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**Vibrio cholerae** accessory colonisation factor AcfC: a chemotactic protein with a role in hyperinfectivity

Esmeralda Valiente1, Cadi Davies1, Dominic C. Mills1,2, Maria Getino3, Jennifer M. Ritchie3 & Brendan W. Wren1

**Vibrio cholerae** O1 El Tor is an aquatic Gram-negative bacterium responsible for the current seventh pandemic of the diarrheal disease, cholera. A previous whole-genome analysis on **V. cholerae** O1 El Tor strains from the 2010 epidemic in Pakistan showed that all strains contained the **V. cholerae** pathogenicity island-1 and the accessory colonisation gene **acfc** (VC_0841). Here we show that **acfc** possesses an open reading frame of 770 bp encoding a protein with a predicted size of 28 kDa, which shares high amino acid similarity with two adhesion proteins found in other enteropathogens, including Paa in serotype O45 porcine enteropathogenic *Escherichia coli* and PEB3 in *Campylobacter jejuni*. Using a defined **acfc** deletion mutant, we studied the specific role of AcfC in **V. cholerae** O1 El Tor environmental survival, colonisation and virulence in two infection model systems (*Galleria mellonella* and infant rabbits). Our results indicate that AcfC might be a periplasmic sulfate-binding protein that affects chemotaxis towards mucin and bacterial infectivity in the infant rabbit model of cholera. Overall, our findings suggest that AcfC contributes to the chemotactic response of WT **V. cholerae** and plays an important role in defining the overall distribution of the organism within the intestine.

**Vibrio cholerae** O1 El Tor is an aquatic, Gram-negative, single flagellated bacterium responsible for the current seventh cholera pandemic1. After entering the human body by ingestion of contaminated food or water, bacteria migrate towards the surface of the small intestine. Colonisation of the intestine leads to the release of cholera toxin (CT) and the development of profuse, watery diarrhoea that is characteristic of the disease. Cholera is treatable with oral rehydration therapy, but if left untreated, can quickly lead to severe dehydration, toxic shock, and death in less than 24 hours post infection2. Cholera remains an important global health problem, mainly affecting areas with poor water sanitation. Each year cholera affects an estimated 3 million people, resulting in over 100,000 deaths, particularly young children3, causing considerable adverse economic consequences to countries in Asia, Africa, Oceania and South America4.

CT and the toxin co-regulated pilus (TCP) are well established as the main virulence factors of **V. cholerae**5–7. CT is responsible for the profuse watery diarrhoea that characterises the disease8 whereas TCP, a type IV pilus, is essential for **V. cholerae** colonisation of the small intestine, as demonstrated in both human volunteers9 and animal models9. A myriad of other factors has also been associated with **V. cholerae** pathogenicity10. Of these, motility and chemotaxis have been the subject of considerable study given their proposed role in controlling the intestinal localisation of the organism. Historically Freter and colleagues proposed that chemotactic-based motility was responsible for the preferential localisation of **Vibrio cholerae** to the crypts of the distal small intestine10,11. However, recent studies10,11 suggest that the distribution of *Vibrio cholerae* in the intestine is as a result of a complex interplay between motility, chemotaxis and host mucin. Chemotaxis is controlled by numerous inner membrane-localised methyl-accepting chemotaxis proteins (MCPs) and soluble chemotaxis proteins11,12. However, at the molecular level, the chemotaxis network is incompletely understood. Chemotaxis also has a role

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in the aquatic environment, where it allows bacteria to find suitable environmental and host surfaces, made, for example, of chitin. Chitin - a polymer of N-acetylglucosamine, the second most abundant polymer in nature - is present in the exoskeleton of crustaceans and serves as both a carbon source and as a chemoattractant of marine Vibrio species.  

An important genomic island involved in cholera disease is the V. cholerae pathogenicity island 1 (VPI-1), which contains the TCP gene cluster. VPI-1 also possesses a cluster of four accessory colonisation factor (acf) genes, designated as acfA, acfB, acfC and acfD. Both TCP and acf genes are regulated by ToxR, the cholera toxin transcriptional activator. The acf genes in V. cholerae O395 (O1 Classical biotype) appear to be required for efficient intestinal mice colonisation and biogenesis of the toxin-associated pilus of V. cholerae. In addition, AcfB was shown to be an inner membrane protein that affects motility in V. cholerae O395. However, in the case of V. cholerae O1 El Tor, acf genes do not seem to have a role in virulence.

In August 2010, Pakistan experienced the worst flooding in its recorded history, affecting more than 20 million people, followed by a cholera epidemic. A genetic analysis of V. cholerae O1 El Tor strains from the floods identified two distinct groups, isolated from different geographic regions: Pakistan subclades 1 (PSC-1) (isolates from the coastal city of Karachi) and 2 (PSC-2) (in countrywide areas). Amongst other differences, genome analyses showed that PSC-2 strains had a frame-shift mutation in afD introducing several stop codons, whereas acf was encoded by a full open reading frame in PSC-1 strains. In addition, comprehensive data base searching revealed that AcfC-like proteins are only found in two other bacterial species, which are both enteropathogens (Campylobacter jejuni and Escherichia coli), suggesting a new family of proteins that maybe important for survival in the mammalian gut. We therefore hypothesized that the presence of a functional acfC in V. cholerae may confer an advantage to strains in PSC-1 to survive in the environment and/or to colonise the human small intestine.

In this work, we investigated the biological role of AcfC in V. cholerae O1 El Tor and found that it might be a periplasmic sulfate-binding protein, which affects chemotaxis towards mucin and has a direct impact on bacterial hyperinfectivity in the infant rabbit model of disease.

Results

Sequence analysis of AcfC suggests a periplasmic localisation and reveals high identity to PEB3 and Paa proteins. The V. cholerae acfC is an open reading frame of 770 bp encoding a polypeptide of 256 amino acids that is predicted to be localised to the periplasm, according to the Protein Subcellular Localization Prediction for Gram-negative Bacteria (http://www.csbio.sjtu.edu.cn/biouni/Cell-PLoc-2/). A protein blast search indicated that AcfC protein has high similarity to E. coli Paa and C. jejuni PEB3. The amino acid sequences of AcfC, PEB3 and Paa were aligned by ClustalW (Fig. 1a). AcfC has 48.4% identity to PEB3 and 47% identity to Paa. After cleavage of the first 24 amino acids, which likely encodes a signal sequence, the mature AcfC protein is predicted to have a size of 28 kDa (http://www.uniprot.org/uniprot). We confirmed the periplasmic localisation of AcfC by cellular fractionation and western blot with anti-AcfC antibodies (Fig. 1b).

V. cholerae AcfC is a sulfate-binding protein. The presence of lysine- and arginine-rich regions in the amino acid sequence suggests AcfC may have sulfate-binding properties. We thus tested the ability of His6-tagged
AcfC to bind to plastic surfaces coated with sulfated proteins - such as heparin sulfate, chondroitin sulfate and porcine mucin type II or the non-sulfated protein hyaluronic acid as a negative control. Using an ELISA assay, we found that His6-tagged AcfC bound to both heparin, chondroitin sulfate and mucin, but significantly less to hyaluronic acid (Fig. 1c). These results suggest that AcfC might prefer binding to sulfate-containing proteins (Fig. 1c).

AcfC is not involved in Caco-2 or HT-29MTX-E12 cell adhesion.

Given that orthologous AcfC proteins are involved in epithelial cell adhesion, we tested if that was also the case for AcfC in V. cholerae. The role of AcfC in adhesion of V. cholerae was assayed using Caco-2 cells, a cell line well known to produce comparatively low amounts of mucin. The adherence of wild type (S2CHK17 strain) and V. cholerae ΔacfC to Caco-2 cells was similar (Fig. 2a). We next repeated the experiment with a previously described mucin hyper-producer cell line, HT-29MTX-E12 cells. However, we found no differences in adherence between V. cholerae wild type (S2CHK17 strain) and V. cholerae ΔacfC (Fig. 2a). These findings indicate that AcfC, unlike its protein homologues (PEB3 or/and Paa)20,21, is not involved in cell adhesion in the conditions of our experiment.

To confirm this result, we investigated whether recombinant expression of AcfC in a non-adherent E. coli strain (LMG194) would be able to alter its adhesion properties. In line with our results obtained with V. cholerae, expression of His6-tagged AcfC in E. coli LMG194 did not alter adhesion to Caco-2 or HT-29MTX-E12 cells compared to the empty vector control (Fig. 2b and c). The positive control strain, E. coli ATCC25922, however adhered effectively to Caco-2 cells (10% ± SD) and HT-29MTX-E12 cells (3% ± SD) indicating the validity of the assay. Based on these results, we concluded that AcfC is not an adhesion to intestinal cell lines.

Biofilm formation. To survive and colonise both the aquatic environment and the human intestine, V. cholerae forms biofilms on different abiotic and biotic surfaces. We thus studied the ability of V. cholerae S2CHK17 and ΔacfC to form biofilms on 24-well plates with or without mucin and/or chitin. Our results demonstrate that the presence of mucin or chitin enhances biofilm formation in both V. cholerae S2CHK17 and ΔacfC. However, the absence of AcfC does not affect biofilm formation neither on mucin nor on a chitin surface (Fig. 3a).
AcfC mediates chemotaxis to mucin but not chitin. The initial stages of colonisation of the human intestinal epithelium often involve the efficient movement and taxis towards mucin covering epithelial cells. We studied bacterial chemotaxis by filling a syringe with the chemoattractant, chitin or mucin, and inserting it into a pipette tip containing a bacterial suspension. We found that *V. cholerae* Δ*acfC* is significantly less chemotactic towards mucin compared to the wild type strain; complementation of the *acfC* gene restored normal chemotaxis (Fig. 3b). However, we found no significant differences in chemotaxis towards chitin between *V. cholerae* wild type and Δ*acfC* mutant (Fig. 3b). Our results suggest that AcfC is required for chemotaxis towards mucin, but does not affect chemotaxis towards chitin.

AcfC deletion does not affect flagellar assembly or motility. To rule out that the attenuated chemotactic phenotype in the Δ*acfC* mutant was not due to a defect on motility or flagella assembly, we measured *V. cholerae* motility and flagellar length. Both wild type *V. cholerae* and the Δ*acfC* mutant were similarly motile (Fig. 4a) and bacteria of both strains possessed a single polar flagellum (Fig. 4b) of similar length (Fig. 4c), suggesting that AcfC has no direct role in motility or flagellar assembly.

AcfC does not contribute to virulence in *Galleria mellonella*. *G. mellonella* larvae have been used previously as a simple, invertebrate model to study the virulence of different bacterial pathogens, including *V. cholerae*. Pathogenic potential, as reflected by larvae mortality, has been found to correlate with virulence established using mammalian models for several pathogens. Injection of *G. mellonella* with 2.6 ± 1.0 × 10^6 CFU of each strain in 10 μl of sterilised PBS and incubated at 37 °C. Survival was assayed by response to touch or discoloration. Killing by WT and Δ*acfC* was observed after 24 h. No killing was observed in the PBS injection control for the length of the experiment. Error bars represent the standard deviation (n = 3). Statistical analyses using the Tukey’s multiple comparisons test showed no significant differences in *V. cholerae* survival.

**Figure 4.** (a) *V. cholerae* S2CHK17 and Δ*acfC* motility; (b) TEM pictures; (c) Flagella measurements: The flagella length of one hundred bacteria were measured in each strain and TEM images were analysed using the line measuring tool of Image J software (National Institutes of Health, USA); (d) *G. mellonella* infection results. Representative data of survival rate of 3 biological replicates of 10 individual *G. mellonella* injected with 2.6 ± 1.0 × 10^6 CFU of each strain in 10 μl of sterilised PBS and incubated at 37 °C. Survival was assayed by response to touch or discoloration. Killing by WT and Δ*acfC* was observed after 24 h. No killing was observed in the PBS injection control for the length of the experiment. Error bars represent the standard deviation (n = 3). Statistical analyses using the Tukey’s multiple comparisons test showed no significant differences in *V. cholerae* survival.
**AcfC mutant exhibits increased infectivity and colonization of the proximal small intestine of infant rabbits.** To further explore the virulence of the Δacfc mutant and correlate the response seen in G. mellonella larvae to that of a mammalian host, we used the infant rabbit model of cholera. Oral infection of rabbits with ~1 × 10⁸ CFU of WT bacteria caused diarrhoea or intestinal fluid accumulation in ~67% of animals (Table 1), similar to that found previously for other V. cholerae O1 El Tor strains, although this was evident by 12h rather than 18h post infection. By contrast, the Δacfc mutant caused watery diarrhoea in all (100%) infected rabbits. Furthermore, the rabbits appeared sicker with a trend towards a more rapid and severe infection compared to those given WT organisms. While fluid accumulation was evident in the mid to distal regions of the small intestine of WT-infected animals, this extended proximally into the upper third of the small intestine of animals infected with Δacfc mutant. Moreover, the cecal fluid accumulation ratio (FAR), a surrogate measure for diarrhoea, tended to be greater in rabbits infected with Δacfc mutant compared to WT (Table 1). Thus, overall the Δacfc mutant appeared to exhibit increased (or hyper) infectivity compared to the WT strain.

Hyperinfectivity of V. cholerae has previously been associated with non-chemotactic smooth swimming strains that are better able to colonise the entire length of the small intestine. In order to investigate whether enhanced colonisation occurred in rabbits infected with the ΔAcfc mutant, we determined the number of WT and ΔAcfc mutant bacteria present in tissue homogenates taken from the proximal, mid and distal regions of the small intestine, and from the mid colon of infected rabbits. Higher numbers of the ΔAcfc mutant were recovered in each of the intestinal sections taken from these single strain infected animals, reaching statistically significant differences in the proximal (P < 0.01) and mid (P < 0.05) small intestine, and the colon (P < 0.05) (Fig. 5). In the tissue samples, ~1 log more bacteria were recovered in Δfcf-infected rabbits than in those given WT V. cholerae. Furthermore, significantly higher numbers of the mutant were recovered in the cecal fluid (WT – 3.6 × 10⁷ CFU/ml versus Δacfc mutant 3.6 × 10⁸ CFU/ml; P < 0.05), consistent with the higher burden of V. cholerae present in tissue homogenates.

**Discussion**

In this study, we provide a detailed characterisation of the V. cholerae O1 El Tor accessory colonisation factor, Acfc, which might be a sulfate-binding protein that enhances chemotaxis towards intestinal mucin and contributes to promoting the ‘correct’ localisation of WT V. cholerae in the small intestine. As mucus is rich in sulfated molecules we hypothesize that Acfc might act as a ‘sulfate sensor’ of V. cholerae O1 El Tor that senses intestinal mucus and facilitates penetration of the mucus layer by directing chemotaxis towards sulfated molecules at the intestinal surface. The V. cholerae acfc gene is part of a polycistronic operon downstream of acfb in Vibrio pathogenicity island I. Moreover, acfb gene is under the control of ToxR-ToxT regulatory cascade and is structurally and functionally related to methyl-accepting chemotaxis enteric protein. It has been suggested that V. cholerae Acfb and Acfc interaction activates a chemotaxis signal transduction cascade mediated by Che proteins. Acfc has also been reported that Acfb is another accessory colonisation factor, which affects chemotaxis but does not affect V. cholerae virulence in mice. This may be due to overlapping or redundant chemotaxis roles with other proteins, such as toxin co-regulated pili (TcpI) and two orthologues of Acfc, PEB3 from C. jejuni and Paa from porcine-pathogenic E.coli. These are considered to be adhesins involved in attachment to human epithelial cells. However, our work suggests that the primary role of Acfc in V. cholerae is that of a chemotaxis-related protein, rather than being directly involved in adhesion to cell lines. Acfc has been also hypothesized to be a secreted protein in V. cholerae O395 (O1 El Tor) and a secreted protein in V. cholerae O1 El Tor using cellular fractionation and immunoblotting. In C. jejuni, PEB3 is also known to be a periplasmic glycoprotein.

Acfc mediates chemotaxis towards mucin but does not have a role in mucin or chitin induced biofilm formation. However, other proteins in V. cholerae with a role on chitin induced biofilm formation such as the toxin co-regulated pili, which mediates bacterial interactions during biofilm formation on chitinaceous surfaces have been described.

Motility is necessary for chemotaxis in V. cholerae, but mutants in motility and chemotaxis behave differently in vivo. V. cholerae chemotaxis mutants exhibited enhanced colonisation in infant mice particularly in the proximal intestine. Our Acfc mutant has an altered chemotaxis phenotype and increased infectivity with enhanced

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<td>19</td>
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<tr>
<td>P valueb</td>
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<td>FARc</td>
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<tr>
<td>P valuec</td>
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Table 1. Diarrhoea status of rabbits infected with wild type V. cholerae or the Δacfc mutant. aNumber of rabbits with diarrhoea as described in the text. bFisher’s exact test was used to compare the number of WT-infected rabbits with disease versus the Δacfc mutant. cFluid accumulation ratio (FAR) is calculated from the weight of the cecal fluid to the tissue for each animal. dStudents 2-sided T test.
fluid accumulation in AcfC-infected rabbits comparing to wild-type infected animals. All these data are in accord with other non-chemotactic “smooth swimming” mutants which have also increased infectivity\(^{11,12}\). In contrast, Kamp and colleagues reported that \(acf\) genes in \(V.\) cholerae O1 El Tor are not important in pathogenesis, dissemination and transmission\(^{32}\). However, in the study of Kamp et al., they sampled the distal small intestine of infant rabbits and did not investigate the other parts of the small intestine, which might explain their contrasting results.

We studied the role of AcfC in virulence by using two \textit{in vivo} model systems. In our \textit{G. mellonella} infection model, AcfC mutant has no effect on pathogenesis. This insect model may only be suitable for crude virulence screening of strains. However, for colonisation studies, an animal model with a more developed intestine would be more appropriate, such as the case of the infant rabbit model.

Taken together, this is the first study showing the accessory colonisation factor AcfC of \(V.\) cholerae O1 El Tor might act as a periplasmic sulfate-binding protein, affecting bacterial chemotaxis towards mucin. Furthermore, our results show that AcfC has a significant role in governing \(V.\) cholerae distribution in the small intestine of infant rabbits. It is intriguing to speculate why PSC-2 strains, which lack a functional \(acfC\) gene, are less prominent in the Pakistan cholera outbreak than PSC-1 strains, given that the former may exhibit increased infectivity. Perhaps despite their increased infectivity, other genetic or phenotypic differences between the lineages aid survival outside of the host, an important aspect of the \(V.\) cholerae lifecycle.

\section*{Methods}
\subsection*{Bacterial strains and growth conditions.} Strains, plasmids and oligonucleotides used in this study are listed in Table 2. \textit{Vibrio cholerae} S2CHK17 and \(acfC\) mutant were routinely grown on Luria agar (LA), broth (LB) or thiosulfate citrate bile salts sucrose agar (TCBS) at 37°C. All cultures were grown from glycerol stocks in aerobiosis. \textit{Escherichia coli} DH5\(\alpha\) were used for cloning experiments. \textit{E. coli} MFD \(\lambda\)-pir was used as a conjugation donor. \textit{E. coli} LMG194 was used as host strain for protein expression. Where appropriate media was supplemented with ampicillin (100 \(\mu\)g/ml) or chloramphenicol (30 \(\mu\)g/ml).

\textit{Vibrio cholerae} cell fractionation and AcfC antibody production. \textit{V. cholerae} cells were fractionated following the protocol as described\(^{33}\). Each protein fraction was run on a Nu-Page 4–12% Bis-Tris SDS-PAGE...
gel and analysed by immunoblotting using anti-AcfC antibodies. Experiments were performed in three separate biological replicates and measured as two technical replicates each. AcfC rabbit polyclonal antiserum was obtained from CovalAB UK (Cambridge, UK) against the following peptides: CAESFEKSQSKRVNIT and CFYLTSDKAEEIFQHY.

**Expression of C-terminally His₆-tagged AcfC in Escherichia coli and Vibrio cholerae.** The acfC ORF with an optimized ribosomal binding site (RBS) and a C-terminally His₆-tag were amplified using primer VC0841RBS_EcoRIF and VC0841RBS_XbaI (Table 2) and Phusion polymerase (NEB). The amplicon was cloned into pEXT20 with optimized RBS and AcfC open reading frame (xxxbp) and Phusion polymerase (NEB). The amplicon was cloned into pEXT20 using EcoRI and XbaI cloning sites producing pEXT20-acfC. This construct was transformed into DH5α until the OD₆₀₀ was 0.5–0.7. Protein expression was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 1 mM for 3 h and 24 h in V. cholerae or E. coli and then incubated with 0–200 pmol purified AcfC (in PBS) for 1 h at room temperature and then incubated with 200 µl of rabbit anti-AcfC antibodies (1:1000) in 0.1% BSA in PBS for 1 h at RT. After several washes, bound AcfC was then incubated with alkaline phosphatase conjugated goat anti-rabbit antibody (Sigma, UK) in 0.1% BSA in PBS. Reactions were developed with 50 mM p-nitrophenylphosphate (Sigma, UK). The reactions were stopped by adding 3 M NaOH to each well and absorbance at 405 nm was measured. Coating efficiency of the glycoaminoglycans and non-glycoaminoglycans and porcine mucin used in this study, was analyzed by measuring the protein concentration in each well after PBS washes using a Bradford assay. Wells without coating were used as negative controls. Experiments were performed in three separate biological replicates, measured as three technical replicates each.

**Western blot.** Proteins were transferred to a nitrocellulose membrane using iBlot system (Life Technologies). A rabbit anti-Histag (ab9108, Abcam, UK) or anti-acfC antibody were used as primary antibodies and a fluorescently labelled goat anti-rabbit IR Dye 680RD secondary antibody (Li-cor) was used as secondary antibody. The membrane was visualized using an Odyssey fluorescent imager (LI-COR). The un-cropped image of Fig. 1b is in Supplementary Figure 1.

**Sulfate binding assay.** Flat-bottomed 96-well plates were coated with 200 µl of 100 µg ml⁻¹ glycosaminoglycans (chondroitin sulfate and heparin sulfate), type II porcine mucus (Sigma) and non-glycoaminoglycan (hyaluronic acid), at 4°C for 18 h. The wells were washed with PBS and blocked with 0.5% bovine serum albumin (BSA) for 1 h at 37°C. The wells were washed and then incubated with 0–200 pmol purified AcfC (in PBS) for 1 h at room temperature and then incubated with 200 µl of rabbit anti-AcfC antibodies (1:1000) in 0.1% BSA in PBS for 1 h at RT. After several washes, bound AcfC was then incubated with alkaline phosphatase conjugated goat anti-rabbit antibody (Sigma, UK) in 0.1% BSA in PBS. Reactions were developed with 50 mM p-nitrophenylphosphate (Sigma, UK). The reactions were stopped by adding 3 M NaOH to each well and absorbance at 405 nm was measured. Coating efficiency of the glycoaminoglycans and non-glycoaminoglycans and porcine mucin used in this study, was analyzed by measuring the protein concentration in each well after PBS washes using a Bradford assay. Wells without coating were used as negative controls. Experiments were performed in three separate biological replicates, measured as three technical replicates each.

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**Table 2.** Strains, plasmids and oligonucleotides used in this study.
Allelic exchange mutagenesis and complementation. In-frame deletion mutants were constructed using the splicing by overlap extension (SOE) PCR and allelic exchange technique. We used the V. cholerae S2CHK17 genome sequence as a template. Primers are listed in Table 2. The SOE PCR fragment was cloned into the suicide vector pDS13235 (a diaminopimelic acid (DAP) auxotroph) and then conjugated into V. cholerae λ- pir via cross streaking on LB plates containing 0.3 mM diaminopimelic acid (DAP). Growth from these plates was then transferred to LB plates containing 30 μg/ml chloramphenicol to select for V. cholerae with the suicide vector only. Double-crossover deletion mutants were then screened by PCR using flanking primers (Table 2) and confirmed by colony PCR and sequencing. The complemented strain was made by electroporation (1.8 kV) of the pEXT20-αcfc construct to αcfc mutant strain. The complemented strain was grown in LB with the corresponding antibiotic and 1 mM IPTG induction for 24 h.

Cell adhesion to Caco-2 and HT-29MTX-E12 mucus secreting cell lines. The low mucin producer, human Caco-2 colon adenocarcinoma cell line was obtained from the National Type Culture Collection. The human HT-29MTX-E12 mucus secreting cell line was obtained from Sigma, UK. The cells were maintained in Gibco RPMI 1640 (Life technologies, USA) or Dulbecco's modified Eagle's high glucose medium (Sigma-Aldrich, UK) respectively, both supplemented with 10% (v/v) fetal calf serum, 1% (v/v) non-essential amino acids and 1% (v/v) penicillin-streptomycin (Sigma-Aldrich) in a 5% CO2 humidified atmosphere at 37°C. Escherichia coli LMG194 and V. cholerae were grown in LA from a glycerol stock for 24 h at 37°C and then LB was inoculated with a single colony and grown 20 h at 37°C. A secondary culture was inoculated, and protein expression was induced by adding IPTG 1 mM for 24 h at 37°C.

For adhesion assay, medium was replaced with 1 ml of 1 × 10^6 CFU/ml of E. coli LMG194 or V. cholerae suspended in the previously described antibiotic free tissue culture media (human Caco-2 colon adenocarcinoma and HT-29MTX-E12 mucus secreting cell line with Gibco RPMI 1640 (Life technologies, USA) or Dulbecco's modified Eagle's high glucose medium (Sigma-Aldrich, UK) respectively, both with 1% (v/v) fetal calf serum and 1% (v/v) non-essential amino acids). The adhesion assay was performed for 2 h. In parallel, we grew E. coli and V. cholerae in the antibiotic free tissue culture media we used for the adhesion assay (described above) and checked AcfC expression after 2 h and we were able to detect AcfC expression in V. cholerae wild type and E. coli with pEXT20-AcfC construct. This could be due to the pTac promoter on the medium copy pEXT20 vector which does lead to leakiness. However, we did not detect AcfC expression in the acfC mutant or pEXT20 empty vector.

For adhesion assays, the medium was replaced with antibiotic free medium supplemented with 1% (v/v) fetal calf serum and 1% (v/v) non-essential amino acids 24 hours prior to experiments. Both cell lines were seeded at 2.5 × 10^5 cells/ml into 24-well plates (Corning Glass Works, Netherlands) using 1 ml of cell suspension per well.

Adhesion assays were performed on Caco2 or HT-29MTX-E12 monolayers that had reached 80–90% confluence in 24-well plates. Infections were carried out for 2 h. For infections, medium was replaced with 1 ml of 1 × 10^6 CFU/ml of E. coli LMG194 or V. cholerae suspended in the previously described antibiotic free tissue culture media. After co-incubation, the monolayers were washed 3 times with 1 ml of PBS 1X and incubated with 200 μl of 0.25% trypsin EDTA to break up the monolayer which was subsequently diluted and plated onto LB agar with or without corresponding antibiotic to calculate CFU/ml. E. coli ATCC25922 was used as adhesion positive control. Experiments were performed in three separate biological replicates, measured as three technical replicates each.

Motility and electron microscopy (EM). Bacteria were grown on LA plates for 24 h, at 37°C. One colony was inoculated in LB with 0.3% agar (BD Bacto-agar) and incubated at 37°C for 18 h. Photographs were captured after the incubation time with a Canon 600D SLR. The diameter of the halo was also determined. Experiments were performed in three separate biological replicates, measured as three technical replicates each.

For V. cholerae visualization by EM, strains were grown in LB tubes at 37°C, overnight under aerobic conditions. Each strain was grown in a total volume of 1 ml and was incubated for 18 h. 500 μl of 2.5% paraformaldehyde/2.5% glutaraldehyde/0.1 M Na cacodylate pH 7.4 was added to 500 μl of the bacterial culture. Five ml were placed onto a platform coated 300 mesh copper grid for 1 min. The sample was then stained with 10 µl of 0.3% phosphotungstic acid (PTA) pH 7 for 1 min. The PTA was drained and the grid was air-dried before examining on the Jeol 1200EX Transmission Electron Microscope. Digital images were recorded using a side-mounted AMT 2K CCD Digital camera supplied by Deben UK Ltd, IP30 9Q8.

Chemotaxis towards mucin and chitin. Chitin (Sigma, UK) and Type II mucin from porcine stomach (Sigma, UK) were prepared freshly for each assay by suspending at 2% (wt/vol) in chemotaxis buffer (sterile PBS 1X with 0.01 mM filter sterilised EDTA). V. cholerae cells were grown to an OD_600 of between 0.4 and 0.6 in LB broth. Chemotaxis was measured following the protocol as described. Results were compared to control needles containing only chemotaxis buffer to determine the chemotaxis response. The chemotaxis response was calculated as the ratio of test CFU/ml to control CFU/ml. Comparisons were made between strains using at least three biological assays containing two technical replicates each.

Biofilm formation. V. cholerae strains were grown in Marine Sea Water Yeast Extract (MSWYE) agar and broth overnight at 37°C. Two ml of culture was inoculated into low evaporation 24-well plates at 1:200 dilution. The cultures were grown statically for one day after which the supernatant was carefully removed and the wells washed with 1 ml of PBS 1X. Each well was incubated with 1% crystal violet (wt/v) at room temperature for 20 min. The crystal violet was dissolved with methanol for 15 min at room temperature. The OD_500 was measured in a Spectrophotometer (Biotek, UK). Experiments were performed in three separate biological replicates, measured as three technical replicates each.
Galleria mellonella virulence assay. This assay was performed as previously described\textsuperscript{41,42} using a micro-injection technique whereby 10 μl of V. cholerae was injected into the haemocoel via the right foreleg, using a Hamilton syringe. G. mellonella larvae were bred in sterile conditions at 37°C. After infection of bacteria, caterpillars were incubated at 37°C and survival and macroscopic appearance were recorded at 24 h post-infection. Caterpillars were considered dead when they were nonresponsive to touch. Three biological replicates of ten individual G. mellonella were performed.

Infant rabbit infections. All experimental protocols were approved by the local Animal Welfare and Ethical Review Body, the Home Office and carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Adult New Zealand White females were obtained from a commercial breeder (Harlan Laboratories, UK) and time-mated when required to produce litters of infant rabbits. Individual litters, typically containing 6–8 pups, were housed as a group in a nest box with the lactating doe for the duration of the experiments. Experiments were performed generally as described previously\textsuperscript{25}. Briefly, two- to three day old infant rabbits were treated with ranitidine hydrochloride (GlaxoSmithKline) by intraperitoneal injection (2 mg/kg bodyweight) 2 hours prior to infection. Bacteria were given orally by gavage using a Size 5 French catheter (Arrow International). Infant rabbits were infected with \(1 \times 10^9\) CFU WT V. cholerae or the Δacf mutant. The inoculum was prepared from stationary phase (18 h) cultures of the bacteria grown in LB broth supplemented with 100 μg/mL streptomycin at 37°C (250 rpm). To prepare the inoculum, bacterial cultures were centrifuged (5 min, 8 rpm) to pellet the cells and the supernatant was removed. Cell pellets were resuspended in sodium bicarbonate solution (2.5 g in 100 mL; pH 9) adjusted to yield a final cell density of \(2 \times 10^9\) CFU/mL. Diarrhoea was scored using the following scale: none (rabbits were dry with no signs of faecal contamination or wetness on their ventral surfaces; upon dissection, the colon contained digesta that appeared normal (dark green, hard and formed)); mild (soft yellow stools and/or limited areas of wetness were evident on the rabbits’ fur; upon dissection, digesta was missing from the colon or appeared yellow, soft and unformed), and severe (rabbits exhibited extensive areas of wetness on their tails and ventral surfaces; upon dissection no digesta was found in the colon and the cecum and small intestine contained large quantities of clear fluid). Infant rabbits were anaesthetised with inhalational isoflurane prior to euthanasia by cervical dislocation.

At euthanasia, intestinal samples and cecal fluid were collected as described previously\textsuperscript{25}. The number of V. cholerae CFU in tissue samples was determined after homogenisation in PBS by plating on LB supplemented with 100 μg/mL streptomycin. The detection limit of the assays was \(100\) CFU/g. Rabbits, which contained no WT V. cholerae colonies in any of the intestinal samples were considered ‘uncolonised’ and excluded from subsequent analysis; the reasons why we fail to recover any colonies in a small number (<1 in 10) of rabbits orally-infected with wild type bacteria are unknown. However, rabbits, in which viable V. cholerae were detected in at least one intestinal sample, were included in order to capture the natural variation of colonisation and disease that occurs in this host. In these animals, samples that yielded no bacteria at the lowest dilution tested were included in the calculation of the mean values presented in Fig. 5 by using the lower limit of detection as a value.

Statistical analysis. Data were expressed as mean ± standard deviation. The statistical test used was Tukey’s multiple comparisons test using Prism software 9 version 4.0 (GraphPad Software, Inc, San Diego, CA). p > 0.05 was considered statistically significant. The diarrhoeal state of the rabbits were compared using Fisher’s exact test. Cecal fluid accumulation ratios (FARs) and colonisation data (after log transformation) were statistically analysed using unpaired 2-sided Student t test (GraphPad Prism, San Diego, CA).

Data availability statement. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References


39. Mazumder, R., Phelps, T. J., Krieg, N. R. & Benoit, R. E. Determining chemotactic responses by two subsurface microaerophiles, analysed the results and wrote the paper: C.D. conducted the Caco-2 and HTX-29 cell adhesion and assisted in the chemotaxis experiments; D.M. initiated the project and carried out mutagenesis and cloning experiments. J.R. performed infant rabbit experiments. All authors reviewed the manuscript. We thank Maria Mc Crossan for assistance with the TEM images.

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Author Contributions E.V. and B.W.W. conceived the experiments; E.V. conducted mutant complementation, phenotypic assays and G. mellonella experiments, analysed the results and wrote the paper; C.D. conducted the Caco-2 and HTX-29 cell adhesion and assisted in the chemotaxis experiments; D.M. initiated the project and carried out mutagenesis and cloning experiments. J.R. performed infant rabbit experiments. All authors reviewed the manuscript.

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