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Cryo-EM Structural Studies of the *Vibrio cholerae* Flagellum

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Vibrio cholerae is a pathogenic bacterium, found in salt and brackish water, responsible for the diarrheal disease cholera [1]. This endemic-causing bacterium uses a single polar flagellum to swim to a surface and colonizes the surface with the help of its pili, forming communities called biofilms. *V. cholerae* forms sessile biofilms or biofilm-like microcolonies both in the environment and intestine during infection as a survival tactic [2]. *V. cholerae* is also capable of leaving a sessile community as a planktonic, hyper-infective, bacterium. *V. cholerae* flagella play a large role during the initial stages of biofilm formation and have a role in *V. cholerae* pathogenicity that is still being explored [1, 2].

Studies of the *Vibrionaceae* flagella have been limited in part due to challenges in purifying the flagellar sheath, which is an extension of the cell membrane, from the flagellar filament [3, 4]. Some bacteria have a single flagellin protein that makes up its flagella, while others have multiple flagellins, forming a helical flagellar filament [5]. *V. cholerae* has 5 flagellins denotated FlaA, FlaB, FlaC, FlaD, and FlaE. FlaA is required to form a flagellar filament, and any ΔFlaA mutant is incapable of forming a filament. The 5 flagellin proteins in *V. cholerae* share approximately 60-80% identity and are redundant in structure but can differ in function [6, 7]. While the purpose of a multi-flagella filament is still being explored, different flagellins are thought to play a role in biofilm formation and toxin expression in *V. cholerae* [6, 8]. Here we show an optimized method for purifying the sheath off the *V. cholerae* flagella while keeping the flagellar filament intact, as well as reconstruction methods for helical assemblies imaged with cryo-electron microscopy (cryo-EM).

V. cholerae ΔFlaBCDE (FlaA only) is used to find the FlaA flagellin structure first due to difficulties resolving differences in flagellin structure in a multi-flagellin filaments during helical reconstruction. Flagellar mutants containing single flagellins can be used to solve these structures in a systematic way. *V. cholerae* flagellar filaments were purified as follows. *V. cholerae* ΔFlaBCDE was grown at 37°C and 250 RPM shaking in LB broth (Fisher BioReagents) until the culture reached an OD_{600} of 0.7. Cell culture was passed through a 20-gauge needle using a 60 mL syringe to remove the sheath from the filament. Cultures were then centrifuged at 10,000 x g to remove flagella from cells and pellet cell debris. Next the supernatant containing detached, unsheathed flagella was centrifuged at 48,000 x g to pellet the flagella. The flagellum pellets were resuspended in 50 mM Tris-HCl, 20 mM NaCl, 0.1 mM Dodecyl-D-Maltoside. The centrifugations were repeated in 3 iterations to further remove cell debris. Cryo-EM grids were prepared as follows: purified flagella were plunge frozen onto glow-discharged, 200 mesh R2/1 Quantifoil grids (Quantifoil, Germany) in liquid ethane using a Vitrobot Mark IV (FEI,

Hillsboro, Oregon). Vitrified grids were imaged on a Titan Krios TEM operated at 300 kV. Three-dimensional reconstructions of flagella were generated using RELION 4.0 [9, 10].

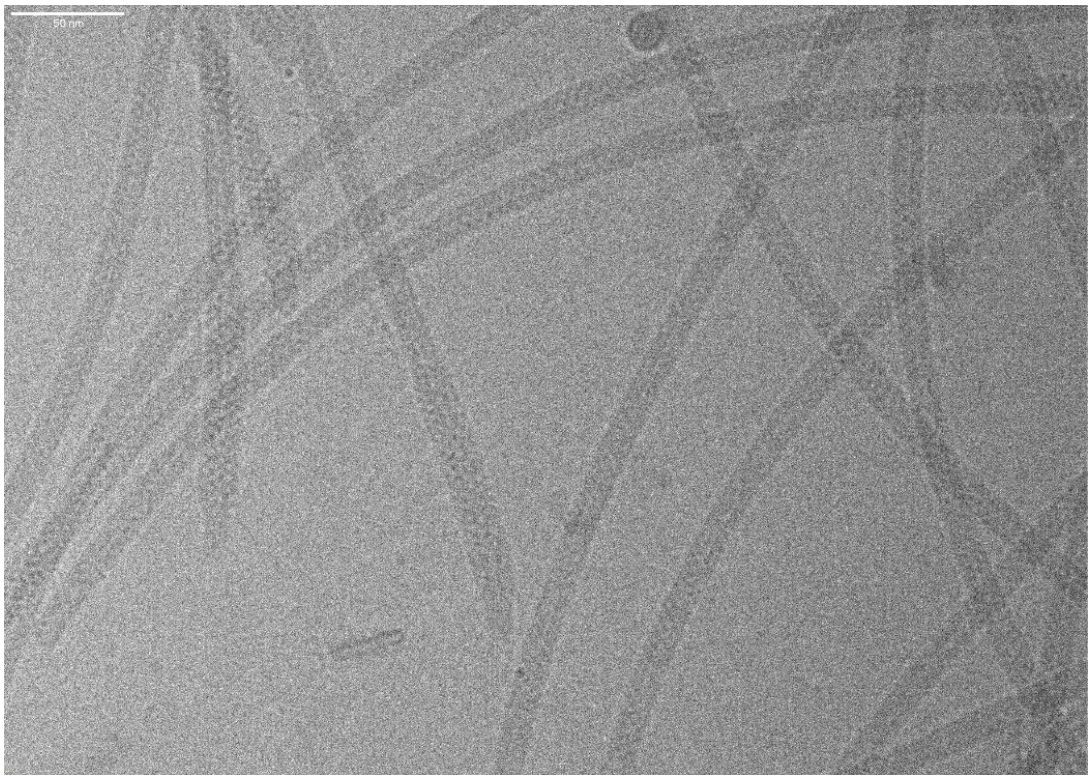

Figure 1. Cryo-EM image of *V. cholerae* ΔFlaBCDE purified flagellar filaments. Scale bar is 50 nm.

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