Structural analysis of the hair-like projections in *Clostridioides difficile* spores by employing high resolution Cryo Electron Microscopy

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Project Abstract

C. difficile spores are essential for the transmission and recurrence of *C. difficile* infections, and their outermost layer, called exosporium, possesses surface-proteins resembling **hair-like projections** that are likely the first pathogen molecules to interact with its host making them crucial for virulence. The Hair-like projections formed by three collagen-like proteins of the BclA-family of proteins (BclA1, BclA2 and BclA3). Preliminary investigations from our lab showed that exosporium projections are made of at least BclA2 & BclA3 proteins, and we know that their N-terminal domain is oriented towards the exosporium and their C-terminal domain in an outside orientation. However, the ultrastructural organization, molecular structure and spatial distribution remains poorly understood. This application is associated to the NIH grant under review **1R01AI192920-01.**

Aim and Impact:

To elucidate the above, we will pursue the following aims:

Aim 1. Determine the atomic structure of recombinant BclA proteins.

Aim 2. Define the ultrastructure of the hair-like projections in *C. difficile* spores.

These aims address a major gap in structural spore biology: the organization and attachment of filamentous surface structures in *C. difficile*.

Feasibility & Data

Initial results to conduct the proposed project highlight the feasibility of this proposal:

1. Initially, we obtained stable and purified recombinant BclA2 and BclA3 proteins at a concentration of 1 mg/ml (**Figure 1**)

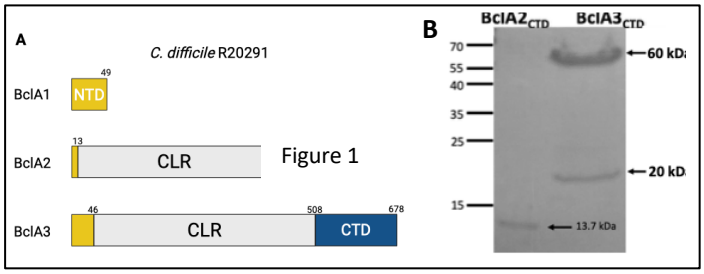

Figure 1

Figure 1. A) Schematic representation of the collagen-like proteins in the epidemically virulent strain R20291. B) Coomassie stained SDS-Page gel showing 5 µg of purified C-terminal domains of the exosporium proteins BclA2 and BclA3. BclA2-CTD migrates as a 13.7 kDa band while BclA3-CTD migrates as a monomeric ~20 kDa and a trimeric of ~60 kDa.

2. Negative staining of recombinant BclA3 reveals a trimeric form.

3. Samples of fixed wild-type spores contain equal propositions of thick and thin exosporium spores, making visualization of both exosporium types feasible in the same grid

4. Our TEM micrographs of ultra-thin sections of *C. difficile* spores confirms the detection of hair-like projections (**Figure 2**).

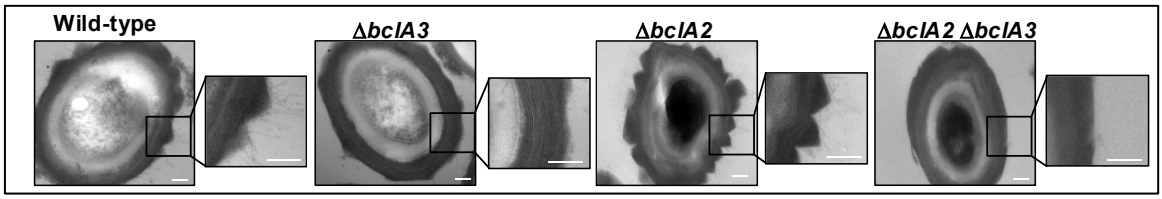

Figure 2. The collagen-like exosporium protein BclA3 is required for the formation of the hair-like projections and electron-dense bumps. TEM of wild-type (Δ*pyrE*/*pyrE*$^{+}$), Δ*bclA3,* Δ*bclA2* and Δ*bclA2* Δ*bclA3 C. difficile* R20291 spores. Scale bar, 100 nm. Data adapted from our published work Castro-Córdova et al. (2021) Nat. Comm. 12, 1140, and from unpublished work (wild-type and *ΔbclA3* mutant strains) and unpublished results (*ΔbclA2* and *ΔbclA2 ΔbclA3* mutant strains).

5. Negatively stained electron micrograph of the particles formed by the C-terminal domain of BclA3 (**Figure 3**). We propose to determine the atomic structure of these particles from the cryo-EM data sets requested with this project. Our initial single particle image analysis of negatively stained dataset of these particles suggest cryo data sets of 400,000 particles would potentially provide us electron density maps of resolution better of 3.5Å, sufficient resolution enough to determine the atomic structure of the C-terminal domain of BclA3.

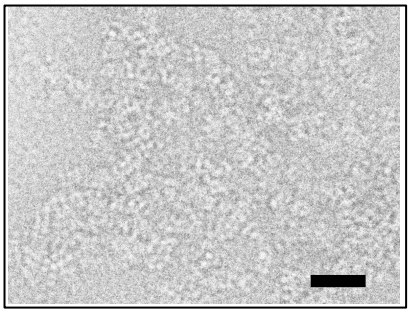

Figure 3. Particles depicting f recombinant C-terminal domain of BclA3. Recombinant CTD-BclA3 was purified to a concentration of 1 mg/ml, diluted to 10^{12} molecules per mL, loaded into carbon coated grid and negatively stained prior to imaging in a FEI TECNAI G2 F20 Cryo-FE-TEM. Bar indicates scale 100 nm.

Proposed experiments.

For Aim 1: Purified recombinant BclA will be incubated under assembly-permissive conditions, and Negative-stain EM will be used to rapidly assess filament formation, morphology, and sample homogeneity. Auspicious samples will proceed to cryo-EM. Helical reconstruction will be used to determine the filament structure at sub-nanometer resolution, which will provide crucial molecular structural information on how these projections are assembled.

Expected Outcomes: Visualization of BclA filament architecture, including diameter, periodicity, and presence of lumen. Results will define the molecular building blocks of spore surface filaments.

For **Aim 2**: purified *C. difficile* spores of wild-type and *bclA* mutant strains will be vitrified on EM grids using plunge-freezing. As a complementary approach, spores may also be lightly fixed and stained for negative-stain EM tomography to increase contrast, if necessary. Tilt series will be acquired on a 300 kV Cryo-TEM with a direct electron detector. Tomograms will be reconstructed to visualize spore surface layers in 3D. Hair-like projections will be identified, segmented, and aligned for subtomogram averaging. Anchoring points at the exosporium will be analyzed to determine attachment sites and structural continuity.

SPA analysis of the C-terminal domain of BclA3 will yield its atomic structure. The analysis of the atomic structure of the whole filament and that of the C-terminal domain would provide us insights about the protein folding during the filament formation.

Expected Outcomes include 3D reconstructions of native spore surfaces, with clear visualization of filamentous projections, projection density, orientation, and length variability across the spore surface. Subtomogram-averaged models of individual projections and anchoring structures at the exosporium.

Goals and Expectations.

The goal of this project is to define the structure and assembly of hair-like projections on *C. difficile* spores, focusing on the collagen-like protein BclA. We aim to (1) determine the molecular architecture of recombinant BclA filaments using negative-stain EM and Cryo-EM, and (2) visualize the native ultrastructure of spore surface projections using Cryo-electron tomography (Cryo-ET). These studies will clarify how BclA contributes to filament formation and anchoring to the exosporium.

We request access to a 300 kV Cryo-TEM with direct electron detection and tomography capabilities, essential for high-resolution reconstruction of native spore surfaces and subtomogram averaging. Negative-stain EM will be used for initial screening of recombinant samples. An estimated 40% to Cryo-EM/negative-stain imaging of recombinant BclA filaments and 60% of instrument time will be allocated to Cryo-ET of spores.

Computational resources are also required for tomogram reconstruction and 3D classification. The combined imaging and structural analysis will provide critical insight into spore surface biology and inform future mutational and functional studies.
Importantly, these results are expected to provide preliminary data for the current **R01 application that is under review (1R01AI192920-01)**.

Expertise and Resources.

Dr. Anindito (Andy) Sen, a Co-PI on the RO1 grant under review (1R01AI192920-01) is structural biologist and electron microscopist with more than 25 years of experience. He will contribute to resolve by Cryo-EM, Cryo-FIB and Cryo-ET a high-resolution molecular landscape of the exosporium layer as well as when assembly of this layer is initiated and differentiates into both types, the structure of the hair-like projections.

Dr. Stanislav Vitha a senior scientist at MIC has expertise in cryo-microtome sectioning of samples and will contribute to this project with the preparation of cryo-sectioning of fixed-inactivated *C. difficile* spores and inclusion bodies of CdeC.

Dr. Daniel Paredes-Sabja is an expert in the spore surface layers of *Clostridioides difficile* spores, he has expertise in ultrastructural analysis of the spore surface.

Francisca Cid is a PhD student, and an expert in overexpression and purification of recombinant BclA proteins and will prepare biological samples. She is acquiring expertise in Cryo-EM sample preparation, analysis, and interpretation.

The Microscope and Imaging Center is a core use facility and is equipped with a LEICA EM GP2 CRYO PLUNGER for freeze frozen samples, a Leica UC7 Ultramicrotome with FC7 Cryo Chamber, and cryo-transmission electron microscope. Computational resources include: a PC set-up with Gatan Latitude S & T for single particle counting and tomography.