transcripts are expressed in the light organ (6, 39), there is likely to be generation of soluble chitin oligosaccharides as breakdown products. V. fischeri requires functional chemotaxis to enter the pore and reach this location (Fig. 3H): pore entry does not occur robustly in a chemotaxis-defective mutant (cheA⁻) or in the presence of exogenous (GlcNAc)₂, which lead to phenotypically similar effects (Fig. 3). The same compound is sufficient to elicit a chemotactic response in culture. We have shown that the chitin disaccharide (GlcNAc)₂ is sufficient to serve as a chemoattractant, chemotaxis is necessary for V. fischeri entry into the pore, and chitin (and likely chitin oligosaccharides) is naturally present in a location consistent with a role in signaling for chemotaxis at the pore. We therefore conclude that bacterial chemotaxis toward chitin oligosaccharides is necessary for the bacteria to efficiently transit through the light-organ pore. There is precedence in vibrios that chitin oligosaccharides are perceived differently than the monosaccharide in V. cholerae chitin oligosaccharides elicit a specific transcriptome response (21), and chitin oligosaccharides and insoluble chitin (e.g., crab shells) can induce genetic competence, whereas GlcNAc cannot (20). The identification of differential chemotaxis responses highlights an additional level of specialization in the spectrum of vibrio chitin signaling.

Previous work identified multiple stages for the initial entry into the squid host; here, we articulate those stages to delimit at what point the chemotaxis phenotype described in this report may be acting.

**Stage 1: aggregate formation.** V. fischeri cells aggregate by attaching to cilia in the host mucus (27; M. A. Altura, E. A. C. Heath–Heckman, A. A. Gillette, and M. J. McFall-Ngai, unpublished data), and we found that the chitin-oligosaccharide signal was not involved in this stage. This conclusion is consistent with previous data that indicate that both nonmotile V. fischeri and other Gram-negative bacteria (alive and dead) can aggregate in the mucus field (27).

**Stage 2: movement through the mucus to the external face of the pore.** Bacterial migration to the light-organ pore occurs in a manner such that V. fischeri forms so-called “tight aggregates” and dominates over other species (27, 29, 29). This process requires viable V. fischeri (29), and our data demonstrate that this behavior takes place in a manner that is independent of chemotaxis toward GlcNAc or (GlcNAc)₂. The ability of a cheA::Tn5 mutant to localize at the external side of the pores indicates that chemotaxis is not a required behavior in stage 2. This stage occurs outside the organ, within a ciliated mucus field. The resulting turbulence from the ciliary beat (28) is likely to constrain solution-based signaling such as a chemotaxis gradient. The V. fischeri genome contains multiple type IV pilus loci (34) that may also contribute to twitching-like movement through the mucus field. In addition, proteases that have been shown to be both quorum-sensing induced (2) and unique to squid symbionts (compared to fish symbionts) (19) may help digest the mucus.

**Stage 3: migration through the pore into the duct.** Migration into the duct via the pore has been well documented (see, for example, Fig. 4c in reference 27) and observed in our experiments as described above. It is at this stage, bacterial movement from the aggregates into the pore and down the duct, that we observe an effect of chitin oligosaccharide chemotaxis. E. scolopes produces chitin, which is present in the tissue lining the light-organ ducts (Fig. 1). Our data support a model whereby host-encoded chitinases (39) degrade the chitin to chitin oligosaccharides, which can diffuse from the tissue through the pores, creating a gradient by which V. fischeri cells that arrive at the pore can recognize and transit directly into the symbiotic organ. If the response to the predicted duct-derived chitin oligosaccharide gradient is disrupted—either by exogenous addition of high (GlcNAc)₆ levels that impair the ability of wild-type bacteria to discern the host gradient or by colonizing squid using chemotaxis-deficient bacteria (cheA::Tn5) that are incapable of responding to the natural gradient—then most aggregates progress only to the external face of the pore (Fig. 2) and K; Fig. 3C, G, and H).

When individual mutations were made in 19 of 43 V. fischeri MCPs, no evidence of a loss of chemotaxis to chitin monomer or dimer was observed. Studies of chemoeffector-MCP assignments in Pseudomonas aeruginosa PAO1 (26 MCPs) and other bacteria (1, 14, 40) have revealed that many microbes can have a more complicated picture of chemoattractant sensing than is found with the much smaller MCP repertoires of enteric bacteria (31). Therefore, two possibilities are plausible to explain the lack of assignment of a particular MCP to the response toward chitin oligosaccharides. First, the chemotactic response may be mediated by the gene product of 1 of the remaining 24 MCPs, and further investigation is required to test all of the 43 V. fischeri MCPs. Second, it may be that either (i) more than one MCP transduces the chitin signal or (ii) there is cross talk within the signal transduction system. Such cross talk would mean that the simultaneous mutation of a dominant response is required to produce this outcome (e.g., as is observed in P. aeruginosa with the Aer receptor [1]). This kind of genetic redundancy would preclude detection of a phenotype when only a single gene is disrupted.

Two other possible explanations for our results should be considered. The first is that the bacteria encode a chitin-oligosaccharide-sensitive protein that typically binds host tissue; the addition of excess ligand blocks this binding, somehow decreasing colonization efficiency. However, imaging of symbiont behavior (Fig. 3) has demonstrated that it is the presence (not absence) of added chitin-oligosaccharide that causes bacteria to stay in place, failing to enter the pore. Furthermore, the natural chitin is present beyond the pore (i.e., internal to the duct; Fig. 1), and a chemotaxis-defective mutant arrests in the mucus field prior to entry into the pore (Fig. 3H). The second explanation is that addition of (GlcNAc)₂ leads to a reduction in the motility of the cells. However, in culture we did not observe such an effect (Fig. 2B and direct imaging [data not shown]), and it seems unlikely that an effect on the bacterial physiology could result in a specific developmental arrest unless it were connected to that stage of development. Thus, we believe that a more parsimonious explanation is that squid-derived chitin oligosaccharides serve as a synomone—a compound that is produced by one species and evokes...
a behavioral response in another that is favorable to both (26). Such host-derived synomones play important roles in the establishment of other horizontally transmitted microbial symbioses (15), but present particular challenges in aquatic environments (36).

Perhaps the best-described example of interdomain communication is that mediated by chitin derivatives—lipo-chitooligosaccharide Nod factors—that are produced by bacteria and used as synomones to determine specificity in rhizobial symbioses (15). The Vibrio-squid mutualism has adapted preexisting capabilities—squid production of chitin and bacterial chemotaxis toward chitin oligosaccharides—to direct Vibrio fischeri into the light-organ ducts, a critical step in colonization. In addition, because only bacteria that have already (i) attached to the squid cilia, (ii) aggregated, and (iii) migrated to the pore will be positioned to receive this signal, only a subset of marine bacteria capable of chemotaxis toward chitin are likely to enter the light organ. Thus, the stepwise nature of the colonization process contributes to the association’s remarkable specificity (17). Future work will seek to address how the squid host regulates production and release of chitin oligosaccharides, how aggregated V. fischeri migrate to the pore, and how chemotaxis influences later stages of host colonization.

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