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The Physical Basis of Type 4 Pilus-Mediated Microcolony Formation by *Vibrio cholerae* O1

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Abstract

The *Vibrio cholerae* toxin co-regulated pilus (TCP) is a type 4b pilus that mediates bacterial microcolony formation, which is essential for intestinal colonization. Structural analyses have defined a surface domain of the TcpA pilin subunit that is displayed repeatedly around the pilus filament surface and forms the molecular basis for pilus-pilus interactions required for microcolony formation. The physical attributes of this domain that lead to pilus-pilus association between bacteria are not known. Mutational analysis has revealed alterations within this domain that allow pilus-pilus interactions among pili expressed by individual bacteria, but do not allow pilus-pilus mediated association between bacteria. We characterized these altered strains using conventional microscopy, as well as three-dimensional high-resolution field emission scanning electron microscopy (FESEM), to reveal the physical difference between nonproductive and productive pilus associations that lead to interactions among multiple bacteria and result in microcolony formation. These findings pave the way towards investigation of the biophysical parameters involved in this basic bacterial property that promotes colonization of intestinal and other biological surfaces.

Keywords

Colonization; Diarrhea; Toxin co-regulated pilus

Introduction

The disease cholera is caused by the Gram-negative bacterium *V. cholerae*. A hallmark of cholera infection is the typical rice water stool that is a direct result of a potent secreted bacterial enterotoxin. Delivery of this toxin is dependent on bacterial colonization of the host intestinal epithelium. The most notable colonization factor utilized by *V. cholerae* is the toxin co-regulated pilus (TCP), a type 4b pilus (Taylor et al., 1987, Herrington et al., 1988). Type 4 pili represent a class of virulence factors commonly expressed on the surface of Gram-negative bacteria. This class of highly antigenic fimbrial structures is involved in an array of cellular activities ranging from bacterial attachment to surfaces to specific modes of surface motility (Strom and Lory, 1993, Mattick, 2002, Swanson, 1972). *In vitro*, bacterial-

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bacterial interaction via TCP is visualized as bacterial autoaggregation. Autoaggregation is completely correlated with the ability to colonize the infant mouse (Kirn et al., 2000). Each TCP filament is a homopolymer of TcpA pilin subunits assembled as a three-start helix with six subunits per turn (Craig et al., 2003). Within the pilus filament, a portion of the C-terminal domain of TcpA is positioned to face outward, forming a repeating patch around the filament in a position that should make it available for interaction with other pilus filaments. The associations between pilus filaments converge to form a bundled pilus structure. Previous genetic analysis has shown that certain charged residues in the C-terminal domain mediate bundle interactions in a manner specifically required for interbacterial association that leads to autoaggregation and colonization of the host (Kirn et al., 2000).

TCP is expressed by strains of both biotypes of the virulent *V. cholerae* O1 serogroup, classical and El Tor. The amino acid sequence of the TcpA pilin subunit is 82% identical between the biotypes (Rhine and Taylor, 1994). In this study, we utilized derivatives of classical biotype strain, O395, that are either wild-type (WT) and express classical TcpA (TCP^{cl}), or are engineered by allelic exchange to express El Tor TcpA (TCP^{ET}), so that the studies could be conducted using isogenic strains of a single biotype expressing comparable amounts of pili.

Studies initiated by Kirn et al. analyzed the role of charged residues of the classical biotype TcpA with respect to TCP structure and function (Kirn et al., 2000). A panel of alanine mutants was characterized on the basis of pilin and pilus production, level of autoaggregation, and physical features of the pilus observed by transmission electron microscopy (TEM). Although the majority of altered strains produced wild type levels of pili, many did not autoaggregate or colonize. Interestingly, two of these non-autoaggregating strains retained the ability to produce pilus bundles that appeared identical to those observed in WT strains when examined by TEM (Kirn et al., 2000). This presented a paradox, in that the pili bundled together, yet the bacteria failed to autoaggregate. In the current study we have attempted to resolve this paradox by utilizing confocal microscopy, in addition to FESEM, to establish a hierarchy of interactions that occur during the process of formation of higher order pilus structures.

Materials and Methods

Bacterial strains and media

Strains utilized in this study are described in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) broth, with aeration at 37°C for 12–16 hours (Maniatis et al., 1982). *V. cholerae* (O395 Ogawa derivative) strains were grown in LB, at a starting pH of 6.5, with aeration at 30°C for 12–16 hours (TCP inducing conditions) as previously described (Taylor et al., 1987, Kirn et al., 2000). As required, strains were grown with antibiotics at the following final concentrations: streptomycin 100 µg/ml, ampicillin 100 µg/ml, carbenicillin 50 µg/ml, gentamicin 30 µg/ml, and kanamycin 45 µg/ml. 5-Bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) was added to LB agar at 40 µg/ml when necessary. Bacterial manipulations, matings, and transformations were carried out as previously described (Maloy et al., 1995, Miller, 1992). To construct a control plasmid for confocal analysis, pGreenTIR was digested at *EcoRI* sites flanking the TIR and GFP cassette (Miller and Lindow, 1997). The plasmid fragment was ligated using T4 Ligase (New England Biolabs), forming pBro52 (Ap^R). pGreenTIR or pBro52 were transformed into *V. cholerae* MgCl₂ competent cells of desired strains (Miller, 1992).

Construction of a classical biotype strain expressing El Tor TCP

Plasmid pTK1, which carries a 2kb Hind III fragment containing classical *tcpA*, was used as a template for inverse PCR employing primers facing outward from the exact ends of *tcpA*. This construct was used to delete the chromosomal copy of *tcpA* using allelic exchange protocols described previously (Skorupski and Taylor, 1996). A portion of the inverse PCR product was blunt end ligated with the El Tor *tcpA* gene, which had been amplified using inward facing primers corresponding to its exact ends. The resulting plasmid was then used to exchange the El Tor *tcpA* gene into the classical *tcpA* deletion strain, resulting in strain RT4045.

Confocal microscopy

Following growth under TCP inducing conditions, cultures were allowed to settle on the benchtop for 30–60 minutes. 30 μ L of culture from the bottom of the tube was pipetted onto a glass microscope slide, a coverslip was placed on the top of the sample, and excess fluid surrounding edges of coverslip was blotted with a Kimwipe. Coverslip edges were sealed with nail polish, and the sample was imaged with an Olympus IX70 confocal microscope, using the FluoView software package to operate the confocal microscope and obtain images. Quantitation of fluorescence, and resulting analysis of the images was completed using ImageJ software (NIH) and Prism software (GraphPad).

Field emission scanning electron microscopy (FESEM)

Bacteria were adhered to 13 mm diameter formvar coated thermanox coverslips (Electron Microscopy Supplies) for 5 hours at 30°C. Formvar coating of the coverslips was initiated by washing coverslips in a detergent solution of 1% TritonX-100 in H₂O. Following thorough rinsing and drying, coverslips were additionally rinsed with 70% ethanol. Debris removal was achieved by gentle sonication of washed coverslips in a solution of 1% HCl, 70% ethanol in a bath sonicator, pulsing for 30 seconds, twice. Coverslips were rinsed following sonication with 70% ethanol, followed by 100% acetone, and repeated with 70% ethanol, drying coverslips completely between rinsing treatments. Coverslips were coated with formvar by submerging once in 0.5% formvar solution (EMS), removing excess with filter paper, and allowed to dry completely. Coated coverslips were rinsed with 70% ethanol in H₂O, allowed to dry, and stored between layers of 3MM filter paper (Whatman) until ready for use. Following incubation with bacterial cultures, coated coverslips were fixed in 0.5% glutaraldehyde (Electron Microscopy Supplies) in 0.1 M sodium cacodylate buffer pH 7.4 (Electron Microscopy Supplies) overnight at 25°C. Following fixation, coverslips were washed in 0.1 M sodium cacodylate buffer pH 7.4 three times for 3 minutes. The samples were treated with 1% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.4 for 1 hour at room temperature. The samples were dehydrated in a graded series of ethanol (50%, 70%, 100%) for 10 minutes each, and an additional two times for 10 minutes each in 100% ethanol. The samples were then further dried in 50% hexamethyldisilazane (HMDS) (Electron Microscopy Supplies) in ethanol, followed by two 10 minute 100% HMDS washes. The majority of HMDS was removed to just coat the coverslip, and the samples were air dried under vacuum overnight at 25°C. Coverslips were mounted, and plasma coated with OPC-60 (Filgen, Inc.) with a 3 nm coat and imaged using a XL30 ESEM FEG scanning electron microscope (FEI Nanotech). Coverslips were scanned for pili structures at 2000 $\times$ magnification (0.003 mm² field of view). Hundreds of fields of view were scanned of at least 3 coverslips per sample type. Images were taken at a range of 2000–40,000 $\times$.

Autoaggregation assays

Strains were tested to examine the level of interaction between two co-inoculated strains. Bacterial strains were grown up separately in LB pH 8.5 at 30°C for 16 hours. Strains were

co-inoculated in LB pH 6.5 at varying ratios, and incubated at 30°C for 16 hours. Microcolonies were prepared and imaged as previously described.

Quantitation of fluorescence level expressed by microcolonies

Quantitation was accomplished using ImageJ software package (NIH). A region of a set area was drawn and the relative level of fluorescence was measured within various random regions within the microcolony. Fluorescence levels were averaged for N values ranging from 12–30 per sample, and plotted using the Prism software package (Graphpad software).

Results

Visualization of pilus structure interactions

It has previously been demonstrated that strains expressing TCP^{cl} form large microcolonies comprised of interacting bacteria (Fig. 1A) (Kirn et al., 2000). The non-clonal nature of the interbacterial interaction was demonstrated by the co-inoculation of strains expressing TCP^{cl}, which were distinguishable by differential expression of green fluorescent protein (GFP), resulting in microcolonies that contain even mixtures of fluorescent and non-fluorescent bacteria when visualized via confocal microscopy (Fig. 1B) (Kirn et al., 2000). The interaction experiment was also performed with strains expressing different fluorophores, specifically yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP), and strains were observed intermixing within the microcolony structure (data not shown). Additionally, the level of interaction and ratio of bacteria within microcolony structures was consistent with the ratio of input bacteria initially inoculated (Fig. 2). These experiments revealed that pili from different bacteria are able to interact with each other to form microcolony structures because non-piliated bacteria are excluded from the microcolony in mixed culture experiments (Kirn et al., 2000). Using this same assay, Kirn et al. demonstrated previously that $\Delta tcpA$ strain does not incorporate into microcolonies composed of wild type TCP^{cl} strains, and are excluded from the microcolony structures when cultured together (Kirn et al., 2000). However, when two TCP^{cl} or TCP^{ET} strains that express distinct fluorescent proteins are cultured together, microcolonies are composed of an even mixture of both bacterial types (Sup. Fig. 1). To examine the physical characteristics of the pilus structures formed by these WT bacteria, bacterial samples were plasma coated with a thin 3 nm coat of osmium, and imaged via field emission scanning electron microscopy (FESEM). This protocol of sample preparation and instrumentation yields an image with more three-dimensional character at a much greater resolution than can be achieved with standard SEM with a platinum coating (Osawa and Nozaka, 1998). This imaging revealed for the first time that the bundles of TCP pili actually form twisted structures and that these structures interact with one another to form a higher order supertwisted structure (Fig. 3A, B). These structures are reminiscent of the striking images of twisted and supertwisted bundles of T4P formed by *Neisseria gonorrhoeae* in colonies published by John Swanson in the 1970s. Similar bundle-forming and twisting structures have been reported in enteropathogenic *E. coli* as well and other T4P-producing bacteria (Swanson and Zeligs, 1974, Giron et al., 1991). These supertwisted structures were observed on the coverslips, lying near bacterial cells (Fig. 3A), as well as participating in microcolony structures that are often observed adhered to the coverslips (Fig. 3B).

TCP^{clD129A} and TCP^{clE183A} arrest at the twist stage

Previous studies examined the phenotype of strains expressing pili comprised of TcpA containing single amino acid changes in its C-terminus. Although the majority of altered strains produced wild type levels of pili, many did not autoaggregate or colonize *in vivo* (Kirn et al., 2000). Interestingly, two of these non-autoaggregating strains, with an alanine replacing either the residue at position 129 (aspartate) or 183 (glutamate) (TcpA^{clD129A} or

TcpA^{clE183A}) (Fig. 4B) produced normal bundled pilus structures observed by TEM (Kirn et al., 2000). It is interesting to note that in this same study, the CTX phage transducing abilities of these strains, mediated by the TCP pili structures, were near to, or exceeded the levels observed in wild type strains (Kirn et al., 2000). This lead us to investigate the basis of why the pili could form normal structures, as viewed by TEM, yet fail to function.

Both confocal and fluorescence microscopy established that strains expressing TCP^{clD129A} and TCP^{clE183A} did not form microcolonies *in vitro* (Fig. 1E, G). To determine whether the lack of interaction between the strains was due to altered pilus interactions, the bacteria were analyzed by FESEM. The pilus structures formed by both point mutants appeared similar to those formed by TCP^{cl}, comprised of thick, twisted bundles (Fig. 5A–H). However, using FESEM we were able to detect one major difference between the WT and point mutant samples that had not been detected by TEM (Kirn et al., 2000). There was no evidence of interactions between individual pilus bundles. Both strains expressing TCP^{clD129A} and TCP^{clE183A}, were observed to produce pilus bundles that remained unassociated with other bundles and no supertwisted structures were visualized, in contrast to what was observed in WT strains (compare Fig. 5A–H with Fig. 3A). Even when bundles were close to one another, they did not intertwine to form supertwists (Fig. 5B,D,F).

We hypothesized that although TCP bundles produced by TcpA^{clD129A} and TcpA^{clE183A} appeared to be similar to those formed by WT strains, sequence alteration of TcpA resulted in a minor physical disruption within the pilus filament that negated pilus-mediated interbacterial interactions due to a lack of interbundle interactions (Fig. 5A–H, Fig. 4A). We reasoned that if the presence of WT pilus structures could promote interactions with the pili produced by the mutant, that it would support the pilus-mediated interbacterial interaction aspect of the model. This could be visualized as a restoration of microcolony formation to either mutant when it was co-cultured with a WT pilus producing strain.

TCP^{cl} and TCP^{ET} structures physically interact to mediate microcolony formation

To visualize whether pili produced by the point mutants were able to physically interact with the WT pilus, we needed to be able to morphologically distinguish between the defective pili and the WT pili, each expressed from different, but otherwise identical, bacteria in the same culture. We found that the bacteria expressing TCP^{ET} autoaggregated to a much lesser degree than those expressing TCP^{cl}, and formed microcolonies that were much smaller in size (compare Fig. 1A, B with Fig. 1C). When these small microcolonies were examined by FESEM, a supertwisted structure was observed, but there was also a great deal of disarrayed small bundles, in contrast to the straight, rigid pilus twist without disarray produced by strains expressing TCP^{cl} (compare Fig. 3A, B with Fig. 3C–E). Of greater importance for the current study, the TCP^{ET} were found to be morphologically distinct from TCP^{cl}, having a bumpy rather than a smooth surface, a feature consistent throughout every sample preparation. We used this property to directly visualize the interaction between pilus bundles from different bacteria resulting in the formation of supertwisted higher order structures. When strains expressing TCP^{cl} or TCP^{ET} were grown together, with one strain containing a fluorescent plasmid and the other a non-fluorescent control plasmid, a similar pattern of intermixing resulted within the microcolony as seen when imaged using confocal microscopy (Fig. 1D). This was similar to the microcolonies that were observed previously when the differentially labeled strains expressing TCP^{cl} were co-inoculated (Fig. 1B). As was observed when differentially labeled strains expressing TCP^{cl} strains were co-inoculated (Fig. 2), the level of interaction between the strains expressing TCP^{cl} and TCP^{ET} within microcolonies was dependent on the ratio of input bacteria within the initial inoculum (Sup. Fig. 2).

To determine whether a physical interaction can occur between the TCP^{ET} and TCP^{cl} pilus bundles, FESEM was utilized. FESEM examination of the microcolonies revealed an amalgam pilus structure (Fig. 3F–H). This intertwined supertwist maintained a thickness similar to that of TCP^{cl}, yet retained some of the curvature and surface irregularities of the TCP^{ET} pili. Additional studies were also conducted to test whether the TCP^{ET} pilus structure was able to function in pathogenesis *in vivo*. When isogenic strains, differing only in the gene encoding the pilin subunit, were directly competed for colonization in the infant mouse model, results indicated that the strains expressing TCP^{cl} and TCP^{ET} were able to colonize the mouse small intestine equivalently (Sup. Fig. 3).

TCP^{clD129A} and TCP^{clE183A} structures interact with TCP^{ET} via pilus-pilus interactions

Having established that the WT El Tor pilus was capable of physically interacting with WT TCP^{cl}, we wanted to test the ability of classical altered pili, TCP^{clD129A} and TCP^{clE183A}, to interact with the strain expressing TCP^{ET}. In contrast to what was observed when the strains expressing TCP^{clD129A} and TCP^{clE183A} or the strain expressing TCP^{ET} were grown alone, when the mutants were grown together with the strain expressing TCP^{ET}, autoaggregation was observed. These microcolonies were further examined by confocal microscopy. When strains expressing TCP^{ET} and TCP^{clD129A} were grown in culture together, the microcolonies containing the mutant strain were formed and were found to be small and ovoid in shape, with a reproducible tendency to cluster together during microscopic examination (Fig. 1F, Fig 6A–C, Supplemental Fig. 4, Fig. 7C, D), a distinct phenotype from that observed when either of these strains is cultured alone (Fig. 1C, E). The size and composition of the microcolonies remained relatively constant when the ratios of the bacteria in the inoculation were similar, although some smaller microcolonies were observed when the strain expressing TCP^{ET} outnumbered the strain expressing TCP^{clD129A} in the initial inoculum by more than 5 fold (Supplemental Fig. 3C). The majority of the bacteria within the microcolony were those expressing TCP^{ET}, with the bacteria expressing TCP^{clD129A} scattered sparsely and randomly throughout the microcolonies (Fig. 1F, Supplemental Fig. 3, Fig. 7C, D, G).

When strains expressing TCP^{ET} and TCP^{clE183A} were co-cultured, the strains producing TCP^{clE183A} were able to participate in microcolony formation, a phenomenon not observed when either of the two strains are cultured individually (compare Fig. 1C, G to Fig. 1H, Fig. 7E–G). Confocal observations determined that both inoculating strains were participating in microcolony formation. The ratio of the strains within the microcolonies correlated with the input ratio (Fig. 7E, F, Supplemental Fig. 5). The presence of microcolonies when the two strains were co-inoculated demonstrated an association between the bacteria expressing the TCP^{ET} and those expressing the defective TCP^{cl} derivatives, which were unable to form microcolonies on their own.

FESEM was utilized to examine whether there was a physical interaction between the two phenotypically distinct pili types. When the microcolonies, produced by co-inoculation of strains producing TCP^{ET} and TCP^{clD129A} or TCP^{clE183A} were examined by FESEM, pili supertwists were visible (Fig. 6). The pili supertwist structures displayed regions of pili with the distinct phenotypic characteristics of each of the inoculating strains. This is suggestive of a direct physical interaction between pilus types that lead to the formation of microcolonies observed using confocal microscopy. These images provide evidence as to the requirement for supertwist formation, involving a higher order interaction of twists from distinct bacteria, to achieve bacterial microcolony formation (Fig. 4A).

Discussion

The aim of this study was to investigate the physical basis of how TCP mediates autoaggregation. We have demonstrated that the process leading up to autoaggregation occurs in stages. This process initiates with the elaboration of bundled pili, forming structures referred to here as twists, and progress to form supertwisted structures to achieve autoaggregation and coincident microcolony formation. In order to study this process, we have utilized strains expressing altered TCP structures, TCP^{clD129A} and TCP^{clE183A}, which are unable to autoaggregate, yet produce pili that appear to resemble WT TCP^{cl} when examined by TEM. Examination of interaction of these strains with bacteria expressing TCP^{ET} by confocal microscopy, as well as examination of the physical associations made by the pili produced by these strains by FESEM allowed us to characterize the distinct stages leading up to microcolony formation.

Craig et al. analyzed the D129 and E183 residues with respect to their roles in pilus filament interactions. These residues are located in positions that allow them to mediate interaction between TcpA subunits within pilus filaments as well as between filaments (Fig. 4B). It was found that residue D129 is available to form hydrogen bonds with other TcpA monomers in the filament. This observation is in contrast to the predicted role of E183, which extends outward from the TCP filament towards other neighboring filaments (Fig. 4) (Craig et al., 2003). The observation that microcolonies formed by TCP^{clD129A} and TCP^{clE183A} strains in conjunction with TCP^{ET} are morphologically distinct and are composed of different ratios of input strains (Sup. Fig. 4, 5), supports the hypothesis that these residues participate in different aspects of autoaggregation. Although only a small number of bacteria expressing TCP^{clD129A} were observed within the mixed microcolonies, the presence of the classical type TCP^{clD129A} pili observed within the mixed supertwists via FESEM indicates that their participation is required for successful microcolony formation. Additionally, the contrast between the lack autoaggregation and lack of microcolony formation observed when the strains were cultured alone (Fig. 1C, E) and the formation of microcolonies when cultured together (Fig. 1F) suggests that the TCP^{clD129A} pili can participate in microcolony formation, if they have the opportunity to interact with WT TCP. The mechanism of supertwist formation will be examined further by altering residues at positions 129 and 183 to retain negatively charged amino acids, as well as switching to positively charged amino acids. This may indicate if the local charge is responsible for the loss in supertwisting capability observed in the strains expressing TCP^{clD129A} and TCP^{clE183A} or if it is due to a different property required at these positions. If we are able to isolate strains with suppressors of TcpA^{clD129A} or TcpA^{clE183A}, it may shed further light on the types of interactions that these residues participate in.

The studies presented here identify the physical basis that distinguishes between the ability of a *V. cholerae* pilus filament to simply bundle, and its ability to promote bacterial interactions and microcolony formation through development of a supertwisted higher order structure. Other pathogenic bacterial strains including enteropathogenic *Escherichia coli* (EPEC) (Bieber et al., 1998) and *Salmonella enterica* serovar Typhi (Bieber et al., 1998) express type 4b pili that mediate autoaggregation and virulence. The structures of the EPEC bundle forming pilus BfpA (Ramboarina et al., 2005) and *S. enterica* serovar Typhi pilus subunit PilS (Ramboarina et al., 2005, Xu et al., 2004, Balakrishna et al., 2006) have been solved by NMR studies. Structural based sequence alignments have revealed some conservation among the three dimensional structures of these pilin subunits (Ramboarina et al., 2005). Residues 129 and 183 of TcpA are located in the $\alpha 3$ and $\alpha 4$ helices, respectively, which are present in all three types of 4b pilin subunits. Specifically, the glutamic acid at residue 183 of TcpA correlates with glutamic acid residues in similar locations of the $\alpha 4$ helix of BfpA and PilS subunits (Ramboarina et al., 2005). Future studies to examine the

role of charged residues within these similar regions in BfpA and PilS may reveal these structures as general regions involved in type IVb pilus-pilus interactions. These interactions, and the ability for pilus structures to physically interact with each other in a supertwist formation with the TCP of *V. cholerae* should lead to general structural characteristics of type IVb pilus function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Confocal analysis of microcolony formation.

Images of microcolonies produced when fluorescent strains are grown under TCP inducing conditions. (A–D, F, H) Panels include representative images obtained from the center of the microcolony (with reference to the Z axis), as well as the stacked image from the XZ and YZ dimensions, as indicated. (E, G) Panels display single bacterial cell suspension, representative of the non-autoaggregating phenotype of these strains. All images were taken at 60× magnification. Strains marked with an asterisk (*) express green fluorescent protein (GFP), produced constitutively from a plasmid.

Fig. 2.

Visual examination and quantitation of interaction between differentially labeled TCP^{cl} strains.

(A–I) Representative image from a Z-series of images obtained via confocal microscopy. Strains (annotated by the various type of TCP expressed), TCP^{cl}(pGFP) and TCP^{cl}(pVector), were inoculated at various initial ratios (TCP^{cl}(pGFP): TCP^{cl}(pVector)). Ratios of input strains are indicated in the lower right of each panel. (J) Quantitation of mean fluorescence intensity observed within defined area of the microcolonies (N=30) (obtained using Image J software (NIH)).

Fig 3.

Morphology of wild type TCP structures.

FESEM images bacterial strains expressing TCP^{cl} or TCP^{ET}. (A–B) Representative image of bacteria expressing TCP^{cl}. (C–E) Representative images of bacteria expressing TCP^{ET}. TCP^{ET} pili are thinner in width compared to TCP^{cl}, and have a surface that appears rough. Images are representative of samples imaged and prepared on multiple days. (F–H) Representative images of co-inoculated TCP^{cl} and TCP^{ET} expressing bacteria. Solid, white arrows indicate examples of TCP^{cl} pili. Dashed white arrows indicate examples of TCP^{ET} pili. Black arrows with white arrow heads (F,G,H) indicate the amalgam pilus structure observed when strains expressing TCP^{cl} and TCP^{ET} are co-inoculated. Black arrows indicate flagella. TCP are rarely observed while attached to bacterial cells when imaged via this technique.

Fig. 4.

A model of supertwist formation and the crystallographic location of the altered residues used in this study.

(A) In WT strains expressing TCP^{cl} and TCP^{ET}, the pilus filament forms a twist (resulting in pilus bundles), which progress to form supertwists, a structure that mediates bacterial autoaggregation. Mutants expressing TCP^{cl}D129A and TCP^{cl}E183A arrest at the twist stage. The current studies cannot discern whether the interactions between twists in the higher order supertwist structure are parallel or antiparallel in nature. (B) Crystal structure of the globular head domain of the TcpA pilin monomer (Protein Data Bank 1OQV) with residues involved with the supertwist formation highlighted, D129 in green and E183 in red.

Fig. 5.
Morphology of TCP structures expressed by *tcpA* point mutants
FESEM images of strains expressing TCP^{clD129A} or TCP^{clE183A}. (A–D) Representative images of bacteria expressing TCP^{clD129A}. (E–H) Representative images of bacteria expressing TCP^{clE183A}. Panels A and H have been electronically magnified to increase detail, and do not have scale bars associated with these images. White arrows indicate pili. Black arrows indicate flagella.

Fig. 6.

Pilus morphology in mixed inoculum containing strains expressing TCP^{ET} and TCP^{clD129A} or TCP^{clE183A}

(A–H) Representative images of microcolonies and pili structures formed when strains expressing TCP^{ET} and TCP^{clD129A} were co-inoculated. White boxes in panels A, C, and E demarcate the boundaries of panels B, D and F, respectively. (I–P) Representative images of pili structures formed when strains expressing TCP^{ET} and TCP^{clE183A} were co-inoculated. White boxes in panels K, M and O demarcate the boundaries of panels L, N and P, respectively. Panels D, F, and P are electronically magnified images and therefore have no scale bar indicated. Solid white arrows indicate examples of TCP^{cl} pili. Dashed white arrows indicate examples of TCP^{ET} pili. Black arrows indicate flagella.

Fig. 7. Reciprocal labeling of strains used for autoaggregation. (A,B) Confocal images of microcolonies formed when TCP^{cl} and TCP^{ET} strains are co-inoculated; strains express either pVector control or pGFP (labeled on panel with *). (C,D) Confocal image of microcolonies formed when TCP^{clD129A} and TCP^{ET} strains are co-inoculated; strains either express pVector control or pGFP (labeled on panel with *). (E,F) Confocal images of microcolonies formed when TCP^{clE183A} and TCP^{ET} are co-inoculated; strains express either pVector control or pGFP (labeled on panel with *). (A–F) Panels include a representative image from the center of the microcolony (with respect to the Z axis), as well as the stacked image from the XZ and YZ dimension, as indicated. (G) Quantitation of mean fluorescence intensity observed within set area of microcolony (N=12) (obtained using Image J software (NIH)).

Table 1**Bacterial Strains and Plasmids used in this study**

Strain/Plasmid	Description	Reference
Strain		
<i>V. cholerae</i>		
O395	Ogawa derivative, Sm ^R	(Taylor et al., 1987)
CG842	Ogawa derivative, O395Δ <i>lacZ</i> , Sm ^R	(Gardel and Mekalanos, 1996)
TJK70	O395 + pGreenTIR, Sm ^R /Ap ^R	(Kirm et al., 2000)
BAJ239	O395 + pBro52, Sm ^R /Ap ^R	This study
RT4045	O395 TCP ^{ET} , Sm ^R	This study
BAJ184	RT4045Δ <i>lacZ</i> , Sm ^R	This study
BAJ240	RT4045 + pBro52, Sm ^R /Ap ^R	This study
RT4013	O395 TcpA ^{D129A} , Sm ^R	(Kirm et al., 2000)
BAJ230	RT4013 + pGreenTIR, Sm ^R /Ap ^R	This study
BAJ235	RT4013 + pBro52, Sm ^R /Ap ^R	This study
RT4060	O395 TcpA ^{E183A} , Sm ^R	(Kirm et al., 2000)
BAJ233	RT4046 + pGreenTIR, Sm ^R /Ap ^R	This study
BAJ238	RT4046 + pBro52, Sm ^R /Ap ^R	This study
<i>E. coli</i>		
S17-1λ <i>pir</i>	<i>thi pro rec hsdR</i> [RP4-2Tc::Mu-Km::Tn7] λ <i>pir</i> Tp ^R , Sm ^R	(de Lorenzo and Timmis, 1994)
Plasmid		
pGreenTIR	GFP expressing plasmid, Ap ^R	(Miller and Lindow, 1997)
pBro52	pGreenTIR-based control plasmid, Ap ^R	This study

Abbreviations used: GFP- green fluorescent protein; Sm^R- streptomycin resistance; Ap^R-ampicillin resistance, Tp^R- trimethoprim resistance