

Correlative Microscopy of the *Caulobacter crescentus* Flagellum Reveals How Changes to the Flagellin Protein Sequence Regulate Structure and Function.

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The bacterial flagellum is a complex propeller and is composed of three major units: the motor (basal body), the hook, and the filament. As a whole, the components work together to drive the bacterium through its environment. A number of studies of many bacterial species, including *Caulobacter* and *Vibrio* species, indicate that flagellar filaments are assembled from several flagellin proteins as opposed to a singular flagellin. Many of the flagellins are differentially regulated, redundant in molecular weight, and function [1]. While there may be some redundancy, there appears to be evidence that a single flagellin is essential for flagellar filament synthesis and motility. *Caulobacter crescentus* expresses six flagellin proteins, and all the flagellins are present along the length of the flagellar filament [1]. In this structural study of the *Caulobacter* flagellum, we sought to ascertain which flagellin was essential and determine the high-resolution structure of both the full-length flagellum and the flagellin protein. We used a combination of mutagenesis experiments, fluorescence microscopy, and cryo-electron microscopy (cryo-EM) to generate and identify the appropriate mutants for structure determination.

Caulobacter crescentus flagellum labeling was done using Alexa Fluor 495 C₅ Maleimide (AF495-mal) (ThermoFisher Scientific). *C. crescentus* cultures were grown to mid-log phase before labeling. Labeled cultures were gently centrifuged and washed once with PYE before being concentrated. Cells were imaged using phase contrast microscopy, while the labeled flagella were imaged using fluorescence microscopy.

Negative stain TEM of the bacteria and isolated flagella followed standard procedures. Briefly, 400 mesh carbon-formvar-coated copper grids were glow discharged for 30-60 seconds. Four μ L of the sample was applied to the grid and allowed to adsorb for 1 min before staining. Negatively stained specimens were imaged on a JEOL JEM-1400 TEM (JEOL Ltd., Tokyo, Japan) equipped with a LaB₆ filament and operated at an accelerating voltage of 120 kV. Images were digitally captured on a Gatan US1000 CCD camera (Gatan, Pleasanton, CA, USA).

Aliquots of bacterial cells were flash frozen onto glow-discharged Quantifoil carbon grids in liquid ethane with either a ThermoFisher (FEI) Mark III Vitrobot or a Leica EM-GP. Cryo-EM and cryo-ET data collection was performed with a JEOL JEM-2200FS 200 kV FEG-TEM equipped with an in-column energy filter (slit width 20 eV), a cryo-transfer specimen holder (Model 914, Gatan), a 4k x 4k Gatan Ultrascan CCD camera, and a Direct Electron DE-20 direct electron detector. Images were acquired with a pixel size ranging from 0.614 to 0.294 nm on the specimen. For tilt series, a total electron dose of ~ 120 e⁻/Å² was fractionated over tilt series ranging from -62° to +62°. Tilt series images were taken automatically with 2° tilt increments by using Serial EM [2].

In our study, we have been able to generate a series of mutant *Caulobacter* strains, each able to synthesize and assemble straight flagella. We used a combination of imaging methods to validate the

presence of straight flagella extending from the cell body and upon isolation (Figure 1).

References:

- [1] A Faulds-Pain, et al. *Journal of Bacteriology* **193(11)** (2011), p. 2695.
[2] JR Kremer, DN Mastrorarde, and JR McIntosh, *Journal of Structural Biology* **116(1)** (1996), p. 71.
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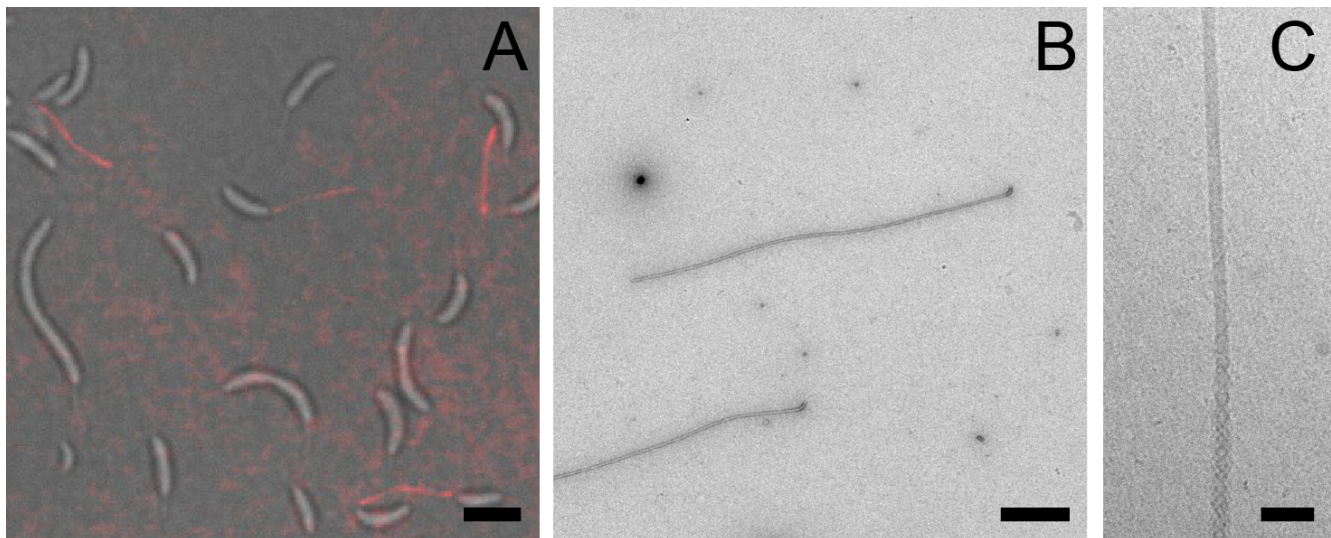


Figure 1. Imaging studies of the *Caulobacter crescentus* flagellum. (A) Fluorescence microscopy of Alexa Fluor 495 C₅ Maleimide-labeled flagella, cell bodies were imaged via phase contrast. Note that the flagellar filaments are straight. (B) Negative stain TEM imaging of isolated flagella from the *Caulobacter* strain identified in (A). (C) Cryo-electron microscopy (cryo-EM) 2D image of an isolated *Caulobacter* flagellum. Scale bars are 2 μm , 500 nm, and 50 nm.