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Rachel Storms April 8, 2016

Demonstrating Correlations Between Structural And Functional Changes In The Three *Vibrio Cholerae* Pili

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a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Vibrio cholerae, the pathogenic bacteria responsible for the deadly disease cholera, produces three functional type IV pili: the MSHA-type pilus; the Chitin regulated pilus; and the Toxin co-regulated pilus. It was hypothesized that: i) the three pili would be morphologically distinguishable from each other; ii) mutations in the MSHA pilin subunit and hydrolyzing ATPase would be structurally and functionally similar, iii) that strains mutated in ChiRP and MSHA will not form a biofilm in non-Tcp inducing conditions. Negative staining and immunogold labeling was utilized to visualize the pili with transmission electron microscopy. It was determined that the MSHA pilus is a moderately bundled, wispy filament; the chitin-regulated pilus is a relatively unbundled, wispy, web-like filament; and the toxin co-regulated pilus is a highly bundled, fibrous, rigid filament. In order to correlate the structure of each pilus with its function during biofilm formation, strains mutated in each pilus were assessed for their ability to form biofilms on abiotic surfaces. It was found that the MSHA-pilus is sufficient for biofilm formation – though deletions of this pilus do not completely inhibit formation. The chitin-regulated pilus aids in the fitness of the biofilm, but is not crucial for the formation of the biofilm. The toxin co-regulated pilus may inhibit the formation of a biofilm, for deletions in this pilus produced a more robust biofilm.

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Table of Contents

Table of Figures

Introduction:

Cholera:

Vibrio cholerae is the causative agent of the deadly disease cholera. Cholera is characterized by extreme diarrhea, and when left untreated, can lead to severe dehydration and possible death within hours of infection (World Health Organization, 2015). According to the World Health Organization, cholera affects 1.4 to 4.3 million people a year, and kills 28,000 to 142,000 (World Health Organization, 2015). It is transmitted both from environment-human interactions and human-human interactions. Environmental reservoirs include primarily warm waters in which chitinaceous organisms thrive, and human-to-human transmission occurs through oral contact with the feces of an infected individual. The prevalence of the disease is highest in warm, moist environments in which access to clean drinking water is restricted and sanitation is poor ("Cholera, Areas Reporting Outbreaks, 2010-2014," 2015). Sub-Saharan Africa consistently reports high rates of cholera, as do poor Caribbean countries such as Haiti, Bangladesh and India ("Cholera," 2015). Countries in which there is access to clean drinking water typically report low rates of cholera, and most will have no reportable cases of cholera. In total, there have been seven cholera pandemics ("Cholera, Areas Reporting Outbreaks, 2010-2014," 2015).

The World Health Organization emphasizes the importance of prevention strategies when tackling cholera outbreaks. These strategies include providing access to clean drinking water and proper sanitation, with a focus on high-risk populations such as refugee camps, where providing clean water becomes more problematic. A vaccine is available for cholera, and is administered in high-risk areas. The vaccine aids in the

prevention of the disease, but is not totally effective, and should not be a substitute for prevention methods such as providing access to clean water (Abba et al., 2010). In the event that these prevention strategies fail, the typical treatment for cholera is rehydration therapy. Antibiotics may aid in the treatment of severe infections – however, with the increasing emergence of antibiotic resistant bacteria, antibiotics are typically avoided (Jaiswal et al., 2015). Most infected individuals will recover with proper treatment. ("Cholera - Vibrio Cholerae Infection," 2014)

Vibrio cholerae:

Vibrio cholerae is the gram-negative pathogen responsible for the disease cholera. Two strains are clinically relevant: the classical and El Tor strains – both of which belong to the serogroup O1 (Son et al., 2011). The El Tor strain has become the predominant pathogenic strain found worldwide, with the exception of outbreaks in Bangladesh (Siddique et al., 1991), where strains in the serogroup O139 have remained relevant (Finkelstein, 1996). While other serogroups exist and may cause diarrhea and other characteristic symptoms of cholera, they do not cause pandemics (Siddique et al., 1991; Finkelstein, 1996). The bacteria exists both in a planktonic state and in biofilms formed on primarily, though not limited to, chitinaceous surfaces (Lutz, 2013; Antonova et al., 2012; Meibom, 2004). They infect the small intestine of its host, and excrete the cholera toxin upon infection. This toxin is responsible for cholera symptoms.

Biofilms:

The formation of biofilms on surfaces confers an advantage to the aquatic bacteria by providing access to nutrients otherwise unavailable in the planktonic state, and giving protection from environmental stresses such as antibiotics and predation by protozoa (Lutz, 2013; Teschler, 2015; Sanchez, 2016). They may form biofilms on biotic or abiotic surfaces, with biotic surfaces such as chitin providing nutrients to the bacteria through enzyme degradation. Shells of crustaceans, plankton, and other organisms serve as chitinaceous surfaces for which *V. cholerae* can attach and form biofilms. As *V. cholerae* thrives on chitin, often outbreaks of cholera are observed during plankton booms (Teschler, 2015).

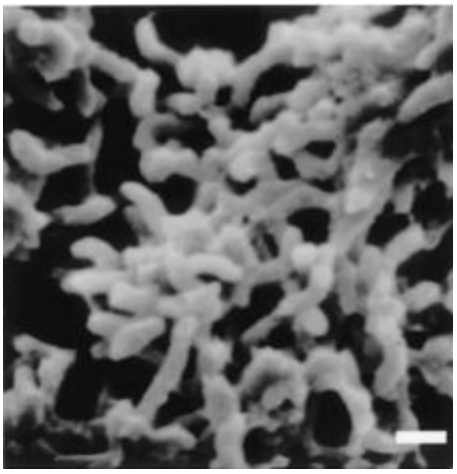


Figure 1. SEM of *Vibrio cholerae* biofilm (Bomchil et al., 2003)

Furthermore, recent evidence has pointed to biofilm formation within hosts. Microscopic examination of *V. cholerae*-infected suckling mouse small intestine has found the bacteria in microcolonies attached to the villi. Stool from a cholera-infected individual typically contains *V. cholerae* in biofilm-like structures and in the planktonic state. Additionally, biofilms require a lower infective dose than *V. cholerae* in the planktonic state. (Silva and Benitez, 2016)

The process by which these biofilms form involves surface attachment, *Vibrio* polysaccharide (VPS) secretion, and cell-cell interactions and attachments (Teschler, 2015; Reguera, 2005). Together, the exopolysaccharide produced by *V. cholerae* (VPS) and the cell-cell interactions by the chitin regulated pilus and the toxin co-regulated pilus

produce the three-dimensional biofilm with water ducts for the distribution of nutrients and diffusion of waste (Watnick and Kolter, 1999). The pili found on *V. cholerae* are involved in both surface attachment and cell-cell attachment in order to form these complex and dynamic biofilms (Teschler, 2015; Reguera, 2005; Roelofs, 2015; Jones, 2015). The three pili involved are the mannose-sensitive haemagglutinin (MSHA) pili; the chitin regulated pili (ChiRP), which is made of the pilin PilA; and the toxin co-regulated pili (Tcp), made from the subunit transcribed from the gene *tcpA*. The three pili are classified as type IV pili, which are involved in motility, cell-surface interactions, and cell-cell adhesions (Melville, 2013; Teschler, 2015; Antonova, 2012; Abuaita, 2009).

Type IV Pili:

Type IV pili are generally conserved among gram-negative bacteria, and are made of pilin subunits that are similar in amino acid sequences and assembled by an ATPase and secreted through the plasma membrane (Melville, 2013; Strom and Lory, 1993). The pili are thin, long filaments, and are involved in non-flagellar mediated motility (Craig et al., 2006). This motility is characterized by its twitching – a jerky movement unlike the smooth gliding movements mediated by the flagellum (Wall and Kaiser, 1999). The pilin

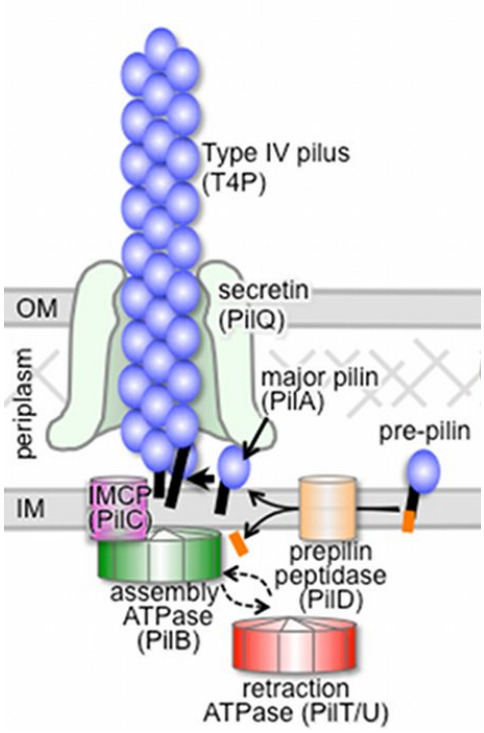


Figure 2. Schematic of a gram-negative TypeIV pilus (Melville and Craig, 2013)

subunits form flexible alpha helices from the base of the pilus, which accounts for flexibility required for twitching (Wall and Kaiser, 1999). Movement results from the quick assembly and disassembly of the pilus from pilin subunits within the inner membrane of the bacteria by the polymerizing ATPase and the retraction ATPase, respectively (Craig et al., 2006). Variations in the globular head domain of the pilin subunits create diversity among type IV pili both within and among bacterial species, and allow for functional variability and a continual evolution against anti-pilus responses (Craig et al., 2006; Craig et al., 2004). These differences in the pilin subunits create differential charges on the outside of the pilus, and alter the ability of the pilus to bundle together to create thicker fibers. Pili from the same bacteria that form thick, tight bundles decrease the amount of surface area available to interact with pili from other bacteria or surfaces. (Craig et al., 2004) *V. cholerae* produces three unique type IV pili, the three pili have different roles in biofilm organization, and they are involved in different steps in the biofilm formation process.

Vibrio cholerae Pili:

The MSHA pilus is associated with cell to surface attachment during biofilm formation (Teschler, 2015). The primary form of motility for *V. cholerae* is its flagellum. It uses the flagellum to move along a surface, while the MSHA pili mechanically 'scan' the surface. Different interactions between a surface and the pili produce varying movement and behavior: strong interactions produce an orbiting type pattern, whereas weak interactions lead to a roaming type pattern (Jones, 2015; Teschler, 2015). Tight, circular motions characterize orbiting patterns, where roaming patterns exhibit a long-

directional movement with small curvatures (Teschler, 2015). A mutation in the MSHA pilus ablates these movements and ultimately inhibit biofilm formation to the level of wild type, demonstrating that the MSHA pilus is important for cell to surface attachment during the first phase of biofilm formation (Teschler, 2015). The El Tor strain and strains belonging to serogroup O139 carry and express the MSHA gene, whereas the classical strain of serogroup O1 carries the gene but does not express the MSHA pilus. It is thought that this difference is the major cause of the emergence of the El Tor strain as the predominant strain in outbreaks of cholera (Chiavelli, 2001). However, while the MSHA pilus is central for attachment to environmental surfaces, it does not seem to have a role in the colonization of the host's small intestine (Chiavelli, 2001).

The toxin co-regulated pilus (Tcp) is responsible for colonization of the host. Tcp has been highly studied as a model for type IV pili, and has been characterized as a highly bundled pilus, creating thick fibers (Li et al., 2012). Tcp is primarily involved in virulence and the formation of microcolonies in the upper digestive tract of its host. Without this pilus, virulence is not possible. Tcp also form pilus-pilus interactions in order to form a more stable and differentiated biofilm (Reguera, 2005; Lim, 2010; Abuaita, 2009). The pilus is necessary for attachment to the vili of the small intestine; however, there appears to be a different mechanism in which the pilus attaches to biotic and abiotic surfaces, for the Tcp pilus is not necessary and confers no advantage for the formation of biofilms on abiotic surfaces such as chitin or borosilicate (Watnick et al., 1999). This may be due to differences in bundling outside and within the host. At temperatures around 22ºC, the pilus is highly bundled and presumably unable to interact strongly with surfaces or other pili. However, at 40ºC (temperatures close to host body

temperatures), the pili are completely unbundled, and presumably able to interact with host vili and other pili. (Li et al., 2012) To express Tcp genes in high amounts, bicarbonate must be present in the culture media – a compound that is present in the small intestine of its host (Abuaita, 2009).

The last pilus expressed by *V. cholerae* is the chitin co-regulated pilus, which is composed of the PilA pilin subunit. The expression of the gene encoding this subunit, and subsequently the pilus itself, is induced by the presence of chitin. For this reason, the pilus that PilA assembles is designated ChiRP (Meibom, 2004; Antonova, 2012). The ChiRP aid in the fitness of a biofilm or colonies formed on chitin, but does not confer any advantage to colonies formed elsewhere, or bacteria in planktonic growth. It does not appear, however, that ChiRP mediates surface attachment to chitin (Meibom, 2004).

Hypotheses:

Three hypotheses have been formed:

1. The three *Vibrio cholerae* pili will be distinguishable from each other by their bundling characteristics.
2. Deletions in the major pilin subunit and the polymerizing ATPase of the MSHA pilus (MshE) will be functionally similar, and that either deletion will be equally effective in ablating biofilm formation.

It is first hypothesized that the three *Vibrio cholerae* pili will be distinguishable from each other by their bundling characteristics. Each pilus has a different function and a different role in the formation of biofilms, with the toxin co-regulated pilus and the chitin-regulated pilus being involved in pilus-pilus interactions, and the MSHA-type pilus

involved in pilus-surface interactions. Given that different levels of pilus filament bundling proportionally alter surface area available for the pilus to bind to other pili or surfaces, and that each of the pili are made from different major pilin subunits, pili should be discernable based upon their function. It is hypothesized that the MSHA-type pilus will be less bundled than the toxin co-regulated pilus in environments outside of the host, for pilus-surface interactions mediated by the MSHA-type pilus are most important for the formation of biofilms outside of the host, and less bundling will open up available surface area on the pilus for surface interactions. It is also hypothesized that the chitin-regulated pilus will be less bundled than the toxin co-regulated pilus in environments outside of the host. The toxin co-regulated pilus functions primarily in the host small intestine, and is highly bundled outside of the host, whereas the chitin-regulated pilus functions on chitin outside of the host, and should be less bundled than Tcp outside of the host in order to perform these functions.

Secondly, it is hypothesized that deletions in the major pilin subunit (MshA) and the polymerizing ATPase of the MSHA pilus (MshE) will appear structurally and functionally similar, and that either deletion will be equally effective in ablating biofilm formation. Both the pilin subunit and the polymerizing ATPase are necessary for the assembly of the MSHA pilus, and without either of these structures, the MSHA pilus will not be formed. It is known that the MSHA pilus is the most important pilus for biofilm formation out of *V. cholerae*'s three pili, deletions in MshA and MshE will be equally effective in ablating biofilm formation. This will be shown through the use of biofilm assays and negative staining with antibodies for MshA.

Methods:

Bacterial Strains:

The wild type El Tor strain A1552, strains with deletions in mshA, mshE, pilA, and tcpA, as well as the double mutants ΔmshAmshE, ΔmshApilA, and ΔpilAtcpA were received from Professor Fitnat Yildiz's lab at the University of California, Santa Cruz. A deletion in mshA results in the loss of the major pilin subunit of MSHA, where a deletion in mshE confers a loss in the polymerizing ATPase. A loss of the major pilin subunit in the ChiRP results from ΔpilA, and ΔtcpA renders *V. cholerae* without the pilin subunit for the toxin co-regulated pilus, and therefore the pilus itself. Double mutants lack both structures encoded by the mutated gene. Double mutants include the ΔmshAmshE, ΔmshApilA, and ΔpilAtcpA strains.

Biofilm Assays:

Bacteria were streaked on 1% NaCl lysogeny broth agar plates, and then cultured overnight in 20 mL of 1% NaCl lysogeny broth at 29° Celsius and shaken at an RPM of 250. The culture was diluted 1:200, and then normalized to the strain with the lowest absorbance (read at a wavelength of 595 nm). One hundred and twenty microliters of each normalized culture was added to a 96 well NunclonΔ surface plate, with four replicates for each strain and a column of blank LB media between each column of culture to minimize quorum sensing between strains. The plate was allowed to sit 24 or 48 hours in the hood at room temperature. Each well was washed three times with sterile water in order to remove planktonic cells, and 150 microliters of 1% crystal violet stain

(Sigma) was added to each well for 15 minutes. A column of LB was stained as a control. The wells were again washed three times with sterile water to remove excess stain, and allowed to dry for 30 minutes. Images of the gross biofilm formed by each strain were acquired at this time. To wash the stain from the attached cells, 150 microliters of 100% ethanol was added to each stained well for 15 minutes, and then transferred to a separate plate for reading. Ethanol was used as the blank, and the plate was read at a wavelength of 595 nm. Absorbance readings for the ethanol blanks were subtracted from the absorbance readings of each sample, and each mutated strain was recorded as percent biofilm formation of the wild type A1552 strain. Therefore, wild type was included in every assay as a way to control for variance between experiment conditions.

Negative Stain Transmission Electron Microscopy (TEM) of Strains:

Experiments were performed to determine which stain to use for the best conventional TEM imaging results. Variables included the type of stain, time of staining, and washing of the sample. Phosphotungstic acid (PTA), methylamine tungstate (MAT), and uranyl acetate (UA) were all tested, with 2% PTA giving the highest quality staining with the least amount of staining artifact, such as crystallization or large grain size. Fifteen seconds was the optimal amount of time for staining, with a water wash of ~2 seconds between sample application and staining. The end protocol for negative staining of *Vibrio cholerae* is as follows:

An overnight culture was grown as specified above. Five microliters of 1:2 diluted culture was applied to a carbon-coated, glow-discharged 400 μm mesh copper grid for 5 minutes. A cut pipette tip was used during pipetting of the bacterial cultures to

minimize the disruption of pili and flagellum. After application of the sample, the grid was then quickly dried with Whatman filter paper, washed in sterile water for about two seconds, quickly dried again with Whatman filter paper, and then stained for about fifteen seconds with 2% phosphotungstic acid. Images were acquired on a Hitachi HT-7700 120kV transmission electron microscope (TEM) and a JEOL JEM-1400 120 kV TEM.

Immunogold Labeling and Imaging:

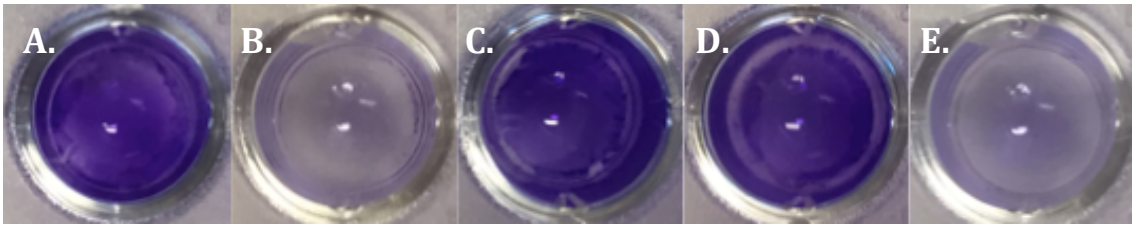
Primary antibodies for the mshA pilin subunit of the MSHA pilus were graciously given by Professor Fitnat Yildiz's lab at the University of California, Santa Cruz. Antibodies are rabbit polyclonal in 2% milk PBST.

An overnight culture was grown as specified above. Ten microliters of a 1:2 diluted sample were added to a carbon-coated, glow-discharged 400 μm mesh nickel grid for 10-15 minutes. It was then washed three times in sterile water for 30 seconds each, and then incubated in blocking buffer (5% normal goat serum in PBS) for 30 minutes. Grids were washed twice in PBS for five minutes each, and then incubated in the primary antibody for two hours. The grid was washed six times in rinsing buffer for 5 minutes each and then incubated with the secondary antibody for one hour. Grids were washed three times for five minutes each in rinsing buffer, and then stained for 15 seconds in 2% PTA and dried. Images were acquired on a Hitachi HT-7700 120kV transmission electron microscope (TEM).

Results:

Biofilm Assays:

Twenty-four hour biofilm assays were performed on the four single mutant strains 3-4 times and the three double mutant strains two times. Biofilm formation in strains with mutations in the pilin subunit and polymerizing ATPase was qualitatively indistinguishable, and there was an insignificant different between the two mutations quantitatively (Figure 3). Strains with mutations in the MSHA pilus formed roughly 45% of the biofilm of the wild type strain. Strains with mutations in the pilin subunits of the chitin regulated pilus and the toxin co-regulated pilus were similarly indistinguishable, and formed roughly 80% of the biofilm of the wild type strain. Mutations in the toxin co-regulated pilus conferred a small advantage one out of four repeats in the single mutated strain, and large advantage one out two repeats of the strain mutated in both Tcp and ChiRP (Figure 3 and Figure 4). Biofilm formation by the strain mutated in both the pilin subunit and polymerizing ATPase of MSHA (ΔmshAmshE) did not reflect the amount of biofilm formed by strains mutated in either the pilin subunit of polymerizing ATPase (ΔmshA or ΔmshE) (Figure 3 and 4). However, biofilm formation by the ΔmshApilA strain reflected biofilm formation by the ΔmshA and ΔmshE strains (Figure 4). Standard deviations for the double mutants strains remain high, due to the lack of repeat experiments performed.



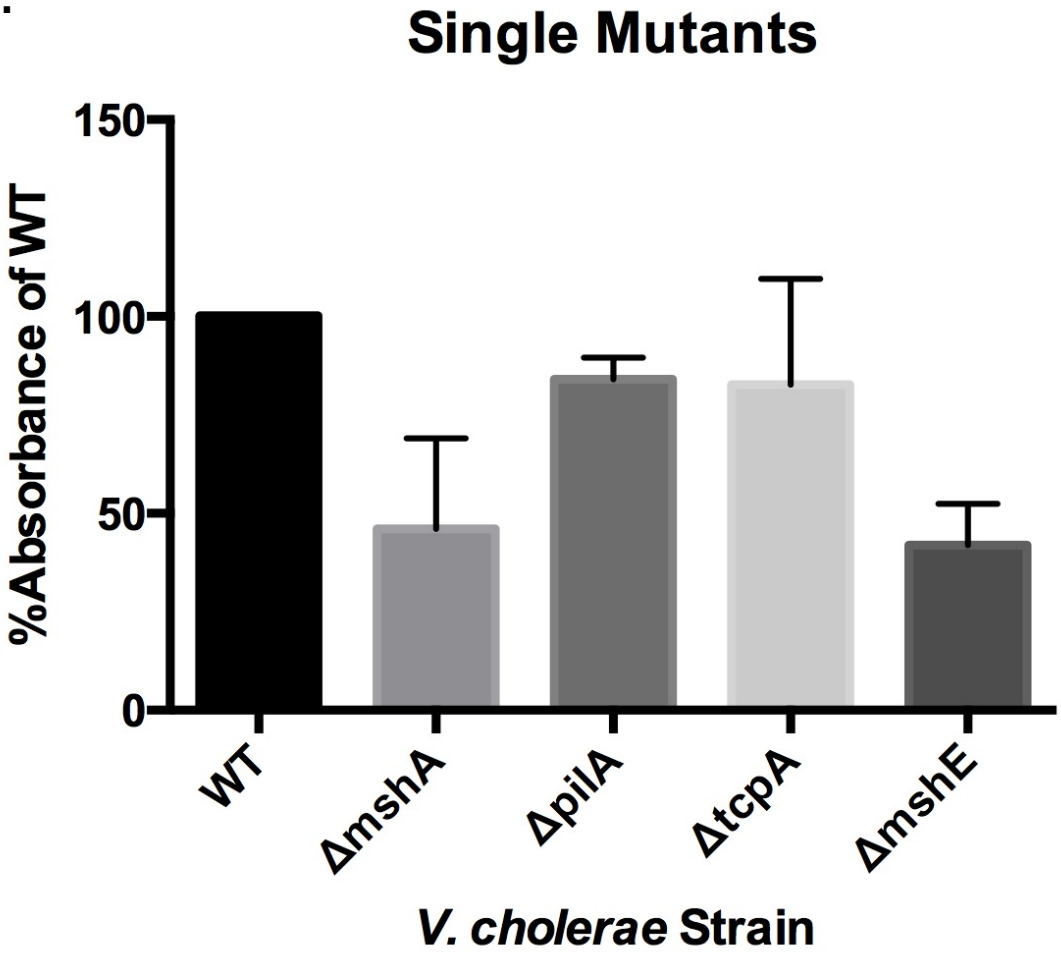
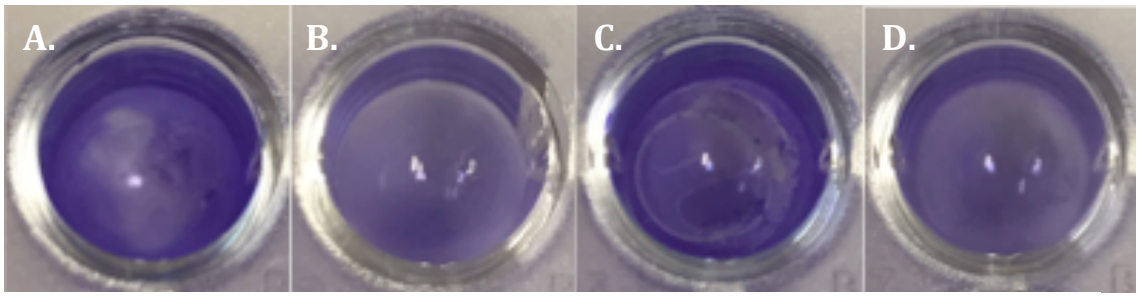


Figure 3. Biofilm assay results: 24 hour assay of strains with one deletion in a single pilus. (A-E): Crystal violet staining of (A) WT; (B) ΔmshA; (C) ΔpilA; (D) ΔtcpA; (E) ΔmshE. (F) Quantitative biofilm formation. Bars represent the average percentage of WT and standard deviation.



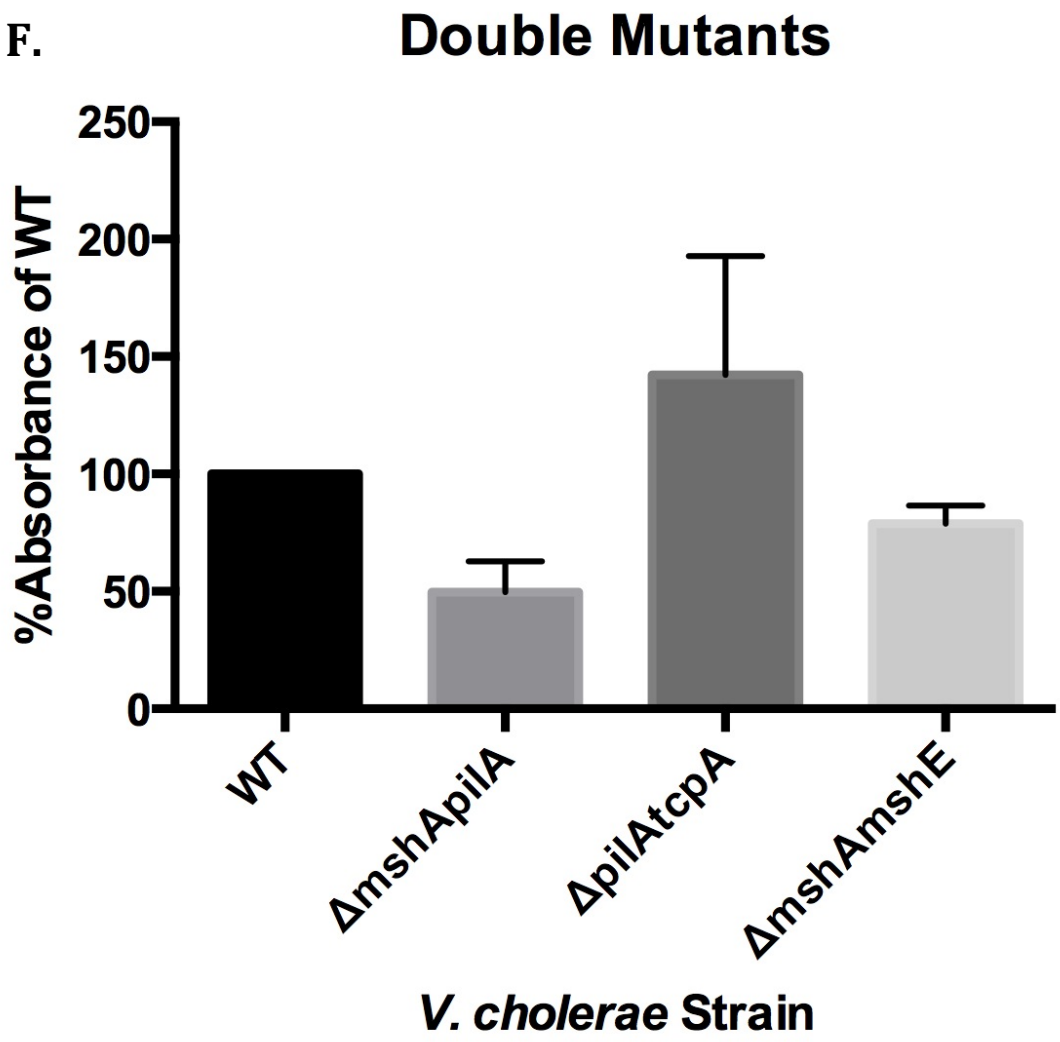


Figure 4. Biofilm assay results: 24 hour assay of strains with two deletions in a single pilus or multiple pili. (A-D): Crystal violet staining of (A) WT; (B) ΔmshApilA; (C) ΔpilAtcpA; (D) ΔmshAmshE. (F) Quantitative biofilm formation. Bars represent the average percentage of WT and standard deviation.

Negative Staining:

Negative stain imaging revealed three distinct pili on the wild type strain. Pili were present as thin filaments extending from the surface of the bacteria; many of them often form bundles whereas others were present as individual pilus filaments. These differences gave evidence for the presence of all three pilus types on wild type *V. cholerae.* (Figure 5)

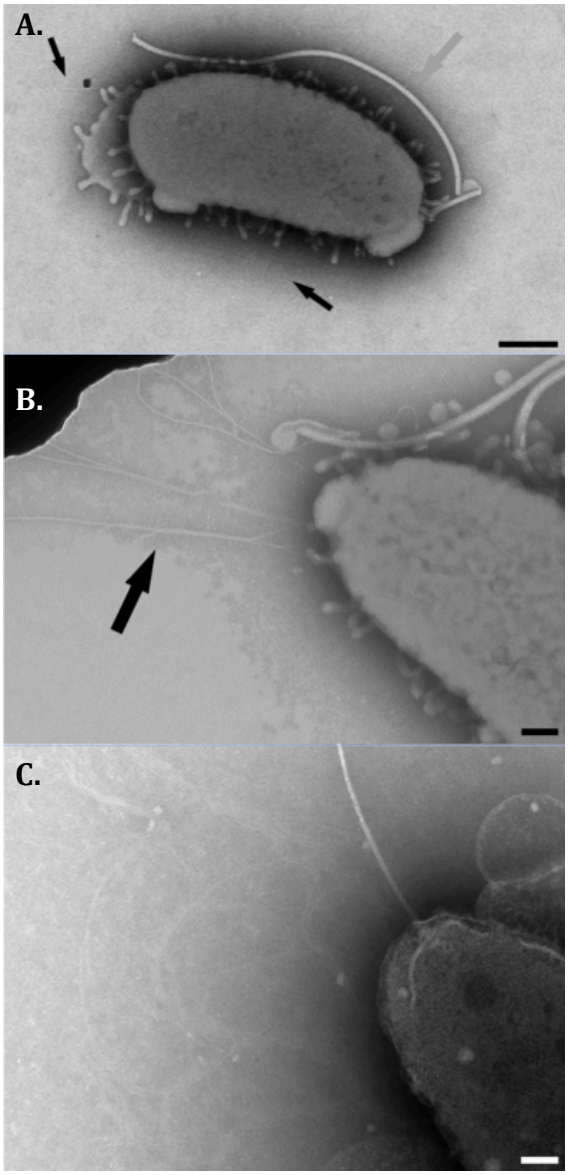


Figure 5. Negative staining of wild type *V. cholerae*. Three pilus types are present, and pointed to with black arrows. Grey arrow labels the flagellum. (A) Moderately bundled, wispy pilus. Scale bar = 1µm. (B) Bundled, rigid pilus. Scale bar = 1nm (C) Relatively unbundled, wispy pilus. Scale bar = 1nm

Negative stain imaging of the ΔmshA and ΔmshE strains showed similarities in structure, in that both strains were especially difficult to stain. Few bacteria remained on the grid after three separate attempts at staining, and what bacteria did remain were mainly devoid of pili. This may be due to their lack of the MSHA pili, for the MSHA pilus is solely responsible for cell-surface interactions on abiotic surfaces such as the carbon-coated copper and nickel grids. (Figure 6)

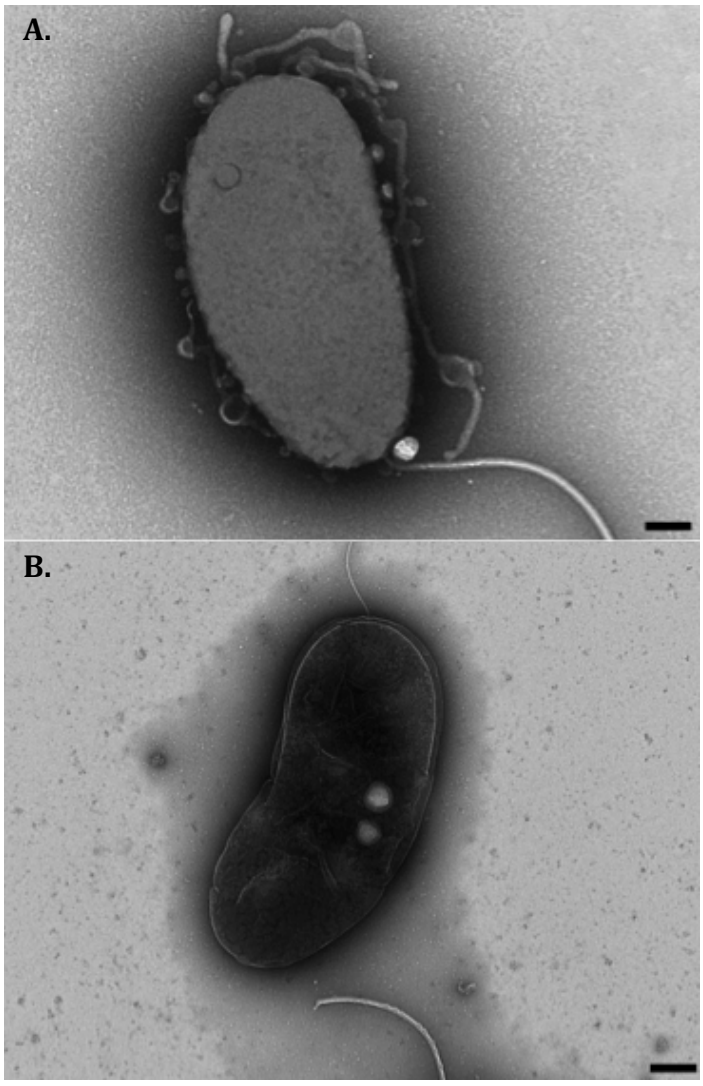


Figure 6. Negative stain images of strain with deletion in the MSHA pilus. Both strains were devoid of pili. Scale bars = 1μm (A) ΔmshE (B) ΔmshA

The strain with a deletion of ChiRP pilin subunit (PilA) displayed two phenotypically different pili: a moderately bundled, wispy pilus displayed in Figure 7A, and a highly bundled fibrous pilus displayed in Figure 7B. These pili were seen consistently on multiple samples from various cultures. No relatively unbundled, wispy pili were observed. (Figure 7)

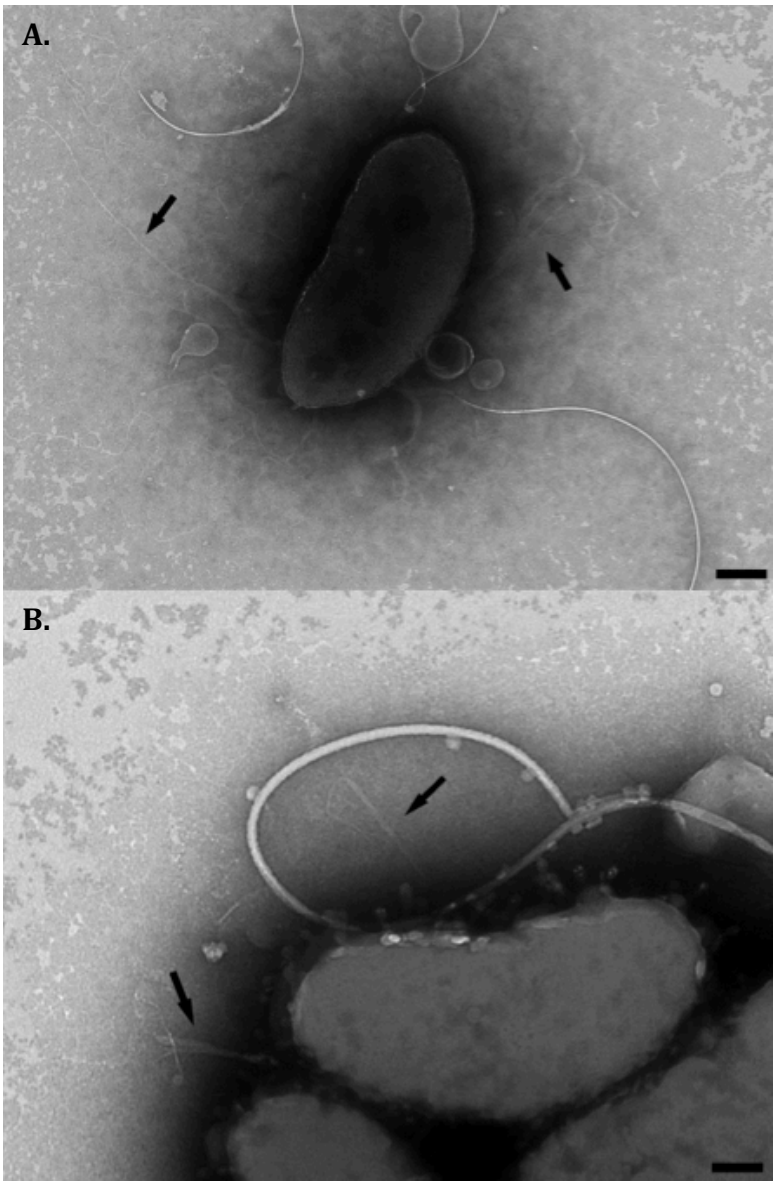


Figure 7. Negative stain images of ΔpilA strain in which the major pilin subunit of the ChiRP has been deleted. Two phenotypically different pili are present, and labeled with black arrows. Scale bars = lμm (A) Bottom arrow labels moderately bundled wispy pilus (B) Arrows label highly bundled, fibrous pili.

Negative stain imaging of the strain with a deletion in the major pilin subunit of the toxin co-regulated pilus (ΔtcpA) showed bacteria with two phenotypically distinct pili. Unbundled, wispy pili were prevalent, as well as bundled, fibrous pili. The unbundled pili is seen in Figure 8A, and the bundled, fibrous pili is seen in Figure 8B. No moderately bundled, wispy pili were observed.

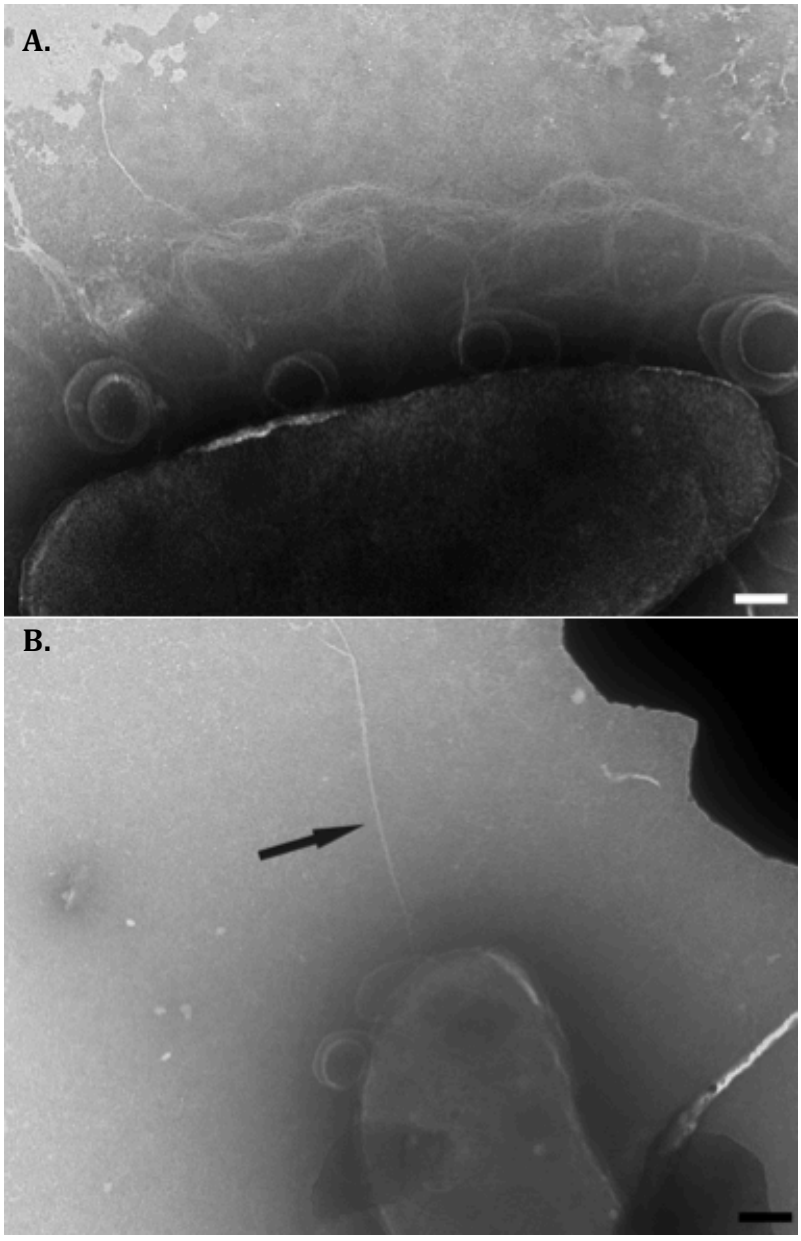


Figure 8. Negative stain images of the ΔtcpA strain. (A) Unbundled, wispy pili. Scale bar = 1nm (B) Highly bundled, fibrous pili labeled with black arrow. Scale bar = 1μm

Immunogold Labeling:

Immunogold labeling was successfully performed on the wild type, ΔmshA, ΔpilA and ΔtcpA strains with an antibody for the MSHA pilin subunit. Again, TEM imaging and staining of the ΔmshA strain proved difficult, and no pili were observed on any bacteria. Labeling in every other strain occurred on pili that are phenotypically similar. Few unbundled, wispy pili were observed on any strain; however, immunogold labeling should be repeated before any conclusions are drawn about the presence of these pili on the bacteria. All immunogold labeled pili are moderately bundled and wispy in their phenotype. (Figure 9)

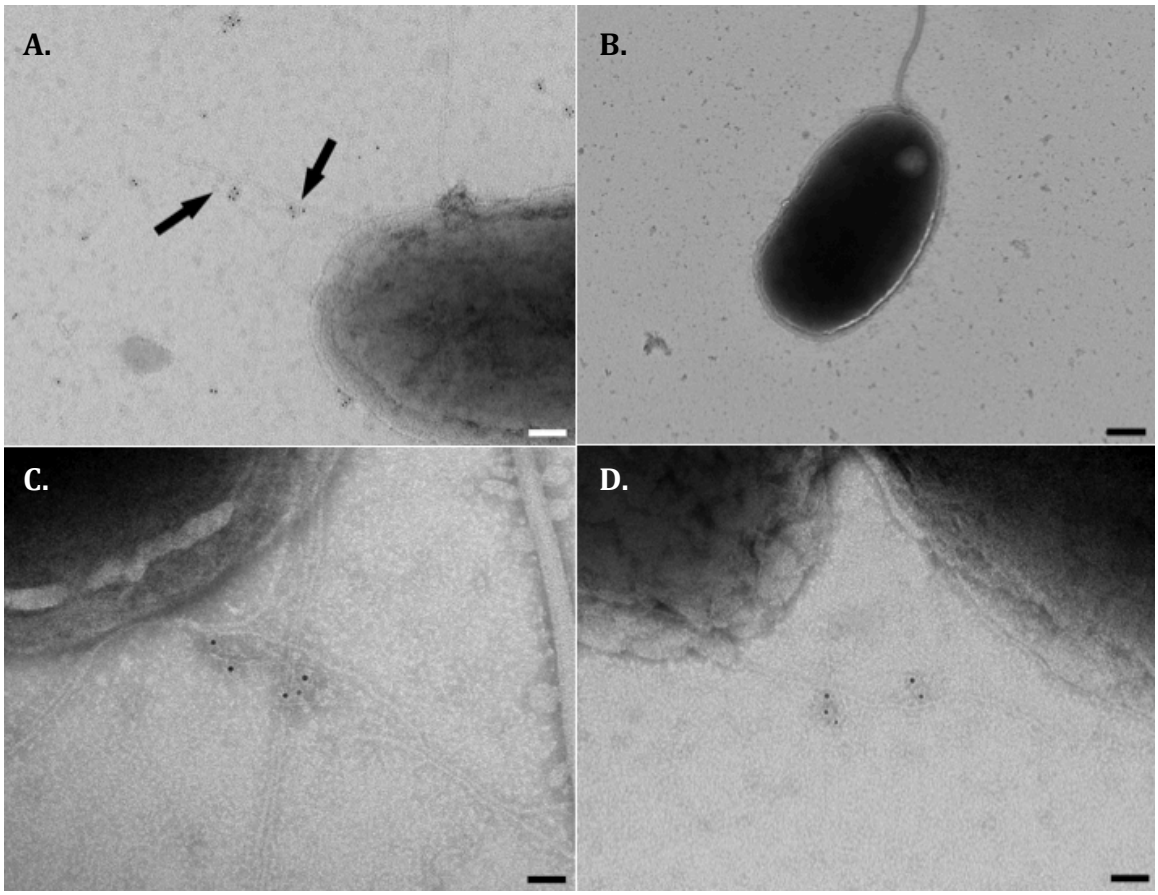


Figure 9. Immunogold labeling and negative staining of (A) WT (Scale bar = 1nm), (B) ΔmshA (Scale bar =1μm), (C) ΔpilA (Scale bar =1nm) and (D) ΔtcpA (Scale bar =1nm). Immunogold labeling is pointed out with black arrows in WT. All labeled pili are moderately bundled and wispy – not fibrous.

Discussion:

The first hypothesis – that the three *Vibrio cholerae* pili will be distinguishable by their bundling characteristics – was supported. However, it was difficult to determine with the negative stain TEM data the structural consequences to the pilus assembly complex of deleting either ΔmshA or ΔmshE. It could be inferred that a deletion of either of these genes produced a strain devoid of the MSHA-type pilus filament, and therefore strains in which cell-to-surface attachments were minimal. This may explain why imaging of these strains by negative stain was difficult.

By immunogold labeling, it was determined that the MSHA-type pilus is a moderately bundled, wispy filament – distinguished from the rigid, highly bundled filaments of Figure 5A and from the relatively unbundled, wispy filaments of Figure 5B. These filaments were visualized by negative staining and by immunogold labeling on all strains except for the ΔmshA and ΔmshE strains. However, as these strains were not successfully stained and imaged, it is not possible to make any conclusions on the presence of the MSHA pilus in these strains.

By process of elimination through negative stain imaging, it could be inferred that the chitin-regulated pilus is a relatively unbundled, wispy pilus that fans out like a web from the bacteria, and that the toxin co-regulated pilus is a highly bundled, rigid filament. These were distinguishable by their bundling characteristics, supporting the first hypothesis.

The second hypothesis, that deletions in the major pilin subunit and the polymerizing ATPase of the MSHA pilus (MshE) will be functionally similar, and that either deletion will be equally effective in ablating biofilm formation, was supported. The

MSHA-type pilus was functionally shown to be integral to biofilm formation. *V. cholerae* strains mutated in either the major pilin subunit or the polymerizing ATPase were significantly depreciated in their ability to form a biofilm; however, they were still able to form a biofilm. These strains were still able to form around 45% of the biofilm of wild type, which suggests that other mechanisms for surface attachment may be relevant. Strains mutated in the pilin subunit, the polymerizing ATPase or both were functionally indistinguishable, suggesting that both the pilin subunit and the ATPase are equally critical to the formation of the MSHA pilus.

The biofilm results pointed to the ChiRP aiding to the fitness of the overall biofilm on abiotic surfaces, but may not be as critical to biofilm formation. Strains mutated in the ChiRP and MSHA-type pilus formed as little of a biofilm as strains mutated only in the MSHA-type pilus, suggesting that deletions of the MSHA-type pilus mask deletions in the ChiRP.

Biofilm assay results suggested that deletions in the toxin co-regulated pilus may confer an advantage in *V. cholerae* biofilm formation on abiotic surfaces. While not all replicates demonstrated this, those that did had an advantage almost twice that of the wild type strain. This suggested that the Tcp may hinder biofilm formation under certain conditions, and push the bacteria towards a preference for the planktonic state. More replicates should be performed in order to assess this claim.

Limitations and Future Directions:

Biofilm assays were performed at room temperature in the hood – a condition not ideal for *Vibrio cholerae* growth. In order to validate our results, these assays should be performed in an incubator at 29° Celsius.

Biofilm assays should be performed on chitinaceous surfaces and in media containing bicarbonate in order to assess the functional contributions of each pilus under conditions in which each of them are preferentially expressed. It is very possible that under different conditions, different pili may be more functionally important in the formation of biofilms. In fact, it has been shown that the toxin co-regulated pilus is crucial to the formation of microcolonies in the villi of the small intestine – an environment in which bicarbonate is present (Watnick et al., 1999).

Assays should be performed for 48 hours and samples acquired and read at 6 hour intervals in order to validate the protocol and the importance of each pilus at different stages of growth. Two assays were performed for strains with single mutations, and similar ratios of biofilm formation were observed, though significantly different amount of biofilm formation was seen. These experiments should be replicated and performed on the double mutants.

Lastly, immunogold labeling should be performed using an antibody for each pilus type in order to characterize the structure of each pilus. Mutations in the toxin co-regulated pilus should be imaged with immunogold labeling to the Tcp and a Western blot should be run with antibodies for Tcp in order to validate a successful deletion of *tcpA* and the results found in which the Tcp hinders biofilm formation.

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