The *Vibrio cholerae ToxR* Regulon Encodes Host-Specific Chemotaxis Proteins that Function in Intestinal Colonization

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Abstract

Virulence gene regulation in *Vibrio cholerae* is under the control of the *ToxR*-ToxT regulatory cascade. Chemotaxis and net motility have been shown to influence the infectivity of *Vibrio cholerae*. *V. cholerae toxR* mutants do not synthesize proteins required for chemotaxis towards mucus. The inability of the *toxR* mutant strain to recognize and swim towards mucus is due to their failure to synthesize AcfB, a methyl–accepting chemotaxis protein. AcfB has previously been shown to be involved in intestinal colonization using the infant mouse model of cholera infection. Wild type *V. cholerae* recognizes galactose-6-sulfate in the capillary tube assay whereas *V. cholerae acfB* mutants fail to migrate into the capillary tubes. Vibrio strains carrying a mutation in *tcpI, a ToxR* regulated gene found within the Vibrio Pathogenicity Island (VPI), which encodes a methyl accepting chemotaxis protein are fully chemotactic towards mucus and galactose-6-sulfate.

Keywords: *Vibrio cholerae*; *ToxR* Regulon; Accessory colonization factor; Chemotaxis

Introduction

The *Vibrio cholerae ToxR* regulon encodes host specific chemotaxis proteins that function in intestinal colonization. *Vibrio cholerae* is the etiologic agent of the severe diarrheal disease cholera in humans. Following the ingestion of contaminated food or water, *V. cholerae* colonize the intestinal epithelium of the small intestine via a complex and poorly complex regulatory circuit that results in the spatial and temporal expression of the cholera toxin and toxin-coregulated-pilus genes. The end result of this process is efficient colonization of the small intestine and the acute watery diarrhea associated with Asiatic cholera [1-7]. To successfully colonize the human small intestine, the vibrios must penetrate the mucus gel and attach to and colonize the brush borders of the micro villi. A key component of this early phase of intestinal colonization is vibrio motility and chemotaxis [8-11]. Non motile vibrios are severely attenuated in the infant mouse model of intestinal colonization whereas, non-chemotactic *V. cholerae* mutants are not defective in intestinal colonization [8-11]. Remarkably, non-chemotactic *V. cholerae* dramatically out-compete wild type organisms. Non-chemotactic *V. cholerae* also demonstrate an altered colonization phenotype, i.e., wild type *V. cholerae* colonize only the lower small intestine whereas the non-chemotactic mutants colonize both the upper and lower small intestine [8]. This out-competition phenotype during in vivo infection requires the presence of Counterclockwise (CCW) biased flagellum [11]. Chemotaxis may also play an important role in the ability of *V. cholerae* to cause epidemics. Passage of *V. cholerae* through the gastrointestinal tract results in a transiently non-chemotactic state that increases their infectivity [12,13]. Understanding the precise role of *V. cholerae* chemotaxis in intestinal colonization is complicated by the presence of three chemotaxis operons and at least 43 methyl-accepting chemotaxis proteins [14]. Only one of the chemotaxis operons has been shown to be required for standard chemotaxis [15,16]. The function of the other two chemotaxis operons is not presently known.

We have previously shown that two Vibrio Pathogenicity Island (VPI) genes (*acfB, tcpI*) under the control of the *ToxR-ToxT* regulatory cascade encode members of the methyl-accepting chemotaxis family of proteins [17,18]. *V. cholerae* strains with mutations in *acfB* were initially identified in a transposon library as being slightly defective in intestinal colonization [19]. TcpI was identified as a negative regulator of pilus synthesis [5,18]. In this report we find that *V. cholerae* strains bearing mutations in *acfB* were defective for chemotaxis towards mucus whereas *tcpI* mutants displayed wild type levels of chemotaxis towards mucus.

Materials and Methods

Media

Luria broth (LB) was used for to culture both *V. cholerae* and *E. coli* strains. The LB was supplemented with antibiotics (ampicillin at 50 ug mL^{-1}, streptomycin at 100 µg mL^{-1}) or 0.1% arabinose as needed. Vibrio motility was confirmed using 0.3% LB soft agar [17].

Bacterial strains

Table 1 lists the bacterial strains employed in this study.

Chemotaxis assay

V. cholerae strains were cultured in LB broth pH 6.5 at 30°C

Table 1: Bacterial strains and plasmids used in this study.

Strains	Genotype	Source/reference
Vibrio cholerae		
0395	01 classical, Str^r	(19)
0395 Δ*acfB*	Δ*acfB*, Str^r	This study
0395 Δ*acfB*/pACFB	Δ*acfB*/p*acfB*, Str^r, Amp^r	This study
0395 Δ*toxR*	Δ*toxR*, Str^r	(19)
0395 Δ*tcpI*	Δ*tcpI*, Str^r	This study
0395 Δ*cheW1*	Δ*cheW1*, Str^r	This study
0395 Δ*cheW2*	Δ*cheW2*, Str^r	This study
0395 ΔcheW3	ΔcheW3, Str^r	This Study
Escherichia coli		
Top10	F$^-$*mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1deoR araD*139 Δ(*ara-leu*)7697 *galUgalKrpsL* (Str^r) *endA1 nupG*	Invitrogen
DH5α (λ*pir*)	*supE*4 D*lac*U169 (Φ80 *lac*ZDM15) *hsdR17 recA1 endA1 gyrA96thi-1 relA1* λ*pir*	Invitrogen
Sm10 (λ*pir*)	*thi-1 thrleutonAlacYsupErecA*::RP4-2Tc::Mu λ*pir* R6K	(37)
Sy327(λ*pir*)	Δ(*lac-pro*) *arE*(Am) *rifnalA recA56* λ*pir*	(37)
Plasmids		
pBAD	*colE1 ori*; *araBAD* promoter; Amp^r	Invitrogen
pACFB	*acfB in* pBAD; Amp^r	This study
pCVD442	*R6K ori;, mobRP4, bla, sacB*	(38)
pGP704	*R6K ori;* Amp^r	(37)

containing the appropriate antibiotics overnight. The vibrios were then diluted 10-fold in LB broth and cultured for 3 hours to maximize the number of motile cells. Vibrios were centrifuged at 5000X g and resuspended in Krebs-Ringer-Tris (KRT) buffer [8] to which 0.1 % Triton X-100 was added to prevent excessive adherence of the bacteria to the glass surface. The vibrios were suspended in KRT at a concentration of 10^7 bacteria/ ml and dispensed in 200 μl aliquots into 1.5 ml polypropylene tubes. A 25 μl capillary tube (Drummond Scientific) heat sealed at one end and containing the taxin suspended in KRT was placed in the polypropylene tube approximately 0.5 cm below the surface of the Vibrio suspension. Following 60 minute incubation at 30°C, the capillary tubes were removed and rinsed three times in KRT. The contents of the capillary tubes were then diluted in KRT and spread on agar plates to determine the number of colonies. All experiments were performed in triplicate on three separate occasions. Capillary tubes containing KRT were used as controls. The chemotactic response of *V. cholerae* to a specific taxin is expressed in terms of the relative response (R_{che}), i.e., the ratio of mean accumulation of vibrios in taxin containing capillaries to the mean accumulation of vibrios in control capillaries [8].

Results

Chemotactic response of *V. cholerae* to mucus

Chemotaxis represents an important mechanism whereby *V. cholerae* is able to colonize the small intestine. Thirty five years ago, Freter, discovered the importance of chemotaxis in the association of cholera vibrios with the intestinal mucosa using an elegant combination of in vitro/in vivo experiments [8-10]. More recently, the Camilli laboratory demonstrated that flagellar-mediated chemotaxis contributes to *V. cholerae* colonization and infectivity [11]. Our finding that two *ToxR/ToxT* regulated genes (*acfB, tcpI*) found within the *Tcp/Acf* pathogenicity island encode members of the enteric methyl-accepting chemotaxis proteins [17,18] prompted us to investigate their role in chemotaxis. Previous studies by Freter showed that motile bacteria guided by chemotactic gradients within mucus gel allowed the vibrios to penetrate efficiently into the deep layers of intervillous spaces [8]. To determine the roles of AcfB and TcpI in vibrio chemotaxis, *V. cholerae* strains containing in-frame mutations in *acfB* and *tcpI* were constructed. The Δ*acfB* and Δ*tcpI V. cholerae* strains were assayed for chemotaxis towards mucus using a capillary tube assay and the results are shown in Table 2.

The role of the V. cholerae general chemotaxis machinery in response to mucus

The completed genome of *V. cholerae* predicts a large number of proteins with amino acid similarity to known enteric related chemotaxis proteins [14]. The complexity of the *V. cholerae* chemotaxis system makes understanding its precise contribution to intestinal colonization an important undertaking. One characteristic of enteric MCPs that is shared by *AcfB* is its ability to interact with the chemotaxis proteins of the general chemotaxis pathway. The interaction of enteric chemosensors with CheW

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Table 2: Chemotactic response *of Vibrio cholerae* to chemoattractants.

Strain	Serine	Mucus	Galactose-6-sulfate
0395	18	12	12
0395 Δ*toxR*	18	3	4
0395 *acfB*::Cm^R	17	2	4
0395 *acfB*::Cm^R/pACFB	17	11	11
0395 Δ*tcpI*	17	12	11
0395 Δ*cheW1*	2	3	3
0395 Δ*cheW2*	18	11	13
0395 ΔcheW3	18	12	12

Chemotactic response of *V. cholerae* to chemoattractants expressed in terms of relative response (Rche). This is the ratio of vibrio accumulation in chemoattractant-containing capillaries compared to capillaries containing buffer alone.

occurs via a Highly Conserved Domain (HCD) present in the cytoplasmic tail of these proteins [20-22]. The highly conserved domain of *AcfB* is almost identical to other MCPs [17]. The possibility that AcfB interacts with *V. cholerae CheW* homologues seems likely since we know that *AcB* influences vibrio swarm plate activity. Genome sequencing identified three *V. cholerae* genes encoding CheW homologues (*cheW1, cheW2* and *cheW3*). It has also been shown that *Chew1* is the dominant homologue (*cheW1* null mutants fail to chemotax whereas *cheW2* and *cheW3* mutants are still chemotactic) [15,16]. To determine which of the three chemotaxis systems *AcfB* signaling is coupled to, we generated *V. cholerae* strains containing mutations within *cheW1, cheW2* and *cheW3*. Only the *V. cholerae* strain with a disruption in the *cheW1* gene was found to be defective for vibrio chemotaxis to mucus. As can be seen in Table 2 strains with disrupted *cheW1* fail to chemotax towards mucus.

Chemotactic response to galactose-6-sulfate

Mutations in *acfC* the gene directly downstream of *acfB* yields Vibrio strains with the same colonization defect as strains bearing *acfB* mutations [19]. Interestingly *acfC* encodes a protein related to sulfate binding proteins in *E. coli*. Since mucus is rich in sulfated sugars we tested the ability of *V. cholerae* to migrate towards a gradient of galactose-6-sulfate. Wild type *V. cholerae* is chemotactic towards galactose-6-sulfate whereas vibrios bearing an in-frame *acfB* deletion are defective for movement towards this taxin. As was noted above regarding *V. cholerae* chemotaxis towards mucus, only vibrios with a disruption of the *cheW1* gene were defective for chemotaxis to galactose-6-sulfate (see Table 2).

Discussion

Motility and chemotaxis are utilized by pathogenic bacteria to colonize and invade the host [21]. Chemotaxis allows for *Helicobacter pylori* to invade the mucus lining of the stomach [23,24] and *Vibrio anguillarum* uses chemotaxis to access the mucus of fish intestines [25,26]. *V. cholerae* which normally inhabits aquatic environments, possesses a remarkable repertoire of both inner membranes localized Methyl-Accepting Chemotaxis Proteins (MCPs) and soluble chemotaxis proteins [14,15]. Although *V. cholerae* is able to utilize chemotaxis to efficiently

colonize the small intestines of humans the contribution of individual components of the vibrio chemotaxis system to this process is not well understood. This is especially true of the over 40 methyl-accepting chemotaxis proteins encoded by the *V. cholerae* genome [14]. The presence of 46 MCP-like proteins in *V. cholerae* suggests an impressive ability to respond to environmental signals. A key component of the *V. cholerae* virulence machinery lies within the TCP/ACF (VPI) pathogenicity island [27,28]. The toxin co-regulated pilus is absolutely required for intestinal colonization whereas the Acf proteins are needed for efficient colonization [19]. Two genes found within the VPI (*acfB, tcpI*) encode proteins related to methyl-accepting chemotaxis proteins [17,18,29]. MCPs form homodimers which span the bacterial inner membrane in order to transduce chemotactic signals to the cytoplasmic Che proteins such that the bacteria can respond with directed motility toward the toxins recognized by the individual MCP's [30,31]. Insertion mutations within *acfB* yield vibrio strains that are slightly deficient in colonization. Mutations within *tcpI* relieve pH mediated repression of TCP synthesis [19]. Strains containing *tcpI* mutations however are not defective for colonization in the infant mouse model of cholera infection [19]. We demonstrate that AcfB contributes to the ability of *V. cholerae* to recognize and swim toward a gradient of mucus whereas TcpI does not promote vibrio chemotaxis towards mucus.

We and others have used computer algorithms to predict that AcfB is structurally related to methyl accepting chemotaxis proteins [17,29]. Over expression of *acfB* in both *V. cholerae* and *E. coli* alters the swarm plate response of these strains. This suggests that AcfB is capable of interacting with the general chemotaxis machinery of both organisms. In this report we demonstrate for the first time that AcfB functions in *V. cholerae* chemotaxis. *V. cholerae* bearing mutations in *acfB* fail to respond to a gradient of mucus and also fail to recognize galactose-6-sulfate as a chemoattractant. Wild type *V.cholerae* respond with a positive chemotactic response to both of these substances. As with *acfB*, over expression of *tcp*I in *V. cholerae* alters the swarm plate response of these organisms in LB soft agar plates suggesting that *tcpI* is able to interact with the general chemotaxis machinery. Unlike AcfB, however; TcpI does not appear to participate in the chemotactic response of *V. cholerae* to mucus/galactose-6-sulfate. TcpI is a pH dependent,

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negative regulator of TCP biogenesis. TcpI permits maximum synthesis of TCP in response to the pH of the culture medium. These findings and the relatedness of TcpI to MCPs, suggest that TcpI may "sense" pH. Capillary tube chemotaxis experiments in which *V. cholerae* 0395 and *V. cholerae* 0395Δ*tcpI* were exposed to KRT pH 6.5, KRT pH 7.4 and KRT pH 8.5 failed to demonstrate directed vibrio motility in response to pH (data not shown). Although regulation of TCP synthesis by TcpI appears to involve the ability of this inner membrane sensor protein to recognize pH, the mechanism by which it affects this regulation and any possible role for Che proteins in this regulation remains obscure. Chemotaxis in bacteria is accomplished via a complex signal transduction system that permits sensory adaptation and relates the input signal to the flagellar motor [20-22, 30,31]. In many bacteria the signal transduction apparatus contains multiple sets of the proteins required for signal transduction [32]. The genome *V. cholerae* is predicted to encode 22 open reading frames that are homologous to *che* genes, most of the *V. cholerae che* genes are clustered in three regions on both chromosomes. The precise role of multiple sets of *che* genes in *V. cholerae* and other bacteria is not known [15]. Several *che* genes have been shown to affect cellular functions not related to chemotaxis. HlyB and TcpI were shown to be involved in hemolysin secretion and pilus biogenesis respectively [17,33]. Previous work examining the role of *V. cholerae che* paralogues in chemotaxis have shown that genes located in the *che* cluster II are responsible for *V. cholerae* chemotaxis [15,16]. The role of the genes encoded in clusters I and III have not been elucidated. In order to examine the role of the three *che* gene clusters in the *V. cholerae* chemotactic response to mucus we generated vibrio strains with mutations in *cheW1*, *cheW2* and *cheW3*. Only mutations within *cheW1* abolished vibrio chemotaxis towards mucus and galactose-6-sulfate.

The *V. cholerae acfC* gene is predicted to encode a periplasmic sulfate binding protein and is part of a polycistronic operon downstream of *acfB*. Given its role in intestinal colonization, it seems likely that AcfC is involved in chemotaxis. Other periplasmic solute binding proteins such as the maltose binding protein and ribose binding protein have been shown to play a role in *E. coli/Salmonella* chemotaxis via interactions with methyl-accepting chemotaxis proteins [34]. Mucus is rich in sulfated molecules [35,36] and thus we hypothesized that the ACF proteins may represent a sulfate "sensing" mechanism whereby *V. cholerae* could sense intestinal mucus and promote penetration of the mucus layer by directing chemotaxis toward sulfated sugars. Galactose is a common sulfated sugar found in mucus [36] and thus we tested galactose-6-sulfate for its ability to act as a chemoattractant. Parental *V. cholera 0395* was capable of chemotaxis towards this sulfated sugar whereas 0395Δ*acfB* and 0395Δ*cheW1* were non-chemotactic towards galactose-6-sulfate. These findings support out the hypothesis regarding the role of AcfB and AcfC in intestinal colonization such that AcfC binds galactose-6-sulfate followed by interaction with AcfB which in turn activates the chemotaxis signal transduction cascade mediated by Che proteins encoded by the *che* II gene cluster.

Recent efforts aimed at generating live-attenuated *Vc* vaccine strains suggest that that motility may play a role in the residual virulence of *Vc* strains lacking cholera toxin genes [39,40]. These studies emphasize the importance of understanding the role of vibrio motility and chemotaxis proteins in intestinal colonization, as this might have a practical impact on the development of efficacious vaccines for the prevention of cholera and may also give new direction to vaccine research for other enteric pathogens. The results presented here, shed light on a novel aspect of *Vc* pathogenesis and promote a clearer understanding of the contribution of the *Vc* chemotaxis signaling proteins in the intestinal colonization process.

Acknowledgments

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