AN ABSTRACT OF THE THESIS OF

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Title: Identification of an Aerotaxis Transducer in *Vibrio cholerae*.

Abstract approved:

Claudia Häse

The ability to move towards favorable environmental conditions, called chemotaxis, is common among motile bacteria. In particular aerotaxis has been extensively studied in *Escherichia coli*. Three putative *aer* gene homologs were identified in the *V. cholerae* genome designated VCAer-1 (VC0512) VCAer-2 (VCA0658), and VCAer-3 (VCA0988). Deletion analyses indicated that only one of them, VCAer-2, actively mediates an aerotaxis response, as assayed in soft agar plates as well as a capillary assay. Complementation studies showed that VCAer-2 is in fact responsible for guiding *V. cholerae* along an oxygen gradient. In addition, overexpression of the gene resulted in a marked increase of the aerotactic response in succinate soft agar plates. No observable phenotypes in mutants deleted in the *V. cholerae aer-1* and *aer-3* genes were detected under standard aerotaxis testing conditions. Furthermore, the *V. cholerae aer-1* and *aer-3* genes, even when expressed from a strong independent promoter, did not show any phenotypes. Several lines of evidence suggested differences in the mechanism of aerotactic signal transduction between *V. cholerae* and *E. coli*.

First, a key amino acid residue involved in the binding of the FAD prosthetic group in the *E. coli* Aer protein is not conserved in the *V. cholerae* VCAer-2 protein. Moreover, unlike other chemotaxis genes, the *V. cholerae aer-2* gene did not complement the heterologous *E. coli* Aer mutant, although a weak activity of the *E. coli aer* gene in the *V. cholerae* VCAer-2 mutant was observed. In the absence of oxygen and any other chemoattractants, *V. cholerae* does not display any chemotactic behavior, making it tempting to speculate that the VCAer-2 protein senses oxygen directly.

As in other bacterial species, the results presented in this study indicate the presence of a secondary aerotaxis transducer in *V. cholerae.* Two putative *V. cholerae* MCP homologs with high sequence similarity to the Tsr protein, found to be a secondary mediator of aerotaxis in *E. coli,* were analyzed for involvement in aerotaxis. Neither gene, deleted either by itself or in combination with *aer-2,* seemed to be important for aerotaxis of *V. cholerae.* Thus, one of the many other MCPs of *V. cholerae* is expected to be part of the complex pathways underlying the aerotaxis signal transduction.

Although, the role of chemotaxis and particularly aerotaxis in the biology of *V. cholerae,* including its environmental and infectious life stages, remains to be fully understood, this study provides a solid foundation for future studies into functions of the multiple chemosensors found in this organism. The enormous complexity of the potential signals perceived, including oxygen, makes *V. cholerae* a particularly interesting model organism to study chemotactic behavior.

Master of Science thesis of Markus A. Boin presented on January 5, 2007.

APPROVED:

Major Professor, representing Microbiology

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Dean of the Graduate School

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Markus A. Boin, Author

Identification of an Aerotaxis Transducer in *Vibrio cholerae.*

by

Markus A. Boin

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INTRODUCTION

A brief history of cholera.

Cholera has been endemic to south Asia since humans have kept written records. A description in the *Sushruta Samhita*, a book written by an Indian physician in the 6th century B.C., details a typical case of cholera (7). The first reported pandemic of cholera started in 1817, when the disease spread out of the Ganges delta, carried along trade routes, and found its way as far west as southern Russia and east as far as the Philippines (47). The second pandemic, beginning in 1829, reached all the major European cities and caused widespread casualties, leading to the establishment of local Boards of Health (61). During this time, cholera also reached the Americas onboard European vessels, and it quickly spread throughout the United States. It was termed “America’s greatest scourge”, due to its ravaging of several cities including New York, Philadelphia, and New Orleans, and reached a point were people had to be buried in mass graves (14, 61). During the third pandemic which began in 1852, a major advance in the understanding of the disease was made by John Snow. In an epidemiological study, Snow was able to ascertain the connection between contaminated drinking water and the disease (71). However, limited sanitation and implementation of sanitary guidelines still caused large outbreaks. A lesser known discovery at the time was the observation of large numbers of curved bacteria in the intestinal

contents of cholera victims by Filippo Pacini. His discovery remained largely unknown due in part to him publishing his findings in a little known local journal and his lack of showing a convincing etiological relationship (5). Due to the fear of cholera, international cooperation in health began during this time; with the first international meeting held in Paris (6).

Three more pandemics followed, claiming many lives throughout the world. In 1883, during the fifth pandemic, Robert Koch demonstrated conclusively that cholera was caused by a curved bacterium, which he called *Kommabazillen* (44). The 5th and 6th pandemics were caused by the classical biotype *Vibrio cholerae* O1, and although the causative agent of the first four was never isolated, it is widely assumed to have been the same biotype (6). During the sixth pandemic a new biotype of *V. cholerae*, designated El Tor (after El Tor quarantine camp in Egypt where it was isolated) was found in 1905, but believed to be incapable of causing pandemics (6, 57). This notion was obliterated, when in 1961 the 7th pandemic was caused by the El Tor biotype. A major difference in the two biotypes is the severity of the disease caused, with El Tor producing a larger number of mild or inapparent infections, allowing people to travel and spread the disease with greater ease (6). In 1992, a non-O1 serogroup began causing large outbreaks in India and Bangladesh (15, 58). This serogroup, designated O139, was the first non-O1 serogroup to cause large epidemics. It now co-exists with the O1 serogroup in endemic areas in India and Bangladesh and continues to cause large outbreaks. The history of cholera has seen many advances in epidemiology and public health, but its elusive nature, unpredictable appearances as well

as disappearances, has been and will continue to be a challenge for epidemiologists.

Epidemiology

The description of cholera as the classic fecal-oral water-borne disease oversimplifies its transmission. The bacterium can also be transmitted by contaminated food. While transmission via contaminated drinking water is most common in underdeveloped countries, food, particularly undercooked seafood, is the usual route in more developed countries (30, 69). Cholera occurs in seasonal outbreaks; in the Indian subcontinent, outbreaks are usually associated with the warm seasons before and after the monsoon rains (65). In South America, epidemics are strictly confined to the warm season (75). This seasonality is thought to be due to the bacterium's ability to rapidly grow in the environment during warm temperatures and also due to seasonal changes in salinity (42).

V. cholerae's interaction with marine copepods and algae and their seasonal increases also may be a cause for outbreaks (42). In cholera-endemic areas children are most severely affected by the disease, in contrast, newly invaded areas show similar attack rates among all age groups (15, 29). A large infectious dose (10^8 bacteria) is needed to cause disease, although in volunteer studies, this can be lowered to about 10^5 bacteria if antacids are given at the same time to neutralize the stomach's acidity (41, 63). The inoculum size in real life settings may be even lower and many patients have been found to have low gastric acid production (64).

Clinical Features

The incubation period of *V. cholerae* may be as short as a few hours or be as long as five days, generally being two to three days. In volunteer studies the incubation period depends on the inoculation size, suggesting the same maybe true in a real life setting (41, 53). The disease is usually characterized by vomiting and large amounts of the typical rice water stool. Rapid dehydration occurs if lost fluids are not replaced quickly. In its most severe form, cholera can rapidly lead to a patient's demise; inadequately re-hydrated patients can develop hypovolemic shock and may die in less than 24 hours. Survival rates of untreated patients are only around 30% (46).

Treatment

It is imperative for patients with cholera to have all lost fluids replaced by an equal or greater amount, either given orally or in severe cases by intravenous fluids (6). Monitoring of a patient's fluid output is usually done by placing the patient on a cholera cot, a camping bed with a hole in the middle covered with a plastic sheet and a calibrated bucket underneath it, allowing for the collection of the passed stool (67). One of the most significant medical developments of the last century, was the discovery that glucose facilitates sodium and water transport in the intestines (19). This led to the development of the oral rehydration therapy (ORT), which is a simple, effective, inexpensive and most of all universally available means of treating patients with cholera. The World Health Organization recommends the ORT contains 90mmol sodium, 20mmol potassium, 80mmol chloride, 30mmol bicarbonate and a ratio of glucose to sodium of at least 1:1 (www.who.int/en/). Methods of

preparing ORT from rice and other grains are also available, and are used in regions of limited medical supplies. For severely dehydrated patients presenting with shock, rapid rehydration by I.V. fluids is life saving. Ringer's lactate is the best available solution, but due to its low potassium content should be supplemented with ORT (6). The occurrence of cholera outbreaks in areas with limited medical resources makes ORT the treatment of choice for most patients, allowing for the conservation of the limited I.V. supplies for the most severe cases. Antibiotic treatment for cholera serves only to shorten the duration of diarrhea (32). It also limits the volume of diarrhea and the time a patient sheds *V. cholerae* bacteria, allowing for a shorter treatment time with ORT and I.V. fluids (6).

Virulence factors

V. cholerae secretes a wide range of extracellular products that are harmful to eukaryotic cells (78). The main virulence factor for *V. cholerae* is the potent cholera toxin (CT), which when administered to volunteers causes cholera symptoms. This prototypical AB_5-type toxin is responsible for the large volume of diarrhea produced by patients (23). The B-subunit binds to the GM_1 ganglioside, whereas the A-subunit enters the cell and activates adenylate cyclase (72). This increases intracellular cyclic AMP levels, which results in an efflux of chloride and sodium ions, as well as water; leading to the profuse diarrhea associated with cholera (72). Colonization of the intestines is a crucial factor in the pathogenesis of *V. cholerae*. The toxin-coregulated pillus (TCP) has been identified to play a major role in the colonization step (77). Deletion mutants in this pilus were found to be

completely attenuated in human volunteers at doses that allowed colonization of the *tcp*$^{+}$ parent strain (38). Expression of the two main virulence factors, CT and TCP, is influenced by environmental signals including pH, temperature, osmolarity and growth medium composition(50, 56, 77). *V. cholerae's* virulence factors are under the control of a regulatory cascade in which ToxR/ToxS and TcpP/H proteins coordinately control expression of ToxT, itself a transcriptional activator that in turn controls the CT and TCP genes as well as other virulence genes (17, 18, 36)

The unusual localization of these regulators in the bacterial membrane suggests possible interactions with the components of the motility and chemotaxis systems and is suggested by several lines of evidence. At least two genes in the ToxR regulon, *tcpI* and *acfB,* encode MCPs and loss of either of these genes resulted in increased swarm circles in semisolid medium as well as reduced colonization abilities of the bacteria (21, 34). Furthermore, *toxR* mutant strains displayed a hypermotile phenotype, whereas some spontaneous hypermotile strains lack expression of CT and TCP under normally inducing conditions (27). However, in most cases, it is not yet clear if these effects are due to hypermotility per se or to an increase in chemotaxis-directed motility. Although, some non-motile mutants show constitutive expression of CT and TCP and increased *toxT* transcription, deletion of the *cheA* genes did not alter virulence gene expression (31). An *in vivo* screening and further characterization revealed that several chemotaxis genes [*mcpX* (VC2161), *cheZ*, *cheA-2*, and *cheY-3*] appear to be required for the induction of the cholera toxin gene (*ctx*) and *toxT* promoters upon

infection of mice, although the induction of the *ctx* promoter *in vitro* does not require any of these genes except *cheZ* (45). These findings suggest a complex interplay between the chemotaxis-signaling system and virulence gene regulation *in vivo.*

Chemotaxis

Extensive structural and genetic analyses of the chemotaxis behavior of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium have deciphered the complexity of the coordination of movement in response to environmental stimuli (reviewed in (4, 16) and (73)). In *E. coli*, the signal for a chemical attractant or repellent is received by one of four membrane-spanning methyl-accepting chemotaxis proteins (MCPs) that respond to a change in concentration of a limited number of periplasmic chemoeffectors. When an attractant leaves or a repellent binds the periplasmic domain of the MCP, a conformational change occurs (22). A fifth MCP, named Aer, is somewhat more unconventional, in that it responds to oxygen by monitoring the cell' s intracellular energy state (9). The signal generated by MCPs is transmitted through the cytoplasmic linker protein CheW to the soluble protein kinase CheA (28, 68). CheA autophosphorylates and this phosphate is transferred to a response regulator, CheY. CheY-P binds to the flagellar motor causing a switch from counterclockwise to clockwise rotation, resulting in reorientation of the cell (12). Adaptation to a stable background level of attractant is accomplished by varying the degree of methylation of specific residues in the cytoplasmic signaling domain of the MCP. Methyl groups are transferred to the MCP by a constitutively active methyltransferase, CheR,

and removed by a methylesterase, CheB, whose enzymatic activity is increased on phosphorylation by CheA-P The genomes of *E. coli* and *Salmonella* contain only single copies of genes that play a role in the chemotaxis machinery. However, chemotaxis in several other bacteria is more complex. The genomes of a large number of bacterial species, including *Vibrio cholerae*. *Pseudomonas aeruginosa*, *Rhodobacter spaeroides*, *Myxococcus xanthus*, *Borrelia burgdorferi*, and *Yersinia pestis*, encode for multiple gene paralogues of the various chemotaxis genes found in *E. coli* (reviewed in (74)). In most cases, the detailed functions of these redundant gene paralogues have not been elucidated. In *V. cholerae* only CheY-3, one of five CheY paralogues, switches flagellar rotation and only CheA-2, one of three CheA paralogues, was found to be essential for chemotaxis (31, 43).

Chemotaxis and virulence in *V. cholerae*

Although the role that motility plays in virulence of *V. cholerae* has not been fully elucidated, but it has been identified as an important factor in some animal models (60). Non-motile strains of *V. cholerae* have been found to be attenuated in the infant mouse model (27, 45). Similarly, the role of chemotaxis during human infection remains largely unknown. Under normal conditions motile *V. cholerae* bacteria enter the mucus gel and reside in the intervillous spaces, where they direct themselves to the mucosal surface, most probably in response to chemoattractants (26). In the rabbit ileal loop model, chemotactic vibrios outgrow non-chemotactic mutants, most likely due to their greater association with the intestinal mucosa (25). In contrast, non-chemotactic mutants outperformed the wild-type strain in the infant mouse

model; causing a more rapid and severe disease (24). Non-chemotactic mutants were found to colonize the entire intestine and not just the distal half where most of the wild type strains are found (45). An interesting finding was the observation that the greater colonization fitness depended on biased counterclockwise (CCW) flagellar rotation and was independent of the main adhesion factor TCP (13). A CCW biased mutant has a diminished tumbling ability and remains in longer smooth swimming runs, whereas as clockwise (CW) mutant tumbles excessively, confining it to the lumen of the intestine (13). The out-competition phenotype of non-chemotactic mutants seems not to be due to an inability to chemotact, but rather expanded colonization distribution due to smooth swimming. The differences in animal models make it hard to interpret some findings and to get a clearer picture of the role chemotaxis plays in virulence. However, all these results were obtained in animal models and human studies might show different results.

The genome sequence of *V. cholerae* revealed a large number of chemotaxis related genes (37). A total of 46 open reading frames have been annotated as putative MCP proteins, however, there is only limited knowledge about their functions in *V. cholerae.* As a first step towards assigning specific functions to some of the MCPs, we targeted three putative MCPs showing significant homology to the *E. coli* aerotaxis transducer, Aer, in *V. cholerae.* Deletion constructs for each gene were created and introduced into the chromosome of *V. cholerae* via homologous recombination. Mutants were analyzed for aerotaxis in a variety of assays. The intact genes were provided *in trans* for complementation studies. Our results strongly suggested the

presence of an additional protein involved in aerotaxis in *V. cholerae*. Based on findings in other bacterial species, two additional genes were examined for their possible involvement in aerotaxis in *V. cholerae*. In summary, we did identify one of the putative Aer proteins in *V. cholerae* as an aerotaxis transducer similar to Aer in *E. coli.* This study reports the first functional analysis of any of the *V. cholerae* MCPs.

Chapter 2

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *V. cholerae* O1 classical strain 0395N1 (designated hereafter c-WT) and El Tor strain Bah-2 (Bah-2) were grown on Luria Bertani (LB) (BIO101, Carlsbad, CA) solidified with 1.5% agar (wt/vol) (Acros Organics, Geel, Belgium) containing 100μg ml^{-1} streptomycin sulfate (EMD Biosciences, Gibbstown, CA). Liquid cultures of *V. cholerae* were grown in culture tubes containing 3 ml of LB broth at 37°C in a roller drum (New Brunswick Scientific, Edison, NJ). *E. coli* strains RP437 (54), UU1117 (9), UU1250 (10) and SM10λ*pir* (70) were grown on LB plates without antibiotics. *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA) and DH5α cells were used for routine cloning and grown on LB supplemented with appropriate antibiotics. All inoculated plates were incubated at 37°C unless otherwise noted. Liquid cultures of all *E. coli* strains were grown in LB broth at 37°C in a roller drum. Antibiotics were dissolved in sterile H_2O and kept as 100mg ml^{-1} stock solutions at 4°C. Antibiotics were added to LB agar cooled to 50°C at a final concentration of 100μg ml^{-1}.

Table 1. Strains and Plasmids used in this study.		
Strain or plasmid	Relevant characteristics	Source or reference
Vibrio cholerae strains Classical O395N1	Wild-type for chemotaxis	(48)
El Tor Bah-2	Wild-type for chemotaxis	(55)
ΔVCAer-1	Bah-2 with deletion in VC0512	Häse lab
ΔVCAer-2	O395 with deletion in VCA0658	Häse lab
ΔVCAer-3	O395 with deletion in VCA0988	Häse lab
ΔVCTsr-1	O395 with deletion in VC0098	This study
ΔVCTsr-2	O395 with deletion in VCA1092	This study
ΔVCAer-2/ ΔVCAer-3	O395 with deletion in VCA0658 &VCA0988	This study
ΔVCAer-1/ ΔVCAer-2/ ΔVCAer-3	Bah-2 with deletion in all 3 *aer* homologs	This study
ΔVCAer-1/ ΔVCTsr-1	O395 with deletion in VCA0658 & VC0098	This study
ΔVCAer-1/ ΔVCTsr-2	O395 with deletion VCA0658 & VCA1092	This study
ΔVCTsr-1/ ΔVCTsr-2	O395 with deletion VC0098 &VCA1092	This study

Table 1 continued

Plasmids		
pWM91	suicide vector	(49)
pBAD24	arabinose inducible promoter	(33)
pBAD TOPO	arabinose inducible promoter	Invitrogen
pWM91ΔVCAer-1	suicide vector with VC0512 deletion construct	Häse lab
pWM91ΔVCAer-2	suicide vector with VCA0658 deletion construct	Häse lab
pWM91ΔVCAer-3	suicide vector with VCA0988 deletion construct	Häse lab
pWM91ΔVCTsr-1	suicide vector with VC0098 deletion construct	This study
pWM91ΔVCTsr-2	suicide vector with VCA1092 deletion construct	This study
pBADVCAer-1	expression plasmid carrying VC0512	Häse lab
pBADVCAer-2	expression plasmid carrying VCA0658	Häse lab
pBADVCAer-3	expression plasmid carrying VCA0988	Häse lab
pBADAer	expression plasmid carrying *E. coli aer*	This study

Construction of *V. cholerae* Aer and Tsr deletion mutants.

Primers used for PCR are listed in Table 2. Deletions in VCAer-1, VCAer-2, and VCAer-3 were made by PCR amplifying 1000bp fragments of the up- and downstream regions of the genes. These fragments were cloned into pWM91 (49) and later spliced together using restriction sites engineered into the primers using standard procedures (66). In frame deletions of VC0098 and VCA1092 were made by using overlap extension PCR (OE-PCR) (39). Two primer sets are designed to amplify regions adjacent to the gene of interest. Primers I and II amplify a region from the start codon to approximately 1500bp upstream of the targeted gene. Primers III and IV amplify a region from the stop codon to approximately 1500bp downstream of the gene of interest. Primers II and III have 12 nucleotides that are complementary to each other at their 5' ends. This nucleotide sequence is not normally found in the *V. cholerae* genome and was designed arbitrarily. Two separate PCR reactions using primer I together with II and primer III together with IV, respectively, were performed using high fidelity Platininum PCR Supermix (Invitrogen). 3µl of a suspension of a *V. cholerae* colony in 100µl dH_2O was used as the DNA template. The following reaction conditions were used.

Step 1: 94°C - 2:00 min
Step 2: 94°C - 0:30 min
Step 3: 55°C - 0:30 min
Step 4: 68°C - 1:40 min
Step 5: go to step 2 – 29 times
Step 6: 68°C – 5:00 min
Step 7: 4°C

5µl of the PCR products were run on a 1% agarose gel in 1X TAE buffer at 100V for 40 minutes. The gel was stained using ethidium bromide and

visualized on a BioDocIt gel imaging station (UVP Inc, Upland , CA). This analysis was performed to confirm the reaction yielded the expected product without the formation of any other non-specific products. Four new PCR reactions were performed using primers I and IV and 3µl of the previous reactions, either undiluted or diluted 10-, 100-, or 500-fold, as the DNA template. The same PCR protocol was used as above with the exception of increasing the time in step 4 of the PCR protocol to 3:20 minutes. The resulting I/IV product was gel electrophoresed as described above. A 3000bp fragment, which is expected to be present, was cut from the gel and extracted using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The manufacturer's manual was followed except for a change in the elution volume; only 30µl of elution buffer was used to collect the final sample. Approximately 1µg of the purified I/IV product was digested sequentially with *Not*I and *Spe*I restriction enzymes at 37°C for 2 hours. Restriction sites for these enzymes were incorporated into primer I and IV, respectively, at their 5' ends (Table 2.). The suicide vector pWM91 (49) was linearized with the same restriction enzymes. The digested I/IV product and linearized vector were combined in a 3:1 ratio and ligated at 16°C overnight with T4 ligase (New England BioLabs, Ipswitch, MA).

SM10λ*pir* cells were made chemically competent prior to transformation with the ligation products. The following protocol for generating competent cells was used: 5ml of fresh LB was inoculated with 100µl of an overnight culture of SM10λ*pir* cells and grown to an OD_{600} of 0.6. The cells were placed on ice for 10 minutes and spun down in pre-chilled microfuge tubes in a table

top centrifuge at 13,000 rpm for 1 minute. The supernatant was discarded and the cells were re-suspended in 500µl of ice cold 01.M $MgCl_2$. The cells were centrifuged again and the supernatant discarded. Next, the cells were re-suspended in 500µl of ice cold 0.1M $CaCl_2$ and left for 10 minutes on ice. After a final centrifugation step the cells were re-suspended in 60µl of ice cold $CaCl_2$ containing 15% glycerol. Cells were kept on ice as much as possible during the entire procedure. The cells were used on the same day they were prepared.

To introduce the vector into the host strain, 5 µl of the ligation product were mixed with the chemically competent SM10λ*pir* cells and left on ice for 20 minutes. The cells were heat shocked at 42°C for 30 seconds and placed immediately back on ice for 3 minutes. After addition of 200µl of SOC medium (Invitrogen, Carlsbad, CA), the cells were allowed to recover at 37°C for 1 hour with shaking. 100µl of the cells were plated onto LB agar plates containing 100µg/ml of ampicillin and 50µl of top spread 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal). Plates were incubated overnight at 37°C and white colonies were screened by PCR to detect the presence of the insert. Frozen stocks of clones containing the insert were prepared in LB media containing 16% (vol/vol) glycerol and kept at
-80°C.

Table 2 . PCR primers used in this study.

Target	Primer	PCR product
VCAer-1	F: 5' TGTAAATGGAGAGCATAATG R: 5' AGTGTTAACCTACATCTG	~1600bp
VCAer-2	F: 5' CCCGAAATGTCAGCCTAT R: 5' CGCGCCTATTTTTGTGC	~1600bp
VCAer-3	F: 5' CTCTTTTATGCGCAATAACC R: 5' GCTCTTGCGCTTTGTTTA	~1600bp
Aer	F: 5' TCTTCTCATCCGTATGTCACCCAGC R: 5' TTAATGCAGTACCCGTCACCG	~1600bp
Upstream of VCTsr-1	I: 5' GGGGACTAGTGTAAGCGCGCGCTTTTACAGC II: 5' TGAGCCTGGGTTGGATGAAAC	~1500bp
Downstream of VCTsr-1	III:5'ATGGTTTCATCCAACCCAGGCTCAAACCTGCTCTC CTTGGATGGGACC IV: 5'AAAAAAGCGGCGCGCGCTTAACCAAGGATTTGA GAGG	~1500bp
Upstream of VCTsr-2	I: 5' GGGGACTAGTAAGAACCCATCCAGTATG II: 5' TGCATGCATGCATAATCTAGGGATAACGTATG TTGG	~1000bp
Downstream of VCTsr-2	III: 5' TGCATGCATGCACATGTGTCTTCCCTCGTTATATGC IV: 5' GGGGGCGGCCGCTATGAATTCAGCGAATTTGAC	~1000bp
Upstream of VCAer-1	F: 5' CTCGAGGGGATGGAATATAAGTAATG R: 5' TTACATTTGATTGGTATAAATTAAG	~1000bp
Downstream of VCAer-1	F: 5' AGCTTAGTGGCAAACGCAGGTG R: 5' TCTAGACTCAGATATTATTTCAGTATTT	~1000bp
Upstream of VCAer-2	F: 5' GGATCCCGAAAAAGCTGAGCGTATGG R: 5' CTCGAGGTTTGCACAAAAATAGGCG	~1000bp
Downstream of VCAer-2	F: 5' CTCGAGGGGAGTATAGGCTGACATTT R: 5' TCTAGAGCGATGACTTTCCCCCGT	~1000bp
VCAer-3 plus flanking regions	F: 5' TCTAGAGCCGAATTGCAAACGCAGA R: 5' GAATTCCTTTGCGAGATATTGAGGTCTTGCCACT	~3500bp

Bacterial conjugation and counterselection.

SM10*λpir* cells carrying the various deletion constructs were mated with *V. cholerae* by cross-streaking on LB plates without antibiotics and allowed to conjugate for about 7 hours at 37°C (62). The cells were harvested by washing the plates with 2ml of LB media and plating serial dilutions on LB plates containing 100µg/ml streptomycin (to select against the *E. coli* donors) and 100µg/ml ampicillin (to select for presence of integrated plasmid). Surviving colonies were streaked onto LB plates without antibiotics, to allow for a second homologous recombination event to occur, and incubated overnight at 37°C. Colonies were then streaked onto LB plates containing 10% sucrose (wt/vol) (Sigma, St. Louis, MO) and incubated at room temperature for 72 hours. This step selects against the integrated plasmid, due to the *sacB* gene, which is lethal in the presence of sucrose. Individual colonies were patched onto LB plates containing streptomycin or ampicillin and incubated overnight at 37°C. Streptomycin resistant, but ampicillin sensitive colonies (pWM91 has been excised) were screened by PCR to determine if the wild-type gene was replaced by the deletion construct.

Construction of complementation plasmids.

PCR was used to amplify the target genes using the high fidelity enzyme *Pfx* (Invitrogen, Carlsbad, CA). VC0512, VCA0658, and VCA0988 were cloned into vector pBAD24. The *E. coli aer* gene was cloned into vector pBAD TOPO.

V. cholerae cells were made electrocompetent by harvesting cells mid log phase and washing them three times with ice cold 1mM $CaCl_2$. Plasmids were electroporated into *V. cholerae* using 0.2cm GenePulser cuvettes (BioRad Laboratories, Hercules, CA) and the pre-programmed setting EC2 (2.5kV) of a MicroPulser Electroporator (BioRad Laboratories, Hercules, CA).

Aerotaxis assays.

Soft agar swarm plates. Minimal media soft agar swarm plates were used to assess aerotactic behavior (3, 9). The composition of the medium per 100ml is as follows: 20ml 5x M9 medium (Amresco, Solon, OH), 100µl thiamine HCl, 100µl 0.1M $CaCl_2$, 100µl 1M $MgSO_4$ (Amresco) , 5ml 1M sodium succinate (Fisher Chemicals, Fair Lawn, NJ), 0.28g agar, and 75ml dH_2O. Plates were made fresh for each experiment and used the same day. Sterile toothpicks were used to inoculate swarm plates by touching a bacterial colony on a LB plate and stabbing the toothpick to the bottom of the swarm plate, which was placed on a grid to ensure adequate spacing between stabs. The plates were incubated at 30°C for 24 hours. Plates were photographed using a UVP BioDocIt imaging station. Swarm diameters were measured using ImageJ analysis software (http://rsb.info.nih.gov/ij/). Statistical analysis was performed using Microsoft Excel Sofware. To asses swarming behavior under anaerobic conditions, KNO_3 (Mallinckrodt Baker Inc., Phillipsburg, NJ) was used as an alternative electron acceptor at a final concentration of 50mM in the swarm plate. Plates were inoculated as described above and placed in an anaerobic chamber together with a gas pack and incubated at 30°C for 4 days.

For complementation assays, arabinose was added to the above described medium at a final concentration of 0.05% (vol/vol) for promoter induction, as well as 100µg/ml of ampicillin to maintain the expression plasmids in the host strains.

Capillary assays. A flat capillary (Vitro Dynamics Inc., Rockaway, NJ) was placed in a cell suspension (mid log phase) and the liquid was allowed to rise to within 1cm of the end of the capillary (79). Cells for this assay were grown in the same medium as was used for the swarm plates, except that no agar was added. The ends of the capillary were sealed using melted wax to avoid evaporation of the liquid. Capillaries were mounted on a microscope slide to allow for easier handling and observation under a microscope. Cells were checked for motility within 5 minutes using an inverted microscope. Capillaries were left at room temperature overnight and observed the following day. Images were captured using a digital camera attached to a Leica microscpope.

Agarose-in-plug bridge assay.

To check whether chemicals had a positive chemoattractant effect on *V. cholerae*, an agarose-in-plug bridge assay was performed (80). Microscope slides were prepared by applying two small strips of tape parallel to each other approximately 15 mm apart. 7.5µl of melted 2% agarose, supplemented with any chemoeffectors to be tested, was pipetted in between the two strips of tape. A glass cover slip was then placed immediately over the drop of agarose. After a 5 minute cool down period, a bacterial suspension was pipetted between the coverslip and the microscope slide; fully surrounding the

agarose plug. To prepare the bacterial suspension, cultures from an overnight growth in M9 minimal medium supplemented with succinate were washed twice with M9 minimal medium containing no carbon source. To avoid damage to bacterial flagella, cells were centrifuged at only 5500 rpm and vigorous pipetting was avoided during re-suspension. Slides were observed with a microscope after 10 minutes and photographed.

Chapter 3

RESULTS

Identification and sequence analysis of *V. cholerae aer* homologs.

Analysis of the published genome sequence of *V. cholerae* (37)(www.tigr.org), revealed the presence of three potential sensory signal transducer genes with high similarity to the *E. coli* aerotaxis transducer *aer* (Fig.1). This identification was made on the basis of the highly conserved domain (HCD) of chemotaxis transducer genes (11), as well as the presence of a PAS domain (76). Furthermore, the two transmembrane regions found in conventional MCPs are fused into one, similarly to Aer, placing their sensing domains inside the cytoplasm, while anchoring the proteins in the cell membrane. VCA0658 and VCA0988 are located on chromosome II in the *V. cholerae* genome. VC0512 is found on chromosome I and is part of a 26.9kb genomic island in the El Tor biotype, but is absent in the classical biotype strains (52).

Mutagenesis of *V. cholerae aer* homologs.

The three genes were inactivated by introducing the vector pWM91, carrying one of the three deletion constructs, via conjugation with *E. coli* strain SM10*λpir.* The presence of the mutated copy of the gene on the *V. cholerae* chromosome was confirmed by PCR (data not shown).

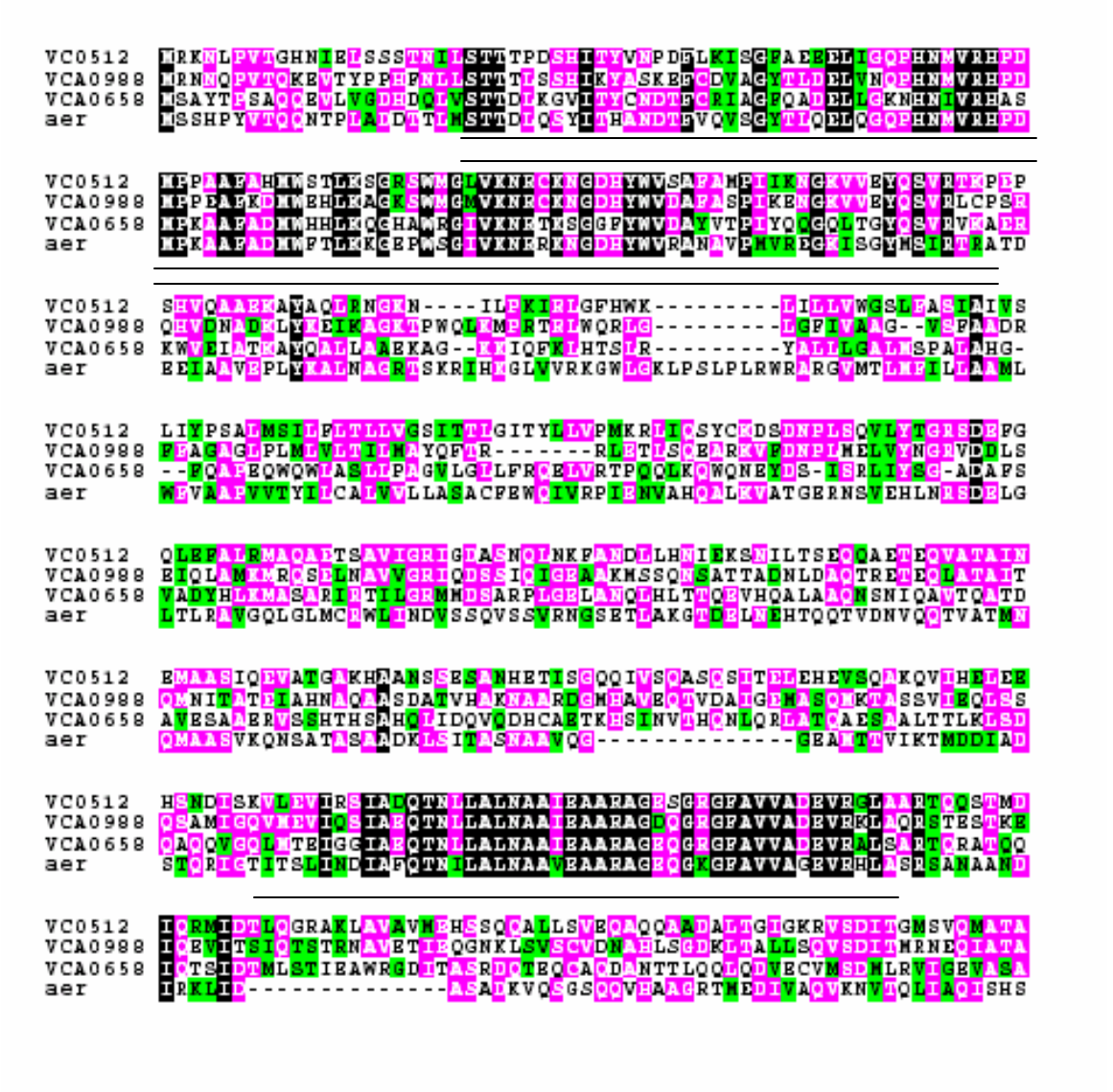


Figure 1. Amino acid sequence alignment of *E. coli* Aer to the three *V. cholerae aer* homologs. The HCD is shown underlined and the PAS domain is double underlined. Globally conserved residues are shaded black.

Characterization of the *V. cholerae* Aer deletion mutants.

Several methods were used to assess whether the three constructed mutant strains showed a defect in aerotaxis, including soft agar swarm plates. (Fig. 2 and Fig. 3). Only ΔVCAer-2 showed a reduced swarm circle diameter ($p \leq 0.05$) compared to the O395N1 parent strain after 24 hour incubation at 30°C. Deletion of VCAer-3 caused a slight, but statistically insignificant increase in diameter ($p=0.08$). A double mutant in VCAer-2 and VCAer-3 had a reduced diameter when compared to the parent strain, but was slightly larger than ΔVCAer-2 alone ($p \leq 0.05$). In the El Tor biotype, deletion of VCAer-1 had no effect on swarm circle diameter ($p=0.25$). Deletion of all three genes in the El Tor biotype showed similar results to the classical biotype double mutant (data not shown). To ensure that the smaller swarm circle diameter for strain ΔVCAer-2 was not due to a growth defect, growth rates of WT and ΔVCAer-2 in liquid succinate minimal medium were compared and found to be identical (data not shown).

A striking difference between the O395N1 parent strain and ΔVCAer-2 was the notable absence of the outermost swarm ring in the mutant, which was largest at the bottom of the plate in the parent strain. This ring was also absent in the ΔVCAer-2/ΔVCAer-3 double mutant, as well as the El Tor ΔVCAer-1/ΔVCAer-2/ ΔVCAer-3 triple mutant. Several attempts were made to document this difference with a camera, but ultimately failed. Swimming behavior of all strains was found to be similar when observed under a microscope.

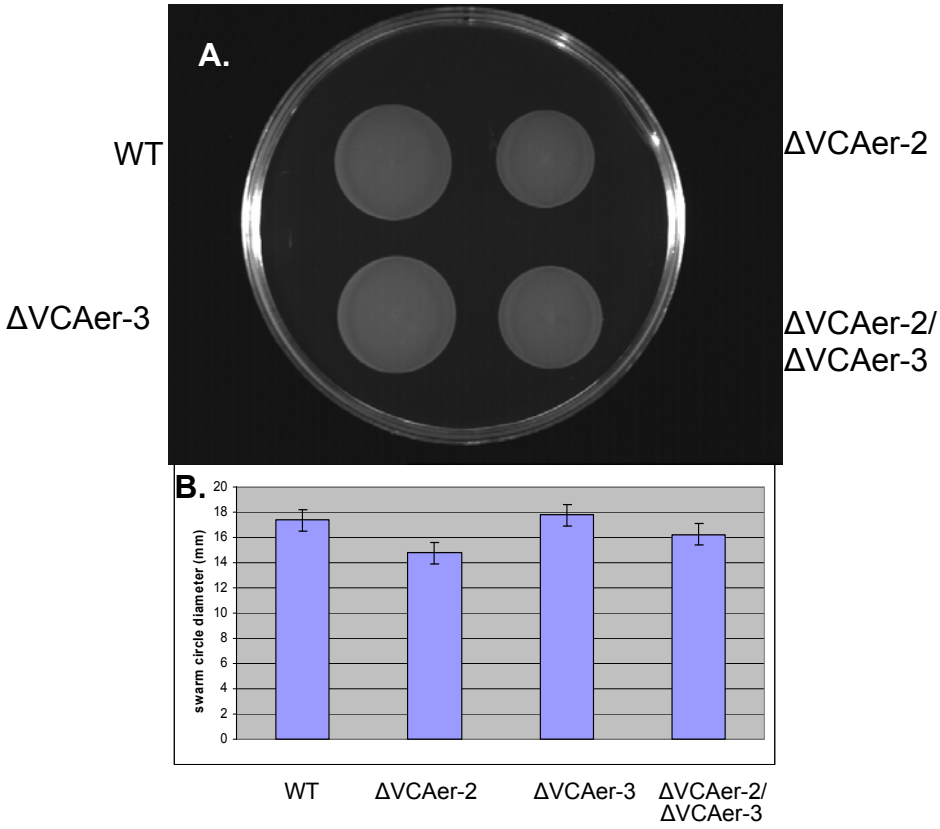


Figure 2. A. Swarm patterns of wt O395N1, ΔVCAer-2, ΔVCAer-3, and ΔVCAer-2/ΔVCAer-3 in succinate soft agar swarm plates. B. Histogram comparing averages of swarm circle diameters.

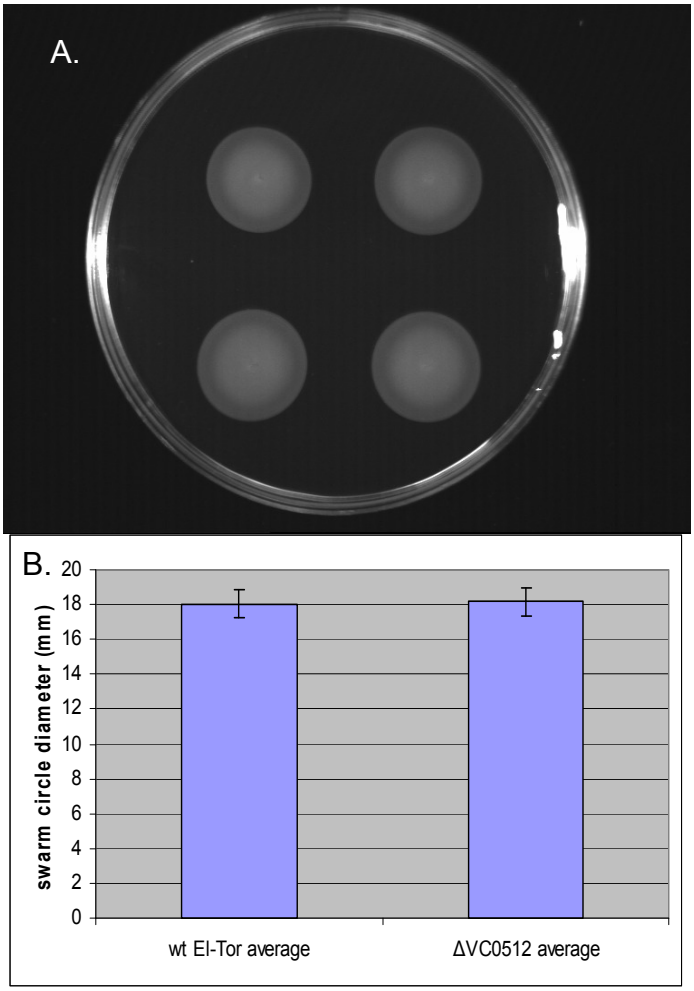


Figure 3. A. Swarm patterns of wt El Tor and ΔVCAer-1 in succinate soft agar plates (duplicates of each strain are shown). B. Histogram comparing averages of swarm circle diameters of wt El Tor and ΔVCAer-1.

Complementation of the *V. cholerae* VCAer-2 mutant.

In order to ensure that the observed phenotype of ΔVCAer-2 was due to the deletion of the gene, plasmids carrying various *aer* genes were constructed and introduced into ΔVCAer-2. Only in the presence of arabinose, did pBADVCAer-2 fully complement ΔVCAer-2, increasing the swarm circle diameter even beyond that of the parent strain (Fig. 4A). It also fully restored the outermost swarm ring that was notably absent in the knock-out strain. In contrast, neither pBADVCAer-1 nor pBAD24VCAer-3 had any effect on the swarm circle diameter of ΔVCAer-2 (Fig. 4A). The complementation of ΔVCAer-2 with the *E. coli aer* gene seemed to increase the diameter slightly; however no difference between the inducing and non-inducing conditions were observed (Fig. 4B) Also, the outermost swarm ring was still absent in ΔVCAer-2 pBADAer. In *E. coli* strain UU1117, an *aer* knock-out strain, pBADAer restored aerotaxis in succinate swarm plates completely (data not shown). Introduction of the *V. cholerae* aerotaxis transducer VCAer-2 into into UU1117 and UU1250 (*E. coli* strain lacking all MCPs) did not restore aerotaxis in either strain (data not shown).

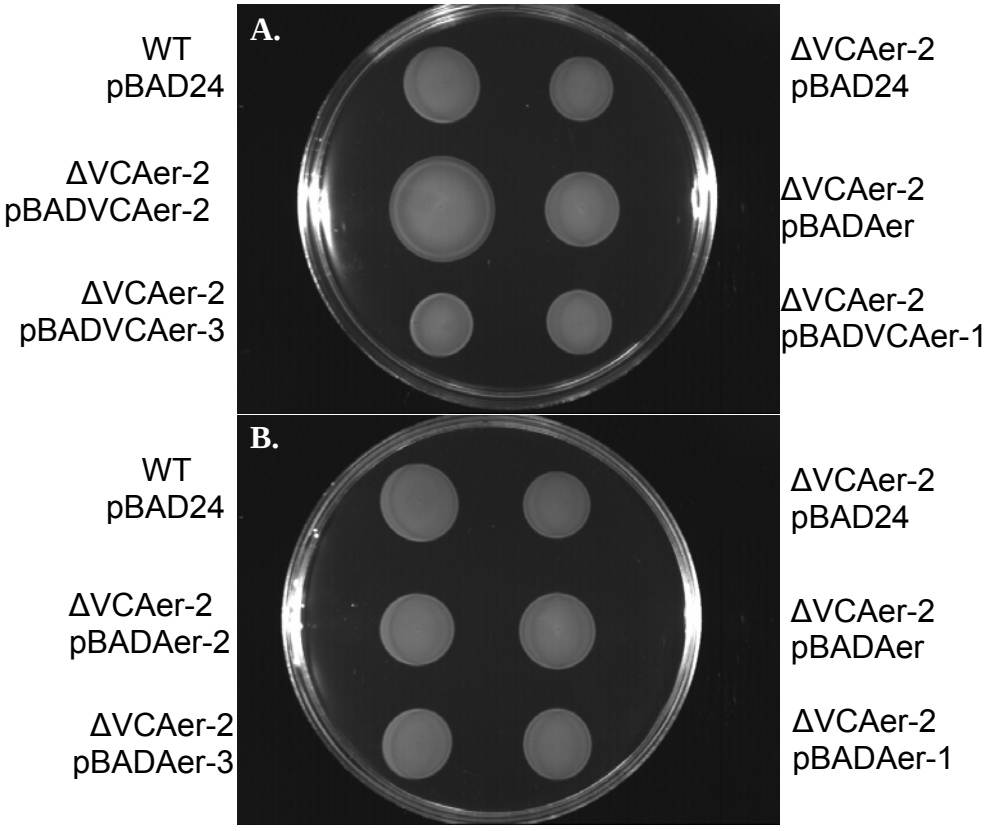


Figure 4. Swarm patterns of WT pBAD24 and ΔVCAer-2 carrying different *aer* expression plasmids in the presence of arabinose.(A.) or without arabinose (B.).

Anaerobic chamber

To test that the outward movement of *V. cholerae* cells in semi solid succinate agar plates is due to the cells responding to an oxygen gradient, anaerobic conditions were used to eliminate the oxygen stimulus. To allow the cell to perform anaerobic respiration, KNO_3 was used as an alternative electron acceptor in place of oxygen. Due to the slow growth under anaerobic conditions in minimal medium the incubation time was increased from 24 to

96 hours. In the absence of oxygen, the outward movement from the initial inoculation site was severely limited in all strains and no difference in swarm circle sizes was observed between the parent and mutant strains (Fig. 5).

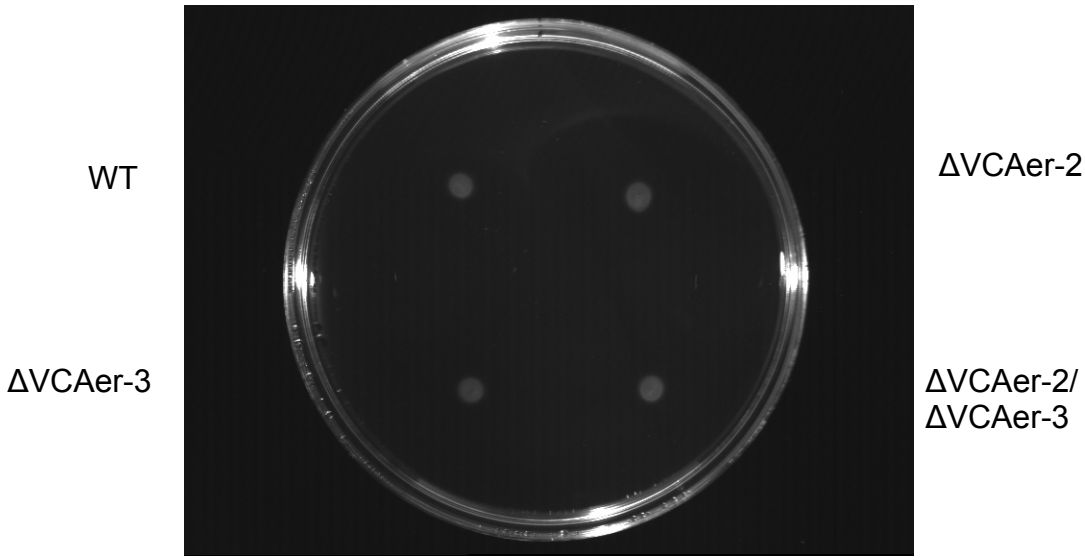


Figure 5. Swarm patterns in semi solid succinate agar during anaerobic conditions. KNO_3 was used as the alternative electron acceptor.

To ensure that the addition of KNO_3 to the medium did not cause the limited outward movement, the plates were also tested under normal aerobic conditions. Figure 6 shows that the bacterial swarm circles formed in the KNO_3 supplemented plates, were essentially the same as in regular succinate plates.

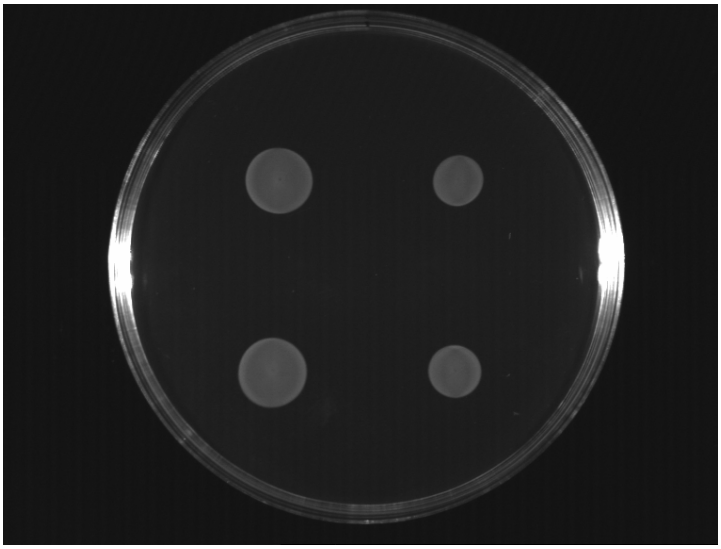


Figure 6. Swarm patterns in KNO_3 supplemented succinate plates under aerobic conditions.

To check that the swarming ability of the bacteria under anaerobic conditions is not impaired, succinate plates supplemented with 0.1mM of the amino acids histidine, leucine, threonine, and methionine (known to be chemoattractants (26)) were used under anaerobic conditions. All the strains formed chemotactic rings in the swarm plates, and no difference between the swarm circles of the different strains was observed (Fig. 7).

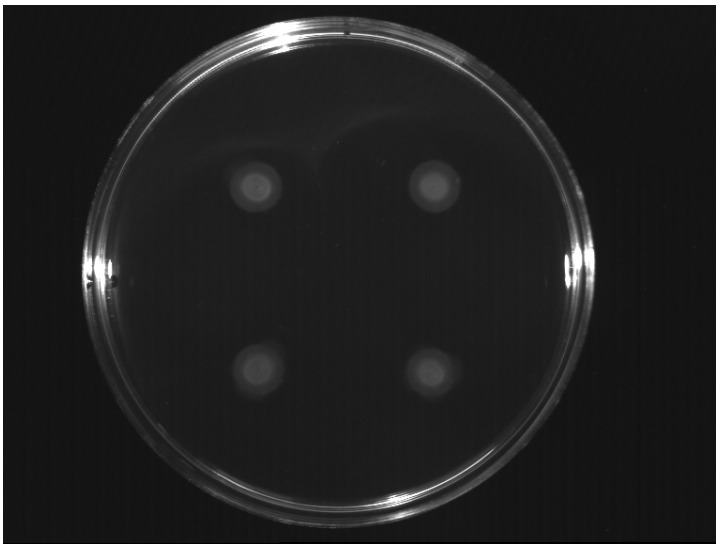
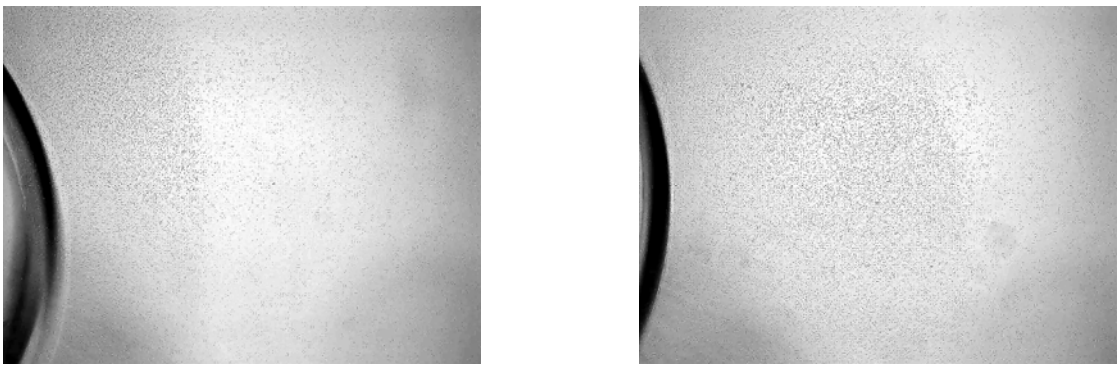


Figure 7. Swarm patterns in succinate plates supplemented with amino acids under anaerobic conditions.

Capillary assay

To further establish VCAer-2 as an aerotaxis transducer, a capillary assay was performed (79). Wild-type cells clustered closer to the meniscus of the liquid-air interface compared to ΔVCA0658, which was more diffuse and further back (Fig. 8). Actively swimming bacterial cells were observed in the dense zones of bacteria in both strains. For both strains, behind this zone of dense bacterial concentration was a region where no bacteria were present. ΔVCAer-1 and ΔVCAer-3 did not show an altered response in the capillary assay (data not shown).



WT ΔVCAer-2

Figure 8. Capillary assay showing normal aerotactic behavior of the parent strain and the altered response of the ΔVCAer-2 strain. The dark line on the left is the meniscus of the liquid-air interface.

Identification and characterization of two putative *V. cholerae* Tsr homologs.

A Blast search of the *E. coli* chemoreceptor and aerotaxis transducer Tsr against the *V. cholerae* genome revealed two likely candidates, VC0098 and VCA1092, designated VCTsr-1 and VCtsr-2 respectively. These genes were also identified by Heidelberg *et. al.* during the sequencing of the two *V. cholerae* chromosomes to be phylogenetically most closely related to *E. coli.*. In-frame deletion mutants were constructed using OE-PCR (39). The deletions were introduced into the *V. cholerae* genome by homologous recombination using a suicide vector and confirmed by PCR (data not shown). Analysis of the mutants in succinate soft agar plates revealed that loss of either gene results in a small, but statistically insignificant increase in swarm circle diameter (Fig.9). Double mutants in VCAer-2 and either VCTsr-1 or VCTsr-2 showed no significant difference compared to ΔVCAer-2 alone. A double mutant in VCTsr-1 and VCTsr-2 did not change the swarm circle diameter anymore than the single mutants (Fig.9).

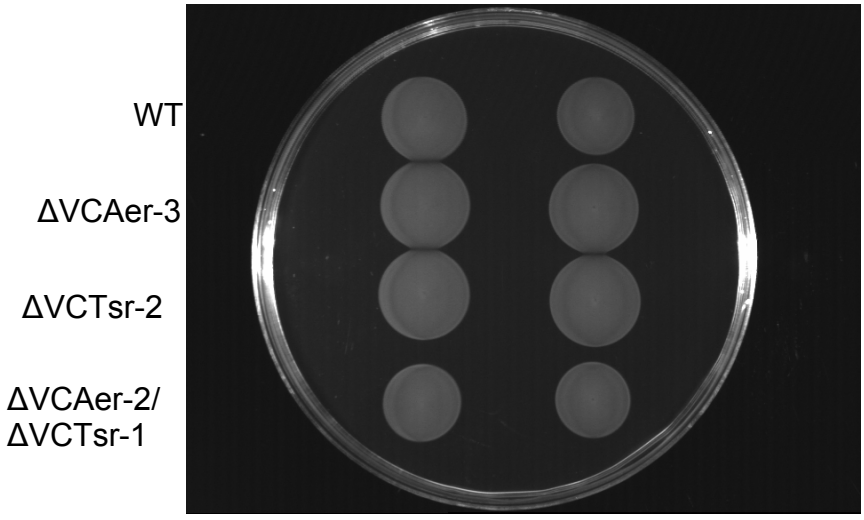


Figure 9. Swarm patterns of various putative aerotaxis related mutant strains.

Agarose-in-plug bridge assay

To ensure that the reduced swarm circle diameter of ΔVCAer-2 in succinate soft agar plates is not due to a chemotactic defect, an agaraose-in-plug bridge assay was performed. Wild-type *V. cholerae* cells were strongly attracted to the agarose plug supplemented with LB, forming a dense band around the plug (Fig.10B). Around the amino acid supplemented plug, bacteria formed a less dense band that was not as pronounced as the band around the LB plug (Fig.10A). Bacteria accumulated around the succinate plug to a much lesser extent than the LB or amino acid supplemented plugs, but more bacteria were present than the control plug (Fig.10C&D). When observed with the naked eye the bands around the LB and amino acid plugs were visible, this was in contrast to the control and succinate plugs (data not shown). ΔVCAer-2 showed no difference to the parent strain in its response to the chemoeffectors tested (data not shown). VCTsr-1 and VCTsr-2 were also specifically tested for their ability to respond chemotactically towards serine as a stimulus. Neither strain showed a defect in its response in the agarose-in-plug bridge assay compared to the parent strain (data not shown).

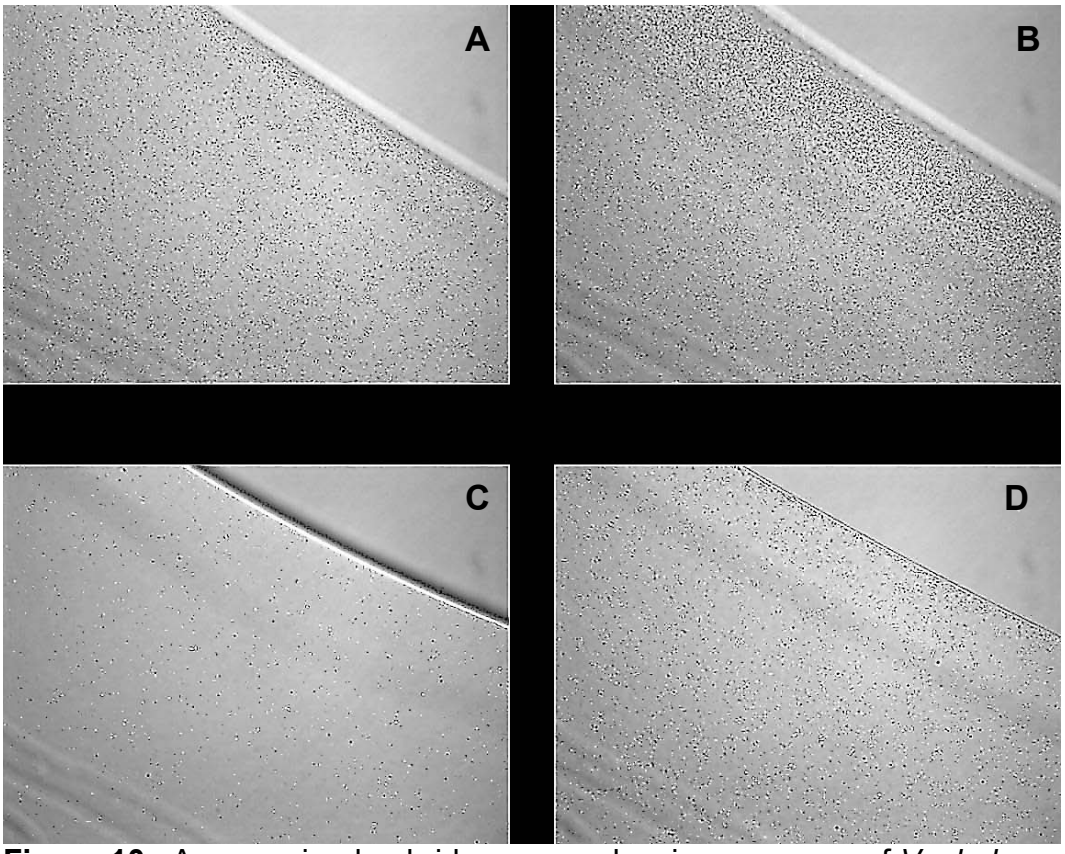


Figure 10. Agarose-in-plug bridge assay showing response of *V. cholerae* to amino acids (A), LB (B), negative control (C), and succinate (D). The line in the upper right corner is the edge of the agarose plug.

Chapter 4

DISCUSSION

Identification and sequence analysis of *V. cholerae aer* homologs

Three potential aerotaxis transducer genes in the genome of *V. cholerae* were identified based on their high sequence similarity to the *E. coli aer* gene (Fig.1). This multiplicity of genes is not surprising as *V. cholerae* seems to have a large repertoire of chemotaxis related genes (37). The *V. cholerae* open reading framesVC0512, VCA0658, and VCA0988 (www.tigr.org) redesignated VCAer-1/-2/-3, respectively, all contain the highly conserved signaling domain (HCD) common to all MCPs at their carboxy-terminus (11). In fact, this domain is the hallmark region that is used to annotate putative *mcp* genes. Furthermore, in their amino-terminus aa sequence each of the *V. cholerae aer* homologs contain a PAS domain, common in a large family of proteins that sense oxygen, redox potential, or light (76). Contrary to most sensing modules of traditional MCPs , these PAS domains are located in the cytosol. Another feature of the *V. cholerae* Aer proteins have in common with other Aer proteins, is the fusion of the two transmembrane domains of typical MCPs into one central membrane-anchoring domain (9, 40, 51). In the *E. coli* Aer protein, the PAS domain binds flavin adenine dinucleotide (FAD) and produces aerotactic responses by monitoring internal redox changes in the cell (8). Closer examination of the PAS domain of the three *V. cholerae* Aer homologs showed that three residues (Arg-57, His-58, and Asp-60), which

are required for FAD binding in the *E. coli* Aer (8), are conserved in VCAer-1 and -3, but not in VCAer-2. The aspartic acid residue in VCaer-2 is replaced by a serine residue. This might indicate that something other than FAD is bound by VCAer-2 and warrants future studies to indentify the prosthetic group used by VCAer-2 to monitor the redox state of the cell. The classical biotype of *V. cholerae*

Characterization of the *V. cholerae aer* deletion mutants.

Soft agar succinate plates were used to asses the aerotactic ability of the three putative *V. cholerae aer* deletion mutants. An oxygen gradient, leading outward from where the initial stab occurred, is created by the cells as they consume the succinate during cellular respiration (1, 2). In *E. coli* deletion of *aer* causes a large decrease in swarm circle diameter in succinate soft agar plates (9). It is reasonable to assume a similar phenotype should be observed in an Aer mutant in *V. cholerae*. Howerver, only one of the three identified Aer homologs, ΔVCAer-2, showed a reduced swarm circle diameter compared to the parent strain (Fig.2). *V. cholerae* has many paralogs of chemotaxis related genes in its genome, and only one of them is actively involved in the traditional chemotactic phenotype (31, 43). The other gene paralogs might not be expressed under the conditions tested, or might encode for proteins that are not functional, or might be involved in other cellular functions. One possible explanation of the absolute lack of an aerotaxis phenotype in ΔVCAer-1(Fig. 3) is the fact that its presence in the El Tor biotype is due to a lateral gene transfer (52). Commonly, foreign genes are not expressed or the

protein might not be functional. The small increase in swarm diameter of ΔVCAer-3 could be due to the CheW/CheA proteins causing longer smooth swimming runs when they are no longer able to interact with the signaling domain of VCAer-3. This might be an indication that this gene has some cellular function that has not been identified. Microscopic observation of the cells did not reveal a noticeable difference in swimming behavior of any of the constructed mutant strains. Future experiments measuring exact the length of smooth runs versus tumbling frequency might reveal a slight difference in the mutant and parent strain that could explain the small increase in swarm diameter.

We noted that the leading edge of the spreading colony is largest at the bottom of the plate. This could be due to the rapid consumption of oxygen, which is slow to diffuse from the atmosphere into the medium. This observation has been reported for aerotaxis mutants in other bacterial species (9, 51). The aerotactic band formed at the leading edge of the colony by the wild-type parent is absent in ΔVCAer-2, indicating that the response to oxygen is diminished.

Complementation of ΔVCAer-2

The various *aer* genes were supplied *in trans* under the control of an arabinose inducible promoter in ΔVCAer-2 as this was the only mutant that showed an observable phenotype. As expected, pBADVCAer-2 fully restored aerotaxis in ΔVCAer-2. Only in the presence of arabinose, the swarm circle diameter of ΔVCAer-2 pBADVCAer-2 was drastically increased even compared to the parent strain (Fig. 4A), indicating that the aerotactic

response is depending on the level of expression of VCAer-2. These results are comparable to findings in *E. coli* and *P. aeruginosa* were gene expression levels also corresponded to the aerotactic responses (9, 40). In constrast, overexpression of VCAer-1 and VCAer-3 had no effect on the swarm diameter of ΔVCAer-2, giving further indication that they do not play a role in aerotaxis, at least not under the conditions tested in this study. Somewhat unexpected was the fact that VCAer-2 did not complement an *E. coli* Aer mutant. Previous studies of chemotaxis in *V. cholerae* have shown that *V. cholerae* the CheA-2 and CheY-3 proteins can complement their *E. coli* homologs (31, 43). This lack of complementation combined with the noted amino acid change in a crucial FAD binding residue, strongly suggest that VCAer-2 does not bind FAD to monitor the cell's redox potential. It is possible that VCAer-2 binds a prosthetic group other than FAD or it might sense oxygen directly by its PAS domain as has been reported for other PAS domain containing proteins that can sense oxygen (76).

Interestingly, complementation of ΔVCAer-2 with the *E. coli aer* gene seemed to partially restore the phenotype to wild-type levels (Fig. 4A). However, the aerotactic ring in the swarm plate was not restored in ΔVCAer-2 by the *E. coli aer* gene. This indicates that the *E. coli* aer gene does function in the *V. cholerae* background, but is not capable of properly transmitting oft he aerotactic signal. Again, this could be due differences in prosthetic group binding or might be due to its inability to properly intact with the heterologous CheA/CheW proteins. Further studies are needed to elucidate these complementation phenotypes.

Anaerobic chamber

An anaerobic chamber was used as another method to assess the role of VCA0658 in aerotaxis transduction. It was reasoned that without the oxygen stimulus, no observable difference between wild-type and mutant strains should be found. Addition of KNO_3, to serve as an alternative electron acceptor, to the in the succinate soft agar plates allowed the cells to perform anaerobic respiration. As was hypothesized, minimal outward movement from the site of inoculation was observed and no difference between wild-type and mutant strains was discernible (Fig. 5). This strongly indicates that oxygen either directly or indirectly is the signaling molecule for the *V. cholerae* Aer-2 protein. This result also served to show that succinate itself is not a strong chemoattractant for *V. cholerae*, as chemotactic outward movement should have occurred in response to a chemoattractant. This observation is further substantiated by the formation of chemotactic rings under anaerobic conditions, when the soft agar plates are supplemented with amino acids, which are known to be chemoattractants (26). Thus even under anerobic conditions the bacteria are fully capable of performing chemotaxis in the presence of an appropriate attractant.

Under the current model of how Aer functions in *E. coli*, redox changes in the cell are the signal sensed by Aer. Possible ways how this is accomplished are direct reduction of Aer by a member of the respiratory complex, a cytosolic electron donor, or a diffusible redox component (20). Since redox changes should still occur even under anaerobic respiration, the lack of a strong outward movement in soft agar plates under anaerobic conditions by both the

parent as well as the ΔVCAer-2 strain, indicates a possible difference between VCAer-2 and the *E. coli* aerotaxis transducer in how the aerotactic signal is transmitted

Capillary assay

In the aerotaxis capillary assay bacteria form bands of concentrated cells in regions of their preferred oxygen concentration (9). Wild-type *V. cholerae* cells formed a band close to the meniscus of the air-liquid interface, whereas the ΔVCAer-2 strain formed a more diffuse band further away from the meniscus (Fig. 8). The fact that the aerotactic response in ΔVCAer-2 is not completely abolished suggests the presence of a second transducer, as has been observed in *E. coli* (59)*, P.aeruginosa* (40)*,* and *P. putida* (51). The moderate reduction in swarm diameter of the VCAer-2 mutant in succinate soft agar plates also suggested the presence of a second transducer for aerotaxis. Since *V. cholerae* has a large number of MCP encoding genes, the possibility of other MCPs being involved in aerotaxis is very likely.

Analysis of two putative *V. cholerae* Tsr homologs

The combined findings of the soft agar plates and the capillary assay strongly suggested the presence of a second transducer for aerotaxis in *V. cholerae.* In *E. coli,* the MCP Tsr is a secondary aerotaxis transducer as the combined loss of both Aer and Tsr completely abolished the aerotactic response (59). Possible candidate ORFs in the *V. cholerae* genome for a secondary aerotaxis transducer are VC0098 and VCA1092. These ORFs are

phylogenetically more closely related to the *E. coli* Tsr protein than any other *V. cholerae* MCP (37). Although, sequence analysis revealed that neither one of these MCPs are predicted to have any transmembrane domains, a Tsr-like transducer that was found to be involved in aerotaxis in *P. aeruginosa* also did not show any detectable transmembrane domain (40). Thus the possibility for either one the gene products of VC0098 or VCA1092 to play a role in aerotaxis in *V. cholerae* was examined. However, neither ΔVCAer-2/ΔVCTsr-1 nor ΔVCAer-2/ΔVCTsr-2 showed an altered response compared to ΔVCAer-2 alone (Fig. 9). This suggests that any secondary aerotaxis transducer in *V. cholerae* is yet to be identified and might be a more conventional MCP with two transmembrane spanning domains anchoring it in the bacterial membrane. This would be more in line with the current model of Tsr, where being an integral membrane protein is important for sensing changes in the proton motive force (PMF) (20). An intriguing possibility for *V. cholerae* is that instead of sensing changes in the PMF, it might sense changes in sodium motive force. This hypotheses is based on the fact that the *V. cholerae* flagellum is powered by sodium ions (35). Further studies are needed to elucidate the molecular mechanisms of aerotaxis in *V. cholerae*.

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