Published in final edited form as:
Nat Rev Microbiol. 2005 August ; 3(8): 611–620. doi:10.1038/nrmicro1207.

GOING AGAINST THE GRAIN: CHEMOTAXIS AND INFECTION IN *VIBRIO CHOLERAE*

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Abstract

Chemotaxis is the process by which motile cells move in a biased manner both towards favourable and away from unfavourable environments. The requirement of this process for infection has been examined in several bacterial pathogens, including *Vibrio cholerae*. The single polar flagellum of *Vibrio* species is powered by a sodium-motive force across the inner membrane, and can rotate to produce speeds of up to 60 cell-body lengths (~60μm) per second. Investigating the role of the chemotactic control of rapid flagellar motility during *V. cholerae* infection has revealed some unexpected and intriguing results.

The Gram-negative bacterium *Vibrio cholerae*, the causative agent of the severe diarrhoeal disease cholera, is responsible for the deaths of approximately 120,000 people annually[1]. Cholera is contracted by ingestion of contaminated water or food and is therefore associated with inadequate sanitation and poverty. As a result, cholera is endemic mainly in the developing world, despite the fact that *V. cholerae* is present in temperate zones around the planet. Although there are at least 200 known serogroups of *V. cholerae*, cholera has generally been associated only with the O1 and O139 serogroups[2]. The O1 serogroup is divided into two major serotypes, Inaba and Ogawa, and these can be further divided into two biotypes, classical and El Tor. Throughout history, cholera has been associated with explosive epidemics and, since the nineteenth century, pandemics.

Humans are the only known vertebrate host for *V. cholerae* and following ingestion, the organism must survive passage through the gastric barrier of the stomach. *V. cholerae* is not particularly resistant to low pH[3], and this is believed to contribute to the relatively high infectious dose that is required to produce infection in healthy human volunteers[4]. However, *V. cholerae* can adapt to mildly acidic pH, and these acid-adapted bacteria have a huge competitive advantage during experimental infection when compared with unadapted *V. cholerae*[5]. Following passage through the stomach, the bacteria then enter the small intestine,

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Competing interests statement
The authors declare no competing financial interests.

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which is the main site of infection. After reaching the small intestine, chemotaxis could conceivably be required to locate the appropriate intestinal niche for colonization and virulence-factor expression, an idea that will be explored in this review.

Two well characterized virulence factors of *V. cholerae* are cholera toxin and the toxin co-regulated pilus (TCP). The TCP is required for colonization in both humans and animal models of infection[6,7], and these pili are believed to mediate microcolony formation on the intestinal epithelium[8]. Cholera toxin is a ribosylating enterotoxin[9] that is responsible for the profuse watery diarrhoea associated with cholera, known as rice-water stool. The genes that encode these virulence factors are tightly regulated, so that expression does not occur inappropriately, such as during extra-intestinal growth in rich media[10,11]. By eliciting diarrhoea, *V. cholerae* cells are shed from the host and back into the environment in the rice-water stool. It is estimated that cholera patients can shed as many as ten trillion *V. cholerae* cells per day[12], and these shed *V. cholerae* are highly motile, as observed by microscopy[13]. There are two possible fates for *V. cholerae* cells that are shed from the host. First, the bacteria are ingested relatively soon after shedding by another human. This might occur within an explosive epidemic scenario, such as an outbreak in a refugee camp. Second, shed bacteria settle into the environmental-reservoir stage of the life cycle, and might or might not be ingested by another human host at a future point in time.

In addition to being an important cause of morbidity and mortality in the developing world, *V. cholerae* is also a natural inhabitant of freshwater, brackish and coastal-water habitats[14]. A model of the pathogenic and environmental aspects of the *V. cholerae* life cycle is shown in FIG. 1. In the environmental stage, *V. cholerae* can exist in a free-living, planktonic form or associate with several environmental hosts. *V. cholerae* can colonize zooplankton, such as copepods[15,16], phytoplankton, such as cyanobacteria[17,18], and the egg masses of Chironomid insects[19,20]. Bacterial cells might enter a viable but non-culturable state[21], although the requirements for this state are not well defined. *V. cholerae* also associates with abiotic and chitinous surfaces in the form of BIOFILMS[22,23], and motility is required for this process[24]. Recent studies have shown that *V. cholerae* that are present in biofilms have improved survival through the stomach and are probably more infectious than PLANKTONIC CELLS[25]. Virtually all environmental (and clinical) *V. cholerae* isolates that have been described are motile, and although it has not been formally proven, it is reasonable to assume that chemotaxis is vital to the fitness of *V. cholerae* in aquatic environments.

Chemotaxis in *V. cholerae* and other bacteria

The single polar flagellum of *V. cholerae* is covered by an extension of the outer membrane to form a sheath[26]. By using a sodium-motive force across the inner membrane to power the flagellar motor[27,28], rotation of 100,000 revolutions per minute can be achieved in liquid environments [29], generating speeds of up to 60 cell-body lengths (~60 μm) per second[30]. The rotation of flagella results in a propulsive force that imparts motility on the bacterial cell (FIG. 2). However, several modes of flagellum-independent bacterial motility have also been identified, including twitching motility (mediated by cycles of extension, adherence and retraction of type IV pili)31,32 and gliding motility (for example, mediated by secretion of slime)[33]. These forms of motility are important for moving over surfaces, and chemotaxis homologues have a role in regulating various aspects of these motilities, including direction of movement and gene expression32.

In the case of flagellar motility, chemotaxis is achieved by modulating the direction or speed of flagellar rotation in response to the surrounding environment (BOX 1). Owing to the work of many laboratories, a tremendous amount of structural and functional information is available on the signal-transduction cascade that results in the alteration of flagellar rotation in the

peritrichously flagellated enteric bacteria *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (*S. typhimurium*). This makes chemotaxis one of the most studied and well-understood signalling pathways **i**n biology.

The Gram-positive soil bacterium *Bacillus subtilis* has several additional chemotaxis components — CheC[34], CheD[35] and CheV[36] — that are also present in *V. cholerae*[37], but which are not reviewed here. However, although *E. coli* and *B. subtilis* have a single gene encoding each chemotaxis component, genome sequencing has revealed that this situation might be the exception, instead of the rule[38]. Many bacteria possess multiple paralogues of each component, often organized into independent systems. *V. cholerae* is no exception and has an impressive number of chemotaxis paralogues (shown in the table in BOX 1), indicating either that chemotactic signalling in *V. cholerae* might be complex or that other systems in addition to chemotaxis are regulated by these signalling proteins[39].

With the exception of the *mcp* (methyl-accepting chemotaxis protein) genes, which are scattered throughout the genome, most *V. cholerae* chemotaxis genes are organized into three operons[37]. Only one of these, operon 2, is important for chemotaxis *in vitro*. The *cheA-2* (REF. 40) and *cheY-3* (REF. 41) genes in operon 2 are required for chemotaxis, whereas *cheA-1* and *cheA-3*, from operons 1 and 3 respectively, are dispensable for chemotaxis[40]. Furthermore, there does not seem to be redundancy between operons 1 and 3 with respect to chemotaxis, as the simultaneous deletion of all the *cheY* paralogues except the one in operon 2 has no effect on chemotaxis *in vitro* (S.M.B. and A.C., unpublished data). These data are consistent with the absence of strong homology to the FliM-binding region in these CheY paralogues (I. Kawagishi, personal communication).

As mentioned, multiple chemotaxis systems within the same bacterium are common, and in some cases the function of these additional systems is known. The soil bacterium *Myxococcus xanthus*, which initiates a developmental programme under starvation conditions that culminates in the formation of a fruiting body, has four chemotaxis systems. This organism has S-motility, which is a form of gliding motility that requires two cell-surface components: type IV pili and extracellular matrix fibrils. One system of chemotaxis homologues, Dif, is involved in fibril biogenesis[42], and the Frz and Che4 chemotaxis systems control the frequency of reversal of gliding direction[43,44]. The Che3 cluster regulates entry into the spore-formation developmental programme by controlling developmental gene expression[45]. The opportunistic pathogen *Pseudomonas aeruginosa* uses one set of chemotaxis genes to control flagellar motility and another to control twitching motility, which is mediated by type IV pili[46]. As *V. cholerae* chemotaxis operons 1 and 3 are not required for chemotactic control of flagellar motility, it is possible that genes in these operons could regulate flagellum-independent motility in this organism. One such mode of motility has been observed; however, the requirements for this process remain to be determined[47].

The role of chemotaxis in virulence

Although the role of motility during infection has been examined in several bacterial pathogens, the importance of chemotaxis in this process has not been as extensively studied[48]. *A priori*, chemotaxis would be predicted to work hand-in-hand with motility to enable bacteria to swim towards preferred colonization sites. However, in the case of enteric pathogens, the requirement of motility and chemotaxis ranges from being crucial to being dispensable for infection (TABLE 1). For example, *Shigella* species are non-motile but are highly infectious, with an infectious dose as low as 10 cells. Clearly, the absence of motility in this organism is not an impediment to infection. Among motile bacteria, it seems that invasive enteric pathogens might not require motility for infection[49]. Some *Shigella*, *Listeria*, *Yersinia* and *Salmonella* species fall into this category, and each of these cross the epithelial barrier by translocation through M

cells (reviewed in REF. 50), which are specialized antigen-sampling cells that lack an overlying mucus layer51. The ability to invade the epithelium in this manner apparently abrogates the need for motility. By contrast, both flagella and flagellar motility are necessary for *Yersinia enterocolitica* to bind and invade enterocytes in tissue culture[52].

Alternatively, some non-invasive pathogens, such as *Helicobacter pylori* and *Campylobacter jejuni*, require chemotaxis for infection: neither species are thought to attach to the epithelium, but instead use chemotaxis to stay within the mucus layer that lines the stomach[53] and caecal crypts[54], respectively. Like *H. pylori* and *C. jejuni*, *V. cholerae* does not invade the epithelium; however, unlike these species, *V. cholerae* does attach to epithelial surfaces, so that it is not clear whether motility would be expected to be important for infection.

Chemotaxis and *V. cholerae* infection

Surprisingly, chemotaxis actually inhibits the ability of *V. cholerae* to colonize the small intestine of infant mice. Defined non-chemotactic mutants of El Tor biotype strains that lack *cheY-3* or *cheA-2* were shown to out-compete the wild-type strain 70-fold *in vivo*[41] (TABLE 1). This out-competition phenotype correlates with an order-of-magnitude increase in infectivity, as defined by a 10-fold reduction in the number of bacteria that are required to cause an infection[55]. No competitive advantage of these mutants is observed during growth *in vitro*, showing that the advantage is specific to the host small intestine. This unusual phenotype is explored further below.

Do the other chemotaxis systems present in *V. cholerae* have a role during infection? The answer to this question seems to be no. Strains with single or combined mutations in each of the *cheA* and *cheY* paralogues outside of operon 2 retain full virulence in competition assays with the wild-type strain in the infant-mouse model (S.M.B. and A.C., unpublished data). Interestingly, *cheA-1* and *cheR-1* from operon 1 were identified as being highly expressed during infection in humans[56]. However, as these genes are not required for infection in mice, the significance of these data are unclear. At present, it is unclear what role, if any, these additional *V. cholerae* chemotaxis systems have in virulence, or for that matter, for life in the environment. The expression of two genes that encode proteins with homology to MCPs, *acfB* (accessory colonization factor B) and *tcpI*, is co-regulated with virulence genes[57,58]. However the roles of these proteins during infection is unknown.

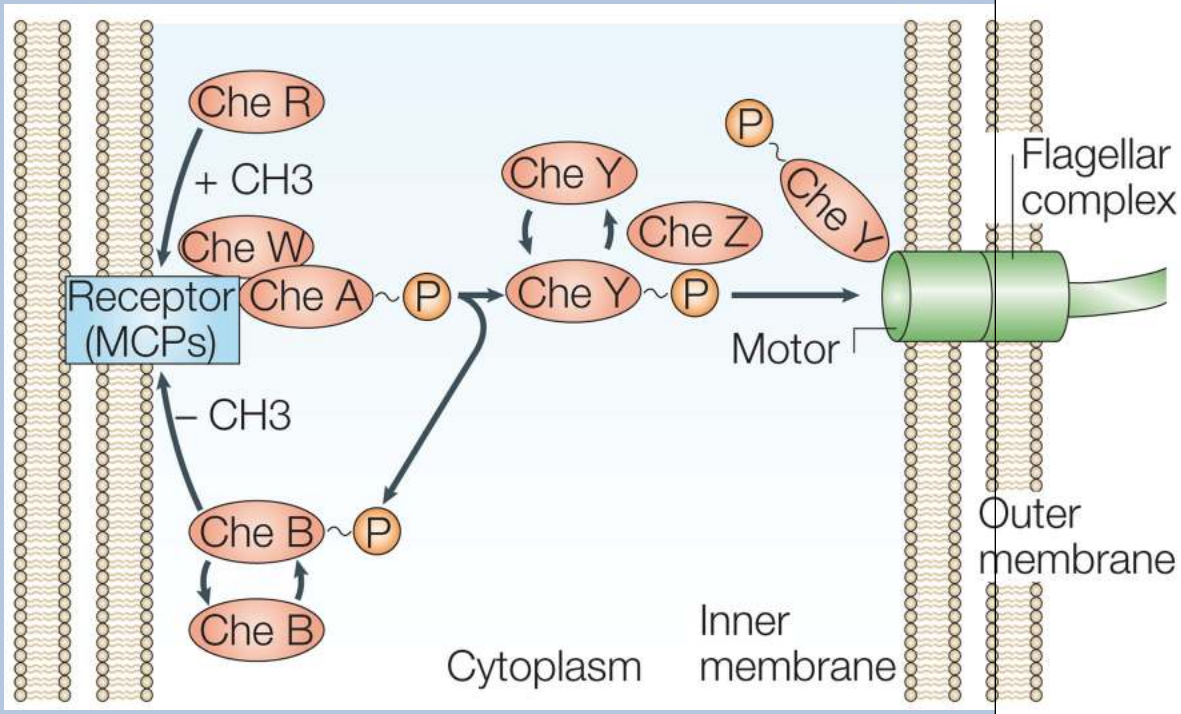
The strong competitive advantage that is observed in the absence of chemotaxis in *V. cholerae* is unusual and interesting. Even within the *Vibrio* genus, non-chemotactic mutants of polarly flagellated *Vibrio anguillarum*, a pathogen of marine and estuarine fish, were attenuated 400-fold for infection[59]. The squid symbiont *Vibrio fischeri* requires motility for colonization of its host *Euprymna scolopes*[60], and chemotaxis has been predicted to be important in establishing the symbiosis, although this hypothesis still needs to be tested[61]. Given that several non-invasive enteric pathogens require chemotaxis for infection, why is chemotaxis not only dispensable, but an impediment, to experimental infection by *V. cholerae*? Part of the answer to this question is that non-chemotactic *V. cholerae* mutants show aberrant distribution within the infant-mouse small intestine.

Box 1

Chemotactic signalling in *Escherichia coli*

This box provides a brief, non-comprehensive overview of chemotaxis in *Escherichia coli* (see the figure, which shows the main signal transduction events that occur during chemotaxis in *E. coli*). For more detailed information, the reader is referred to REFS [95–100]. In the figure, all the Che proteins are shown free in the cytoplasm for the sake of clarity, although, with the exception of Che Y-P, all of these proteins are located at the receptor

complex. In the table, the chemotaxis paralogues of *E. coli* and *Vibrio cholerae* are listed. The large number of paralogues suggests substantial complexity in chemotactic signalling by *V. cholerae*.



In *E. coli*, flagellar rotation alternates between the default direction of counter-clockwise (CCW) and clockwise (CW) rotation. In a chemically homogeneous environment, the cells change direction approximately once per second, and there is no bias for net movement in any particular direction[100]. However, in the presence of a concentration gradient of chemoattractant or chemorepellent, this frequency is altered, enabling bacteria to swim up concentration gradients of attractants and down concentration gradients of repellents101. Net movement is achieved by lengthening the period of runs as a cell is experiencing an increasing concentration of attractant, and decreasing the period of runs when there is a decreasing concentration of attractant. The process by which bacteria control the frequency of switching between CCW and CW flagellar rotation in response to chemical gradients is called chemotactic signalling.

The first step in this process is signal reception by chemoreceptors, known as methyl-accepting chemotaxis proteins (MCPs)[102]. These are generally transmembrane proteins, although cytoplasmic MCPs have also been found, for example in *Rhodobacter sphaeroides*[103]. An important feature of MCPs is their clustering at the cell poles[104,105], or in the cytoplasm in the case of cytoplasmic MCPs[103]. MCPs bind specific ligands and ligand occupancy is communicated to the flagella through a signal-transduction cascade. Bacteria can respond to changes of only a few molecules of ligand[106,107] and clustering is thought to be important not only for this sensitivity but also for the signal amplification that is required to achieve efficient chemotaxis[108].

A decrease in attractant binding to MCPs is communicated to the auto-histidine kinase CheA through the protein CheW, resulting in auto-phosphorylation of CheA to CheA-P[109]. CheA and CheW form a ternary complex with the MCPs[110] at the cell poles[104,105]. Two soluble response regulators, CheY and CheB, compete for phosphorylation by CheA-P[111]. As CheY-P binds to the flagellar switch protein FliM[112] and causes CW rotation, the

intracellular ratio of CheY to CheY-P controls the direction of flagellar rotation[113]. Tumbles are kept brief through rapid CheZ-stimulated dephosphorylation of CheY-P, resulting in turnover of this CW-promoting signal[114].

The ability of the chemotaxis system to respond to further increases or decreases in attractant binding is crucial for chemotaxis along gradients. Such adaptation is achieved in *E. coli* by modulating the methylation state of the MCPs using two proteins, a constitutively active methyltransferase CheR[115] and a methylesterase CheB116, the activity of which is stimulated after phosphorylation by CheA-P[117]. Increased methylation of the MCPs dampens the response to ligand binding, whereas decreased methylation sensitizes this response. This allows for adaptation because the decision-making reaction (CheA auto-phosphorylation) can be set to approximately the pre-stimulus level. Null mutations in any of the *che* genes result in a motile but non-chemotactic (swimming blind) phenotype: loss of *cheA*, *cheY*, *cheW* or *cheR* causes CCW-biased flagellar rotation, whereas loss of *cheZ* or *cheB* results in CW-biased rotation.

***che* gene**	***E. coli***
MCPs	4
cheA	1
cheW	1
cheY	1
cheZ	1
cheR	1
cheB	1
cheV	0
cheC	0
cheD	0

Intestine colonization by *V. cholerae*

Whereas wild-type *V. cholerae* predominantly colonize the lower half of the small intestine, corresponding approximately to the lower jejunum and ileum, non-chemotactic mutants colonize equally well throughout the small intestine[41]. This relaxation of tissue specificity allows a greater surface area to be colonized.

Why do non-chemotactic *V. cholerae* mutants colonize the upper small intestine? A breakthrough in answering this question came from the examination of different types of non-chemotactic mutants *in vivo*. Both counter-clockwise (CCW)-biased and clockwise (CW)-biased flagellar mutants are non-chemotactic. However, the out-competition phenotype is observed only in the presence of CCW-biased flagellar rotation: a CW-biased mutant has the opposite phenotype, being attenuated 10-fold for infection[55]. As the CW-biased mutant changes the direction in which it is swimming extremely frequently, producing a zig-zag pattern of movement, it is defective in its ability to make net progress in any direction. By contrast, the CCW-biased mutant swims in straight runs for relatively long periods of time. Although the direction in which cells swim is random (with respect to chemical gradients), they nonetheless cover a reasonable distance (~40 $\mu m\ s^{-1}$)[55]. As the diameter of the lumen in the infant-mouse small intestine is approximately 400 μm, it is easy to see how the CCW-biased mutant can make contact with the mucus layer and villi, and how the CW-biased mutant would be impaired in this process. It is unknown whether the same outcome would be observed during infection in humans.

Interestingly, CW-biased mutants of *S. typhimurium*, as well as flagellated but non-motile mutants, show decreased invasion of Peyer's patches, which are intestinal lymphoid aggregates that contain M cells. Non-flagellated mutants, however, can invade Peyer's patches at wild-type levels. The attenuation of both the CW-biased mutants and the flagellated but non-motile mutants is not caused by the lack of chemotaxis or motility but by the resulting unbundled peritrichous flagella which prevent interaction of adherence factors on the bacterial cell surface with host cells[62].

The expanded tissue range of CCW-biased *V. cholerae* might only account for part of the 70-fold competitive advantage that is seen *in vivo*. Previously, it was observed that wild-type *V. cholerae* use chemotaxis to penetrate the mucus layer and move to the intestinal crypts, which are located at the base of the villi (FIG. 3). Non-chemotactic mutants do not accumulate in this site, and instead accumulate in the mucus and on the luminal side of the villi. This was observed

in sections dissected from rabbit ileum[63,64], as well as in the infant-mouse intestine[65]. Based on these observations, and to explain the out-competition phenotype of non-chemotactic *V. cholerae*, Freter *et al.* proposed that wild-type *V. cholerae* (but not non-chemotactic strains) move by chemotaxis to the intestinal crypts, and that antimicrobial substances that are present in this location kill a large fraction of the bacteria. Since this initial observation, antimicrobial peptides named defensins (cryptdins) have been identified. They are released from Paneth cells, which are located at the base of the intestinal crypts[66]. However, as defensins are not expressed in mice until 20 days after birth67, the identity of the proposed antimicrobial substances in the infant-mouse small-intestine crypts remains unclear.

There are several possible explanations as to why *V. cholerae* might 'recklessly' follow a chemotaxis gradient to the crypt epithelia. First, the signals for maximal expression of cholera toxin might be present at this site, as proposed by Lee *et al*.[41] Second, *V. cholerae* that penetrate into the intervillous spaces and colonize epithelia might be better protected from the forces of PERISTALSIS. Third, this migration might be crucial for infection in humans, and *V. cholerae* uses the same strategy during experimental infection of mice and rabbits, which have more potent antimicrobial mechanisms. Arguing against the first explanation is the finding that non-chemotactic mutants that colonize the upper small intestine are competent for transcriptional induction of TCP and cholera-toxin genes[55]. Moreover, no differences were observed in TCP production itself (S.M.B. and A.C., unpublished results). Although these experiments are not conclusive, they might indicate that the signals for virulence gene expression are not confined to the crypt epithelium. Arguing against the second explanation is the fact that non-chemotactic mutants, which are predicted to fail to enter the intervillous spaces, can out-compete the wild-type strain. Future experiments that visualize the locations and gene-expression patterns of wild-type and non-chemotactic *V. cholerae* in the infant-mouse small intestine might resolve these issues.

The ability of non-chemotactic *V. cholerae* mutants to colonize the upper small intestine of infant mice indicates that chemotaxis is used by the wild-type bacteria to avoid colonizing this tissue. As the infant mouse small intestine is ~13 cm in length and peristalsis creates a downward flow of luminal contents, *V. cholerae* cannot respond to distal small-intestine-specific attractants when transiting the upper small intestine. Therefore, there must be an attractant or repellent gradient that is confined to the upper small intestine and directs *V. cholerae* to the lumen (FIG. 4). This gradient could consist of a repellent that is at a higher concentration near the intestinal wall, or an attractant that is at a higher concentration in the lumen. Presumably, by the time *V. cholerae* reaches the distal small intestine, this gradient has dissipated and the bacteria can now respond to another gradient that draws them into the intervillous spaces. We have observed that *V. cholerae* are chemotactically attracted to the epithelial surface of the upper small intestine in primary tissue culture assays, suggesting a lack of chemorepellents emanating from the intestinal surface[55]. These data point to the presence of a chemoattractant gradient in the lumen of the upper small intestine (arrows in FIG. 4).

One potential source of attractants in the lumen of the upper small intestine are sugars, peptides and amino acids. As food is digested and moved by peristalsis, absorption occurs at the epithelial surface, potentially generating a gradient. A second possible source of chemoattractant is bile. After consumption of food, bile is released into the lumen of the upper small intestine from the bile duct. Bile acids are vital for digestion and absorption of fats and fat-soluble vitamins, and are absorbed by the small-intestine epithelium. Although bile is a repellent for *H. pylori*[68], it is an attractant for *V. cholerae* at physiological concentrations[69]. Also, although bile represses flagellar synthesis in *S. typhimurium*[70], it increases the motility of *V. cholerae* and represses virulence gene expression[71,72]. In this scenario, chemotactic *V.*

cholerae that are retained in the lumen because of a bile gradient would be predicted to repress virulence gene expression until the local concentration of bile decreases.

Motility and *V. cholerae* virulence

When considering bacterial pathogens, it is reasonable to assume that the absence of flagella in a flagellated organism would reduce the ability of that organism to initiate an infection[48,73]. As mentioned previously, invasive pathogens are known exceptions to this intuitive rule. To determine the role of motility during infection, the requirement of motility *per se* must be separated from the ability of a bacterium to use flagella as an adherence factor, as occurs in enteropathogenic *E. coli* infection[74]. This can be achieved by comparing the ability of a non-flagellated (fla^-) and a flagellated but non-motile (fla^+ mot^-) mutant to colonize. The latter category of mutants is conveniently isolated by disrupting genes that encode the motor proteins that are necessary for flagellar rotation. As the *V. cholerae* flagellum is sheathed, the flagellar subunits themselves are unlikely to function as adhesins, and consistent with this, no differences with respect to infection have been observed for fla^- versus fla^+ mot^- mutants[41].

Is motility *per se* important for *V. cholerae* infection? Based on early studies, it was proposed that the ability of *V. cholerae* to swim into the mucus layer might be an important factor in colonization of the intestinal surface[75,76]. However, recent studies using the infant-mouse model of infection have produced conflicting data regarding the importance of motility for infection by the classical biotype[77–79]. In the case of the El Tor biotype, the situation is more clear; both fla^- and fla^+ mot^- mutants are attenuated 10-fold compared with the wild-type strain[41]. Therefore, for the current pandemic strains, which are of the El Tor biotype, motility is important for colonizing the wall of the small intestine.

Perhaps the most convincing argument for a *bona fide* role of motility in infection comes from live-attenuated *V. cholerae* vaccine trials in human volunteers. Although live-attenuated vaccines are more immunogenic than whole-cell killed vaccines, they are associated with the production of moderate side effects, including nausea, cramps and diarrhoea; this undesired property is referred to as reactogenicity[80]. Mekalanos and colleagues found that non-motile derivatives of live-attenuated vaccine strains are less reactogenic, without compromising immunogenicity[81,82]. These non-motile derivatives are more attenuated than the parent live-attenuated strain in infant mice, but nevertheless seem to be able to colonize the small intestine of human volunteers[80,81]. The reduced reactogenicity is believed to be due to the inability of the non-motile derivatives to come into close contact with the intestinal epithelium[83], consistent with the hypothesized role of motility in penetrating the mucus layer in animal models.

One complicating factor in measuring the role of motility in *V. cholerae* virulence is the link between motility and virulence gene expression that is observed in many pathogens. These properties are reciprocally regulated in several organisms, so that motility is repressed upon initiation of virulence gene expression. This occurs in *S. typhimurium*[84,85] and *Bordetella bronchiseptica*[86] and was suggested to occur in *V. cholerae*[78]. Häse and colleagues showed that increasing the viscosity of the medium, as would occur in mucus, as well as adding inhibitors of flagellar motility resulted in increased expression of the major virulence-gene transcriptional activator ToxT in the classical biotype[28]. It was therefore proposed that the *V. cholerae* flagellum might respond to increased viscosity to induce virulence gene expression. This type of response has precedence in the *Vibrio* genus; the polar flagellum of *Vibrio parahaemolyticus* serves as a mechanosensor of increased viscosity[87,88]. In addition, a recent study by Klose and colleagues indicates that the *V. cholerae* flagellum might have a mechanosensor function that operates during biofilm formation[89]. However, the effects of increased viscosity on virulence gene induction in *V. cholerae* were later shown to be

independent of the flagellum and instead are probably due to changes in the membrane potential[90]. In another study, differences in virulence gene expression in the El Tor biotype were noted during infection in the presence of Δ*flaA* and Δ*motAB* mutations[41]. However, no differences were observed using a more quantitative technique (S.M.B. and A.C., unpublished results), and therefore motility does not seem to be linked to virulence gene expression in this biotype.

Additional roles for motility and chemotaxis

In addition to its role in the penetration of the mucus layer and for chemotaxis into the intervillous spaces, motility might serve other functions during the infectious process. It was observed that *V. cholerae* shed in rice-water stools are highly motile[13]. Because *V. cholerae* are unlikely to use flagellar motility within microcolonies on the intestinal epithelium, motility must be switched back on prior to exit from the host. Although this might simply be in preparation for life outside the host, it might also facilitate movement from the epithelium into the lumen and therefore in subsequent shedding from the host. It is not known whether chemotaxis is involved in this process, although it is hypothesized below that rice-water-stool *V. cholerae* are phenotypically non-chemotactic. If this hypothesis is correct, then perhaps chemotaxis is repressed to allow for more efficient accumulation of motile *V. cholerae* in the lumen for subsequent expulsion in stool. In this speculative scenario, bacteria would be blind to any potential chemoattractant gradient that might otherwise draw them back into the intervillous spaces. Looking beyond the human host, it is probable that the motile state of shed *V. cholerae* promotes survival in aqueous environments. Motility coupled with chemotaxis would be predicted to have a crucial role in dissemination of shed *V. cholerae* in the environment and in the location of suitable environmental hosts and surfaces (FIG. 1).

The heightened infectivity of non-chemotactic *V. cholerae* mutants in experimental infection lends support to a recently proposed mode of natural transmission of cholera. A competitive advantage similar in magnitude is observed with rice-water-stool *V. cholerae*, which out-compete an *in vitro*-grown wild-type strain 10–100 fold in infant mice[13]. This competitive advantage is retained after 5 hours of incubation in pond water but is completely lost following overnight growth *in vitro* of the stool *V. cholerae*, showing that the phenotype is transient. Despite the fact that rice-water-stool *V. cholerae* are motile, analysis of their transcriptome reveals repression of several of the chemotaxis paralogues[13], including *cheW-1* and *cheR-2*, that are required for chemotaxis (S.M.B. and A.C., unpublished data). In addition, 18 of the 43 MCPs that are encoded in the genome were also repressed. If any of these MCPs are receptors for chemo-attractants in the lumen, a chemotaxis defect would also be observed. These data indicate that the stool *V. cholerae* might be in a transiently non-chemotactic CCW-biased state, and if so, this might in part account for the competitive advantage observed *in vivo* with the stool bacteria. A second microarray study using a different internal reference did not, however, detect this repression of chemotaxis genes[91]. Given the difference with respect to internal standard, as well other methodological differences between these microarray experiments, the two studies cannot be directly compared. It is quite possible that other physiological attributes of the stool *V. cholerae* contribute to the competitive advantage, and therefore it is important to determine whether stool *V. cholerae* are non-chemotactic at the phenotypic level to validate the above hypothesis. Whether the competitive advantage of stool *V. cholerae* in infant mice translates to an increase in infectivity in humans has not been tested.

Conclusions

In terms of pathogenesis, chemotaxis is often thought to be required for efficient colonization. This is true of several non-invasive enteric pathogens, but chemotaxis is either absent, or present but dispensable, in several invasive pathogens. The increased infectivity of non-

chemotactic *V. cholerae* might in fact contribute to an important stage of the *V. cholerae* pathogenic life cycle. For example, if rice-water-stool *V. cholerae* truly exist in a transient state of non-chemotaxis, then *V. cholerae* might have evolved to take advantage of this to improve its chances of infecting new human hosts. It is not yet known whether other pathogens modulate chemotaxis to optimize particular stages of infection. In the case of *V. cholerae*, it is unlikely that such a motile but non-chemotactic state would persist in aqueous environments for an extended period, although even if maintained for only a few hours, such a state might confer a fitness increase by allowing it to take better advantage of an important growth medium and vehicle of dissemination — us.

Acknowledgments

We thank M. Angelichio for the scanning electron micrographs of *Vibrio cholerae* during infection.

Glossary

BIOFILM	Microbial biofilms are populations of microorganisms that are concentrated at an interface (usually solid–liquid) and typically surrounded by an extracellular polymeric substance matrix. Aggregates of cells that are not attached to a surface are sometimes termed ‘flocs’ and have many of the characteristics of biofilms.
PLANKTONIC CELLS	Single cells in suspension, instead of in a biofilm.

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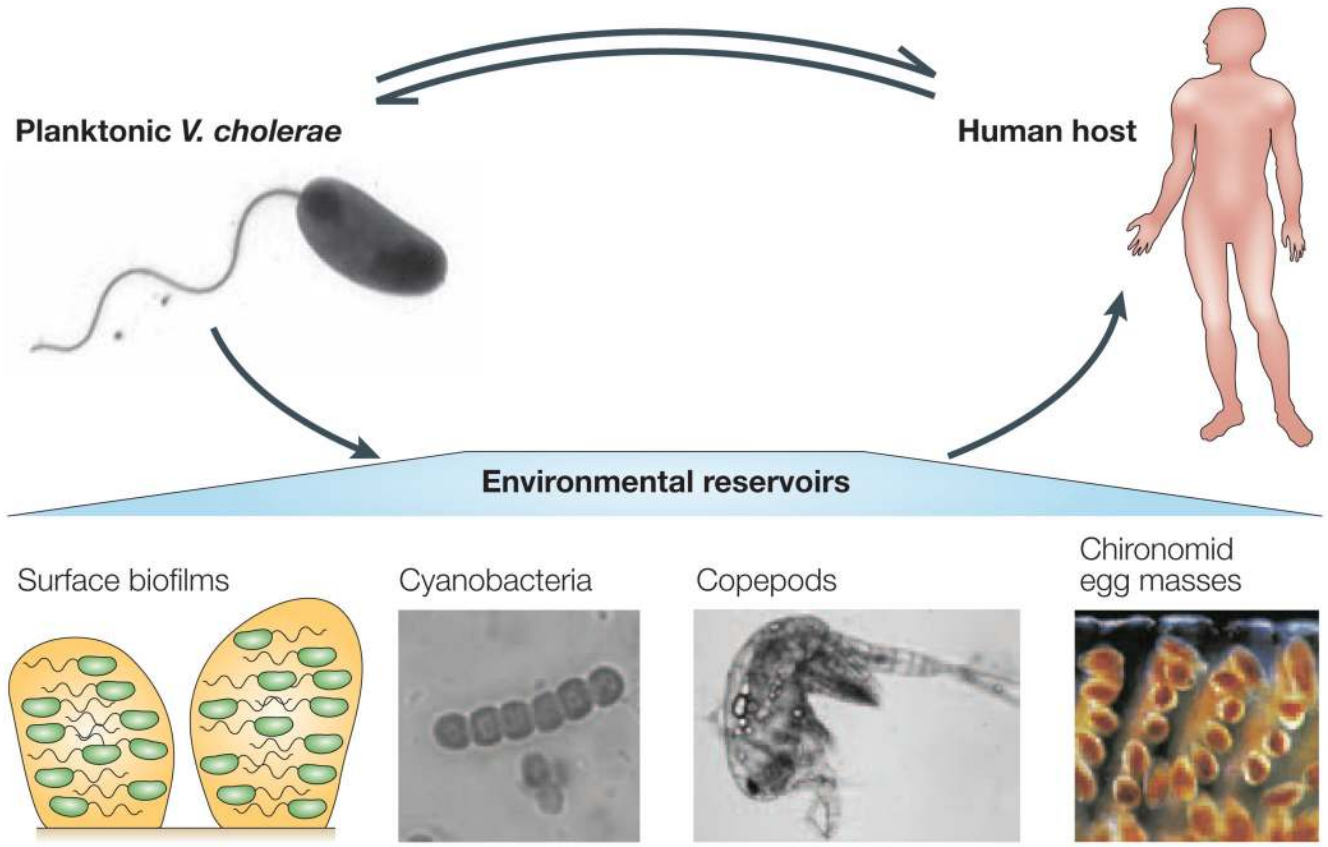

Figure 1. The life cycle of pathogenic *Vibrio cholerae*

The planktonic *V. cholerae* that are shed from humans in rice-water stool are central to the life cycle. These highly motile bacteria can be ingested by a new human host after shedding, or can associate with abiotic surfaces (possibly forming biofilms), copepods, algae and Chironomid egg sacs in the environment. These environmental *V. cholerae* can, presumably, disassociate from these hosts and be ingested by humans or form associations with a new environmental host. Alternatively, or in addition, environmental host-associated *V. cholerae* can be ingested by humans, causing infection and resulting in shedding of planktonic *V. cholerae*, therefore completing the life cycle. Chironomid egg mass panel reproduced with permission from REF. [126] © (2003) American Society for Microbiology.

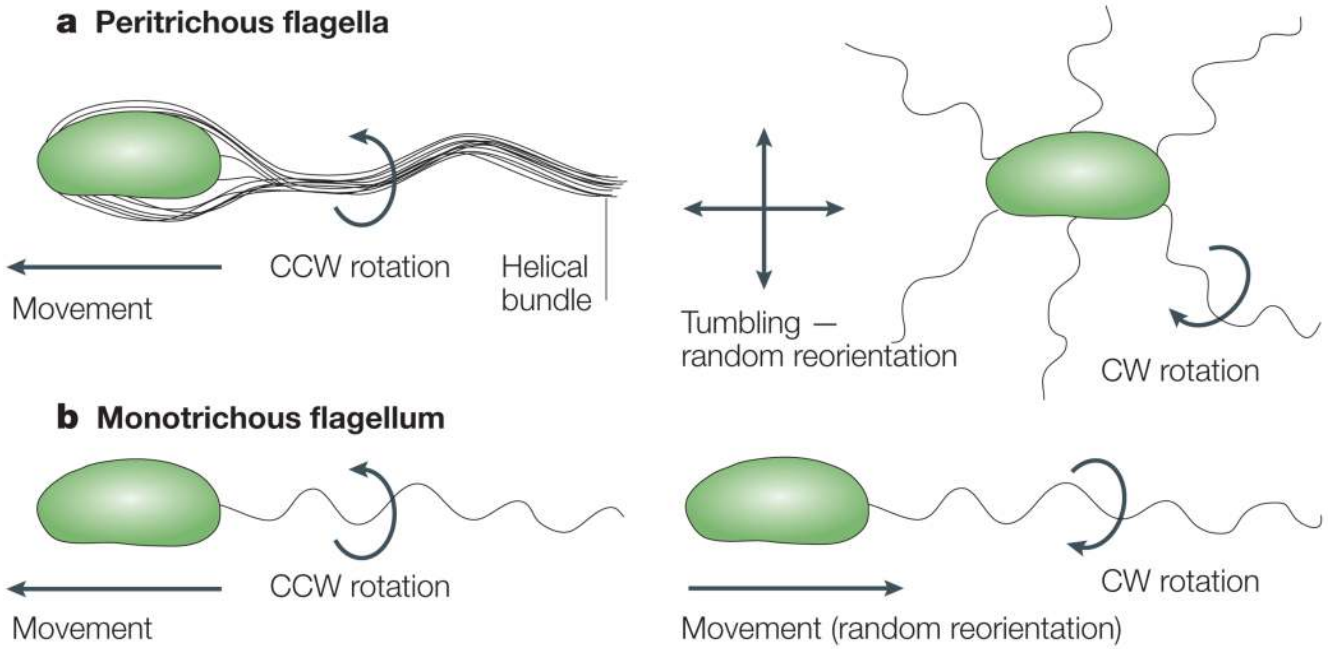

Figure 2. Flagellar-based motility

There are many schemes for flagellation in bacteria, of which peritrichous flagella and a single polar (monotrichous) flagellum are two types. **a** | In the case of peritrichous flagella, such as those found in *Escherichia coli*, counter-clockwise (CCW) flagellar rotation results in the formation of a helical bundle that propels the cell forward in one direction in a smooth-swimming motion (a 'run'). By contrast, the presence of clockwise (CW) rotation causes unbundling of the helical bundle, allowing the bacterium to randomly reorient its direction (a 'tumble'). **b** | In the case of a single polar flagellum, CCW rotation propels the cell forward in a run, whereas CW rotation propels the cell backward with a concomitant random reorientation.

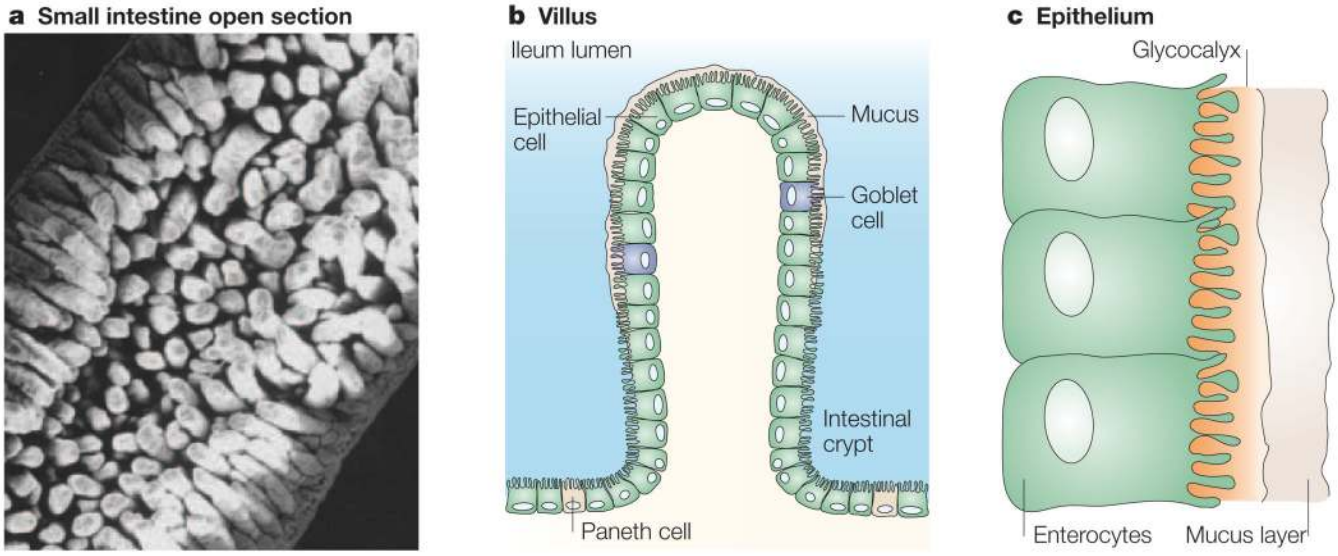

Figure 3. Architecture of the small intestine

Vibrio cholerae colonizes the small intestine in infant and adult humans and in experimentally infected infant mammals. Although several animal models of *V. cholerae* infection have been developed, the most widely used is colonization of the infant-mouse small intestine following oral inoculation[92]. Important factors for human colonization have been identified using this model, lending credence to its use as a viable model of infection. Furthermore, virulence genes that are crucial for infection have been shown to be expressed in infant mice[93]. A schematic of the small intestine is shown. **a** | In the scanning electron micrograph (SEM) of an opened section of infant-mouse small intestine, the numerous and closely packed villi are seen. The villi surround a central lumen (not shown), and intervillous spaces are present between villi. At the base of the villi are the crypts of Lieberkühn. **b**,**c** | The villi are composed of absorptive epithelial cells (enterocytes) as well as Goblet cells, which produce mucus. This mucus forms a gel covering the villi, which concentrates at the tips. As *V. cholerae* is known to colonize epithelial surfaces on the villi and crypts, it must therefore penetrate not only the mucus layer present at the tips of the villi, but also any mucus gel covering the intestinal epithelium.

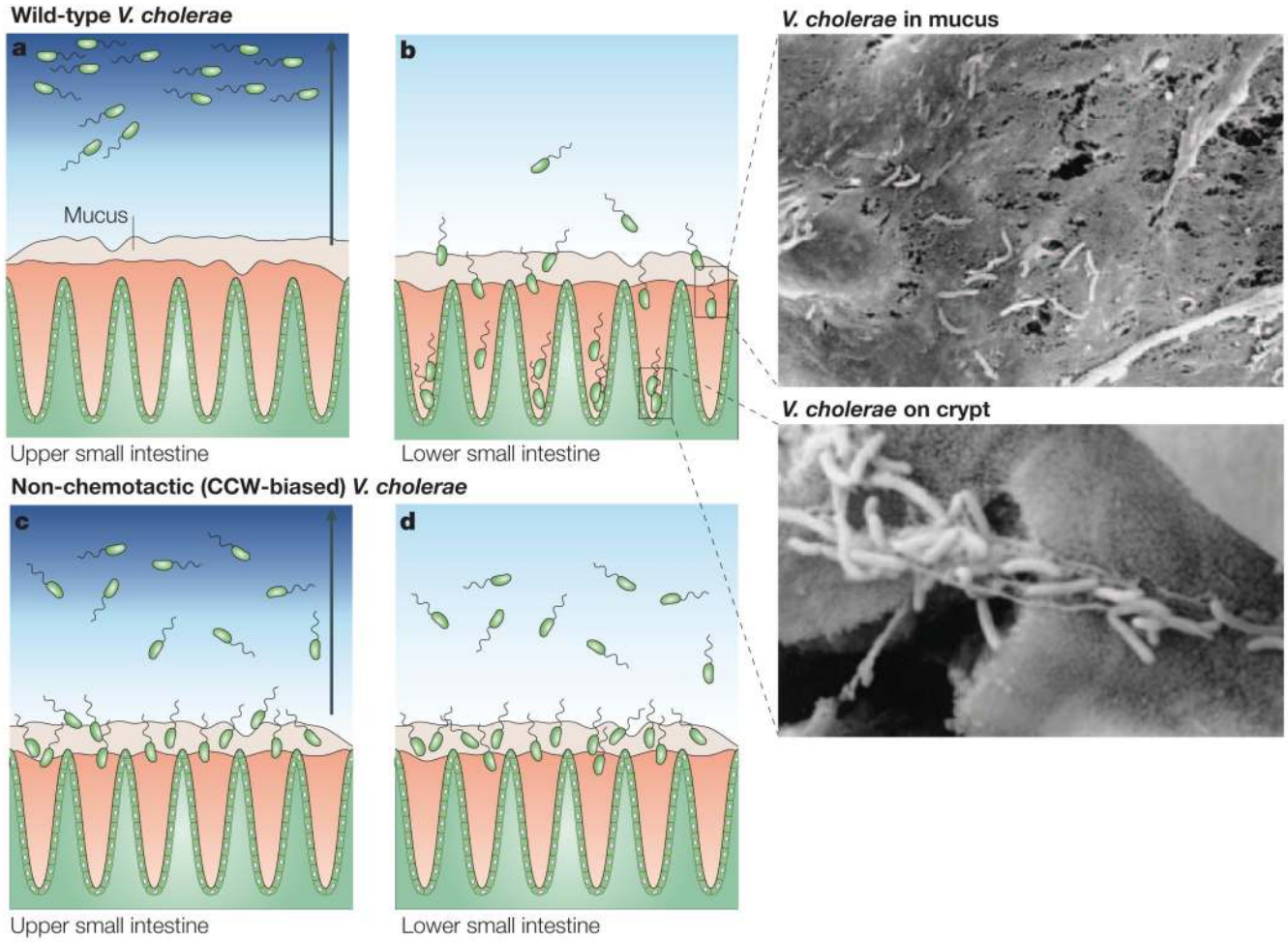

Figure 4. Model for the effect of chemotaxis in limiting *Vibrio cholerae* colonization

A gradient of chemoattractant is present in the lumen of the upper small intestine, oriented away from the villi (shown by arrows, panels **a**,**c**). Using chemotaxis, wild-type *V. cholerae* responds to this gradient by concentrating within the lumen. However, this chemoattractant gradient is absent from the lumen of the lower small intestine (panels **b**,**d**), allowing wild-type *V. cholerae* to respond to a different chemoattractant gradient that directs them into the intervillous spaces and onto epithelial surfaces (see scanning electron micrograph (SEM) panels). By contrast, non-chemotactic counter-clockwise (CCW)-biased *V. cholerae* are blind to these gradients and therefore colonize both the upper and lower small intestine, presumably on the luminal side of the mucus gel and villi (panels **c**,**d**). The *V. cholerae* that get stuck in mucus can also multiply, as evidenced by the presence of dividing bacteria in the SEM of mucus. SEM lower panel reproduced with permission from REF. [94] © (2002) American Association for the Advancement of Science.

Table 1

Outcome of infection for non-chemotactic mutants

Pathogen/animal model	Outcome of infection	Ref.
Campylobacter jejuni		
Mouse intestine colonization	*cheY* mutant absent from stool after 6 days of infection	118
Ferret disease	*cheY* mutant does not cause diarrhea	118
Chick colonization	*cheY* mutant present in caecum at levels 10,000-fold less than the wild-type strain	119
Helicobacter pylori		
Gnotobiotic-pig stomach Colonization	*cheY1* mutant fails to colonize	120
Mouse stomach colonization	*cheY1* and *cheAY2* mutants fail to colonize	120
Mouse stomach colonization	*tlpA* and *tlpC* (putative chemoreceptors) mutants attenuated 50-fold in competition experiments	121
Infant-mouse stomach colonization	*cheY* mutant attenuated 5-fold in competition experiment	122
Listeria monocytogenes		
Mouse systemic infection (oral inoculation)	Δ*cheYA* mutant showed no defect for recovery from spleen but showed decreased recovery from liver	123
Proteus mirabilis		
Mouse ascending urinary-tract infection	*cheW* mutant is attenuated 10^6-fold for infection of the urinary tract and bladder	124
***Salmonella enterica* serovar Typhimurium**		
Murine ligated-loop tissue invasion	*cheB* (CW-biased) mutant attenuated 10-fold for invasion of Peyer's patches	62
Mouse colitis and systemic infection	*cheY* mutant attenuated 100-fold in caecum-colonization competition experiment; no attenuation observed in spleen or liver	125
Vibrio anguillarum		
Rainbow-trout tissue invasion (immersion inoculation)	*cheR* mutant has a 400-fold higher LD_{50} than wild-type; no difference between strains when inoculated intraperitoneally	59
Vibrio cholerae		
Infant-mouse small intestine	*cheY-3* mutant out-competes wild-type strain 70-fold; CW-biased non-chemotactic mutant attenuated 10-fold in competition experiment	55

CW, clockwise.