

Ultrastructure of Clostridial endospores

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Submitted for the degree of Doctor of Philosophy

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The University of Sheffield, United Kingdom

February 2021



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University
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Sheffield.

Abstract

The overall theme of this thesis is on the application of electron microscopy techniques to interrogate bacterial cell ultrastructure and assemblies. The main focus is on Clostridial spore ultrastructure but work on proteorhodopsin assemblies and micro compartments is also briefly covered in the appendix.

Clostridia include many significant human pathogens such as *Clostridioides difficile* and *Clostridium botulinum*. *C. difficile* is a major nosocomial pathogen that affects patients with compromised intestinal microbiota, typically caused by antibiotic treatment. *C. difficile* infections cause a range of symptoms from diarrhoea to potentially fatal pseudomembranous colitis, toxic megacolon and sepsis. *C. botulinum* is the causative agent of botulism, a severe and life-threatening disease, classified as a bioterrorism threat based on the toxicity of the neurotoxin released. In this work, the widely accepted surrogate *Clostridium sporogenes* was used to gain a greater understanding of group I *C. botulinum*.

These pathogens utilise the highly resilient and metabolically dormant spore state for the transmission and persistence of infection. The outermost layers of the spore including the exosporium, a paracrystalline layer composed of cysteine-rich protein in *Bacillus*, dictates the spore's interaction with its environment. As the outermost layers are the first point of contact between the pathogen and its host, they are pivotal for colonisation and establishing infection - providing attractive targets for therapeutics and epitopes for vaccine development. However, our understanding of Clostridial spores, particularly compared to Bacilli, is limited. The work described in this thesis attempts to address this by studying the structure and composition of the *C. sporogenes* and *C. difficile* outermost layers.

- Chapter 3 – The involvement of cysteine-rich proteins in the outermost layers of *C. sporogenes* was investigated by screening mutants and their crystal assemblies using transmission electron microscopy and electron crystallography.
- Chapter 4 – CsxA, a cysteine-rich protein from *C. sporogenes*, was heterologously expressed in *E. coli* and found to self-assemble into crystal sheets with the same structure as native exosporium. Structural studies of CsxA crystals were conducted by electron crystallography and

atomic force microscopy. Cryo-electron crystallography was used to generate the first cryo 3D reconstruction of an exosporium basal layer at 9Å lateral resolution and capped to 20Å axially.

- Chapter 5 – Advanced sample preparation techniques, including high pressure freezing and freeze-substitution, were combined with electron tomography to gain novel insights of *C. sporogenes* spore ultrastructure. This permitted the identification of new assemblies such as the ‘dense body’ and characterisation of parasporal layers.
- Chapter 6 – Characterisation of outermost layers in *C. difficile* spores, including the involvement of cysteine-rich proteins in their assembly, by screening WT and mutant spores using transmission electron microscopy of negatively stained whole spores, chemically fixed thin sections and electron crystallography.

Publications

Janganan, T.K., Mullin, N., Dafis-Sagarmendi, A., Brunt, J., Tzokov, S.B., Stringer, S., Moir, A., Chaudhuri, R.R., Fagan, R.P., Hobbs, J.K., et al. (2020). Architecture and Self-Assembly of *Clostridium sporogenes* and *Clostridium botulinum* Spore Surfaces Illustrate a General Protective Strategy across Spore Formers. *mSphere* 5, 1297–17.

Acknowledgements

The work in this thesis was far from a lone individual effort, it is the result of an agglomeration of collaborations, group meetings and many fruitful discussions over coffee. First of all, I would like to thank Prof. Per Bullough for giving me the opportunity to explore the field of electron microscopy and its many branches and techniques. His guidance and expertise were invaluable for this research. I would also like to thank Prof. Neil Hunter for all the valuable discussions and suggestions and the BBSRC for funding this project.

This research would not have been possible without the continuous support from the EM facility at Sheffield; Chris Hill for all his help with chemical fixation samples and thin sections, and Dr Moumita Dutta for all the tomography suggestions early in my PhD journey. A special thanks to Dr Svetomir Tzokov for the training with all thing's microscopy related, the late evenings on the microscope and advice throughout.

I am incredibly lucky to have been a part of many collaborative efforts throughout my PhD studies and had the opportunity to work with researchers from all scientific backgrounds. For the *C. sporogenes* research I would like to thank Dr Thamarai Janganan (University of Bedfordshire, UK) for laying the groundwork for the CsxA project, Dr Nic Mullin (University of Sheffield, UK) for all the AFM research and Dr Jason Brunt (University of Cambridge, UK) for providing the Csx mutant spores. I would also like to thank Dr Adriano Henriques and Dr Monica Serrano (ITQB NOVA, PT) for their collaboration on the *C. difficile* project and many useful discussions. I also consider myself very fortunate to have worked so closely with Dr Eleonora Marini (ITQB NOVA, PT) on the *C. difficile* project; she provided the spores and mutants, a wealth of information about the world of spores, advice and suggestions. With regards to the microcompartments and proteorhodopsin projects, I would like to thank Dr Craig MacGregor-Chatwin and Dr Paul Davidson (University of Sheffield, UK) for all their training, help and support throughout the projects and for providing the bacteria for both projects.

I would also like to acknowledge Prof. Emer. Anne Moir, Dr Robert Fagan, my advisor Dr John Rafferty (University of Sheffield, UK) and Dr Julien Bergeron (King's College London, UK) for their help and recommendations.

The EM lab has been very dynamic over the last few years, I am very grateful for all my colleagues that have come through during my time: Caitlin Brumby, Dr Oishik Banerji, Alex Taylor, Natalie Al-Otaibi, Dr Dan Mann, Dr Raquel Faba-Rodriguez, Sarah Gratton, Sasha Evans, Becky Farthing, Shamy Lale, and Dr Giusy Mariano for their support, encouragement and all the lively coffee discussions. A special thanks to Dr Jason Wilson for his continuous support especially when it came to image processing and anything computer related, and Hannah Fisher for all her help and enthusiasm. I am very grateful to Lucia Lafage for all her help with the freeze substitution work and sectioning, and Alex Parker for keeping me sane when it came to Chimera and to both for many reenergizing coffee breaks.

On a personal note, I would also like to thank the many friends and family that have been there throughout the journey. To my parents, Arthur and Carolina, for all their encouragement and support. To Megan, Leire, Deio and Cai for providing the best distraction from work and always knowing how to make me laugh. To Da circle: Katerina, Beth and Eli for all our adventures over the years! Diolch o galon i Mamgu a Dadcu am eich cefnogaeth, am ddarllen trwy fy ngwaith a gwirio fy grammadeg.

Last but definitely not least to my husband Aleksa, you have ridden this journey of highs and many lows with me. Words cannot describe how grateful I am for your continuous support, for picking me up when I am down, keeping me focussed on what is important and providing a continuous supply of cake and coffee while writing this up. Ha llovido mucho desde que nos conocimos (sobretudo en Yorkshire), todas las risas, las tristezas, viajes por el mundo, nuestra boda...y por muchos mas que vendrán, se que siempre estarás a mi lado. Ништа од овога не би било могуће без тебе.

Abbreviations

2D	Two-dimensions
3D	Three-dimensions
xg	Relative centrifugal force
Å	Angstrom
AFM	Atomic force microscopy
ATCC	American type culture collection
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
CCP4	Collaborative computational programming 4
CDI	<i>Clostridioides difficile</i> infection
CEMOVIS	Cryo-electron microscopy of vitreous sections
CLEM	Correlative light and electron microscopy
CTF	Contrast transfer function
Cryo-EM	Cryogenic electron microscopy
Cryo-ET	Cryogenic electron tomography
DED	Direct electron detector
DPA	Dipicolinic acid
DQE	Detective quantum efficiency
DTT	Dithiothreitol
EC	Electron crystallography
ECT	Electron cryotomography
EDB	Electron dense body
EM	Electron microscopy
ET	Electron tomography
LB	Luria-Bertani broth
MC	Microcompartments
FEG	Field emission gun
FIB-milling	Focused ion beam milling
FT	Fourier transform
FFT	Fast Fourier transform

GCW	Germ cell wall
GFP	Green fluorescence protein
GO	Graphene oxide
GUI	Graphical user interface
HPF	High pressure freezing
IPGT	Isopropyl-beta-D-thiogalactoside
IQ	Signal to noise ratio of reflection amplitude given by $7/\text{grade}$ (Henderson et al., 1986)
kDa	Kilodalton
keV	Kiloelectron volts
min	Minute(s)
MRC	Medical Research Council
NAM	N-acetyl muramic acid
NAG	N-acetyl glucosamine
NCIMB	National Collection of Industrial, Food and Marine Bacteria
NDM	Negative difference map
NMR	Nuclear magnetic resonance
OD	Optical density
PMSF	Phenylmethylsulfonyl fluoride
PG	Peptidoglycan
PDM	Positive difference map
PM	Plasma membrane
pR	Proteorhodopsin
RMS	Root means square
rpm	Revolutions per minute
s	Second(s)
SASP	Small acid-soluble proteins
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SIM	Structured Illumination microscope
SIRT	Simultaneous iterative reconstruction technique

SNR	Signal to noise ratio
SOD	Super oxide dismutase
SPA	Single particle analysis
TBS	Tris buffer saline
TEM	Transmission electron microscopy
TGY	Tryptose Glucose Yeast extract
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet
UA, UF	Uranyl Acetate/Formate
w/v, v/v	weight/volume, volume/volume
WBP	Weighted back projection
WT	Wild type

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Chapter 1 – Introduction

1.1 Ultrastructure of the bacterial cell

Bacterial species are found in all environments on our planet and play an essential role in ecosystems from the soil to our gut microbiota. Some bacteria are also pathological agents that can cause life-threatening disease. Bacterial cells were previously considered to be “amorphous reaction vessels with chromosomes that wandered freely and randomly throughout the cell”(Shapiro *et al.*, 2009). However, research on prokaryotic cells has demonstrated that intricate cellular organisations are present within bacterial cells. The subsystems and structures are important for the localisation of cellular components which mediate fundamental processes such as chromosome organisation and cell division (Shapiro *et al.*, 2009). Bacterial cells also possess dynamic 3D organisations to enable rapid responses to changes in environments (Shapiro *et al.*, 2009). By using a range of different technology, we are increasing our understanding of bacterial ultrastructure and how they function. Consequently, these insights have provided diagnostic tools and therapeutics; in addition to forming the basis of the biotechnology industry.

A range of different techniques can be used to study bacterial ultrastructure. Light microscopy has been used to study populations of bacteria to visualise their morphology and phenotypes. The localisation of particular proteins can be elucidated using fluorescence microscopy, which gives insight into their function and involvement in cellular processes. Structural biology techniques, such as X-ray crystallography and NMR have been widely used to generate high resolution structural information of proteins and macromolecular machines, providing a detailed understanding of structure-function relationship. These techniques have been pivotal for our current understanding yet are limited in different respects. Light and fluorescence microscopy are capped by their attainable resolution. Whereas, X-ray crystallography and NMR depend on the isolation of proteins or complexes, a non-native state that does not enable the study of interactions that occur in the cells.

Electron microscopy (EM) is unique in that it allows both the study of individual protein complexes and whole cells using different techniques, as a result EM has been instrumental in understanding

bacterial assemblies. Single particle analysis (SPA) can be employed to attain detailed reconstructions of macromolecular complexes. Electron tomography (ET) allows 3D understanding of whole cells and assemblies, bridging the gap between high resolution structures and their native assembly within the cells. This can be used to gain insight into the organisation and architecture of bacterial ultrastructure, providing remarkable understanding.

1.1.1 Development of Electron Microscopy for Ultrastructure Analysis

In the 1950s, techniques including negative staining and chemical fixation were being used to study biological samples (Brenner and Horne, 1959). These approaches provided significant information of bacterial cells, their layers, cytoskeletons and large cytoplasmic particles including ribosomes. To this day, most of our cellular understanding of bacterial ultrastructure is based on the knowledge gathered using these techniques.

These methods evolved in 1968 as De Rosier and Klug published their ground-breaking paper that described how 3D molecular reconstructions could be generated from EM projections (De Rosier and Klug, 1968). This was presented with the first 3D reconstruction of a bacteriophage T4 tail and provided the fundamental principles for 3D reconstruction across electron microscopy techniques (De Rosier and Klug, 1968).

Yet the methodology was restricted to low resolutions due to the use of heavy stains in sample preparation. These harsh chemicals were also believed to affect structural integrity and cause artefacts. The introduction of sample vitrification in 1988, a novel sample preparation technique, was game changing (Dubochet *et al.*, 1988). Plunge freezing samples into liquid ethane at -195°C , allowed the preservation of biological samples to near native state (Dubochet *et al.*, 1988). This provided access to higher resolution structural information including the first structure of a membrane protein to $2.7\text{\AA}$ resolution by cryo-electron crystallography (cryo-EC) (Ceska and Henderson, 1990). Sample vitrification also revolutionised the obtainable information of bacterial ultrastructure from whole cells by ET. Various studies of whole *Caulobacter crescentus* cells over the years have demonstrated the potential of cryo-ET. It has provided insight into all the layers that compose the cell, mapping of

particles including ribosomes (Briegel *et al.*, 2006) and enabled the localisation and structural elucidation of chemoreceptors (Briegel *et al.*, 2008). More recently it has also been used to resolve the structure of the outermost layer, the S-layer, to 7.4Å resolution (Bharat *et al.*, 2017). Additionally, cryo-ET also revealed that bacterial cells possess cytoskeletal structures, that run the whole length of the cells, and play an important role in cellular processes and motility (Kurner, 2005).

Due to sample thickness, not all samples are amenable for whole cell tomography. Thus, techniques have been developed for sectioning and retaining the frozen hydrated state including: cryo-electron microscopy of vitreous sections (CEMOVIS) and focused ion beam (FIB) milling. CEMOVIS involves the sectioning of frozen samples at -195°C. It has provided completely novel insights into assemblies, including the bacterial cell layers in *Staphylococcus aureus* (Matias and Beveridge, 2006) and *Mycobacteria* (Hoffmann *et al.*, 2008). The near native-state has also revealed never before visualised features including the periplasmic space in *S. aureus* (Matias and Beveridge, 2006). In 2007 the FIB-milling approach was developed, which produces thin lamella of frozen hydrated sample by gallium ion beams (Marko *et al.*, 2007). This was used to visualise *Escherichia coli* cells with remarkable detail and revealed never before identified vesicles near the cytoplasmic membrane (Marko *et al.*, 2007). Since the development of FIB-milling, it has been used to yield ground-breaking detail of both prokaryotic and eukaryotic systems such as cellular assemblies in the algal *Chlamydomonas* cells (Engel *et al.*, 2015). More recently, this has included a complete characterisation of the thylakoid membrane network in *Chlamydomonas* and the organisation of key protein complexes on the membranes (Wietrzynski *et al.*, 2020).

The lack of EM protein tags has meant that localisation or identifying proteins in ET can be challenging. To overcome this, efforts have been placed to develop correlative light and electron microscopy (CLEM) technology. This allows the visualisation of fluorescent tags coupled with the detail obtained from EM, which can be used to study cellular processes such as cell division in *Streptomyces* (Koning *et al.*, 2014).

All of these findings demonstrate the intricate cellular organisations present within bacterial cells and how powerful EM technology can be to elucidate them. Technological and software developments within the cryo-EM field, makes it an exciting time to study bacterial ultrastructure. This research aims to use a range of different electron microscopy techniques to understand bacterial endospore ultrastructure for clinically relevant *Clostridium sporogenes/botulinum*, and *Clostridioides difficile*. We will particularly focus of the outermost layers, including the exosporium, which dictates the spores' interaction with its environment and the host.

1.2 Electron Microscopy

EM can be used to study a wide variety of biological samples including individual proteins and whole cells. A range of EM techniques can provide substantial information that can be used to understand the structure of biological material, their properties and function. One of the first developed sample preparation approaches was the negative stain technique, which involved coating samples in heavy atom compounds, such as uranyl acetate/formate, to provide high levels of contrast (Amos, 1982). This approach required sample dehydration, which could cause artifacts, and the stain capped the attainable resolution to a maximum of $\sim 20\text{\AA}$ (Amos, 1982). An alternative approach is cryo-EM, where samples are fixed in vitreous ice, retaining the hydrated state of the sample. The lack of stain yields low sample contrast levels but enables access to high resolution information (Dubochet *et al.*, 1988).

In the past eight years, the EM field has experienced an upsurge in users due to the cryo-EM 'resolution revolution' (Callaway, 2015; Kühlbrandt, 2014; Thompson *et al.*, 2016). This is the result of a wave of technological advancements in both hardware and software. Developments in optics and column stability has provided a new generation of electron microscopes being installed across the globe. The biggest leap was provided by the development of the direct electron detector (DED) in 2012, which provided ground-breaking signal detection compared to prior technology (Kuijper *et al.*, 2015; Schröder, 2015). More recently, it has also demonstrated a potential of surpassing X-ray crystallography in terms of resolution, with near atomic resolution structures of apoferritin and the

GABA receptor revealing solvent molecules and hydrogen bonding networks (Nakane *et al.*, 2020; Yip *et al.*, 2020).

EM does not come without its own set of limitations. Obtaining high resolution structural information is still challenging and not achievable for some EM techniques. Sample preservation is one of the biggest problems as both the vacuum conditions in the microscope and exposure to electron beam damages biological material. Additionally, sample preparation for cryo-EM requires extensive optimisation to provide grids with good sample and vitreous ice distribution (Dubochet *et al.*, 1988; Thompson *et al.*, 2016).

EM techniques used in structural biology include SPA, ET, electron crystallography (EC) and micro-electron diffraction. As this thesis focusses on ET and EC, the following sections will provide a brief description of these techniques. More in-depth accounts can be found as follows: for ET (Frank, 2008; Harapin *et al.*, 2013; Jensen and Briegel, 2007; Koning *et al.*, 2018; Lučić and Baumeister, 2013; McIntosh *et al.*, 2005) and EC (Amos *et al.*, 1982; Glaeser, 2016; Glaeser and Downing, 1993; Henderson *et al.*, 1990; Renault *et al.*, 2006).

1.2.1 Principles of electron microscopy

EM utilises a beam of electrons that travels in a vacuum down a column which is controlled by electromagnetic lenses. The electron discharged possess ‘wave particle duality’. The wavelength of the electrons (λ) is determined by the acceleration voltage (V). The higher the voltage, the smaller the wavelength and thus the greater the theoretical attainable resolution. Depending on the microscope, the electron beam will be accelerated to voltages of 100-300keV, thus wavelengths range from 3.70pm-1.96pm.

1.2.1.1 Image formation

At the sample plane, the beam of electrons can either interact with the sample and scatter or remain unscattered and pass straight through (Figure 1.1 and Figure 1.2). Scattered electrons interact with the

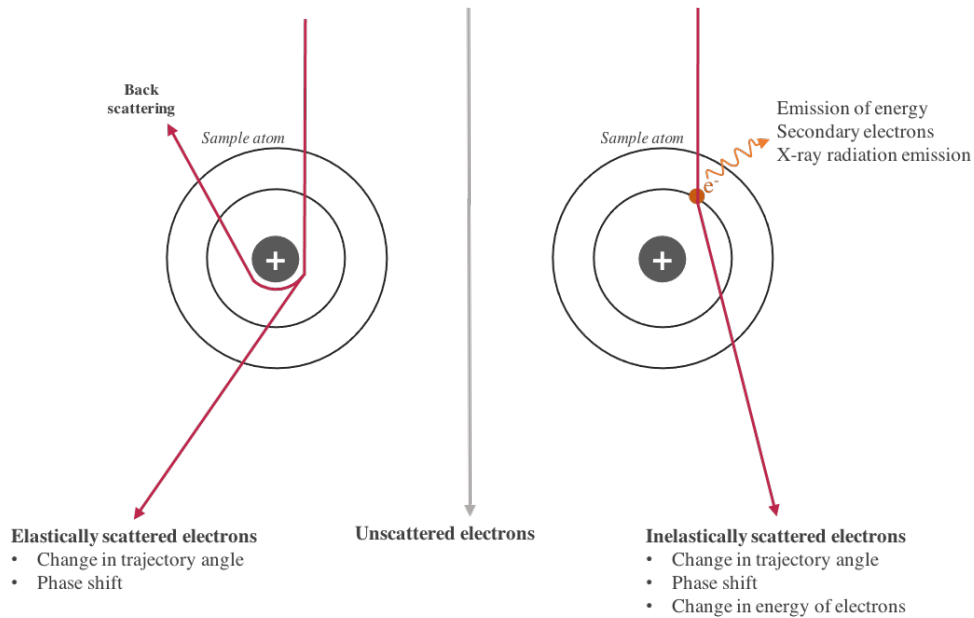


Figure 1.1 Interaction of electron beam with sample

At the sample plane the electron beam is either scattered or unscattered (grey arrow). Elastically scattered electrons (LHS) undergo a change in trajectory angle and phase shift from interacting with the atom's nuclei, some electrons may also be back scattered. Inelastically scattered electrons (RHS) interact with the electron cloud causing radiation damage. This also induces a change in trajectory and a phase shift, in addition to a change in wavelength due to the loss of energy.

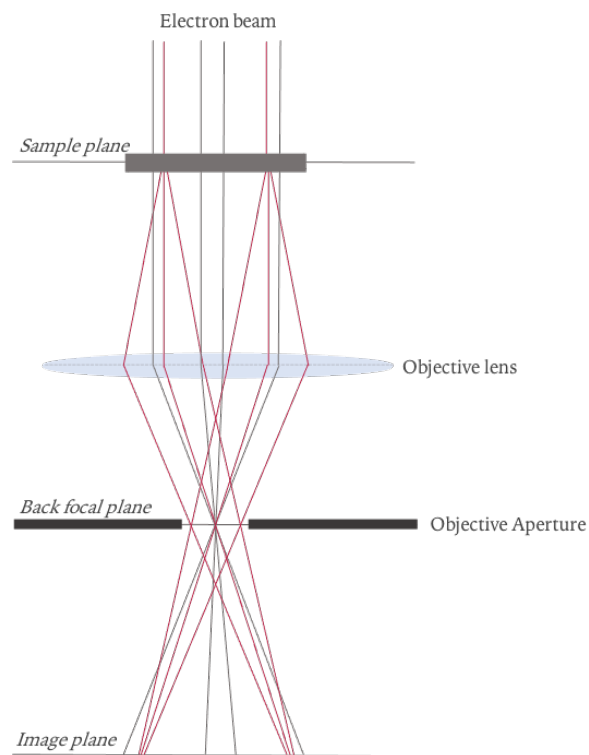


Figure 1.2 Schematic diagram of optics involved in image formation

The electron beam is scattered (red) or remains unscattered (grey) at the sample plane. The electrons are redirected by the objective lens for image formation. At the back focal plane, also known as the diffraction plane, the scattered electrons interfere to generate a diffraction pattern. The objective aperture is inserted at the back focal plane to prevent high angle scattering events from contributing to the final image, thus providing greater amplitude contrast. The electrons recombine at the image plane where an image is recorded.

sample either elastically or inelastically (Figure 1.1), described in the following sections. Figure 1.2 displays a schematic diagram of image formation in the microscope. After scattering events at the sample plane, the beam is redirected by the objective lens for image formation. At the image plane, diffracted beams interfere with unscattered electrons and recombine to produce an image with amplitude and phase contrast, which allows features to be distinguished.

1.2.1.1.1 Elastic scattering

Elastic scattering events occur when the electrons interact with the atoms' nuclei (Figure 1.1). This induces a change in beam trajectory and a $\sim 90^\circ$ phase shift without a change in energy (Amos, 1982). Electrons are scattered at a range of different angles (θ). High angle scattering events may hit the internal wall of the column or the objective aperture thus do not contribute to image formation, providing amplitude contrast - contrast at low or medium resolutions. The objective aperture is used to block high angle scattering events from reaching the detector and this enhances amplitude contrast in the image, but also reduces the attainable resolution.

Phase contrast is the primary form of contrast at high resolution. The change in wave fronts, phase shift, due to electron scattering causes phase contrast. At the image plane, the scattered electrons will interact to generate an image. This includes constructive and destructive interference of both elastic and inelastic scattered waves to generate contrast. The microscope's transfer function is formed by spherical aberrations of objective lenses (C_s) and defocus (Δf), which induces an additional phase shift that affects phase contrast – this is further described in section 1.2.1.2.

1.2.1.1.2 Inelastic scattering

Inelastic scattering arises when electrons interact with the atoms' electron cloud, causing a transfer of around 25 eV of energy to the sample (Amos, 1982). These events cause a change in trajectory, phase shift and changes to the wavelength. As a result, the biological material becomes damaged due to radiation causing changes in temperature, the loss of mass and alterations of chemical composition. Reactive ions and free radicals are also released causing secondary damage. Exposure to 100 electrons/nm² causes damage to protein secondary structure which effects tertiary structure (Amos,

1982). Inelastically scattered beams contribute to background noise as they are not focused in the same way as zero loss electrons (unscattered and elastically scattered electrons), which reduces the signal-to-noise ratio (SNR) in the image. Equation 1 describes the ratio of elastic (σ_e) to inelastic (σ_i) scattering cross-sections, which is dependent on the atomic number of the element (Z).

Equation 1

$$\frac{\sigma_e}{\sigma_i} = \frac{Z}{19}$$

Data collection at cryogenic temperatures with a low electron dose can reduce the beam induced damage (Dubochet *et al.*, 1988). Energy filters can also be used to remove inelastically scattered electrons from contributing to the final micrograph (Verbeeck *et al.*, 2004). The SNR of data can be improved by averaging multiple low dose images of identical molecules or complexes (Amos *et al.*, 1982; De Rosier and Klug, 1968; Unwin and Henderson, 1975).

1.2.1.2 CTF

The phase contrast transfer function (CTF) $\sin\gamma(s)$, is a function that describes the contrast observed in the image based on spatial frequency (equation 2). At different spatial frequencies (ν) the contrast may contribute positively, negatively or not at all (Figure 1.3). The wavelength of electrons (λ) is also considered in this function, although the axial asymmetry terms have been ignored for simplicity. The effect of CTF is observed in the FFT (Figure 1.4) as Thon rings. The amplitude information is zero at the coordinates where the wave intercepts the x axis, thus information at these coordinates are lost and can only be obtained by redundancy – merging images with varying defoci. As demonstrated by the graph in Figure 1.3 and 1.4, the CTF oscillates between a positive and negative sign, which is dependent on defocus (Δf) and spherical aberration (C_s) (equation 2). Any astigmatism will affect the CTF also distorting the Thon ring shape.

Equation 2

$$\sin\gamma(s) = 2\pi \left\{ \frac{C_s}{4} \lambda^3 \nu^4 - \frac{\Delta f}{2} \lambda \nu^2 \right\}$$

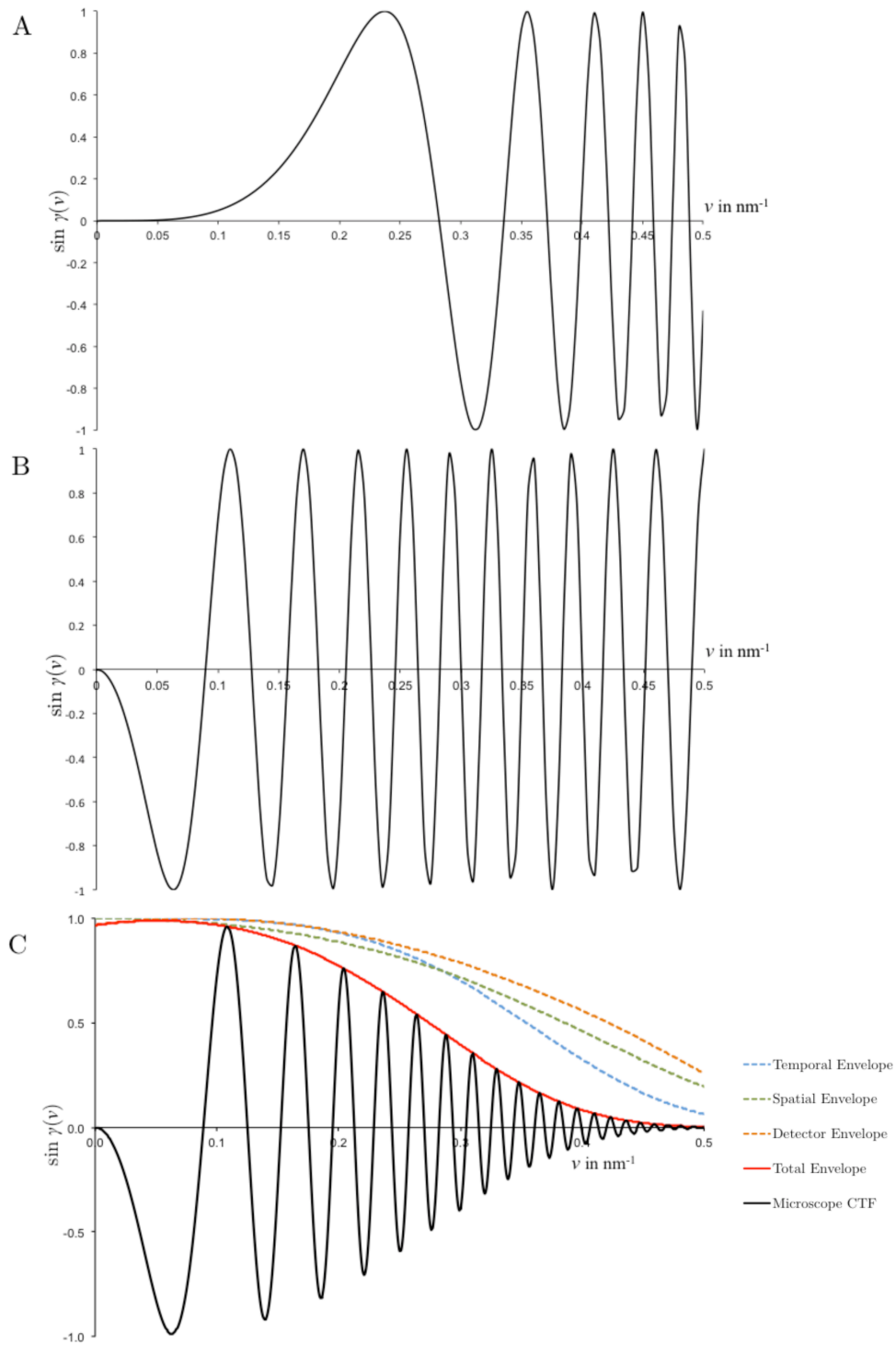


Figure 1.3 Contrast transfer function over spatial frequency

A, B display CTF against spatial frequency in a microscope operating at 200keV and spherical aberration constant of 2mm. The effects of the envelope function are not displayed in A,B. A) Data collected at a defocus of 0nm (in focus) B) Data collected at defocus 500nm (underfocus) C) Displays graph B with the effect of envelope function that dampens contrast at higher resolutions. Spatial envelope includes incoherent beam and instabilities in the lens. Temporal envelope includes energy spread, focal spread, chromatic aberrations.

Figure with permission from Oishik Banerji Thesis (2018)

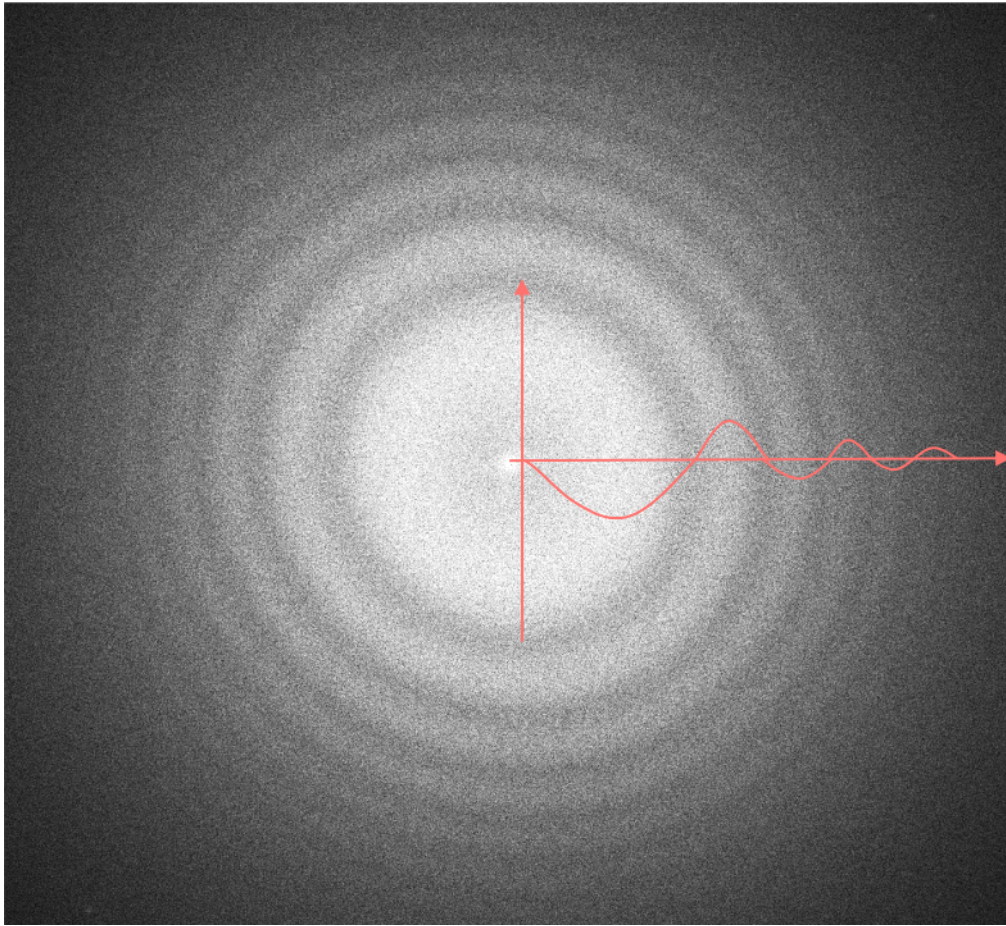


Figure 1.4 The effect of CTF on FFT

The FFT displays CTF oscillations as Thon rings, the point at which the CTF crosses zero and information is absent. The graph superimposed on the FFT display the CTF fluctuations across different spatial frequencies. After crossing each minima, the sign of the maxima alternates from positive to negative. The CTF decreases in wavelength and increases in frequency at high spatial frequencies, limiting the attainable information.

EM micrographs are recorded in underfocus which increases the low resolution contrast in the image.

Figure 1.3A demonstrates the effect of recording images in focus, $\sin\gamma(s)$ values are near zero thus provide low contrast at low spatial frequencies. By recording images with defocus, the $\sin\gamma(s)$ values are greater at low resolution and shifts the position at which the first minimum is encountered, thus more information is attained of low spatial frequency features (Figure 1.3B). Defocusing images also increases the frequency of the CTF oscillations, thus increasing the number of times the wave crosses zero, and so resulting in missing information in the Fourier components at those spatial frequencies. High frequency of the CTF oscillations can also affect the results of image processing. The optimum defocus needs to be found to attain maximum information with minimum data loss.

At high spatial frequency the envelope function dampens the contrast, limiting the attainable information at high resolution levels (Figure 1.3C). The envelope function arises from temporal and spatial incoherence such as incoherent beam and instabilities in the lens, energy spread, focal spread and chromatic aberrations.

CTF oscillations means that the information changes from a positive sign, through zero, to a negative sign. CTF can also vary within an image, especially when collecting tilted data as areas in the image are at different heights relative to the focal plane of the microscope. The CTF of each image needs to be determined and corrected for.

1.2.1.3 Image recording

Image recording devices include film, CCD cameras or DED. The performance of a recording device is measured by its detective quantum efficiency (DQE), described in equation 3 as the ratio between the SNR input and output. Film was the original method used for recording images. Even though it involved long delays between exposure and image analysis, it was the superior data collection option compared to its more convenient counterpart, the CCD camera, based on DQE. This was until the development of the DED which was a ground-breaking technological advancement for the EM field. DED technology possessed improved DQE, which enhanced the SNR of recorded images (McMullan *et al.*, 2009). It also provided the ability to collect data in ‘movie-mode’ which allowed for the correction of beam induced movement, primarily observed in the first frames upon exposure of the sample. The last frames of exposure are where most of the sample damage is recorded and limits the attainable resolution. Movie-mode enabled the removal of these frames and the correction of positional changes of molecules from frame to frame, which provide data that can access high resolution structural information. Since their development, DED have become an essential component for high quality data collection.

Equation 3

$$DQE = \frac{(S/N)_{out}^2}{(S/N)_{in}^2}$$

1.2.1.4 3D Reconstruction

Images collected by EM can be used to generate 3D structural reconstructions. For techniques such as EC and ET, information of different planes in reciprocal space is obtained by tilting the sample in the microscope (Figure 1.5). Each micrograph will provide different 2D projection information in real space, which is perpendicular to the direction of incident electron beam. The central section theorem or projection theorem describes the relationship between a 2D projection and its FT (Figure 1.6). A 3D object possesses a 3D FT. The FT of 2D projections correspond to central sections in the 3D FT and can be reconstructed by sampling different views in 3D space. The data collected at different tilt angles are then merged in 3D space. By taking the inverse FT of the merged 3D FT a 3D density map can be generated.

However, there are mechanical and optical constraints of the tilt angles that can be collected. The grid holder is limited to a maximum tilt angle of $\pm 60^\circ$ or 70° , depending on the microscope. From a single axis tilt series, the resulting effect is referred to as the missing wedge of information (Figure 1.6–pink). The lack of information in the z axis causes structural distortions and results in anisotropic resolution. The effective sample thickness also increases at higher tilts which causes multiple scattering events, and so the number of electrons that get through is much lower making the relationship between the image and electron density non-linear. Additionally, a thicker sample causes more inelastic scattering events that further damage biological sample and reduce attainable information.

EC and ET techniques differ in their data collection and processing workflows, all of which will be discussed in the following sections. However, the principles of generating 3D reconstructions are the same.

1.2.2 Electron Crystallography

EC involves the use of EM to study 2D crystals. Historically, this involved studying membrane proteins synthetically packed into 2D crystals within lipid bilayers (Glaeser *et al.*, 1986; Henderson

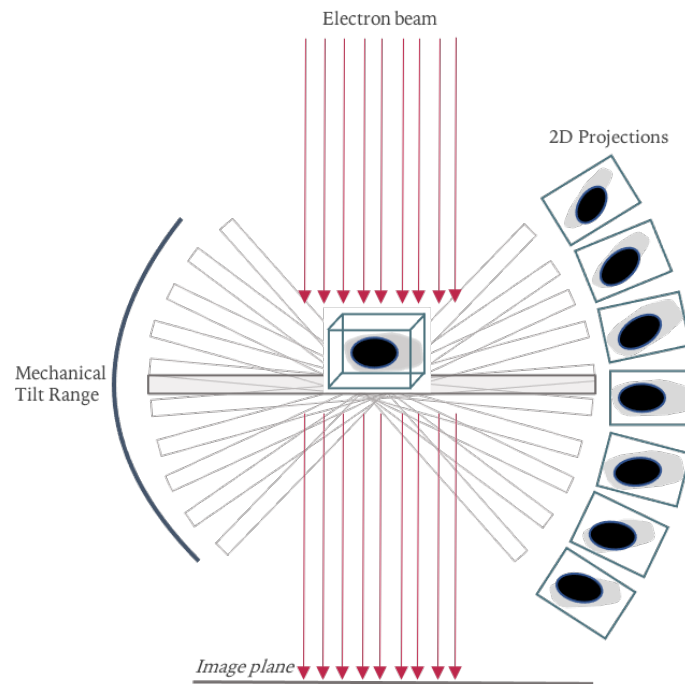


Figure 1.5 Collecting 3D data in the microscope

The grid holder is tilted to collect 2D projections at different angles. The mechanical tilt range is typically limited to $\pm 60^\circ$, depending on the microscope.

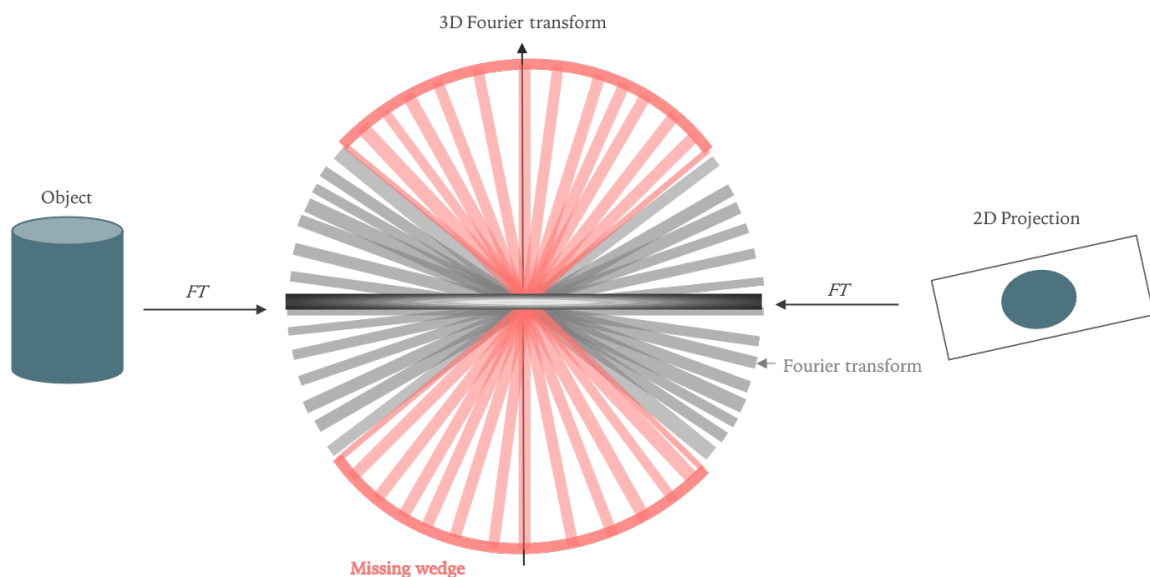


Figure 1.6 Projection theorem

A 3D object possesses a 3D Fourier transform. 2D projection of the 3D object provides a 2D FT, corresponding to a central section through the 3D FT. By tilting the object in the microscope, we can sample different angles in the 3D FT. Due to mechanical tilting limitations, angles greater than 60 or 70° are not obtained. This results in a missing wedge of information (pink) in the 3D FT.

and Unwin, 1975; Henderson *et al.*, 1990; Kühlbrandt, 1992; Stahlberg *et al.*, 2015; Unwin and Henderson, 1975). There are also many examples of proteins that natively pack into 2D sheets including: purple membrane of *Halobacterium halobium* (Henderson and Unwin, 1975; Unwin and Henderson, 1975) bacterial S-layers (Fagan and Fairweather, 2014) and exosporium (Janganan *et al.*, 2020; Kailas *et al.*, 2011; Terry *et al.*, 2017). 2D crystals do not readily pack into 3D crystals thus are not suitable for X-ray crystallographic analysis. Prior to the ‘resolution revolution’ discussed in section 1.2, the highest achievable resolutions of biological samples by EM were by EC, including 1.9Å structure of aquaglyceroporins (Gonen *et al.*, 2005). Although the attainable resolution of EC is limited by crystal quality, biological crystals can be disordered, and the missing cone of information causes anisotropic resolution of 3D reconstructions in the z axis.

1.2.2.1 2D crystals

2D crystals are layers composed of repeating unit cells (Figure 1.7). A unit cell corresponds to the smallest volume that repeats within a crystal. The dimensions of a unit cell are described in terms of lengths a and b , and angle denoted γ , the included angle between a and b . 2D crystals can be classified into plane groups based on the symmetry elements within the unit cell (Amos, 1982). 17 plane groups are possible for biological crystals (Amos, 1982).

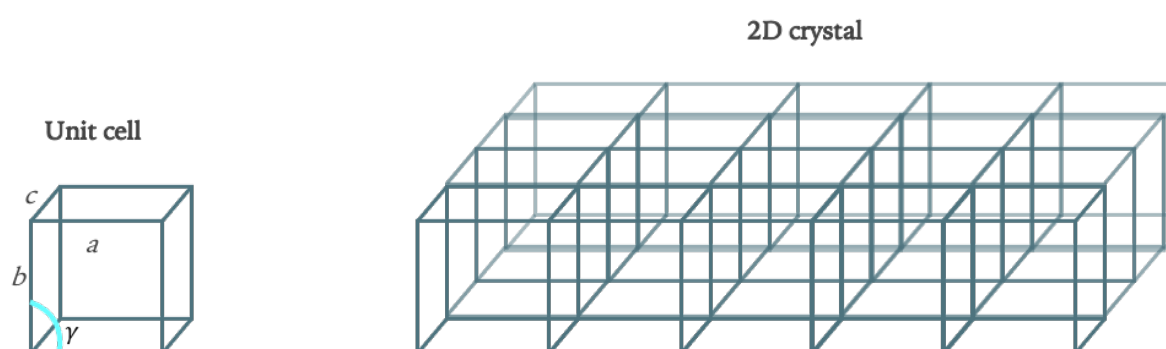


Figure 1.7 2D crystals are layers formed of repeating unit cells

The parameters of each unit cell are described in terms of length $=a, b$ and angle $=\gamma$ corresponding to the angle between a and b . In a 2D crystal these unit cells are repeated to form a single molecule thick crystal.

An electron beam that passes through a 2D crystal obeys Braggs law and generates a diffraction pattern at the back focal plane (Figure 1.2) of the microscope. The diffraction recombines at the imaging plane to generate an image. The diffraction pattern can be visualized in Fourier space by calculating a Fourier transform (FT), although this is not a direct representation of the diffraction pattern at the back focal plane as it includes the effects of the CTF. The diffraction pattern reveals a series of spots that correspond to the reciprocal lattice; throughout this thesis the peaks in the FT will be referred to as diffraction spots. Thus, the FT diffraction spots can be used to calculate the unit cell dimensions of the crystal by assigning miller indexes h and k , and also extract the amplitude and phase information (Amos, 1982).

2D crystals can have disordered properties, which are very common in biological crystals, as crystals tend to assemble in patches with varying sizes and quality. Further disorder is provided by the lack of flatness of crystal sheets on EM grids, as most will bear folds or wrinkles. The disorder of crystals can be visually observed in the micrographs, as patches in different orientations, they also affect the diffraction pattern by reducing the SNR, blurring the diffraction spots, or displaying multiple lattices. Image processing is employed to identify the crystal lattice and account for crystal distortions, a detailed account of these processes is described in chapter 2.6.1. SNR of the crystal lattice is improved by using ‘crystal unbending’ which aims to flatten and track crystal distortions. However, the unbending algorithms are limited and cannot account for large crystal distortions, in these cases the images are not suitable for further processing.

1.2.2.2 3D electron crystallography

As discussed in 1.2.1.4, 3D data are collected by tilting crystals in the microscope. The FT from a 2D crystal includes periodicity in the x, y plane as the crystals are single molecules thick. By tilting the sample, we still attain x, y periodicity but with a z^* variable. Thus the FT of tilted data includes amplitudes and phases (h, k, z^*) , given by a third index (z^*) - corresponding to the difference in height between tilted and untilted reciprocal space data.

As stated by the projection theorem, the 2D FT correspond to a section through the 3D FT (Figure 1.6). The 2D FT will intercept the lattice lines at different points in z^* , which are perpendicular to the plane of the crystal (Figure 1.8). The resulting FT of tilted 2D projections will possess apparent distortions of the projected crystal lattice with respect to the tilt axis (Figure 1.8– blue). These distortions can be used to calculate the tilt angle of the crystal.

The 3D reconstruction is generated by merging amplitude and phase information from different 2D planes, beginning with the lower tilt angles and incrementally adding higher tilts. Symmetry in the sample can aid reconstruction by reducing the volume of the asymmetric units in the transform, thus reducing the number of independent lattice points required. Mechanical limitations of data collection, described in 1.2.1.4, leads to the missing cone of information in 3D space which can distort or stretch information in the z direction.

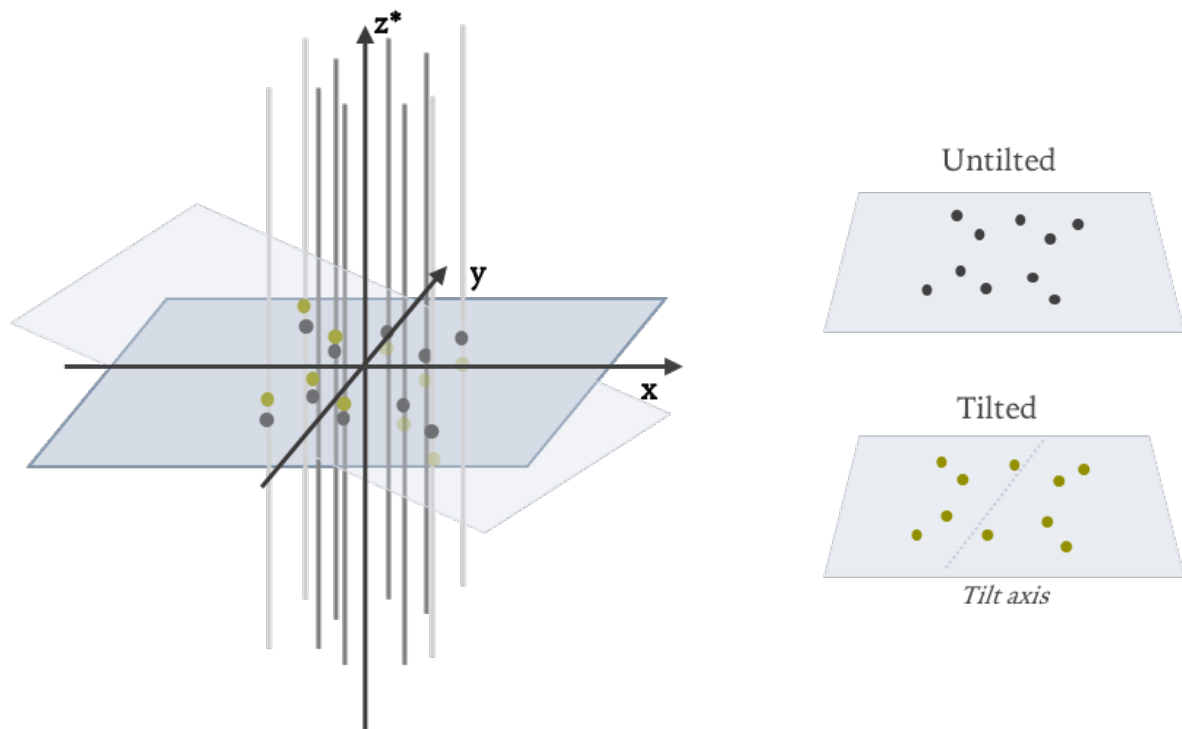


Figure 1.8 Lattice Lines

The 3D FT of a crystal possesses lattice lines in the z^* axis (grey lines). A 2D projection of untilted crystal will possess phase and amplitude information for (h,k) . As the sample is tilted the projections will sample through z^* , thus become complex values for (h,k,z^*) . The FFT of the tilted projection will display a distorted lattice with respect to the tilt axis.

1.2.2.3 High Resolution Electron Crystallography

The roots of high resolution EC came from the bacteriorhodopsin paper that resolved its structure to 7-9Å resolution (Henderson and Unwin, 1975; Unwin and Henderson, 1975). Since then, records of 1.9Å resolution for aquaglyceroporins have been reported (Gonen *et al.*, 2005), although the high disorder of biological crystals makes these resolutions unattainable for most samples. Due to technological advances, more recent EC studies of disordered crystals are reaching resolutions in the range of ~4.5Å, as was demonstrated for relatively disordered 2D crystals of the potassium channel Mlok1 (Kowal *et al.*, 2018). This is the result of advances at various fronts of the EC workflow.

Cryo sample preparation is essential for obtaining high resolution structural information. There are many commercially available instruments that facilitate this by blotting grids at specified humidity and temperature, followed by plunge freezing grids into liquid ethane at liquid nitrogen temperatures (-196°C). The process of rapidly freezing samples ensures vitreous ice and prevents the formation of cubic and hexagonal ice that damages biological samples (Dubochet *et al.*, 1988). Vitrification retains the sample hydrated state, thus preserving biological structure (Dubochet *et al.*, 1988). Vitrified samples lack stain thus resolutions of much greater than 20Å are attainable, although reduced contrast levels can pose a challenge for identifying samples at low magnifications. However, the cold temperatures also reduce the effect of beam induced radiation damage; thus, samples can be exposed to a greater electron dose (Dubochet *et al.*, 1988). The increased dose allows increased signal-to-noise for visibility of features.

The new generation of EM instruments provides access to higher resolution structural information. These microscopes are operated at 200-300keV and equipped with DED. As already discussed, DED allow data collection in 'movie-mode' which enables tracking of beam induced movement. As a result, increased levels of exposure can be used and the frames displaying damage removed from the dataset. Additionally, phase plate technology improves the contrast of low spatial frequency features at defocus levels closer to focus (Danev *et al.*, 2014). Phase plates have provided a low molecular

weight breakthrough for the cryo-EM field as structures including haemoglobin (64kDa) were resolved to 3.2Å resolution (Khoshouei *et al.*, 2016).

New methods of data collection have demanded further software developments. This has included algorithms that account for sample movement in a movie such as: MotionCorr, movie mode unbending and new CTF correction scripts (Biyani *et al.*, 2018; Zheng *et al.*, 2017). Novel image processing software has had considerable effect on structure reconstruction. EC data previously capped to ~10Å have now been demonstrated to reach ~5Å (Biyani *et al.*, 2018). However, software developments for EC are lagging behind other EM techniques such as SPA and ET. Researchers have resorted to using alternative techniques to study 2D crystals. The potassium channel Mlok1 was reported at 4Å resolution using SPA, where more advanced software such as RELION was used to pick individual crystal subunits and generate a 3D reconstruction (Righetto *et al.*, 2019). This study has demonstrated the potential of SPA, especially for studying disordered crystals that can be problematic by EC. ET has been employed to study native 2D crystals such as the S-layer from *Caulobacter crescentus* (Bharat *et al.*, 2017; Kugelgen *et al.*, 2020). ET allowed data collection of S-layers on the vegetative cell by collecting data at the stalk, a more electron transparent part of the cell (Bharat *et al.*, 2017; Kugelgen *et al.*, 2020). By sub-tomogram averaging a reconstruction to 4.8Å resolution was obtained (Kugelgen *et al.*, 2020). Although unlike SPA, the attainable resolution of ET is intrinsically lower.

EC has benefitted from all the advances within the EM field. However, many aspects of the EC workflow are falling behind its counterparts. This is particularly apparent in data collection as no software is easily accessible for automated EC data collection, providing a major bottleneck for EC studies. EC image processing software is also limited, further reducing the accessibility of these techniques without prior knowledge and experience. As efforts are focused on techniques such as SPA and ET, it is unlikely that major EC specific advances will be developed in the years to come. Thus, more researchers are likely to turn to alternative techniques for structure determination of 2D crystals.

1.2.3 Tomography

ET allows the visualization of cellular complexes in 3D. Its potential for studying bacterial architecture has already been discussed in section 1.1. It is a powerful technique that bridges the gap between studying individual proteins or complexes to understanding their role and assembly *in vivo*. In combination with other techniques such as X-ray crystallography or SPA, it can provide novel insights into complex structure and function.

ET involves collecting multiple 2D projections of a unique object at different angles. This is achieved by tilting the sample in the microscope (Figure 1.5) and collecting different views. This approach can be used to study a range of different samples such as purified protein, viruses or bacterial cells. However, attaining data from samples greater than 500nm in thickness is challenging due to their electron dense nature (discussed in 1.2.1.4). This is particularly problematic in ET as effective sample thickness increases at higher tilt angles. Consequently, a range of sectioning approaches have been developed to study thicker samples. Energy filters are frequently employed for data collection to filter out inelastic scattering events and improve visibility of features (Verbeeck *et al.*, 2004).

Beam induced sample damage is a particular challenge for ET as multiple exposures are required to collect 3D information. To keep sample damage at an acceptable range, where no visible alterations are recorded, the electron dose for each image is low. As a result the images possess low SNR, which is particularly problematic in the absence of stain as features become very difficult to identify.

ET technology is often limited to low resolutions. This is due to the low SNR already described. A trade-off between magnification and resolution is also frequently observed. Data collected at low magnification can provide information of global cellular ultrastructure but is capped at low resolution. The missing wedge of information (Figure 1.6) also results in anisotropic resolution in the z axis. The development of grid holders that allow dual axis tomography has reduced the missing wedge to a missing pyramid of information (Mastronarde, 1997). However, data collection in dual axis means that electron dose exposure of each image is further reduced. Sub tomogram averaging techniques are employed to improve the SNR and resolution of the object of interest (Mastronarde, 1997). This relies

on the presence of repeating structures that can be averaged, which is not always the case (Koster and Barcena, 2008).

1.2.3.1 Sample preparation

Conventional sample preparation EM techniques such as negative stain or cryo-EM can be used with samples up to ~500nm thick. As established, samples greater than ~500nm thick typically need to be sectioned which can be conducted using a range of different approaches. Historically, thin sections were prepared by chemical fixation which involved treating samples with chemical fixatives such as osmium tetroxide or glutaraldehyde (Bozzola, 2007). Samples are then dehydrated in ethanol and embedded into plastic resin blocks (Bozzola, 2007). However, the slow process of chemical fixation and dehydration can damage the biological sample, leading to the loss of structural integrity and misleading artefacts, limiting the attainable results and analysis (Luther, 2008).

Sample freezing has already been established as a method of preserving biological material (Dubochet *et al.*, 1988). Unfortunately, due to the thickness of these samples plunge freezing methods are not suitable, and so high pressure freezing (HPF) was developed as an alternative sample preparation approach. HPF involves the vitrification of sample in liquid nitrogen at high pressure (>2000 bar) (reviewed in Thompson *et al.*, 2016). These samples can be sectioned by CEMOVIS and FIB milling, discussed in 1.1.1. These techniques have proven to generate detailed insights of bacterial cells (section 1.1.1) but require high skill level for sample preparation.

Alternatively, samples can be freeze-substituted where solvents slowly substitute the frozen water content followed by chemical fixation and plastic embedding (McIntosh *et al.*, 2005). Freeze-substitution yields samples with better structural preservation due to the initial high pressure freezing. Additionally, samples possess improved contrast levels due to the stain, which also limits resolution. However, the limited resolution is not a problem when studying assemblies on a cellular level.

1.2.3.2 Alignment of tilt series

A tilt series corresponds to the stack of images collected at different angles. Tomography processing is required to take the raw projections and construct a 3D reconstruction of the object, referred to as a tomogram (Frank, 2008). The alignment of the tilt series is one of the most critical parts of tomography processing. It allows for the correction of image shift, rotation, magnification changes and shrinkage of sections (Frank, 2008).

Gold fiducial markers are frequently used for tomography alignment. These markers, around 10nm in diameter, are added to the sample and due to their electron dense properties they can readily be identified and tracked through the tilt series (Mastronarde, 2008). For optimal use they need to be well distributed across the sample, especially the object of interest, and trackable throughout the tilt series. This is particularly challenging for plastic sections; thus fiducials are normally loaded above and below the section and not in the sample plane which limits the attainable alignment (Mastronarde, 2008). The gold fiducial markers produce artifacts in the reconstructions thus need to be erased after processing, this can be done computationally although can pose many difficulties (Mastronarde, 2008).

An alternative approach is to use patch tracking, which is employed when no fiducials are present or an insufficient number of fiducials (Brandt, 2008; Sorzano *et al.*, 2009). This involves generating a model of patches across the tilt series (Sorzano *et al.*, 2009). Patch tracking requires a lot of parameter refinement including patch sizes and using boundary models. The approach can be used to correct for sample shrinkage or magnification changes but is not as robust as the use of gold fiducial markers (Brandt, 2008).

1.2.3.3 Tomogram reconstruction

Once the tilt series have been aligned the data are prepared for the next part of image processing, the tomogram reconstruction. Here the information from the 2D projections will be merged to generate a 3D volume of the object. Tomogram reconstruction can be done using two approaches: by weighted back projection (WBP) or by the simultaneous iterative reconstruction technique (SIRT).

WBP involves taking the 2D projections and calculating the FT of each image which corresponds to a central section in the object's 3D FT (Figure 1.9) (Radermacher, 2008). To avoid over sampling of low spatial frequency information, the Fourier components of each section are weighted depending on spatial frequency (Mastronarde, 2017; Radermacher, 2008). The FT from 2D projections is then merged to form a 3D FT; the inverse FT is then back projected to generate the 3D reconstruction in real space (Figure 1.9) (Radermacher, 2008). WBP is the least computationally intensive approach, however can yield tomograms with high noise levels (Koning *et al.*, 2018; Radermacher, 2008).

SIRT is an alternative approach which involves real space reconstruction (Gilbert, 1972). The tomograms generated by SIRT are less noisy compared to WBP, however it is a computationally very demanding process (Koning *et al.*, 2018). It starts by generating a 'trial tomogram' that is reprojected at the original tilt angles (Figure 1.10). In an iterative process, the densities are modified using data from all projections simultaneously, this improves the SNR of the reprojection until close resemblance is reached with the raw projections (Gilbert, 1972; Mastronarde, 2017). Processing starts with low spatial frequency information and with more iterations moves to higher spatial frequency where the noise level is much higher (Mastronarde, 2017). The greater the number of iterations applied to SIRT processing, the higher spatial frequency it will cover (Mastronarde, 2017). However, too many iterations will lead to oversampling of high spatial frequency features including noise, which will start dominating the tomogram and worsen the reconstruction (Gilbert, 1972; Mastronarde, 2017). Thus, a known number of iterations is required prior to processing to generate an optimal tomogram (Mastronarde, 2017). This value varies across samples, thus requires trial and error to find the right margin (Mastronarde, 2017).

An alternative approach is to use WBP with SIRT-like filters. This has been demonstrated to improve the SNR of features, equivalent to SIRT without the intensive computational processing and less low frequency artefacts (Zeng, 2012). However, prior knowledge of the number of iterations is also required for the SIRT-like filter which will take trial and error to find (Mastronarde, 2017).

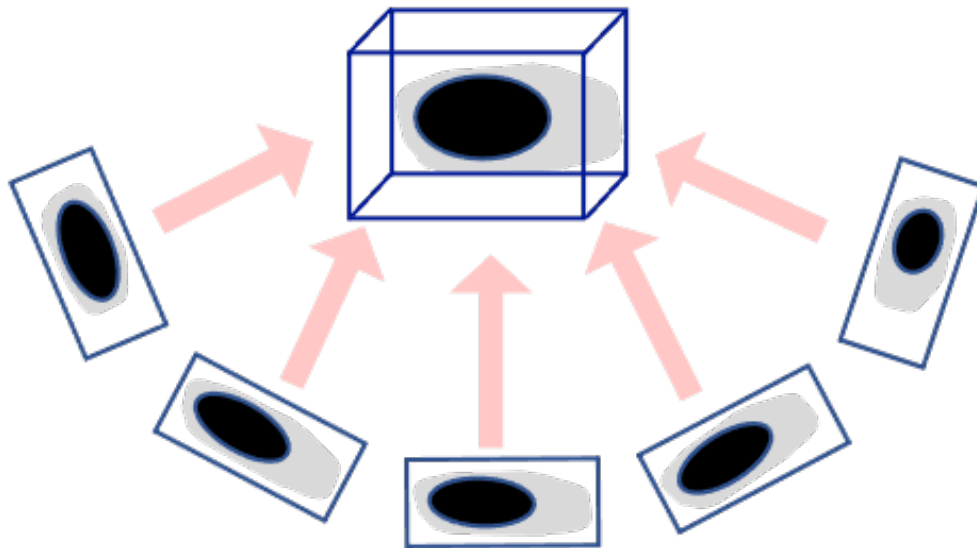


Figure 1.9 Weighted back projection
2D projections are merged to generate 3D reconstruction

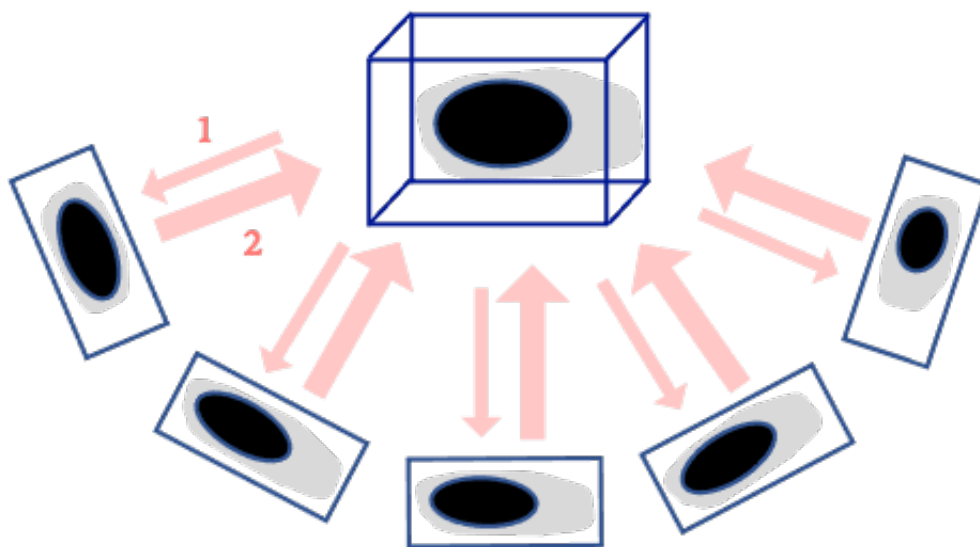


Figure 1.10 SIRT
A trial tomogram is generated and reprojected to the original tilt angles (arrow 1). Reprojection and original projection are adjusted to improve SNR which is then merged to the 3D reconstruction (arrow 2)

1.2.3.4 Advances in tomography

The advances in the EM field have also improved the attainable information from tomography data. As with EC, this has included hardware and software developments that improve all aspects of the ET workflow (reviewed in (Hagen *et al.*, 2017; Koning *et al.*, 2018)). Developments in sample preparation technology, particularly for freezing samples, have given novel insights into cellular complexes and assemblies. The commercialisation of instruments for high pressure freezing and freeze substitution have made these techniques more accessible (Thompson *et al.*, 2016). However, technologies such as FIB-milling are still in their early stages, with expert skill level required for using such instruments (reviewed in Koning *et al.*, 2018). Further developments are required to make these approaches more streamlined.

Improvements in microscopy technology have also advanced ET data collection. The low exposure levels required to record an image, with minimum cumulative damage, means that low SNR is obtained. DED technology, with its increased sensitivity, has resulted in great improvements of SNR (Kuijper *et al.*, 2015; Schröder, 2015). Phase plate technology is also employed to improve the contrast levels of low SNR to make features more visible (Grange *et al.*, 2017; Khoshouei *et al.*, 2016). Other technology including energy filters for improved data collection of thicker samples has already been discussed (reviewed in (Koning *et al.*, 2018)). The automatization of data collection provided by a range of different platforms has been pivotal for improving ET. This allows large volumes of tomography data to be collected with much more controlled parameters of tilting, exposure and focus with ease. Future problems come from storage of these high volumes that readily add up to terabytes of data.

There are many software platforms available for image processing of tomography data. Due to large volumes of data, there has been a push to automate the processing pipeline (reviewed in (Koning *et al.*, 2018)). However, the current automatised tools are far from robust and still require a lot manual input. After generating a tomogram, segmentation software packages are frequently employed to label features (Frangakis and Hegerl, 2008). Tomograms can be very noisy and difficult to interpret without

expert eye, thus segmentation aids visualisation and analysis (Frangakis and Hegerl, 2008). There have also been efforts to automate tomogram segmentation, yet the technology still requires manual input (Frangakis and Hegerl, 2008; Koning *et al.*, 2018).

ET is still in early stages of development, especially compared with SPA. There is a lot of margin for improving all areas of the workflow (reviewed in (Hagen *et al.*, 2017 and Koning *et al.*, 2018)).

Sample preparation needs to become more accessible, especially promising techniques such as FIB-milling (reviewed in by Thompson *et al.*, 2016). Data collection has drastically improved with the new generation of microscopes and detectors however further developments are still required. This includes faster data collection of tilt series, even with a fully automated pipeline the collection of each tilt series takes approximately an hour which limits the amount of data that can be recorded in a session (Hagen *et al.*, 2017). ET would also benefit from integrated automated image processing, which would prevent the need to store terabytes of raw data (Koning *et al.*, 2018). The automatization of tomography segmentation would also aid the visualisation and interpretation of tomography data to get the most out of each tomogram (Frangakis and Hegerl, 2008; Koning *et al.*, 2018). Many of these efforts are currently in development and it will be very exciting to see how they come to play in the years to come.

1.3 Bacterial Endospores

1.3.1 Firmicutes bacteria

The Firmicutes phylum consists of mostly Gram-positive bacteria and includes Bacilli, which are obligate or facultative aerobes, and Clostridia, which are anaerobes. Despite the diversity of the phylum, they all share the ability to form endospores (spores) under unfavourable environmental conditions such as nutrient limitation. The spore state allows survival in a range of conditions including UV, radiation, desiccation, heat and organic solvents (Setlow, 2006), by maintaining a metabolically inactive state for prolonged periods of time. There have been accounts of spores revived from dormancy ranging from tens to thousands of years (Cano and Borucki, 1995; Vreeland *et al.*,

2000) which has led to a 500-year-experiment to test the extent of spore longevity (Ulrich *et al.*, 2018).

Spore formers are primarily soil-dwelling yet have been found in environments all around the world. Many Bacilli and Clostridial species have been identified as playing an important role in the human gut microbiota (Browne *et al.*, 2017; Schroeder and Bäckhed, 2016). Some species are also significant pathogens, these include *Bacillus anthracis* (causative agent of anthrax) and *Bacillus cereus* (cause of food poisoning) (Logan, 2011). Other significant pathogens include *Clostridium botulinum* and *Clostridioides difficile* which are the spores of interest for this research and are further described in 1.3.2. These pathogens utilise the highly resilient spore state for the persistence and transmission of infection.

1.3.1.1 Firmicutes Life cycle

Figure 1.11 displays a schematic of the Firmicutes life cycle. The vegetative cell state is metabolically active, thus undergoing bacterial growth and division by binary fission. Upon encountering harsh growth conditions, Firmicutes undergo a separate cell cycle for spore generation. This involves the process of sporulation which occurs over different stages from 0-VII as illustrated in Figure 1.11 (1.3.1.2). The released spore can remain in a metabolically dormant state for prolonged periods of time, while constantly sensing the environment for signals of suitable cell growth conditions. This triggers germination, where vegetative cells grow out of the spores and the vegetative cell cycle is reinstated.

B. subtilis has been widely used as a model for studying the Firmicutes life cycle (Piggot and Hilbert, 2004). The following section will describe our understanding primarily in *B. subtilis* and provide examples established by research in Clostridia.

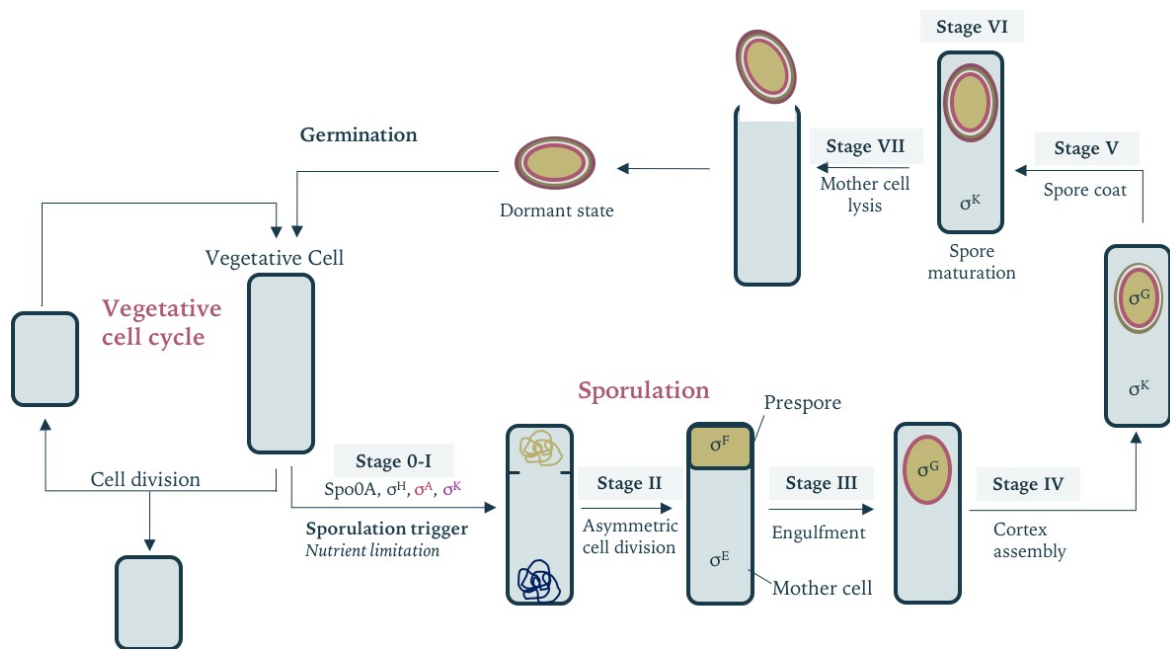


Figure 1.11 Life cycle of *Firmicute* bacteria

Vegetative cells undergo cell growth and division. Sporulation triggers will initiate the sporulation pathway via Spo0A which is regulated by σ^H in Bacilli and Clostridia spore formers. Other transcription factors are also involved in early stages of sporulation including σ^A in *C. difficile* (red) and σ^K in *C. botulinum* (purple). Stage 0-I involved DNA replication and anchoring to cell poles, stage II: asymmetric cell division, stage III: engulfment of forespore to yield a prespore: transcription factors involved include σ^F in prespore and σ^E in the mother cell, stage IV-VI: transcription factors involved include σ^G in forespore and σ^K in the mother cell cortex, coat and exosporium assembly, species dependent, and maturation before spore release by mother cell lysis in stage VII. Spores remain in the metabolically dormant state until they encounter germinants. This triggers a cascade of reactions for vegetative cell outgrowth, by germination, and returns to original vegetative cell cycle.

1.3.1.2 Sporulation

As described, the sporulation pathway involves various stages from 0-VII. The initial stages, 0-I, involve vegetative cell growth and DNA replication. Sporulation triggers asymmetric cell division, stage II, which leads to the formation of the mother cell and prespore. This requires the polar localisation of FtsZ (Levin and Losick, 1996), DNA translocation by SpoIIIE to the forespore and peptidoglycan removal from the septum for division (Piggot and Hilbert, 2004). After asymmetric division, the forespore is engulfed by the mother cell in a process akin to phagocytosis – this is stage III. The following stages (IV-VI) involve the assembly of multiple layers important for maintaining dormancy and resilience. These assemblies include the cortex (a modified peptidoglycan layer) the coat (a proteinaceous layer) and, depending on species, the exosporium. Once a fully mature spore has been formed it is then released to the environment through the lysis of the mother cell in stage VII.

Sporulation is a highly coordinated process involving sigma factors, transcription factors, proteases and phosphatases which ensure a timely schedule of processes. Most of our understanding of

sporulation stems from accounts of *Bacillus subtilis* (Piggot and Hilbert, 2004). While many details of sporulation mechanisms in *Clostridia* remain unclear, well characterised sigma factors from *B. subtilis* have been identified in clostridial species (Al-Hinai *et al.*, 2015). The main players include: Spo0A, σ^H , σ^F , σ^E , σ^G , σ^K . Whilst many of the important sporulation genes and sigma factors appear to be conserved there are many significant differences between Bacilli and Clostridia (Al-Hinai *et al.*, 2015). Some clostridial species even have differences in sporulation mechanisms amongst themselves (Al-Hinai *et al.*, 2015).

The master transcript, Spo0A, initiates sporulation (Figure 1.11 - Stage 0-I). In *B. subtilis* Spo0A is activated by phosphorylation from a phosphorelay system (Piggot and Hilbert, 2004). Spo0A is estimated to regulate around 121 genes, including sporulation specific RNA polymerase sigma factors σ^F (prespore) and σ^E (mother cell) (Figure 1.11) (Piggot and Hilbert, 2004). Spo0A has an analogous role in Clostridia although it appears to be phosphorylated directly by orphan histidine kinases (Al-Hinai *et al.*, 2015). In *B. subtilis*, the alternative sigma factor σ^H is required for transcription of *spo0A*, as the promoter of *spo0A* is σ^H dependent (Figure 1.11– Stage 0-I) (Piggot and Hilbert, 2004). In *C. difficile* σ^H and σ^A (Figure 1.11– Stage 0-I) are required for *spo0A* transcription, its involvement in other Clostridia remains unclear (Saujet *et al.*, 2011).

In both Bacilli and Clostridia classes, σ^F is the first sigma factor expressed in the forespore (Figure 1.11 – Stage II). It becomes active after asymmetric cell division, shortly after which σ^E , the first mother cell specific sigma factor, is expressed (Figure 1.11– Stage II) (Al-Hinai *et al.*, 2015; Pereira *et al.*, 2013; Piggot and Hilbert, 2004). Genes under control of σ^E are involved in engulfment, cortex formation and initiation of coat assembly (Eichenberger *et al.*, 2003; Piggot and Hilbert, 2004). In stage III, σ^G is expressed (Figure 1.11– Stage III) which in *C. difficile* has been identified to regulate more than 50 genes including those involved in dipicolinic acid (DPA), cell wall synthesis and cortex (Saujet *et al.*, 2013).

The later stages of sporulation (stage IV-VI) involve the assembly of outer layers including coat and exosporium. In *Bacillus* these assemblies are under the regulation of σ^K (Figure 1.11– IV) in the

mother cell and the proteins are laid down by the mother cell (Piggot and Hilbert, 2004).

Equivalently, σ^k has also been identified in Clostridia at later stages of sporulation (Al-Hinai *et al.*, 2015). It has also been associated with early sporulation stages (prior to stage II) in *C. botulinum*; here transcription of *sigK* and activation of σ^k is likely to occur upstream of Spo0A production, although the details of this remain unclear (Al-Hinai *et al.*, 2015). For *C. difficile*, this was not the case and σ^k accumulation was only identified in late sporulation stages (Fimlaid *et al.*, 2013; Pereira *et al.*, 2013).

1.3.1.3 Germination

Stimuli in the environment, termed germinants, trigger a sequence of events that lead to the breakdown of the dormant spore state and outgrowth of a vegetative cell. In *Bacillus*, the germinants include L-alanine or inosine (Barlass *et al.*, 2002). For Clostridia including *C. sporogenes* or *C. botulinum*, germinants include L-alanine or L-lactate (Brunt *et al.*, 2014) and bile salts such as taurocholate for *C. difficile* (Sorg and Sonenshein, 2008). In *Bacillus*, the germinants interact with receptors on the inner membrane of the spore (Barlass *et al.*, 2002; Brunt *et al.*, 2014; Moir *et al.*, 2002), similar receptors have also been identified in *C. sporogenes* and *C. botulinum* (Brunt *et al.*, 2014). This triggers a series of reactions beginning with the rapid rehydration of the core followed by the degradation of cortex and some coat layers. The inner membrane expands and becomes more fluid allowing the further hydration of the core and excretion of calcium and DPA. This re-establishes the ion flux and oxidative metabolism resumes. Any DNA damage suffered in the spore state is then repaired and small acid-soluble proteins (SASPs) bound to DNA in the spore state are degraded. The resulting amino acids are then used as a source for outgrowth synthesis of the vegetative cell. A similar mechanism has also been identified in *C. sporogenes*, thin sections reveal a core rehydration followed by a cortex and coat breakdown (Brunt *et al.*, 2015; Hoeniger and Headley, 1969).

In *C. difficile*, germination differs from *Bacillus* with an ‘outside-in’ approach as the cortex is degraded before calcium and DPA release from the core (reviewed in Lawler *et al.*, 2020). *C. difficile* does not encode germination receptors homologous to *Bacillus*, instead they possess bile salt specific

subtilisin-like pseudoprotease germination receptors including Csp proteins CspA, CspB and CspC (reviewed in Lawler *et al.*, 2020). CspB is able to proteolytically cleave SleC which activates its cortex hydrolysis activity. Cortex degradation allows cell wall expansion for rehydration of the core followed by calcium and DPA release (reviewed in Lawler *et al.*, 2020).

Germination is an asynchronous process, thus bacteria within a population are always at different stages of the cell cycle. This may have an importance for pathogens such as *C. difficile* when colonising the host gut (Antunes *et al.*, 2018).

1.3.2 Clostridia

The Clostridia class includes anaerobic bacteria, capable of forming heat resilient spores. Clostridia are found in either a vegetative or spore state across environments such as soil or marine sediments. Clostridia also includes species important to human and animal microbiota and some significant pathogens (Paredes *et al.*, 2005). They are considered the predecessors of aerobic Firmicutes which diverged into the genus *Bacillus* during the great oxidation (Paredes *et al.*, 2005). Research into this class has demonstrated how phylogenetically and morphologically diverse they are (Paredes *et al.*, 2005). Most of the fundamental research, including sporulation, germination and spore structure and composition, have primarily been focussed on *Bacillus*, thus our understanding of the class remains limited.

1.3.2.1 *Clostridium botulinum*

C. botulinum includes many phylogenetically and physiologically distinct species, all capable of producing botulinum neurotoxins (Peck *et al.*, 2011). Based on the type of neurotoxin variant produced, the species have been classified into four *C. botulinum* groups, *Clostridium baritii* and *Clostridium butyricum* (Peck *et al.*, 2011). Groups I and II *C. botulinum* include agents of human botulism, classified according to their proteolytic or non-proteolytic toxins respectively (Carter and Peck, 2015).

The most common form of botulism is foodborne botulism that involves both groups I and II (Peck *et al.*, 2011). This arises from inadequate septic conditions of both home prepared and commercially

prepared foods, such as canned and bottled foods or cured meats and fish. For the highly heat resistant spores of group I, this can arise from insufficient heat treatment to destroy the spores. As a result, there are strict industry regulations and safety measures with which the food industry needs to comply in order to avoid outbreaks. Thus, botulism cases are rare in 2014 123 cases were reported and 91 confirmed in 16 EU/EEA countries (ECDC, 2017). Yet large outbreaks of botulism are still a risk, such as that from home-canned bamboo shoots in Thailand (2006), involving 209 cases, 134 hospitalised and 43 requiring mechanical ventilation (Wongtanate *et al.*, 2007).

The botulism disease is caused by the botulism neurotoxin, released by the vegetative cell, which binds to neuronal membranes and blocks synaptic transmission (Peck *et al.*, 2011). The resulting disease includes nerve paralysis that can escalate to respiratory paralysis and death (WHO, 2002). The current treatment for botulism is the anti-toxin from equine serum (WHO, 2002). The botulism neurotoxin is one of the most potent, as 30-100ng is potentially fatal it is classified as a potential bio-terrorism threat (Peck *et al.*, 2011) and a schedule 5 organism in the UK (Anti-terrorism, crime and security act 2001). However, the neurotoxin also possesses pharmaceutical applications, for instance in the treatment of spastic muscular conditions and hyperhidrosis (WHO, 2002).

1.3.2.2 *Clostridium sporogenes*

C. sporogenes is found in soil, marine and freshwater sediments. Due to its highly resilient spores, it is a concern in food safety and spoilage problems (Bradbury *et al.*, 2012; Warren *et al.*, 1994). *C. sporogenes* is very rarely of pathogenic concern, yet it has been implicated in some clinical cases (Abusnina *et al.*, 2018; Inkster *et al.*, 2011). *C. sporogenes* is also used as a model organism for studying group I *C. botulinum* (Bradbury *et al.*, 2012; Carter *et al.*, 2009; Peck *et al.*, 2011). Their high relatedness provides a non-toxigenic alternative to gain insight into the lethal group I *C. botulinum* strains. The spores are also considered more resilient than *C. botulinum* and are therefore widely used to test the efficacy of sporocides to prevent foodborne botulism (Bradbury *et al.*, 2012; Warren *et al.*, 1994).

1.3.2.3 *Clostridioides difficile*

Originally named *Clostridium difficile*, it was recently reclassified to *Clostridioides difficile* due to the great differences found from other clostridial species (Lawson *et al.*, 2016). *C. difficile* is a global health concern as one of the main nosocomial antibiotic associated diseases (Rupnik *et al.*, 2009), especially since the appearance of more virulent strains which have caused an increase in mortality rates (Rupnik *et al.*, 2009). It is transmitted via the faecal-oral route and easily spread within a healthcare environment. In 2011, almost half a million infections were associated with *C. difficile* in the USA, with approximately 29,000 resulting in death (Lessa *et al.*, 2015). In 2018-2019, 12,275 cases were identified in the UK alone (Public Health England, 2019)

C. difficile infection (CDI) commonly arises after antibiotic treatment, which causes dysbiosis of the native gut microflora. This provides optimal conditions for *C. difficile* to grow and, in its vegetative cell state, release the disease inducing toxins (Rupnik *et al.*, 2009). *C. difficile* toxins include TcdA, TcdB and a binary toxin which cause cell death of the colonocyte, disrupting the epithelial gut barrier, and triggering intestinal inflammation. The resulting disease causes a range of symptoms from mild diarrhoea to more severe pseudomembranous colitis, toxic megacolon, sepsis and even death (Rupnik *et al.*, 2009). CDI treatments include antibiotics such as vancomycin and metronidazole, although increased levels of antibiotic resistance are being recorded (Rupnik *et al.*, 2009). The spore state is a key part of the persistence of CDI and is a major problem for treatment. For complicated CDI, the only effective treatment is to surgically remove infected parts of the colon. Thus the field is seeking alternative therapeutics including faecal microbiota transplants to re-establish the gut microbiota and prevent recurrence (Mullish *et al.*, 2018).

1.3.3 Spore ultrastructure

The general spore ultrastructure is conserved across Bacilli and Clostridia (Figure 1.12). The spore architecture includes membranes, along with peptidoglycan in both germ cell wall (GCW) and cortex

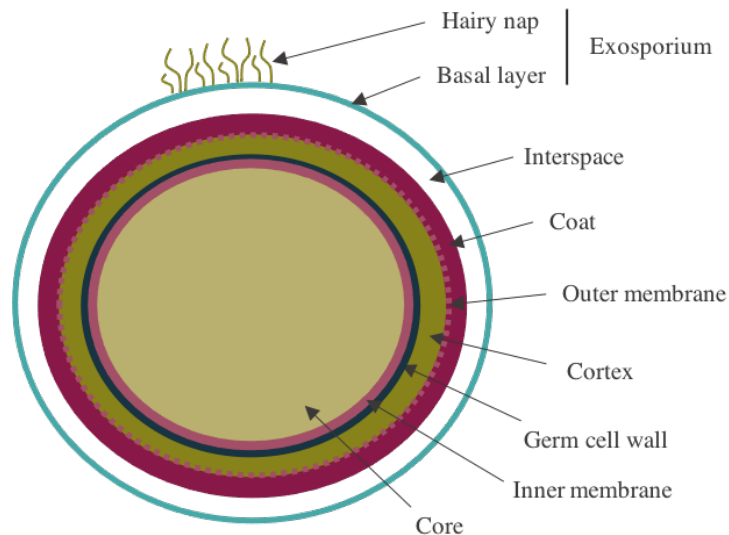


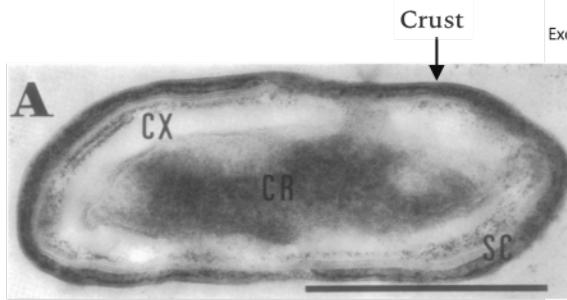
Figure 1.12 Schematic diagram of general spore structure

The metabolically dormant state is composed of multiple layers that surround the dehydrated spore core. This includes membranes, peptidoglycan, germ cell wall and cortex, and proteinaceous layers – coat and exosporium. The exosporium is species dependent and is separated from the coat by an interspace region. The exosporium includes a para-crystalline basal layer and a hairy nap formed of glycosylated collagen-like proteins.

(modified peptidoglycan), and proteinaceous layers consisting of coat and exosporium (species dependent). These layers are important in maintaining the metabolically inactive state for prolonged periods of time and conferring resistance to heat, desiccation, UV and biological or chemical insults (Setlow, 2006; Stewart, 2015). For pathogens the spore state is important for the dissemination and recurrence of infection (reviewed in Stewart, 2015).

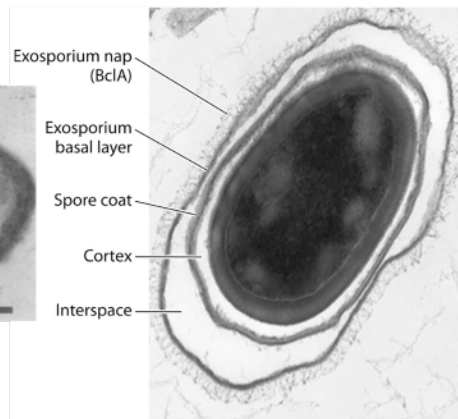
Spore formers display some differences in their architecture, especially in the outermost layers including the coat and exosporium, likely to reflect adaptations to different environments (Figure 1.13). Like many spore formers, *B. anthracis* possesses an exosporium as the outermost layer, a baggy-flexible assembly with an interspace separating it from the coat (Figure 1.13B). A similar assembly is also observed in *C. sporogenes* (Figure 1.13C). However, *C. difficile* does not appear to have an analogous assembly; it is suggested that its ‘exosporium’ is assembled close to the coat (Figure 1.13D) (Permpoonpattana *et al.*, 2011) similar to the crust in *B. subtilis* (Figure 1.13A). A key difference in *C. difficile* from other spores of both Bacilli and Clostridia, including the *B. cereus* group and *C. sporogenes*, is the formation of the ‘appendage’, a proteinaceous assembly elongated at one of the spore poles - believed to play an important role in gut colonisation (Antunes *et al.*, 2018).

Bacillus subtilis

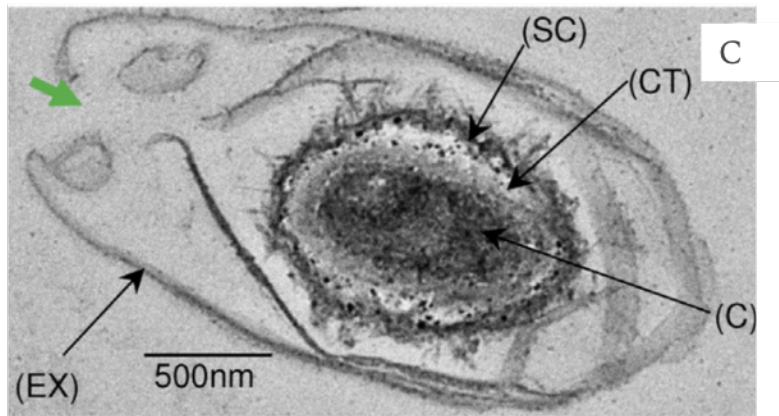


Bacillus anthracis

B



Clostridium sporogenes



Clostridioides difficile

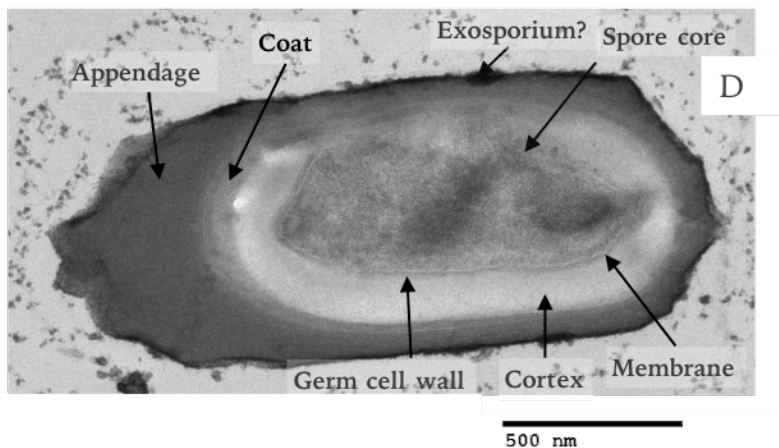


Figure 1.13 Sections of *Bacillus* and *Clostridia* spores

A) *Bacillus subtilis* * B) *Bacillus anthracis* ** C) *Clostridium sporogenes* *** D) *Clostridioides difficile* ****. All the spores possess a core (C), membrane, germ cell wall, cortex (CT), coat (SC) – not all layers are visible in these sections. *B. anthracis* and *C. sporogenes* possess a ‘classical exosporium’ with an interspace from the coat. *C. difficile* has an extension of the coat at one of the spore poles, an appendage. A ‘classical exosporium’ is not observed in *C. difficile*. The exosporium, if present, appears to be assembled close to the coat with no interspace similar to *B. subtilis* crust.

Figures from: *Kozuka *et al.*, (1983). ** Stewart, G.C. (2015). ***Brunt *et al.*, **** provided by Eleonora Marini (ITQB, Lisbon)

In the following sections the different spore layers and their functional importance in maintaining the spore state will be described, including key differences and similarities between relevant species. Most of the fundamental research is based on *Bacillus*, especially *B. subtilis*, thus this introduction will outline the systems in Bacilli and provide examples established by research in Clostridia.

1.3.3.1 Core

The core consists of DNA, enzymes, ribosomes and tRNA. It possesses a low water content, which reduces the movement of macromolecules and maintains metabolic inactivity. The dehydrated state is preserved due to high DPA, or pyridine-2, 6-dicarboxylic acid, and calcium content in the core (Setlow, 2006; 2007). DPA makes up 5-15% dry weight of both Bacilli and Clostridia spores and chelates with divalent cations, including calcium, in a 1:1 ratio (Setlow, 2006; 2007). Spores lacking DPA are much more susceptible to heat, especially wet heat (Setlow, 2006). A correlation between DPA and UV photochemistry protection of spore DNA has also been identified, although the exact mechanism behind this remains unclear (Setlow, 2006). DPA is synthesised in the mother cell compartment under σ^K control (Piggot and Hilbert, 2004) and during germination it is released within minutes to allow the rehydration of the core (Setlow, 2006).

Another property of the core that confers resistance to heat, chemicals and UV radiation is small acid-soluble spore proteins (SASPs) bound to DNA (Setlow, 2006; 2007). Both α and β types form a complex with the DNA that alters its structure and properties. The amino acid sequence of SASPs appear to be conserved across Bacilli and Clostridia (Setlow, 2007). Their involvement in resilience has also been documented for Clostridia including *C. perfringens* (Raju *et al.*, 2006). *ssp* genes encoding the SASPs are expressed under σ^G control in the forespore during late stages of sporulation (Setlow, 2007). The SASPs are also the first to be degraded during germination and used as a source of amino acids for vegetative cell outgrowth (Setlow, 2007).

The implications of a dehydrated state also mean that no DNA damage repair can take place in the dormant state. DNA damage is accumulated and repaired as one of the primary stages of germination. DNA damage levels which are too high lead to the death of the germinating spore (Setlow, 2006).

1.3.3.2 Membrane layers

The dehydrated core is encapsulated by an inner membrane, which possesses the same lipid composition as the mother cell (Cortezzo and Setlow, 2005). The inner membrane is considered one of the main permeability barriers that stops insults from reaching the core and damaging DNA (Cortezzo and Setlow, 2005; Setlow, 2006). The dehydrated spore state results in a membrane with low fluidity levels (Cowan *et al.*, 2004), contributing to the impermeability, an important feature in chemical resistance (Setlow, 2006).

The inner membrane is also the location of germination receptors across Bacilli and Clostridia (Barlass *et al.*, 2002; Brunt *et al.*, 2014; Moir *et al.*, 2002; Setlow, 2014). In response to germination triggers the inner membrane rapidly expands in size, important for accommodating the rehydration of the core. CEMOVIS of *B. subtilis* spores revealed sub core membrane reservoirs that likely allow rapid insertion into the plasma membrane (Laue *et al.*, 2018). These membrane reservoirs have also been identified in *C. difficile* and *C. botulinum* (Laue *et al.*, 2018).

Based on the phagocytic-like approach of spore engulfment in stage III, a second membrane layer is believed to be assembled on the cortex. This layer is the outer membrane, considered to have an important role in sporulation (Setlow, 2006) by dictating the localisation of important proteins involved in the assembly of spore layers (Piggot and Hilbert, 2004). The second membrane is not associated with spore resilience, and it remains unclear as to whether the layer maintains integrity in dormant spores (Nicholson *et al.*, 2000; Setlow, 2006).

1.3.3.3 Peptidoglycan assemblies

In Bacillus vegetative cells possess a peptidoglycan (PG) cell wall composed of alternating N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM). The cell wall is heavily crosslinked between NAM glycan strands and both D-alanine and diaminopimelic acid (Popham, 2002). The spore state possesses two PG layers: the GCW and cortex. The GCW possesses a similar composition to the vegetative cell wall as around 40% of NAM glycan strands are crosslinked with both D-alanine and diaminopimelic acid (Meador-Parton and Popham, 2000; Popham, 2002). This layer has no proposed

role in spore resistance but is an important template for synthesis of the cell wall on outgrowing spores during germination (Popham, 2002; Setlow, 2006).

The cortex is a thicker, modified PG layer assembled between the GCW and the outer membrane. Unlike the GCW, it has an important role in maintaining the dehydrated state by preventing the expansion of the core in response to hydration (Setlow, 2006). Cortex mutants have also revealed increased heat sensitivity (Eichenberger *et al.*, 2003). In the cortex 50% of NAM are converted to muramic- δ -lactam which provides important specificity for lytic enzymes to breakdown the cortex during germination and not the GCW (Meador-Parton and Popham, 2000). The degree of crosslinking in the cortex is much lower than in the GCW with only 4% NAM crosslinked. A crosslinking gradient has also been described in the cortex, with a higher abundance in outermost layers and reduced levels closer to the spore core (Meador-Parton and Popham, 2000). It has been suggested that this confers a degree of flexibility in the cortex to changes in environmental hydration (Meador-Parton and Popham, 2000). In *B. subtilis*, the cortex is assembled after engulfment of the forespore under σ^E regulation, involving SpoVM, SpoVB, SpoVD and SpoVE (Eichenberger *et al.*, 2003).

The cortex composition (Atrih and Foster, 2001) and genes involved in synthesis and assembly, are conserved across Bacilli and Clostridia (Eichenberger *et al.*, 2003). However, enzymes involved in cortex modifications or hydrolysis differ (Díaz-González *et al.*, 2015; Paredes-Sabja *et al.*, 2014). In *Bacillus* cortex hydrolysis enzymes, for germination, include *gerM*, *cwlJ* and *ywdL* which are not present in *Clostridium* species (Díaz-González *et al.*, 2015; Paredes-Sabja *et al.*, 2014). Instead *C. difficile* possesses *sleC* for cortex breakdown (Paredes-Sabja *et al.*, 2014).

1.3.3.4 Coat

1.3.3.4.1 Coat structure

The coat is a proteinaceous assembly observed in EM sections to be electron dense, especially compared to the cortex (Figure 1.13). It is estimated that up to 30% of the spore's protein content is in the coat (Henriques and Moran, 2007). Genomic analysis suggest that coat proteins are species specific and likely adapted to particular environments (Driks and Eichenberger, 2016). Of the 70

known proteins that form the coat in *B. subtilis*, only 20 have been identified in clostridial genomes (Henriques and Moran, 2007).

The diversity in proteins expressed across species equate to varying coat structures. The coat is normally divided into sub-coat layers which, depending on the species, can vary from 1-3 defined layers. In *B. subtilis*, three layers have been identified as making up the coat: the undercoat, probably involved in bridging coat and cortex, in addition to the inner coat that assembles into laminations and the outer coat, which is a thick electron dense layer (Henriques and Moran, 2000; 2007). In *B. anthracis* two coat layers are observed, with the outermost more densely stained than the innermost layer (Driks and Eichenberger, 2016). Structural analysis of outer layer fragments from *B. cereus* and *Bacillus thuringiensis* by electron crystallography identified a type III crystal (Figure 1.15), possibly corresponding to the coat (Ball *et al.*, 2008). This ‘pitted layer’ revealed an assembly of hexagonal rings with $p6$ symmetry and a core diameter of around 60Å (Ball *et al.*, 2008). Thus, indicating that outer coat layers have a para-crystalline assembly (Ball *et al.*, 2008).

C. sporogenes appears to have two defined coat layers (Brunt *et al.*, 2015; Hoeniger and Headley, 1969; Panessa-Warren *et al.*, 1997; Warren *et al.*, 1994), some homologues to *Bacillus* coat proteins have been identified (Janganan *et al.*, 2016) but further work is required for full characterisation. The coat in *C. difficile* includes two main layers (Permpoonpattana *et al.*, 2011). Identified coat proteins include: CotA, CotB, CotCB, CotD, CotE, SpoVM, CotF, CotG, CotJB and SodA, although it is suspected that there are many more (Díaz-González *et al.*, 2015; Permpoonpattana *et al.*, 2011; 2013).

1.3.3.4.2 Coat Assembly

Coat assembly is complex, requiring hours and even days for full maturation (Henriques and Moran, 2007). Both σ^E and σ^k are involved in synthesising coat and morphogenetic proteins (Henriques and Moran, 2007). Around 50% of coat proteins are under control of the σ^E promoter in the mother cell shortly after division. This includes SpoIVA, a protein essential for coat assembly, conserved across Bacilli and Clostridia (Henriques and Moran, 2007). SpoIVA localises at the mother cell proximal pole anchored at the septum division site and recruits SpoVM (Figure 1.14), also under σ^E regulation,

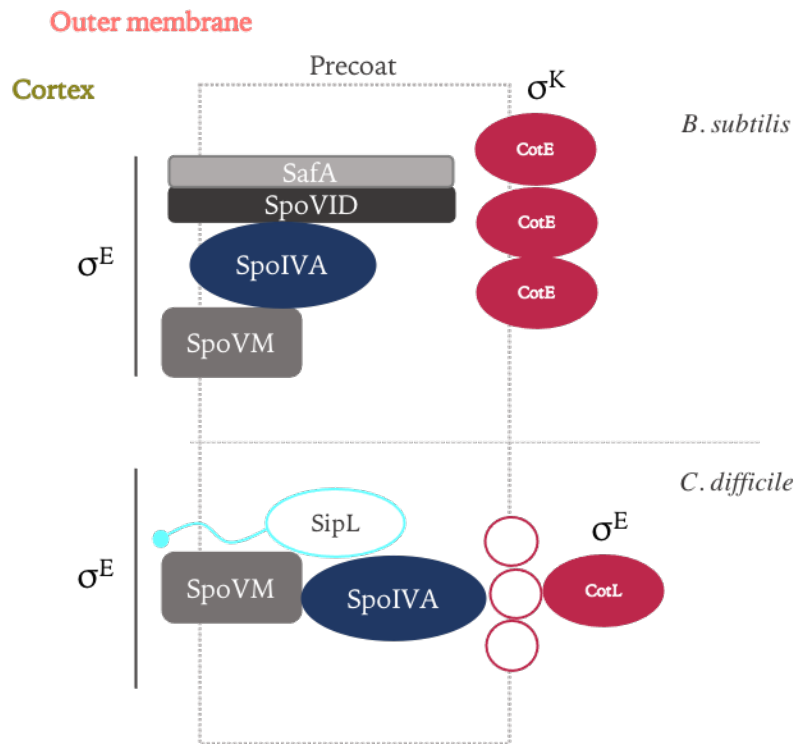


Figure 1.14 Assembly of the spore coat in *B. subtilis* and *C. difficile*

SpoIVA is localised by SpoVM, to the mother cell proximal pole. In *B. subtilis* SpoIVA also localizes SpoVID and SafA and recruits CotE, all of which are important coat assembly proteins. In *C. difficile* SpoVM is dispensable instead SipL, which is bound to the cortex peptidoglycan via its LysM domain (blue dot), interacts with SpoIVA. CotL, a novel morphogenetic protein is also recruited for coat assembly in *C. difficile*. σ^E regulates the expression of SpoVM, SpoIVA, SipL, CotL in *C. difficile* and SpoVM, SpoIVA, SpoVID and SafA in *B. subtilis*. CotE is under σ^K regulation.

to mark the site of cortex synthesis and coat attachment (Henriques and Moran, 2007). In *B. subtilis*, SpoIVA localisation also leads to the localisation of key coat morphogenetic proteins SpoVID and SafA, both of which are under σ^E regulation (Figure 1.14) (Henriques and Moran, 2007). The second group of coat proteins are expressed under σ^K in the mother cell after engulfment. In *B. subtilis* around 75 genes are under σ^K regulation including coat morphogenetic proteins such as CotE (Figure 1.14) (Driks and Eichenberger, 2016; Henriques and Moran, 2007) and outer layer proteins CotA and CotC (Driks and Eichenberger, 2016). The number of conserved genes under σ^K regulation across species is fewer than under σ^E . This reflects the species dependent diversity of the outermost layers which are adapted to different environments (Henriques and Moran, 2007). In *C. difficile*, SpoIVA, SipL and CotL are morphogenetic coat proteins under σ^E regulation (Figure 1.14) (Alves Feliciano *et al.*, 2019; Putnam *et al.*, 2013; Touchette *et al.*, 2019). SpoVM is conserved yet it is dispensable during spore

formation (Ribis *et al.*, 2017). All known coat proteins in *C. difficile* are controlled by σ^k (Fimlaid *et al.*, 2013; Pereira *et al.*, 2013; Saujet *et al.*, 2013).

Once the coat is assembled it is then matured by various post-translational modifications such as glycosylation, proteolytic processing and crosslinking (Henriques and Moran, 2000). The full extent of coat maturation can continue for days after spores have been released (Henriques and Moran, 2007).

1.3.3.4.3 Role of the coat

As already established, differences in coat structure are likely adaptations to different environments (Driks and Eichenberger, 2016). This includes variability in number of layers, thickness and even shape - as some possess folds believed to provide space for core expansion in response to high humidity conditions (Sahin *et al.*, 2012). The coat is an important assembly for conferring resistance against biological and chemical insults. This network of layers provides a physical barrier that prevents harmful compounds from reaching inner spore layers. Coat chemical resistance has been demonstrated against chlorine dioxide, hypochlorite, ozone, peroxydinitrite and a minor role in hydrogen peroxide resistance (Setlow, 2006). It also provides important lysozyme protection (Henriques and Moran, 2000). The coat acts like a molecular sieve that allows smaller molecules, including germinants, to pass through in order to reach the receptors on the inner membrane (Barlass *et al.*, 2002; Brunt *et al.*, 2014; Gerhardt and Ribi, 1964; Moir *et al.*, 2002).

Catalases and other enzymes have also been identified associated with the coat. Manganese catalase, which provides protection against oxidative stress, and super oxide dismutase (SOD) involved in inactivating toxic agents are present across Bacilli and Clostridia (Driks and Eichenberger, 2016). The coat protein CotA, is also believed to confer UV resistance by generating a melanin like pigment that protects spores in *B. subtilis*, although it is present in other Clostridia, its function is not conserved (McKenney *et al.*, 2013).

The coat is also important in germination, in addition to acting as a molecular sieve for germinants, many proteins involved in germination are also located in the coat (Driks and Eichenberger, 2016).

Alanine racemase is involved in preventing premature germination by converting L-alanine, a germinant, to D-alanine. This enzyme is found associated with the coat across *Bacillus* and identified in *C. difficile* (Driks and Eichenberger, 2016). Cortex lytic enzymes are also present in the coat, in *B. subtilis* they include CwlJ and YaaH (McKenney *et al.*, 2013), and SleC in *C. difficile* (Paredes-Sabja *et al.*, 2014).

1.3.3.5 Exosporium

Many spores across Bacilli and Clostridia have an exosporium as their outermost proteinaceous layer. This is not an assembly conserved across all spore formers, as *B. subtilis* lacks an exosporium and instead has a crust assembled close to the coat (Figure 1.13 A). As most fundamental spore research has been conducted in *B. subtilis*, there is less information concerning exosporium in comparison with other assemblies.

As the outermost layer, the exosporium dictates the spores' interaction with its surroundings and has many functions in host cell targeting, germination, adherence and protection (1.3.3.5.5). Thin sections in Bacilli (Beaman *et al.*, 1972; Gerhardt and Ribi, 1964) and Clostridia (Hoeniger and Headley, 1969; Warren *et al.*, 1994) revealed a layer with apparent flexibility separated from the coat by an interspace region. Depending on the species, the basal layer was decorated with filaments termed the 'hairy nap', some spores also possessed longer surface appendages (Driks, 2007; Panessa-Warren *et al.*, 2007; Pradhan *et al.*, 2020). Negative stain EM and freeze etching studies revealed a honeycomb para-crystalline assembly of the basal layer across Bacilli (Gerhardt and Ribi, 1964; Wehrli *et al.*, 1980) and Clostridia (Lund *et al.*, 1978; Takumi *et al.*, 1979).

Most exosporium research has been conducted on the *Bacillus cereus* group including: *B. cereus*, *B. anthracis* and *B. thuringiensis* (Ball *et al.*, 2008; Henriques and Moran, 2007; Kailas *et al.*, 2011; Stewart, 2015; Terry *et al.*, 2017). The structure, composition and assembly of the exosporium across *Bacillus* will be described in the following sections and extended to both *C. botulinum/sporogenes* and *C. difficile*.

1.3.3.5.1 Exosporium in *Bacillus*

The *B. cereus* group has a ‘classical exosporium’, a baggy layer separated from the coat by an interspace region and in some cases elongated at one of the spore poles. An electron crystallography (EC) study of isolated outermost layer fragments from *B. cereus* and *B. thuringiensis* revealed three crystal types 1 (Figure 1.15) (Ball *et al.*, 2008). The basal layer of the exosporium was deemed composed of type II crystal: a honeycomb lattice with central enclosed rings with sixfold symmetry (Ball *et al.*, 2008). The type II crystal in *B. cereus* was decorated with hairy nap, also revealed by atomic force microscopy (AFM) analysis (Kailas *et al.*, 2011). The *B. thuringiensis* 4D11 strain possesses little to no hairy nap, which was also observed on type II crystals (Ball *et al.*, 2008). The other two crystal types were proposed to correspond to inner spore layers (Ball *et al.*, 2008).

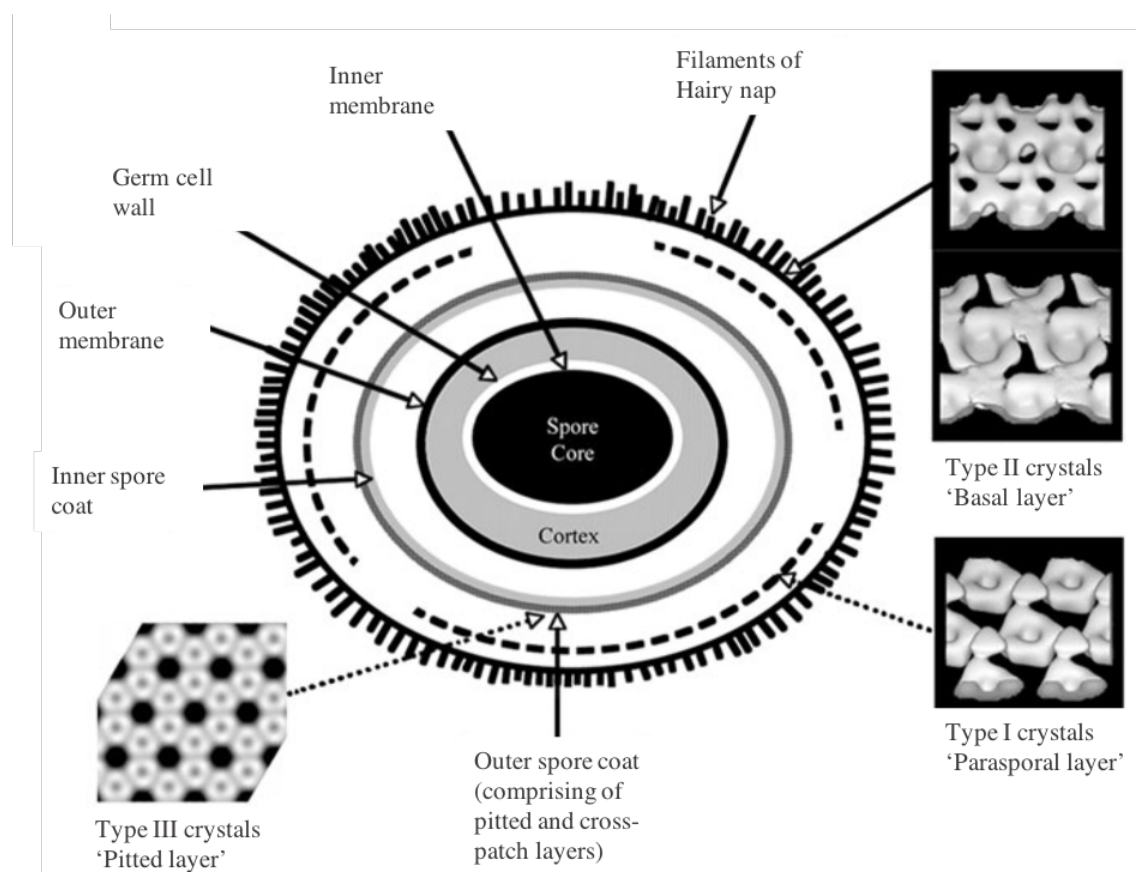


Figure 1.15 Schematic diagram of *Bacillus thuringiensis* spore crystalline layers

Three crystal types observed: Type I crystal proposed to correspond to ‘parasporal layer’. Unit cell: $a = b = \sim 65 \text{ \AA}$, $\gamma = 120^\circ$. Type II: analogous to the most abundant crystal type observed in *B. cereus*, likely corresponds to ‘basal layer’. Unit cell: $a = b = \sim 80 \text{ \AA}$, $\gamma = 120^\circ$. Type III rarely observed and likely corresponds to the coat. Unit cell: $a = b = \sim 100 \text{ \AA}$, $\gamma = 120^\circ$.

Figure from Ball *et al.*, (2008).

Type I crystal possessed a smaller unit cell size and central enclosed ring with $p3$ symmetry, corresponding to the parasporal layer in the interspace (Ball *et al.*, 2008), which will be discussed in 1.3.3.6. Type III crystal was suggested to correspond to outer coat and also revealed a central ring consistent with $p6$ symmetry (1.3.3.4.1) (Ball *et al.*, 2008).

The basal layer of the exosporium in the *B. cereus* group is composed of a hexagonal array formed of central ring structures with sixfold symmetry. 3D reconstructions revealed that the ring is enclosed to form a ‘cup’ structure. The subunits appeared to be partially made up of α -helices, suggested by a cryo-EC 2D projection map at 8Å resolution and circular dichroism spectroscopy (Kailas *et al.*, 2011). In *B. cereus* the rings are interconnected by threefold linkers, a likely attachment point for the hairy nap (Ball *et al.*, 2008; Kailas *et al.*, 2011; Terry *et al.*, 2017). The central ring is also surrounded by six peripheral pores with up to 20Å diameter, large enough to allow germinants to pass through but not enzymes or antibodies (Ball *et al.*, 2008; Terry *et al.*, 2017).

Various proteins were identified in exosporium fragments of *B. cereus* and *B. anthracis* including: ExsY, CotY, BclA, BclB, ExsFA and ExsFB (Redmond *et al.*, 2004; Todd *et al.*, 2003). Mutant analysis of *B. cereus* spores revealed that both cysteine rich proteins ExsY and CotY are involved in the exosporium basal layer (Johnson *et al.*, 2006). This was further confirmed by the heterologous expression of ExsY and CotY in *E. coli*, which self-assembled into crystals with the same structure as native exosporium (Terry *et al.*, 2017). Interestingly, a similar assembly was also observed for the cysteine rich crust protein CotY from *B. subtilis* which bears low sequence homology (~35%) to *B. cereus* proteins (Jiang *et al.*, 2015). This suggests that there may be a conserved assembly approach across spore outermost layers, even between proteins with low similarity, described in section 1.3.3.5.4.

Both ExsY and CotY share 90% amino acid sequence identity (Redmond *et al.*, 2004; Todd *et al.*, 2003) and have been identified associated with different regions of the spores’ exosporium described in section 1.3.3.5.4. Thermal and redox experiments of ExsY and CotY reveal disulphide bond

crosslinking across the exosporium, likely both within and across subunits, conferring the exosporium with highly resilient properties (Terry *et al.*, 2017).

B. anthracis and *B. cereus* possess a hairy nap, an important component of spore interaction with surfaces and host cells (Sylvestre *et al.*, 2002). The filaments are formed of glycosylated BclA or BclB, proteins with collagen-like repeats with 70 GXX triplet regions similar to mammalian collagen (Sylvestre *et al.*, 2002). BclA or BclB are covalently anchored to the basal layers via N-terminal interaction with basal layer proteins ExsFA or ExsFB (Kailas *et al.*, 2011; Stewart, 2015; Terry *et al.*, 2017).

1.3.3.5.2 Exosporium in *Clostridium botulinum/sporogenes*

Previous work on *C. sporogenes* exosporium by electron microscopy identified a honeycomb crystalline layer (Janganan *et al.*, 2016), similar to the exosporium from the *Bacillus cereus* family (Kailas *et al.*, 2011; Terry *et al.*, 2017). Mass-spectrometry analysis of these layers in *C. sporogenes* identified three cysteine rich proteins: CsxA, CsxB and CsxC (Janganan *et al.*, 2016) which provides potential candidates for the composition of the exosporium. Janganan and co-workers (2016) looked at the similarity between these proteins and those in *C. botulinum* group I and II, a summary of their findings is outlined in Table 1.2. CsxA is the most conserved protein with 100% identity with *C. botulinum* Prevot 594, a representative group I toxigenic strain, and 41% identity with group II *C. botulinum* protein (Janganan *et al.*, 2016). Whereas CsxB and CsxC are both highly conserved in *C. botulinum* Prevot 594 and *C. sporogenes* PA 3679, and present across other group I strains, they are absent from group II (Janganan *et al.*, 2016). The high similarity identified across Csx proteins reinforces that *C. sporogenes* is a good model organism for studying the exosporium layers in *C. botulinum* group I (Janganan *et al.*, 2016). However, the exact function of these layers remains unclear.

C. sporogenes NCIMB 701792 strain revealed a highly decorated exosporium with hairy nap and surface appendages (Figure 1.16) (Janganan *et al.*, 2016). Mass-spectrometry analysis of *C. sporogenes* exosporium also identified two proteins with collagen-like repeats, BclA and BclB

(Janganan *et al.*, 2016). Based on their role in *Bacillus hairy nap*, BclA and BclB in *C. sporogenes* are likely to have an analogous function. Only BclB was found to have homology to BclB in *Bacillus anthracis* at 68% identity (Table 1.3) The only homology between BclA and its counterpart in *Bacillus anthracis* is its collagen-like repeats. Both proteins were identified in *C. botulinum* Prevot 594 and *C. sporogenes* PA 3679 but not conserved across other group I strains (Table 1.3) (Janganan *et al.*, 2016). Janganan and co-workers (2016) also found that BclA forms complexes with CsxA and CsxB, analogous to the BclA, CotY, ExsY and ExsFA in *Bacillus* (Redmond *et al.*, 2004). This indicates a likely involvement of BclA in the hairy nap of *C. sporogenes*, although unlike *Bacillus* it is by association directly with the basal layer proteins (Janganan *et al.*, 2016). Further work is required to elucidate mechanisms of hairy nap assembly.

Table 1.1 Amino acid sequence of cysteine-rich proteins present on *C. sporogenes* endospore surface

Protein	Amino acid sequence	Expected MW	% cysteines
CsxA	MFLLYEKEDIYMAINSKDFPRPGFVNKQG CLPDPVEITCIQVPKVFQCLIKECLKPTD DCEQLCKQIPNITDPAQVRCVGCCKDLKVKVNSVTKCPV SNGKPGHKKVTINFVTFDQCLIKECLKPTD RTITASNLYCPDAIAKTIIGKECTSAEEIDQQFIKLEVVGEC LSTDISKIDCDNDCCSCSCTCEDNGDKKVFLCITLGLFIIHK CEIVVQLMVPAYGYCPVPEECKCSHDPCKEFMERELPTL YPPQEMDNLFDDYDERQDERHIHDRKHIEEEEERGNLVT SSIIASN	34kDa	7.1%
CsxB	MSKSSEEMENKEVLNINSFNISEFCNAEEGSNFIHFKPCE ICKRAILDPIINVADTSRLQVNVALRNVCIGKELTVGCILI DRTGTVLAFKSQTFTVGHGGSGCGCEDKHGSPCTNTSR RFSFILPTRDLCSSMDLKVKIIANYTHPCN	16.6kDa	6.6%
CsxC	MMSMDEMGRNYDSNSYKSADHDCHKDCGRVIESQTLPL CEGTNITPTAVTTPVVAKIPVVLAEKEVQIDVEARMKLKE KFFEIKRIKKDVFLTQCELLPRAGVIENGVPITGKFLSGF VRKNIEFATADCVKHDVVSSEIKHTTEKIPFTCVTEVTYI TPPILGNRGIQKTDLFCGRCDCECEEEERLGLTCEEF LEDSITLVEKPFCELLGARIFEADIQRKPCYEHGEKVFDEL LEKMOVHVRVKVLQLQQVAVNDTDAGTGSMCRK	30kDa	5.5%

Table 1.2 Analysis of Csx proteins identified in *C. sporogenes* NCIMB 701792 compared with other *C. botulinum* and *C. sporogenes* strains (Janganan *et al.*, 2016)

	Comparing sequence identity of proteins from <i>C. sporogenes</i> NCIMB 701792 with other <i>C. botulinum</i> and <i>C. sporogenes</i> strains		
Protein from <i>C. sporogenes</i> NCIMB 701792	<i>C. botulinum</i> Prevot 594 ¹ and <i>C. sporogenes</i> PA 3679 ²	Other group I strains	Group II <i>C. botulinum</i> Eklund 17B ³
CsxA	100%	78-87%	41%
CsxB	98-100%	78%	No homologues
CsxC	100%	61-77%	No homologues

¹ *C. botulinum* Prevot 594: toxigenic strain, ² *C. sporogenes* PA 3679: alternative strain with available partial genome sequence, ³ *C. botulinum* Eklund 17B: representative taxonomically distinct group 2 (non-proteolytic strains)

Table 1.3 Analysis of Bcl proteins identified in *C. sporogenes* NCIMB 701792 compared with Bcl proteins from *Bacillus anthracis* and other strains of *C. botulinum* and *C. sporogenes* (Janganan *et al.*, 2016)

	Comparing gene presence/ sequence identity		
Protein from <i>C. sporogenes</i> NCIMB 701792	<i>C. botulinum</i> Prevot 594 ¹ and <i>C. sporogenes</i> PA 3679 ²	Other group I strains	<i>Bacillus anthracis</i>
BclA	Gene present in both strains	No homologues	No homology to BclA in <i>C. sporogenes</i> NCIMB 701792
BclB	82% identity to protein	Not homologues	66% homology to BclB in <i>C. sporogenes</i> NCIMB 701792

¹ *C. botulinum* Prevot 594: toxigenic strain, ² *C. sporogenes* PA 3679: alternative strain with available partial genome sequence

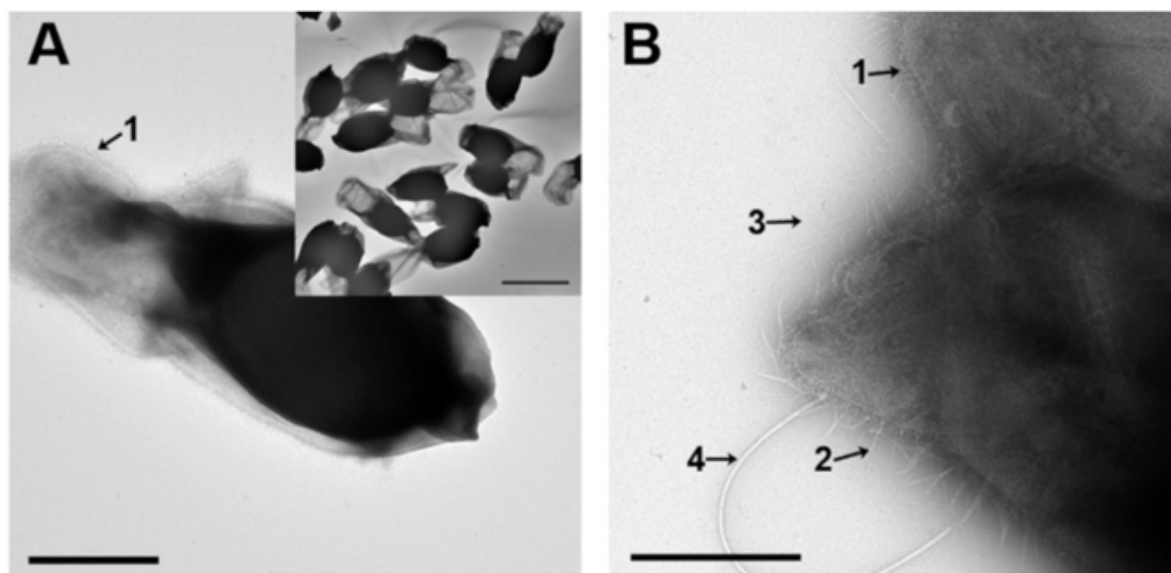


Figure 1.16 Negative stain electron microscopy of *C. sporogenes* NCIMB 701792

A) Low magnification image and population of spores (inset- scale bar =2μm) B) High magnification of exosporium at the pole. 1: hairy nap present on the exosporium, 2: intermediate size fibrils, 3: beaded fibrils, 4: large appendage. Scale bars = 0.5 μm.

Figure from Janganan *et al.*, (2016).

1.3.3.5.3 Exosporium in *Clostridioides difficile*

Unlike other spore forming bacteria, *C. difficile* spores do not appear to possess a ‘classical’ exosporium (a baggy, loosely fitted layer with an interspace region). Thin sections revealed that the exosporium is present tightly fitted to the spore body (Permpoonpattana *et al.*, 2011), although the lack of structural information makes this inconclusive. This layer is proposed to be an exosporium based on its hairy nap association (Díaz-González *et al.*, 2015), different from the *B. subtilis* crust.

Mass spectrometry of the outermost layers identified cysteine-containing proteins, displayed in Table 1.4 (Calderón-Romero *et al.*, 2018; Díaz-González *et al.*, 2015). The encoding genes of these proteins appear to be regulated by σ^K including CdeC and CdeM, or σ^G for CdeA (Saujet *et al.*, 2013), whereas *cdeB* expression has not yet been studied. Based on cysteine content, CdeA, CdeC and CdeM are classified as ‘cysteine-rich proteins’. The cysteine-rich proteins are of particular interest due to their involvement in exosporium assembly in *Bacillus* and *C. sporogenes* (Boydston *et al.*, 2006; Johnson *et al.*, 2006; Janganan *et al.*, 2016; Terry *et al.*, 2017). No sequence similarity is present among

Table 1.4 Amino acid sequence of four cysteine-rich proteins present on *C. difficile* endospore surface

Protein	Amino acid sequence	MW	% cysteines
CdeA	MKNNNLNCAATNCAYNNTSGYCYAGSIKVDGMQATTENTYCASFEDK YTSGITSRSNETNQVDTDNIHCE AVKCKYNKNELCKAEKVHINERNASCETFEMK	11kDa	7.8%
CdeB	MKGNIQYNFADIEEEVYSLDYAIAWNTNEENVNIIPFTNKFCKESIESF CLGKINNFEILNEGIVENH HYVHLDKMISVPKKKVNLYVQQDTHGYLLRDDNDNLIPAKITSEQSKSI SSKMELFCAGEEKCLINILLK ADPSYILDVDSIKDKNILNLGYESIDRYKEYNFDDDKILIFFINKKRYSVI MKKTNSDNDLVSRNNAIK ELFTNKAGNLN	27kDa	1.8%
CdeC	MQDYKKNKRRMMNQPMSTMNEEEVYTDEINSEDMRGFKKSHHHNGC NTDNKCECHDDCNPCNPCNPKPN PCNPCKPNPCDDNCGCHDNCKCDCEPCEMDSDECENKCGPECCNPIS PRNFSVSNAVPFAIEANRIFDT MQFQTFTDATGPNGEPLTFETEVVEVFGSVPSAGQASVTIEKICLSNDGI VIDTGMTLEDFDLPLGDI VGRNCETTFEFAVCGERNSECCRQKGKSVAYKQRLTVAVRNVLVE LRGRCGCTEFVALAFPAVRAGGG CKRRVDYVEFTFNTLSAPICLPADGRAVTLRQEYQTNLTVDCIGKSILK LECNECCEPFYELIIPNDIDL VLCLETSTLISEQIVVLASPNPIQPRLVDTFSKVCDFSQCGPNHGS GK PSCHR	49kDa	8%
CdeM	MENKKCYSEDWYERGESTAKWFQNDREEYERAAAYDEDRERRGSNCG CSDSGGNRPRNCERFRREAEIRER EAREAFCESEKKKEALAYECEARKLWKEAEKYWDEYSKYNQYGIEY LAEAAARLFDEGMECEARRNGNG GNNNNCCHKCHKCNCNCCRK	17kDa	8.8%

exosporium proteins from *Bacillus* and *C. sporogenes*, thus unsurprisingly no similarity is found with the Cde proteins.

Previous research has primarily focused on CdeC and CdeM, with very little research on CdeA and CdeB. As the most abundant protein (Díaz-González *et al.*, 2015), CdeC was proposed to make up the exosporium layer (Barra-Carrasco *et al.*, 2013). Spores from a $\Delta cdeC$ strain indicated reduced germination and adherence to epithelial cells (Calderón-Romero *et al.*, 2018). They were also more sensitive to ethanol, heat, macrophages and lysozyme treatment (Calderón-Romero *et al.*, 2018). This

suggests that CdeC is involved in the assembly of the outermost layers of the spore, but it is not necessarily part of a true exosporium. CdeM has been found associated with the appendage, discussed further in section 1.3.3.7.

The hairy nap in *C. difficile* appears to be strain dependent, as R20291 strains possess a nap but 630 does not (Díaz-González *et al.*, 2015). Paralogues of the *B. anthracis* BclA were identified in *C. difficile* 630 strain, *bclA1*, *bclA2* and *bclA3*, under σ^K control (Abhyankar *et al.*, 2013; Díaz-González *et al.*, 2015). Apart from the collagen like repeats, no sequence homology is shared between these proteins and other hairy nap proteins (Díaz-González *et al.*, 2015). BclA1 was identified in fragments of outer spore layers in *C. difficile* 630 strain, although a direct link between the BclA proteins and the hairy nap is yet to be established in *C. difficile* (Díaz-González *et al.*, 2015).

1.3.3.5.4 Assembly of exosporium

The mechanism of exosporium assembly has primarily been studied in *Bacillus* (reviewed in (Henriques and Moran, 2007; Stewart, 2015)). Exosporium components are under σ^K regulation in the mother cell during late sporulation. The primary stages of assembly likely involve the formation of a ‘sac layer’, a template layer upon which proteins assemble, although the composition and mechanism of this layer remains unclear (Henriques and Moran, 2007).

The assembly of the basal layer is then initiated by a ‘bottle cap’ mechanism (Figure 1.17A) (Steichen *et al.*, 2007). This involves the formation of a cap, at the mother cell facing pole, after which a switch occurs that leads to the assembly of the rest of the exosporium. The cap is suggested to be formed of CotY and maybe ExsY proteins, whereas the rest of the exosporium is formed of ExsY (Johnson *et al.*, 2006). Both regions are decorated with hairy nap, although only BclA is found in the cap whereas BclA and BclB are present in the rest of the exosporium (Thompson *et al.*, 2012). High structural similarity between exosporium in Bacilli and Clostridia suggests there may be orthologous assembly approaches, but no bottle cap has been confirmed. An aperture assembly has been observed at one of the *C. sporogenes* exosporium poles that could correspond to a cap, but this is yet to be proven (Brunt *et al.*, 2015).

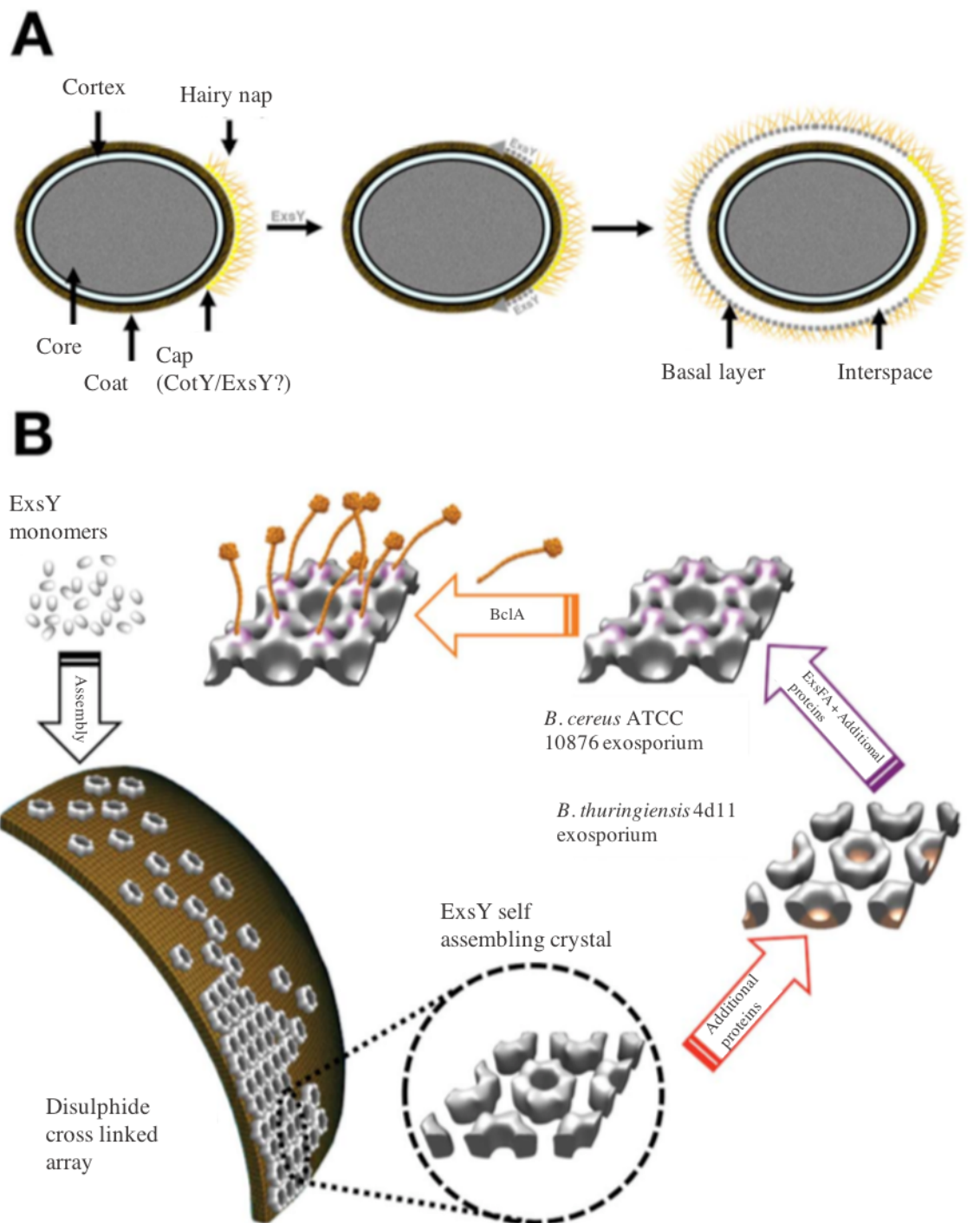


Figure 1.17 Schematic diagram assembly of exosporium in *B. cereus* group

- Exosporium assembly is initiated at the cap: composed of CotY (maybe also ExsY?), which is decorated by BclA hairy nap. ExsY assembly then extends across the whole spore, decorated by BclA and BclB hairy nap.
- ExsY is the primary composition of the basal layer, it self-assembles into a para-crystalline layer with a honeycomb lattice structure. Following assembly stages involve the addition of proteins that enclose the central ring structure and form three-fold linkers. BclA proteins then assemble onto the basal layer to form the hairy nap.

Figure from: Terry *et al.*, (2017).

The ability of ExsY and CotY to self-assemble is likely to drive crystal packing of the basal layer at the cap and the rest of the exosporium (Figure 1.17B) (Terry *et al.*, 2017). The mechanism of limiting cap length remains unclear, Terry *et al.* (2017) suggested it may be due to kinetic and thermodynamic favourability that leads to ExsY dominating the exosporium assembly which is yet to be established.

The ability of crystal self-assembly within *E. coli* cytosol, a reducing environment that likely has no elements to aid ExsY or CotY assembly is remarkable (Terry *et al.*, 2017). It also indicates that even in unfavourable environments for disulphide bond crosslinking, such as the mother cell cytosol which is also a reducing environment, the high symmetry packing of ExsY or CotY provide favourable conditions for cooperative disulphide bond formation (Terry *et al.*, 2017).

Additional proteins are assembled to the basal layer in *B. cereus* to form the ‘cup’ and the trimeric linkers that interconnect the hexameric rings; ExsFA (BxpB) and ExsFB are suggested to form the latter (Kailas *et al.*, 2011; Terry *et al.*, 2017). Consequently, the hairy nap is then formed as BclA or BclB and covalently binds to the ExsFA or ExsFB (Kailas *et al.*, 2011; Terry *et al.*, 2017). BclA has only been observed in its glycosylated state in the cell, suggesting that both synthesis and glycosylation are tightly linked.

1.3.3.5.5 Role of the exosporium

As the outermost layer, the exosporium is the first point of contact between the spore and its surroundings (Stewart, 2015). The baggy-like structure can deform to increase spore contact with surfaces. The hairy nap provides increased hydrophobicity - both of which improve spore attachment to different surfaces including soil types, food preparation material and stainless steel (Stewart, 2015). For pathogens, the exosporium dictates the spore’s interaction with the host including immune cells and gut epithelial cells where many *Bacillus* and *Clostridia* reside (reviewed in Stewart, 2015). Surface appendages have been associated with increased adherence to a range of surfaces including human cell lines such as the Caco-2 colon cell line for *C. difficile* and fibroblast cells for *C. sporogenes* (Panessa-Warren *et al.*, 2007). The exosporium is also decorated with highly immunogenic proteins such as elongation factor TU, identified in *B. anthracis* (Delvecchio *et al.*,

2006), *C. difficile* (Díaz-González *et al.*, 2015) and *C. sporogenes* (Janganan *et al.*, 2016). The exosporium may also increase the persistence of infections, although the exact mechanism of the exosporium involvement in pathogenicity remains unclear (Henriques and Moran, 2007).

Exosporium layers are also involved in conferring resistance. Treatment of exosporium fragments from *C. botulinum* revealed resistance to 1M Guanidine HCl, DTT, B-mercaptoethanol, urea and enzymes including trypsin and pronase E (Takumi *et al.*, 1979). 8M Urea and 1% B-mercaptoethanol in alkaline conditions was required to completely disintegrate the layers (Takumi *et al.*, 1979). Heterologously expressed ExsY crystals also required harsh conditions to disassemble crystals including: 8M urea, 200mM DTT, 2% (w/v) SDS at 90°C for 20 minutes (Terry *et al.*, 2017). An analogous resistance was also observed in CotY crystals of *B. subtilis* crust (Jiang *et al.*, 2015) which suggests that the assembly of highly resilient outer layer crystals may be a conserved feature across spore formers.

Similar to the coat, the exosporium is a porous layer that may have filtering properties as a molecular sieve. Various enzymes are also associated with the exosporium in *Bacillus* including super oxide dismutases (SODs) and arginases that reduce the effect of free radicals (reviewed in (Henriques and Moran, 2007; Stewart, 2015)). Arginases in *B. anthracis* have also been associated with a protective role from host cell nitric oxide, important for microbial killing, by reducing the pool of arginine for nitric oxide synthesis (reviewed in (Stewart, 2015)). Clostridia also possess ferredoxin/ferredoxin oxidoreductase, involved in oxidative stress response (Díaz-González *et al.*, 2015; Janganan *et al.*, 2016).

The bottle cap structure of the exosporium is believed to play an important role in germination. The composition of the cap differs from the rest of the exosporium, making it a possible point of weakness. This would allow for rapid accommodation of outgrowing vegetative cells without having to hydrolyse the exosporium (Steichen *et al.*, 2007). Alanine racemase is also present associated with the exosporium, likely involved in germination modulation discussed in section 1.3.1.3, although it is excluded from the cap region in *B. anthracis* (Steichen *et al.*, 2007). The significance and mechanism

of this alanine racemase exclusion from the cap remains unclear. As already mentioned, a cap has not been identified in *Clostridia*, although the aperture observed at one of the spore poles may correspond to this (Brunt *et al.*, 2015; Warren *et al.*, 1994).

1.3.3.6 Interspace

The interspace corresponds to the region between the exosporium and the coat. Due to the flexible nature of the exosporium, the interspace varies in width around the spore (Henriques and Moran, 2007). The exact composition and significance of the interspace is uncertain.

Thin sections of spores have revealed a network of layers within the interspace termed the ‘parasporal layer’. In *Bacillus*, both *B. megaterium* (Beaman *et al.*, 1972) and *B. thuringiensis* (Ebersold *et al.*, 1981; Wehrli *et al.*, 1980) possess layers that stack into distinct laminae across the spore. Freeze-fracture studies demonstrated that the layers are also composed of para-crystalline assemblies (Beaman *et al.*, 1972; Ebersold *et al.*, 1981; Wehrli *et al.*, 1980). EC of exosporium fragments of *B. thuringiensis* and *B. cereus* revealed a crystal type I believed to correspond to the interspace layers, termed parasporal layers (1.3.3.5.1) (Ball *et al.*, 2008; Kailas *et al.*, 2011). Type I crystal (Figure 1.15) possessed a central enclosed structure with $p3$ symmetry and lacked hairy nap (Ball *et al.*, 2008). 2D projection maps of the crystal to 6Å resolution from cryo-EC demonstrated that this crystal is composed of α -helices, also confirmed by circular dichroism spectroscopy (Kailas *et al.*, 2011), although the protein composition of this layer is still unknown.

Both *C. sporogenes* (Hoeniger and Headley, 1969) and different *C. botulinum* strains (Stevenson *et al.*, 1972) possess layers in their interspace but the structure and composition of these layers remain unstudied.

1.3.3.7 *C. difficile* appendage

Longitudinal sections of *C. difficile* spores reveal a distinct outermost structure, the appendage (Figure 1.18D). This assembly is a thick proteinaceous layer that appears continuous around the whole spore and extends out from one of the spore poles. Studies of spore population by phase

contrast microscopy and TEM of thin sections identified subpopulations with varying appendage lengths (Figure 1.18) (Antunes *et al.*, 2018). Antunes and co-workers (2018) proposed that the length of the appendage plays an important role in the colonisation and persistence of infection by dictating the germination rate (Antunes *et al.*, 2018). The model proposed suggests that morphotype C, which has no appendage (Figure 1.18C,E), is the first to respond to germinants thus rapidly forming the

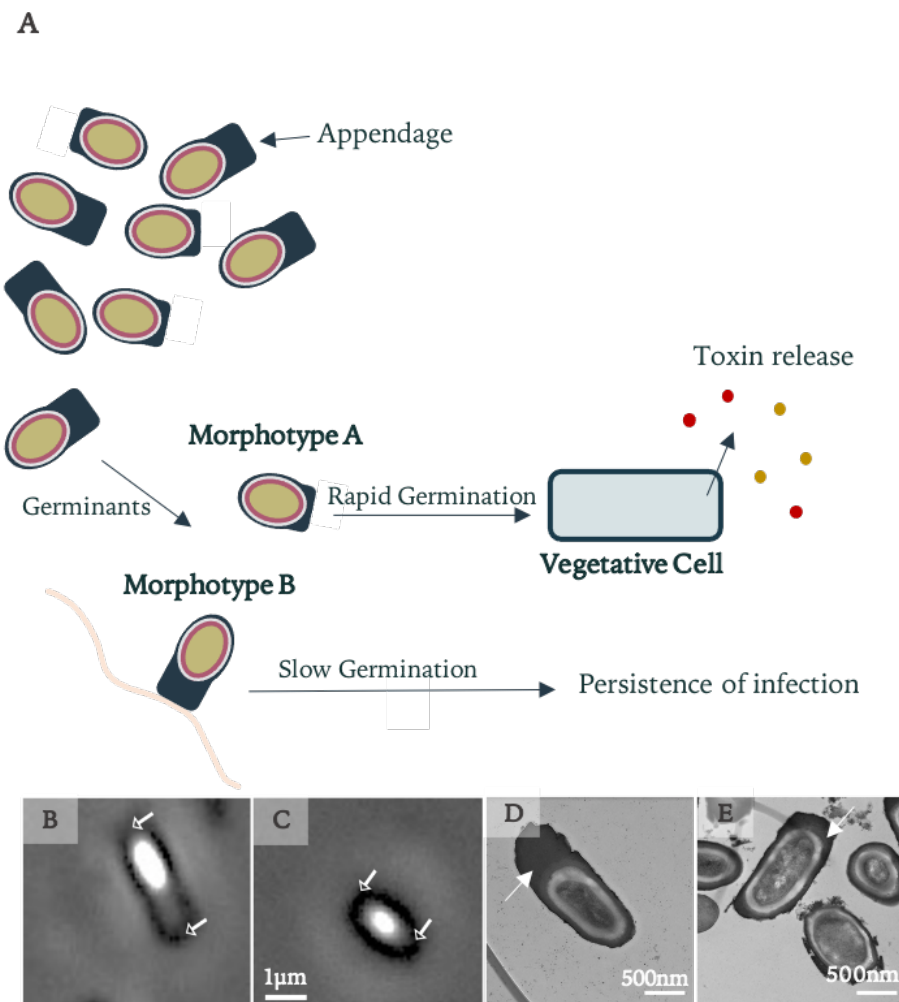


Figure 1.18 *C. difficile* infection model

A) Schematic of different *C. difficile* endospore morphotypes and their role during infection. Primary difference between morphotype A and B is the length/presence of appendage, depicted as a dark blue layer that protrudes from one pole of the spore. During gut colonisation the endospores will encounter germinants and thus begin to germinate. Morphotype A germinates rapidly, forming the vegetative cell state that releases toxins. Morphotype B germinates more slowly, believed to be involved in the persistence of the infection. B-C) Phase bright images and D-E) Thin-section TEM of morphotypes A and B: display spores with long and short/absent appendage (arrows).

Figures B and C and schematic in Figure A from: Antunes *et al.*, (2018) D and E: images from Eleonora Marini (ITQB, Lisbon)

vegetative cell that releases toxins to initiate the infection. Morphotypes A and B have a range of lengths of appendage, involved in the delayed response to germinants. These morphotypes are considered to be important in the persistence of the infection (Antunes *et al.*, 2018).

Antunes and co-workers (2018) proposed that CdeM is associated with the appendage, as phase contrast, fluorescence and TEM of thin-sections of $\Delta cdeM$ displayed a less defined polar appendage. Functional analysis of $\Delta cdeM$ found no effect on spore sensitivity and reduced adherence (Antunes *et al.*, 2018; Calderón-Romero *et al.*, 2018; Pizarro-Guajardo *et al.*, 2020), yet an increase in virulence and germination. These results are consistent with the colonisation model, suggesting that the presence of CdeM, and so the appendage, can delay response to germinants (Antunes *et al.*, 2018).

1.3.4 Studying spore structure

Whole spore studies are challenging due to the highly electron dense spore body. Whole spore electron cryotomography (ECT) has been employed to study the Gram-negative Firmicute, *Acetona longum*, for the visualisation of spore layers during various stages of the spore cycle (Tocheva *et al.*, 2011). *A. longum* is much thinner compared to other spores, thus less electron dense enabling layers to be identified when imaged on a 300keV microscope equipped with an energy filter. Based on spore thickness, this approach could not be extended to spores of Bacilli and Clostridia (Tocheva *et al.*, 2011), thus sectioning is required to study their internal layers by electron microscopy.

Thin section analysis of chemically fixed samples is an established method to study spores. As discussed in section 1.2.3.1, this involves fixation with harsh chemical fixatives, dehydration in ethanol and embedding into plastic resin blocks, which can lead to damage of biological samples and misleading artefacts. High pressure freezing (HPF) is an alternative sample preparation approach, which preserves the biological specimen. These samples can then be sectioned at -160 degrees by cryo-electron microscopy of vitrified sections (CEMOVIS), bypassing the use of any chemical fixatives, dehydration or plastic embedding. By using this approach, membrane deposits were discovered in the spore core (Laue *et al.*, 2018). These were identified across Bacilli and Clostridia spores and believed to play an important role in the expansion of the plasma membrane during

germination yet were not visible by regular thin sectioning methods (Laue *et al.*, 2018). CEMOVIS poses many challenges, especially as it is very laborious and requires high level of skill to be conducted successfully. The lack of any plastic or preservatives makes the sections very delicate and prone to knife marks, compression in the direction of cutting and crevasses, grooves perpendicular to the cutting direction (Michael *et al.*, 2008). Alternatively, HPF samples can be FIB-milled to generate lamella that do not have any knife marks or crevasses. This is an approach that has been used to get novel insights into *B. subtilis* spore engulfment during sporulation (Khanna *et al.*, 2019). However, the technique also requires a high degree of skill and throughput is low.

HPF can also be combined with freeze substitution (described in 1.2.3.1) which provides improved biological sample preservation. HPF and freeze substitution on *Bacillus thuringiensis* spores demonstrated the potential of the methodology (Ebersold *et al.*, 1981). The degree of contrast, definition and detail of each layer was far superior than any chemical fixation conducted. It provided novel insight into a network of laminations present in the interspace, between the exosporium and the coat (Ebersold *et al.*, 1981).

1.3.4.1 Spore structure in Clostridia

Our understanding of Clostridia spore ultrastructure was based solely on chemical fixed spores. They have provided insight into spore layers, establishing high similarity to other spore formers.

Thin section work on *C. sporogenes*, has been used to study sporulation (Hashimoto and Naylor, 1958), the dormant spore state and germination (Brunt *et al.*, 2015; Hoeniger and Headley, 1969). Due to chemical fixation approaches, the innermost layers of dormant spore sections lacked definition and detail, limiting the observational analysis (Brunt *et al.*, 2015; Hoeniger and Headley, 1969; Knudsen *et al.*, 2016). However, previous sections of *C. sporogenes* exosporium revealed an opening on some of the spores at the elongated pole (Brunt *et al.*, 2015; Knudsen *et al.*, 2016). This indistinct pole was confirmed by scanning electron microscopy (SEM) and termed the sporiduct or aperture in the literature, proposed to be important in germination (Brunt *et al.*, 2015).

The spore layers become more visible upon screening germinating spore sections (Brunt *et al.*, 2015; Hoeniger and Headley, 1969) – likely due to the rehydration of the spore that is more amenable to chemical fixatives. Sections of germinating spores have also provided insight into important changes in spore structure during germination, including the rehydration of the core, the breakdown of the cortex, breaks in the coat and outgrowth of vegetative cell (Brunt *et al.*, 2015; Hoeniger and Headley, 1969).

1.4 Aims and Objectives

Our understanding of spore ultrastructure in *Bacillus* and *Clostridia* is restricted to chemically fixed thin sections that provide limited 2D information of spore assemblies. Prior research on the *Bacillus cereus* group have given insight into the structure and composition of outer layers including the exosporium and revealed the involvement of cysteine-rich proteins in their assembly. However, the current structural understanding is based on resolution-limited reconstructions of the basal layer. Additionally, little is known of these assemblies in *Clostridia*. The aim of this research is to gain a greater understanding of spore ultrastructure and composition, with a primary focus on outermost layers including the exosporium, on the clinically relevant *Clostridium sporogenes/botulinum*, and *Clostridioides difficile* spore.

The main objectives of this research were to:

- Determine the involvement of cysteine rich proteins in outer spore assemblies in *C. sporogenes* and *C. difficile* by combining TEM mutant screening and EC
- Express CsxA in *E. coli* and investigate the structure and properties of the crystal
- Optimise CsxA crystal preparation for cryo EC to generate high resolution 3D structural information of exosporium basal layer
- Develop advanced sample preparation methods for sectioning *C. sporogenes* spores to investigate spore assemblies and for collecting 3D ultrastructure information

Chapter 2: Materials and methodology

2.1 General Methodology

2.1.1 Bacterial strains

Table 0.1 *Escherichia coli* strains

Strain	Source/Reference	Notes	Genotype/Phenotype
NEB 5-alpha	New England BioLabs	Modified DH5- α strain	<i>fhuA2</i> Δ (argF-lacZ) U169 <i>phoA</i> glnV44 Φ 80 Δ (lacZ)M15 <i>gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>
BL21 pLySs	Novagen	Used for CsxA crystal expression	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) gal dcm (DE3) pLyS (Cm ^R)
BL21 DE3	Novagen	Used for <i>C. difficile</i> heterologous expression of Cde proteins	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) gal dcm (DE3)

Table 0.2 *Clostridium sporogenes* strains

Strain	Source/Reference	Notes
ATCC15579	Dr Jason Brunt (University of Cambridge)	Wild type strain
Δ csxA ATCC15579	Dr Jason Brunt (University of Cambridge)	Δ csxA mutant generated using Clostron
Δ csxB ATCC15579	Dr Jason Brunt (University of Cambridge)	Δ csxB mutant generated using Clostron
Δ csxC ATCC15579	Dr Jason Brunt (University of Cambridge)	Δ csxC mutant generated using Clostron

Table 0.3 *Clostridioides difficile* strains

Strain	Source/Reference	Notes
630 Δ erm	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Wild type strain
630 Δ erm Δ cdeC	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Δ cdeC mutant
630 Δ erm Δ cdeM	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Δ cdeM mutant

630 Δ erm Δ cdeC Δ cdeM	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Δ cdeC and Δ cdeM mutant
630 Δ erm containing P _{colE} cdeM in pMTL84121	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	cdeM+ over expression
630 Δ erm containing P _{colE} cdeC in pMTL84121	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	cdeC+ over expression
630 Δ erm containing P _{colE} cdeB in pMTL84121	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	cdeB+ over expression
630 Δ erm containing P _{colE} cdeA in pMTL84121	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	cdeA+ over expression

Table 0.4 *Escherichia coli* with plasmid for Cde expression

Strain	Plasmid	Source/Reference	Selectivity	Notes
AHEC 683 BL21 (DE3) containing pCAF18	pCAF18 - cdeC in pACYCDuet-1	Dr Adriano Henriques (ITQB, University Nova Lisbon)	Cm ^R	cdeC expression
AHEC 682 BL21 (DE3) containing pCAF17	pCAF17 - cdeM in pET-33b	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Km ^R	cdeM expression
AHEC 783 BL21 (DE3) containing pEM44	pEM44 - cdeA in pET-33b	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Km ^R	cdeA expression
AHEC 787 BL21 (DE3) containing pEM47	pEM47 - cdeB in pET-33b	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Km ^R	cdeB expression
AHEC 685 BL21 (DE3) containing pCAF18+pCAF17	pCAF18 - cdeC in pACYCDuet-1 and pCAF17 - cdeM in pET-33b	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Cm ^R + Km ^R	cdeC and cdeM expression

AHEC 798 BL21 (DE3) containing pEM44+pCAF18	pEM44 - <i>cdeA</i> in pET-33b and pCAF18 - <i>cdeC</i> in pACYCDuet-1	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Cm ^R + Km ^R	<i>cdeA</i> and <i>cdeC</i> expression
AHEC 880 BL21 (DE3) containing pEM44+pEM59	pEM44 - <i>cdeA</i> in pET-33b and pEM59 - <i>cdeM</i> in pACYCDuet-1	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Cm ^R + Km ^R	<i>cdeA</i> and <i>cdeM</i> expression
AHEC 881 BL21 (DE3) containing pEM47+pEM59	pEM47 - <i>cdeB</i> in pET-33b and pEM59 - <i>cdeM</i> in pACYCDuet-1	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Cm ^R + Km ^R	<i>cdeB</i> and <i>cdeM</i> expression
AHEC 799 BL21 (DE3) containing pEM47+pCAF18	pEM47 - <i>cdeB</i> in pET-33b and pCAF18 - <i>cdeC</i> in pACYCDuet-1	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Cm ^R + Km ^R	<i>cdeB</i> and <i>cdeC</i> expression
AHEC 829 BL21 (DE3) containing pEM44+pEM52	pEM44 - <i>cdeA</i> in pET-33b and pEM52 - <i>cdeC</i> + <i>cdeM</i> in pACYCDuet-1	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Cm ^R + Km ^R	<i>cdeA</i> , <i>cdeC</i> and <i>cdeM</i> expression
AHEC 830 BL21 (DE3) containing pEM52+pEM47	pEM52 - <i>cdeC</i> + <i>cdeM</i> in pACYCDuet-1 and pEM47 - <i>cdeB</i> in pET-33b	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Cm ^R + Km ^R	<i>cdeB</i> , <i>cdeC</i> and <i>cdeM</i> expression
AHEC 861 BL21 (DE3) containing pEM58+pCAF18	pEM58 - <i>cdeA</i> + <i>cdeB</i> in pETDuet and pCAF18 - <i>cdeC</i> in pACYCDuet-1	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Cm ^R + Amp ^R	<i>cdeA</i> , <i>cdeB</i> and <i>cdeC</i> expression
AHEC 869 BL21 (DE3) containing pEM58+pEM59	pEM58 - <i>cdeA</i> + <i>cdeB</i> in pETDuet and pEM59 <i>cdeM</i> in pACYCDuet-1	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Cm ^R + Amp ^R	<i>cdeA</i> , <i>cdeB</i> and <i>cdeM</i> expression
AHEC 846 BL21 (DE3) containing pEM58+pEM52	pEM58 - <i>cdeA</i> + <i>cdeB</i> in pETDuet and pEM52 - <i>cdeC</i> + <i>cdeM</i> in pACYCDuet-1	Eleonora Marini - Dr Adriano Henriques (ITQB, University Nova Lisbon)	Cm ^R + Amp ^R	<i>cdeA</i> , <i>cdeB</i> , <i>cdeC</i> and <i>cdeM</i> expression

Cm^R = Chloramphenicol selective resistance

Km^R = Kanamycin selective resistance

Amp^R = Ampicillin selective resistance

2.1.2 Growth Media

Table 0.5 Growth Media

Media	Ingredients	Quantities for a litre	Note
Luria-Bertani (LB) broth	LB broth, Miller (Thermo Fisher Scientific)	25 g	For <i>E. coli</i> cultures
Luria-Bertani (LB) agar	LB broth, Miller (Thermo Fisher Scientific) Difco bacto agar	25 g 15 g	For <i>E. coli</i> cultures
Brain heart infusion (BHI) agar	Brain heart infusion (Oxoid) Yeast extract (Bacto) L-Cysteine (Sigma-Aldrich) Difco bacto Agar	25 g 5 g 1 g 15 g	For <i>C. sporogenes</i> cultures
TGY broth	Tryptic soy broth (Bacto) Glucose (VWR)* Yeast extract (Bacto) L-Cysteine *Add as a filter sterilised solution after autoclaving	30 g 2% (w/v) 10 g 1g	For <i>C. sporogenes</i> cultures
SMC	Bacto peptone (Bacto) Proteose peptone (Sigma-Aldrich) (NH ₄) ₂ SO ₄ (Sigma-Aldrich) Tris-(hydroxymethyl) aminomethane (VWR) *Difco bacto agar	90 g 5 g 1 g 1.5 g *15 g	For <i>C. sporogenes</i> cultures *Agar (1.5%) added to make plates

2.1.3 Buffers and stocks

Table 0.6 Buffers and stocks

Name	Ingredients	Note
TBS buffer	25 mM Tris pH 8 150 mM NaCl 1 mM PMSF*	*Added fresh from stock [†]

TBS buffer with 1M Imidazole	25 mM Tris pH 8 150 mM NaCl 1 mM PMSF* 1 M Imidazole	*Added fresh from stock [†]
TBS buffer with 500mM Imidazole	25 mM Tris pH 8 150 mM NaCl 1 mM PMSF* 500 mM Imidazole	*Added fresh from stock [†]
TBS and 0.1% Triton X100	25 mM Tris pH8 150 mM NaCl 0.1% (v/v) Triton X100 from 4% stock ^{††}	
TBS2 buffer	50 mM Tris pH8 150 mM NaCl	From (Terry <i>et al.</i> , 2011)
TBS2-T buffer	50 mM Tris pH8 150 mM NaCl 10% (v/v) Tween-20	From (Terry <i>et al.</i> , 2011)
[†] phenylmethylsulfonyl fluoride (PMSF)	100 mM PMSF	Container wrapped in aluminium foil to prevent light exposure at -20°C.
^{††} 4% Triton X-100	4% (v/v) Triton X-100	
20% HistoDenz	20% (w/v) of Histodenz (Sigma-Aldrich)	
50% HistoDenz	50% (w/v) of Histodenz (Sigma-Aldrich)	
Isopropyl-beta-D-thiogalactoside (IPTG)	1 mM IPTG*	*working concentration Filter sterilised with 0.2 µm Millex filters (Milipore)
Ampicillin	100 µg/ml Ampicillin*	*working concentration Filter sterilised with 0.2 µm Millex filters (Milipore)

Chloramphenicol	15 µg/ml Chloramphenicol* in 70% (v/v) ethanol	*working concentration Filter sterilised with 0.2 µm Millex filters (Milipore)
Kanamycin	50 µg/ml Kanamycin*	*working concentration Filter sterilised with 0.2 µm Millex filters (Milipore)

2.1.4 Antibodies

Table 0.7 Antibodies

Antibody	Raised in	Dilution used from stock	Source
Anti-CdeC (<i>primary polyclonal antibody</i>)	Rabbit	1:500	Eleonora Marini, Dr Adriano Henriques (ITQB, University Nova Lisbon)
Anti-CdeM (<i>primary polyclonal antibody</i>)	Rabbit	1:1000	Eleonora Marini, Dr Adriano Henriques (ITQB, University Nova Lisbon)
Goat anti-rabbit (<i>secondary antibody</i>)	Goat	1:100	BB International

2.1.5 Nucleic acid methodology

2.1.5.1 Plasmids

Table 0.8 Plasmids

Plasmid name	Vector backbone	Markers	Note
csxA plasmid	pET21a	Ampicillin (100 µg/ml) and chloramphenicol (15 µg/ml)	C-terminal His ₆ fusion

2.1.5.2 Chemically competent *E. coli* cells

Stocks of chemically competent cells were made as follows: *E. coli* cells were inoculated in 10ml of LB broth and incubated overnight at 37°C. The overnight culture was inoculated into 100 ml of LB broth and grown to an OD₆₀₀ of 0.4-0.6 at 37°C. The cultures were then kept on ice for 20 mins and cell pellets harvested by centrifugation at 6,000 × g for 8 mins. The pellets were resuspended in cold CaCl₂ solution (60 mM CaCl₂, 15% (v/v) glycerol, 10 mM PIPES at pH 7) and left on ice for a further

30 minutes. The cells were centrifuged again, pellets were resuspended in 4 ml of cold CaCl₂ solution, incubated overnight in ice and stored at -80°C in aliquots of 100 µl.

2.1.5.3 Transformation into *E. coli* cells

E. coli cell transformations were conducted using chemically competent cells. An aliquot of chemically competent cells was thawed from -80°C and mixed with 0.01-1 µg of plasmid DNA. This mixture was left on ice for 30 mins, followed by a 45 s heat shock at 42°C. The mixture was then left on ice for 5 mins, after which LB was added up to 1 ml volume and left at 37°C in a shaking incubator for an hour. 50-100 µl of cells were then plated onto LB agar with respective antibiotics. The plates were incubated overnight at 37°C and stored at 4°C for up to three weeks.

2.1.5.4 Plasmid purification

Plasmid purification was conducted using the commercially available QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's protocol.

Briefly, transformed *E. coli* NEB 5-alpha cells were grown overnight in a shaking incubator at 37°C in 1-5 ml of LB media with respective antibiotics. Cells were harvested by 6,000 × g for 2 minutes. The supernatant was discarded, and the cell pellet resuspended in 250 µl of buffer P1. This was followed by repelleting and resuspending in 250 µl of buffer P2 and mixed thoroughly by inverting tubes 4-6 times or until the solution became clear. 350 µl of neutralising Buffer N3 was then added and mixed. The sample was centrifuged at 13,000 rpm for 10 mins - all following centrifugations were conducted at this speed. The supernatant was harvested and applied onto a QIAprep sample column and centrifuged for 30-60 s; the flow through was discarded. The column was washed with 500 µl of buffer PB and centrifuged for 30-60 s, and the flow through discarded. 750 µl of buffer PE was added to the column and centrifuged, the flow through discarded. An additional 60 s centrifugation step was used and flow through discarded. The column was placed in a clean microcentrifuge tube and 50 µl of buffer EB added to elute DNA from column. The mixture was left to stand for 10 mins before centrifuging for 1 min and the flow through harvested.

2.2 Spores methodology

C. difficile and *C. sporogenes* spores were used throughout this project. All *C. difficile* spores were cultivated and harvested by Dr Eleonora Marini (ITQB, University of Lisbon), shipped to the UK and stored at 4°C in water. *C. sporogenes* spores were cultivated and harvested at Sheffield, based on an approach used by Dr Joe Kirk (Dr Robert Fagan's lab, University of Sheffield) for *C. difficile* spores. All broth and agar plates were placed in an anaerobic cabinet for a minimum of 4 hours before inoculation. The anaerobic cabinet (Don Whitley Scientific) was supplied with anaerobic gas mixture (BOC) of 80% N₂, 10% H₂ and 10% CO₂, maintained at 37°C and 70% humidity.

2.2.1 Spore cultivation

C. sporogenes strains were streaked onto BHI agar plates and left overnight at 37°C in the anaerobic chamber. A colony from the plate was then inoculated into 15 ml of TGY broth and incubated overnight at 37°C in anaerobic chamber. 1.5 ml of the overnight culture was then added to 15 ml of SMC broth and incubated until an OD_{600nm} of 0.4-0.7 was reached. 100 µl of the culture was spread onto SMC agar plates and left to incubate for 10 days.

2.2.2 Harvesting spores

After 10 days, the SMC plates were covered in a lawn of spores and vegetative cell remnants. Using an inoculating loop, the spores were collected off the surface of the plates and placed into water. This suspension was washed five times in 500 µl ice cold water and centrifuged at 5,000 × g for 1 min. The spore pellet was resuspended in 20% HistoDenz and loaded on top of 1 ml 50% HistoDenz, being careful not to let them mix. The gradients were then centrifuged at 15,000 × g for 15 mins at room temperature. The spores pelleted at the bottom of the microcentrifuge tube, with vegetative cells and debris accumulating at the HistoDenz gradient interface. The spores were then washed a further five times in cold water.

2.2.3 Immunogold labelling spores

C. difficile spores were immunogold labelled as previously described (Terry *et al.*, 2011). 0.5 mg of spores were resuspended in 1 ml of TBS2-T with 1% Bovine Serum Albumin (BSA) and left on a

gentle shaker for 10 mins. The sample was then centrifuged at $8,000 \times g$ for 5 mins. Spores were then incubated for an hour at room temperature on a gentle shaker with 1 ml of TBS2-T with 1% BSA and primary antibody, either anti-CdeC or anti-CdeM (Table 2.7). The spores were then washed by centrifugation at $8,000 \times g$ for 5 mins and resuspended in TBS2-T three times. Spores were incubated for another hour as previously but with 1 ml of TBS2-T with 1% BSA and secondary antibody, anti-rabbit (Table 2.7). As previously, spores were washed three times by centrifugation resuspending in TBS2-T. Finally, spores were resuspended in TBS2 and loaded onto EM grids for screening (section 2.4.2).

2.3 CsxA crystals

2.3.1 Heterologous expression of CsxA in *E. coli*

A single colony of transformed *E. coli* cells (section 2.1.5.3) was inoculated into 5 ml overnight culture in LB broth with ampicillin and chloramphenicol. The overnight culture was inoculated into fresh LB broth, with ampicillin and chloramphenicol, at an OD_{600} of 0.05. The culture was incubated at 37°C with the shaker at 150 rpm until grown to an OD_{600} of 0.5. Expression was induced with 1 mM IPTG for 3 hours at 37°C , after which cells were harvested by centrifugation at $5,000 \times g$ for 20 mins. Cell pellets were then stored at -20°C .

2.3.2 CsxA crystal purification

A cell pellet of 0.1 g was resuspended in 10 ml of TBS with 10 μl of DNase (500 $\mu\text{g}/\text{ml}$) and 10 μl lysozyme (2 mg/ml) and kept on ice. The sample was then sonicated for 15 s 3-5 times at 10 μm amplitude. The cell lysate was then loaded onto EM grids and negatively stained and screened on the CM100 to confirm crystal presence before further purification.

Crystal purification was carried out by batch purification using Ni-NTA agarose beads (Qiagen). 4 ml of bead slurry was added to a falcon tube with TBS buffer added to the 15 ml mark. The beads were pelleted by centrifugation at $500 \times g$ for 5 mins. 10 ml of supernatant was removed, leaving the beads in 5 ml of buffer, this mixture was split across two falcon tubes each for ~ 20 ml of cell lysate. The beads and cell lysate mixture were left on a rotator at room temperature for 1 hour. To reduce the loss

of sample and beads, all steps that required pelleting the beads were done by gravity. The mixture was left to settle, and supernatant removed to leave 5 ml volume, the beads were then resuspended in TBS to a 15 ml mark- this was repeated 5 times. To the final 5 ml volume, 2 ml of TBS with 1 M imidazole was added, gently stirred and left to settle. The supernatant was harvested and 5 ml of TBS with 500 mM imidazole buffer was added and left to settle. This was repeated until 15 ml of supernatant was recovered.

The harvested supernatant was then loaded onto a Vivaspin 15R (Vivaproducts) and centrifuged at 5,000 rpm until 2-4 ml were obtained. The concentrated sample was further centrifuged in microcentrifuge tubes at 10,000 rpm for 10 mins. The pellet was resuspended in 100 µl TBS with 0.1% Triton X100 and further centrifuged. The final pellet was resuspended in 50 µl TBS with 0.1% Triton X100. 3-5 µl of sample was loaded onto EM grids and negatively stained for screening on the CM100.

2.3.3 Testing CsxA crystal stability

The CsxA crystal sample from 3.2 was centrifuged at 10,000 rpm for 10 mins. The pellet was then resuspended in TBS buffer, TBS buffer with 8 M Urea and TBS buffer with 2 M DTT. Samples in TBS buffer and a second sample with 2 M DTT were placed in a heat block at 95°C. All samples were incubated for 60 mins in respective conditions before loading onto EM grids and negatively stained for screening on the CM100.

2.4 Microscopy Sample Preparation

2.4.1 EM reagents

Table 0.9 EM reagents

Reagent	Ingredients	Note
Uranyl formate (UF) (0.75%)	0.035 g of uranyl formate (Polysciences Inc.) 5 ml distilled water* 8 µl 5M NaOH	Filter sterilise with 0.22 µm Millex filters (Milipore) Store at 4°C wrapped in aluminium foil to avoid light exposure *Add to boiling water

Uranyl acetate (UA) (2%)	0.093 g of uranyl acetate (Polysciences Inc.) 5 ml Distilled Water* 8 µl 5M NaOH	Filter sterilise with 0.22 µm Millex filters (Milipore) Store at 4°C wrapped in aluminium foil to avoid light exposure *Add to boiling water
Reynold's citrate	1.33 g lead nitrate 1.76 g sodium citrate 50 ml CO ₂ free distilled water 7 ml 1M NaOH pH12 +/- 0.1	Store at 4°C for up to 6 months
2.5% Glutaraldehyde	2.5% glutaraldehyde 0.1M phosphate buffer pH 7	Make fresh every week
2% aqueous OsO ₄	Osmium tetroxide (Agar Scientific)	
Araldite resin	10 ml araldite resin CY212 (Agar Scientific) 10 ml dodecenyl succinic anhydride (DDSA, Agar Scientific) 1 ml benzyldimethylamine (BDMA, Agar Scientific)	
Spurr Resin	10 g ERL 6 g diglycidyl ether of polypropylene glycol (DER) 736 26 g nonenyl succinic anhydride (NSA) 0.4 g dimethylaminoethanol (S-1) (DMAE)	
Freeze-substitution cocktail 1	1% (w/v) OsO ₄ Acetone	Make fresh every time
Freeze-substitution cocktail 2	1% (w/v) OsO ₄ 0.5% (w/v) uranyl acetate 3% (w/v) glutaraldehyde	Make fresh every time

2.4.2 EM grids

2.4.2.1 Negative stain grids

Negative stain samples were loaded onto 300 mesh copper palladium grids (EM Resolutions) with a layer of carbon, which provided a hydrophilic surface for greater sample adherence. Carbon coating

of the grids was carried out in house. This involved evaporating high purity carbon (Agar Scientific) onto a freshly exposed sheet of mica (Agar Scientific) on filter paper in vacuum using the Quorum Q150T. The carbon was evaporated under high vacuum ($\sim 10^{-5}$ mbar and lower). The thickness of the carbon film, $\sim 150\text{\AA}$ and $\sim 300\text{\AA}$ thick, was determined by using thickness monitors or visually estimated based on the darkness in colour of the filter paper. Carbon floating was employed to cover the grids with the carbon layer, as described in Harris (2007).

Upon drying the grids, they kept for about a month. However, the carbon layer became more hydrophobic over time. The hydrophilic properties were restored by glow discharging the grids using the Cressington 208 before loading the sample. This involved a 15-25 s plasma exposure at $\sim 10^{-2}$ mbar with atmospheric air.

2.4.2.2 Cryo-EM grids

Cryo-EM samples required lots of sample preparation optimisation, this included testing different grid types. All of the grids used possessed a 300 mesh copper grid base, with a variety of surface layers that conferred different surface properties.

Electron crystallography data collection was conducted on the commercially available Quantifoil R2/2 grids with either an additional layer of carbon support or graphene oxide (GO) support film (EM Resolutions). Quantifoil grids were precoated with an amorphous carbon support film with repeating punctuating holes $2\text{ }\mu\text{m}$ in diameter and $2\text{ }\mu\text{m}$ apart. The grids coated with GO support film improved imaging stability by reducing charging effects and hydrophobic surface to which some biological samples readily adhere (Pantelic *et al.*, 2010).

Tomography samples of whole spores were loaded onto lacey carbon grids (EM Resolutions). These grids possess a carbon layer with an irregular hole pattern mesh and included larger holes than quantifoil grids for sample adhesion.

2.4.2.3 Thin section grids

Sections were loaded onto 300 mesh copper grids (Agar Scientific) coated with formvar. The formvar, a plastic layer, provided a robust layer required for loading sections onto the grids. Formvar coated grids were also prepared in house.

In a small water bath, 10 μ l of 1.5% pyroxylin in amyl acetate was added, which forms a film on the water surface; grids were then loaded onto the film on the water surface and then placed onto a sheet of absorbent paper (Bench Guard). These were left to dry in a petri dish with filter paper under lamp light overnight.

2.4.3 Sample preparation for TEM

2.4.3.1 Negative staining

After glow discharging the grids, as described in 2.4.2.1, 3-10 μ l of sample was loaded onto the carbon side of the grids and allowed to adsorb for 60 s. Excess sample was then removed by blotting with filter paper (Whatman no.1). The grid was then washed in two droplets of water, loaded on Parafilm M (Bemis Company, Inc), and blotted in between each droplet. This was followed by two droplets of 0.75% UF (Table 2.9) and incubated for 20 s in the last droplet before blotting. The grids were then dried using filter paper and a vacuum drier.

2.4.3.2 Cryo sample preparation

The Leica EM GP plunge freezer was used for cryo sample preparation. It permits the automatization of plunge freezing at set conditions. For this work, the chamber was set to 10°C and 80% humidity.

Liquid nitrogen was used to cool the Leica cryo container, and ethane was cooled to liquid nitrogen temperatures. The EM grid was then loaded onto Leica tweezers and mounted onto the plunger hook. 3 μ l of sample was loaded onto the grids and incubated for 60 s, after which the blotting arm removed excess liquid onto filter paper (Whatman no.1) for 5 s. This was followed by rapidly plunging the grid into liquid ethane. Grids were transferred into grid holders and stored in liquid nitrogen.

2.4.3.3 Chemical fixation

Chemical fixation of samples was conducted by Chris Hill (EM facility, University of Sheffield), the protocol was as detailed below.

Pellets of spores were fixed with 2.5% glutaraldehyde and left ON at 4°C. The suspension was pelleted and washed in 0.1 M phosphate buffer, repeated twice with 30 mins intervals. The final pellet was treated with a second fixative, 2% aqueous OsO₄, for 1 hour at room temperature and washed twice with 0.1 M phosphate buffer as described above.

Samples were dehydrated with a series of ethanol dilutions in increasing concentration: 50%, 75%, 95% and 100%. Each step involved 15 min incubation with ethanol, followed by discarding the solution and replacing with the next concentration. The final step at 100% ethanol was repeated twice. An additional dehydration step was conducted with 100% ethanol dried in anhydrous copper sulphate, as described above, this was repeated twice.

The sample was then placed in 100% araldite resin for 15 mins, repeated twice. The araldite resin was then left to evaporate for an hour and the remaining mixture discarded. The sample was then incubated with 100% araldite resin for 4 hours on a rotating mixer; this step was repeated twice. The final sample was embedded in fresh araldite resin for 48-72 hours at 60°.

2.4.3.4 High Pressure Freezing and Freeze substitution

High pressure freezing of spore pellets was done on the Leica EM PACT (Leica). Pellets were loaded into carriers and frozen in liquid nitrogen at pressures >2000 bar. These carriers were transferred into vials with freeze substitution cocktails at -90°C. Freeze substitution was conducted in the Leica AFS2, the protocol used was based on Matias and Beveridge (2006) - outlined in Table 2.10. This permitted a gradual warm up of the frozen sample over 42 hours where the water content of the sample was replaced with the FS cocktail. The FS cocktails used are described in table 2.9, as FS cocktail 2 possessed UA the container was wrapped in aluminium foil to prevent light exposure.

Table 0.10 Program set on the Leica AFS2 for freeze substitution

Time (hours)	Temperature (°C)
12	-90
8	-90 -> -60
5	-60
7	-60 -> -30
5	-30
5	-30 -> 0

Sample pellets were then removed from the carriers by washing in acetone and treated with 1% UA in acetone for an hour in the dark. Samples were then placed in 100% ethanol for 30 mins and supernatant was removed, this was repeated 3 times. A second acetone wash was repeated on samples and removed before treating with increasing concentrations of Spurr resin as detailed in the Table 2.11. In the final 100% Spurr resin, the samples were placed into moulds and polymerised at 60°C for 16 hours.

Table 0.11 Infiltration cycles with Spurr resin

Spurr resin in acetone	Incubation time
30%	4 hours
60%	ON
90%	4 hours
2x 100%	2 hours each

2.4.3.5 Sectioning and post staining

The plastic blocks were sectioned using the Reichert Ultramicrotome to generate 70-90 nm and 200 nm-300 nm thick samples. The sections were loaded onto the formvar coated grids and post stained with 3% UA for 30 mins, washed in water and Reynold's Lead Citrate for 5 mins.

2.5 Electron Microscopy

2.5.1 TEM of negatively stained samples

Negatively stained samples were screened on a Philips CM100 TEM, with a LaB₆ source operated at 100 kV. Images were recorded on Gatan 1k x 1k Multiscan 794 CCD camera. Micrographs of spores were recorded at magnifications of 1,650 x - 3,900 x for lower magnifications and 15,500 x - 28,500 x for higher magnifications. Electron crystallography data were collected at magnifications of 21,000 x - 28,500 x at 0.7-3 μ m under focus. The goniometer was tilted from -60° to +60° to collect tilt series at every 10° interval in low dose mode for 3D EC data.

2.5.2 TEM of sections

Grids of sections were screened on the FEI Tecnai T12 fitted with a tungsten source and operated at 80 kV. Images were recorded on Gatan Orius SC1000B 2672 × 4008 pixels CCD camera.

Micrographs of sections were recorded at magnifications of 1,900 x - 2,3000 x.

2.5.3 Cryo electron crystallography

All cryo-EC data collection was conducted on the FEI Tecnai Arctica with field emission gun operated at 200 kV. The Arctica was equipped with a CEMOS CCD camera and Falcon III direct electron detector (Thermo Fisher Scientific). Grids were loaded into the microscope using the cassette-autoloader system that allows the loading of up to 12 grids.

2.5.4 Electron crystallography

There was no dedicated software for electron crystallography data collection, a workflow for manual collection described in Figure 0.1 was developed using EPU (Thermo Fisher Scientific) and TIA (Thermo Fisher Scientific) to operate the microscope. The EPU platform was used to search, find and set the appropriate locations and settings for data collection. An 'Atlas' of the grid was collected to get an overview of the whole grid and to select regions for data collection. In 'select grid square', sample presence and ice thickness were screened and the eucentric height set. In 'hole selection', the sample presence and position was checked at higher magnification using minimal dose. The defocus was set for low dose data collection using the 'autoCTF' software. Parameters for data collection were

then set and the microscope interface used to adjust camera parameters, including exposure and frames, and record the micrograph. TIA was used to visualise the micrograph and save as an mrc format file.

Electron crystallography data were collected at 35,000 x and 78,000 x magnification with Falcon III direct electron detector (Thermo Fisher Scientific) at a defocus values of 1 μ m-6.5 μ m with a 2s exposure over 79 fractions at ~ 50 e⁻/nm². 3D data were collected by tilting the goniometer to -60° to +60° and collect every 20°.

2.5.5 Tomography

Tomography data were collected on the FEI Tecnai Arctica operated at 200 kV. Whole spore grids were plunge frozen and were loaded using the same approach described in 2.4.3.2. Grids with sections were not frozen prior to loading thus the grids were loaded at room temperature and the system was set to cool to -195° in the autoloader.

The tomo software (Thermo Fisher Scientific) was used to operate the microscope and set up automated data collection. As described for electron crystallography (2.5.4), an ‘atlas’ was collected to visualize the whole grid and select regions for data collection. Different positions in the grid were then selected, each with the eucentric height set and the off-sample focus points for low dose mode. The holder calibration was set from -60° to +60° at 2° increments for whole spores and -20° to +60° and -20° to -60° at 3° increments for sections. The tilt series were collected at a magnification of 16,600 x (0.663 nm) with the Falcon III direct electron detector (Thermo Fisher Scientific) and a total dose of 60e⁻/Å².

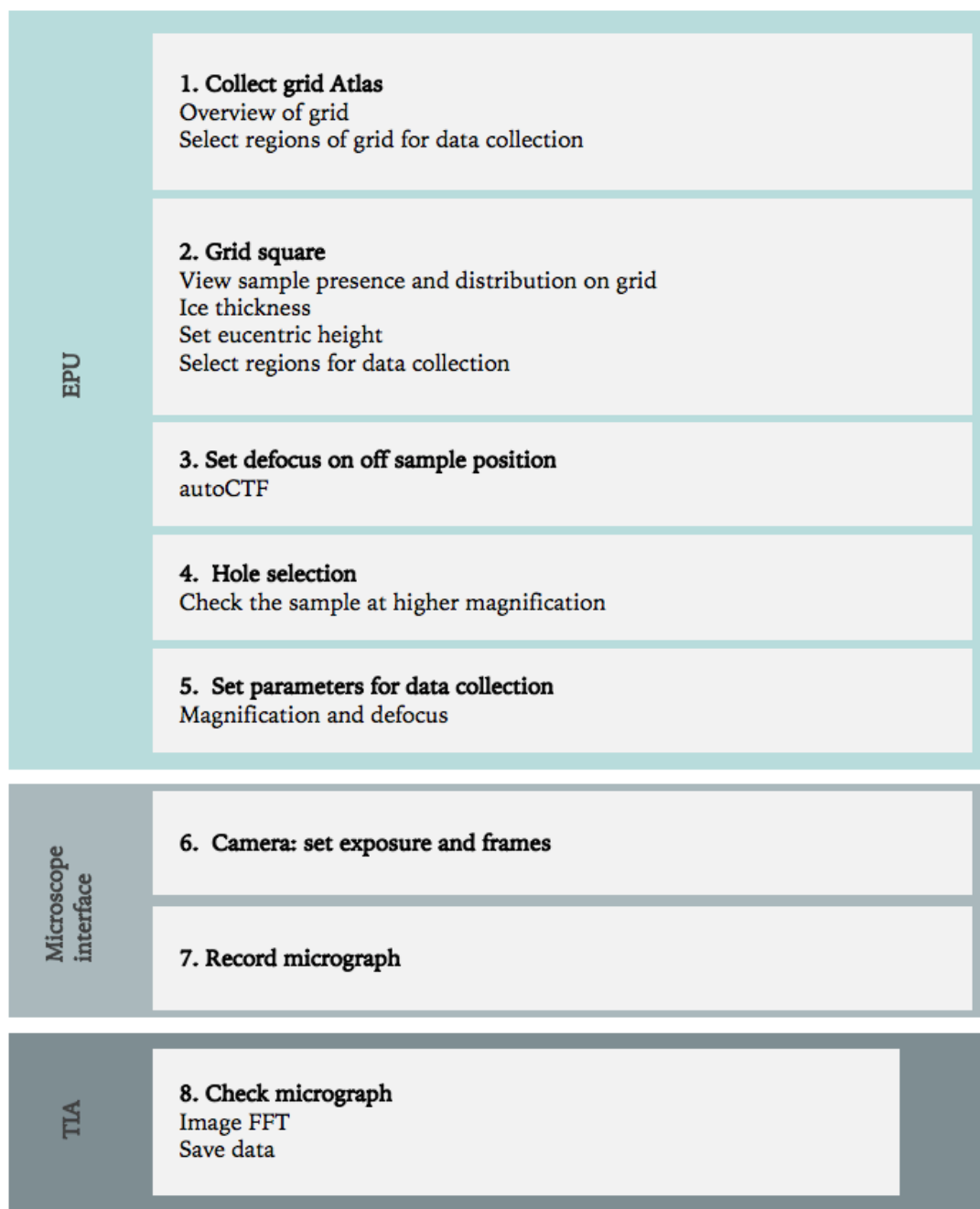


Figure 0.1 Workflow developed for electron crystallography data collection

2.6 Image processing

2.6.1 Electron crystallography

Electron crystallography processing was performed with 2dx and FOCUS (Gipson *et al.*, 2007; Renault *et al.*, 2006), based on the MRC suite of programs (Crowther *et al.*, 1996; Henderson and Unwin, 1975; Henderson *et al.*, 1990; Valpuesta *et al.*, 1994). The workflow used for image processing is described in Figure 2.2. Details of the programs and processes involved in each step will be discussed in the following section. All the programs described in the text are listed on Table 0.12.

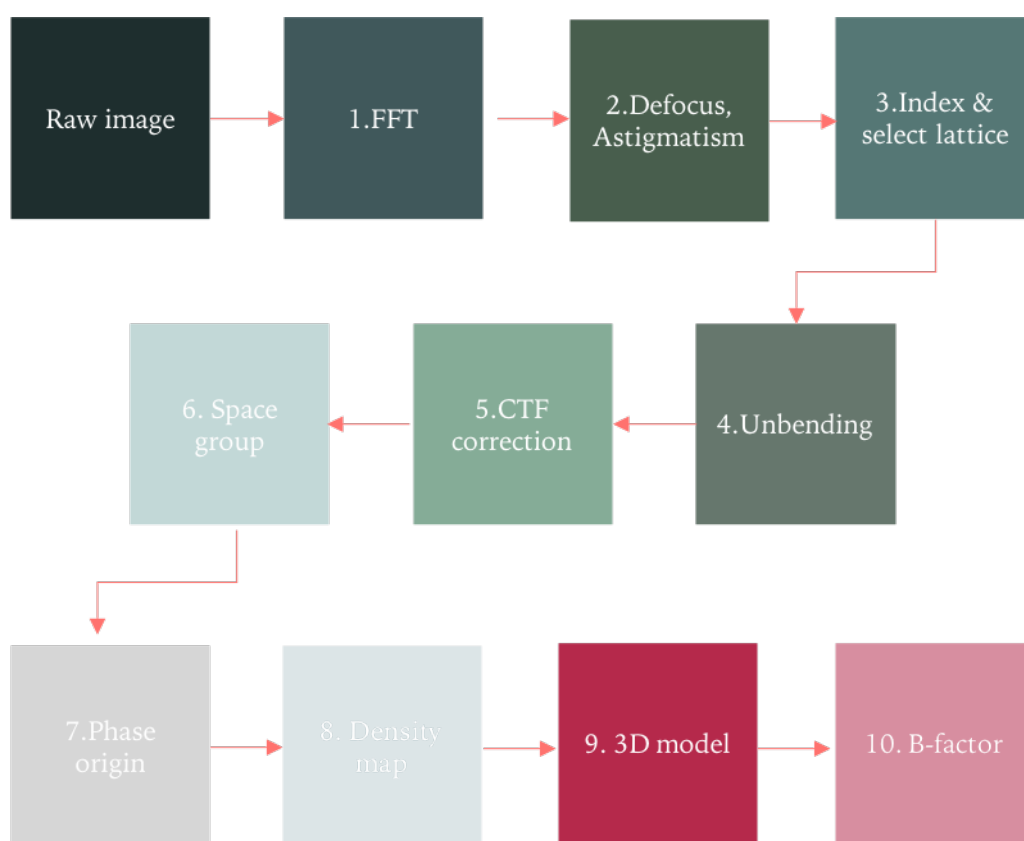


Figure 0.2 Overview of electron crystallography image processing workflow

Table 0.12 List of all scripts used during electron crystallography image processing

Program	Function	Reference
<i>MotionCorr2</i>	Anisotropic correction of beam-induced motion	(Zheng <i>et al.</i> , 2017)
<i>FFTRANS</i>	Generate FFT	(Crowther <i>et al.</i> , 1996)
<i>CTFFIND3</i>	Calculate defocus and astigmatism	(Mindell and Grigorieff, 2003)
<i>GETDEFTILT</i>	Calculates tilt based on 7x7 CTFFIND	(Mindell and Grigorieff, 2003)
<i>GETLATTICE</i>	Automated lattice determination	(Zeng <i>et al.</i> , 2007)
<i>FINDLAT</i>	Lattice determination based on 0,1 and 1,0 positions set manually	(Zeng <i>et al.</i> , 2007)
<i>GETSPOT1</i>	Spotlist generation for unbending	(Zeng <i>et al.</i> , 2007)
<i>2dx_maskCrystal</i>	Generates a mask around optimal crystal patches	(Crowther <i>et al.</i> , 1996)
<i>MASKTRAN</i>	Mask the diffraction spots	(Crowther <i>et al.</i> , 1996)
<i>TWOFILE</i>	Calculate cross-correlation maps	(Crowther <i>et al.</i> , 1996)
<i>QUADSEARCH</i>	Generate map of vectors describing lattice distortions from cross-correlation	(Crowther <i>et al.</i> , 1996)
<i>CCUBEND</i>	Unbend crystal and remove distortions	(Crowther <i>et al.</i> , 1996)
<i>MMBOX</i>	Generate list of amplitudes and phases for map generation	(Crowther <i>et al.</i> , 1996)
<i>CTFAPPLYK</i>	Apply CTF to data from MMBOX	(Crowther <i>et al.</i> , 1996)
<i>ALLSPACE</i>	Space group determination from CTF corrected data	(Crowther <i>et al.</i> , 1996)
<i>NPO</i>	Generate 2D density map	
<i>LATLINE</i>	Plot the phase and amplitude against z*	(Agard, 1983)

<i>SCALIMAMP3D</i>	Correct image amplitudes for resolution dependent factors	(Havelka <i>et al.</i> , 1995)
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2.6.1.1 Cryo electron crystallography data of CsxA

To attain higher resolution electron crystallography data, larger datasets were collected. Efforts were made during this project to set up a workflow for processing high volumes of electron crystallography data in a semi-automated approach. An overview of the workflow is outlined in Figure 0.3.

Cryo-EC data were collected using Falcon III direct electron detector in frames. The alignment of these frames was conducted using *MotionCor2* for anisotropic correction of beam-induced motion (Zheng *et al.*, 2017). The *FOCUS* software was used to automatically import images and run the scripts to calculate fast Fourier transform (FFT)(*FFTRANS*). The FFT of the images could be examined in a swift approach, from which images of suboptimal crystal quality were filtered out without extensive processing. The most optimal crystals were then imported into *2dx* for thorough processing. All *2dx* processing was conducted manually as described in 2.6.1.2 - 2.6.1.11.

2.6.1.2 Fast Fourier Transform

An FFT calculates the diffraction pattern of the crystal in the image. The repeating units in the lattice produce sharp peaks displayed as spots. The edge of the FFT represents the resolution limit, the Nyquist frequency, defined as $1/(2 \times \text{pixel size})$. Effects of CTF are observed in the FFT as Thon rings (chapter 1.2.1.2). The FFT also includes background noise e.g from carbon film on grids and blurriness due to distortions in the lattice. The *FFTRANS* script is used to generate the FFT from micrographs.

2.6.1.3 Defocus and astigmatism

EM micrographs were collected with underfocus applied, which needs to be accounted for during image processing. The degree of underfocus and astigmatism determines the CTF (section 1.2.1.2). *CTFFIND3* was used to calculate the defocus and astigmatism in the image.

The defocus across the image varies in images of tilted samples as a function of height in the microscope column (Mindell and Grigorieff, 2003). *GETDEFTILT* was used to estimate the tilt of the image from the defocus distribution by dividing the image into 7x7 regions and calculating each region by *CTFFIND* (Mindell and Grigorieff, 2003).

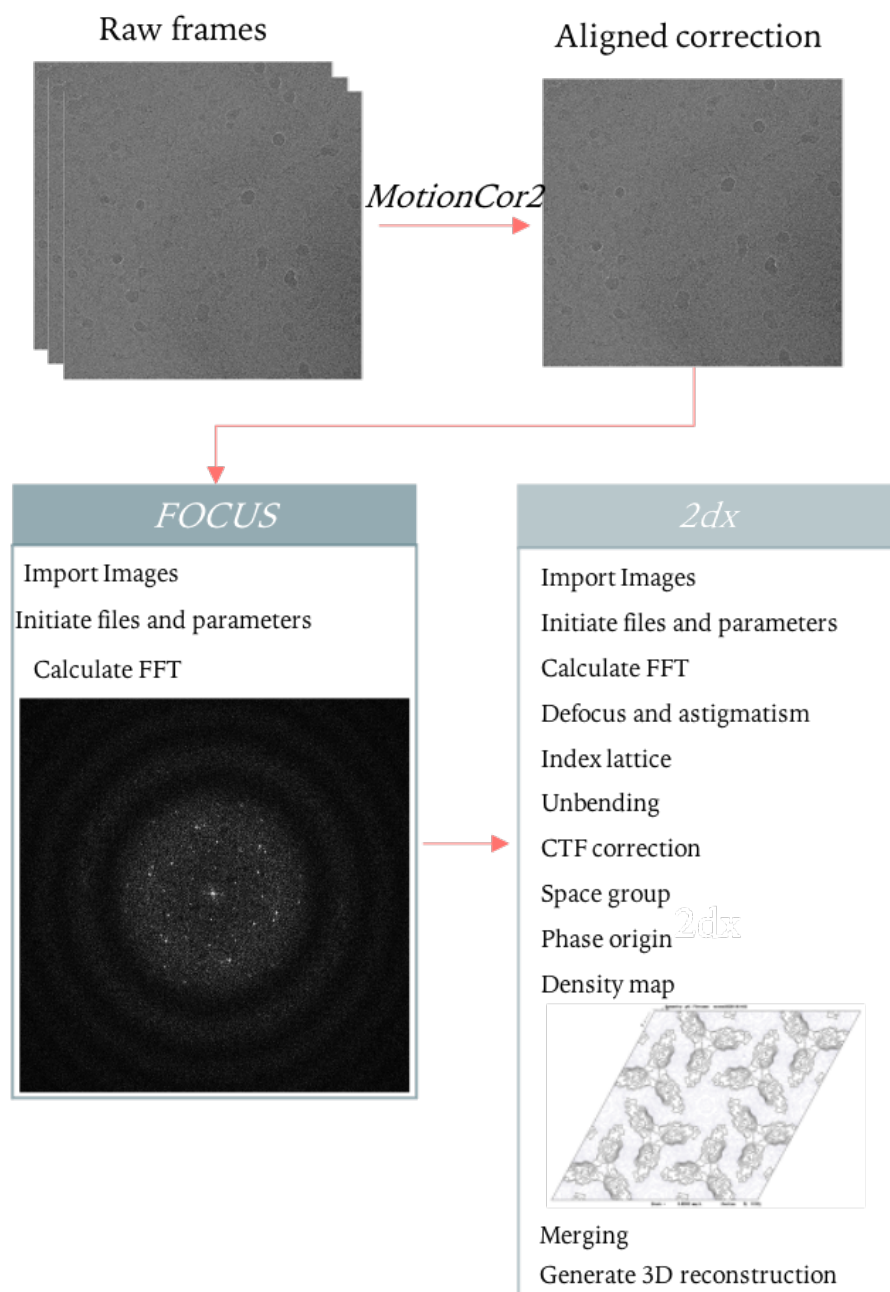


Figure 0.3 Workflow for processing cryo-electron crystallography data

2.6.1.4 Indexing and selecting lattice

The diffraction patterns in the FFTs were indexed using *GETLATTICE*, an automated approach, or manually by indexing h,k with 0,1 and 1,0 coordinates and then running *FINDLAT* to identify the rest of the lattice. *GETSPOT1* was then used to select the spots corresponding to this lattice for further refinement processes.

Once the lattice was defined, it could also be used to estimate the tilt of the crystal based on the skewing of the apparent unit cell axis with respect to the untilted crystal. The *FINDLAT* program was also used to calculate this.

2.6.1.5 Unbending

Crystals will always have imperfections such as lattice distortions including bending or stretching. Biological crystals also possess patches of crystals in different orientations with differing crystal quality. More optimal crystal patches can be used to enhance the signal to noise ratio, thus providing a better map of the crystal by a process referred to as ‘unbending’. Some crystal patches are such low quality that processing cannot improve the signal to noise and thus need to be masked out via *2dx_maskCrystal* script.

The process of unbending is outlined in Figure 0.4. The FFT of the image was masked to filter out noise by *MASKTRAN* (1). An inverse FFT (2) then generated a filtered map (3), a reference patch which appears well ordered is then selected (4). Using *FFTRANS*, the FFT of the reference map was calculated (5) and multiplied with FFT of the full filtered image with *TWOFILE* program (6). The product of the two is then reverse FT to give the cross-correlation image between reference and whole image (7); a peak search algorithm generates a map of correlation peaks. In an ideal lattice, the correlation peaks are related by exact unit cell translations, however distortions in the crystal shift their position, a map of these lattice distortions are described by vectors from *QUADSEARCH* (8) and corrected by interpolation with *CCUBEND* (9). The output FFT displays sharper spots indicating increased SNR (10), which was then used for further unbending. The iterative process of unbending improved the image quality. The amplitude and phase information from predicted diffraction spots

after unbending was provided by *MMBOX* and fed into *ORIGTILT* for further processing and map generation.

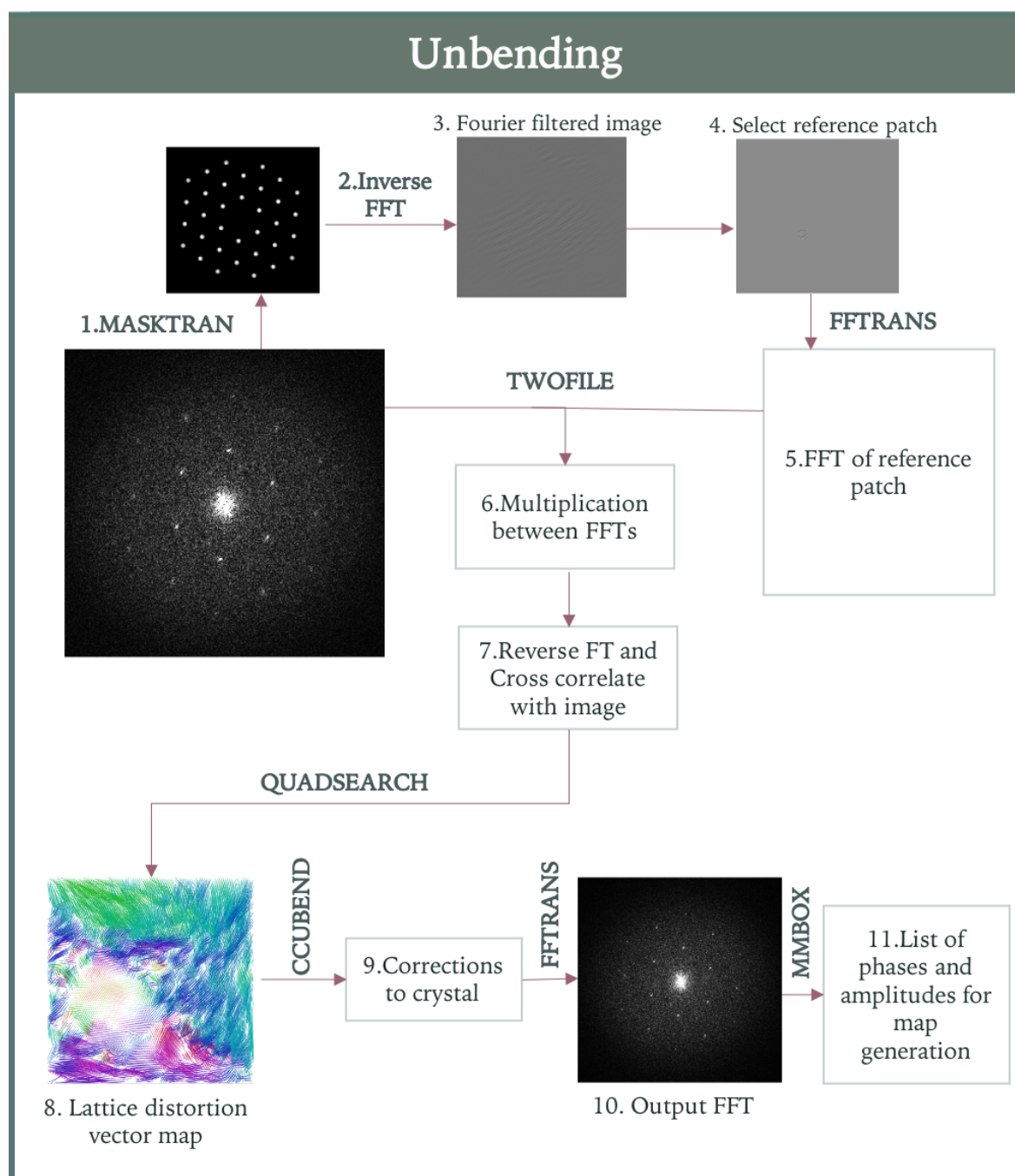


Figure 0.4 *2dx* unbending processing

2.6.1.6 CTF correction

Phase flipping and amplitude correction was performed with *CTFAPPLYK*, which results in all the maxima information being of the same sign (Figure 0.5).

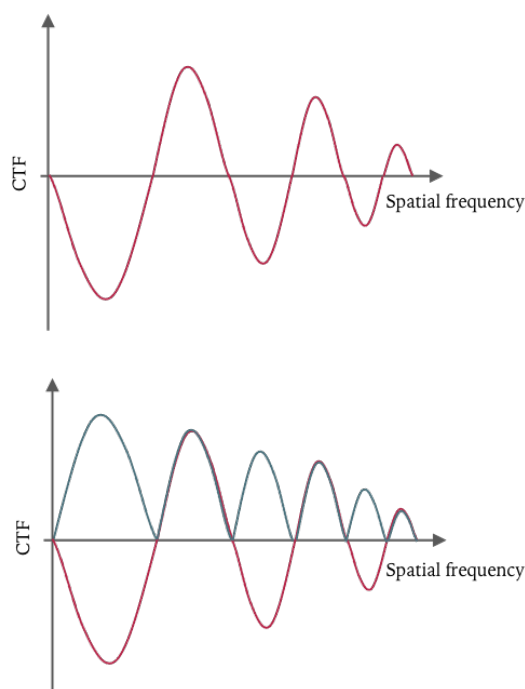


Figure 0.5 CTF correction

A) CTF an image B) CTF after correction using phase flipping: before correction (red) and after correction (blue)

2.6.1.7 Plane group determination

The crystals' space group was calculated using *ALLSPACE*, which compared the final amplitude and phase values of symmetry related diffraction spots (phase residual) against all plane groups (Valpuesta *et al.*, 1994). The predicted symmetry related phases and phase residual is compared against the expected theoretical for a particular plane group, given the SNR. The output generated a phase residual table comparing to the different plane groups; the phase residual should be at or better than the expected theoretical thus, the lowest value in the table. The plane group determination was repeated across images of the same crystal before establishing the symmetry applied for processing of a particular crystal type. The symmetry was applied for further amplitude and phase averaging and thus to enhance the SNR of the final map (Amos, 1982).

2.6.1.8 Phase origin

The software initially sets the phase origin as the position from which the Fourier components were calculated in the original image. This position varies across crystals from different images. For merging of phase information, a common phase origin needs to be calculated for the crystal. For many crystals the phase origin corresponds to the symmetry axis in the unit cell which is normally positioned in the centre of the unit cell (Amos, 1982).

The *ALLSPACE* program was used to calculate the phase origins for each image. The program moves the phase origin around, until it coincides in position with a real symmetry element of the crystal. At this position, the phases of symmetry related spots will be in the best agreement, as well as the correlation to the densities in the real space computed map. Upon merging data, the phase origin will remain the same however the relative position will need to be adjusted by comparing different crystals until the best correlation was found. This was done by using one of the best images from the dataset as a reference and all further images were refined with respect to this. With tilted data, the refinement was conducted initially with untilted data, followed by incrementally adding tilted data (Amos, 1982). This incremental approach is important to ensure the phase origin is not skewed by images that are drastically different from the dataset as it will affect further merging.

2.6.1.9 Density map

For untilted views, the reciprocal lattice points can be used to calculate the projection view of the structure (Amos, 1982) through a Fourier synthesis. The *CCP4* program *NPO* plots these coordinates to generate a 2D density map.

2.6.1.10 3D reconstruction

3D data were merged to a common phase origin. This involved initially merging low angle data and successively adding higher tilts. The tilted data sample different positions of the lattice lines in the z^* axis (chapter 1.2.2.2). The program *LATLINE* was used to plot the interpolated amplitude and phase values along z^* . In order to sample the interpolated lattice lines at discrete regular intervals, a pre-set value of c , the real-space unit cell length in z -axis, was set using the estimated 1.5-2x crystal

thickness. The lattice line curves were used for further refinement of the tilt angle and phase origin by *ORIGTILT*, which refines the tilt angle and phase origin to minimise phase residual in cycles. The overall phase residual provides an indication of the quality of the merged dataset.

Due to the missing cone of information 0,0,1 data were not acquired. These values were estimated by generating a plot of contrast at different planes in the z-sections of the calculated volume map. The 0,0,1 amplitude values were scaled against the 1,0,0 values. These values were added as (0,0,1), (0,0,2) and (0,0,3) indices used for volume map generation.

The complete set of *hkl* data was then converted into *mtz* files to calculate the real-space EM density by plotting the inverse FT in 3D. The reconstruction was displayed in *ChimeraX* and the volume threshold set as described in equation 1. The mass was calculated based on the protein mass (Da) multiplied by the constant for atomic mass (1.66×10^{-24} g). As the output reconstruction displayed a 2x2 unit cell model this value was multiplied by 24. This was then divided by an assumed average protein density (1.35 g cm^{-3}) to calculate the overall volume. An appropriate threshold contour level was set to reflect the volume calculated.

Equation 1

$$Volume = \frac{Mass}{Density} = \frac{Protein\ mass(Da) \times 24\ subunits \times Atomic\ mass\ unit}{Average\ protein\ density}$$

2.6.1.11 Temperature factor or B-factor

The structure factor amplitudes drop at higher resolutions (chapter 1.2.2.3-1.2.2.4), thus a temperature factor or B-factor was added to correct the fading amplitudes at increasing resolutions.

SCALIMAMP3D was used to correct image amplitudes (Havelka *et al.*, 1995) before merging by comparing with a reference structure such as bacteriorhodopsin (Henderson *et al.*, 1990). Individual temperature factors can be assigned to images, thus allowing the correction of images of differing

qualities. A default maximum setting in *SCALIMAMP3D* of 1,000 was applied, any values greater than this were down weighted to prevent amplification of noise.

2.6.2 Tomography processing

The IMOD software package (Mastronarde and Held, 2017) was used for tomography processing and *AMIRA* (Thermo Fisher Scientific) was used to generate the 3D surface model. This section will give an overview of the tomography processing workflow, displayed in Figure 2.6, all the programs used are italicised in capitals.

2.6.2.1 Pre-processing

Before any tomography processing could be conducted, the dataset was screened for aberrant pixels. X-rays and flaws in the detector can cause extreme, high or low, intensity pixels that affect processing. *CCDERASER* was used to identify and remove these pixels from the dataset.

2.6.2.2 Coarse alignment

An initial crude alignment of the tilt series was required before more refined approaches were implemented. This was done by cross-correlation using the *TILTXCORR*, which determines the x and y translations required for aligning each image.

2.6.2.3 Alignment

Accurate alignment of the tilt series is fundamental for generating tomograms. It relies on tracking movement of local areas in the tilt series for correction. Whole spore tilt series were aligned using gold fiducial markers, as shown in Figure 2.6 - each fiducial used is circled in green. The fiducial markers were mapped using *AUTOFIDSEED*, an automated fiducial mapping approach, or manually selected. *BEADTRACK* tracked the mapped fiducial markers throughout the tilt series.

Tilt series of sections did not possess gold fiducial markers; thus, patch tracking was used for alignment. Each patch in the tilt series (Figure 2.6 – patches circles in pink) possessed a trajectory

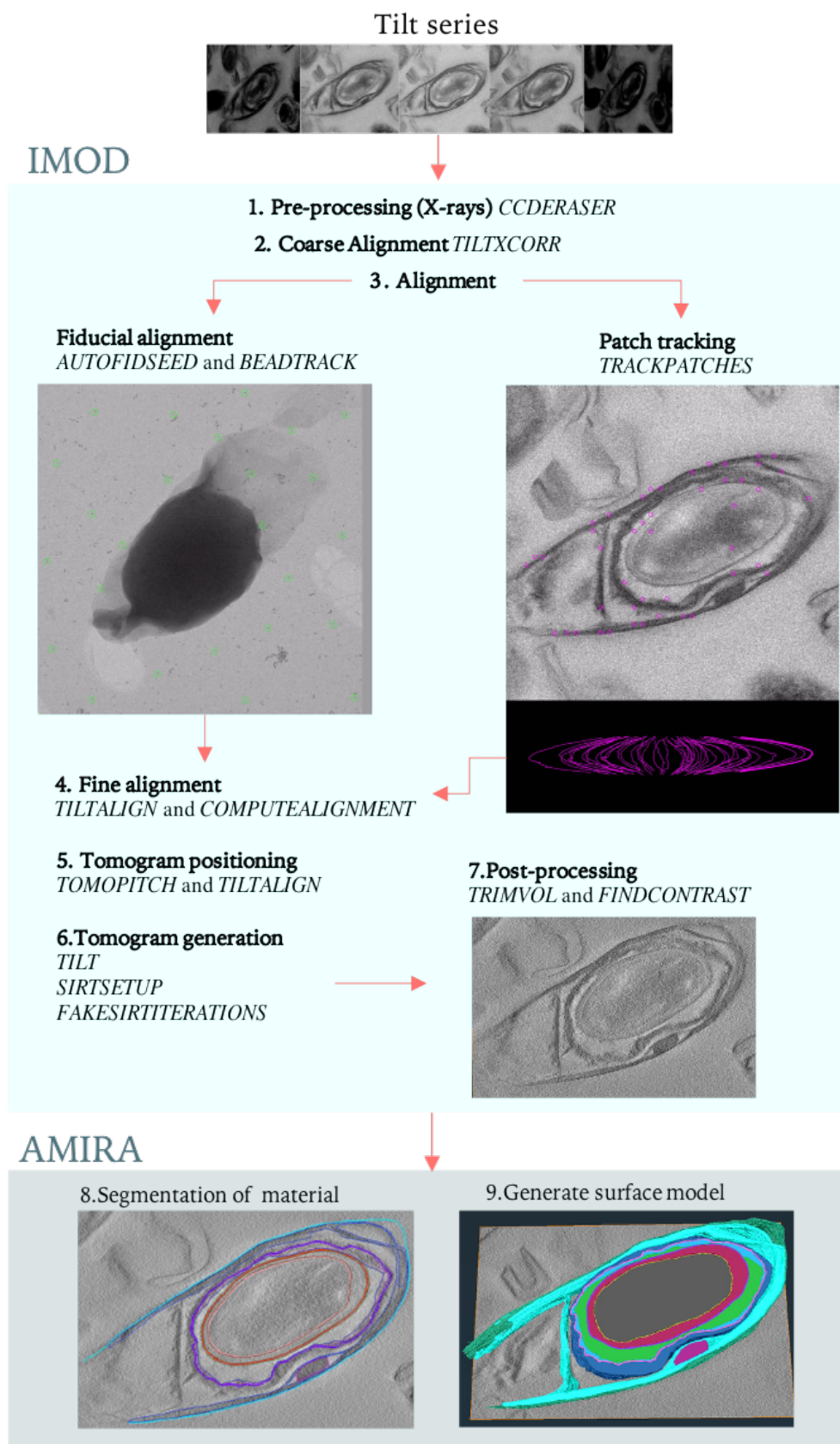


Figure 0.6 Workflow for processing tomography data

through the tilt series (Figure 2.6 – pink trajectory lines) which helped determine how well the tracking was maintained. The patch of interest is referred to as a contour, which can be viewed as points in 2D or trajectory lines in the 3D volume. Many parameters were refined to improve patch tracking including adjusting the patch size and using boundary models - where a boundary was set to only include the sample. Some patches were difficult to track successfully, here contours were broken into smaller trajectories. Note this did not always work and sometimes gave misleading results as it appeared to improve the trajectory lines but not the overall alignment, thus breaking contours should be used with caution. *TRACKPATCHES* was used for tracking patches through the tilt series.

2.6.2.4 Fine alignment

The fiducial or patch tracking model defined in the ‘alignment stage’ was enforced on the full tilt series by running *TILTALIGN*. Using the mean residual error output from *COMPUTEALIGNMENT*, the initial model was adjusted in *3dmod* to remove patches/fiducials with large errors that skewed the data. This was an iterative approach, as refined fine alignments improved the tomogram generated at the end. It is worth noting that patch tracking gave much higher residual errors (~20-30 pixels residual error mean) compared to gold fiducial markers (~0.6-0.9 pixels residual error mean).

2.6.2.5 Tomogram positioning

Tomogram generation is a computationally intensive process, to facilitate this, the reconstruction volume must be made as small as possible to only include the sample of interest. This is done in the *Tomogram Positioning* step, where a boundary of maximum thickness is set across three regions in the tomogram: top, middle and bottom of the y axis. Due to the size of the spores, a whole tomogram was computed from binned images for tomogram positioning. *TOMOPITCH* analyses the boundaries set and recommended pitch and z-shift changes to flatten the sections in z and x axis respectively. *TILTALIGN* generated the final alignment.

2.6.2.6 Tomogram generation

Tomogram reconstruction can be done using two approaches: WBP or SIRT, described in chapter 1.2.3.4. This project employed the option of WBP with SIRT-like filters that provided reduced noise

levels without the computationally intensive scripts. This was run through the *TILT* script with *FAKESIRTITERATIONS* at 50 iterations.

2.6.2.7 Post-processing

After the tomogram generation, post-processing was conducted by trimming the volume and scaling the contrast. Volume trimming generated a tomogram that only included the sample of interest. The boundaries were manually drawn and *TRIMVOL* was used to cut the volume to the specified dimensions. To ensure that the contrast levels in the sample were based on biological features, a square was drawn of a representative region in the sample. *FINDCONTRAST* uses this square, finds and applies the optimal contrast settings for the tomogram.

2.6.2.8 3D surface models

The software used for generating 3D surface models was AMIRA (Thermo Fisher Scientific). A 3x binned tomogram was used for this processing. Segmentation was done from the outermost assemblies working inwards, using different tools depending on the shape and whether the assembly was enclosed. Assemblies such as the dense body and the core were done using the lasso tool and interpolated between every 10-15 sections. All other layers were segmented using the brush tool and interpolated between every 5-10 sections. After interpolation, gaps were filled in manually using the brush tool. For visualisation of the material, the *GENERATESURFACE* tool was used which forms a 3D surface using a polygonal mesh. The final 3D surface was generated from a surface mesh, the mesh density was reduced by 2.6x to create a smooth visual finish.

Chapter 3 –Unveiling *Clostridium sporogenes* external layers

3.1 Introduction

In *C. sporogenes* the outermost layer is the exosporium, a loosely fitted baggy layer with an interspace region, considered to play an important role in germination, vegetative cell outgrowth and colonisation (Brunt *et al.*, 2015; Panessa-Warren *et al.*, 1994; 1997). Its morphology is similar to the exosporium from the *Bacillus cereus* family (Kailas *et al.*, 2011; Terry *et al.*, 2017). However, unlike the *Bacillus cereus* family, the exosporium across *Clostridia* endospores remains understudied.

This chapter will focus on *C. sporogenes*, a model organism for studying group I *C. botulinum* (Carter and Peck, 2015), the causative agent of botulism: a severe and life-threatening disease caused by the release of highly potent neurotoxins (Peck *et al.*, 2011) (Chapter 1.3.2.1). In addition to gaining insight into *C. botulinum* we also hope to gain an understanding of the exosporium across other *Clostridia* particularly its involvement in the germination process and its potential protective role; especially as parallels have been identified between the exosporium of *C. sporogenes* and other *Clostridia* on both a biochemical (Janganan *et al.*, 2016) and structural level (Janganan *et al.*, 2020).

As established in chapter 1.3.3.5.2 three cysteine-rich proteins were identified in *C. sporogenes*: CsxA, CsxB and CsxC (Janganan *et al.*, 2016). All three proteins are conserved across *C. sporogenes* and group I *C. botulinum* strains (Janganan *et al.*, 2016). However, only CsxA appears to have homologues in group II *C. botulinum* (Janganan *et al.*, 2016). Based on the involvement of cysteine-rich proteins in layers such as the exosporium in *Bacillus* (Kailas *et al.*, 2011; Terry *et al.*, 2017), it is likely that these proteins are involved in the assembly of spore layers however their involvement is yet to be characterized.

3.2 Aims

The aim of the work described in this chapter was to interrogate the roles of cysteine-rich proteins in both the structure and assembly of *C. sporogenes* spore envelope, through structural analysis of mutants. In addition to the exosporium, thin-section analysis of *C. sporogenes* demonstrated para-

sporal layers in the interspace (Hoeniger and Headley, 1969; Warren *et al.*, 1994) could these layers also be para-crystalline, as was found for *Bacillus thuringiensis* (Ebersold *et al.*, 1981; Wehrli *et al.*, 1980)? Are the cysteine-rich proteins involved in these assemblies?

Negative stain TEM was employed to examine spore phenotypes across WT and mutants, particularly focused on the outermost layers. Do any of the mutants cause a change in phenotype of enveloping layers? If so, could they have an analogous role to the ExsY or CotY proteins from *Bacillus*? Width and length analyses were also conducted to see if the knockouts drastically affected internal layers, although this would require thin section analysis for confirmation. Electron crystallography was used to identify and classify crystal types observed on enveloping layers across the spores. Image processing of these micrographs provided insight into the structure of these layers. By screening both WT and mutants we aimed to identify the involvement of Csx proteins in these assemblies. We suspected that one of these proteins will compose the exosporium basal layer, and by knocking it out the basal layer will be absent.

3.3 Results

3.3.1 WT *C. sporogenes* ATCC15579 endospore morphology

This WT strain was provided by Jason Brunt (University of Cambridge) as the background strain for the mutant spores. TEM micrographs display a homogenous population (Figure 3.1). All spores have a dense spore body (pink arrow) with a baggy exosporium layer (blue arrow) that encapsulates the spore and extends out from one of the poles. High magnification micrographs of the outermost layers revealed a para-crystalline layer with a hairy-nap on the surface of the spores (Figure 3.2). The hairy nap in this strain appeared as well-defined unstained feature, visible at the edge of the para-crystalline layer. No fibrils or appendages were found associated with these samples.

Table 3.1 demonstrates the average width and length with standard deviation. Calculations based on measurements from 30 spores. Width and length of spore body and length of enveloping layers correspond to the graph in Figure 3.3 and Figure 3.4 respectively. This demonstrates a range in size of the spore body (Figure 3.3) and in the length of enveloping layers (Figure 3.4)

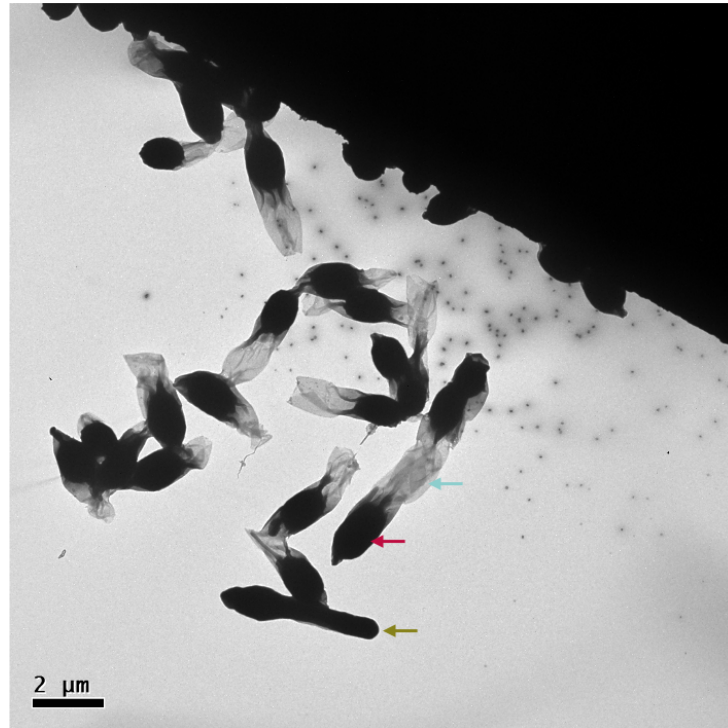


Figure 3.1 *C. sporogenes* ATCC15579 endospores by TEM

Arrows indicate spore body (pink) and exosporium layer (blue). The green arrow highlights a possible germinating spore.

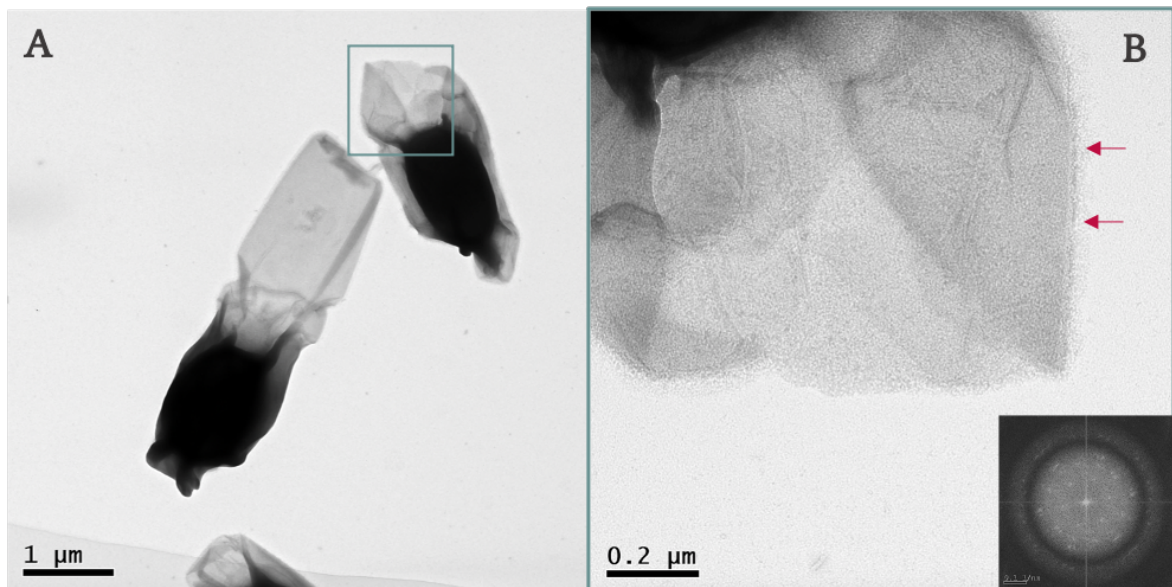


Figure 3.2 *C. sporogenes* ATCC15579 endospore and exosporium by TEM

A) Low magnification of *C. sporogenes* spores display dense spore body surrounded by a baggy translucent layer (exosporium) B) High magnification image of the exosporium extended from the pole of the spore. Crystalline nature of the layer is displayed in FFT as a diffraction pattern. Arrows point out the hairy nap on the surface of the exosporium. Unit cell dimensions $a = b = 106 \text{ \AA}$ and $\gamma = 120^\circ$

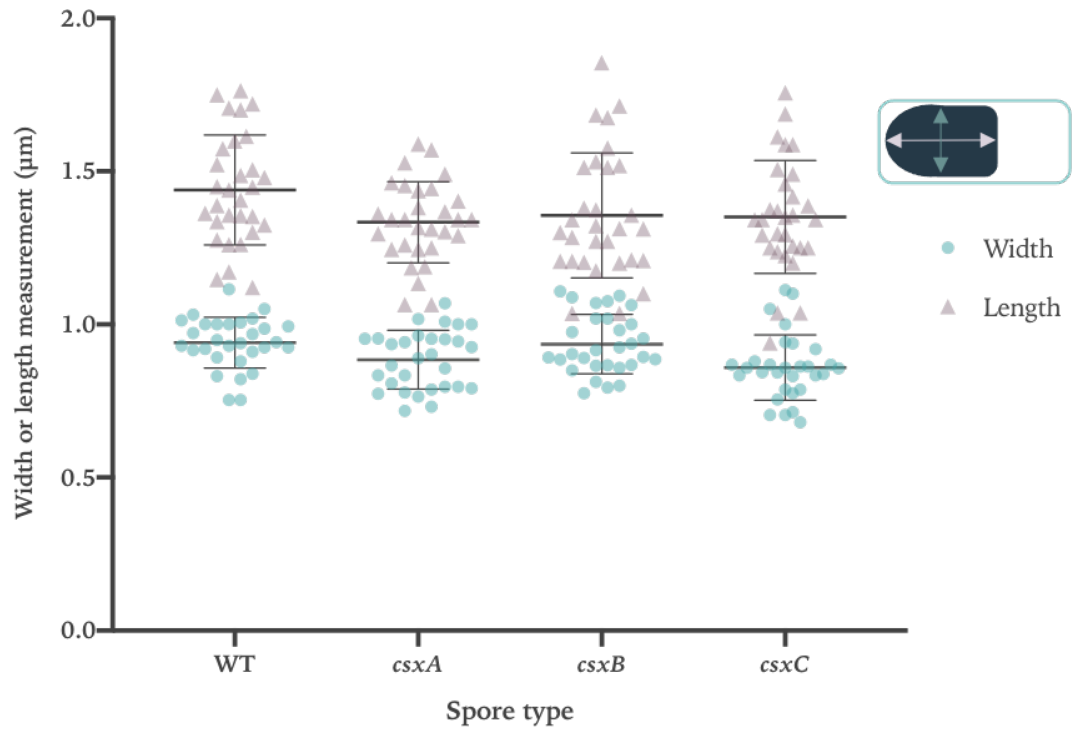


Figure 3.3 Width and length analysis of *C. sporogenes* WT and mutant endospores

~30 spores were subject to measurements of width (green circles) and length (purple triangles) of main spore body. Grey lines display the mean and error bars the standard deviation. WT endospores 0.94 ± 0.08 µm (W), 1.44 ± 0.18 µm (L), $\Delta csxA$ 0.88 ± 0.09 µm (W), 1.33 ± 0.13 µm (L), $\Delta csxB$ 0.94 ± 0.09 µm (W), 1.36 ± 0.2 µm (L), $\Delta csxC$ 0.86 ± 0.11 µm (W), 1.35 ± 0.18 µm (L).

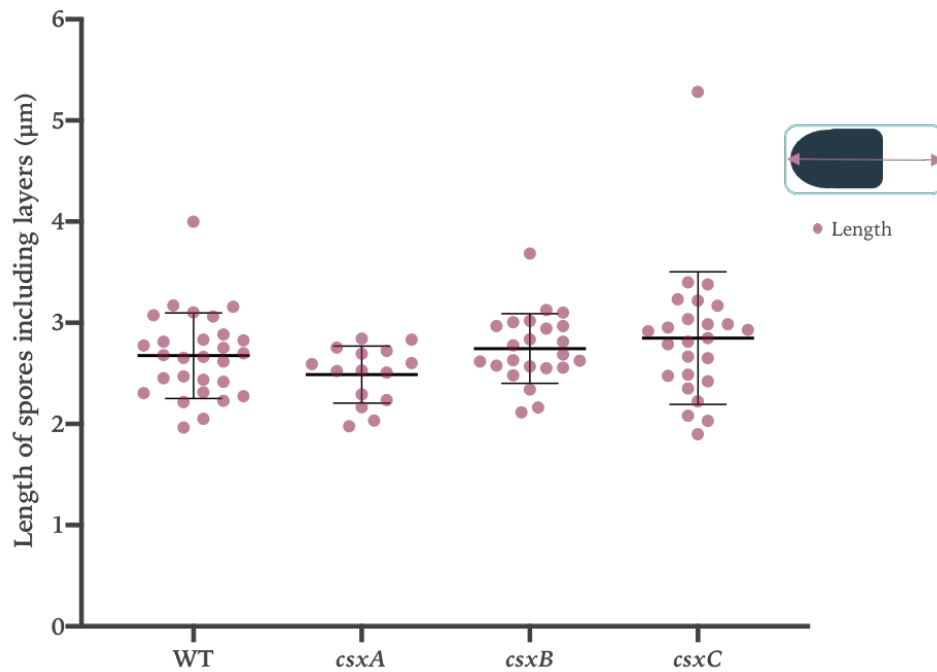


Figure 3.4 Length analysis of *C. sporogenes* WT and mutant endospores outermost envelope

~30 spores were subject to length measurements of their outermost envelope from the short pole to the elongated pole, this was not possible on all spores where the edge of the outermost layer was not visible. Grey lines display the mean and error bars the standard deviation. WT endospores 2.68 ± 0.42 µm, $\Delta csxA$ 2.49 ± 0.28 µm, $\Delta csxB$ 2.75 ± 0.34 µm, $\Delta csxC$ 2.85 ± 0.66 µm.

Table 3.1 Width and length measurements of WT and mutant spores

Spore	Width of spore body (μm)	Length of spore body(μm)	Length including enveloping layers (μm)
WT	0.9 ± 0.1	1.4 ± 0.2	2.7 ± 0.4
ΔcsxA	0.9 ± 0.1	1.3 ± 0.1	2.5 ± 0.3
ΔcsxB	0.9 ± 0.1	1.4 ± 0.2	2.8 ± 0.3
ΔcsxC	0.9 ± 0.1	1.4 ± 0.2	2.9 ± 0.7

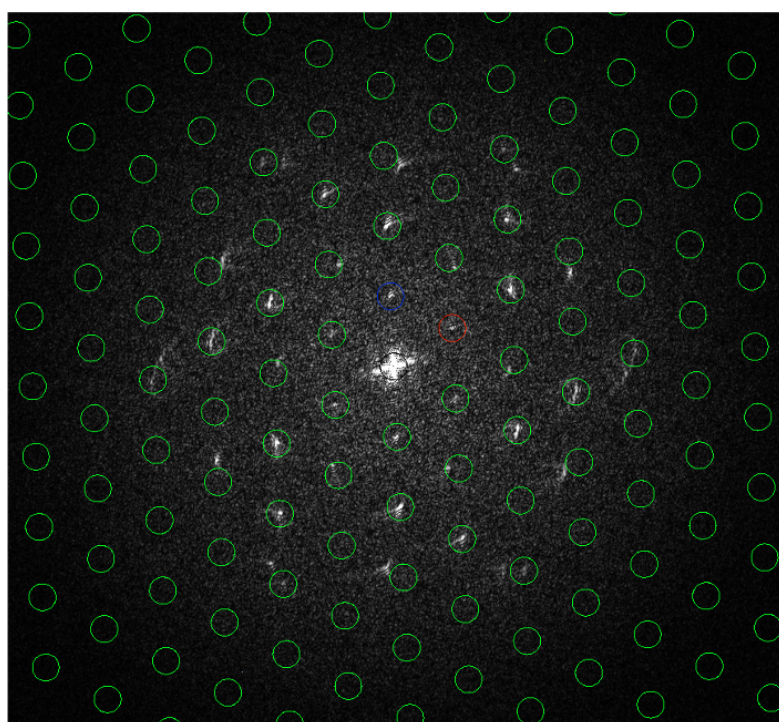


Figure 3.5 Indexed FFT of exosporium from *C. sporogenes* endospores by TEM

Diffraction spots of WT exosporium, the lattice is highlighted with green circles. h, k coordinates highlighted in blue and red circles, respectively. Unit cell dimensions $a = b = 106 \text{ \AA}$ and $\gamma = 120^\circ$

3.3.1.1 *C. sporogenes* ATCC15579 exosporium

High magnification micrographs of the exosporium (Figure 3.2b) were used to obtain structural information by electron crystallography. The FFT displays the diffraction pattern of the crystalline layer – Figure 3.5 displays the h, k coordinates on an indexed FFT. Unit cell parameters were calculated as an average from 3 images; $a \sim b \sim 108 \pm 1 \text{ \AA}$, $\gamma \sim 120 \pm 1^\circ$. Both the diffraction pattern and unit cell reveal consistency with the exosporium from NCIMB 701792 (Janganan *et al.*, 2020).

3.3.2 Mutant structural phenotype screening

Jason Brunt provided $\Delta csxA$, $\Delta csxB$ and $\Delta csxC$ mutant spores generated using the ClosTron technology (Heap *et al.*, 2007). These mutants were imaged by negative stain TEM (Figure 3.6).

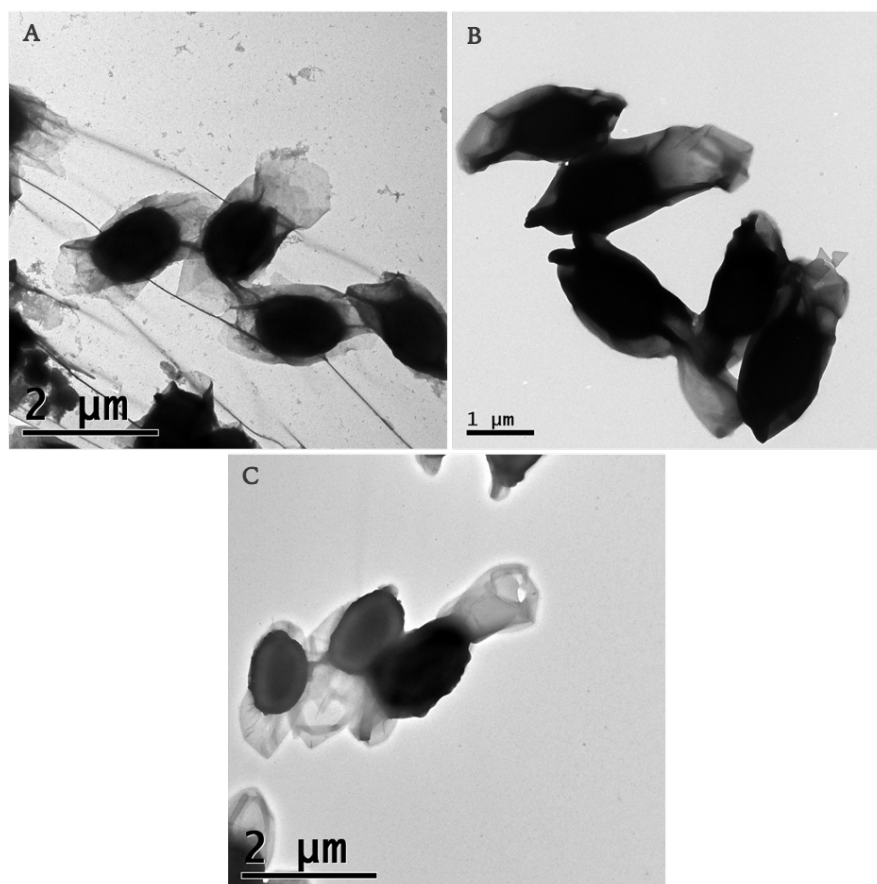


Figure 3.6 *C. sporogenes* mutant endospores in Csx proteins by TEM

A) $\Delta csxA$: outermost layers are broken and frayed, no elongated layer at the pole of the spores B) $\Delta csxB$: outermost layers appear intact, encapsulates whole spore with an elongated layer at one of the spore poles C) $\Delta csxC$: all spores possess a layer extending from one pole, this layer does not appear to encapsulate the whole spore body. Some spores also possess short layers at the opposite pole of the spore.

3.3.2.1 $\Delta csxA$

TEM micrographs of $\Delta csxA$ mutant spores (Figure 3.6A) lack the continuous outermost layer that encapsulates the whole spore. Instead outermost layers present in patches, which appear broken and frayed at the edges. These layers cover the spore body but do not extend from the spore pole. Width and length analysis (Figure 3.3 and 3.4), conducted on 30 spores, suggest a diverse range in sizes displayed in Table 3.1. These are not statistically significant different from WT spores.

High magnification micrographs of these layers indicate a crystalline assembly with no hairy nap (Figure 3.7). Image processing of these layers demonstrates a crystal type that is different from WT exosporium. This crystal type has been named ‘type II’ crystal with a unit cell length of $a = b = 64.8 \pm 0.8 \text{ \AA}$, $\gamma = 119.8^\circ \pm 0.5^\circ$ (calculated from 5 images). An indexed FFT of type II crystal is displayed in Figure 3.8.

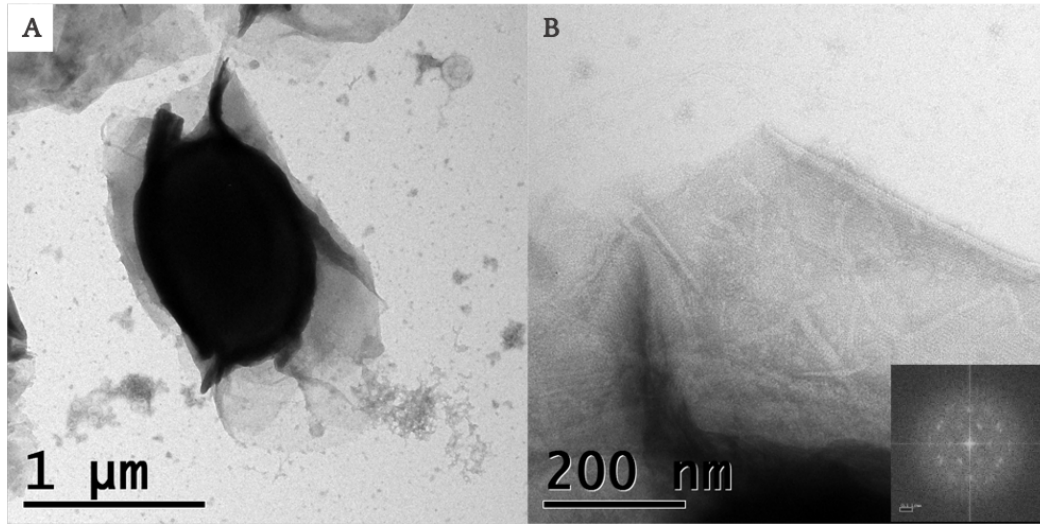


Figure 3.7 $\Delta csxA$ *C. sporogenes* mutant by TEM

A) Low magnification micrograph of the spore B) High magnification micrograph of the outermost layers visible at the edge of the spores. The crystalline nature of the layer is displayed in inset FFT in the diffraction pattern. No hairy nap observed on the edge of these layers. FFT scale bar = 0.1 nm^{-1}

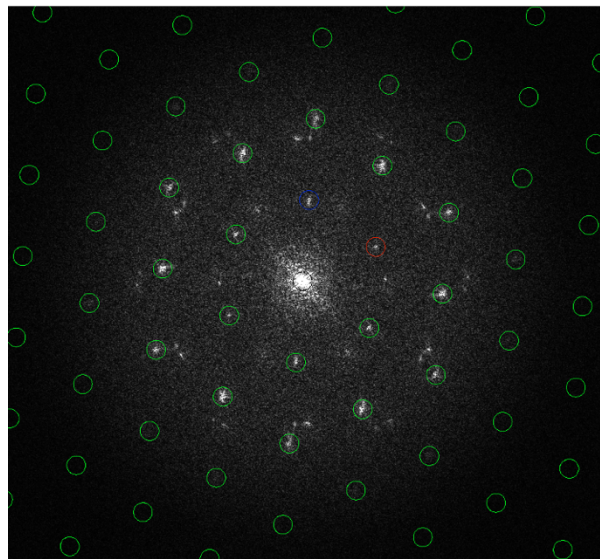


Figure 3.8 Indexed FFT of type II crystal observed on the surface layers of $\Delta csxA$ *C. sporogenes* mutant

Lattice selected in green circles. h , k coordinates highlighted in blue and red circles, respectively. Unit cell dimensions $a = b = 65 \text{ \AA}$ and $\gamma = 120^\circ$

3.3.2.2 $\Delta csxB$

Morphologically, there appears to be little difference between WT and $\Delta csxB$ spores, as they all possess a baggy layer that covers the whole spore body and extends from one of the spore poles (Figure 3.6B). Width and length analysis (Table 3.1) across 30 spores also displays no apparent difference from WT with ranging sizes of spore body (Figure 3.3 and 3.4).

Unlike WT spore, high magnification micrographs of the enveloping layers revealed a disordered crystalline assembly with two main crystal types observed. ‘Type I’ crystal (Figure 3.9) possessed unit cell dimensions of $a = b = 106 \pm 0.7\text{\AA}$ and $\gamma = 120.2^\circ \pm 1.6^\circ$ (calculated from two merged images) (Figure 3.11A), similar to WT exosporium. ‘Type III’ crystal (Figure 3.10) possessed unit cell of $a = b = 52.9 \pm 0.7\text{\AA}$, $\gamma = 120^\circ \pm 0.3^\circ$ (Figure 3.11B). Both crystal types were identified at many positions around the spore including the short pole, edge of the spore body and elongated pole (Figure 3.9 and 3.10). Most spores possessed both crystal types in different positions of the spore or as patches, however some only had either type I or type III.

A hairy nap was also observed on the edge of layers with both crystal type I and III (Figure 3.9 and 3.10). The hairy nap appeared less dense when compared to WT, such as in Figure 3.9B and D. This was not the case on all spores as is observed in Figure 3.9F which possesses a much denser hairy nap.

In addition to this another crystal type was identified, as a patch close to the spore body (Figure 3.10E,F). This crystal possessed a unit cell of $a = b = 64.7 \pm 1.1\text{\AA}$ and $\gamma = 120.2^\circ \pm 1.6^\circ$ (from 3 images) (Figure 3.11C). It was only observed on 3 occasions in combination with other crystal types, thus no correlation with hairy nap was possible. Due to the low abundance of this crystal type no further structural information was obtained.

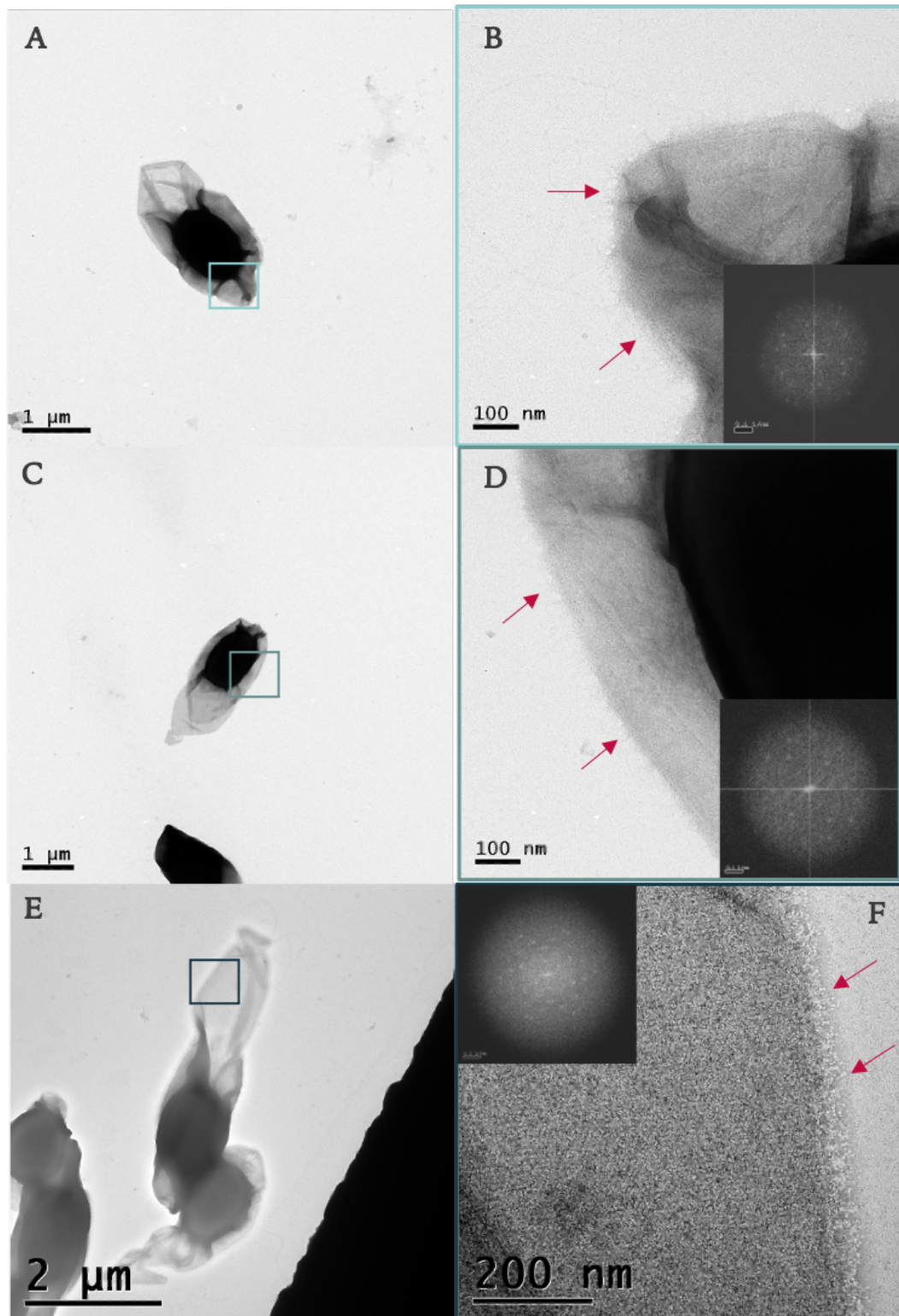


Figure 3.9 Type I crystal on $\Delta csxB$ *C. sporogenes* mutant spores

A,C,E) Low magnification images of $\Delta csxB$ spores, box indicates the location of high magnification displayed in B,D,F. FFT inset displays diffraction pattern of type I crystal B) Crystal present on short pole of spore (A), D) type I crystal present on the edge of spore (C), F) elongated pole of spore (E). Arrows label hairy-nap observed on the edges of outermost layers. FFT scale bars = 0.1nm^{-1}

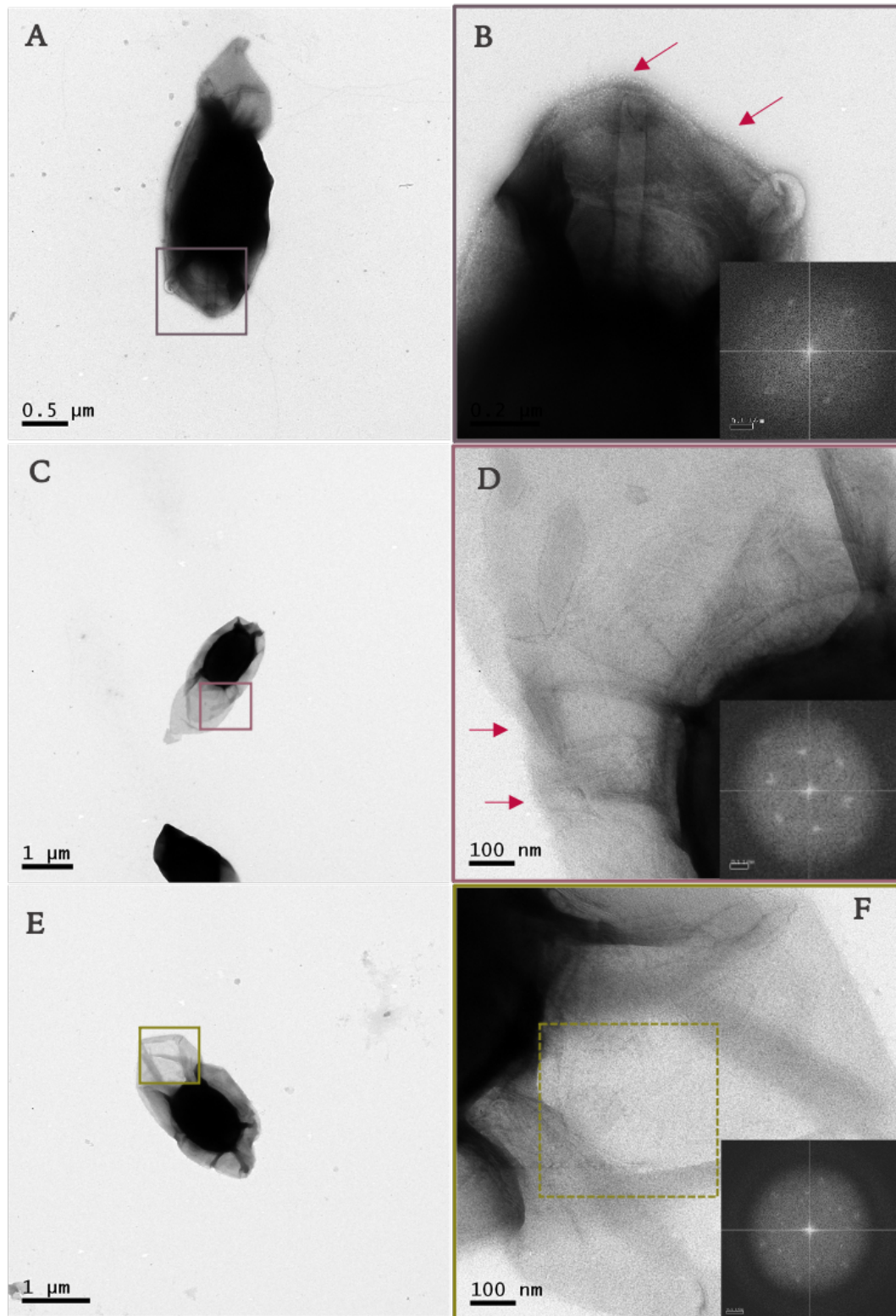


Figure 3.10 Alternative crystal types observed on $\Delta csxB$ *C. sporogenes* mutant spores

A,C,E) Low magnification images of $\Delta csxB$ spores, box indicates the location of high magnification displayed in B,D,F. FFT inset displays diffraction pattern A-D) Endospores with type III crystal at short pole (B) and elongated pole (D) corresponding to spores (A) and (C) respectively. E,F) Endospore with other crystal type $\sim 65\text{\AA}$ unit cell length present as a patch at the elongated pole close to spore body. Inset FFT in (F) displays diffraction pattern from square patch. Arrows label hairy-nap observed on the edges of outermost layers. FFT scale bars = 0.1nm^{-1}

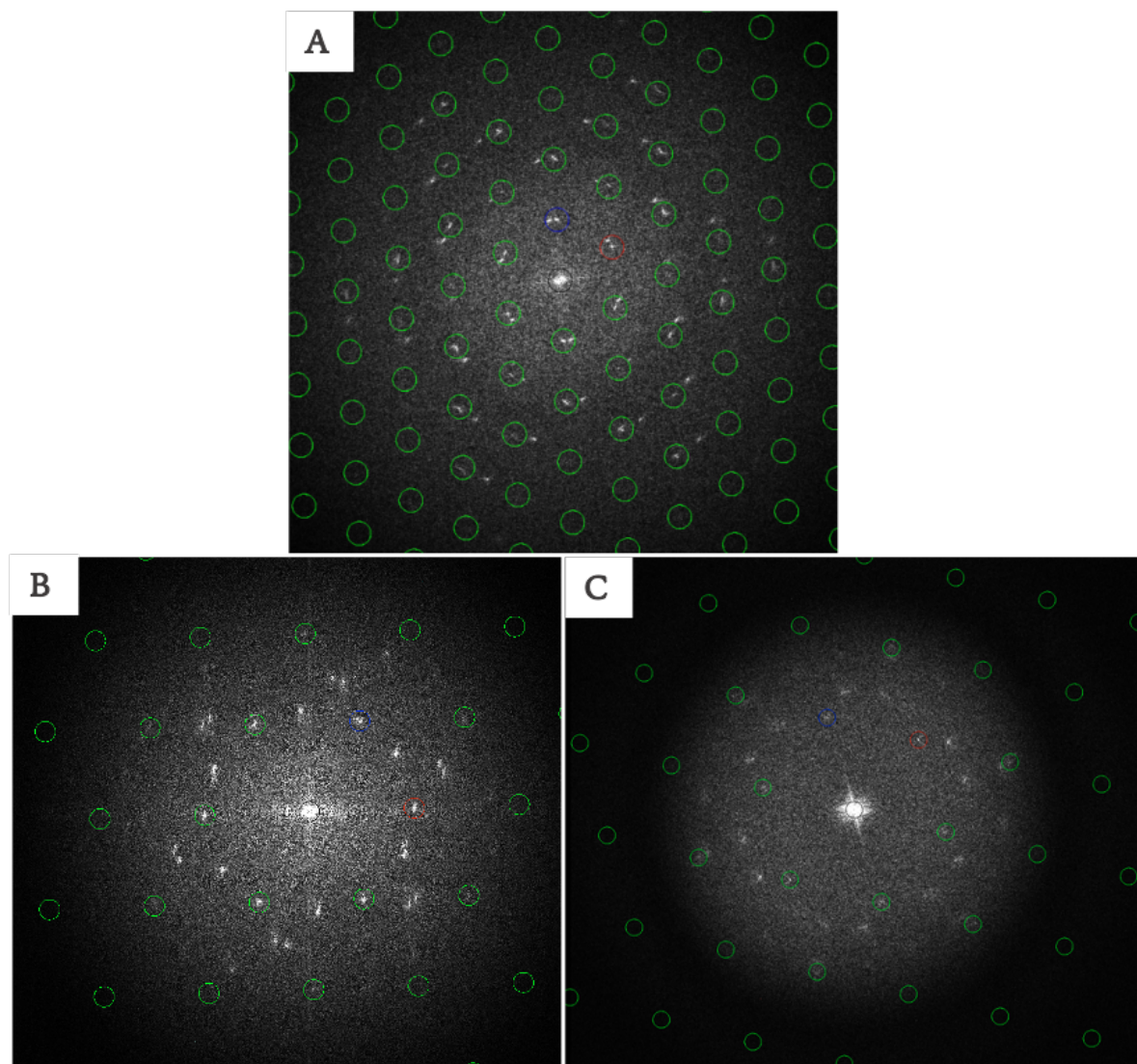


Figure 3.11 Indexed FFT of crystal types observed on the outermost layers of $\Delta csxB$ *C. sporogenes* mutant

Lattice selected in green circles. h , k coordinates highlighted in blue and red circles respectively A) Type I crystal: $a = b = 106 \text{ \AA}$ and $\gamma = 120^\circ$, B) Type III crystal: $a = b = 52 \text{ \AA}$ and $\gamma = 120^\circ$ C) Type V crystal: $a = b = 65 \text{ \AA}$ and $\gamma = 120^\circ$

3.3.2.3 $\Delta csxC$

The phenotypes of this mutant were more diverse (Figure 3.6). Many spores possessed observable layers protruding from both poles of the spore; a short and an elongated pole (Figure 3.12,3.13). Less abundant were spores that only appeared to have an elongated pole. Across all spores few to no layers were observed around the long edges of the spore. Width and length analysis indicated varied sizes, not significantly different from WT spores (Table 3.1) (Figure 3.3 and 3.4).

Two crystal types were also observed in $\Delta csxC$: ‘type I’ (Figure 3.12) with a unit cell of $a=b= 107 \pm 1.3\text{\AA}$, $\gamma = 118.8^\circ$ (Figure 3.14A), and ‘type IV’ (Figure 3.13) with a unit cell of $a=b= 49 \pm 1.2\text{\AA}$, $\gamma = 119.5^\circ \pm 0.9^\circ$ (Figure 3.14B). Type I crystals were named based on their similarity to type I in $\Delta csxB$, whereas type IV was named differently from type III due to their structural differences discussed in the next section. Type I and IV crystals are visibly different on TEM micrographs, as features in type IV appear more heavily stained than type I. Type IV crystals were also more abundant than type I.

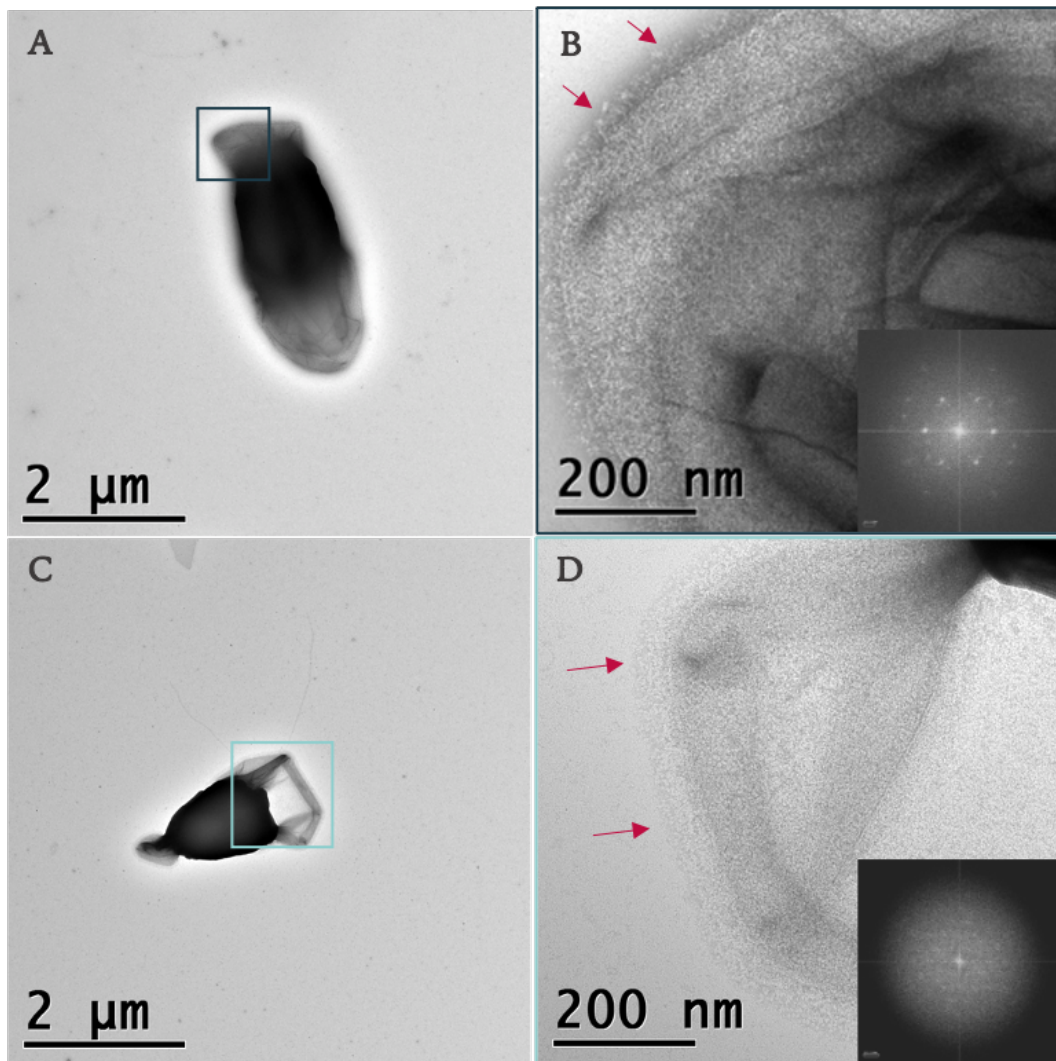


Figure 3.12 Type I crystal on $\Delta csxC$ *C. sporogenes* mutant spores

A,C) Low magnification images of $\Delta csxC$ spores, box indicates the location of high magnification displayed in B,D,F. FFT inset displays diffraction pattern of type I crystal B) Crystal present on short pole of spore (A); D) type I crystal present on the elongated pole of spore (C). Arrows label hairy-nap observed on the edges of outermost layers. FFT scale bars = 0.1nm^{-1}

Both crystal types were present at the short pole and elongated pole of the spores (Figure 3.12, 3.13), although most short poles possessed layers with no crystallinity (Figure 3.13C). The elongated poles were composed of disordered and patchy crystals comprised of either type I and IV alone (Figure 3.12D and 3.13D) or a combination of both. Many of these layers also possessed patches that lacked crystallinity.

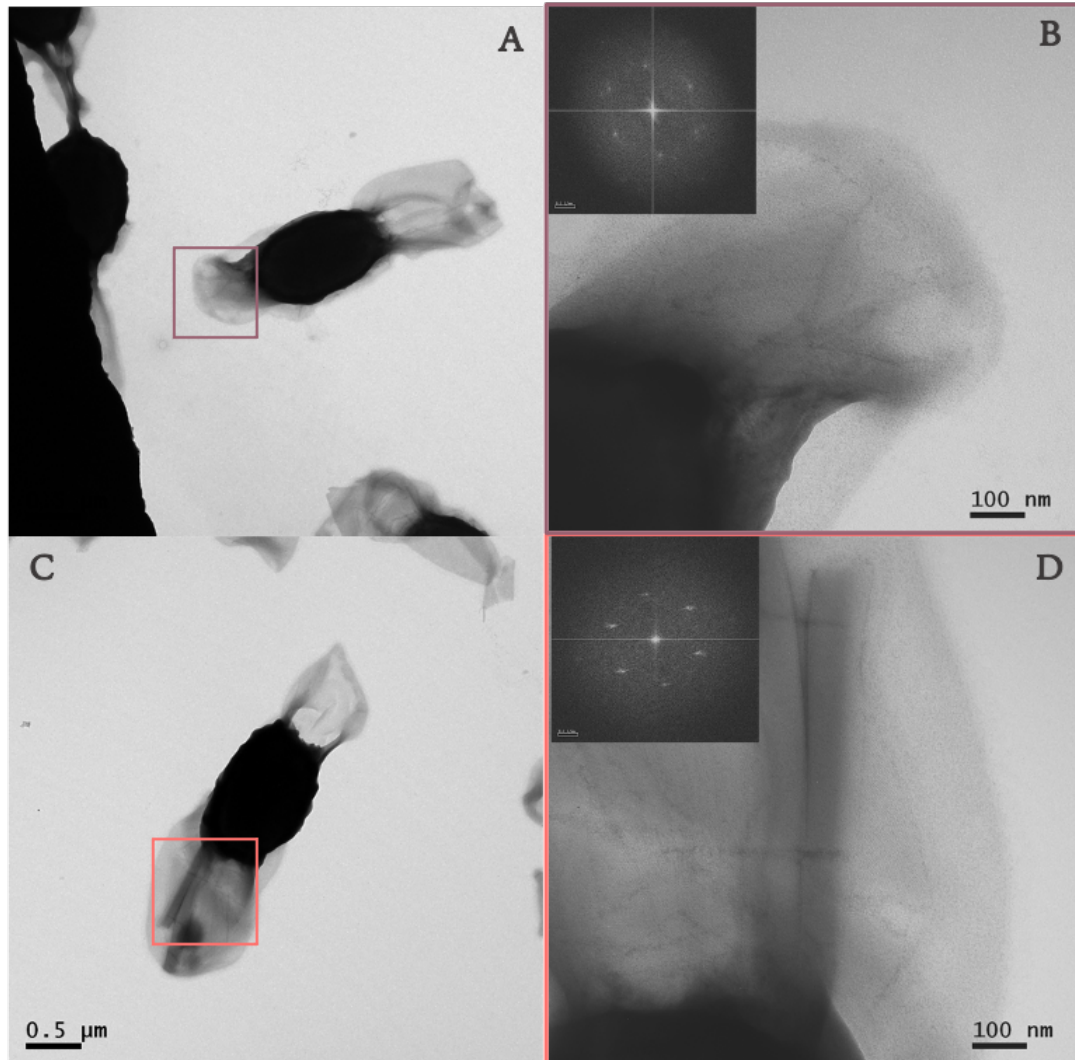


Figure 3.13 Type IV crystal on $\Delta csxC$ *C. sporogenes* mutant spores

A,C) Low magnification images of $\Delta csxC$ spores, box indicates the location of high magnification displayed in B,D,F. FFT inset displays diffraction pattern of type IV crystal B) Crystal present on short pole of spore (A), D) type IV crystal present on the elongated pole of spore (C). FFT scale bars = 0.1 nm^{-1}

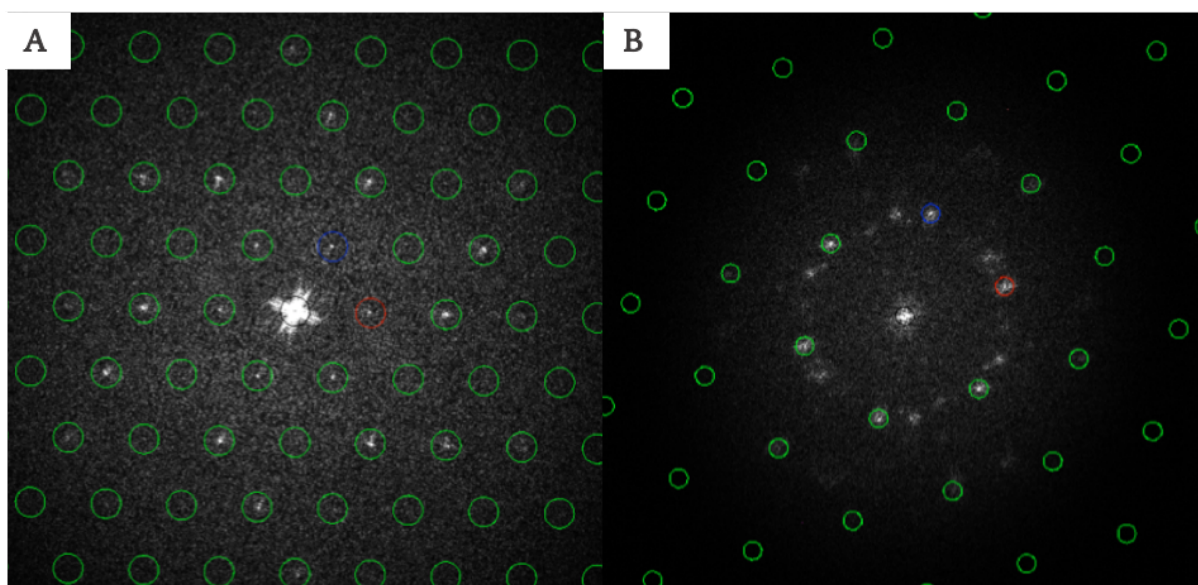


Figure 3.14 Indexed FFT of crystal types observed on the outermost layers of $\Delta csxC$ *C. sporogenes* mutant

Lattice selected in green circles. h , k coordinates highlighted in blue and red circles respectively A) Type I crystal: $a = b = 108 \text{ \AA}$ and $\gamma = 120^\circ$, B) Type III crystal: $a = b = 54 \text{ \AA}$ and $\gamma = 120^\circ$

A well-defined hairy nap, similar to WT spores, was observed on some of these layers. The hairy nap was present on all layers comprised of type I crystal at both the short pole (Figure 3.12B) and elongated pole (Figure 3.12D). Type IV crystal layers did not have hairy nap associated at either pole of the spore (Figure 3.13).

3.3.3 Electron crystallography of layers observed in mutant spores

Four distinct crystal types were identified in mutant spores. These were processed using the $2dx$ software package (chapter 2.6.1) to generate projection maps (Figure 3.15). Due to the highly disordered nature of these crystal patches, processing was particularly challenging. Thus, a low number of images were suitable for merging to generate average maps. Potential subunits have been indicated with a square symbol on Figure 3.15, however further data processing is required to confirm this, including 3D reconstructions.

3.3.3.1 Type I

This crystal type was observed in both $\Delta csxB$ and $\Delta csxC$ spores (Figure 3.9, Figure 3.12) and resembles WT exosporium. Both 2D projection maps for $\Delta csxB$ and $\Delta csxC$ type I were generated from merging 2 images (Figure 3.15B,D). They show a central ring with $p6$ symmetry and an area of low density in

the core of the ring, likely a pore (Figure 3.15B,D – square). Each ring is interconnected by threefold linkers (Figure 3.15B,D – triangle).

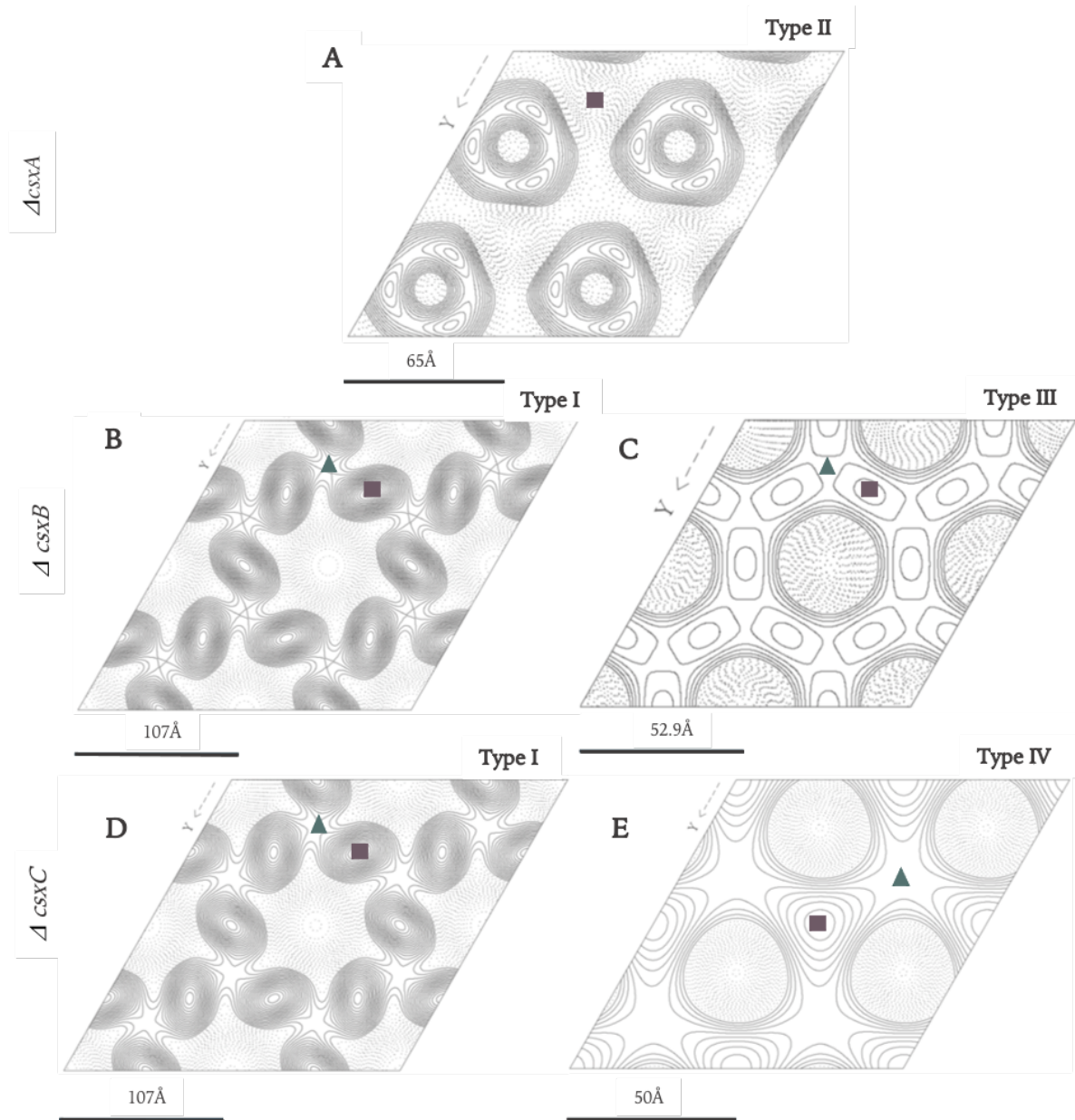


Figure 3.15 2D projection maps of para-crystalline layers observed in *C. sporogenes* mutant spores

The solid contour lines displays stain excluding areas, thus high density whereas dashed lines display low density areas. Potential individual subunits (square) and three-fold linkers where present (triangle). A) $\Delta csxA$ mutant type II lattice ($p3$ symmetry applied): 5 images merged unit cell $a=b= 64.8 \pm 0.8\text{\AA}$, $\gamma = 119.8^\circ \pm 0.5^\circ$ B) $\Delta csxB$ mutant lattice I ($p6$ symmetry applied): 2 images merged unit cell $a=b= 105.96 \pm 0.7\text{\AA}$, $\gamma = 120.2^\circ \pm 1.55^\circ$ C) $\Delta csxB$ mutant lattice III ($p6$ symmetry applied): 3 images merged unit cell $a=b= 52.9 \pm 0.7\text{\AA}$, $\gamma = 120^\circ \pm 0.3^\circ$ D) $\Delta csxC$ mutant lattice 1 ($p6$ symmetry applied): 2 images merged unit cell $a=b= 107 \pm 1.3\text{\AA}$, $\gamma = 118.8^\circ \pm 0.6^\circ$ E) $\Delta csxC$ mutant lattice 2 ($p3$ symmetry applied): 4 images merged unit cell $a=b= 49 \pm 1.2\text{\AA}$, $\gamma = 119.5^\circ \pm 0.9^\circ$.

3.3.3.2 Type II

This was the only crystal type observed surrounding the $\Delta csxA$ spore body (Figure 3.7). Analysis of the plane group suggests the crystal possessed $p3$ symmetry (Table 3.2). The 2D projection map was generated by merging 5 images, which displays a ‘ring’ with threefold symmetry with an area of low density in the core (Figure 3.15A).

3.3.3.3 Type III

This crystal type was only observed on $\Delta csxB$ spores, with roughly half the unit cell length when compared with type I crystal. Space group analysis suggests $p6$ symmetry (Table 3.3). Thus a 2D projection map was generated from 3 images merged with $p6$ symmetry applied (Figure 3.15C). The unit cell possesses a ring with sixfold symmetry with a low-density core and threefold linkers interconnecting each ring.

3.3.3.4 Type IV

$\Delta csxC$ spores possessed type IV crystal in higher abundance than type I. The crystal unit cell length was also roughly half that of type I, however space group analysis suggested $p3$ symmetry (Table 3.4). The final 2D projection map was generated from merging 4 images (Figure 3.15E). This map has a central threefold symmetry subunit that interconnects with neighbouring subunits by threefold linkers (Figure 3.15E – triangle). Regions of low density are present in between each subunit, which could correspond to pores in the layer.

Table 3.2 Space group determination table for type II lattice in $\Delta csxA$

Space group	Phase residual (90° = random)	Target residual ¹
<i>p1</i>	16.3°	
<i>p2</i>	46.8°	23.4°
<i>p3</i>	8.0°*	16.3°
<i>p312</i>	17.3°*	16.7°
<i>p321</i>	27.3°	16.9°
<i>p6</i>	28.6°	17.8°
<i>p622</i>	30.9°	17.1°

¹Target residual: expected phase residual of symmetry groups

* = Acceptable

! = Should be considered

` = Possibility

The internal phase residuals were calculated using *ALLSPACE* (2.8.1.6) for spots IQ1-IQ5 up to 20Å resolution. All acceptable space groups are * in the phase residual column. This is based on the experimental phase residual being better than the expected due to SNR. The most likely symmetry for type II crystal is *p3*, neither *p6* or *p2* are suggested.

Table 3.3 Space group determination table for crystal type III in $\Delta csxB$

Space group	Phase residual (90° = random)	Target residual ¹
<i>p1</i>	17.5°	
<i>p2</i>	3.2°*	25.2°
<i>p3</i>	1.3°*	17.5°
<i>p312</i>	2.0°*	18.4°
<i>p321</i>	1.8°*	18.4°
<i>p6</i>	2.5°*	19.1°
<i>p622</i>	2.5°*	18.3°

¹Target residual: expected phase residual of symmetry groups

* = Acceptable

! = Should be considered

` = Possibility

The internal phase residuals were calculated using *ALLSPACE* (2.8.1.6) for spots IQ1-IQ5 up to 20Å resolution. All acceptable space groups are * in the phase residual column. This is based on the experimental phase residual being better than the expected due to SNR. The most likely is *p6*, thus all other * are also possible.

Table 3.4 Space group determination table for lattice IV from $\Delta csxC$

Space group	Phase residual (90° = random)	Target residual ¹
<i>p1</i>	15.3°	
<i>p2</i>	31.3°	21.9°
<i>p3</i>	4.1°*	15.3°
<i>p312</i>	12.1°*	16.0°
<i>p321</i>	13.6°*	16.3°
<i>p6</i>	23.0°	16.9°
<i>p622</i>	21.5°	16.1°

¹Target residual: expected phase residual of symmetry groups

* = Acceptable

! = Should be considered

` = Possibility

The internal phase residuals were calculated using *ALLSPACE* (2.8.1.6) for spots IQ1-IQ5 up to 20Å resolution. All acceptable space groups are * in the phase residual column. This is based on the experimental phase residual being better than the expected due to SNR. The most likely is *p3*, not likely to be *p6* as neither *p6* or *p2* are suggested.

3.4 Discussion

3.4.1 WT *C. sporogenes* spores – similarity with other strains

The NCIMB 701792 strain used to characterise *C. sporogenes* exosporium structure and composition (Janganan *et al.*, 2016; 2020) was not amenable for mutational studies by ClosTron (Heap *et al.*, 2007) due to the strain's erythromycin resistance. Alternatively, mutants were generated in ATCC15579, an appropriate model strain for studying *C. botulinum* group I (Carter and Peck, 2015).

Thin sections of this strain revealed a 'classical exosporium' morphology (Brunt *et al.*, 2015), a large baggy outermost layer with an interspace, as observed in characterised NCIMB 701792 strain (Janganan *et al.*, 2016). This work aimed to confirm structural similarities between the *C. sporogenes* strains, so that our current understanding from NCIMB 701792 could be extended to ATCC15579. As CsxA is widely conserved across *C. sporogenes* strains (Janganan *et al.*, 2016), no significant difference was expected between the basal layer structure. It also meant that any difference observed in the mutants were likely relevant across the strains.

As in other *C. sporogenes* strains, the spore population is phenotypically homogenous with an exosporium layer that encapsulated the spore and extended out from one of the poles. The ATCC15579 exosporium was decorated with a hairy nap although, unlike the strain NCIMB 701792, these spores do not appear to possess other appendages or fibrils on their outermost layers (Janganan *et al.*, 2016). Diffraction pattern and unit cell dimensions were consistent between the ATCC15579 exosporium basal layer unit cell dimension with that of NCIMB 701792 (Janganan *et al.*, 2020).

3.4.2 Exosporium basal layer is comprised of CsxA

$\Delta csxA$ mutant spores lacked exosporium basal layer and did not possess a crystal type similar to WT exosporium. The outermost layer observed around the spore body was in patches and appeared broken at the edges. It was also composed of a different crystal type with a smaller unit cell and $p3$ symmetry – significantly different from WT exosporium. These findings suggest that CsxA is essential for the assembly of *C. sporogenes* exosporium basal layer.

As established in chapter 1.3.3.5.1, *B. cereus* exosporium is composed of EsxY and CotY, the latter making up the cap structure. $\Delta esxY$ spores reveal a para-crystalline layer, similar to WT exosporium, limited to one of the spore poles corresponding to CotY (Johnson *et al.*, 2006). These observations in $\Delta csxA$ spores suggest no comparable cap structure is present in *C. sporogenes*. If there is a cap structure, then the mode of assembly is different from *B. cereus*.

Type I crystal, observed in both $\Delta csxB$ and $\Delta csxC$ mutants, resembles WT exosporium basal layer in both unit cell dimension and structure. As in the WT basal layer, the merged 2D projection maps from both $\Delta csxB$ and $\Delta csxC$ mutants also appeared slightly different from the negative stain 3D reconstruction of NCIMB 701792 strain. The density suggested to correspond to subunits which were rotated with respect to the negative stain reconstruction but consistent with the cryo map (chapter 4). However, the resolutions of these maps are so low that it would need to be confirmed by cryo-EC and 3D reconstructions.

Hairy nap was observed associated with type I crystal in both $\Delta csxB$ and $\Delta csxC$ mutants. Thus, we propose that the type I crystal forms the main component of the WT basal layer. This is consistent with the expression of CsxA in these mutants. As BclA, the most likely candidate for hairy nap, forms a complex with CsxA it provides further evidence that CsxA forms the basal layer (Janganan *et al.*, 2016).

Table 3.5 Summary of crystal types observed in *C. sporogenes* csxA, csxB and csxC mutant spores

Crystal type	Unit cell dimensions	Space group	Spore
I	$a=b=106.3\pm0.1.2\text{\AA}, \gamma = 118.9\pm1.3^\circ$	$p6$	$\Delta csxB, \Delta csxC$
II	$a=b= 64.8 \pm0.8\text{\AA}, \gamma = 119.8^\circ \pm0.5^\circ$	$p3$	$\Delta csxA$
III	$a=b= 52.9 \pm0.7\text{\AA}, \gamma = 120^\circ \pm0.3^\circ$	$p6$	$\Delta csxB$
IV	$a=b= 49 \pm1.2\text{\AA}, \gamma = 119.5 \pm0.9^\circ$	$p6$	$\Delta csxC$
‘Rare crystal’	$a=b \sim 65\text{\AA}, \gamma=120^\circ$		$\Delta csxB$

3.4.3 Type II crystal is a candidate for parasporal layer

This crystal type was observed in $\Delta csxA$ spores. It appeared that a similar crystal was also present in patches on $\Delta csxB$ spores, however due to the low abundance and small patches of this crystal type a structural comparison to type II in $\Delta csxA$ could not be conducted.

The layers of these crystals in the $\Delta csxA$ spores extended across the whole spore body but appeared in patches. Length analysis of the outermost layers in $\Delta csxA$ (Table 3.1) (Figure 3.4) demonstrated a diversity in length. The length distribution calculated was not significantly shorter than WT exosporium layers. Additionally, high magnification micrographs revealed a lack of hairy nap associated with the layer which suggested that it could correspond to an internal spore layer. It remains unclear what this crystal corresponds to. It may be present on WT spores but not visible, possibly as a layer assembled under the basal layer which is yet to be confirmed.

As discussed in chapter 1.3.3.6, thin sections of *Clostridia* spores revealed a network of layers present along the length of the exosporium in the interspace region (Brunt *et al.*, 2015; Warren *et al.*, 1994). These layers are described in greater detail in chapter 5, as the layers assemble into striking laminae.

In *Bacillus thuringiensis* these layers are para-crystalline in assembly (Ebersold *et al.*, 1981; Wehrli *et al.*, 1980). Established in chapter 1.3.3.5.1, different crystal types were identified associated with exosporium fragments of *B. thuringiensis* and *B. cereus* (chapter 1- Figure 1.14) (Ball *et al.*, 2008). One of the layers was labelled the ‘parasporal’ layer suggested to be under the basal layer (Ball *et al.*, 2008). Could this crystal correspond to a parasporal layer in *C. sporogenes*? As crystal type II was not decorated with hairy nap and was similar in size to WT exosporium envelopes this could be a possibility (discussed in Chapter 7). Both the type II crystal in *C. sporogenes* and the parasporal layer in *Bacillus* have an assembly with phases consistent with $p3$ symmetry. As *Bacillus* and *C. sporogenes* share structural similarities in their exosporium basal layer, it would be interesting to see if this also extended to other spore assemblies such as the parasporal layer. Further work is required to establish a connection between type II crystal and parasporal layers in *C. sporogenes*, including subtomogram averaging of the layers in tomograms in chapter 5 to see if they are consistent with this crystal type. This work would also provide an insight into other spore assemblies that are conserved across *Bacillus* and *Clostridia*.

The composition of the type II crystal remains unknown. As type II crystal has been observed on $\Delta csxA$ and potentially in $\Delta csxB$, it is unlikely to be composed of either the CsxA or CsxB proteins. If we consider that CsxA at 33.4kDa forms a crystal with a unit cell length of $a \sim b \sim 108\text{\AA}$, where six CsxA monomers assemble to form a central ring with $p6$ symmetry (chapter 4), then CsxC with a molecular weight of 30kDa (Janganan *et al.*, 2016) could plausibly fit into a unit cell of $a \sim b \sim 64\text{\AA}$ with three monomers to form a $p3$ crystal. Based on the ability of CsxA to self-assemble in *E. coli*, heterologous expression of CsxC should be considered. If CsxC self-assembles into a crystal that resembles type II then the composition of this assembly could be confirmed.

3.4.4 CsxB and CsxC

Both $\Delta csxB$ and $\Delta csxC$ spores possessed type I crystal (Table 3.5) (Figure 3.15), which has been established as resembling exosporium basal layer 3.4.2. The crystal patches in the mutants were present in smaller more disordered patches. As $\Delta csxA$ spores lacked any assembly resembling

exosporium basal layer, it suggests that neither CsxB nor CsxC assemble into a crystal structure similar to WT exosporium basal layer, at least not in the absence of CsxA. However, the effect of CsxB and CsxC on crystal uniformity observed in the mutants could suggest that these proteins are involved in the assembly of the exosporium in some other respect.

3.4.4.1 $\Delta csxB$

The morphology of $\Delta csxB$ spores appears very similar to WT spores. The presence of type I crystals in $\Delta csxB$ spores and their absence in $\Delta csxA$ spores suggests that CsxB is not the primary component of the exosporium basal layer. High magnification micrographs revealed a disordered and patchy envelope layer, composed of two crystal types in equal abundance: type I and type III. EC revealed that type III crystals were half the unit cell length of type I (Table 3.5) and formed a lattice with a central ring and phases consistent with *p6* (Figure 3.15). The hairy nap was found associated with both crystal type I and III, suggesting that the protein composition of this layer was able to interact with the hairy nap proteins, likely BclA or BclB (Janganan *et al.*, 2016).

A ‘rare crystal’ was observed in $\Delta csxB$ spores. Due to its low abundance no structural information could be generated, however the unit cell size was estimated as $a \sim b \sim 65\text{\AA}$, $\gamma = 120^\circ$. These dimensions are very similar to type II crystal from $\Delta csxA$, although the lack of structural information means that this cannot be confirmed. Further work would be required to establish a connection between the ‘rare crystal’ and type II crystal, this could include comparing 2D and 3D EC maps.

3.4.4.2 $\Delta csxC$

CsxC knockouts clearly affected the morphology of the spores. The phenotypes of the $\Delta csxC$ mutant population were more diverse. All spores possessed a thin layer extended from one of the spore poles, whereas some spores also possessed a short thin layer on the opposite pole (Figure 3.12 and 3.13).

The outermost layers along the edges of the main spore body were lacking across $\Delta csxC$ spores. This suggests that CsxC is involved in the assembly around the main spore body. Type I crystal (Table 3.5), the basal layer, was less abundant on these spores when compared with another crystal, type IV, that was observed on the outer layers of $\Delta csxC$ spores. This crystal was also half the unit cell size of

WT exosporium but possessed phases consistent with $p3$ symmetry. The composition and role of type IV crystals remains unknown.

3.4.4.3 Possible roles of type III and type IV crystals

3.4.4.3.1 Are type III and type IV crystals mis-indexed type I?

As both type III and type IV crystals are half the unit cell length of type I it is possible that the lattice corresponds to mis-indexed type I crystal, whereby the second order spots are indexed as the first order. This possibility was considered seriously, especially due to the high disordered nature of these layers. However, there are many examples of diffraction patterns that reveal no evidence of spots that would correspond to type I first order (Figure 3.11 and 3.14), thus it is improbable that this lattice has been mis-indexed. Additionally, type IV crystals were very abundant, lacked hairy nap and possessed phases consistent with $p3$ symmetry (discussed in section 3.4.4.2), making the probability of mis-indexing less likely.

Further research is required to confirm this, including 3D crystal reconstructions. Heterologously expressed crystals tend to be more ordered than native exosporium, thus if the crystal compositions were to be identified and expressed in *E. coli* it would also provide an opportunity to confirm the crystal types.

3.4.4.3.2 Could the crystals be mis-assembled CsxA?

Another possibility considered was that the crystals could correspond to mis-assembled CsxA, unable to pack into its native assembly due to the lack of CsxB or CsxC. For type III crystals it is unlikely that CsxA would pack into a smaller unit cell with the same structure as its native assembly, as this would involve assembling CsxA into a smaller volume which would be thermodynamically unfavourable. It is more likely that CsxA would mis-assemble in a different structure with a smaller unit size, such as type IV crystals with $p3$ symmetry and around half the unit cell size of WT exosporium (Figure 3.15E). Nevertheless, due to the demonstrated ability of CsxA to self-assemble in para-crystalline layer with the same structure as WT basal exosporium (Janganan *et al.*, 2020)

(discussed in chapter 4), without the presence of either CsxB or CsxC, it is highly unlikely that type III and IV crystals correspond to mis-assembled CsxA.

3.4.4.3.3 Are the crystal types localised on the basal layer or sublayers?

Type III crystals are decorated with a hairy nap, suggesting that it could be a basal layer crystal. If this were the case, crystal type III would be observed on WT exosporium, although there have been no accounts of this crystal type on WT spores. However, native exosporium can be patchy, thus small patches of different crystal types may not be readily distinguishable from the basal layer. This is particularly true for FFT analysis, as type III crystal is half the unit cell of basal layer exosporium thus spots would be observed in the region of second order basal layer spots. As no type III crystals have been observed in $\Delta csxA$ it is an unlikely possibility, unless the crystal requires CsxA for its assembly.

Another possibility is that type III crystal is a sublayer, such as an alternative crystal type on the parasporal layer. However, if this was the case, one would expect it to be present on $\Delta csxA$ mutant spores where the basal layer is absent. Another feature that makes this unlikely is the association of hairy nap- this is not something that would be present on innermost layers.

Type IV crystals were not decorated with hairy nap (Figure 3.13), suggesting it could correspond to a more internal layer. Hypothetically, if type II crystal corresponds to a sublayer such as the parasporal layer and is composed of CsxC (discussed in 3.4.4.2), the CsxC knock out would not possess the parasporal layer and would reveal layers under the parasporal layer. Thus, type IV crystal could correspond to a layer under the parasporal layer, possibly the outer coat (discussed in chapter 7) – similar to type III crystal in *B. thuringiensis* (Ball *et al.*, 2008). Further research is required to establish type IV crystal as a layer under the parasporal layer including screening a double mutant for $\Delta csxAcsxC$ to see if the crystal is present. This is further discussed in Chapter 7.6.1.2.

Chapter 4 – CsxA crystal structure and properties

4.1 Introduction

Previous research on *C. sporogenes* exosporium revealed a well-ordered hexagonal lattice (Janganan *et al.*, 2016), analogous to the exosporium from the *Bacillus cereus* family (Kailas *et al.*, 2011; Terry *et al.*, 2017). As discussed in chapter 3, three cysteine-rich proteins were previously identified in *C. sporogenes* exosporium fragments: CsxA, CsxB and CsxC (Janganan *et al.*, 2016). Knockout mutants of these proteins revealed their involvement in outer spore assemblies, particularly Δ csxA spores which had no exosporium basal layer.

Dr. Thamarai Janganan (TJ) (former post-doctoral research assistant in Prof. Per Bullough's lab, The University of Sheffield) overexpressed recombinant CsxA in *E. coli* and identified crystals that resemble exosporium, indicating that CsxA can self-assemble into crystals similar to basal layer exosporium. Originally CsxA crystals were harvested by following the protocol established for analogous crystals from *Bacillus* (Jiang *et al.*, 2015; Terry *et al.*, 2017). However, high levels of aggregation and background material indicated that this approach was not optimal for CsxA. Additionally, crystal abundance was low thus unsuitable for advanced structural studies, particularly cryo-EC.

4.2 Aims and Objectives

This chapter aimed to confirm the involvement of CsxA in the exosporium basal layer of *C. sporogenes* by characterising the heterologously expressed CsxA crystals from *E. coli*. EM and atomic force microscopy (AFM) were employed to establish structural similarity between the CsxA crystals and native exosporium. Negative stain EM was conducted by TJ and AFM by Dr. Nic Mullin (Department of Physics and Astronomy, The University of Sheffield). Based on previous findings in *Bacillus* exosporium proteins, I suspected that CsxA would self-assemble into crystalline sheets with the same structure as native exosporium. As CsxA is highly conserved across *C. sporogenes* and *C. botulinum* strains (Janganan *et al.*, 2016), these findings would also apply to group I *C. botulinum*.

CsxA crystals lacked hairy nap and could be concentrated without the main spore body, that provided obscured views of the crystal, thus were more amenable to structural studies than native exosporium. Here I aimed to use the CsxA crystals for cryo-EC to generate the first 3D cryo reconstruction of exosporium basal layer to lay the groundwork for atomic resolution analysis and complement with future cryotomography on spores.

The properties of the exosporium have been investigated across different spore formers (reviewed in Stewart, 2015). The work described herein aims to characterise the properties of the exosporium in *C. sporogenes* by investigating CsxA crystals. This was conducted by comparing the structural properties of the crystals in hydrated and dehydrated states both by EM and AFM. Additionally, the resilience of CsxA crystals was tested by treating them with harsh conditions and screening by TEM. Based on prior observations of ExsY and CotY crystals (Jiang *et al.*, 2015; Terry *et al.*, 2017), we suspected a similar degree of resilience would also be observed in CsxA crystals which would be conferred to the *C. sporogenes* spores.

4.3 Results

4.3.1 Optimising CsxA crystal harvesting

A pET21a plasmid construct, provided by TJ, with the *csxA* gene C-terminally tagged with His₆ was transformed into *E. coli* BL21 (DE3) pLysS cells. CsxA expression was IPTG induced and cells were lysed by sonication (chapter 2.3) to release crystals (Figure 4.1). The His₆ tag was then used for batch purification of the crystals on Ni-NTA beads. The established protocol from *Bacillus* (Jiang *et al.*, 2015; Terry *et al.*, 2017) was adapted for CsxA crystals, details of which are described in chapter 2.3. Induced cells were sonicated in TBS buffer with 1mM PMSF (chapter 2.3.2). All buffers in the previous protocol included 8M urea in an attempt to reduce sample aggregation. However, CsxA crystal samples displayed increased levels of aggregation at 8M Urea, thus it was excluded from all buffers (Figure 4.3). The original protocol involved ultracentrifugation of samples at 43,000rpm to pellet the crystals. The high centrifugal forces were found to cause crystals to shred and fray (Figure

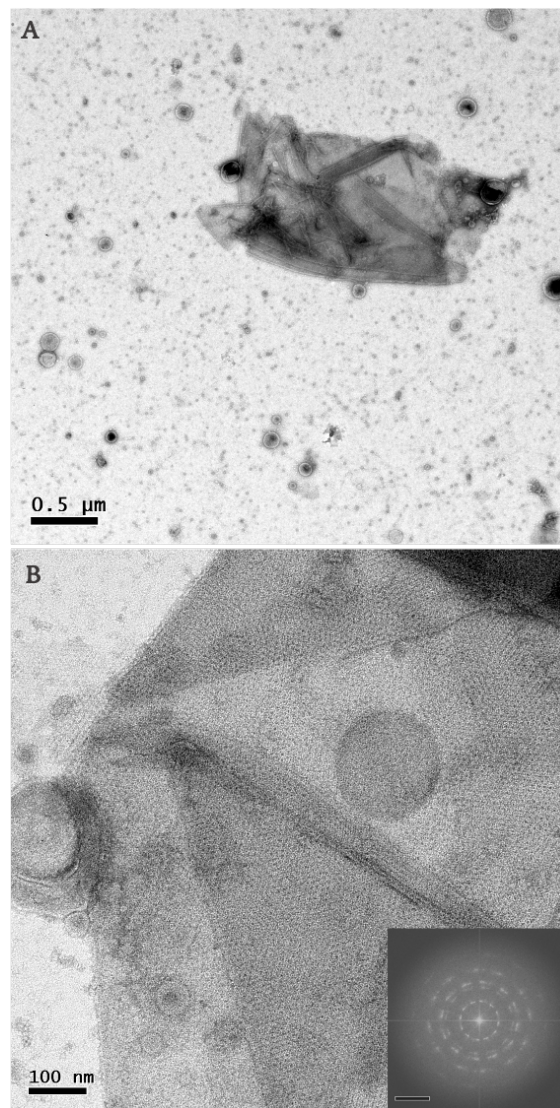


Figure 4.1 CsxA crystals self-assemble when heterologously expressed in *E. coli*

A) Low magnification micrograph of CsxA crystal in cell lysate B) High magnification micrograph of CsxA crystal, inset displays FFT demonstrating the diffraction pattern of CsxA crystals. Scale bar of FFT = 0.2 nm^{-1}

4.4), thus a gentler approach was implemented. This involved centrifugation at 10,000rpm for 10 minutes. These pellets were then resuspended in TBS and 0.1% Triton X100 to remove vesicles and background material. The result of this was a much cleaner background with larger and intact crystals (Figure 4.4B).

The optimised protocols provided a much cleaner prep with higher concentrations of crystals (Figure 4.2B,C). The sample was stable for at least 3 months at 4°C , however, to ensure high crystal quality the samples were best loaded onto grids the same day as crystal harvesting.

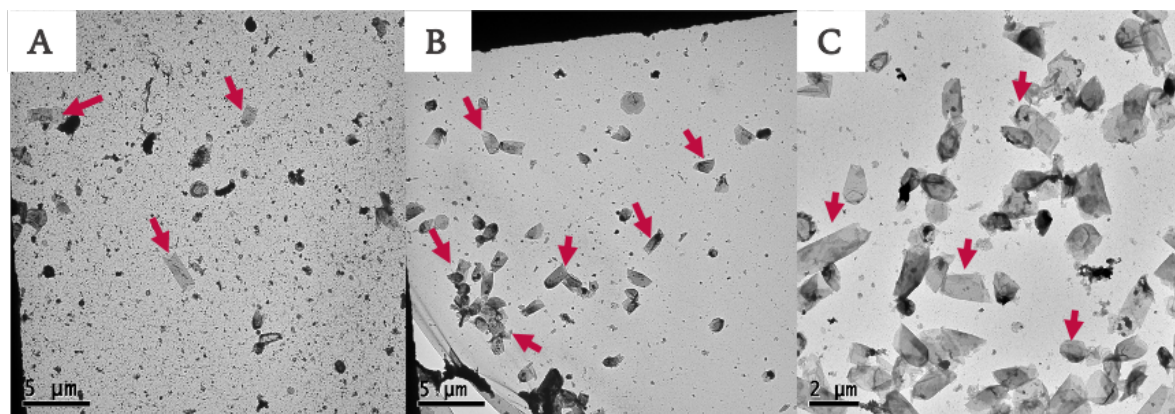


Figure 4.2 Optimisation of CsxA crystal sample preparation

Electron micrographs of negatively stained CsxA samples. Arrows indicate the crystals. A) Previous protocol: sample concentration is low, aggregation observed and high levels of background material B,C) New protocol: aggregation levels are reduced and better concentration of intact crystals.

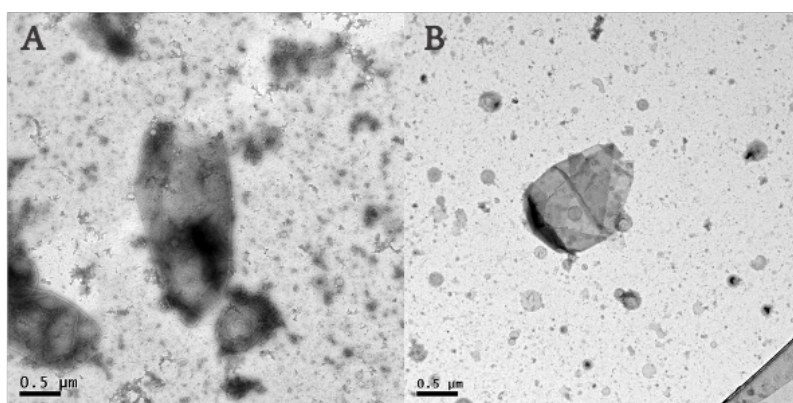


Figure 4.3 Omitting urea from buffer improves crystal harvesting protocol

A) 8M urea: aggregation observed in sample, high background material and crystal integrity reduced B) No urea: no abundance of aggregation

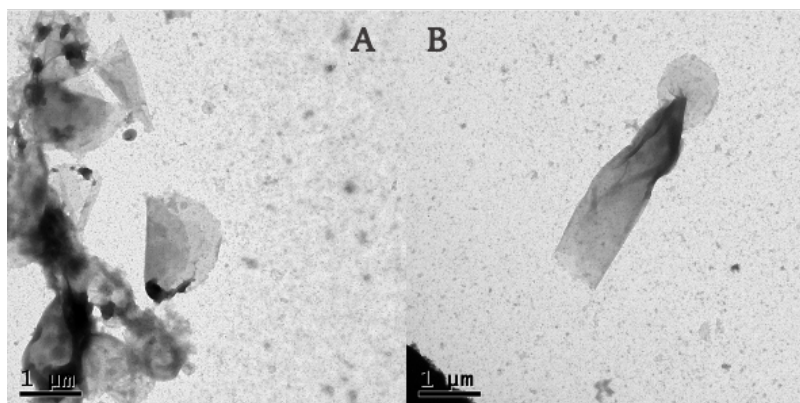


Figure 4.4 Concentrating crystals with lower centrifugal forces preserves crystals

A) Ultracentrifugation at 43,000rpm: crystals ripped apart and clumping together B) Centrifuged at 10,000rpm for 10 minutes and resuspended in 0.1% Triton X100: background of sample is much cleaner, crystals are larger.

4.3.2 CsxA crystals and native exosporium

4.3.2.1 Negative stain EC

Structural comparison between CsxA crystals and native exosporium was conducted by TJ using negative stain EC. A 3D reconstruction of native exosporium was generated from 61 images collected at $\pm 55^\circ$ (Figure 4.5A-C) revealing a honeycomb lattice structure with permeating pores. The central ring possesses sixfold symmetry with a central pore (Figure 4.5 – 1) and peripheral pores (Figure 4.5 – 2). Threefold linkers interconnected the central rings (Figure 4.5 – arrow). The unit cell dimensions are $a = b = 110 \pm 5\text{\AA}$ and $\gamma = 120 \pm 3^\circ$. The 3D reconstruction of CsxA crystal, from 66 images collected at $\pm 55^\circ$, revealed the same crystal structure as native exosporium (Figure 4.5 – yellow). These samples were embedded in negative stain thus the resolution was limited to around $25\text{\AA}$.

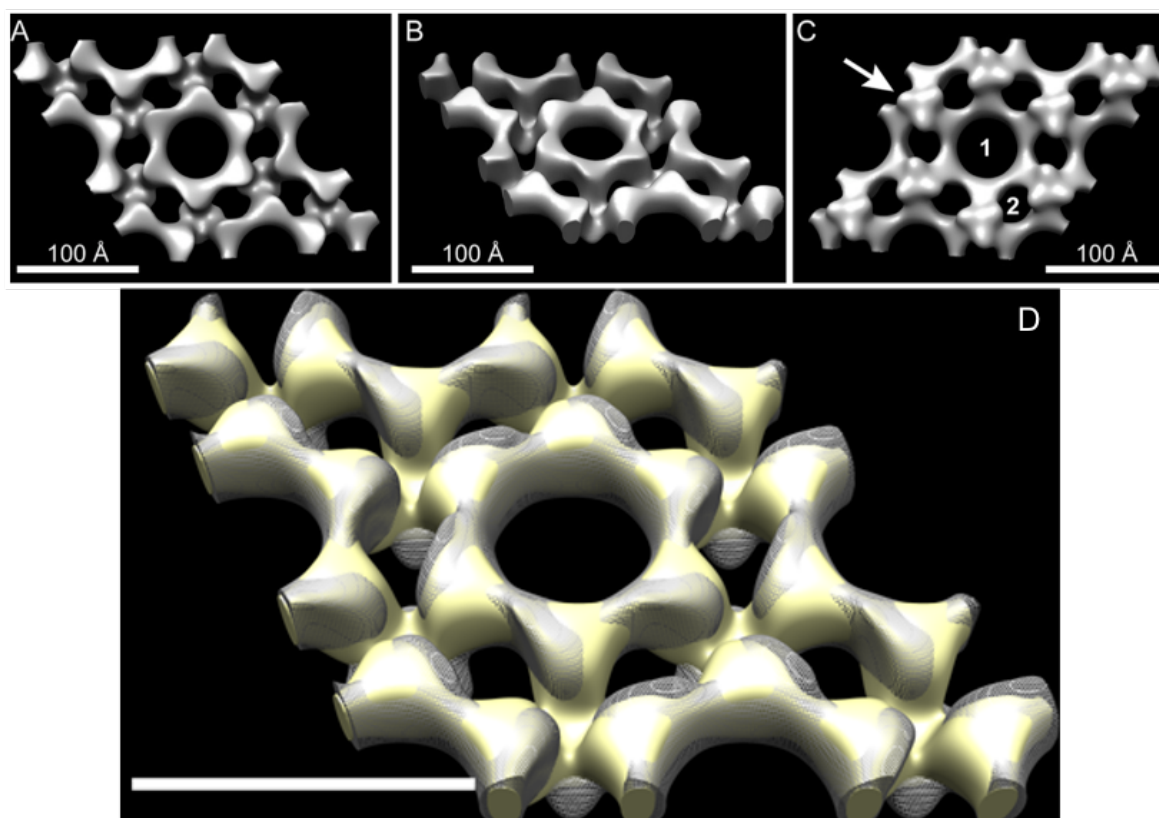


Figure 4.5 3D reconstruction of *C. sporogenes* exosporium and CsxA heterologously expressed in *E. coli*

A-C) 3D reconstruction of *C. sporogenes* exosporium. A,C) Views are perpendicular to the plane: arrow indicates the three-fold symmetry linker and numbers 1 and 2 display the central and peripheral pores respectively. B) View at 40° to the plane. D) 3D reconstruction of CsxA heterologously expressed in *E. coli* (yellow) overlay with *C. sporogenes* exosporium (grey mesh). Scale bar in D corresponds to $110\text{\AA}$.

Images provided by TJ

4.3.2.2 AFM

AFM was conducted by NM on fragments of exosporium and heterologously expressed CsxA crystals. The exosporium fragments were prepared by resuspending *C. sporogenes* spores in TBS buffer (Chapter 2 – Table 2.6), French pressed twice at 16,000psi and pelleted by centrifugation at 10,000xg for 15 minutes. These fragments were then imaged in air with the JPK NanoWizard Ultra AFM. Fragments revealed $\sim 70\text{\AA}$ and $\sim 140\text{\AA}$ thicknesses, as displayed in Figure 4.6 where the centre (box 1) corresponds to a single layer at $\sim 70\text{\AA}$ and the peripheral edges (box 2) are likely to represent a folded-over double layer. Two distinct surfaces were observed by AFM. The first surface displayed a clear honeycomb-like lattice structure with the unit cell measurement of $105 \pm 3\text{\AA}$ (Figure 4.6B). The second surface was disordered, no crystalline order was distinguishable which is likely to correspond to the presence of hair nap that made the basal layer inaccessible for visualisation (Figure 4.6C).

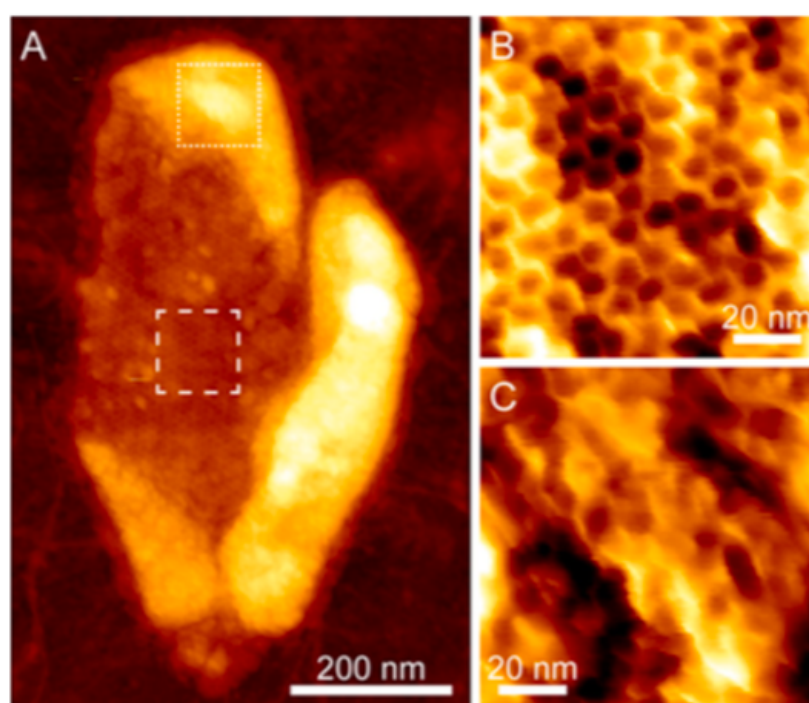


Figure 4.6 AFM of *C. sporogenes* exosporium fragments

A) Height image of fragments images in air, colour scale = 40nm. Central region of the fragment (box 1) is $\sim 70\text{\AA}$ thick and corresponds to a single layer. Brighter peripheral areas (box 2) likely correspond to the folding over of the layer thus revealing both sides of the exosporium layer. B) High magnification of box 1: honeycomb-like lattice with unit cell measurement of $105 \pm 3\text{\AA}$, corresponding to the internal surface of basal layer. Colour scale = 3nm C) High magnification of box 2: disordered structure likely due to the presence of hairy nap thus corresponding to outer surface of the exosporium. Colour scale = 5nm

Images provided by NM

CsxA crystal fragments were also imaged in air by AFM, where both sides of the crystal were accessible for imaging due to the lack of hairy nap (Figure 4.7C,D). This revealed a crystal with a unit cell length of $\sim 110\text{\AA}$, similar to the native exosporium imaged in air (Figure 4.6). The same crystals, upon imaging in water with the Bruker Dimension FastScan AFM revealed two distinct surfaces: one with the established cog-like hexameric ring (Figure 4.7A), the second possessing a petalled structure (Figure 4.7B) – both of which possessed a unit cell length of $\sim 110\text{\AA}$. Hydration also caused the crystal thickness to increase from $40\text{\AA}$ to $65\text{\AA}$. This conformational change was demonstrated as reversible by changing the imaging conditions of the crystal.

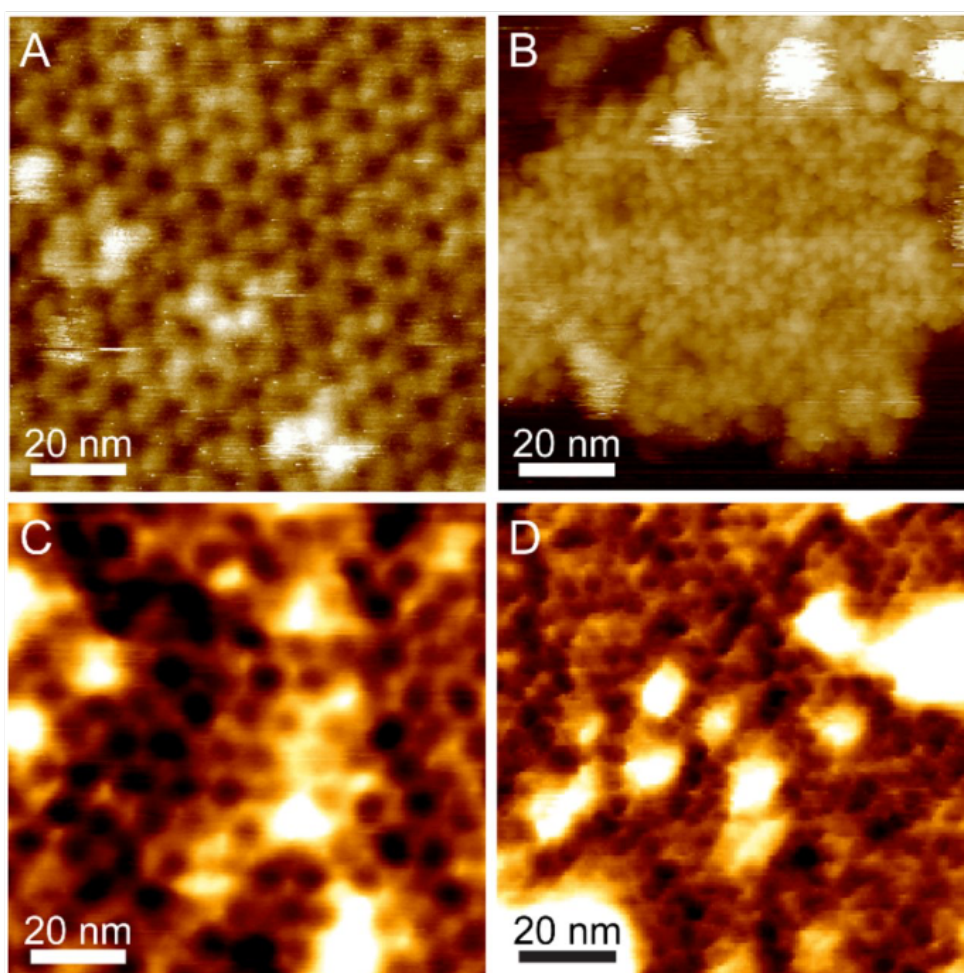


Figure 4.7 AFM of CsxA crystal in hydrated and dehydrated state

A,B) Imaging in water shows two surfaces: A) Honeycomb lattice structure similar to known exosporium layer. Colour scale 6nm B) Second surface composed of ‘petalling’ proposed due to swelling of hexamer. Colour scale 15nm C,D) Crystal imaged in air: porous honeycomb lattice. Both have a colour scale of 2.5nm.

Images provided by NM

4.3.2.3 Testing crystal properties

CsxA crystals were treated with harsh conditions to test their resilient properties. Samples were loaded onto TEM grids by negative stain and screened on the CM100. High magnification micrographs displayed the level of crystal integrity after treatment and their respective FFTs revealed the degree of crystal order (Figure 4.8).

At low magnifications the crystals subjected to heat treatment, 95°C for 60 minutes, were similar in appearance when compared to non-treated crystals (Figure 4.8A, B). Crystal abundance was unaffected and there was no impact on structural integrity, clearly displayed in FFT of the crystals (Figure 4.8B) which possessed sharp diffraction patterns extending out to third order spots.

As described in section 4.3, crystal samples treated with 8M urea showed lower crystal abundance and were more prone to aggregation. Many crystals also possessed frayed edges (Figure 4.8C). Crystals were also more mosaic and disordered; however, some patches of highly ordered crystals were also observed (Figure 4.8C, D).

After treatment with 2M DTT crystals appeared disintegrated in parts, many of them possessed holes in the layers and severed edges (Figure 4.8E). Some structural integrity of the crystal was still maintained although much more disordered – apparent in weaker intensity of the diffraction patterns in the FFT (Figure 4.8F). A combination of heat treatment, 95°C for 20 minutes, and 2M DTT lead to the complete disintegration of the crystals.

4.3.3 Cryo-EM sample preparation

Sample preparation for cryo-EM involves plunge freezing grids in liquid ethane at liquid nitrogen temperatures, the details of this are provided in chapter 2.4.3.2. Extensive optimisation was required to find the best conditions for grids with good crystal distribution and ice. The parameters optimised included grid type and plunge freezing conditions.

As presented in chapter 2.4.2, there are many different types of grids available. Generally, samples are suspended in vitreous ice supported by a holey carbon layer (quantifoil grids) or by lacey carbon

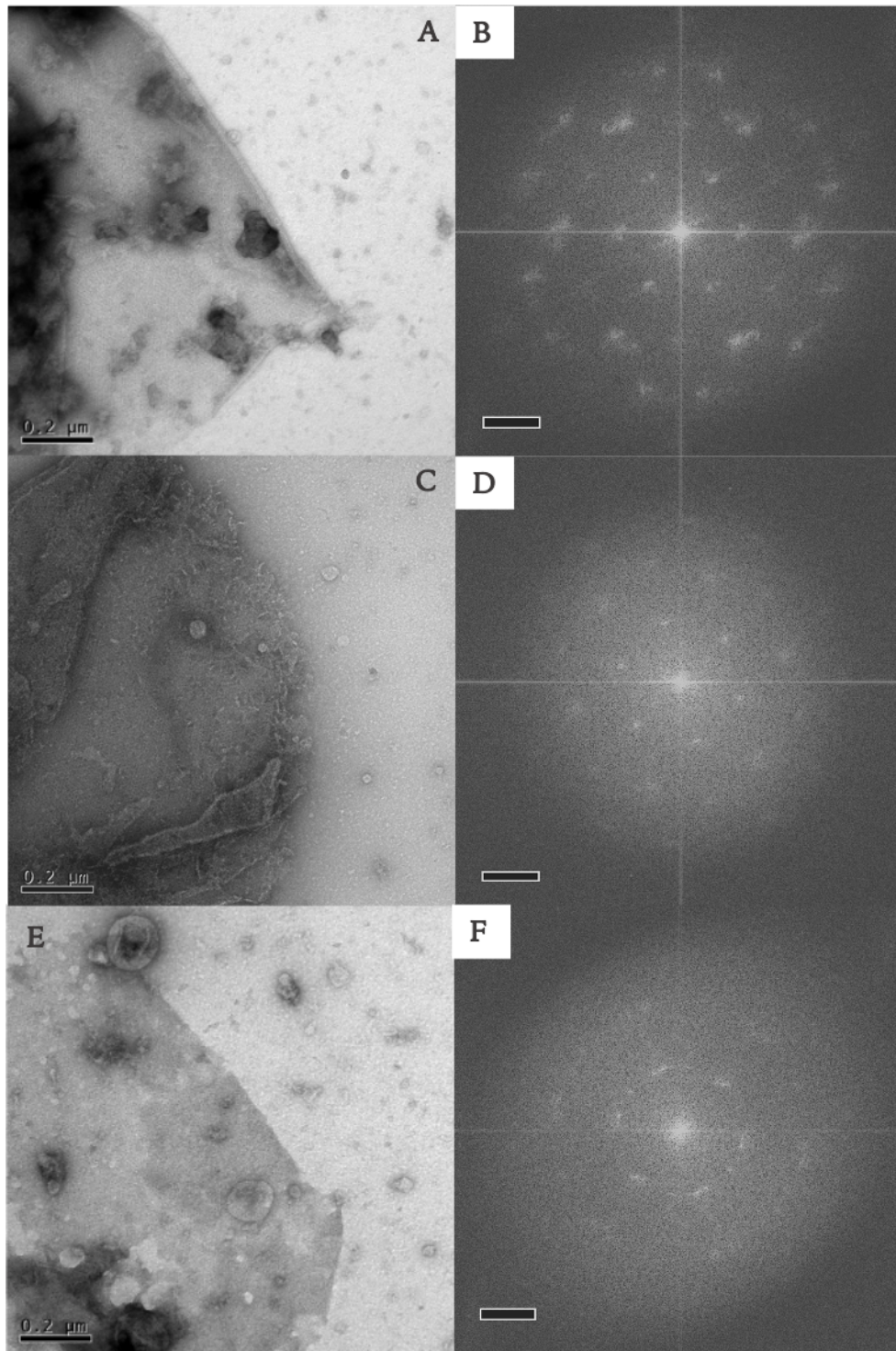


Figure 4.8 Testing CsxA crystal resilience

Micrographs of treated CsxA crystal and FFT displaying diffraction pattern, FFT scale bar = $100 \mu\text{m}^{-1}$ A, B) 95°C treated crystals for one hour: no effect on crystal integrity C, D) 8M Urea: structural integrity is partially conserved in patches across the crystal, fraying observed on the edges of the crystals E, F) 2M DTT: crystals are partially disintegrated but some crystallinity is still observed.

(chapter 2.4.2.2). As the CsxA crystals were very large and electron crystallography required crystals to be flat for optimal image processing, quantifoil or lacey grids were not required. Ideally the most appropriate grids are those with a layer of continuous carbon, however in practice these had a tendency for the carbon to rip and support predominantly thick ice which was unsuitable for imaging.

After a lot of trial and error, the best results were obtained on Quantifoil R2/2 grids with a GO layer. A property of GO layers is an increased sample adherence to the grids (Pantelic *et al.*, 2010), this was also observed for CsxA crystals as a greater abundance and distribution was present on GO grids. However, the quality of grids was very batch dependent, some grids were covered with good ice whereas others supported ice that was too thick for imaging. Nevertheless, when the batch was good this was the best option by far.

The Leica EM GP plunge freezer was used for sample preparation. This provided sample blotting of the grids from a single side (from the front or back) which generated better ice distribution across the grid, compared to the alternative double-sided blotting (from the front and back simultaneously). The optimal conditions for plunge freezing of Quantifoil R2/2 grids with GO were front blotting for 5s at 10 °C and 80% humidity.

4.3.4 Cryo data collection

Data collection was done at the in-house EM facility on the Arctica 200 kV microscope with Falcon III direct electron detector (DED). Unlike for single particle and tomography data collection, there was no automated approach for collecting electron crystallography data. Therefore, the EPU software (normally used for single particle work) was used for operating the microscope: navigating across the grid, switching magnifications, setting positions for data collection and keeping track of exposed areas all in low dose conditions. The microscope interface was then used to record the image, which was visualised and stored using TIA software (chapter 2.5.4).

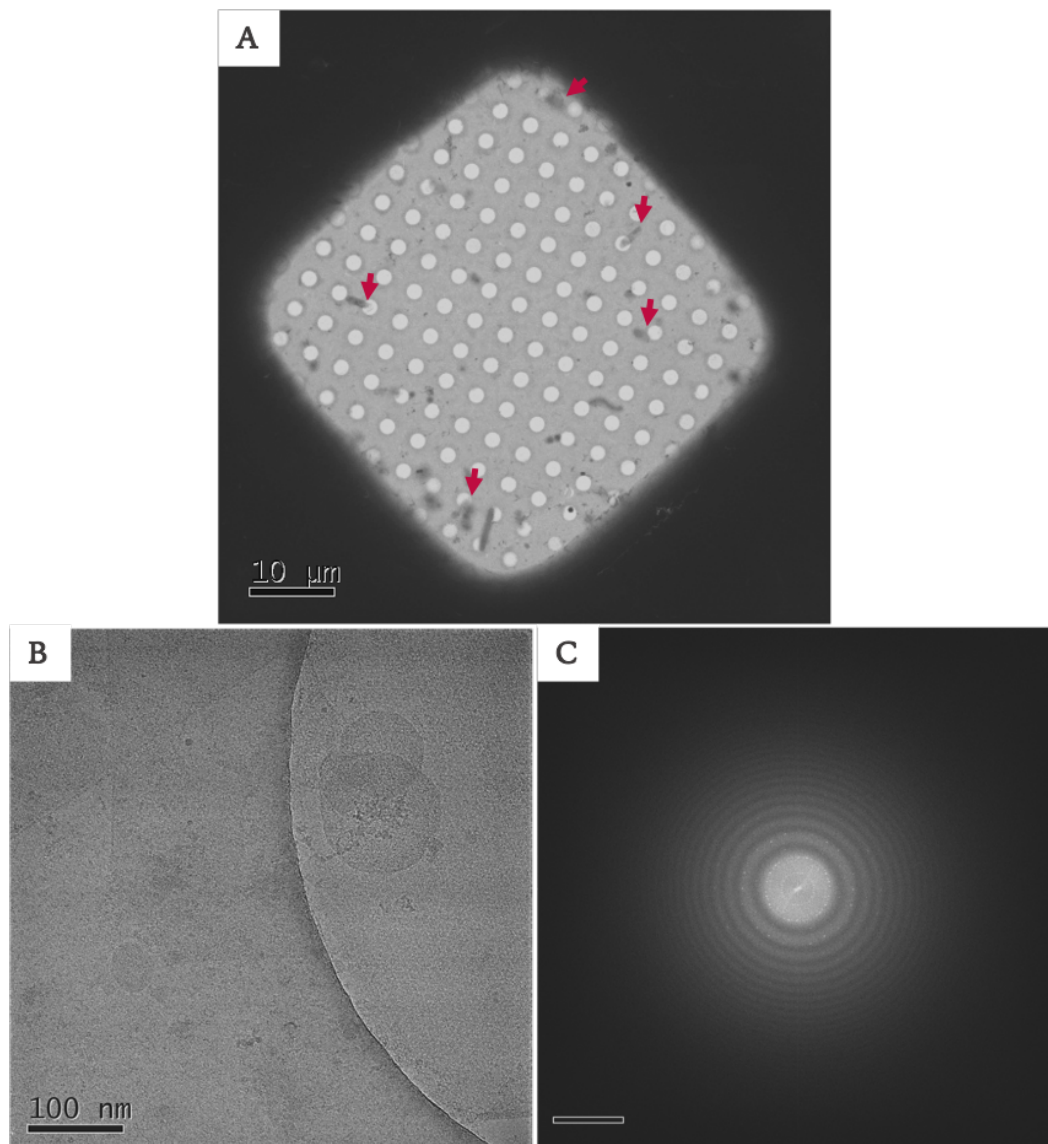


Figure 4.9 Cryo-EM of CsxA crystals

A) Micrograph of grid square showing CsxA crystals at low magnification (red arrows), B) High magnification of crystal in vitrified ice and its FFT (C). Scale bar of FFT = 1 nm⁻¹

To attain the highest resolution, the crystals could only be exposed once. Thus crystals needed to be identified at low magnification, this could be challenging as many did not have defined edges and appeared smudge-like on the grid (Figure 4.9A).

Data were collected at a magnification of 78,000x with a pixel size of 0.134 nm/pixel at sample level (Figure 4.9B). Images were collected with a total electron dose of $\sim 50 \text{ e}^-/\text{\AA}^2$ over ~ 2 second exposure collected over 80 frames and 40 fractions. Collection in ‘movie-mode’ enabled drift correction and

increased the attainable resolution as movement of sample during exposure could be tracked (Kuijper *et al.*, 2015; Liao *et al.*, 2013).

4.3.5 2D Projection map

Electron crystallography image processing involves many different stages. This section describes the overall workflow of processing, a detailed description was covered in Chapter 2.6.1.

Data collected from the Falcon were in frames and required alignment pre-processing with MotionCor2 software. The FOCUS software was then employed, which possesses automated scripts for importing images and calculating FFTs. This allowed for the quick quality check of all micrographs to identify those with high quality crystals. Further image processing was conducted using the *2dx* software, which involved: generating FFTs, unbending crystal distortions, CTF correction and generating projection maps. Figure 4.10 displays a micrograph of a crystal and its respective IQ plot and projection map. The IQ value defines the signal to noise ratio (SNR) of a diffraction spot which was used as measure of crystal quality. The IQ value was calculated as described in equation 4 (Henderson *et al.*, 1990), spots with an IQ value of 1 have the highest SNR. The IQ plot (Figure 4.10B) displays the distribution of IQ values for the crystals' diffraction pattern, *h* and *k* indicates the axes in reciprocal space and the circles represent Thon ring minima. At low spatial frequency, spots possess low IQ values indicating high SNR. Reflections extend out to 4Å resolution; however the IQ values are much higher reflecting the low SNR and the resolution limitations.

Equation 4

$$IQ = 7 \left(\frac{\text{Background corrected amplitude}}{\text{Background near the spot}} \right)$$

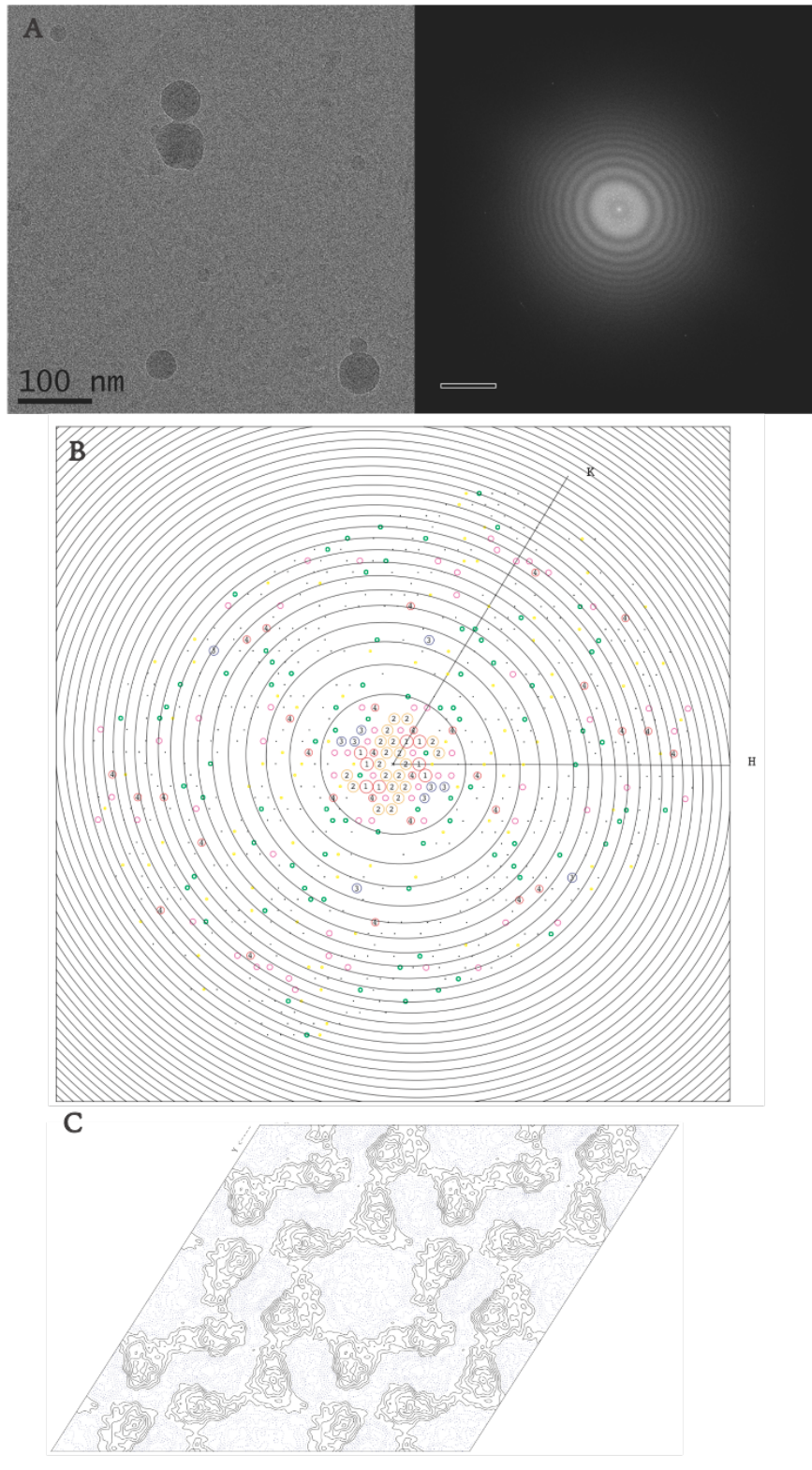


Figure 4.10 Image of CsxA crystal with corresponding IQ plot and final 2D projection map in $p6$

A) Micrograph of CsxA crystal and FFT. Scale bar of FFT = 1 nm^{-1} B) IQ plot after unbending in reciprocal space. The rings represent the Thon rings minima. Diffraction spots are displayed as IQ values, defined as I/grade , indicating the signal to noise ratio of the spot. The lower the value the greater the difference between the amplitude of spot and noise. The axes of reciprocal space are indicated as h and k . Nyquist limit of plot at $3.6 \text{ \AA}$. C) 2D Projection map of crystal (A) no symmetry applied. Scale bar = $105 \text{ \AA}$

Table 4.1 displays the power of the diffraction spots for a single image at different resolution brackets. The power corresponds to the sum of intensities of pixels at the predicted position of diffraction spots. Background noise is scaled to an average of 7, thus for this image significant signal is given up to 9 Å resolution.

Table 4.1 The power of diffraction spots at different resolution brackets for a single CsxA crystal image

Resolution brackets (Å)	Power
15	278
12	17
9	9
7	7
5	7
3	8

The unsymmetrised projection map displays a central ring with 6 subunits (Figure 4.10C). Space group analysis confirms the crystals' *p6* symmetry (Table 4.2), which is the same as previously calculated in TJ's work at low resolution. Thus, *p6* symmetry was applied throughout data processing.

Table 4.2 Space group determination table for CsxA crystal

Space group	Phase residual (90° = random)	Target residual ¹
<i>p1</i>	32.0	
<i>p2</i>	46.4*	47.6
<i>p3</i>	16.5*	32.0
<i>p312</i>	50.1	32.8
<i>p321</i>	44.1	33.3
<i>p6</i>	26.0*	36.8
<i>p622</i>	48.8	34.5

¹Target residual: expected phase residual of symmetry groups

* = Acceptable

! = Should be considered

` = Possibility

All acceptable space groups are * in the phase residual column including *p2*, *p3* and *p6*. The most likely space group is *p6*, in which *p2* and *p3* are also possible.

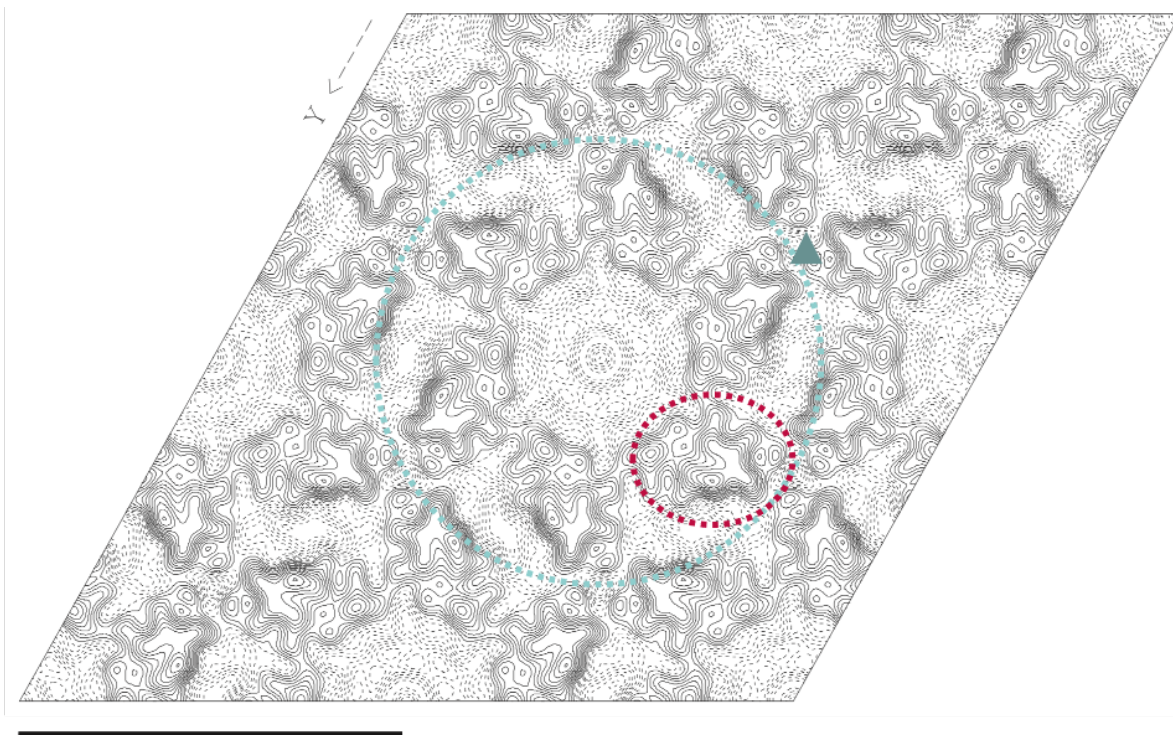


Figure 4.11 Projection map of crystal by cryo-EM at 9Å resolution

Merge map generated from 14 images. Map was contoured at 0.2 x root-mean square (RMS) deviation from mean density. The solid contour lines displays above mean density and the dashed contoured lines below mean density. The unit cell possesses a hexameric ring (blue ring) which encloses a less dense cavity. Each ring possesses six subunits (pink ring) which are interconnected by threefold symmetry linkers (triangle). Scale bar = 105Å.

A 2D projection merge map was generated by combining the data collected on the Arctica with data from TJ (Figure 4.11). The latter dataset was collected on a Phillips CM200 with a CCD camera. 14 micrographs were merged from both datasets (Table 4.3), displaying an average unit cell dimension of $a=b=105 \pm 3 \text{ Å}$ $c=120 \pm 1^\circ$. The temperature factor of the projection maps was set to the uppermost limit of 1,000. This was based on the temperature factors calculated for each image by the *SCALIMMAMP3* script, which determined a similar value across all images.

Table 4.4 breaks down the phase residual error at each resolution bracket. If we consider that a mean phase residual of 45° is equivalent to noise, the 2D projection map is at approximately 9Å resolution. The 2D projection map (Figure 4.11) displays regions above mean density as solid lines and below mean density as dashed lines. This reveals a central ‘cog-like’ ring with $p6$ symmetry (Figure 4.11 – blue dotted circle). The centre of the ring consists of low density which, based on 3D reconstructions in negative stain, corresponds to a pore. Each ring is interconnected by threefold linkers (Figure 4.11- blue triangle).

Table 4.3 Merge table for 2D CsxA crystal

Image directory	Image number	Unit cell dimensions			Overall phase residual (°)	Defocus*		
		a (Å)	b (Å)	γ (°)		X (Å)	Y (Å)	γ (°)
08_c1*	0009	102.7	103.3	119.3	20.47	13978.5	16038.5	48
18_c1*	0067	103.9	103.9	120.3	13.36	15716.9	15816.9	0
22*	0022	101.7	102.3	119.7	11.61	17904.5	17549.5	-12.18
29*	0029	102.7	101.5	120.2	31.36	17703.8	17074.8	338.07
35*	0035	102.1	100.3	118.3	10.49	17230.2	17230.2	0
60*	0060	101.3	100.8	119.6	21.49	18567.8	18157.7	48.68
60_c1*	0064	101	98.7	119.4	44.0	19987.8	17137.7	133.68
61*	0061	102.3	101.5	119.6	27.47	18136.3	18136.3	0
12	1200	106.5	106.9	119.9	32.94	50148.5	47261.2	55.00
1703	1703	106.9	109.3	121.087	16.74	64290.9	62547.5	55.00
14600	14600	111.8	114.9	120.6	33.87	24717	26086	42.16
151	15100	106.9	104.92	121.88	29.92	20787.5	21178.4	29.47
150	15000	106.9	104.9	121.2	30.56	18046	17786.6	303.13
15	1500	106.61	105.93	119.8	18.18	64526.3	66052.1	315.83

Lists all images used to generate 2D merge projection map, their respective unit cell dimensions, overall phase residual from merging and defocus values. The average unit cell dimensions: $a=b=105 \pm 3 \text{ Å}$ $\gamma=120 \pm 1^\circ$. The range of defocus used for data collection: $1.39785 \mu\text{m} - 6.42909 \mu\text{m}$

Table 4.4 Reflections and phase residual error of 2D projection map at different resolution brackets

Resolution bracket (Å)	Number of Reflections	Phase Residual	Error
200-15	25	11.0	4.0
15-12	14	32.0	6.9
12-10	13	25.3	4.4
10-9	18	25.9	5.0
9-8	15	39.3	5.9

4.3.6 3D cryo data

3D data were obtained by tilting the stage of the microscope. Data were collected at nominal tilt angles of 20°, 40° and 60° (Figure 4.12). These micrographs were processed analogously to those at zero degrees, however images of tilted crystals were particularly difficult to process. This is apparent in the FFTs of Figure 4.12 as the diffraction pattern becomes elongated and spots more diffuse. To prevent mis-indexing of diffraction patterns, a zero-degree image was collected after each tilted image as an indexing reference.

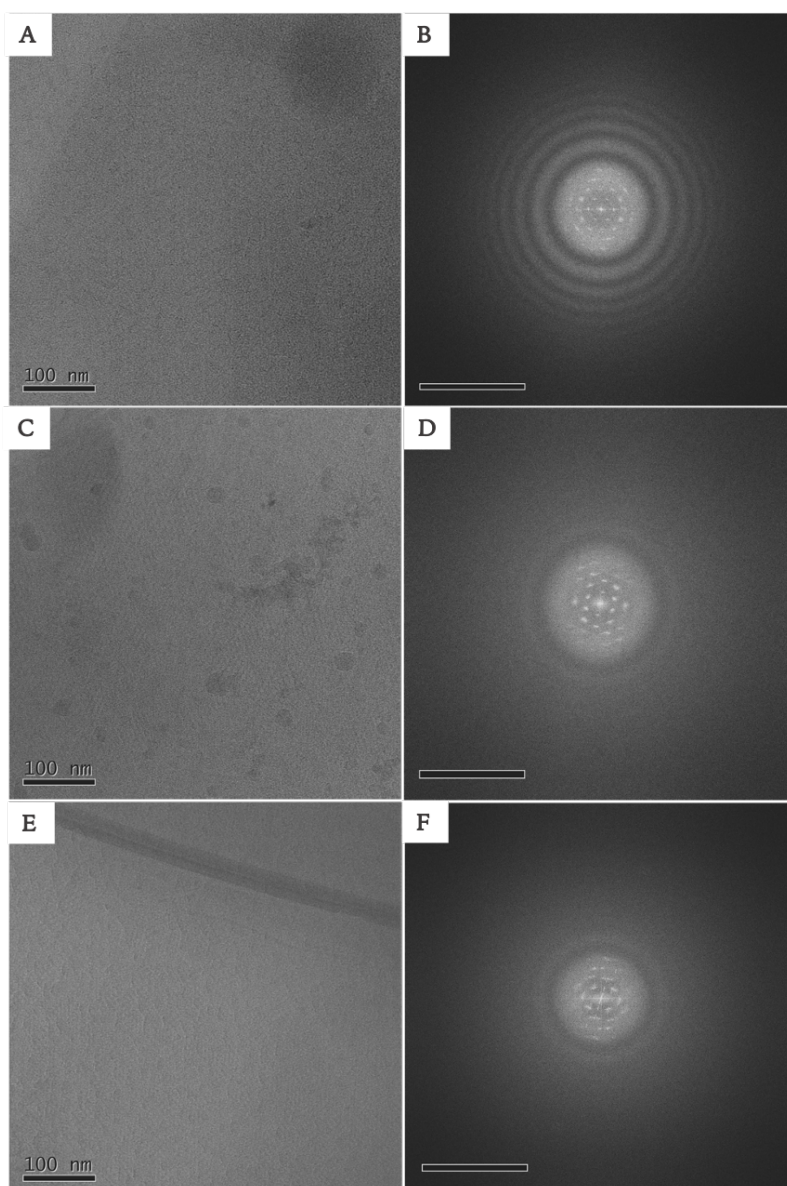


Figure 4.12 Micrographs of tilted CsxA crystals

Tilted crystal at 20 degree (A), 40 degree (B) and 60 degree (C) with their respective FFT. Scale bar of FFT = 1 nm^{-1}

Table 4.5 Statistical summary of 3D data from crystals

3D reconstruction parameters	
Crystal plane group symmetry	$p6$
Crystal unit cell parameters	$a=b=105 \pm 3\text{\AA}$ $\gamma=120 \pm 1^\circ$.
Number of micrographs	38
Range of defocus (μm)	0.87-6.42
IQ range used for 3D refining	1-5
Tilt range used	$0-61^\circ$
In-plane resolution cut-off	$9\text{\AA}$
Vertical resolution cut-off	$20\text{\AA}$
Number of observed reflections	6804
Number of observed unique reflections	3165
Overall R factor	41.3°
Overall phase residual	55.7°
Overall weighted r factor	34.8°
Overall weighted phase residual	23.3°

The final 3D dataset included 38 images – 17 at 0° , 7 at 20° , 6 at 40° and 8 at 60° . The summary for the 3D data is outlined in Table 4.5. It included 3165 unique reflections, merged to an overall phase residual at 55.7° . Latlines in appendix 3 displays the distribution of amplitudes and phases for h, k spots along z^* . The 0,0,l values were modelled into the 3D transform as described in chapter 2.6.1.9. Figure 4.13 displays the final 3D reconstruction of the CsxA crystal. The resolution is at $9\text{\AA}$ resolution laterally and capped to $20\text{\AA}$ axially.

Reconstructions were visualised using ChimeraX software (chapter 2.6.1.9), where the visible volume of the model was adjusted using a volume threshold level. Volume threshold calculations were based on average protein density size as described in chapter 2.6.1.9. Initially, the threshold of the map was set to a volume corresponding to a 33.4kDa protein per subunit, the estimated molecular weight for CsxA (assuming an average protein density of 1.35 g/cm^3). The resulting map is a hexagonal lattice with a central sixfold symmetry ring with a density filled core (Figure 4.14B). The significance of this

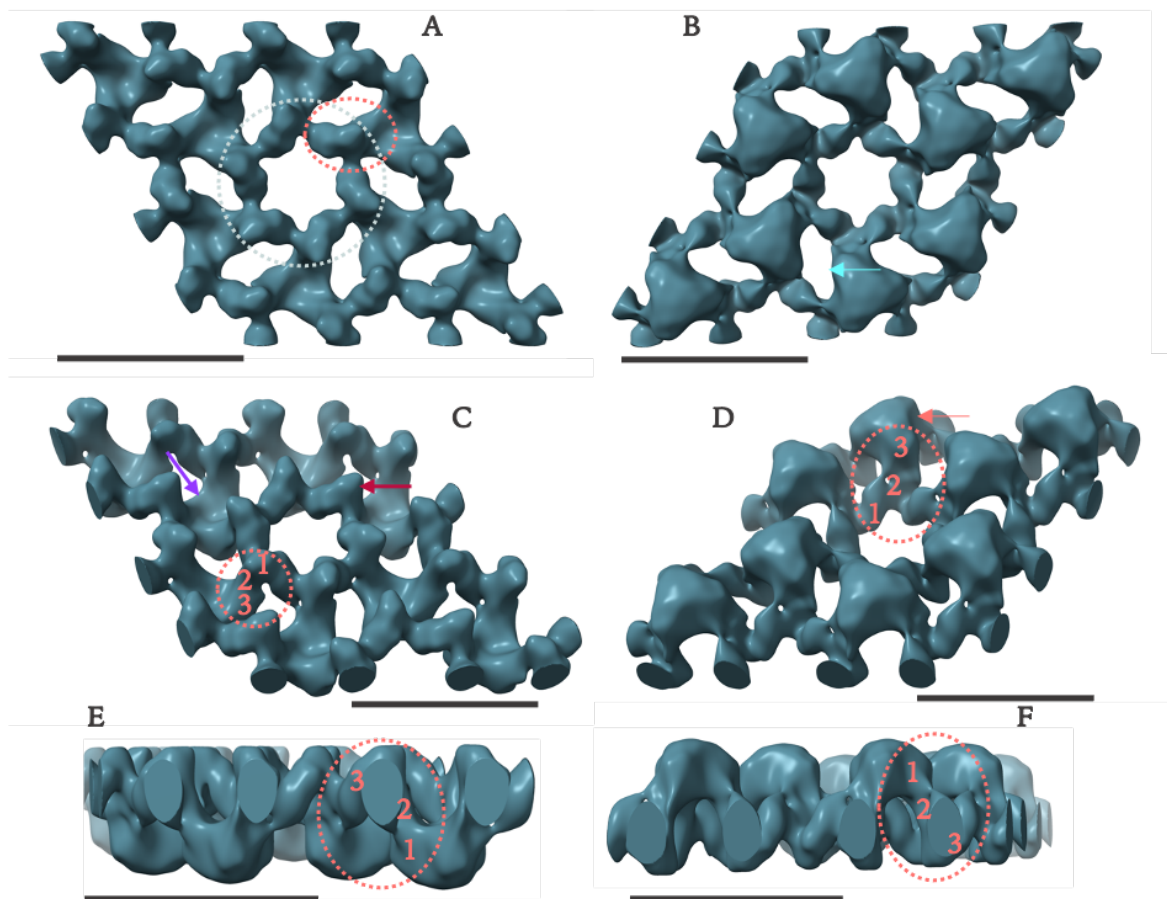
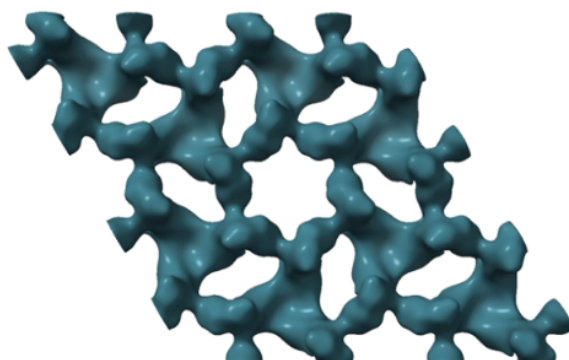


Figure 4.13 Cryo CsxA 3D reconstruction

A,B) Views perpendicular to the plane C,D) Tilted view to the plane E,F) Side on view. Dotted circle (A) reveals the central ring, with raised subunits (red arrow in C). Three-fold linkers (pink arrow in D) appear 'crater' like on opposite side (purple arrow in C). Peripheral pores present around the central ring (cyan arrow in B). Tentative assignment of individual subunits (Pink dotted circle) with three domains corresponding to the three-fold linker (1), the central ring (3) and a domain connecting both (2). Threshold set to volume below the point noise arises (40.6). Scale bar = 105Å. Lateral resolution is 9Å and ~20Å axially. Calculated before absolute hand determination.



Video 4.1 Cryo CsxA 3D

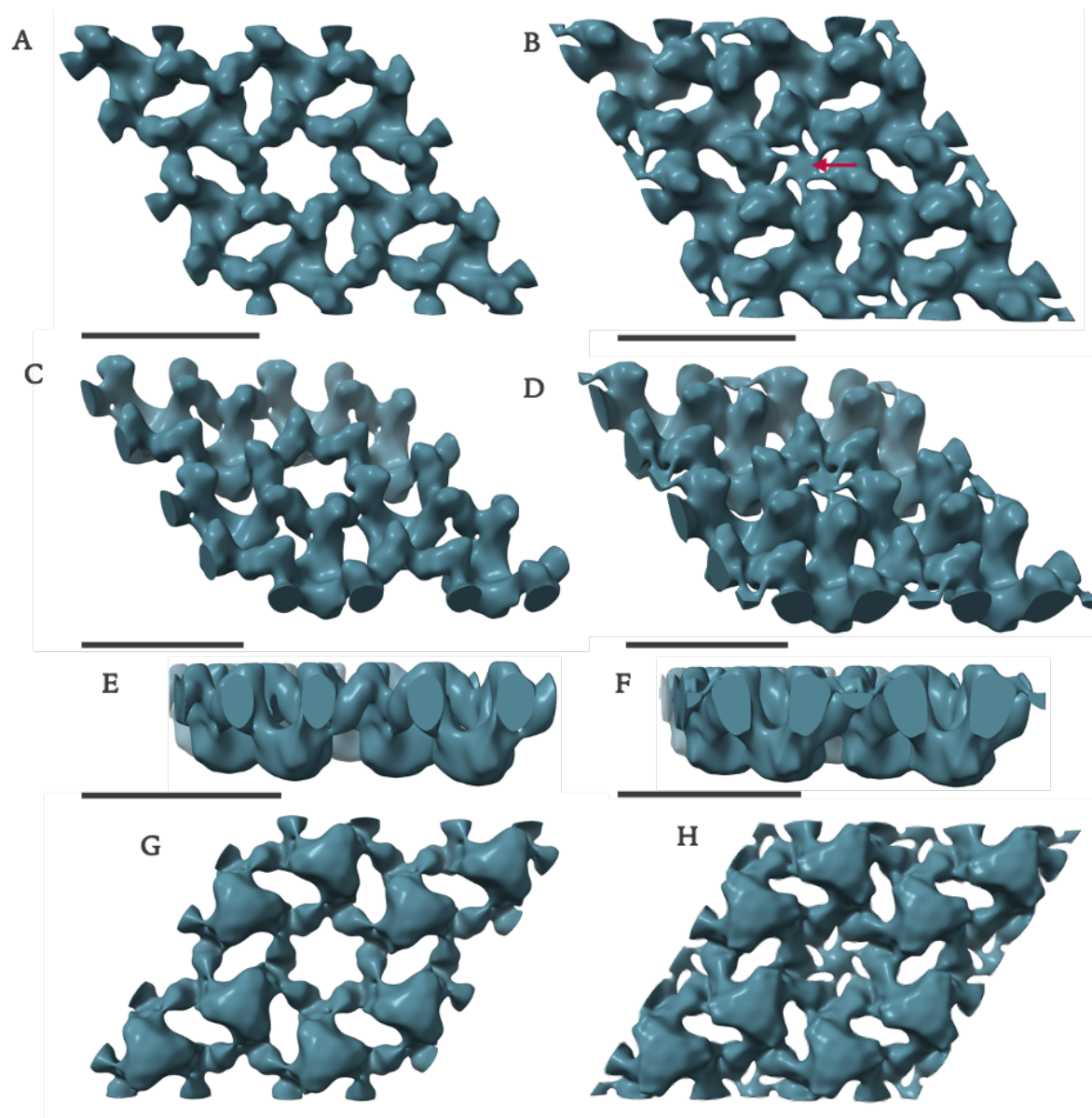


Figure 4.14 Setting adequate threshold to cryo CsxA 3D reconstruction

A,C,E,G) Threshold set to volume below the point noise arises = 40.6 B,D,F,H) Threshold volume of 34kDa per monomer. Primary difference observed in the 'cap' structure in the core of the central ring (arrow). Calculated before absolute hand determination.

central density was considered as the density is on the sixfold symmetry axis where noise can be enhanced. A higher volume threshold level, at 40.6, was reviewed where the core density was absent.

A threshold level of 40.6 is estimated to correspond to a 22.5kDa protein per subunit. To estimate how significant this core density was, I considered the root mean square (RMS) mean density for this map, which was determined at 39. The threshold level at which the core density becomes visible is 40.5, clearly visible at threshold level 39 (Figure 4.15D). For a value to be significant it would need to be observed at 6 times the RMS mean density, as this is not the case, we cannot be confident that the core density is real. Additionally, if we consider the structural differences between the map at threshold levels 24 and 40.6 (Figure 4.14) it reveals that the main basal layer structure does not change significantly either laterally or axially, and so we propose using a threshold of 40.6 for this reconstruction.

Figure 4.15 reveals the map at varying threshold levels, the highest is at 170 (Figure 4.15A) where density corresponding to the threefold linkers is visible (Figure 4.15A – arrow 1). These threefold linkers lie on a threefold symmetry axis; however the density is observed at greater than 3 times the RMS mean density and thus they are significant. At a threshold of 115 the density of the central ring begins to appear (Figure 4.15B – arrow 2). The density in the core of the ring begins to appear at 40.5, visible at 39 (Figure 4.15D – arrow 3) and is enhanced at 24 (Figure 4.15E – arrow 3). At a threshold level of 21, density at the twofold symmetry axis becomes visible (Figure 4.15F – arrow 4), any thresholds below this point start to incorporate noise above and below the crystal plane. Figure 4.15 demonstrates that at higher density thresholds, 40.6-21, the basal layer structure remains the same with enhanced noise artifacts on different symmetry axes.

The final 3D reconstruction reveals a central ring structure (circle), likely composed of 6 subunits (Figure 4.13 and Video 4.1). The central ring possesses a crown-like appearance with elements of the density that stick up (Figure 4.13– red arrow). A central pore is present in the central ring, with a width of 56Å at its widest point and 40Å at its narrowest (Figure 4.16). Each central ring is interconnected by threefold linkers, which appear raised on one side (Figure 4.16- pink arrow) and on

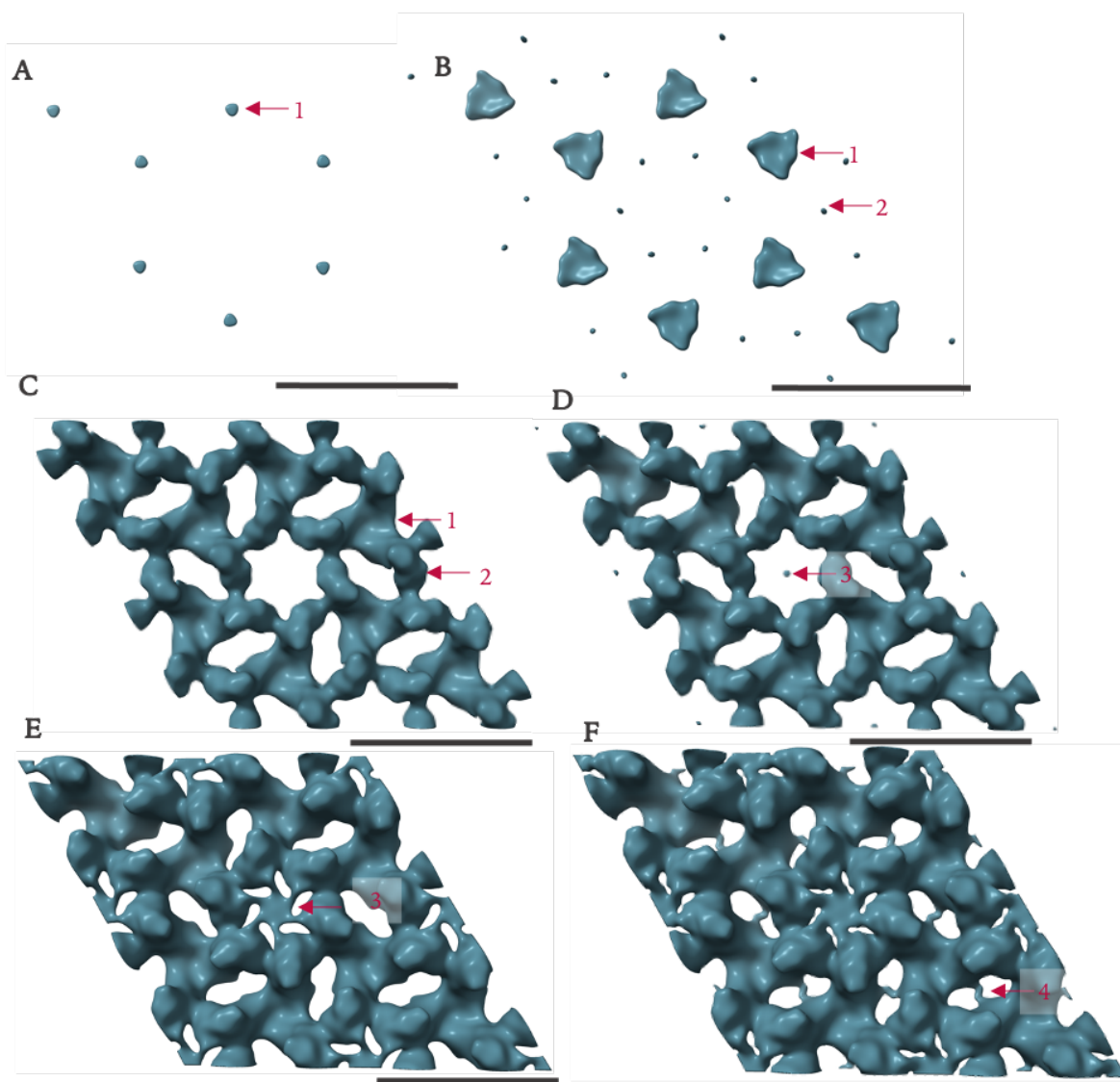


Figure 4.15 Cryo CsxA 3D reconstruction at different volume thresholds

A)170: density at three-fold linkers observed (arrow 1), B)115: densities at central ring appear (arrow 2) and three-fold linkers observed (arrow 1), C) 40.6: linkers between the three-fold linkers (arrow 1) and central ring (arrow 2) observed, D) 39: density 'cap' appears in the center of the pore (arrow 3), also the six-fold symmetry axis, E)24: 'cap' in the center of the pore increase in density (arrow 3). This is the estimated volume threshold for 34kDa monomer F)21: density observed at two-fold symmetry axis (arrow 4). Calculated before absolute hand determination. Scale bar = 105Å

the opposite side they create a ‘crater’ like shape that dips into the structure (Figure 4.16– purple arrow). Peripheral pores are present in between these linkers (Figure 4.16- cyan arrow), these are ovoid in shape with a width of 16.5Å and a length of 47Å (Figure 4.16). The overall crystal thickness was 67Å: consisting of a 32Å ring and 35Å trimeric linkers (Figure 4.16).

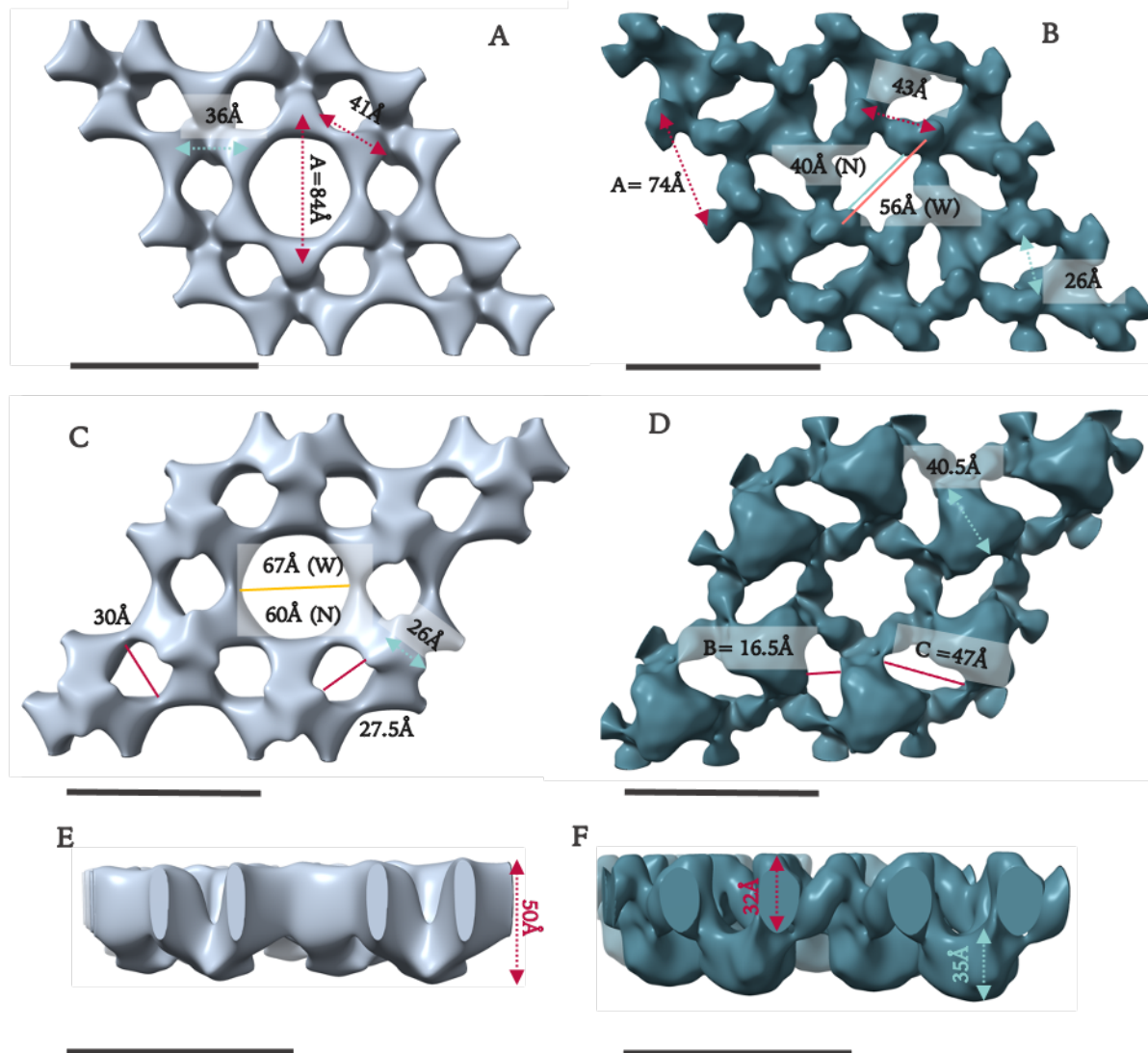


Figure 4.16 Comparing dehydrated and hydrated CsxA reconstructions

A,C,E) Dehydrated state (Negative stain $\sim 25\text{\AA}$ resolution) B,D,F) Hydrated state (Cryo reconstruction) A-D) Perpendicular views to the plane A,B) Central ring possesses 6 subunits, the distance between neighboring subunits (red arrow) is 41Å and 84Å across the ring (measurement A), this is equivalent to 43Å and 74Å (measurement A) in the hydrated state. Distance between subunits of neighboring rings (blue arrow) is 36Å in dehydrated state and 26Å in hydrated. Width of the pore is 56Å at the widest (pink line) and 40Å at the narrowest (blue line) for hydrated state C,D) Width of three-fold linkers is 26.5Å for dehydrated and 40.5Å for hydrated state. Width of pore in dehydrated state is 67Å at the widest point and 60Å at the narrowest (yellow line). Peripheral pores (red lines) in dehydrated state are 30Å and 27.5Å wide, in hydrated state they are 16.5Å and 47Å along dimensions B and C respectively C E,F) Side on view width of layer is 50Å (E) and 67Å (F). The absolute hand was not determined for this comparison due to ambiguity in the hand of the negative stain reconstruction. Scale bar = 105Å

4.4 Discussion

4.4.1 CsxA composes the exosporium basal layer in *C. sporogenes*

CsxA was heterologously expressed in *E. coli* by TJ and, similar to ExsY from *Bacillus cereus*, self-assembled into crystal sheets with the same structure as native exosporium (Figure 4.5). This was further confirmed by AFM analysis of exosporium fragments and CsxA crystals (Figure 4.6 and 4.7), conducted by NM. The ability of CsxA to self-assemble into the same crystal structure as native exosporium combined with the observations of *csxA* spores in chapter 3 which lack exosporium basal layer, establishes that CsxA is the primary protein of the basal layer in *C. sporogenes*. CsxA shares no homology to known exosporium proteins from *Bacillus* (Janganan *et al.*, 2016), yet appears to assemble into a similar honeycomb lattice structure.

The heterologously expressed CsxA crystals provide a representative sample of the native exosporium basal layer, which can be harvested and concentrated and are thus more amenable for further detailed studies. The CsxA crystals were used to gain greater insight into the structure and physical properties of the exosporium basal layer in *C. sporogenes*. As CsxA crystals are more ordered than native exosporium, they were also more suitable for higher resolution structural studies including cryo-EC. The resulting 2D cryo map provides the resolution that individual subunits can start to be tentatively assigned (Figure 4.11– red dotted circle).

4.4.2 Cryo electron crystallography: sample and processing

The installation of the Arctica equipped with a DED provided the opportunity to collect higher resolution structural information of the exosporium basal layer. This required extensive optimisation of sample preparation to ensure that crystals were undamaged and retained high crystal integrity. The methodology for crystal harvesting was developed to yield clean sample with an abundance of crystals. The refined approach is described in chapter 2.3.2.

Cryo sample preparation was challenging due to the lack of reproducibility of grid quality. The best sample was on commercially available GO coated grids, although grid quality varied greatly between batches. The high-quality batches were far superior providing a sample with good CsxA crystal and

ice distribution across the grid. Efforts to develop methods for in-house GO coating of the grids to improve consistency would be beneficial for further work.

More than 400 micrographs were collected across the tilt angle range. However, the final 3D reconstruction included only 38 micrographs. General experience has been that at best 10% of crystal images are of high quality. With capabilities of data collection in 'movie mode' and multi-frame alignment with software such as MotionCorr the percentage of high-quality crystal images is in theory much greater. For these data, the primary limitation was variable crystal quality. CsxA crystal disorder affected the ability to index crystal lattices, in many cases indexing was not possible and thus the image could not be processed further. Unbending patchy crystals was also very challenging, particularly when the best crystal reference patch was small, and the distortions were too great for tracking. Thus, crystal processing heavily relied on masking high quality crystal patches. This became problematic with highly tilted samples as masked patches became much smaller and, in some cases, not processable, limiting the attainable 3D information.

The resolution of the 3D reconstruction is at 9 Å laterally and capped to 20 Å axially. To increase the resolution of this map further sample optimization is required, including improving crystal quality and flatness of crystals on the grid which was beyond the scope of this research. Further efforts should also seek more automated data collection and processing methods to increase the throughput of the workflow so that more crystals can be included in the final structure.

4.4.3 CsxA crystal structure

Figure 4.13 reveals the first ever 3D reconstruction of any exosporium basal layer determined by Cryo-EC; it possesses a honeycomb-like lattice structure with central hexameric rings that are interconnected by threefold linkers. The crystal appears permeable with two main pore structures: the central pore and the peripheral pores around the hexameric ring.

A threshold closer to the expected molecular weight of CsxA included density in the core of the central ring, which could not be confidently assigned as real density due to its position on the sixfold

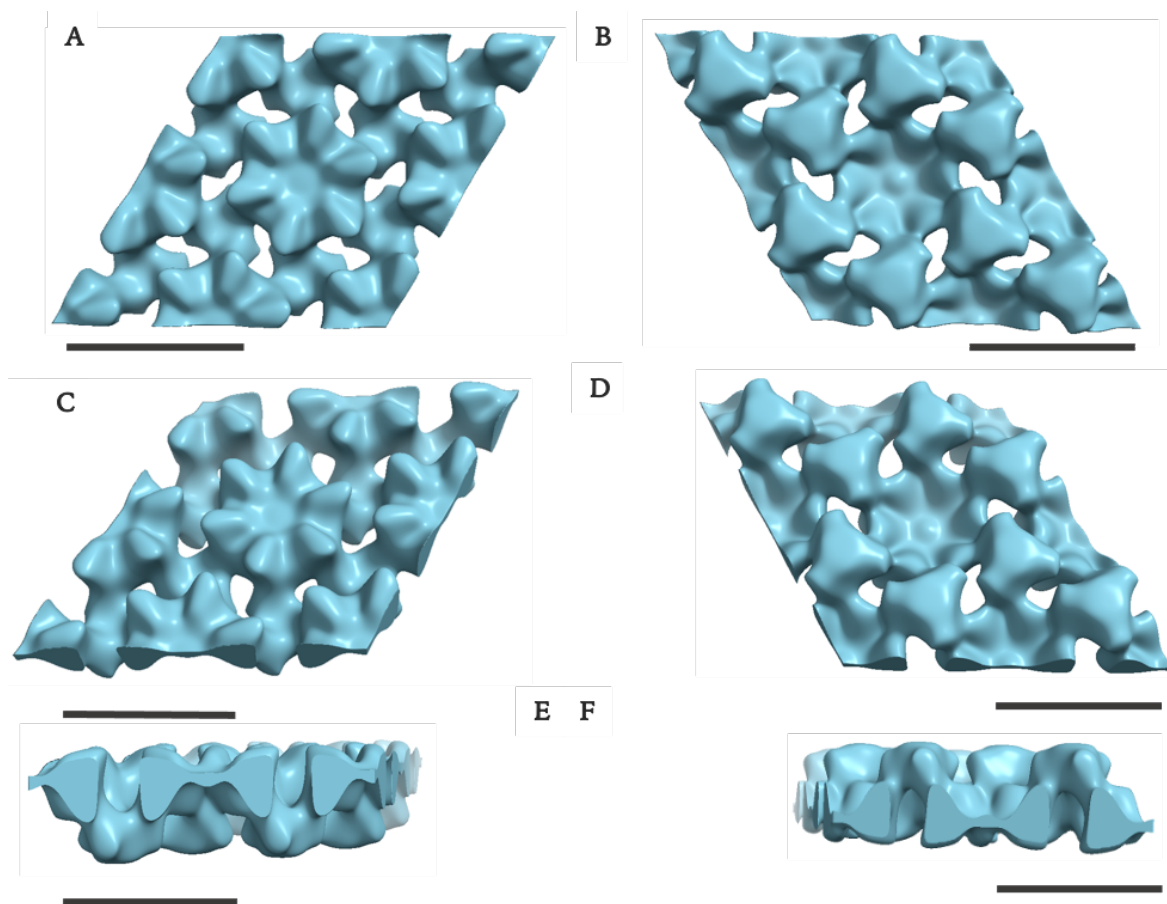


Figure 4.17 Cryo CsxA 3D reconstruction capped at 20Å resolution

A,B) Views perpendicular to the plane C,D) Tilted view to the plane E,F) Side on view. Threshold volume set to ~35kDa per monomer. Reconstruction calculated after absolute hand determination. Scale bar = 105Å

symmetry axes (Figure 4.14). The possibility of a density filled core cannot be discarded based on this reconstruction. It is possible that the exosporium possesses a plug in the central ring as no other noise appears enhanced along the sixfold axis at that threshold. Additionally, the density is localized at the centre, a potentially biologically relevant position. The plug is also observed to branch from the centre to the density of the central ring, as would be expected for such a feature. A 20 Å resolution capped reconstruction of the map (Figure 4.17), set to a threshold level of ~35kDa, also suggests that the density of the core is filled. Further data need to be collected and added to this data set to establish whether this feature is statistically significant or noise amplification on the symmetry axis.

The calculations for estimated protein molecular weight are based on averages of protein density. We do not know if CsxA, or any exosporium protein, complies with these norms as no high-resolution

structural information has been obtained and there are no estimated secondary structure predictions based on the sequence. Thus, the density at a threshold of 40.6 could correspond to a 33.4kDa protein. Alternatively, there may be a flexible domain ~10kDa in size that is not visible in this reconstruction as any flexibility would be averaged out during electron crystallography processing. The only way to confirm either of these would be to generate a high-resolution reconstruction that allowed modelling of the amino acid sequence.

The density observed at the threefold symmetry axis corresponds to the threefold linkers, which was established in 4.3.6 as significant. It is also an assembly that is observed across exosporium from other species including *B. cereus* and *B. anthracis*, which suggests it is an analogous feature (Ball *et al.*, 2008; Kailas *et al.*, 2011; Terry *et al.*, 2017).

In this reconstruction, we can tentatively assign a subunit that corresponds to a CsxA monomer (Figure 4.13– pink dotted circle). These subunits appear to have three distinguishable domains (Figure 4.13E): the threefold linker (1), the central ring (3) and the domain connecting both 1 and 3 (2). As already discussed, if additional domains were present that were flexible they would not be observed in this reconstruction. Prior to this reconstruction, our understanding of *C. sporogenes* basal layer was based on negative stain reconstruction of CsxA crystals (Figure 4.5) and AFM data (Figure 4.6 and 4.7). The negative stain sample preparation involved sample dehydration and caps the resolution to ~25Å. Negative stain can cause positive staining patterns on hydrophilic surfaces which distort features such as pores making them look larger than they are. Thus, exact density and measurements from negative stain reconstructions need to be confirmed using complementary techniques such as AFM (Figure 4.6 and 4.7).

The CsxA cryo map was prepared from samples in vitrified ice, which have no heavy metal stains and retain the hydrated state of the sample, thus provides a potentially more representative map of the native hydrated state. Comparison of the negative stain and cryo 3D reconstructions (Figure 4.18-4.19 and Video 2) demonstrates that the overall lattice is very similar but features across the structure do differ.

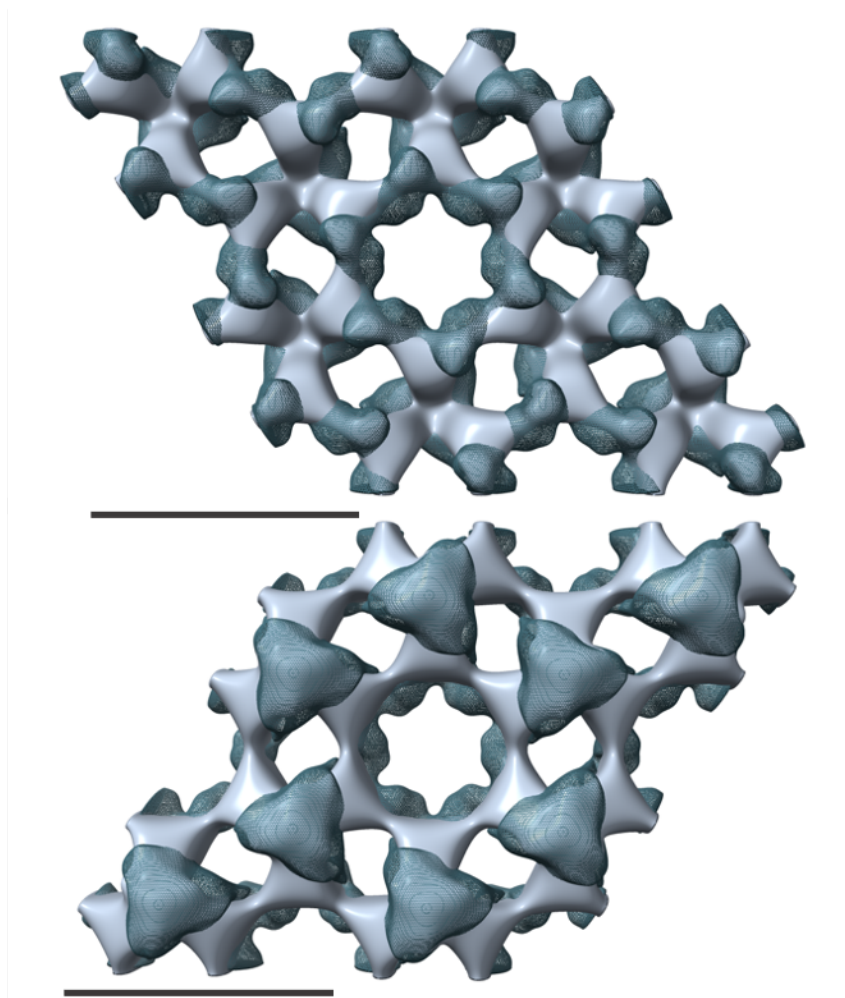
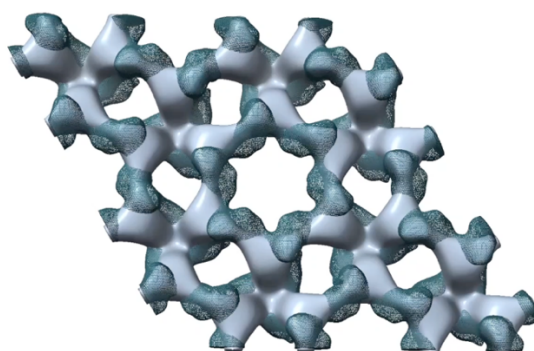


Figure 4.18 Negative stain and cryo CsxA 3D reconstruction

Perpendicular views to the plane of negative stain (grey) overlayer with cryo CsxA (blue mesh) reconstruction. Negative stain map at $\sim 25\text{\AA}$ resolution, cryo map is at a lateral resolution of $9\text{\AA}$ and $\sim 20\text{\AA}$ axially. The absolute hand was not determined for this comparison due to ambiguity in the hand of the negative stain reconstruction. Scale bar = $105\text{\AA}$



Video 4.2 Cryo CsxA (blue mesh) and negative stain 3D reconstruction (grey)

Please find this video in a separate file under the additional material

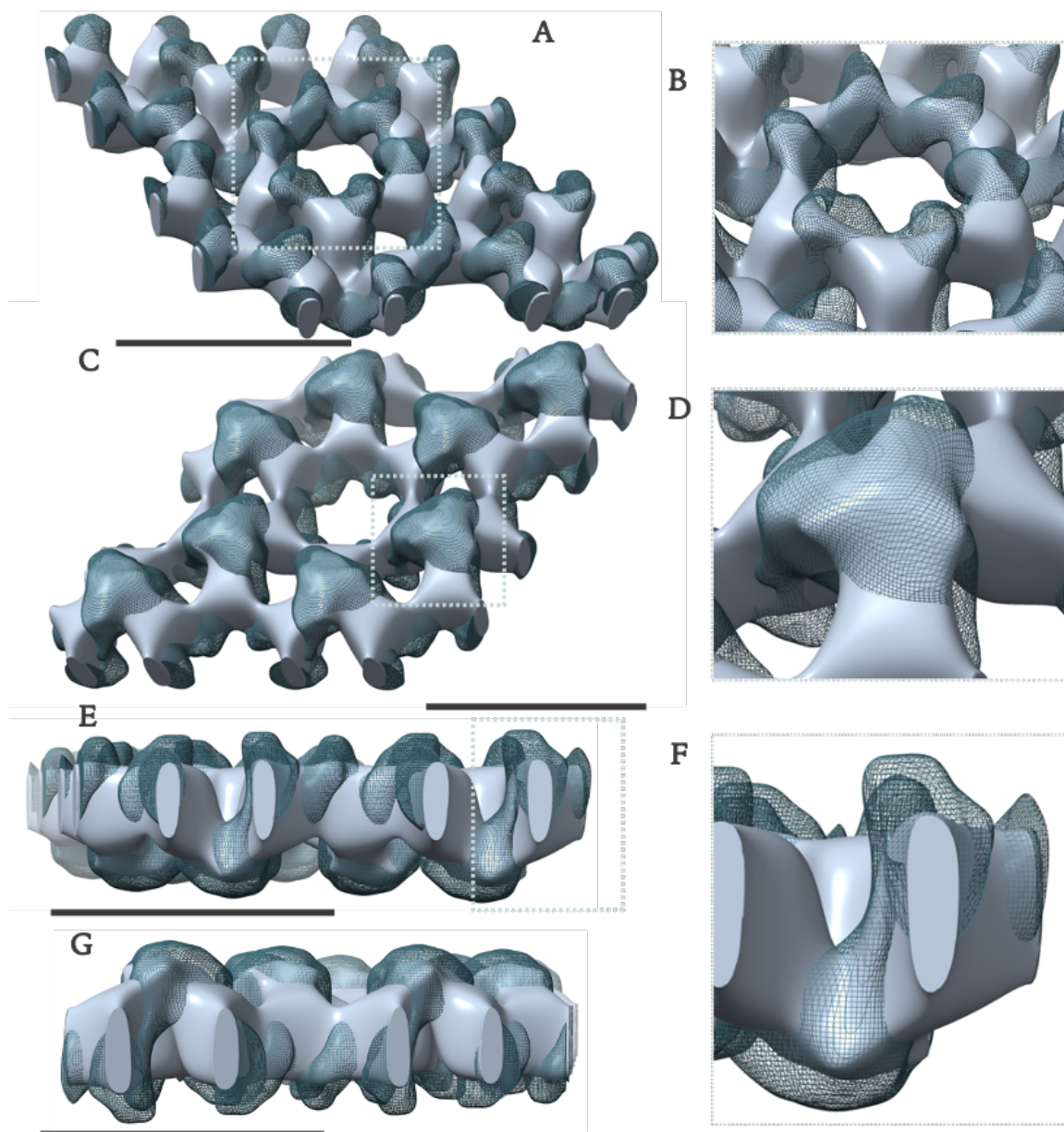


Figure 4.19 Differences between the negative stain and cryo CsxA 3D reconstruction

Different views of the negative stain CsxA map (grey) and cryo CsxA map (blue mesh) overlay presented in Figure 4.18 A,C) Tilted view of maps B) Magnified view of central ring in A D) Magnified view of three-fold linker E,G) Side on view E) Magnified view of subunit from F. The absolute hand was not determined for this comparison due to ambiguity in the hand of the negative stain reconstruction. Scale bar = 105Å

Due to the ambiguity of the hand in the negative stain reconstruction, the absolute hand determination was not needed for the comparison of the cryo and negative stain. However, for comparison with AFM (Figure 4.17 and 4.22) the absolute hand determination was required and conducted as described in Amos *et al.* (1982).

Difference maps were generated to aid visualisation of differences between the negative stain and Cryo-EM maps (Figure 4.20 and 4.21). Positive difference map (PDM) (negative stain map subtracted from the cryo map) is displayed in cyan mesh and the negative difference map (NDM), cryo map subtracted from the negative stain map) is visible in a red mesh (Figure 4.20 and 4.21).

4.4.3.1 Central ring

The central ring in the middle of the unit cell possesses a ‘crown-like’ appearance in the cryo-EM map, with densities protruding out of the crystal plane (Figure 4.19A and B). These protrusions are not observed in the negative stain map which has a flat surface. The additional density from the ‘crown-like’ protrusions are clearly visible in the PDM (Figure 4.20 – arrow 1). Measurements of the distance between each subunit within the ring does not greatly differ between different reconstructions, at 41-43Å distance (Figure 4.16), however the relative position of the subunits in the cryo map have a rotational twist with respect to the negative stain map (Figure 4.19A and B). The twist of the subunits in the central ring is clearly observed in the difference maps (Figure 4.19). Arrow 1 shows the density in the cryo map, whereas arrow 4 is the position in the negative stain map. The difference density maps can be used to calculate this tangential shift from red to blue causing the rotation of sixfold centres of mass (Figure 4.21). An upward tilt is observed in the subunits from negative stain to the cryo map, which corresponds to a translational distance of 25Å and 63° twist with respect to the negative stain structure. This conformational change of the central ring likely corresponds to a structural change in response to hydration, as will be further discussed in section 4.4.3.4.

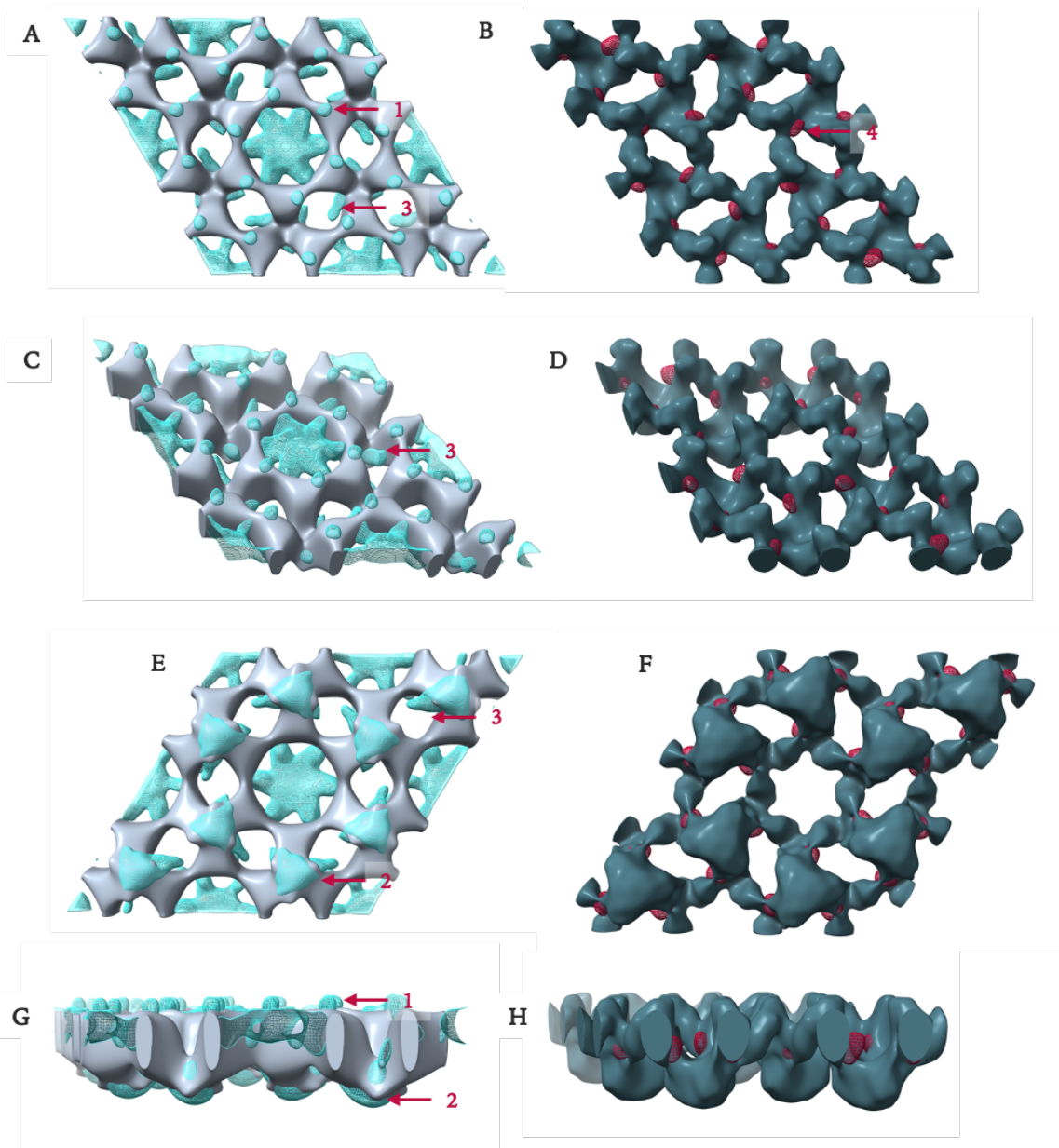


Figure 4.20 Positive and negative difference maps

A,C,E,G) Negative stain map (grey) and positive difference from cryo map (cyan mesh). 1) Density difference at the central ring with an increased height and twist of subunits in the cryo map 2) Three- fold linkers are raised in the cryo map and rotated with respect to the negative stain map 3) Shift in the position of the density connecting three-fold linkers to the central ring. Positive difference map possesses density in the core of the ring corresponding to the magnified difference between negative stain and cryo map. B,D,F,H) Cryo map (blue) and negative difference from negative stain map (red mesh) 4) Difference observed in the subunits of the central ring. The absolute hand was not determined for this comparison due to ambiguity in the hand of the negative stain reconstruction.

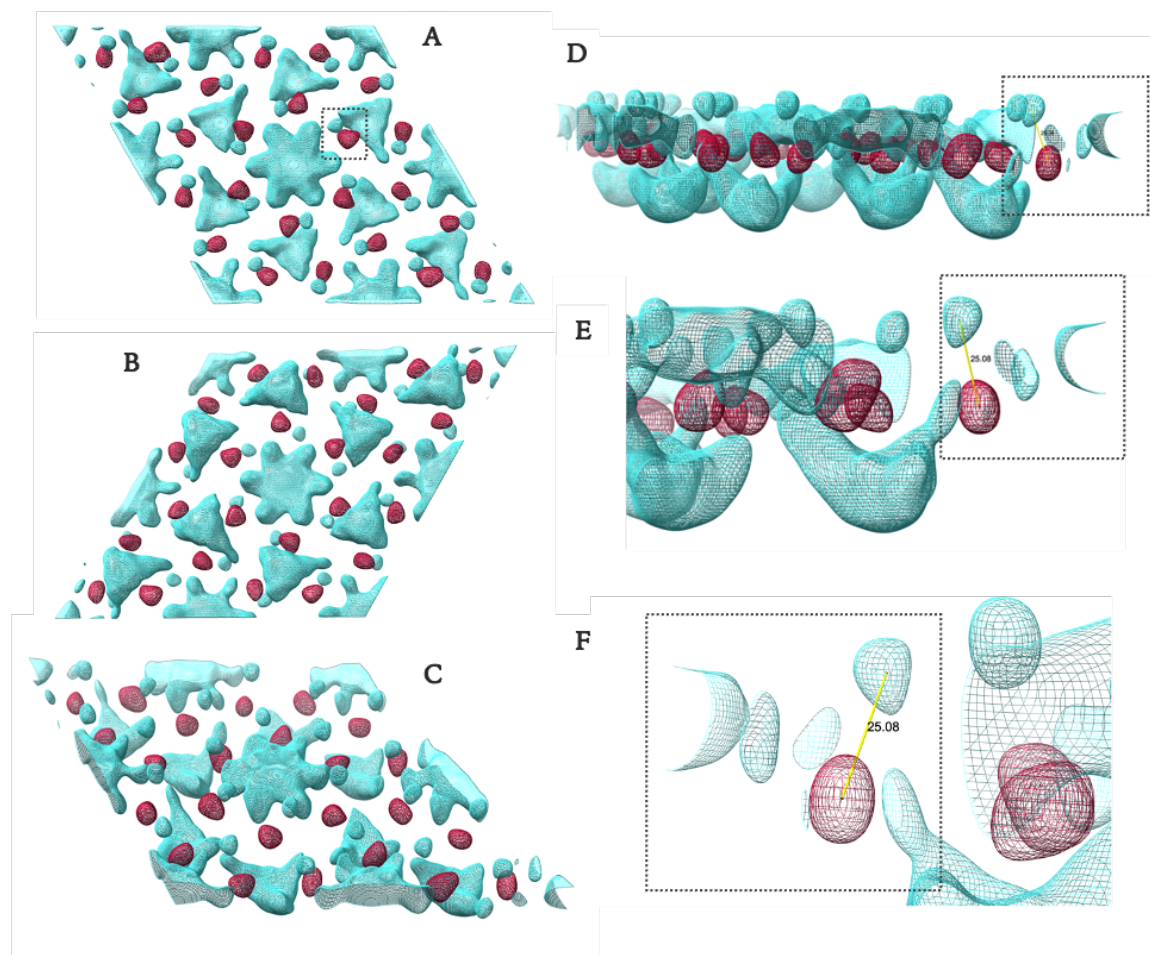


Figure 4.21 Conformational change in central ring observed in positive and negative difference maps

Positive difference map (cryo map - negative stain map = cyan) and negative difference map (negative stain map - cryo map = red). Subunits of the central ring in the cryo map have a tangential shift with respect to the negative stain map, this conformational change can be calculated by measuring the distance between the difference densities corresponding to a single monomer (dotted square). Thus a translational distance of 25Å which corresponds to a 63° twist. The absolute hand was not determined for this comparison due to ambiguity in the hand of the negative stain reconstruction.

Both the negative stain and Cryo-EM map possess a pore in the central ring. The PDM hints at an apparent density filled core (Figure 4.20 and 4.21). The dimensions of the pore in the negative stain reconstruction are 67Å (widest point) and 60Å (narrowest point), quite different from the cryo reconstruction at 56Å (widest) and 40Å (narrowest).

4.4.3.2 Threefold linkers

Figure 4.19C and D highlights the difference in both height and width of the threefold linkers in the reconstructions. A side on view demonstrates an increased height in the cryo map (Figure 4.19E-G) measured at 35 Å (Figure 4.16F). The width of the threefold linkers is also larger in the Cryo-EM map at 40.5 Å, compared to 26 Å of the negative stain (Figure 4.16C,D). The PDM also displays the difference in the threefold linkers (Figure 4.20-arrow 2). This difference in density could correspond

to staining patterns, however analogous differences were also observed in AFM of hydrated and dehydrated state (Figure 4.22), suggesting it is significant.

Figure 4.18 suggests there is a slight rotational twist in the threefold linkers of the CryoEM map with respect to the negative stain map. This rotational change is also visible in PDM (Figure 4.20– arrow 2). The conformational change of subunits in the central ring of the CryoEM map, may induce a slight rotational change in the threefold linkers.

On the opposite side of the threefold linkers is a large dip in the map referred to as a ‘crater’. These craters are observed in the negative stain map but are much smaller than in the cryo-EM reconstruction (Figure 4.19), consistent with the increased width of the threefold linkers. These craters could provide a ‘cup’ like structure, similar to the central ‘cup’ found in *Bacillus* exosporium layers (Ball *et al.*, 2008; Terry *et al.*, 2017). The exact function of the enclosed ring remains unclear, but it has been proposed to adsorb other exosporium associated molecules such as germination moderators and protective enzymes (Ball *et al.*, 2008; Terry *et al.*, 2017). The craters in *C. sporogenes*, in addition to the possibly plugged central ring, could provide an analogous function to the cups in *Bacillus*.

4.4.3.3 Peripheral pores

The negative stain reconstruction reveals peripheral pores that are almost circular in shape with a width of 30 Å and 27.5 Å, respectively. The shape of these pores is strikingly different in the cryo map as they appear more oval in shape and narrower at 16.5 Å and 47 Å along dimensions B and C respectively (Figure 4.16). The PDM reveals that the domain connecting the central ring with the threefold linker is shifted from the negative stain map (Figure 4.16– arrow 3). The twist of the subunits in the central ring is likely to induce a change in this domain, which also twists the threefold linkers (4.4.3.2), forming an elongated pore structure. It is also worth emphasising that the difference from the negative stain map could also be due to staining patterns of the crystal, as discussed in 4.4.3. Nevertheless, the cryo map reveals a representative structure with narrower peripheral pores, likely to

provide a smaller filter for molecules to pass through, although the exact function *in vivo* as a molecular sieve remains unclear, especially with a large central pore.

4.4.3.4 Cryo-EM CsxA reconstruction is the hydrated state of the crystal

Comparison of the cryo CsxA reconstruction with AFM of native exosporium (Figure 4.6) allows the tentative assignment of the crystal surfaces. The inward facing layer corresponds to the raised trimers which were clearly visible by AFM (Figure 4.6B). The outward facing layer corresponds to the raised hexameric rings, this side was decorated with hairy nap on native exosporium and was not clearly visible by AFM (Figure 4.6C).

AFM imaging of crystals revealed a reversible structural conformation when imaged in water or in air (Figure 4.7). The differences observed between the cryo and negative stain CsxA reconstruction appear to resemble this reversible structural conformation, suggesting that the cryo CsxA reconstruction reveals the hydrated state and negative stain the dehydrated state (Figure 4.22).

The inside facing layer by AFM reveals subunits (Figure 4.22- arrow 1) that correspond to raised trimers. These subunits appear larger upon imaging in water than in air. This difference is consistent with the observations of the threefold linkers in negative stain and cryo reconstructions, discussed in 4.4.3. The darker central ‘pit’ (Figure 4.22– arrow 2) indicates a region of less height in AFM, which corresponds to the central pore in the crystal. The peripheral pores are observed as gaps in the AFM (Figure 4.22 – arrow 3) which are more visible and elongated in the hydrated state compared to the dehydrated state, which resembles the difference in shape of peripheral pores observed between the cryo and negative stain reconstruction (4.4.3).

The outward facing layer in CsxA crystals displays a remarkable structural change upon hydration in AFM (Figure 4.22F,H). AFM imaging in air, reveals a hexameric ring structure with a central pore structure. Upon imaging in water, a reversible conformational change is observed, the central ring forms a ‘petalled’ structure (Figure 4.22– arrow 4), suggested to be due to the swelling of subunits in the hexameric ring. In the cryo reconstruction, the ‘petalling’ effect is observed as a ‘crown-like’ assembly of protruding density from the central ring (Figure 4.22– arrow 4). These features are not

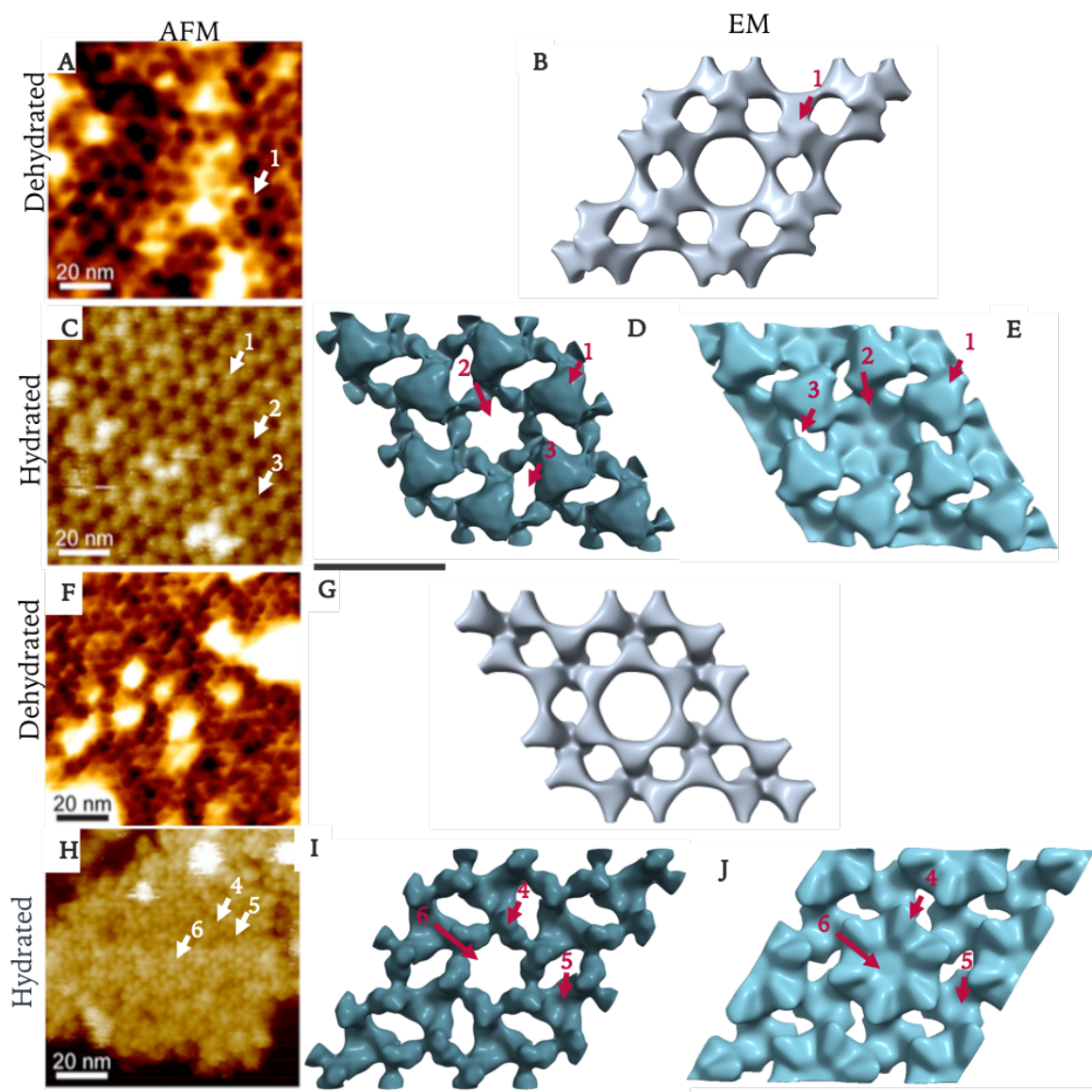


Figure 4.22 AFM and EM data for tentative assignment inwards and outwards faces of basal layer

A-E) Inwards facing layer A,B) Dehydrated state from AFM in air (A) and negative stain EM(B) C,D) Hydrated state from AFM in water (C) and cryo EM (D,E). E) 20Å capped cryo EC reconstruction. Individual subunit in AFM correspond to trimers in EM (arrow 1), central pore corresponds to either a pore (D) or cavity in EM (E) (arrow 2), peripheral pores appears as gaps in between subunits in AFM (arrow 3). F-J) Outward facing layer F,G) Dehydrated state from AFM in air(F) and negative stain EM(G) H,I) Hydrated state from AFM in water (H) and cryo-EM(I,J). J) 20Å capped cryo EC reconstruction. Petalling observed in hydrated state corresponds to hexamer subunits swelling (arrow 4). Trimeric pits observed in AFM correspond to raised trimers on opposite side (arrow 5). AFM indicates cavity filled pore (arrow 6) similar to 20Å capped cryo-EM reconstruction. Due to lower lateral resolution in AFM this may be a pore as observed in 9Å cryo-EM reconstruction. Cryo-EM reconstructions (D,E,I,J) calculated after absolute hand determination Scale bar = 105Å

observed in the dehydrated state, consistent with the negative stain reconstruction. Arrow 5 highlights dips in the hydrated crystal with a trimeric shape (Figure 4.22). These dips correspond to the ‘craters’ in the crystal caused by the raised threefold linkers on the opposite side (Figure 4.22). The central pore is not visible by AFM in water (Figure 4.22– arrow 6), however the lateral resolution by AFM is limited due to the size of the tip. Thus the lack of pore does not necessarily imply that the central ring is density filled, but rather that it might be too small for appropriate detection. As was discussed in 3.3, based on the current cryo CsxA reconstruction it is uncertain whether the central ring structure possesses a density filled core although a 20Å resolution capped 3D reconstruction suggests that it could be (Figure 4.16) and further data are required to confirm this. The 20Å resolution capped cryo CsxA reconstruction (Figure 4.22J) reveals striking resemblance to the AFM images in water (Figure 4.22H), confirming the correct absolute hand determination of the cryo reconstruction.

AFM of CsxA crystals also revealed an increased thickness of the crystal layer from ~40Å in air and ~65Å in water. Measurements of the cryo reconstruction give a thickness of 67Å (Figure 4.16) which is consistent with the AFM data, further confirming that the cryo map is the same crystal structure as observed by AFM in water. The negative stain map suggests a thickness of 50Å, which is thicker than the 40Å measured by AFM in air. The difference in thickness between AFM and the negative stain reconstruction could correspond to discrepancies in staining patterns, discussed in 4.4.3, which may lead to the projection appearing thicker than the true structure. The increased thickness is apparent when comparing the cryo and negative stain reconstructions at both the central ring and the threefold linkers (Figure 4.19). Additionally, it could also be the result of lack of data sampling along z^* causing the elongation of features.

All of these data suggest that the negative stain map corresponds to the dehydrated state of the exosporium and the cryo reconstruction the hydrated state. The hydrated and dehydrated state represent real environmental conditions that spores will encounter. These structures reveal important exosporium states and its ability to adapt to changes in environment, thus have biological significance which will be further discussed in chapter 7.

4.4.4 Crystal properties

Chapter 1.3.3.5.5 introduces the highly resilient properties of the exosporium as identified in *Bacillus* and *Clostridia* exosporium fragments that require high concentrations of reducing agents and heat for their disintegration (Takumi *et al.*, 1979; Terry *et al.*, 2017). These properties were also observed for CsxA crystals as they possessed complete heat resilience and partial resistance to 8M urea and 2M DTT.

Crystals were observed in 8M urea, although they were not in high abundance and crystal fraying was observed. This suggests that crystal resilience to urea was lower than its *Bacillus* counterpart, which used 8M urea in purification buffers for harvesting ExsY crystals (Terry *et al.*, 2017). 2M DTT treatment affected crystal structure but did not destroy crystals completely. The cryo-EC reconstruction of CsxA reveals that packing of hexagonal rings is likely maintained by the threefold linker as distances between the hexameric rings are too large for disulphide bond formation (Figure 4.13). The threefold linkers are a likely location for disulphide bond crosslinking, which explains why some degree of crystallinity is maintained upon DTT treatment at room temperature. However this effect is exacerbated when combined with high temperatures causing the complete disintegration of the crystal.

The cysteine rich content of CsxA suggests high disulphide bond crosslinking across the basal layer. The ability for CsxA to assemble in a reducing environment such as the mother cell cytosol and *E. coli* cytosol, suggests a similar mechanism of assembly as that described in *Bacillus* (Terry *et al.*, 2017). The monomers of cysteine rich proteins are driven to assemble a hexagonal lattice, once assembled it provides greater proximity of cysteine residues to allow cooperative disulphide bond formation in the normally reducing cytosol (Chapter 7).

Chapter 5: *Clostridium sporogenes* spore ultrastructure

5.1 Introduction

Thus far, we have focused on the outermost layers of *C. sporogenes* endospores and the involvement of cysteine-rich proteins in their assemblies. This chapter aims to extend our focus to the spores' ultrastructure, spanning all the layers that compose *C. sporogenes* endospores and place the structures we have revealed in chapters 3 and 4 in their cellular context.

As established in chapter 1.3.3, the dormant spore state is formed from multiple layers that confer resilience properties and maintain a metabolically inactive state (Setlow, 2006; Stewart, 2015). The spore architecture includes both inner and outer membranes, along with peptidoglycan in both GCW and cortex, and proteinaceous layers consisting of coat and exosporium (species dependent). The exosporium, when present, is separated from the coat by an interspace region which in some species contains a network of layers (Ebersold *et al.*, 1981; Warren *et al.*, 1994; Wehrli *et al.*, 1980).

Studying spore ultrastructure can be challenging due to their highly electron dense spore body (chapter 1.3.3). Thus methods have been developed that involve fixing and sectioning the spores to analyse their internal layers. Most of our understanding of spore ultrastructure is from chemically fixed, dehydrated, heavily stained and plastic embedded sections – referred to as chemical fixation (chapter 1.2.3.1). Previous EM research on *C. sporogenes* spore sections has also been conducted using chemical fixation (chapter 1.3.3) (Brunt *et al.*, 2015; Hoeniger and Headley, 1969; Warren *et al.*, 1994). This method can damage biological samples and lead to the loss of structural integrity and generate misleading artefacts. New tools and technology including HPF, CEMOVIS and FIB-milling (chapter 1.2.3.1) provide an alternative sample preparation approach that preserves biological specimens and provides novel insight into bacterial ultrastructure. Unfortunately, CEMOVIS and FIB-milling are both very laborious techniques that require a high level of skill to be conducted successfully and a limited success rate (chapter 1.1.2). HPF combined with freeze-substitution is an

alternative approach that provides superior preservation of biological samples (chapter 1.2.3.1), due to HPF, with easier handling of plastic sections (Studer *et al.*, 2001).

5.2 Aims

This work aimed to gain insight into *C. sporogenes* spore ultrastructure by electron microscopy and place the 2D crystallographic work into its cellular context *in situ*. A greater understanding of *C. sporogenes* spores will also provide insight into group I *C. botulinum* (Carter and Peck, 2015). The current thin-section data of dormant *C. sporogenes* spores lack definition and details of the spore layers. This research focussed on optimisation of sample preparation techniques, including the use of HPF and freeze-substitution, for sectioning to gain novel insights into spore layers. In order to obtain 3D information of spore assemblies ET was employed for both whole spores and sections.

5.3 Results

5.3.1 Whole spore tomography

With the aim of gaining 3D information on the outermost spore layers, whole spores were plunge frozen onto lacey carbon support grids as described in chapter 2.4.2.2. Gold fiducials (10nm diameter) were added to the sample for tilt series alignment. Data were collected from -60° to +60° at 2-degree increments on the Arctica at 200kV.

Figure 5.1 and video 5.1 displays the coarse alignment of a whole spore tilt series. The two main features observed are the spore body (Figure 5.1– purple arrow) and the exosporium (Figure 5.1 – blue arrow). High tilts (Figure 5.1A,E) demonstrate that the exosporium completely surrounds the main spore body. Gold particles were observed on the carbon support and on the sample (Figure 5.1– red arrows), however none of the particles on the sample was trackable due to obstructed views from the dense spore body. Thus, no further tomographic analysis could be carried out on these data.

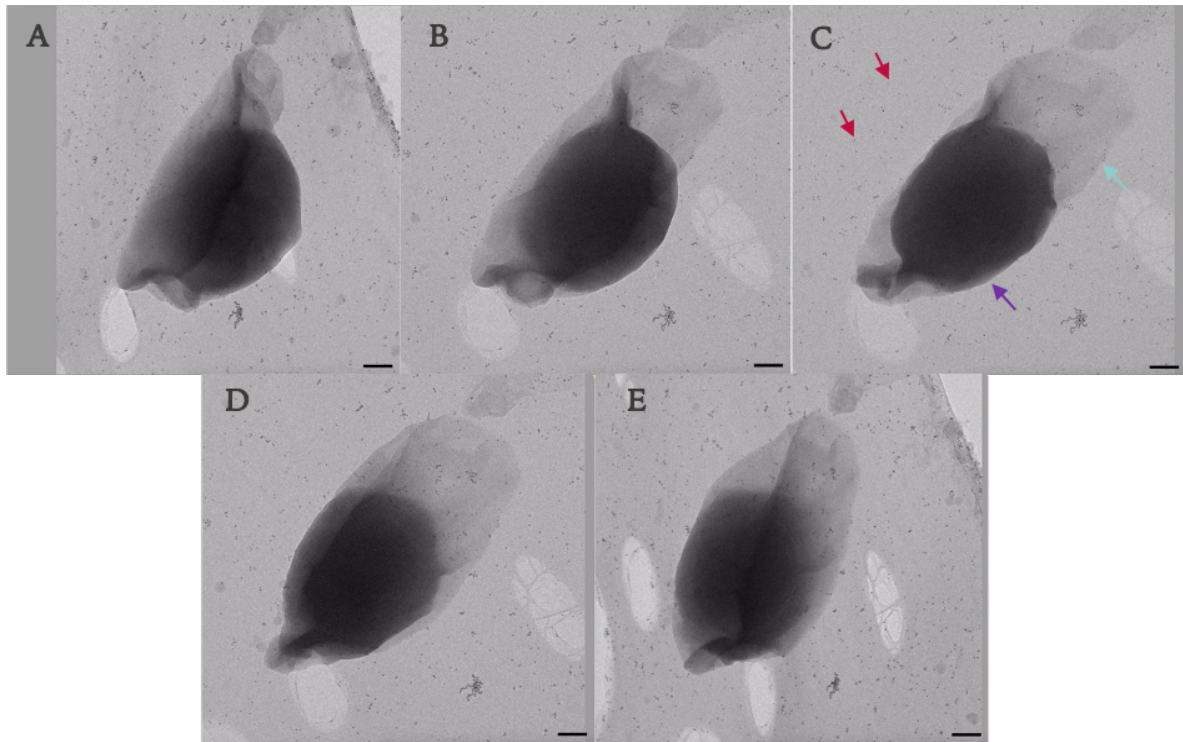
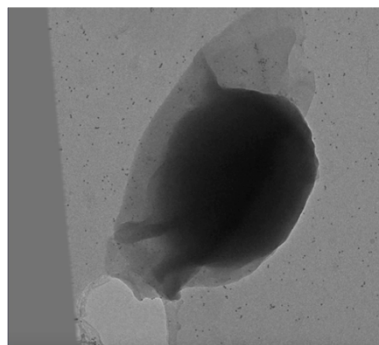


Figure 5.1 Tilt series of whole *C. sporogenes* endospores

Tilt series collected from -60° to $+60^{\circ}$ with 10nm gold fiducial markers (red arrows), main spore body (purple arrow) and exosporium (blue arrow) A) -60° , B) -30° C) 0° D) $+30^{\circ}$, E) $+60^{\circ}$ Scale bar = 200nm



Video 5.1 Tilt series of *C. sporogenes* endospores

5.3.2 Thin sectioning spores

As established, there are different approaches to sample preparation for sectioning. In order to find the optimal methodology both chemical fixation and HPF + freeze-substitution were employed. For a thorough description of the methodology refer to chapter 2.4.3.4.

5.3.2.1 Chemical fixation

The chemical fixation samples were made by Chris Hill (EM facility, University of Sheffield) (chapter 2.4.3.3). Thin sections (70-90nm) of these samples were prepared and screened on the Tecnai T12. The sections revealed heavily stained layers that constitute the spore. Identifiable assemblies are labelled in longitudinal and transverse sections (Figure 5.2). This demonstrated that PM and GCW are not visible in these sections. The cortex appears lightly stained next to the electron dense coat layers. The exosporium is visible as a densely stained outermost layer, although the hairy nap is not apparent on these sections. Layers are observed in the interspace, between the exosporium and the coat, which assemble into laminae (Figure 5.2B,C - pink arrows). These layers appear distinct from the basal layer of the exosporium as thinner and less densely stained, thus will be defined from herein as the parasporal layers. The spore cores across the sections possessed unstained ‘bubbles’ (Figure 5.2– pink star), which are likely caused by problems with fixative infiltration due to the highly dense material in the core (Meek, 1976).

5.3.2.2 High pressure freezing and freeze substitution

HPF + freeze-substitution was employed as an alternative to chemical fixation. HPF was conducted on the EMPACT 2 Leica, which rapidly freezes sample pellets into liquid nitrogen at high pressure (>2000 bar). Due to sample thickness, high-pressure is required for vitrification. Figure 5.3 demonstrates the impact on sample when high pressure was not sustained (800-1400 bar); spore morphology loses structural integrity causing the deformation and the accumulation of aggregation, observed in the background and in the interspace between the exosporium and the coat (Figure 5.3– pink star). This was not observed in samples that had been frozen at adequate pressure levels (Figure 5.3C,D).

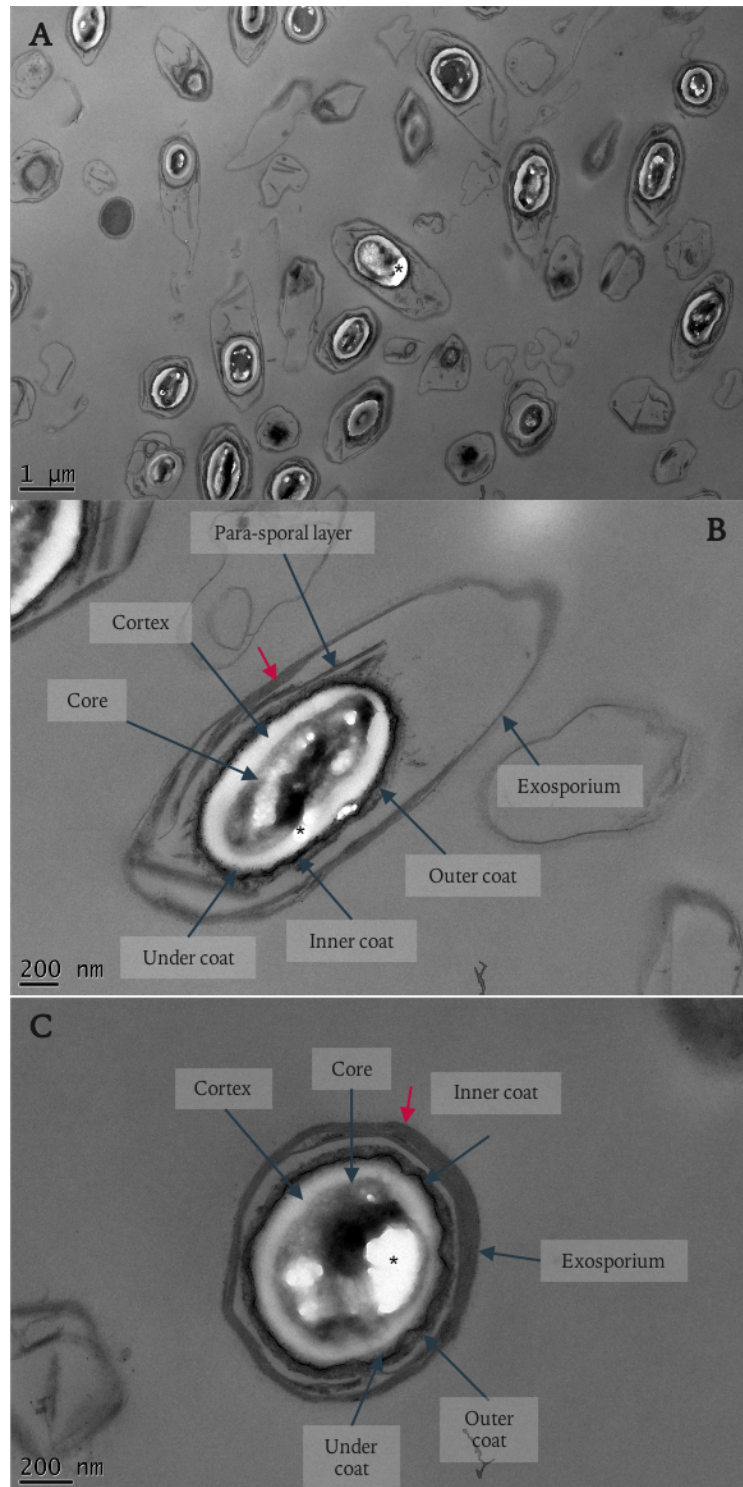


Figure 5.2 Thin sections of chemically fixed *C. sporogenes* endospores

A) Low magnification micrograph of endospores displays longitudinal (B) and transverse (C) sections. All layers that compose the endospore are labelled. 'Bubbles' observed in the core of spores (*) suggest infiltration problems. Cortex is the least stained layer appearing almost white compared to coat and exosporium which are heavily stained. Parasporal layers are layers observed between coat and exosporium. These sections also display laminations on the outermost layers (pink arrow).

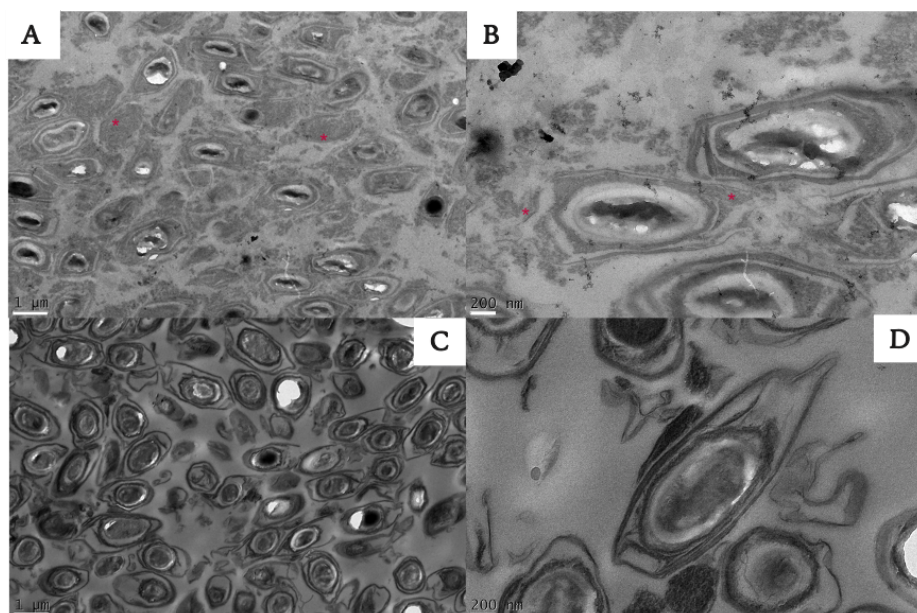


Figure 5.3 High pressure freezing of *C. sporogenes* endospores

Low magnification (A,C) and high magnification (B,D) of sections prepared with differing freezing pressure (A,B) Inadequate pressure levels (800-1400bar) obtained when freezing the samples, affected the structural integrity of the spores and high level of aggregation observed (pink star). (C,D) ~2000bar freezing conditions provided a sample which retained ultrastructure and no aggregation observed.

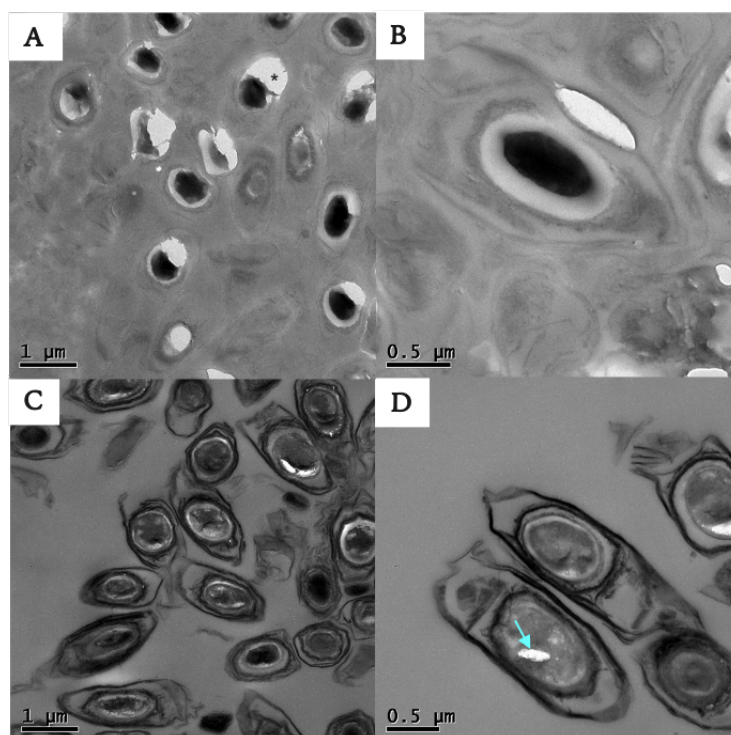


Figure 5.4 Optimising freeze-substitution cocktails for *C. sporogenes* endospores

Low magnification (A,C) and high magnification (B, D) micrographs of sections prepared with different freeze-substitution cocktails (A,B) 1%OsO₄ in acetone, pink star indicates holes in the sections (C,D) 1% OsO₄ in 3% glutaraldehyde and 0.5% uranyl acetate. Blue arrow indicates some infiltration problem in the spore.

Freeze-substitution was conducted on the Leica EM AFS2, following the 42-hour programme described by Matias and Beveridge (2006) (chapter 2.4.3.4). The freeze-substitution cocktails are sample specific and required screening and optimisation. There are many examples in the literature which involve 1% (w/v) OsO₄ in solvent (either acetone or methanol). Spores prepared with 1% (w/v) OsO₄ in acetone yielded sections with low contrast levels making it difficult to distinguish internal layers (Figure 5.4A,B). The sections also possessed holes in the spore core (Figure 5.4 – pink star).

Based on the freeze-substitution cocktail used on *B. thuringiensis* spores (Ebersold *et al.*, 1981) a cocktail of 1% (w/v) OsO₄, 3% (w/v) glutaraldehyde and 0.5% (w/v) UA was used. This cocktail generated a stronger staining pattern providing clear definition of all layers. The holes observed in the spore core of samples with only OsO₄ were not observed in these sections. Unstained ‘bubbles’ in the core of the spores (Figure 5.4– blue arrow), possibly from fixative infiltration problems (Meek, 1976), were observed but were not present throughout the sections. These conditions were chosen as the best for further work.

After freeze-substitution, samples were washed in acetone, dehydrated with ethanol, and infiltrated with Spurr resin (chapter 2.4.3.4). The samples were then placed into plastic tubes and polymerised at 60 °C for 16 hours. Thin sections, approximately 70-90nm thick, were cut using the Reichert Ultracut E ultramicrotome and screened on the Tecnai T12 at 80kV.

As the best freeze-substitution cocktail contains UA, the necessity to further stain the sample after sectioning was considered. As standard, most sections are normally stained with UA for 30 minutes and Reynold’s lead citrate for 5 minutes (chapter 2.4.3.4). The impact of both stains was examined by screening sections with no stain, only UA, only Reynold’s lead citrate and both UA and Reynold’s lead citrate (Figure 5.5,5.6).

Sections omitted from post-staining (Figure 5.5A) retained high levels of contrast on overall spore layers, with the spore core being less stained. These spores also lacked finer details including the inner coat (Figure 5.5A - arrow 1) and laminae observed next to exosporium (Figure 5.5A - arrow 2).

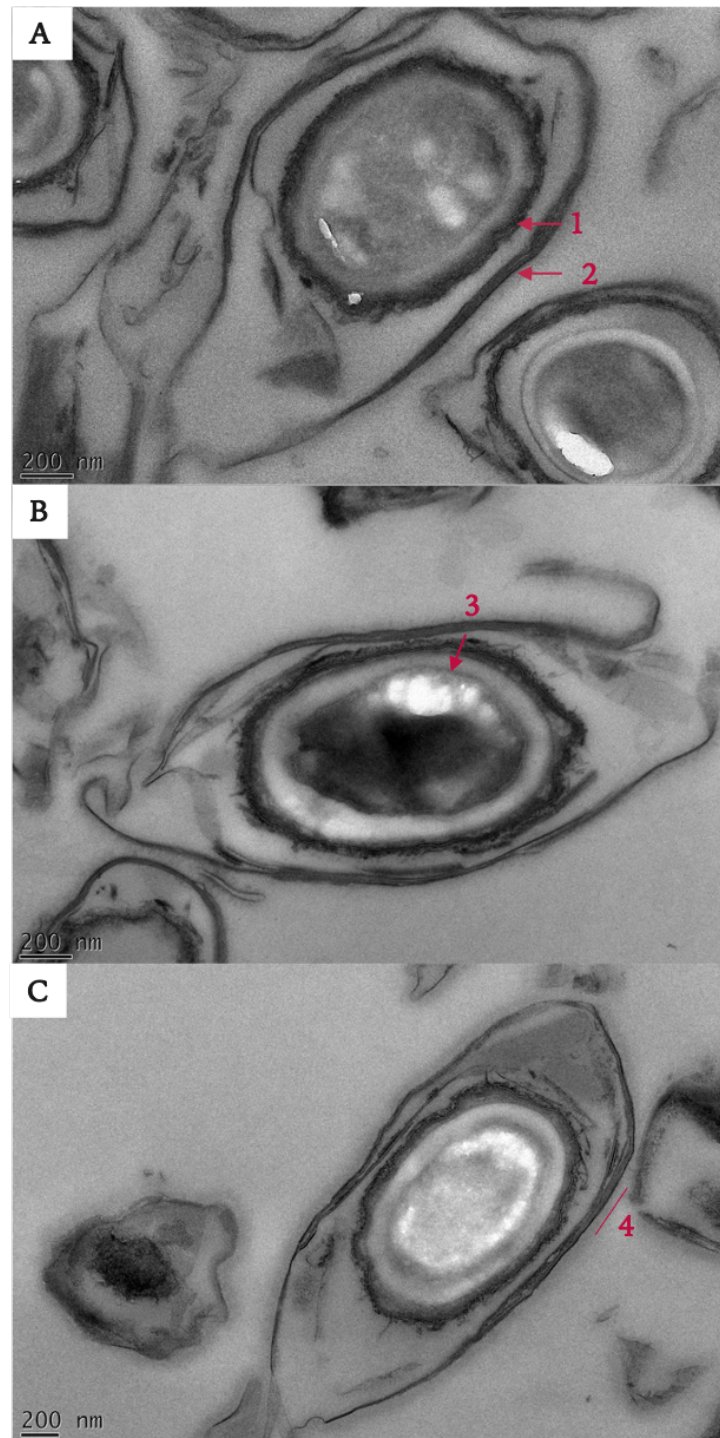


Figure 5.5 Optimising protocols for post-staining of *C. sporogenes* endospore sections

A) No staining: all layers are visible but finer detail including inner coat (arrow 1) and laminae on outermost layer (arrow 2) is lacking B) Uranyl acetate: all layers are visible, core is stained and layers including the germ cell wall (arrow 3) is visible but lacks finer detail on outermost layers C) Reynold's lead citrate: all layers are stained, including laminations which are much better defined (4), apart from the core which is unstained

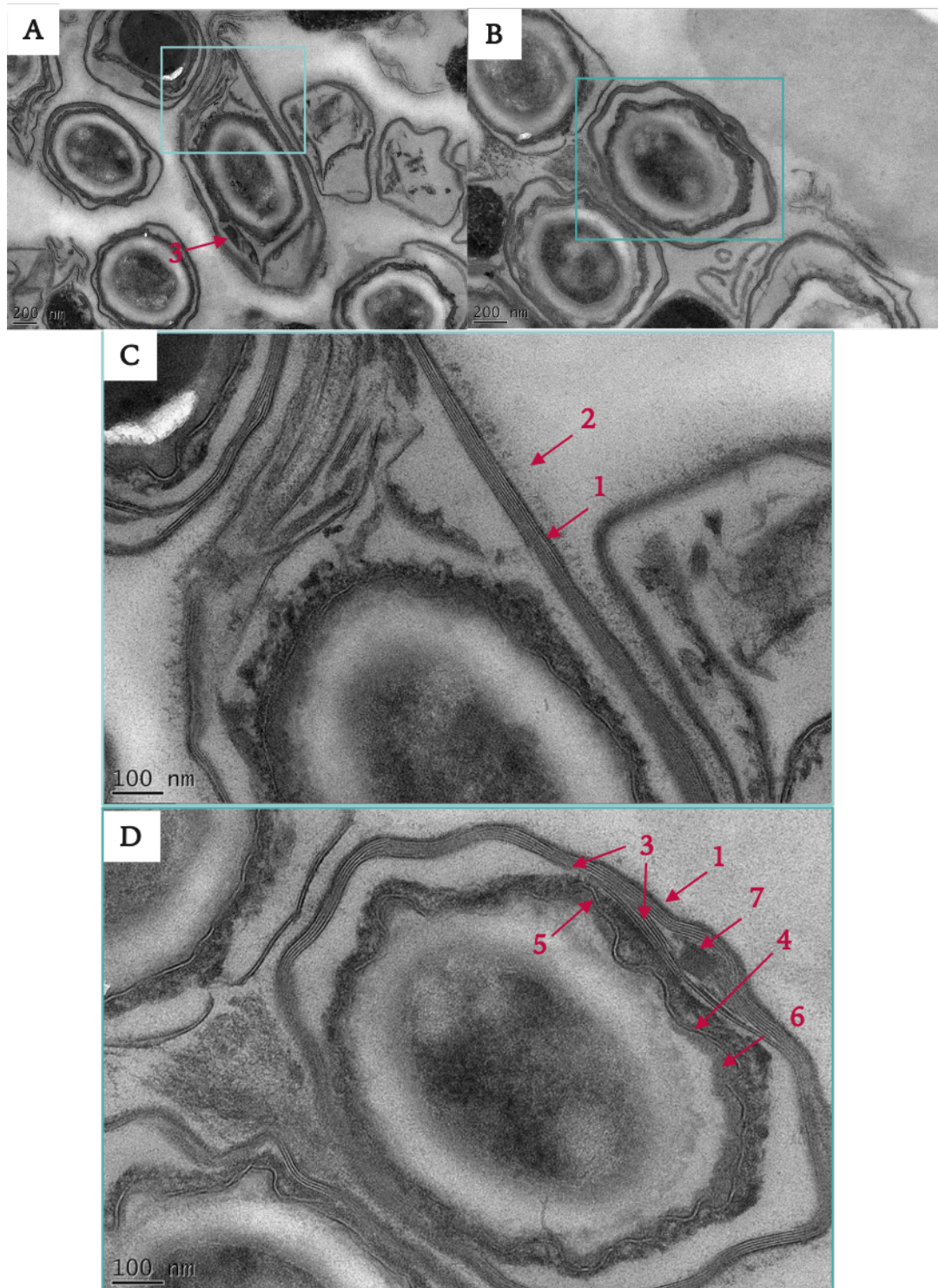


Figure 5.6 Post-staining *C. sporogenes* sections with Reynold's lead citrate and uranyl acetate

A,B) Low magnification micrographs of longitudinal (A) and transverse (B) sections of spores. C,D) High magnification micrographs of spores in A,B respectively. All endospores layers are visible with good contrast levels. Staining reveals highly detailed laminations (arrow 1) along the edge of the spore, some patches also display hairy nap (arrow 2). Repeats are visible within laminations layers in some regions of the sections (arrow 3). A well defined inner coat (arrow 4) with bead-like structure in some regions (arrow 5) and under coat layer (arrow 6) is visible. A dense cuboid is observed in transverse section (arrow 7).

Sections stained with UA (Figure 5.5B) possessed much stronger contrast levels particularly of the exosporium, coat and core. Other layers were also more defined, including the GCW (Figure 5.5B - arrow 3). Strikingly, sections stained only with Reynold's lead citrate possessed much better definition of the outermost layers, including the exosporium basal layer and parasporal laminations (Figure 5.5C– 4), but reduced staining on internal layers including the core.

As demonstrated in Figure 5.6, sections post-stained with both UA and Reynold's lead citrate displayed the sharpest contrast providing definition and detail of all spore layers. High magnification of these spores displays clear laminae next to the exosporium basal layers (Figure 5.6– arrow 1) and hairy nap on some exosporium regions (Figure 5.6– arrow 2). The layers of laminae appear to possess repeats, visible in some areas of sections (Figure 5.6– arrow 3). Clear distinction between previously described outer coat and inner coat was visible (Figure 5.6 – arrow 4). The inner coat also revealed bead-like structure in some regions of the layer (Figure 5.6– arrow 5). A third possible coat layer was consistently observed termed here as the undercoat (Figure 5.6 – arrow 6), present under the inner coat. Whilst screening these sections another feature was identified in some of the spores (Figure 5.6D – arrow 7). This dense material, referred here as the dense cuboid, was observed embedded between parasporal laminae. The exosporium was in some sections cut at an angle that revealed the crystalline patches (Figure 5.7). The unit cell length of these crystal patches was measured from FFTs, for Figure 5.7B the calculated dimensions were $a=101.0\text{\AA}$, $b=105\text{\AA}$ and an angle of 127° , consistent with known WT exosporium which has a unit cell parameter of $a = b = 110 \pm 5\text{\AA}$ and $\gamma=120 \pm 3$.

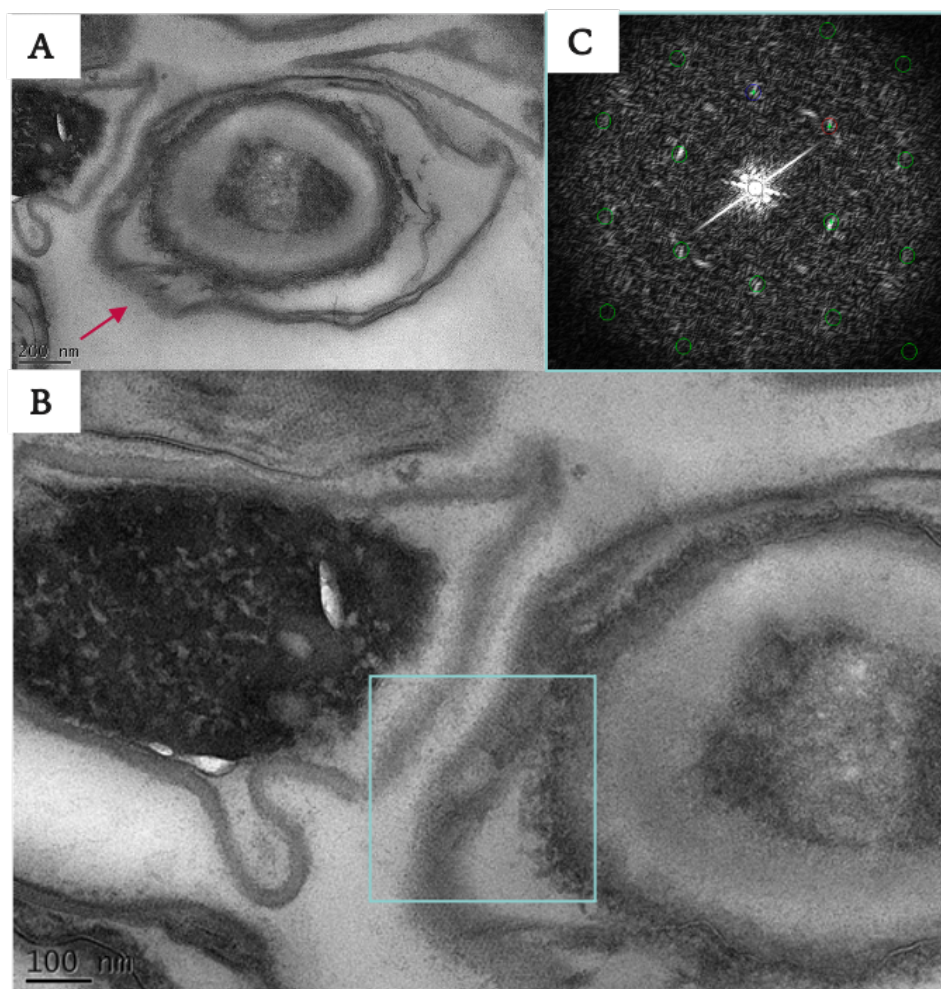


Figure 5.7 Exosporium on HPF and freeze-substitution *C. sporogenes* sections

A) Example of a spore at low magnification where crystalline patch is observed (arrow) B) High magnification of spore with crystalline patch displayed in blue box C)FFT generated from highlighted region in B. The calculated unit cell size of this crystal patch is unit cell length $a=101\text{\AA}$, $b=105\text{\AA}$ and unit cell angle $=127^\circ$.

5.3.3 Tomography data collection

Samples processed by HPF and freeze-substitution with 1% (w/v) OsO_4 , 3% (w/v) glutaraldehyde and 0.5% (w/v) UA were promising candidates for tomography data, as they best preserved the spores and provided contrast for assemblies to be identified. Thin-sections (70-90nm) provide limited 3D information due to small volume sampling, thus thicker sections at 200nm and 300nm were prepared and post-stained with UA and Reynold's lead citrate for optimal contrast levels (chapter 2.4.3.5).

Data collection on a holder at -195°C enables greater exposure to the sample prior to beam damage.

The routine approach of plunge freezing was not conducted on these samples as it was likely to damage or even remove sections from the grids. Instead, the grids were loaded into the Arctica, via

the cassette in the autoloader, at room temperature. Once loaded the whole system was cooled to -195°C. This approach gradually cools down the sample, a gentler approach confirmed from the atlas map of the grid (Figure 5.8). The sections were visible on the grid (Figure 5.8 – blue box), some rips and tears observed (Figure 5.8– orange arrows), but there were sufficient patches of sample (Figure 5.8 – pink arrows) for data collection.

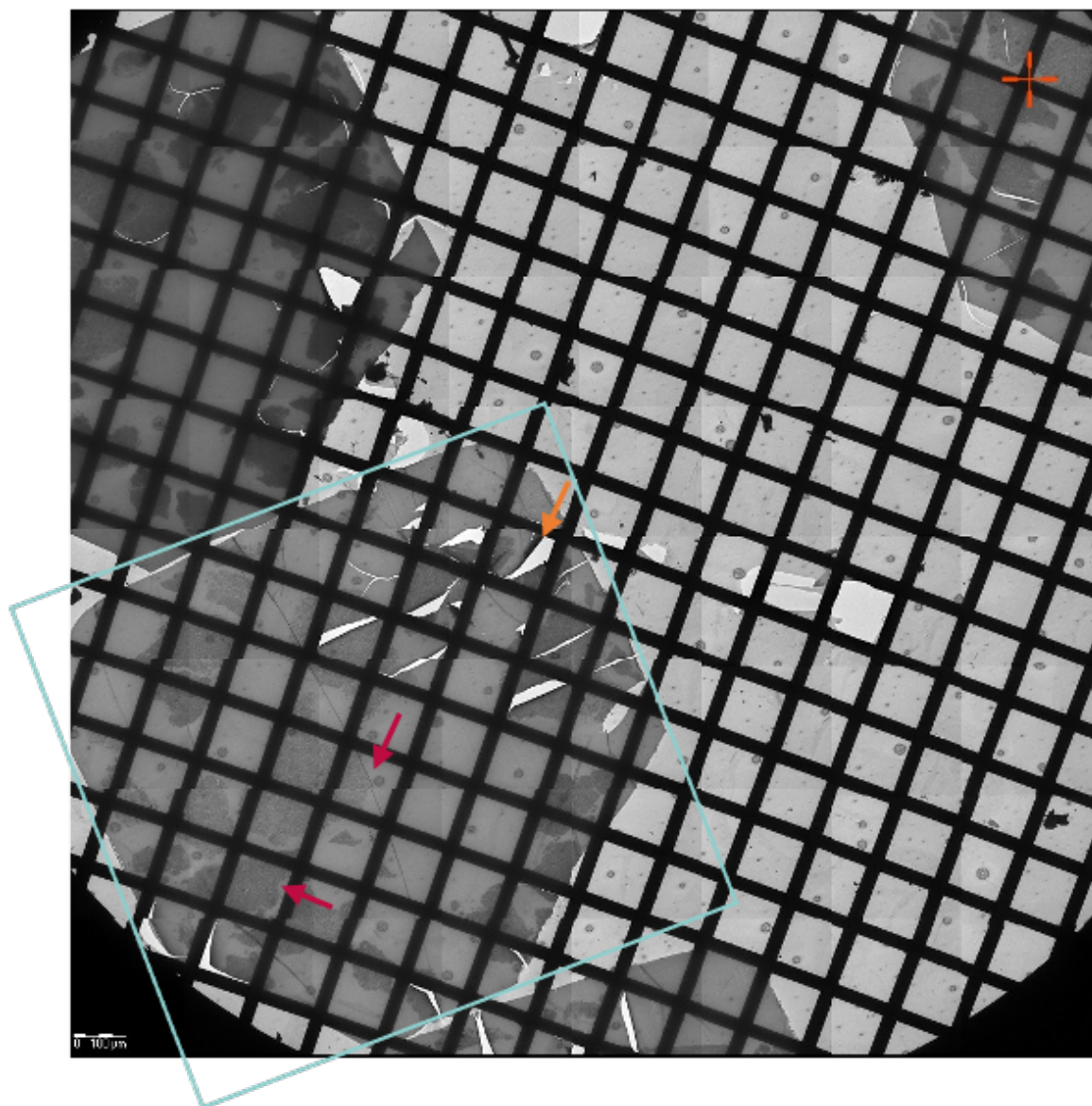


Figure 5.8 Atlas of sections on EM grids

Grid in the microscope after cooling to ~195°C. Blue square indicates one section on the grid, some rips and tears observed (orange arrow). Examples of sample patches are highlighted with pink arrows.

5.3.3.1 Tilt series

Tomography data were collected using the Sheffield in-house 200kV Arctica, equipped with a Falcon 3 direct electron detector. Tilt series collection was done using Tomography software (Thermo Fisher Scientific), which allows automated data collection.

Holder calibration was programmed to collect data from -20° to $+60^{\circ}$ and then from -20° to -60° , with a total dose of 60 electrons/ $\text{\AA}^2$ across the tilt series. The magnification used was 16,600x (pixel size = 0.663nm), sufficient to fit the whole spore in the field of view.

Many tilt series were collected, some of which possessed obstructed views at higher tilts either from the grid bars or other spores. Due to skewed sampling that can cause alignment problems during processing, these tilt series were excluded. Examples of tilt series from -60° to $+60^{\circ}$ are demonstrated in Figures 5.9-5.11 and Videos 5.2-5.4. Figure 5.9 and 5.10 (Video 5.2 and 5.3) are of 200nm sections and Figure 5.11 (Video 5.4) is of 300nm thick sections. Higher tilts have increased effective sample thickness, especially in 300nm sections where features become very dense and difficult to distinguish.

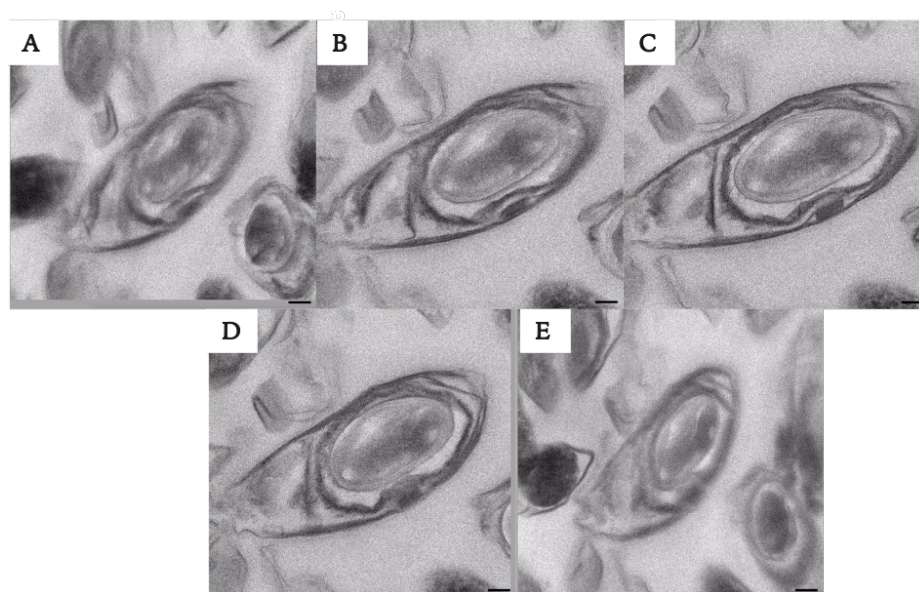


Figure 5.9 Tilt series of 200nm *C. sporogenes* endospore section

Tilt series collected from -60° to $+60^{\circ}$ A) -60° , B) -30° C) 0° D) $+30^{\circ}$, E) $+60^{\circ}$ Scale bar = 200nm

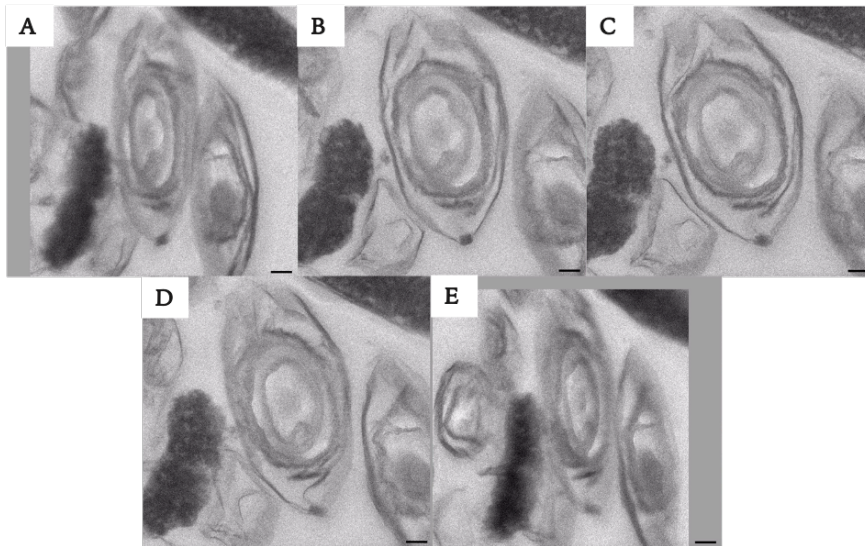


Figure 5.10 Tilt series of 200nm *C. sporogenes* endospore section

Tilt series collected from -60° to +60° A)-60°, B) -31° C)2° D)+29°, E)+59° Scale bar = 200nm

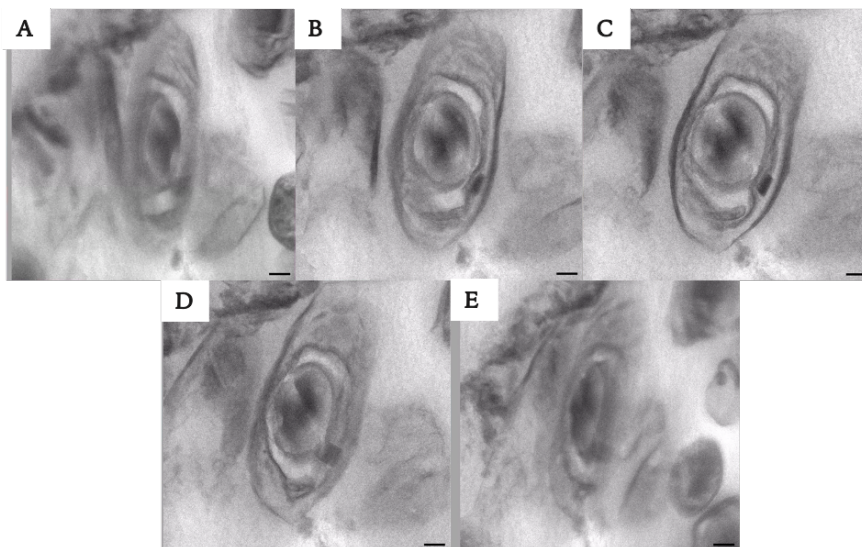
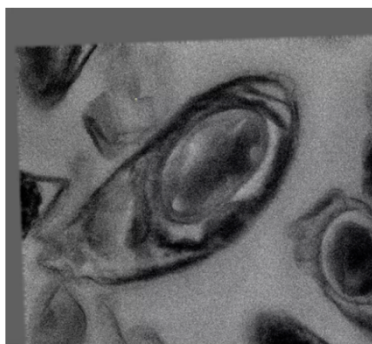
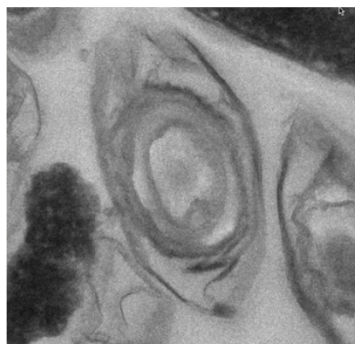


Figure 5.11 Tilt series of 300nm *C. sporogenes* endospore section

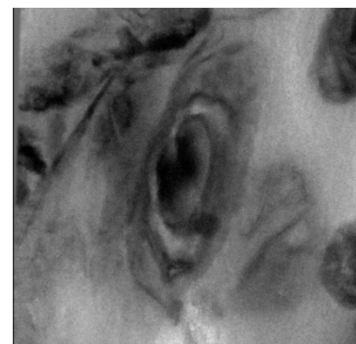
Tilt series collected from -60° to +60° A)-60°, B) -30° C)0° D)+30°, E)+60° Scale bar = 200nm



Video 5.2 Tilt series of 200nm *C. sporogenes* endospore section



Video 5.3 Tilt series of 200nm *C. sporogenes* endospore section



Video 5.4 Tilt series of 300nm *C. sporogenes* endospore section

5.3.3.2 Data processing

Detailed description of processing methodology is described in chapter 2.6.2. In summary, the tilt series alignment and tomogram generation were performed using IMOD software. As samples lacked fiducial markers, patch tracking was used for the alignment of the tilt series. Patch tracking is a less robust method of alignment and yields higher error margins than fiducial based alignment (Chapter 2.6.2.4). The residual error mean for alignment was 20.6 and 31.8 pixels for tomograms in Figure 5.12 and 5.13 respectively. As described in chapter 2.6.2.4, an iterative approach was implemented to improve these error margins; the final alignment of the tomograms was adequate for the identification of spore assemblies.

SIRT-like weighted back projection with 50 iterations was used to generate tomograms. The tomograms were binned by 3 (pixel size = 1.989nm). The tomograms of tilt series from Figures 5.9 and 5.10 are demonstrated in Figures 5.12 and 5.13 (Video 5.5 and 5.6) respectively, which displays slices through the tomographic volume at different positions in z.

Tomograms displayed many layers that compose the spore (Figure 5.15), thus were suitable for 3D surface reconstruction. This was done using Amira software (Thermo Fisher Scientific) for the segmentation and visualisation of surface reconstruction. Each layer was labelled and segmented as displayed in Figure 5.15. The final 3D surface was generated from a surface mesh, the mesh density was reduced by 2.6x to create a smooth visual finish.

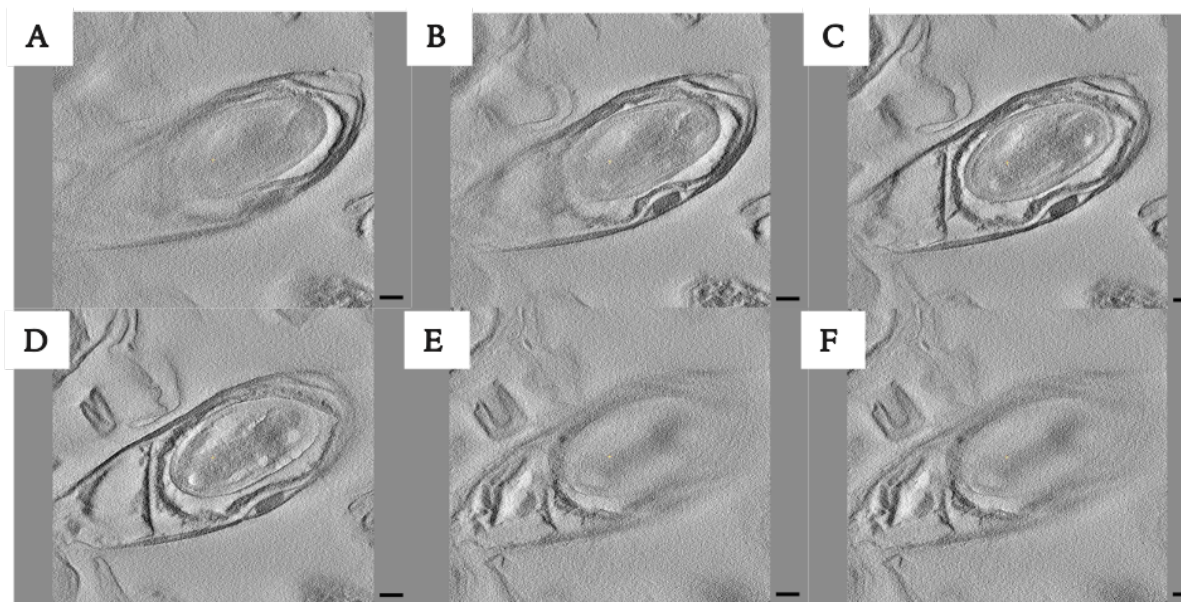


Figure 5.12 Slice through tomogram of 200nm *C. sporogenes* endospore section
6nm tomogram slices at A) 150, B)175, C)200, D)225, E) 250, F) 275. Scale bar = 200nm

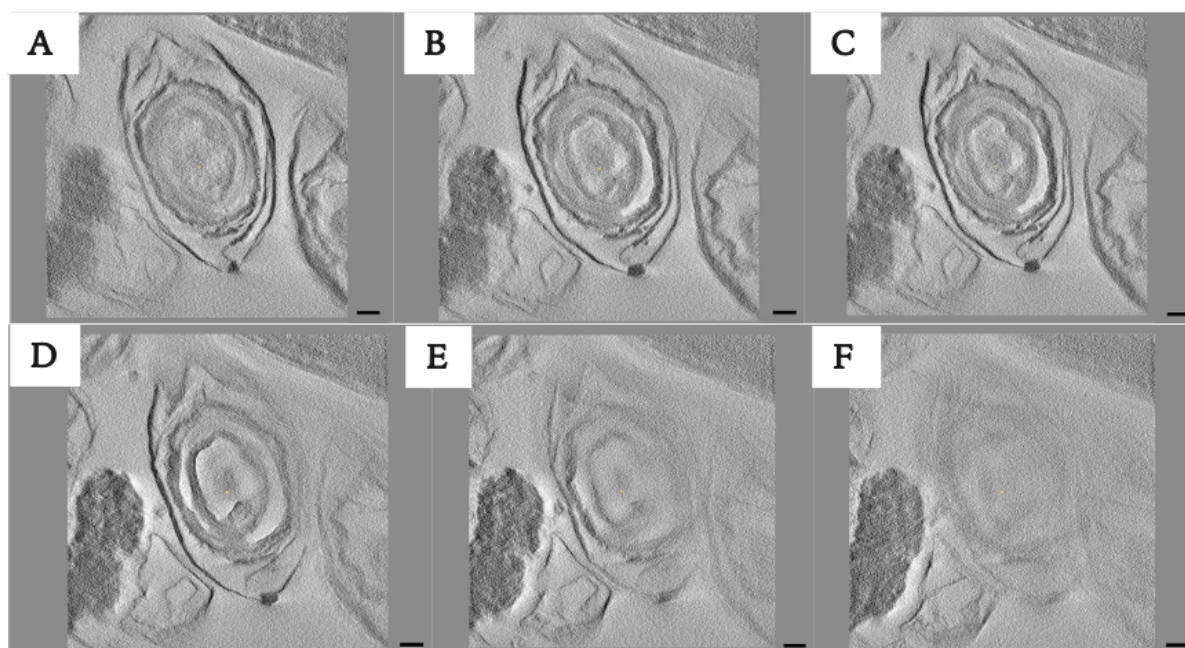


Figure 5.13 Slice through tomogram of 200nm *C. sporogenes* endospore section
6nm tomogram slices at A) 150, B)175, C)200, D)225, E) 250, F) 275. Scale bar = 200nm



Video 5.5 Tomogram of 200nm *C. sporogenes* endospore section



Video 5.6 Tomogram of 200nm *C. sporogenes* endospore section

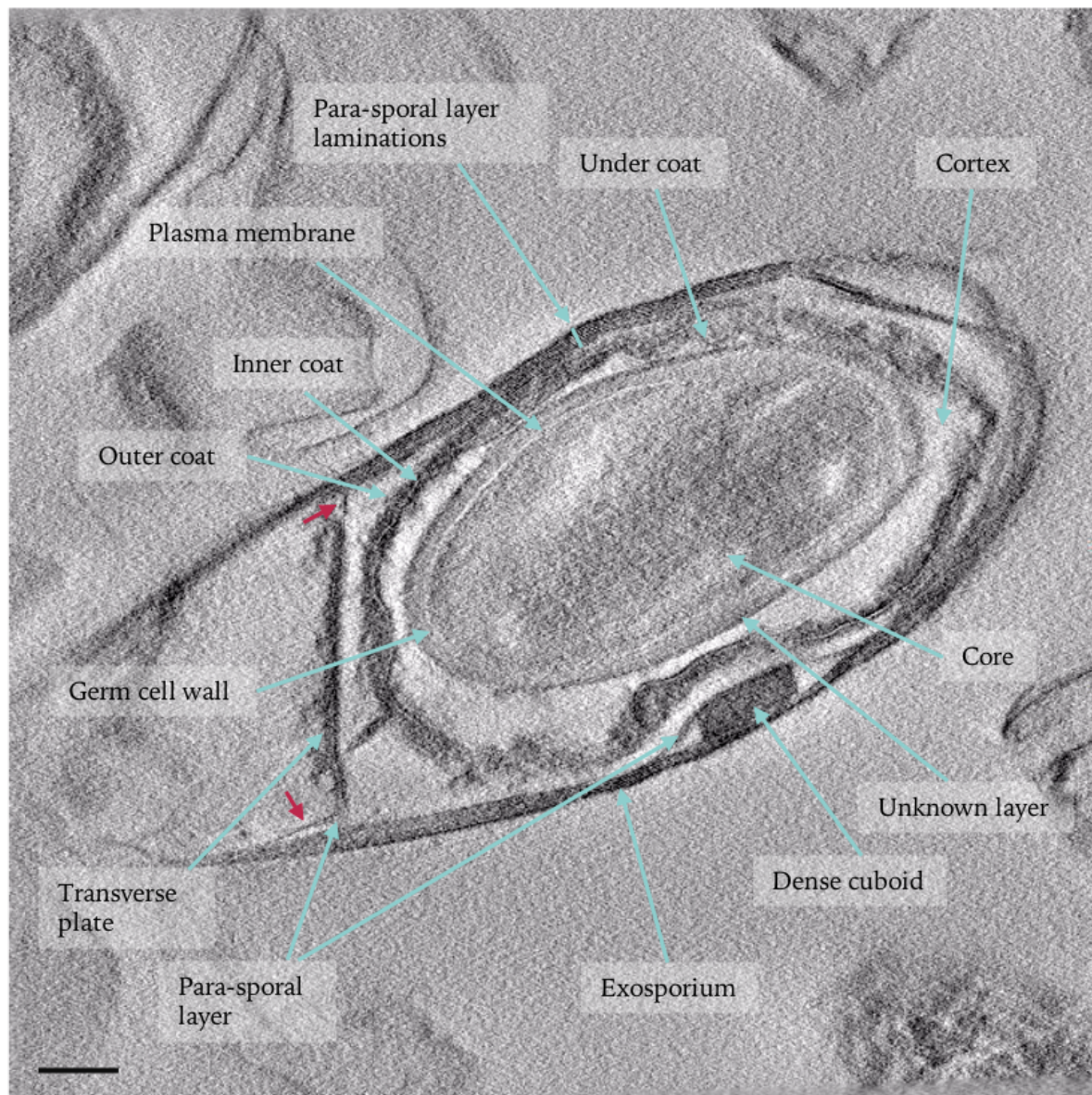


Figure 5.14 Labelled *C. sporogenes* tomogram slice

Tomogram binned by 3 with 6 slices merged (35nm thickness). Scale bar = 200nm

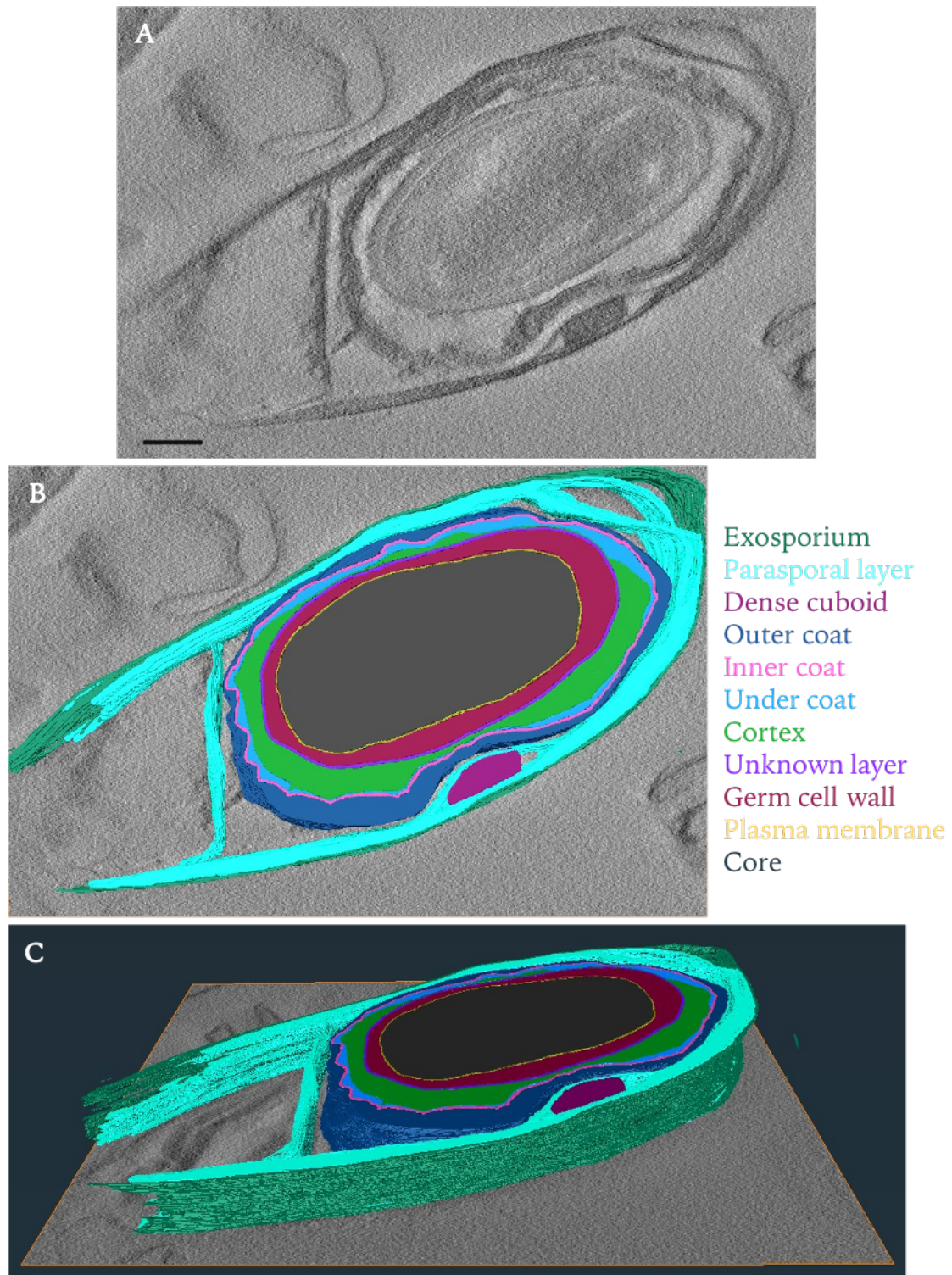


Figure 5.15 Segmentation of *C. sporogenes* tomogram

Tomogram slice scale bar = 200nm (A) with surface model overlay top view (B) and side view (C).

5.4 Discussion

5.4.1 Optimising sample preparation for endospore sections

Previous research on *C. sporogenes* endospore sections was conducted on chemically fixed spores (Brunt *et al.*, 2015; Hoeniger and Headley, 1969; Warren *et al.*, 1994). The best example in the literature of a chemically fixed dormant *C. sporogenes* spore section is displayed in Figure 5.16A, which demonstrates the main elements of the spore, but the layers lack definition and the detail of important subassemblies is not visible (Brunt *et al.*, 2015). In the literature, spore assembly features are more visible in germinating spores (Figure 5.16B) (Brunt *et al.*, 2015), likely due to the rehydration and thus increased accessibility of fixatives to different zones in the spore.

This work was aimed at gaining a better understanding of spore structure by optimising the sample preparation technique for spore sections. The chemical fixation methodology used provided much more definition of the different spore assemblies compared to current literature reports on the same spore strain (Figure 5.16C). Chemical fixation approaches shared many similarities including fixation with glutaraldehyde and OsO₄, dehydration with ethanol and post staining with both UA and Reynold's lead citrate. There were two main differences observed between chemical fixation protocols: the buffer used for fixation with glutaraldehyde and resin type. The glutaraldehyde fixation buffer described in the literature was made up of PIPES buffer, whereas a phosphate buffer was used for this research. PIPES buffer has been demonstrated to aid preservation of lipid assemblies (Schiff and Gennaro, 1979), the effect of this is not apparent in the dormant spores as the PM is not visible (Figure 5.16A,C). The PM becomes visible in germinating spores (Figure 5.16B), thus it would be interesting to compare these features in germinating spores prepared with the protocol used in this project.

Different resin types were also used for plastic embedding of spores, epoxy propane resin was used in this project whereas LR white was used for sections from the literature (Brunt *et al.*, 2015). LR white is a premixed commercially available resin that has been demonstrated to yield inferior definition of assemblies in sections compared to epoxy propane resins (Brilakis *et al.*, 2009). The use of epoxy

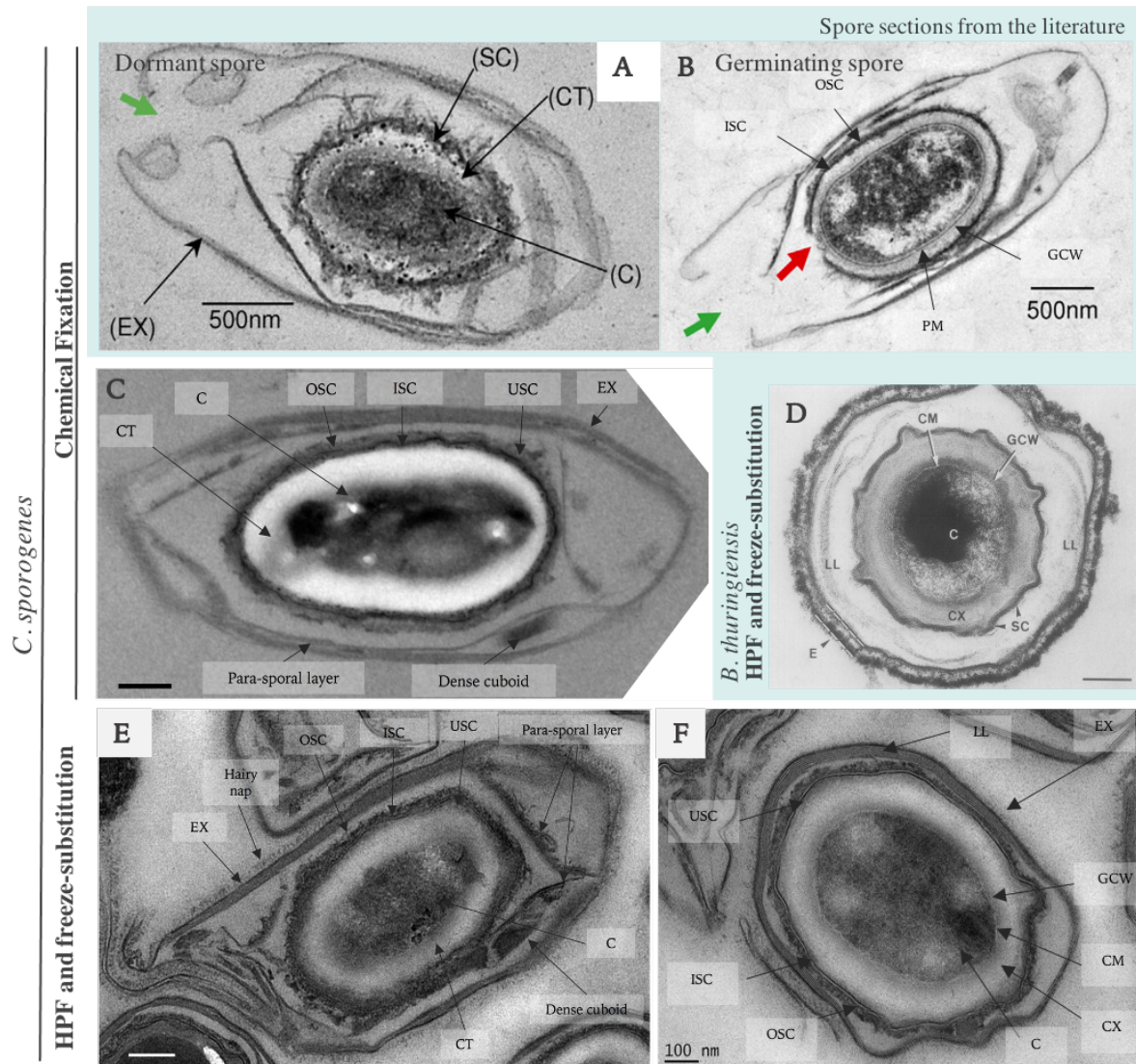


Figure 5.16 Chemical fixation and HPF with freeze-substitution of *C. sporogenes* and *B. thuringiensis* spores from the literature

A,B) 95nm sections of *C. sporogenes* (Brunt *et al.*, 2015) dormant spore (A) and germinating spore (B) treated by chemical fixation (2.5% glutaraldehyde, 1%OsO₄ and post staining with UA and LC) C) 85nm section of *C. sporogenes* from this project treated by chemical fixation (2.5% glutaraldehyde, 2%OsO₄ and post staining with UA and LC). D) *B. thuringiensis* sections from HPF with freeze-substitution (1% OsO₄, 0.5% uranyl acetate, 3% glutaraldehyde in Methanol) and post stained with UA and LC. EX (E in panel D): exosporium, C: core, CT (CX in panel D): Cortex, SC: spore coat (OSC: outer spore coat, ISC: inner spore coat, USC: under spore coat). E,F) Longitudinal (E) and transverse (F) 85nm *C. sporogenes* sections from this project prepared by HPF with freeze-substitution (1% OsO₄, 0.5% uranyl acetate, 3% glutaraldehyde in methanol) and post stained with UA and LC. Figures with permission from * Brunt *et al.*, (2015) ** Ebersold *et al.*, (1981).

propane resin in this project is likely to have contributed to improved results in imaging dormant spores, compared to those in the literature (Figure 5.16A,C).

Microscope settings for data collection also differed, 200kV was reported in the literature compared to 80kV in this project. Lower voltages increase the number of scattered electrons and increase

contrast, thus 80kV would provide more contrast to features compared to 200kV. All of these differences make it difficult to assign a particular aspect to the increased sample quality. Comparison of chemical fixation protocols with germinating spores would be interesting to see if improved detail of assemblies is also observed using our chemical fixation approaches.

Visibility of features in the spore were improved in both chemical fixation and HPF + freeze-substitution approaches when compared with sections in the literature (Figure 5.16C,E). If we consider the outermost layers; the exosporium and the parasporal laminae of layers are visible across all three sections, although definition of these layers was much more apparent in both the chemical fixation and HPF + freeze-substitution conducted during this project compared to previous studies, particularly as laminae were observed in both approaches (Figure 5.16C,E). The hairy nap was also visible in some sections of HPF + freeze-substitution (Figure 5.16E).

Differences are also apparent in the spore coat, the three coat layers cannot be distinguished in dormant spores reported in the literature and only two layers were observed in germinating spores (Figure 5.16A,B), yet all three layers were visible by both chemical fixation and HPF + freeze-substitution (Figure 5.16C,E). The core and cortex regions are clearly visible in all three sections, with an electron dense core and a lightly stained cortex.

Layers in between the core and cortex, the GCW and PM, are not visible in any chemically fixed sections of dormant spores (Figure 5.16A,C) but become visible in germinating spores (Figure 5.16B). The GCW and PM was visible on some HPF + freeze-substitution samples of dormant spores (Figure 5.16F) but not all (Figure 5.16E) this may be due to the to the height or angle at which the spores are sectioned as they can be tracked in the spore by tomography (Figure 5.15).

5.4.2 Improved results from high pressure freezing and freeze substitution

HPF provides increased preservation of biological material which yields greater detail and definition of the sample (Studer *et al.*, 2001). Whilst freeze-substitution involves the use of chemical fixative it also provides increased contrast levels enabling finer details to be visualised. Freeze-substitution is

also an alternative that still involves the use of plastic sections which are much more readily handled compared to CEMOVIS.

The improved sample contrast and detail is apparent across the different spore assemblies, particularly on the outermost layers. The exosporium is the most densely stained outermost layer, in some cases decorated by hairy nap (Figure 5.16C). Unit cell measurements of exosporium patches observed in thin sections (Figure 5.7) correlate with the known dimensions from electron crystallography studied in chapter 4. As a crystal with $p6$ symmetry, the unit cell angle is known to be 120° , the sections revealed a slightly larger $\sim 127^\circ$. The difference of the unit cell angles is likely the result of the crystal being imaged at an angle, especially as it is a section, more than the actual distortion of the crystal itself by $\sim 7^\circ$. Overall, this suggests that HPF and freeze-substitution approaches do not alter the integrity of spore assemblies such as the exosporium.

The improved contrast was also apparent in the laminae layers observed parallel to the exosporium basal layer, which are so strikingly visible, they can be counted manually (Figure 5.20+5.21). The different coat layers are also readily distinguished, particularly the inner coat layer.

The HPF and freeze-substitution protocols were adapted throughout this project to find the best combination to visualise *C. sporogenes* assemblies. High pressure is employed to prevent the formation of ice crystals that damage biological material (Dubochet *et al.*, 1988). The effects of suboptimal pressure levels were evident in the sections as spores presented with deformation and aggregation (Figure 5.3).

The optimal freeze-substitution cocktail was based on the cocktail used to study spores of *B. thuringiensis* (Figure 5.16D) (Ebersold *et al.*, 1981). Glutaraldehyde appeared to be critical for freeze-substitution of spores. In the absence of glutaraldehyde spore cores were compromised causing many to be missing or broken (Figure 5.4). This is likely due to glutaraldehyde's protein cross-linking properties, which aids the fixing of the densely packed spore core. This effect is further enhanced by osmium tetroxide which has a slower reactivity to proteins but does contribute to preservation (Bozzola, 2007).

Contrast levels in the sample were enhanced by the use of heavy metals in the freeze-substitution cocktail and post-staining. The freeze-substitution cocktail included osmium, a fixative that also enhances contrast of features, particularly lipids although it will also react with proteins but more slowly; this results in enhanced contrast levels for TEM. This is apparent in figure 5.4A and B, as samples prepared with only 1%(w/v) OsO₄ in acetone, possessed heavily stained cores. The addition of UA to the cocktail improved the staining distribution across other assemblies in the spore (Figure 5.4C,D), providing greater distinction between the different spore layers.

Post-staining of the sections with both Reynold's lead citrate and UA was required for optimal visualisation of the different assemblies in the spore (Figure 5.6). In the absence of any post-staining the spore layers are still visible but finer detail including laminations on the outermost layers are not (Figure 5.5A). UA stains all the layers including the core and outermost layers, but the finer details are enhanced by the use of Reynold's lead citrate (Kuo, 2007). The improved staining pattern observed with both stains is also due to Reynold's lead citrate's ability to react with UA (Leica Microsystems, 2013), thus enhancing the stained regions making features more visible.

5.4.3 Optimising sample preparation for tomography of endospores sections

Initial work was conducted on whole spores (Figure 5.1) but due to sample thickness, attempts at tomography processing were not successful. The highly electron dense core meant that gold fiducial markers were not visible on the sample and those that could be identified were obstructed at high tilts. This meant that the fiducials could not be tracked throughout the tilt series. Additionally, the highly dense core throws off any attempts of fiducial-less alignment such as patch tracking. Without optimal tilt series alignment, the tomograms are noisy and display 'ghosting' features due to misalignment in the tilt series.

Thicker samples generate more inelastically scattered events; this contributes to an increase in noise causing blurring of features in the image. At higher tilts, such as 60° effective sample thickness doubles, thus the effect is exacerbated in tomography. Energy filters are frequently employed in

tomography to reduce this effect (Schröder, 2015) by removing inelastically scattered electrons so that they don't contribute to the final image (Hawkes., 2008).

Samples around 1µm thick, such as the spore body, possess insufficient elastically scattered electrons available to give high signal to noise ratio. This is demonstrated in the *A. longum* whole spore cryo-ET study (Tocheva *et al.*, 2011) which lacks any information about central regions of the spore. For *C. sporogenes* whole spores, an energy filter would have only improved the visibility of outermost layers, but sectioning would still be required to study the core.

The optimised HPF + freeze-substitution approach for thin sections was used for making tomography samples. As bacterial spores are ~1µm thick, conventional thin sections (70-90nm) provide limited 3D spatial information, thus thicker sections (200nm and 300nm) were used to increase attainable 3D information about spore assemblies. Tilt series of thicker samples (Figure 5.9-5.11) display increased thickness at higher tilts, particularly apparent for 300nm sections (Figure 5.11). This presented many difficulties with alignment, as features became less trackable at higher tilts, distorting the tomogram generated. ET studies of ~300nm sections tend to be collected on 300kV microscopes equipped with energy filters (Briegel *et al.*, 2014). 300kV microscopes are better suited for thicker specimens, the increased voltage reduces the electron's wavelength thus improves sample penetration. It also means that the degree of beam interaction with the sample is less, thus specimens are more stable during exposure. Further work on 300nm sections of *C. sporogenes* spores would require an analogous set up.

ET of 200nm sections has previously been conducted on a 200kV microscope without an energy filter (Jiang *et al.*, 2017), comparable to the set up used for this work. As with previous studies, the increased thickness of the sections at high tilts was within the acceptable range for patch tracking. Thus, tomograms of 200nm sections were successfully generated (Figure 5.12 and 5.13).

In some tilt series, views were obstructed at high tilts, due to grid bars or other spores. These views do not provide any useful information thus need to be removed from the reconstruction. This reduces the

sampling in z which expands the missing wedge of information. The generated tomograms display ‘ghosting’ effects of features in the final tomogram, thus were not used for further analysis.

Due to the thickness limitation, 200nm sections only provide a limited amount of spatial information. Greater insights into spore ultrastructure would require the full volume of a spore, which could be obtained by using other techniques such as serial sectioning (Lučić and Baumeister, 2013; McIntosh *et al.*, 2005). Nevertheless, the current study has laid the groundwork for future studies of thicker sections on a higher voltage energy filtered instrument or serial sections of HPF and freeze-substituted sections for greater 3D sampling.

5.4.4 *C. sporogenes* spore assemblies

A combination of screening 2D spore sections and tomography have been used throughout to study *C. sporogenes* spores. Tomographic information has been used to construct a 3D surface model of a spore. Figure 5.17 displays the surface model from different angles, with all the different layers labelled as described in Figure 5.15. Here we will discuss each feature and observations made.

5.4.4.1 Core

The spore core (chapter 1.3.3.1), modelled in grey (Figure 5.15 and 5.17), consists of densely packed DNA and proteinaceous material maintained in a dehydrated state by high calcium concentration and DPA (Setlow, 2006; 2007). The dense properties of the core are reflected by the heavy staining, particularly with UA, demonstrating its high protein content. Many spore sections revealed patches that are lighter in colour which in some instances appear like ‘bubbles’, a likely effect of problems infiltrating with fixative due to its highly packed contents (Meek, 1976). Glutaraldehyde is a protein cross-linker, thus was required to preserve the integrity of the high-density spore core. Examples without glutaraldehyde lose the core integrity (Figure 5.4).

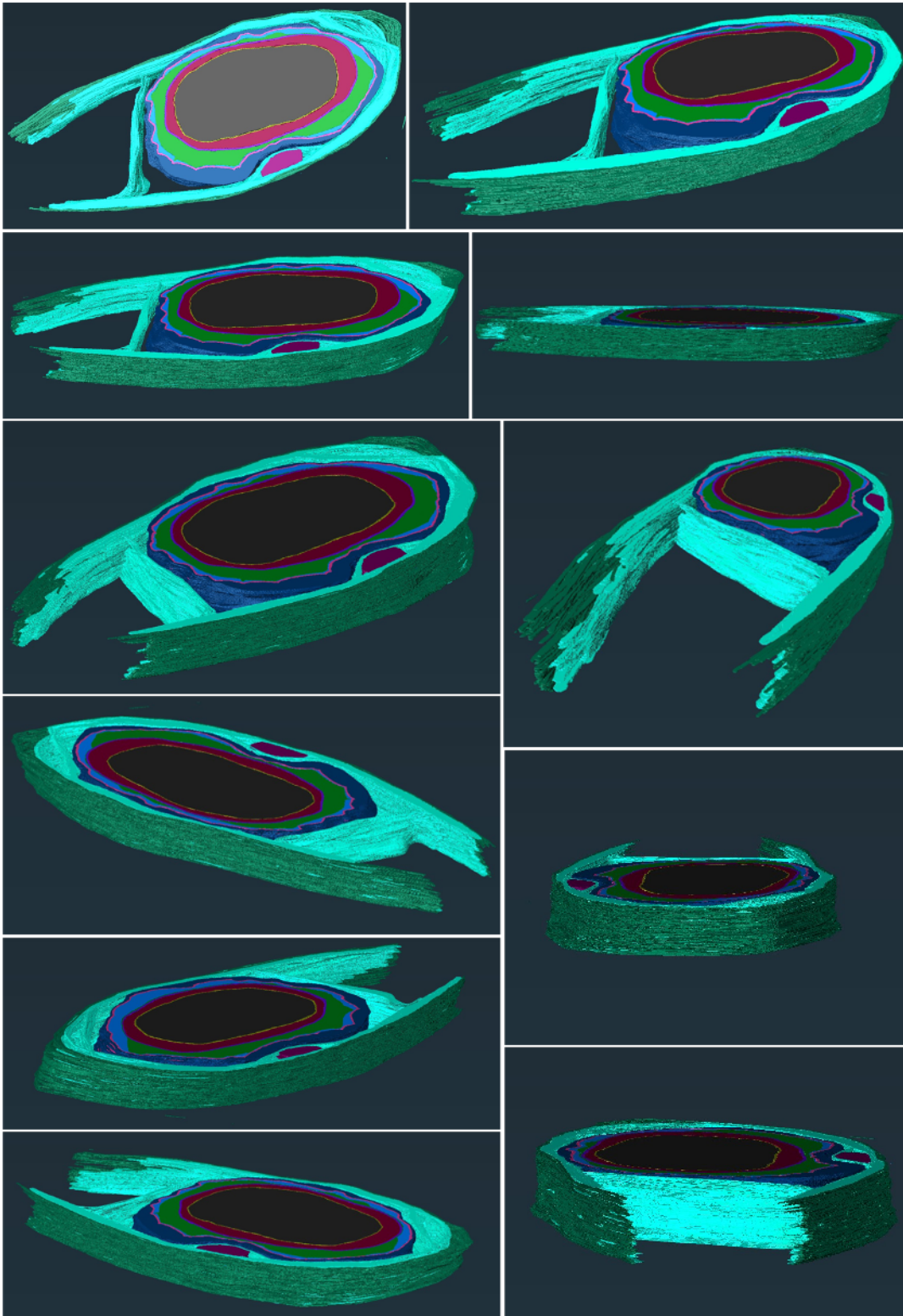


Figure 5.17 *C. sporogenes* 3D surface model

Different views of the surface model. Thickness of model ~200nm

Neither chemical fixation nor HPF + freeze-substitution provided insight into any substructures in the core. CEMOVIS of spores previously revealed membrane deposits in the core (Laue *et al.*, 2018), which were not visible in these sections. Thus, for greater understanding of the core's ultrastructure, HPF with CEMOVIS or FIB-milling will need to be employed.

5.4.4.2 Plasma Membrane

The PM (chapter 1.3.3.2) is depicted in yellow on the surface model (Figure 5.15 and 5.18), an important permeability barrier that confers spore resistance to chemicals (Setlow, 2006). The membrane was visible as a thin unstained layer in the spore sections. This property is not unique to spore sections, examples of membranes across the literature demonstrate an unstained layer (Matias and Beveridge, 2006). Phospholipid bilayers are composed of outwards facing hydrophilic regions that do interact with stain by TEM, however the hydrophobic core does not which give the appearance of a 'white line' (Stoeckenius, 1962).

The PM was identified as previously described in the literature on dormant and germinating spores (Brunt *et al.*, 2015; Hoeniger and Headley, 1969) and segmented respectively. The PM was not

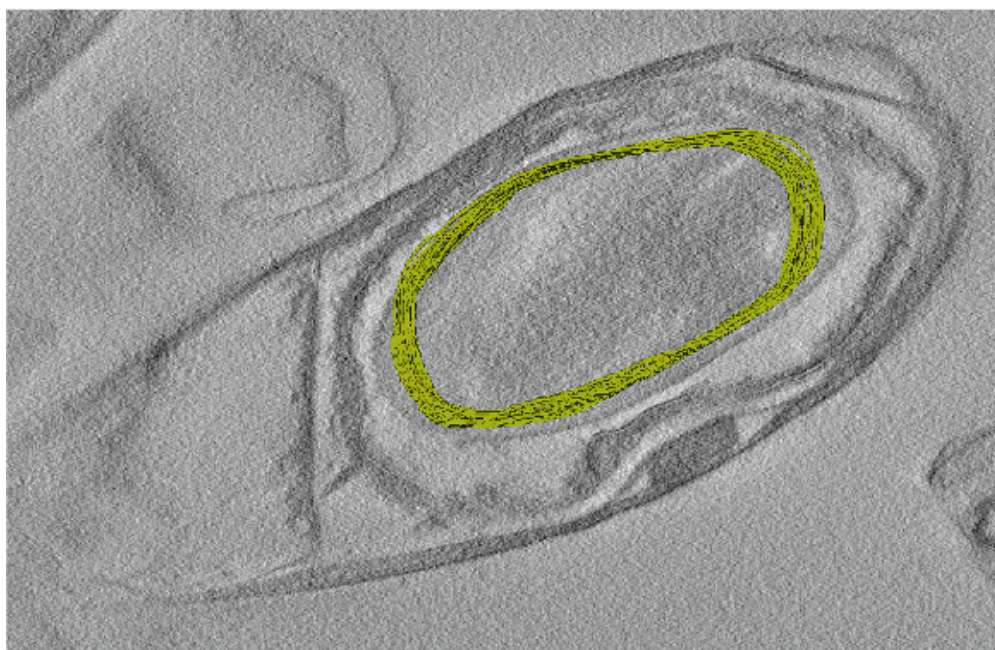


Figure 5.18 Segmentation of plasma membrane in *C. sporogenes*
Face on view of slice through tomogram with PM in yellow

visible in the chemically fixed sections of dormant spores in this research and in Brunt (2015).

However, the PM was clearly visualised previously in germinating spores (Figure 5.16B) (Brunt *et al.*, 2015).

In HPF + freeze-substitution sections, the PM was not always apparent and sometimes only partially visible (Figure 5.21). Tomograms demonstrated that the layer does enclose the spore core (Figure 5.14), but that complete layers are not always visible at a single 2D plane (Figure 5.12). This is likely due to the angle at which spores are sectioned.

5.4.4.3 Germ cell wall

The GCW is assembled on the PM, it has no involvement in spore resilience but is an important template for cell wall formation upon vegetative cell outgrowth during germination (Setlow, 2006).

As with the PM, the GCW was not readily visible in chemically fixed spores in this project, nor was it reported in previous literature on dormant spores but was distinguishable in germinating spores (Figure 5.16) (Brunt *et al.*, 2015; Hoeniger and Headley, 1969). Previous literature of germinating spores labelled the GCW as the layer next to the PM (Figure 5.16). Thus in the HPF + freeze-substitution sections of dormant spores in this study, a partially visible and darker stained layer next to the PM (Figure 5.21) was labelled as the GCW and segmented respectively. Tomograms reveal two layers next to the PM: a thicker inner layer that is lighter stained (Figure 5.14 – Germ cell wall) and a thin darker stained outer layer (Figure 5.14 – Unknown layer), corresponding to maroon and violet layers on 3D surface model respectively (Figure 5.17). The distinction between these two layers is not apparent in 2D micrographs.

It is unclear which layer corresponds to the GCW. However, it has been segmented consistent with the previous labelling of spore section of *C. sporogenes* (Brunt *et al.*, 2015; Hoeniger and Headley, 1969). Based on the likely mechanism of GCW formation during sporulation asymmetric cell division and engulfment, the innermost lightly stained layer next to the PM will correspond to the GCW.

5.4.4.4 Cortex

The cortex is the second PG layer on the spore; it differs from the GCW in its composition as its PG content is modified (chapter 1.3.3.3) (Meador-Parton and Popham, 2000). It is proposed to maintain the spore's dehydrated state by preventing the expansion of the core in response to hydration (Setlow, 2006). The cortex is labelled as previously established (Brunt *et al.*, 2015; Hoeniger and Headley, 1969), a lightly stained thicker layer near the core (Figure 5.15), segmented in emerald green in the 3D surface model (Figure 5.15 and 5.17). The lighter staining properties are conferred by its carbohydrate composition that does not react strongly with any of the stains or fixatives.

The 3D surface model suggests that the cortex encapsulates the spore core, although the model corresponds to a small sampling of the spore volume and further work including serial sections are required to prove this. The surface model also provides insight into variations in thickness around the spore. This may be affected by the thickness of the undercoat, as regions where the undercoat is thicker the cortex appears thinner (Figure 5.19). The significance of this observation remains unclear, and more surface models are required to establish this as a tendency.

A more densely stained layer was observed under the cortex, labelled the 'unknown layer' (Figure 5.15). Sections of *A. longum* spores revealed a similar darker assembly proposed to correspond to a second 'inner' cortex layer (Tocheva *et al.*, 2011). The different staining pattern of this layer compared to the cortex suggests that the layers have different compositions (Tocheva *et al.*, 2011). The inner cortex has not previously been described in *C. sporogenes*. Thus the 'unknown layer' in *C. sporogenes* sections could correspond to the inner cortex, although further work is required to establish this including CEMOVIS or FIB-milling.

5.4.4.5 Outer Membrane

A second membrane layer, the outer membrane, is believed to be assembled between the cortex and the coat and have an important role in sporulation (Piggot and Hilbert, 2004; Setlow, 2006). However, this layer has not been well characterised in mature spores and has even proposed to lack structural integrity (Nicholson *et al.*, 2000; Setlow, 2006). The outer membrane has only been visualised in the

Gram-negative *A. longum* spores by ECT, where the membrane is required to form the outer membrane in outgrowing vegetative cells (Tocheva *et al.*, 2011). The outer membrane was not visualised in either thin sections or tomography data, consistent with previous observations in Gram-positive spore formers.

One possibility was that the ‘unknown layer’ corresponded to outer membrane in Figure 5.15. However, this would mean a different localisation of the outer membrane from what has been previously established (Henriques and Moran, 2007; Piggot and Hilbert, 2004). Additionally, the ‘unknown layer’ is heavily stained, the opposite of what is observed for the PM - a ‘white line’ corresponding to the hydrophobic core (Stoeckenius, 1962). Therefore, we suggest that the outer membrane is not visible in these spore sections, which may be the result of staining patterns and not a true reflection of the spore ultrastructure. CEMOVIS and FIB-milling methodology should be employed to visualise spores with no stain to see if the outer membrane can be visualised.

5.4.4.6 Coat

The coat (chapter 1.3.3.4) is a proteinaceous assembly, an important barrier against enzymatic and chemical agents and environmental stresses such as heat, desiccation and UV (Driks and Eichenberger, 2016; Henriques and Moran, 2000; McKenney *et al.*, 2013). The high proteinaceous content means that it appears more electron dense than the cortex, as fixatives, UA and Reynold’s lead citrate strongly interact with the proteins. Previous chemical fixation of *C. sporogenes* dormant and germinating spores identified two coat layers: an inner coat and outer coat (Figure 5.16) (Brunt *et al.*, 2015; Hoeniger and Headley, 1969). Literature reports on dormant spores revealed little distinction between the inner and outer coat, especially compared to germinating spores (Figure 5.16) (Brunt *et al.*, 2015; Hoeniger and Headley, 1969), however these layers were clearly distinguished in both chemically fixed and HPF + freeze-substitution spores in this research (Figure 5.16 and 5.20). The inner and outer spore coat was segmented (Figure 5.19) based on the labels established in previous research on sections of germinating spores.

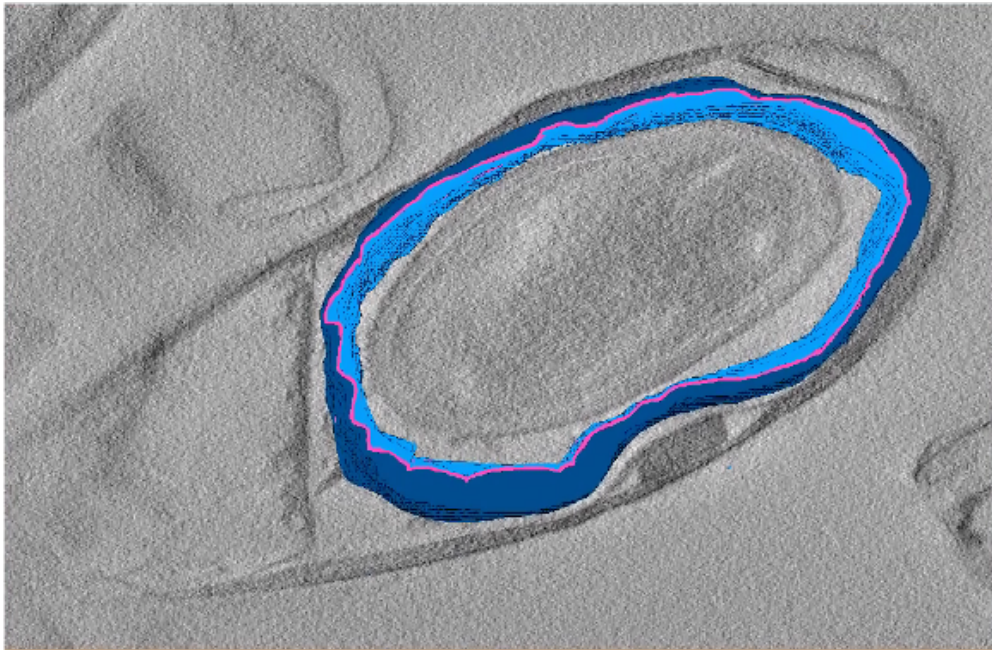


Figure 5.19 Segmentation of coat layers in *C. sporogenes* tomogram

Face on view of slice through tomogram outer coat (dark blue), inner coat (pink) and undercoat (blue) segmented.

The inner coat zone is very distinctive with two densely stained layers flanking a lighter stained middle layer (Figure 5.15). This zone has a constant thickness with an undulating shape around the whole spore; some regions have bead-like structures (Figure 5.6 – arrow 5). Folds in spore coats are considered an important feature for rapid response of the core by expansion in response to changes in humidity (Sahin *et al.*, 2012). Therefore, the undulations of the inner coat in *C. sporogenes* could also enable core expansion in response to environmental humidity levels and prevent premature germination.

The outercoat is the outermost coat layer. It displays a darker staining pattern with varying thickness around the spore, which does not appear thicker or thinner in any particular area of the spore.

The spore sections in this project consistently revealed a possible third spore coat layer, termed the undercoat (Figure 5.16 and 5.20), not previously described in *C. sporogenes* spores. The undercoat is assembled between the inner coat and the cortex; it is not as heavily stained as the other coat layers but distinguishable from the cortex. Tomograms revealed that the layer is not consistent in size across the spore. Displayed by the surface model, the undercoat appears thinner along the edge of the spore body and thicker at the poles (Figure 5.15). As described previously, in these areas the cortex appears

thinner, but further work needs to be conducted to establish whether it is a common feature across spores.

5.4.4.7 Parasporal layers

A network of layers has been described in the interspace region between the coat and exosporium in *Bacillus* (Beaman *et al.*, 1972; Ebersold *et al.*, 1981; Wehrli *et al.*, 1980). These layers may correspond to the ‘parasporal layer’, a para-crystalline assembly, present under the basal exosporium layer (Ball *et al.*, 2008). Previous research on *C. sporogenes* sections revealed a network of laminae under the basal layer (Hoeniger and Headley, 1969), which was observed throughout the spore sections examined in the current work. Hoeniger and Headley (1969) suggested that the laminae were part of the exosporium, however based on our observations we propose that the laminations are a different assembly. The layers are distinct from the basal layer of the exosporium, as they appear thinner and less densely stained and possess different periodicities. Due to the position of these laminae, under the exosporium basal layer, we defined them as parasporal layers. It is important to note that the parasporal layers in *C. sporogenes* were consistently observed assembled parallel to the exosporium basal layer, especially along the flanks of the main spore body. This is different from the parasporal layers in *B. thuringiensis* which were detached from the exosporium in the interspace (Ebersold *et al.*, 1981; Wehrli *et al.*, 1980).

The laminations of the parasporal layers were so well defined that they could be visually counted (Figure 5.20 and 5.21). As demonstrated in figures 5.20 and 5.21, the number of laminations varies within the spores. There also does not appear to be a conserved number of laminations. This is apparent in the transverse sections where some regions have from 2 to 7 layers (Figure 5.20). Other patches are ill-defined with no deducible laminations (Figure 5.20 - blue arrow). Longitudinal sections also reveal variation in number of laminations across the spores, with the most laminations observed close to the main spore body. The laminations appear to taper at the poles of the spore, as is highlighted in figure 5.21B,F (blue arrows) where layers may merge or terminate. The average

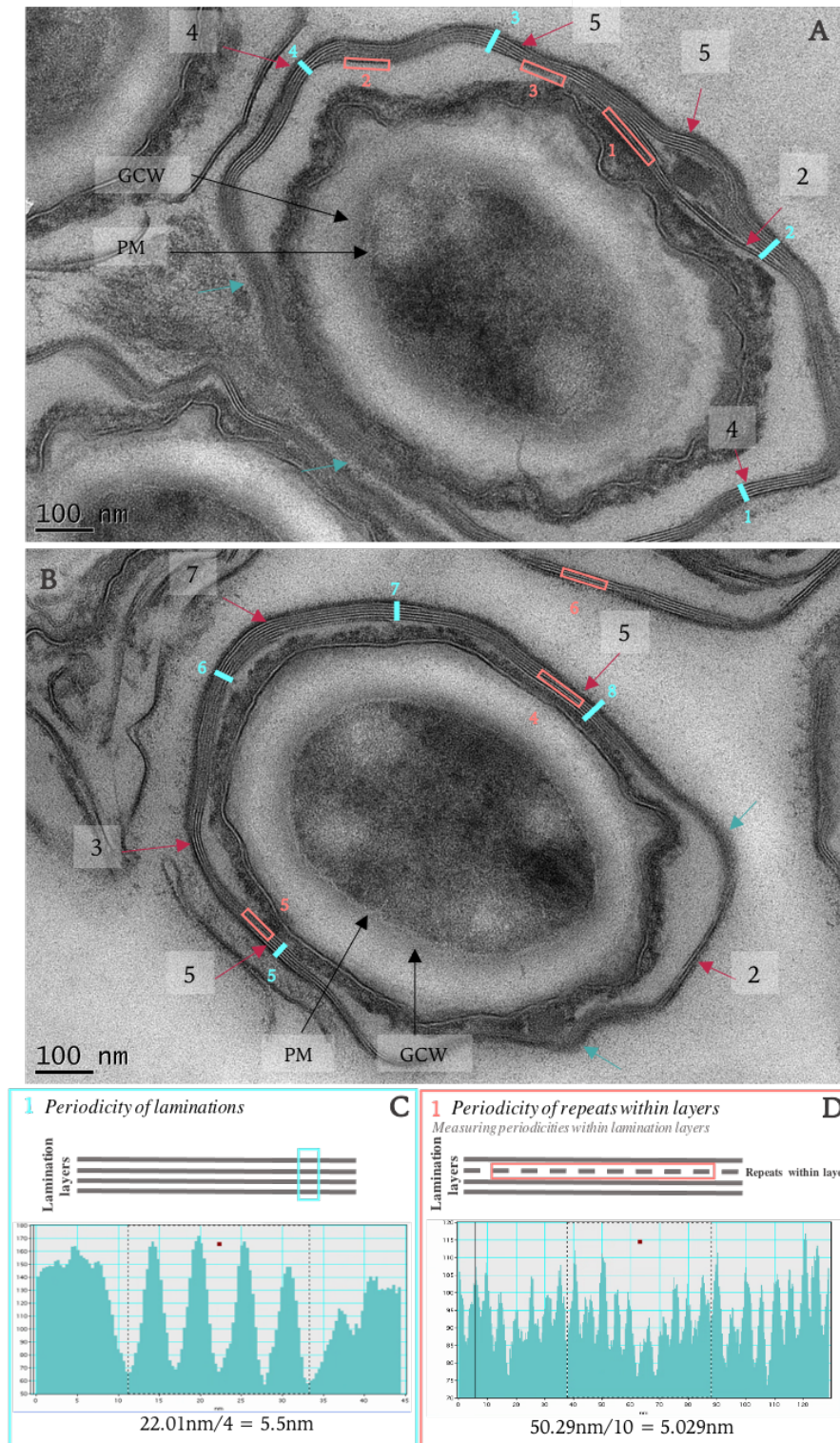
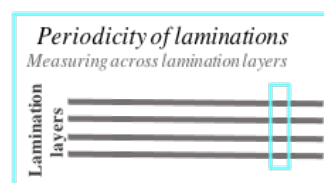


Figure 5.20 Parasporal layer laminations in transverse section of spores from ~85nm thick sections

A+B) Transverse sections: Number of laminations observed around the spore differ (pink arrows) with ill-defined patches where laminations are not observed (blue arrows). Plasma membrane (PM) and GCW (Germ cell wall) are visible in these sections and labelled. C+D) Schematic diagrams of different periodicity in parasporal layers and density profiles of representative patches C) Periodicity of laminations at position 1 in the spore (A- cyan 1): four distinct peaks with average thickness of 5.5nm D) Periodicity of repeats within a layer at position 1 in the spore (A – pink 1): 10 peaks with an average spacing of 5nm. The measurements from positions 1-8 (cyan) and 1-6 (pink) are displayed in table 5.1 and 5.2 respectively.

Table 5.1 Periodicity of laminations from transverse sections in Figure 5.20: Average of thickness is $\sim 54\text{\AA}$

Position	Thickness per layer ($\text{\AA}$)
1	55
2	55
3	55
4	54
5	50
6	54
7	54
8	51
Average	53.5



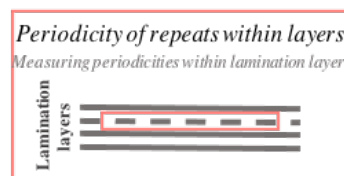
periodicity of the laminations was measured at different points in the spores (Figure 5.20 – cyan blue) and displayed in table 5.1. This indicates that the layers possess an average thickness of $\sim 54\text{\AA}$.

Tomograms have demonstrated that the parasporal layers are present across the whole spore, interconnected at both poles of the main spore body as shown in the 3D surface model. Many spores possess a layer that connects the parasporal layers on both sides of the main spore body, termed here the transverse plate (Figure 5.14). The tomogram revealed that the transverse plate was connected to the parasporal layer (Figure 5.14 – red arrow). A magnified view of the spore section reveals that the transverse plate also possesses laminations (Figure 5.21 – white arrows), although less than those observed along the main spore body.

Based on these observations, I propose that the transverse plate is part of the parasporal network layer and was segmented respectively (Figure 5.15 + 5.17). The 3D surface model reveals that the parasporal layers enclose around the main spore body. In some sections, including the 3D surface models (Figure 5.23), the laminations were shown to encapsulate a previously undescribed dense cuboid. This will be discussed in the following section.

Table 5.2 Periodicity of subunits within parasporal laminations from transverse sections in Figure 5.20: Average of periodicity is $\sim 48\text{\AA}$

Position	Thickness per layer ($\text{\AA}$)
1	50
2	50
3	46
4	46
5	49
6	49
Average	48.3



Freeze-fracture analysis of *B. thuringiensis* spores identified that the parasporal layers were paracrystalline in nature (Ebersold *et al.*, 1981; Wehrli *et al.*, 1980). Based on highly conserved assemblies across *Bacillus* and *Clostridia* spores, these layers are also likely to assemble into paracrystalline sheets. Some of the parasporal laminations in the *C. sporogenes* sections revealed visible repeats (Figure 5.6 – arrow 3). An analysis was conducted on these repeats in order to characterise the periodicity profile within the layers at different positions in the spores (Figure 5.20) and displayed in table 5.2. The average periodicity measured was $\sim 48\text{\AA}$, which might reflect the unit cell length of a crystalline assembly. Mutant analysis of Csx spores in chapter 3 revealed two crystal types, type III and type IV with a unit cell length of $a=b= 52.9 \pm 0.7\text{\AA}$ and $a=b= 49 \pm 1.2\text{\AA}$ respectively; both crystal types could correspond to the parasporal layer.

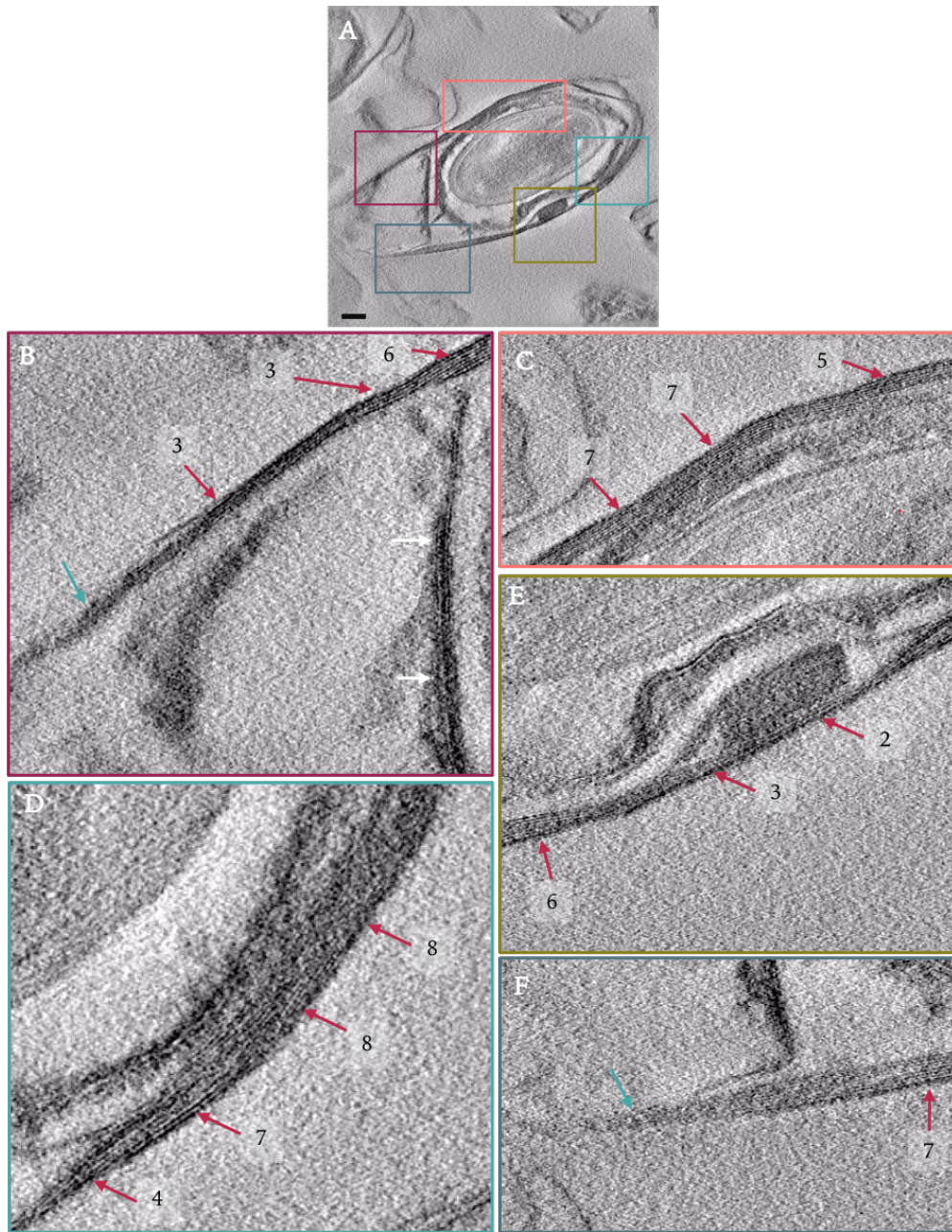


Figure 5.21 Para-sporal layer laminations in longitudinal section of *C. sporogenes* spore

A) Low magnification of micrograph of slice through 3x binned tomogram (3 slices merged). Scale bar = 200nm B-F) High magnification images of different regions of outermost layers corresponding to the coloured boxes in A. Laminations are observed around the spore, which differs in numbers (pink arrows) with ill-defined patches where laminations are not observed (blue arrows). White arrows in B highlights laminations also present in the transverse plate at pole of the spore.

However, based on the mode of sectioning it is highly unlikely that we have sectioned precisely along the unit cell length. The periodicity is more likely to reveal the distance between planes and not the unit cell length, as is observed in the basal layer. The average periodicity of the basal layers was $\sim 79\text{\AA}$ (Figure 5.22+ Table 5.3), this difference in periodicity also consolidates that the parasporal layers are

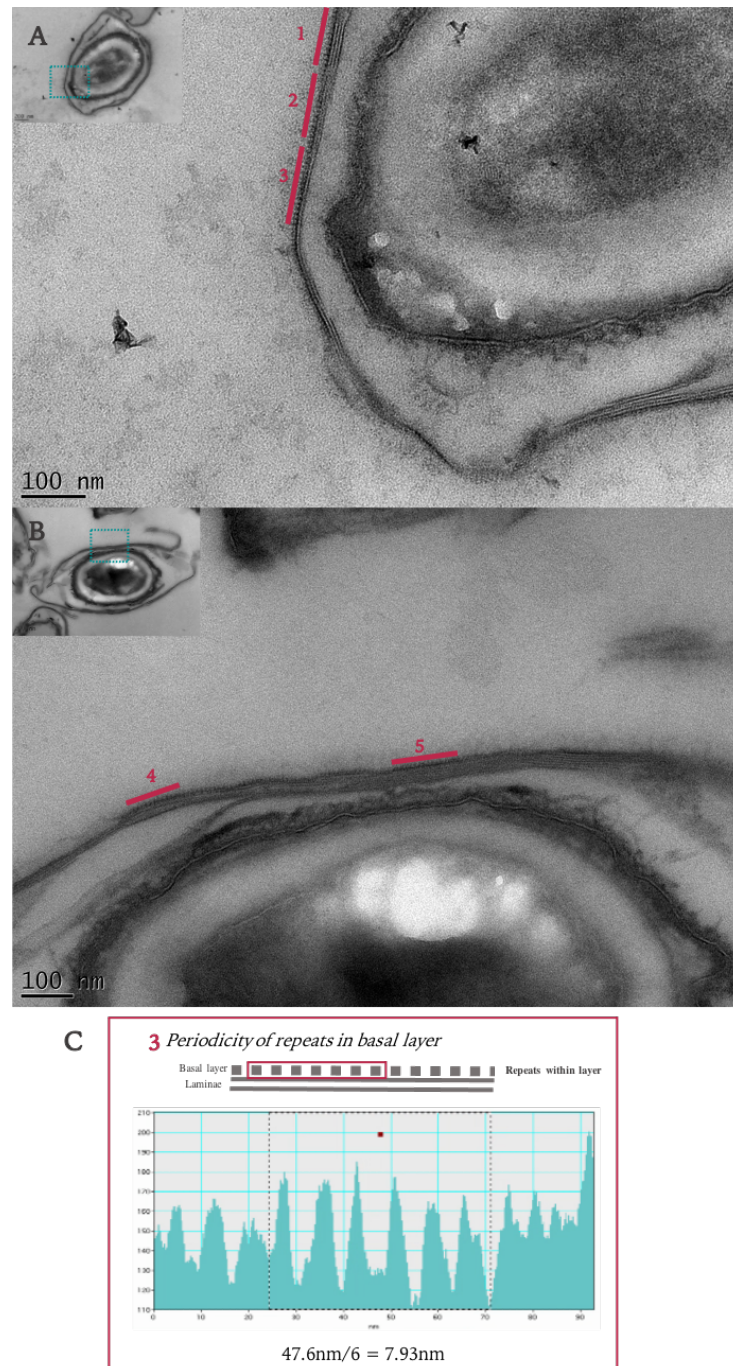
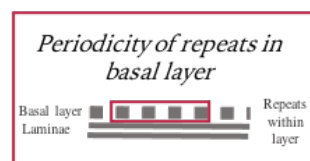


Figure 5.22 Periodicity in basal layer from $\sim 85\text{nm}$ thick spore sections

Transverse (A) and longitudinal (B) sections with inset displaying low magnification of the image C) Density profile of repeats observed on location 3 (A– red 3): 6 peaks with an average spacing of 7.93nm . Measurements from positions of 1-5 are displayed in table 5.3.

Table 5.3 Periodicity of subunits within basal layer spore sections in Figure 5.21: Average of periodicity is $\sim 79\text{\AA}$

Position	Thickness per layer ($\text{\AA}$)
1	78
2	78
3	79
4	86
5	75
Average	$79.2\text{\AA}$



distinct from the exosporium basal layer. The dimensions of the *C. sporogenes* basal layer crystal is well characterised and possesses a unit cell length of $a=b=105\pm 3\text{\AA}$ (Chapter 4), for this unit cell the distance between planes is $\sim 90\text{\AA}$. Considering the effect of shrinkage and compression due to sectioning, the measured $79\text{\AA}$ periodicity could correspond to the distance between planes.

The periodicity in the parasporal layers at $\sim 48\text{\AA}$ is more likely to represent the distance between planes and not the actual unit cell. Type II crystal, observed in $\Delta csxA$ spores, was discussed as a possible candidate for parasporal layer assembly in chapter 3 with a unit cell length of $a=b=64.8\pm 0.8\text{\AA}$. The distance between planes for type II crystal would be $\sim 56\text{\AA}$, considering shrinkage and compression the measurement of $48\text{\AA}$ could correspond to this. Further processing including sub tomogram averaging is required to gain more representative crystal dimensions for localisation of the crystal types. We also cannot discard the possibility that the parasporal layers are composed of multiple crystal types that have not yet been visualised, thus sub tomogram averaging of different layers would also provide insight into this. This analysis should be combined with HPF + freeze-substitution of mutants to help localise the crystal types.

The function of the parasporal network is also unclear, it may be involved in the assembly of the exosporium or maintaining the interspace region. Identification of its composition would allow mutant analysis to screen the effect of parasporal layers on spore ultrastructure. To explore their possible involvement in germination or conferring spore resilience, the germination rates and susceptibility to insults of mutant spores could also be tested.

5.4.4.8 Dense cuboid

Thin sections of *C. sporogenes* by chemical fixation and HPF + freeze-substitution have revealed an electron dense assembly located within the spores' outermost layers (Figure 5.16 and 5.23). This material has never before been described in *C. sporogenes* spores. Due to its highly structured assembly and almost rectangular shape, the material is described here as a dense cuboid. Tomography data demonstrate how prominent the material is with a length of ~300nm and a width of ~130nm (Figure 5.23). Segmentation of the tomogram estimated the dense body had a volume of ~480,000nm³. As this analysis is only based on a single dense body further 3D work is required to see if the dimensions are conserved.

Spore sections reveal the cuboid sandwiches between parasporal layers (Figure 5.16 and 5.23) which is displayed in the 3D surface model (Figure 5.23B). However, many spores of both thin and thick sections did not appear to have a dense cuboid, suggesting that it is not always present in the spore. Alternatively, the dense cuboid may always be present but due to the low sampling of the spore 3D volume it is not always visible, serial sectioning of spores is required to confirm this.

The composition and function of the dense cuboid is unclear, as nothing like it has been described in Clostridia. The toxin crystal in *B. thuringiensis* spores somewhat resemble the dense cuboid based on the structured edges observed (Feng *et al.*, 2001; Sawaya *et al.*, 2014). This crystal is an insecticidal toxin that varies in shape, size and toxicity depending on the endotoxin type. *C. sporogenes* is not known to produce toxins, thus this option is unlikely. The dense cuboid was also much smaller with no clear crystal shape, thus it may not be a crystal assembly.

Mass-spectrometry in *C. sporogenes* identified a range of other proteins and enzymes associated with the exosporium (Janganan *et al.*, 2016). This included soluble proteins suggested to be either trapped in the outermost layers or correspond to remnants of vegetative cell state or coat proteins (Janganan *et al.*, 2016).

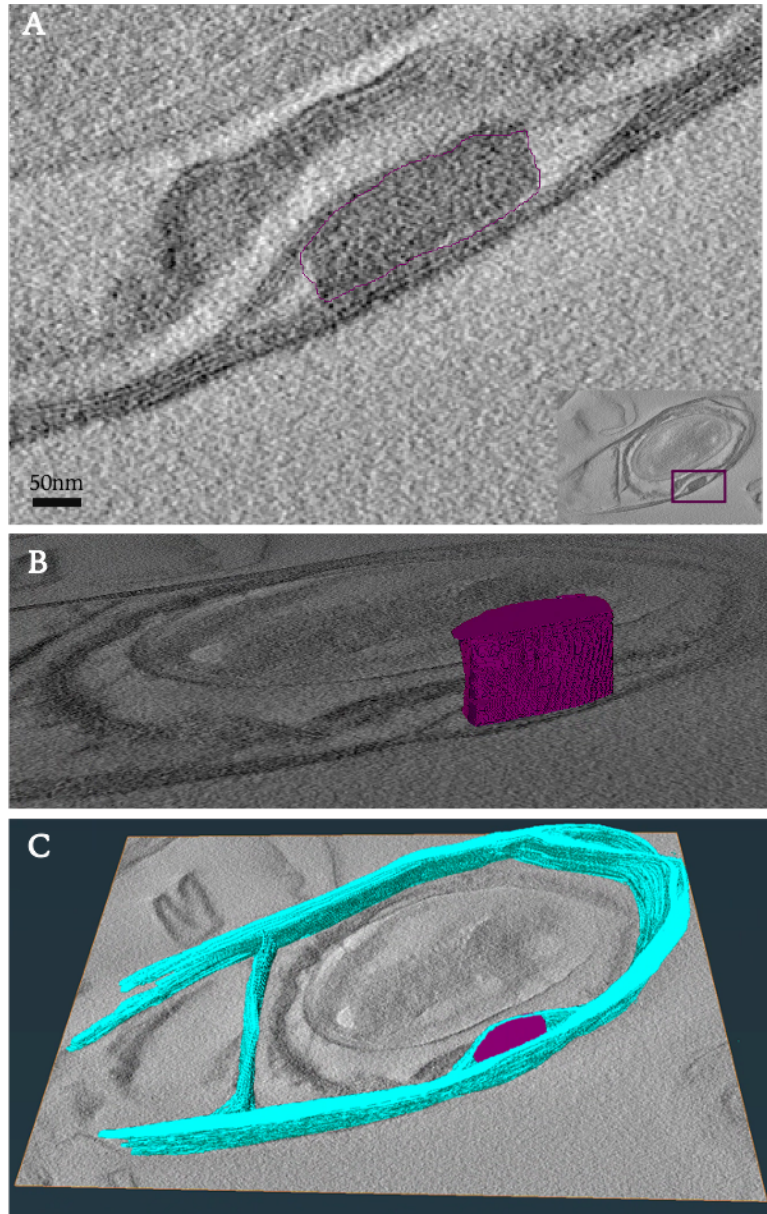


Figure 5.21 Dense cuboid in *C. sporogenes* spores

A) Face on view of tomogram with dense material outlined in purple, inset displays the low magnification image with box corresponding to area of image B)Side view: 3D surface reconstruction of dense cuboid on tomogram slice C)3D surface reconstruction of dense cuboid and para-sporal layer on tomogram slice.

Could the dense cuboid correspond to proteins important for germination? Upon germinant triggers could this body release enzymes important for the breakdown of spore layers? Or similarly for conferring resilience against environmental insults? I have no data to suggest this currently and further work is required to identify its composition and function.

5.4.4.9 Exosporium

The exosporium is the outermost layer, observed as slightly thicker and darker stained in spore sections (Figure 5.15). Demonstrated as a green layer in the 3D surface model (Figure 5.15 and 5.17). As established in previous chapters, the exosporium is involved in: germination, vegetative cell outgrowth and colonisation (Brunt *et al.*, 2015; Panessa-Warren *et al.*, 1994; 1997). It is a paracrystalline layer and depending on the angle at which the spore is sectioned, the crystal can be visualised (Figure 5.7). Side on repeats have also been observed in the basal layer (Figure 5.22), which revealed a periodicity of $79\text{\AA}$. As already discussed, the unit cell length of the basal layer crystal is known to be at $a=b=105\pm 3\text{\AA}$ (Chapter 4), thus this periodicity measurement is likely reflecting the distance between planes in the crystal.

Previous TEM and SEM analysis of *C. sporogenes* spores revealed diversity at the poles; some spores possessed a ‘lipped terminal protrusion’ whereas others appeared to have an aperture (Brunt *et al.*, 2015; Warren *et al.*, 1994). The significance of this remains unclear, however the aperture is possibly essential for the outgrowth of the vegetative cell (Brunt *et al.*, 2015). Sections and tomograms demonstrated that the exosporium was present around the whole spore, but high diversity was observed at the elongated spore pole. Some sections revealed a closed pole (Figure 5.3D), some of which possessed complex topology with many folds (Figure 5.4D) – this may correspond to the ‘lipped terminal protrusion’ (Brunt *et al.*, 2015). There were also many examples of poles where the exosporium could not be tracked in either the thin sections or tomograms, which may coincide with the aperture (Figure 5.5B – arrow 4). The 3D surface model is left open ended at the extended pole as layers could not be tracked definitively. Further work should combine a lower magnification tilt series of the spore for improved tracking of the poles and higher magnification of the poles to determine whether there is structure to this region. This could also include high magnification tomography of the poles of whole spores as the elongated pole is less dense.

It is possible that the *C. sporogenes* pole is sealed in dormant spores but possesses a more fragile cap that may have been disrupted during specimen preparation, thus different handling methods should be

tested to see if the aperture remains. A fragile cap would reflect a different composition at the pole, which was observed in the tomograms as parasporal layers taper at the poles (Figure 5.21). Higher magnification tomography of the poles could be used to gain structural understanding of the cap region.

This strain of *C. sporogenes* (ATCC15579), was less decorated when compared with NCIMB 701792 which possesses hairy nap and surface appendages (Janganan *et al.*, 2016). Some spore sections prepared by HPF + freeze-substitution revealed a decorated hairy nap but no surface appendages. Hairy nap was not visible on all spore sections, likely the result of inadequate staining patterns thus CEMOVIS or FIB-milling methods should be employed to gain a greater understanding of native hairy nap decoration.

Chapter 6: Structure of the *Clostridioides difficile* coat and exosporium

6.1 Introduction

C. difficile is a global health concern as one of the main nosocomial antibiotic associated diseases (chapter 1.2.2.4) (Rupnik *et al.*, 2009). *C. difficile* utilises the spore state for the transmission and persistence of the infection. Thin sections revealed that internal layers of *C. difficile* spores are similar to other Firmicutes in their overall structure and morphology, although the outermost layers display key differences (Paredes-Sabja *et al.*, 2014; Permpoonpattana *et al.*, 2011).

The outermost layers of the spore are the first point of contact with the host thus believed to be involved in: resilience for passage through the gastric tract, attachment to host cells and germination. Perturbation of the outermost layers in *C. difficile* affects the spores' ability to adhere to epithelial cells (Paredes-Sabja and Sarker, 2012) and colonise the gut (Antunes *et al.*, 2018; Calderón-Romero *et al.*, 2018), thus demonstrating the importance of these layers in establishing infection. The outermost layers also present attractive targets for therapeutics against CDI including potential epitopes for vaccine development. However, little is known about the outermost layers in *C. difficile*. Previous research has provided some insight into the protein composition, discussed in chapter 1.3.3.5.3 (Díaz-González *et al.*, 2015), yet their structure and assembly remain unknown.

Unlike other spore formers, *C. difficile* does not possess a 'classical exosporium', a baggy, loosely-fitted layer with an interspace region (chapter 1.3.3.5.3). Thin-section work proposed that the exosporium is present tightly fitted to the spore body (Permpoonpattana *et al.*, 2011), although the lack of structural information makes this inconclusive. The outermost layer can be decorated with a hairy nap, suggesting that the layer is an exosporium, although the presence of a hairy nap appears to be strain dependent (Barra-Carrasco *et al.*, 2013). *C. difficile* spores also possess an appendage, a thick proteinaceous layer that appears continuous around the whole spore and extends out from one of the spore poles (Antunes *et al.*, 2018).

As established in chapter 1.2.3.6.4, *C. difficile* possesses cysteine-rich proteins on its surface including: CdeA, CdeB, CdeC and CdeM (Díaz-González *et al.*, 2015). The involvement of cysteine-rich proteins in outer spore layers has been established in chapter 3 and 4, thus these Cde proteins are of interest for their potential involvement in outer layer assembly in *C. difficile*. Previous work on these proteins in *C. difficile* has focussed on CdeC and CdeM (chapter 1.3.3.5.3). CdeC is proposed to make up the exosporium layer (Barra-Carrasco *et al.*, 2013) and CdeM the appendage (Antunes *et al.*, 2018; Pizarro-Guajardo *et al.*, 2020). Although further research is required to establish this.

6.2 Aims

The outermost *C. difficile* spore layers remain largely uncharacterized. The ultrastructure remains unclear, to the extent that it is not known if the spores possess an exosporium. Cysteine-rich proteins have been identified on the surface of *C. difficile* spores, but their direct involvement in specific spore assemblies is yet to be deduced.

This project aimed to provide new insights into these uncertainties by screening whole spores using a range of electron microscopy techniques, combined with thin-section data conducted by our collaborators from ITQB Lisbon. These approaches were applied to WT and a range of mutant spores (provided by our collaborators from ITQB Lisbon), including: $\Delta cdeC$, $\Delta cdeM$, $\Delta cdeC cdeM$, CdeA+, CdeB+, CdeC+ and CdeM+, with the aim of identifying the role of these cysteine-rich proteins in spore assembly.

Prior work conducted by the Bullough group demonstrated that exosporium and coat proteins can be recombinantly expressed in *E. coli* and will self-assemble to form highly resilient crystals reflecting their respective native *in situ* assemblies (Jiang *et al.*, 2015; Terry *et al.*, 2017). These crystals can be observed in thin section of *E. coli* cells as laminations; upon lysing the cells large crystalline sheets are seen by TEM. Thus, I hoped to follow a similar principle to identify protein composition of crystalline layers in *C. difficile*.

Based on previous findings, I suspect that CdeM is involved in the appendage (Antunes *et al.*, 2018; Pizarro-Guajardo *et al.*, 2020) and CdeC plays an important role in outermost layer assembly (Barra-Carrasco *et al.*, 2013; Calderón-Romero *et al.*, 2018; Díaz-González *et al.*, 2015). The lack of *ΔcdeA* and *ΔcdeB* spores presents a challenge to identifying their roles in spore assembly, thus further work is likely to be required.

Our understanding of the exosporium in *Bacillus* and *Clostridia* (Chapter 1.3.3.5) leads us to speculate that *C. difficile* will have an exosporium layer. Whether it possesses the conserved ‘honeycomb’ lattice structure and if its assembly is similar to the ‘classic’ exosporium or the crust in *B. subtilis* is yet to be revealed.

As we focused on the commonly used laboratory strain 630, the analysis of the hairy nap is beyond the scope of this research. However further work on other strains including R20291 could provide further insight into this.

6.3 Results

6.3.1 Negative stain EM of *C. difficile* endospores

Spores of the 630 *C. difficile* strain were embedded in negative stain and examined by EM (Figure 6.1). Three populations of different spore morphologies were identified; ~72% of spores adhering to the specimen grid were of the ‘elongated spore’ morphology, which appeared as a slightly elongated electron-dense body with a more rectangular shape, particularly at one pole of the spore (Figure 6.1B1). The second most common morphology (~25% of spores adhering to the specimen grid) were more oval in projection and appeared shorter than elongated spores and referred to as ‘oval spore’ (Figure 6.1B2). At ~3% of the population, the third phenotype was observed at low abundance levels. These spores possessed an elongated electron transparent layer extending out from one pole of the spore (Figure 6.1B3).

Widths and lengths of the different morphologies are plotted in Figure 6.2. The average width across the different morphologies was ~0.75 μm . The primary difference was observed in the length, elongated spores had a large variation in length with most spores ranging between 1.5 μm -1.7 μm .

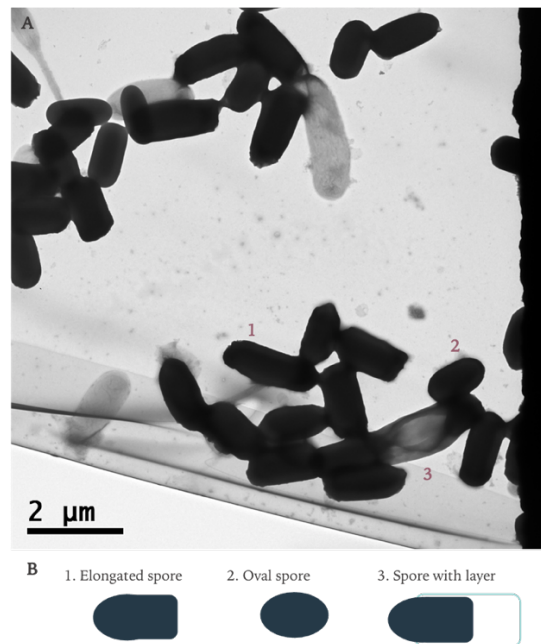


Figure 6.1 *C. difficile* 630 endospores

A) Electron micrograph of negative stained whole spores displaying a diverse population of phenotypes. B) Schematic of main phenotypes present: 1) Elongated spore (most abundant ~72%), 2) Oval spore (~25%): smooth surface, oval shaped and appear smaller, 3) Spore with layer (~3%): visible from one pole of the spore.

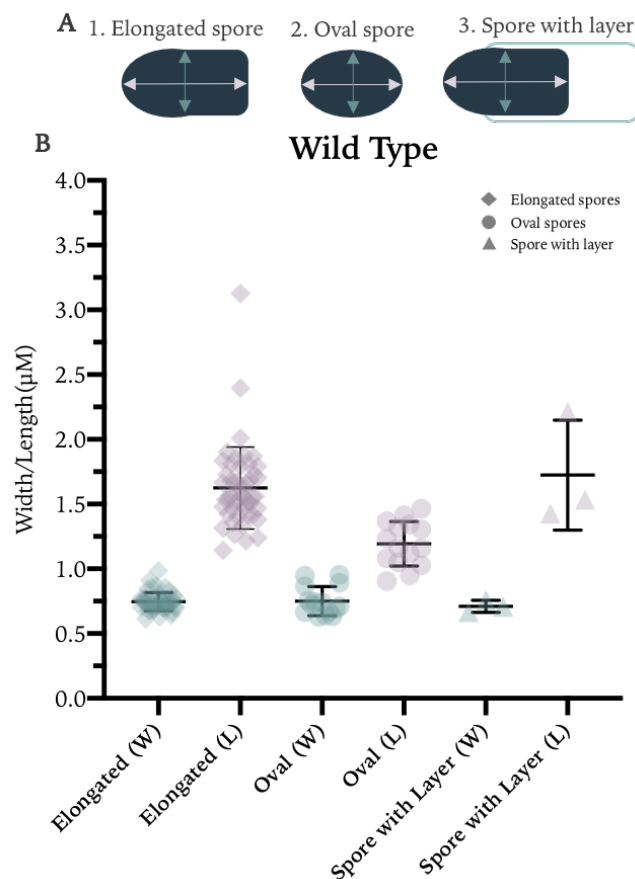


Figure 6.2 Width and length analysis of WT spores

A) Schematic of how width (green) and length (violet) measurements were taken for each spore type B) Scatter plot of individual measurements for width (green) and length (violet) of all phenotypes. The bars display the mean and standard deviation. The mean width across WT spores is very similar: elongated = 0.74 μM , oval = 0.75 μM , spore with layer = 0.71 μM . High differences between the mean length of spores: elongated = 1.6, oval = 1.1, spore with layer = 1.7

The mean length of elongated spores was at $1.6 \pm 0.3 \mu\text{m}$. Oval spores were shorter at $1.1 \pm 0.2 \mu\text{m}$.

Length measurements of the densest part of phenotype spore 3, spores with layers, demonstrated an average length of $1.7 \pm 0.04 \mu\text{m}$ which was comparable to the elongated spore phenotype.

6.3.2 Para-crystalline layer

High magnification micrographs of the layer extending from the pole of the type 3 spores revealed that some of these layers were para-crystalline (Figure 6.3A,B). Unit cell parameters were determined as $a=b = 96 \pm 3 \text{ \AA}$, $\gamma = 121 \pm 2^\circ$.

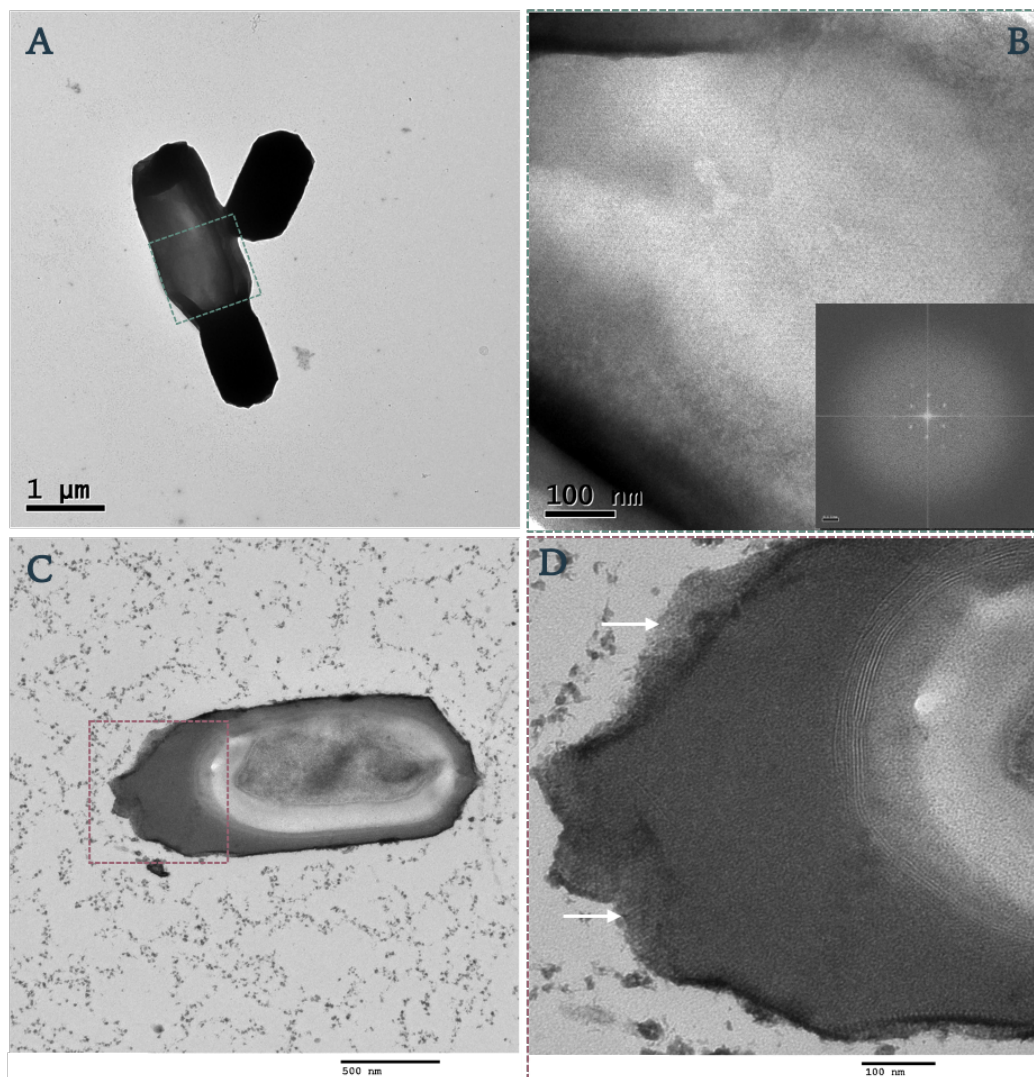


Figure 6.3 Para-crystalline layer on *C. difficile* 630 spores

A,B) Negative stain EM of whole spores C,D) Thin-sections of chemically fixed spores A) Para-crystalline layer visible at one pole of the spore B) Higher magnification of crystalline layer in A (blue box), crystallinity of the layer is displayed in the diffraction pattern of FFT (inset, scale bar 0.1 nm^{-1}) C) Low magnification of spore section with crystalline patches visible (purple box) D) High magnification of pole of spore in C, honeycomb lattice patches observed on the edges of the spore (white arrows).

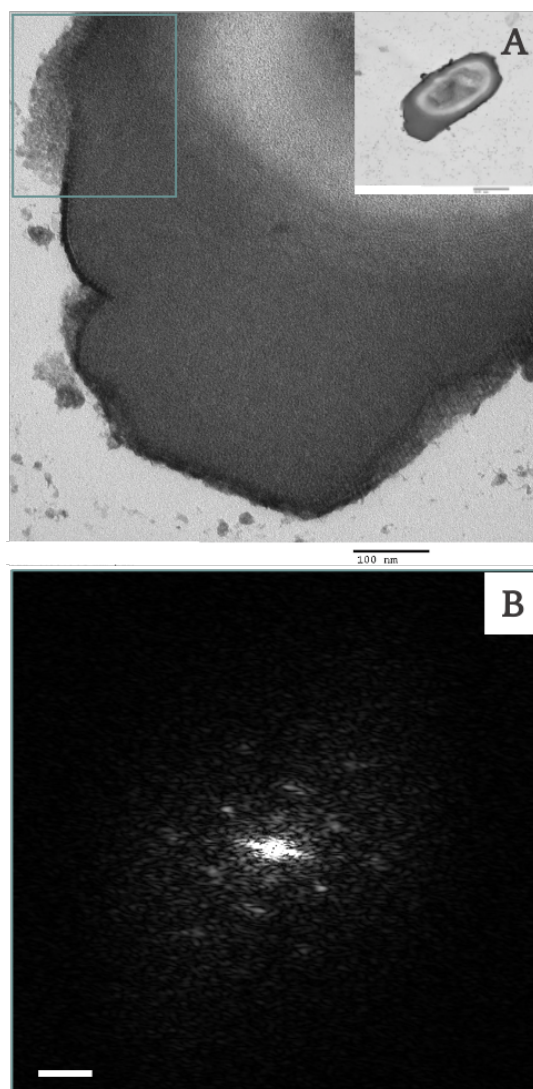


Figure 6.4 Measuring unit cell size of para-crystalline layers on thin-sections of *C. difficile* spores

A) Thin section of spore with para-crystalline layers on the edges of the spores, inset shows low magnification of spore B) FFT of a patch in A (blue box) measured unit cell length $x=y \sim 85 \text{ \AA}$. Scale bar = $0.0135 \text{ \AA}^{-1}$

Upon screening thin sections of endospores, crystalline patches were identified around the periphery of the spores (Figure 6.3C,D) which appeared to have a honeycomb lattice structure. To calculate the unit cell length of crystals observed in thin sections, micrographs were imported into 2dx image processing software. Crystal patches, such as that one shown in Figure 6.4 were masked and the script calculate FFT was run. The generated FFT is displayed in Figure 6.4b, from this, the unit cell length of the crystal is measured at $a \sim b = 85 \text{ \AA}$.

6.3.3 Electron crystallography of crystalline layer

High magnification micrographs of the crystalline layers are suitable for electron crystallography processing. Image processing was conducted using the MRC suite of programmes through the *2dx* software platform. The details of the workflow are described in chapter 2.6.

As the crystalline layers are highly mosaic with variable crystal quality, patches of crystal with high distortions were masked out of the processing. The FFT of images are then calculated and the lattice is indexed by assigning h, k coordinates – these are displayed in Figure 6.5 in blue and red respectively. Computed diffraction spots were visible in the best images up to $\sim 20\text{\AA}$ resolution (Figure 6.6A), due to the resolution limitations of negative stain.

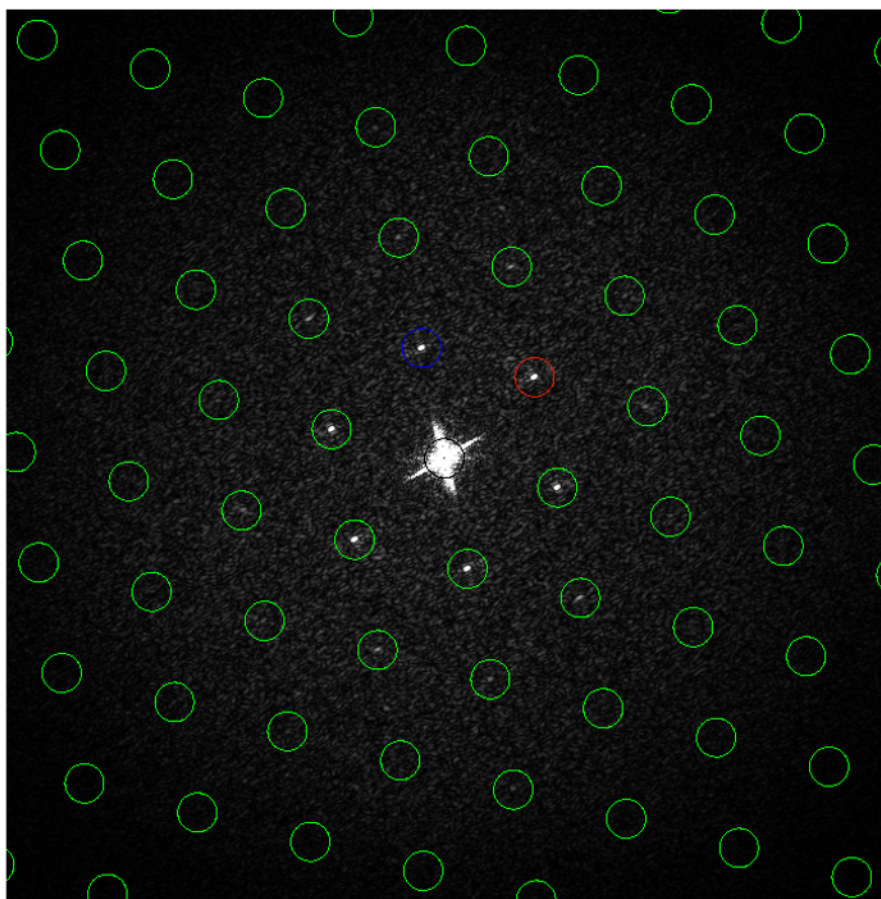


Figure 6.5 FFT of *C. difficile* para-crystalline layer after unbending

Diffraction spots from high magnification image of *C. difficile* para-crystalline layer by negative stain TEM. The lattice is highlighted with green circles, with h (blue) and k (red) coordinates highlighted. The unit cell dimensions of the crystal $a=b = 96\pm 3\text{\AA}$, $\gamma = 121\pm 2^\circ$

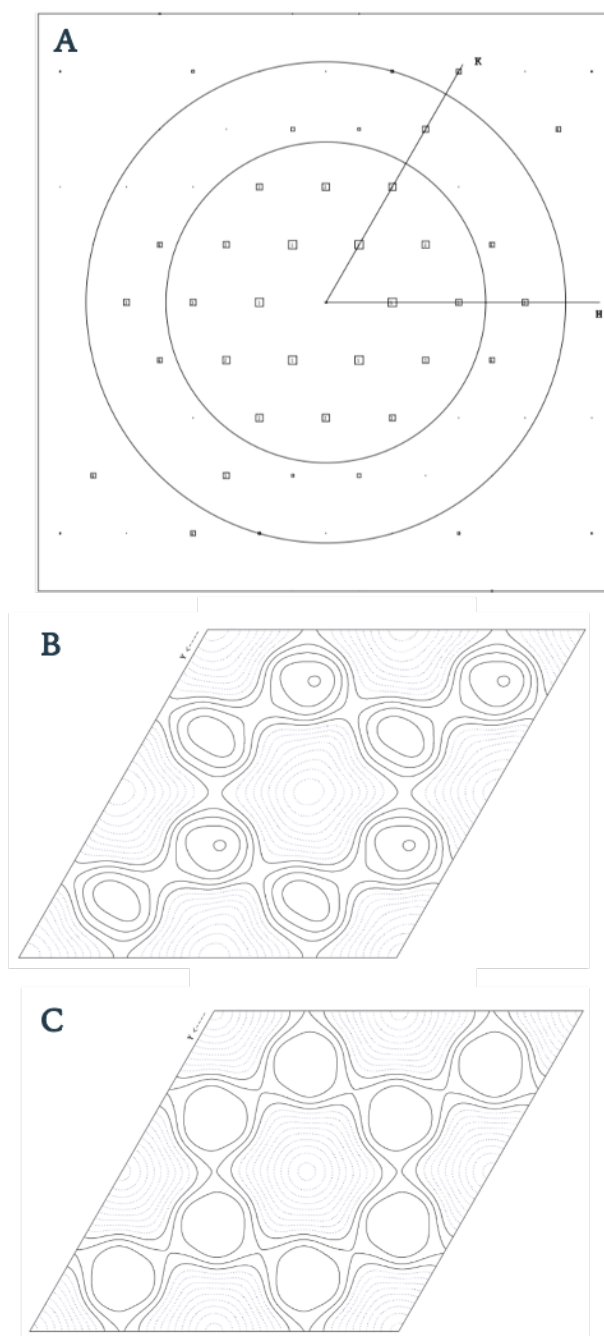


Figure 6.6 Resolution plot and projection maps of *C. difficile* para-crystalline layer

A) Resolution circle plot from single image with reflections labelled based on IQ value. Resolution rings indicate 36 Å and 24 Å with the Nyquist limit at 20 Å. B,C) Projection maps of unit cell from single processed image B) Unsymmetrised C) $p6$ symmetry applied

Table 6.1 Space group determination table of *C. difficile* para-crystalline layer

Space group	Phase residual (90° = random)	Target residual ¹
<i>p1</i>	23.9	34.8
<i>p2</i>	23.2*	27.5
<i>p3</i>	20.2*	23.9
<i>p312</i>	19.8*	24.4
<i>p321</i>	19.4*	25.2
<i>p6</i>	18.3*	26.2
<i>p622</i>	18.7*	25.0

¹Target residual: expected phase residual of symmetry groups

* = Acceptable

! = Should be considered

` = Possibility

All acceptable space groups are * in the phase residual column. The most likely is *p6* and due to this all other * are also possible within *p6*.

A projection map calculated from one image is shown in Figure 6.6, indicating approximate sixfold symmetry at this resolution. Comparison of predicted symmetry related Fourier phases for all possible plane groups suggested that the phases were consistent with trigonal and hexagonal plane groups.

Fourier amplitudes and phases from 6 independent micrographs were averaged. Figure 6.7 shows a projection map calculated from averaged structure factors with *p6* symmetry enforced.

6.3.4 Three-dimensional reconstruction of the crystalline layer

5 tilt series were collected sampling -50° to +50° with 10° intervals where possible. Due to crystal quality and obstructed views, only two of the series were amenable to image processing and merging. The lattice lines (Appendix 4) show the interpolated amplitude and phase values based on observed values as a function of z^* . It demonstrates relatively low phase and amplitude variability for lattice lines 1,0 and 1,1 with a greater variability observed in 1,2. The large error bars for both phase and amplitude variability amongst the second order lattice lines indicates high error in fitting.

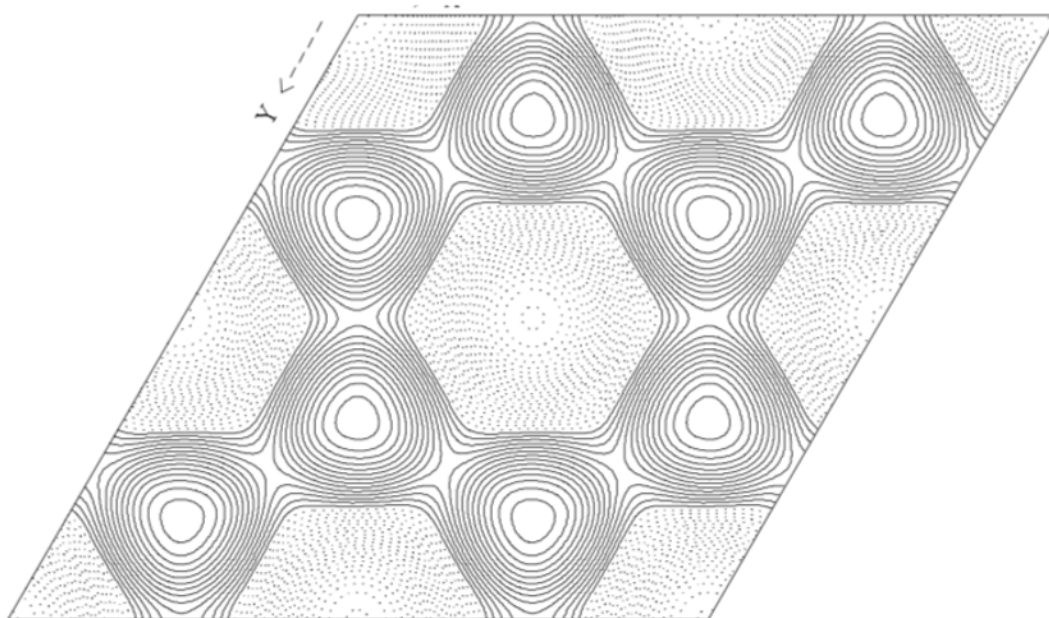


Figure 6.7 2D merge maps of *C. difficile* para-crystalline layer from negative stain electron micrographs

Contour-scale map with sixfold symmetry applied respectively. The maps were generated from six merged images, displaying a six-fold symmetry and a unit cell size of $a=b= 96\text{\AA} \pm 3\text{\AA}$, $\gamma = 121\pm 2^\circ$

Table 6.2 Reconstruction parameters for generating a 3D model

3D reconstruction parameters	
Crystal plane group symmetry	$p6$
Unit cell parameter	$a=97\text{\AA}$, $b=97\text{\AA}$, $\gamma=120^\circ$
Number of crystals	2
Tilting range	-30° to $+40^\circ$
Defocus range	1000nm - 2500nm
IQ range used for 3D refining	1-6
Number of reflections	234
Number of observed unique reflections	208
In-plane resolution cut off	20 $\text{\AA}$
Vertical resolution cut off	20 $\text{\AA}$
Overall phase residual	13.1 $^\circ$

As displayed in Table 6.2, the final 3D reconstruction was generated from two datasets with 234 reflections, sampling -30° to $+40^\circ$. The overall phase residual is 13.1. ALAT limit was set to 300. $0,0,l$ values were calculated by modelling into 3D transform (chapter 2.6.1.9). The resulting reconstruction is shown in Figure 6.8 and Video 6.1. The surface was rendered at a density corresponding to the maximum density gradient.

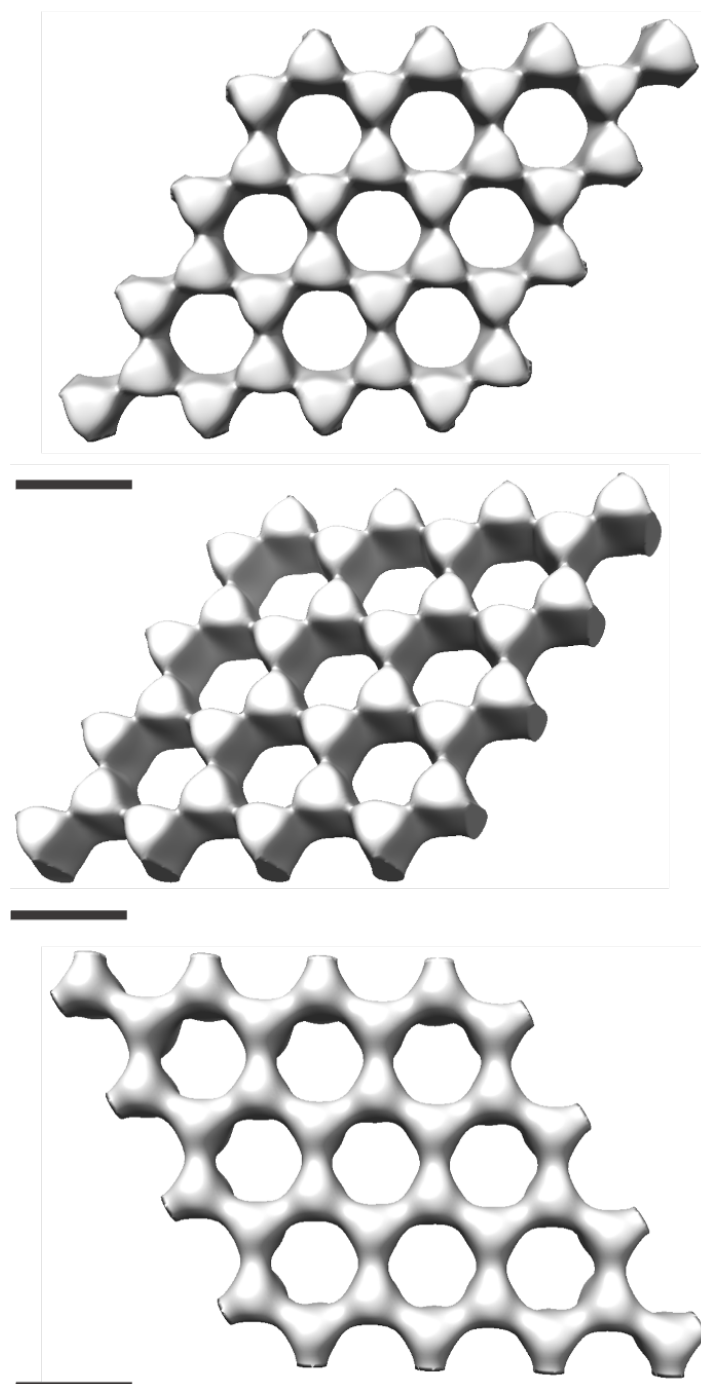
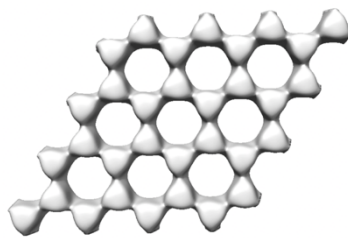


Figure 6.8 3D Model of *C. difficile* para-crystalline layer

Low resolution map generated from 2 tilt series from -30° to $+40^\circ$ (Scale bar = $100\text{\AA}$)



Video 6.1 3D reconstruction of *C. difficile* para-crystalline layer

6.3.5 Protein composition of outermost spore layers

In order to identify the roles that the Cde proteins have in spore assembly, a combination of mutant screening, immunogold labelling and recombinant expression in *E. coli* was conducted.

6.3.5.1 Mutant Screening

Eleonora Marini, from Adriano Henriques' laboratory in ITQB Lisbon, made mutants and spores overexpressing all the Cde proteins (CdeA+, CdeB+, CdeC+ and CdeM+) in the CD630 Δ *erm* strain. Mutants included Δ *cdeC*, Δ *cdeM* and a double Δ *cdeC**cdeM* mutants, no mutants for CdeA and CdeB were available. These spores were screened as whole spores and as thin sections by TEM (Figure 6.9 *C. difficile* spore mutants by electron microscopy Figure 6.10). In both figures, the blue arrows correspond to different spore morphologies and maroon arrows indicate phenotypical differences. All thin section work was conducted by the group in Lisbon. Table 6.3 summarises all the morphologies observed across the spores.

6.3.5.1.1 CdeM

The Δ *cdeM* spore population appears to possess all spore morphologies identified in WT spores (Figure 6.9A,B); elongated spores (Figure 6.9A,B - blue arrow 1) with rough edges around the spore and particularly at the pole (maroon arrow 1). Oval spores with no apparent layers on the surface of the spore are also present (Figure 6.9A -blue arrow 2). There are also spores with layers extended from the pole (Figure 6.9A- blue arrow 3). Thin sections display spores lacking the appendage region at the pole of the spore, apart from a small patch of material (Figure 6.9B,C - maroon arrow 2) present at the pole.

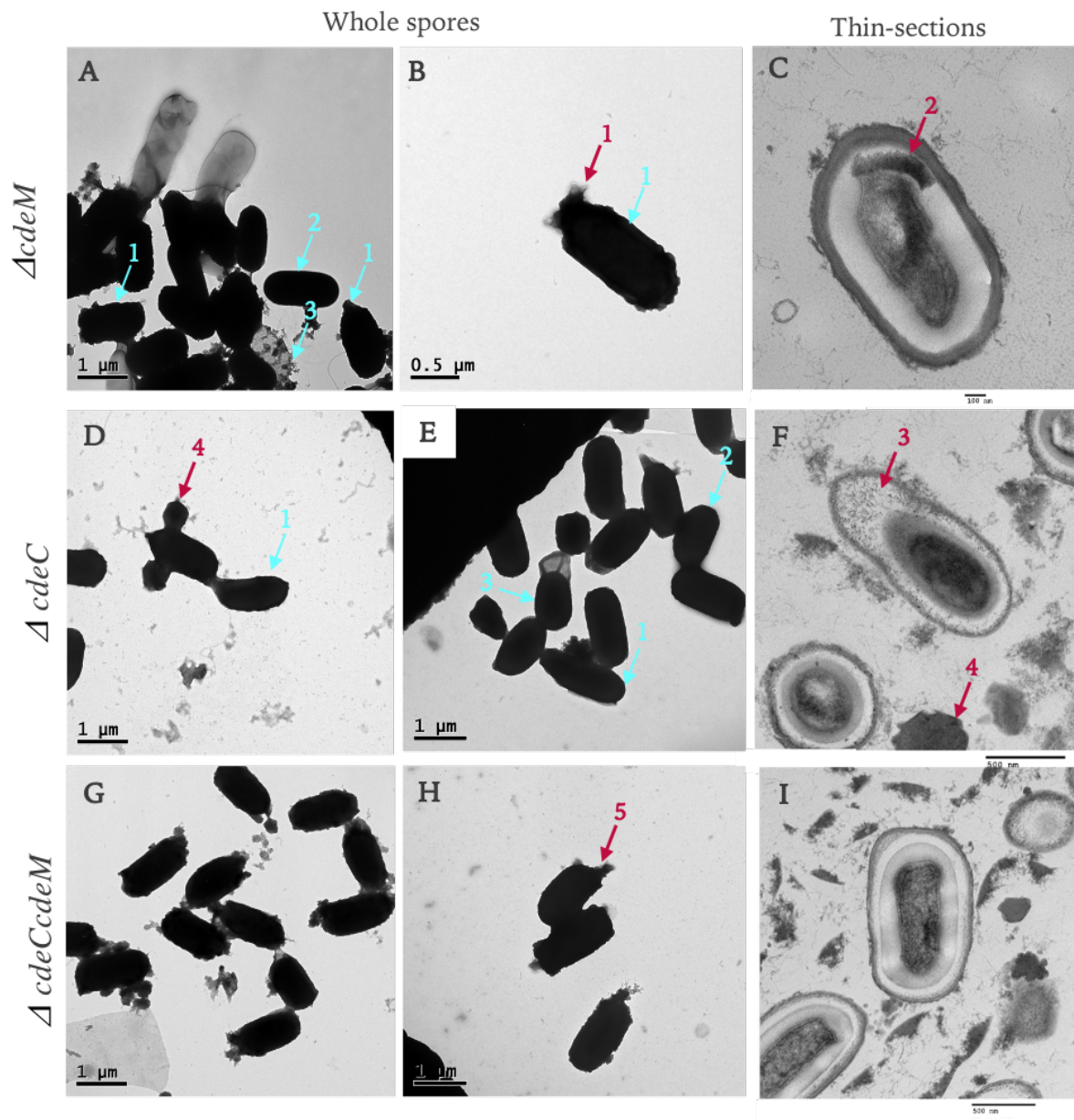


Figure 6.9 *C. difficile* spore mutants by electron microscopy

A, B, D, E, G, H) TEM micrographs of whole spores and C, F, I: Thin-sections of spores (images collected by collaborators in ITQB Lisbon). Blue arrows indicate different spore populations based on morphology: 1: Elongated spores 2: Oval spores and 3: spores with layers extended from poles, maroon arrows highlight phenotype features A, B, C) $\Delta cdeM$: rough edges of spores (1), no appendage present apart from small patch (2) D, E, F) $\Delta cdeC$: undefined appendage (3), proteinaceous material associated with spores (4) G, H) $\Delta cdeC\Delta cdeM$: rough edges on spores (5), no appendage or proteinaceous material associated to spores.

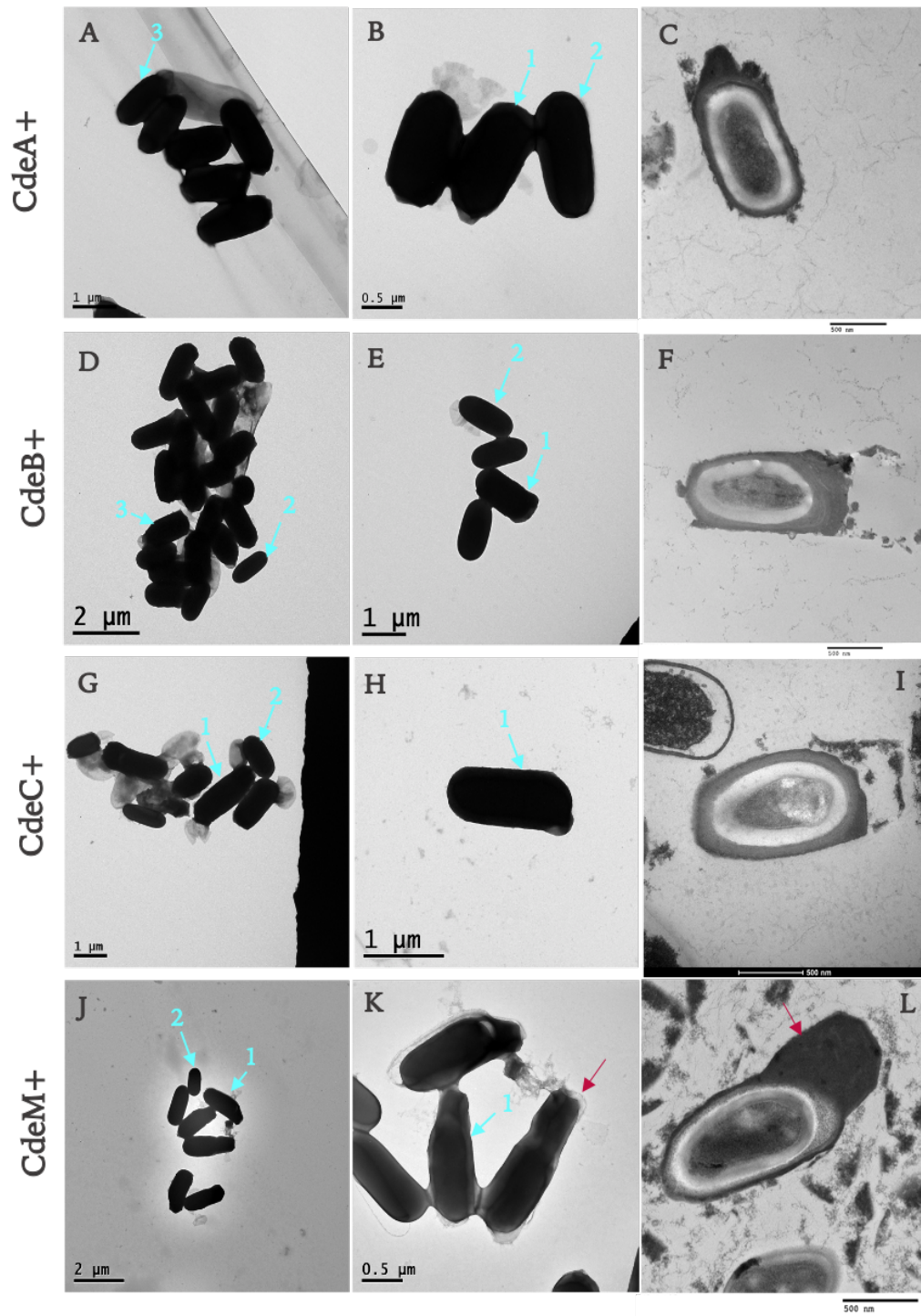


Figure 6.10 *C. difficile* overexpressing Cde proteins by electron microscopy

A, B, D, E, G, H, J, K) TEM micrographs of whole spores and C, F, I, L: Thin-sections of spores (images collected by collaborators in ITQB Lisbon). Blue arrows indicate different spore populations based on morphology: 1: Elongated spores 2: Oval spores and 3: spores with layers extended from poles A, B) CdeA+, D, E) CdeB+, G, H) CdeC+, J, K) CdeM+: Elongated appendage (maroon arrow)

CdeM+ spores also possessed the different spore morphotypes (Figure 6.10J, L). Both whole spore and thin section micrographs of CdeM+ display an elongation of the appendage at the pole (Figure 6.10K and L), highlighted by maroon arrows.

6.3.5.1.2 CdeC

$\Delta cdeC$ spores were primarily composed of elongated spores (Figure 6.9D,E blue arrow 1), with a much lower abundance of oval spores (Figure 6.9E - blue arrow 2). The number of spores with layers extending from the pole, was also very low (Figure 6.9E - blue arrow 3). Thin sections of these spores indicated that $\Delta cdeC$ spores also lack a polar appendage although the outermost layers appeared to extend at one of the poles (Figure 6.9F - maroon arrow 3). The lack of appendage was not apparent in the whole spore screening. An additional feature observed in this mutant was the presence of dense material associated with the spores (Figure 6.9D,F - maroon arrow 4). This material was much smaller than the spores.

CdeC+ spores presented all morphologies, including oval spores (Figure 6.10G,H). Thin sections demonstrate that the spores possess appendages (Figure 6.10I). High magnification of the thin sections reveals that the spores have an increased number of laminations in the coat (Figure 6.11C).

6.3.5.1.3 CdeC and CdeM

Only elongated spores were observed in the double $\Delta cdeC\Delta cdeM$ mutant. These spores possessed rough edges around the spore in whole spore screening (Figure 6.9E), with no material associated with the spores as observed in the $\Delta cdeC$ mutant. Thin sections also revealed no appendage present at the pole of the spore (Figure 6.9F).

6.3.5.1.4 CdeA

CdeA+ spore populations presented all morphologies including elongated and oval spores (Figure 6.10A,B), although abundance of spores with layers extending from the pole was very low. Thin sections revealed that all layers were present (Figure 6.10C), including the appendage, with no apparent difference from WT spores.

Table 6.3 Spore morphologies observed in WT and mutant *C. difficile* spores by whole spore negative stain and chemically fixed thin sections TEM

Spore	Three main morphology populations present*	Distinct morphologies	Crystalline layer dimensions
WT	✓		$a=b = 96 \pm 3 \text{ \AA}, \gamma = 121 \pm 2^\circ$
$\Delta cdeC$	No oval spores Low abundance of spores with layer extending from pole	No appendage Dense material associated with spores	$a=b=96.7 \text{ \AA}$
$\Delta cdeM$	✓	No appendage	$a=b=95.9 \text{ \AA} \pm 3.7 \text{ \AA}$
$\Delta cdeC \Delta cdeM$	Only elongated spores	No appendage No crystalline layers observed	
CdeM+	✓	Elongation of the appendage at the pole	$a=b=95.5 \text{ \AA} \pm 3.2 \text{ \AA}$
CdeA+	✓ Very low number of spores with layers from the pole	Second crystal type observed	$a=b=96 \text{ \AA}$ Second crystal type: $a \sim b \sim 120 \text{ \AA}$
CdeB+	✓ Very low number of spores with layers from the pole	Increased laminations in the coat No crystalline layers observed	
CdeC+	✓	Appendage present Increased laminations in the coat Second crystal type observed	$a=b=96.5 \text{ \AA}$ Second crystal type: $a \sim b \sim 120 \text{ \AA}$

6.3.5.1.5 CdeB

CdeB+ possessed all spore populations although numbers of spores with layers from the pole were also very low (Figure 6.10D,E). Thin sections revealed spores with all layers including the appendage, although high magnification of sections displayed an increased number of laminations in the coat (Figure 6.11B).

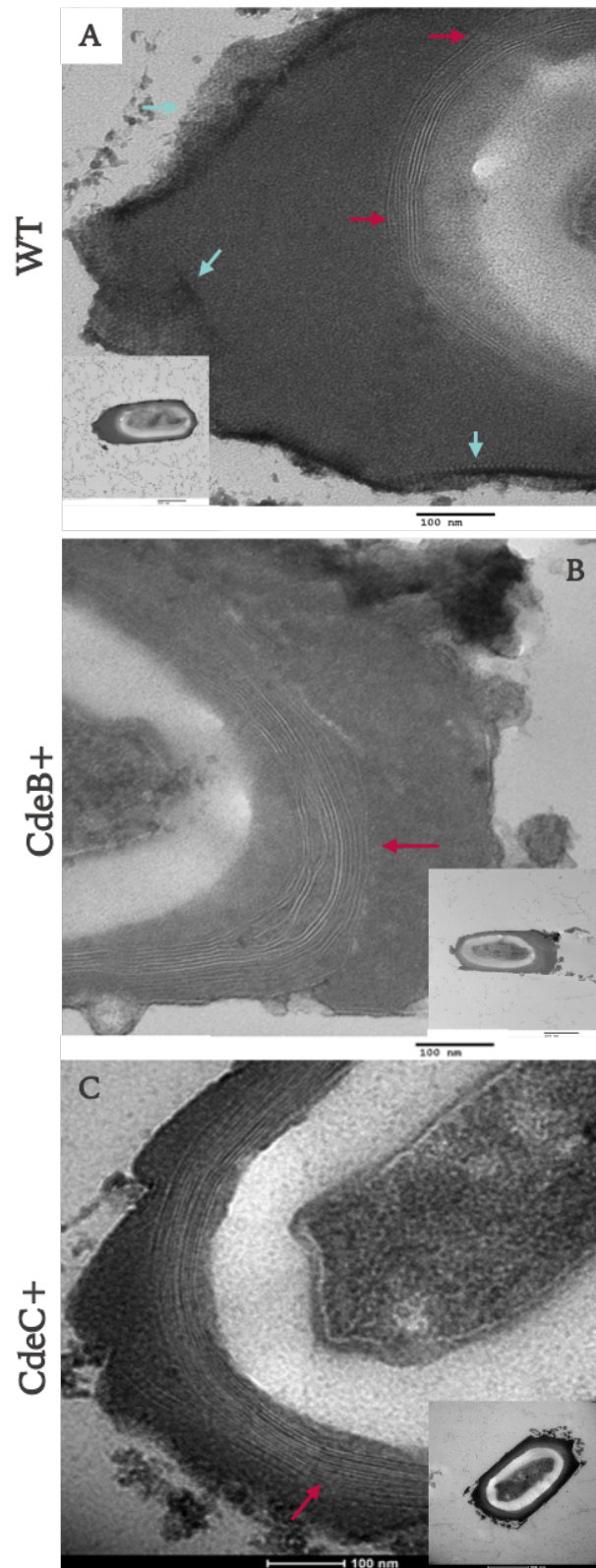


Figure 6.11 Laminations observed in *C. difficile* spores

High magnification of thin-sections and low magnification insets A) WT spores: laminations labelled by pink arrows, blue arrows display crystalline patches on edge of endospores B) CdeB+ spores and C) CdeC+ spores pink arrows highlight laminations, both spore types appear to have increased laminations.

6.3.5.2 Dimensions of wild type and mutant spores

Width and length analysis were conducted across the mutant spores and compared to the WT. The results are displayed in Figure 6.12 which indicated that the average width across all spores ranges between 0.57-0.75µm. The ‘elongated’ morphology was the most common across WT and the mutants. The average length of elongated spores was as outlined in table 6.4.

Table 6.4 Average length of elongated spores across WT and mutant *C. difficile* spores

Spore	Average length (µm)
WT	1.6 ±0.3
$\Delta cdeC$	1.4 ±0.2
$\Delta cdeM$	1.5 ±0.2
$\Delta cdeC\Delta cdeM$	1.4 ±0.2
CdeM+	1.7 ±0.3
CdeA+	1.6 ±0.3
CdeB+	1.4 ±0.2
CdeC+	1.5 ±0.4

Actual differences are not significant due to the high variability in *C. difficile* spore sizes, as seen by large error margins, thus difficult to make any conclusive statements.

‘Oval’ spores were mostly present in $\Delta cdeM$ and CdeM+, insufficient number of oval spores in $\Delta cdeC$ meant that significant analysis could not be conducted. The greatest difference between length was observed between the oval and elongated spores of CdeM+ with an average of 1.1 µm ±0.2 and 1.7 µm ±0.3 respectively. No significant difference was observed between oval and elongated spores of $\Delta cdeM$ at 1.3 µm ±0.1 and 1.5 µm ±0.2 respectively. This suggests that the main difference between oval and elongated spores is the presence of the appendage region.

The width and length analysis highlight a subpopulation with a much shorter length, which corresponds to the ‘mass’ identified in the $\Delta cdeC$ spores. The average length is 0.6 µm ±0.1 - more than half the size of a spore.

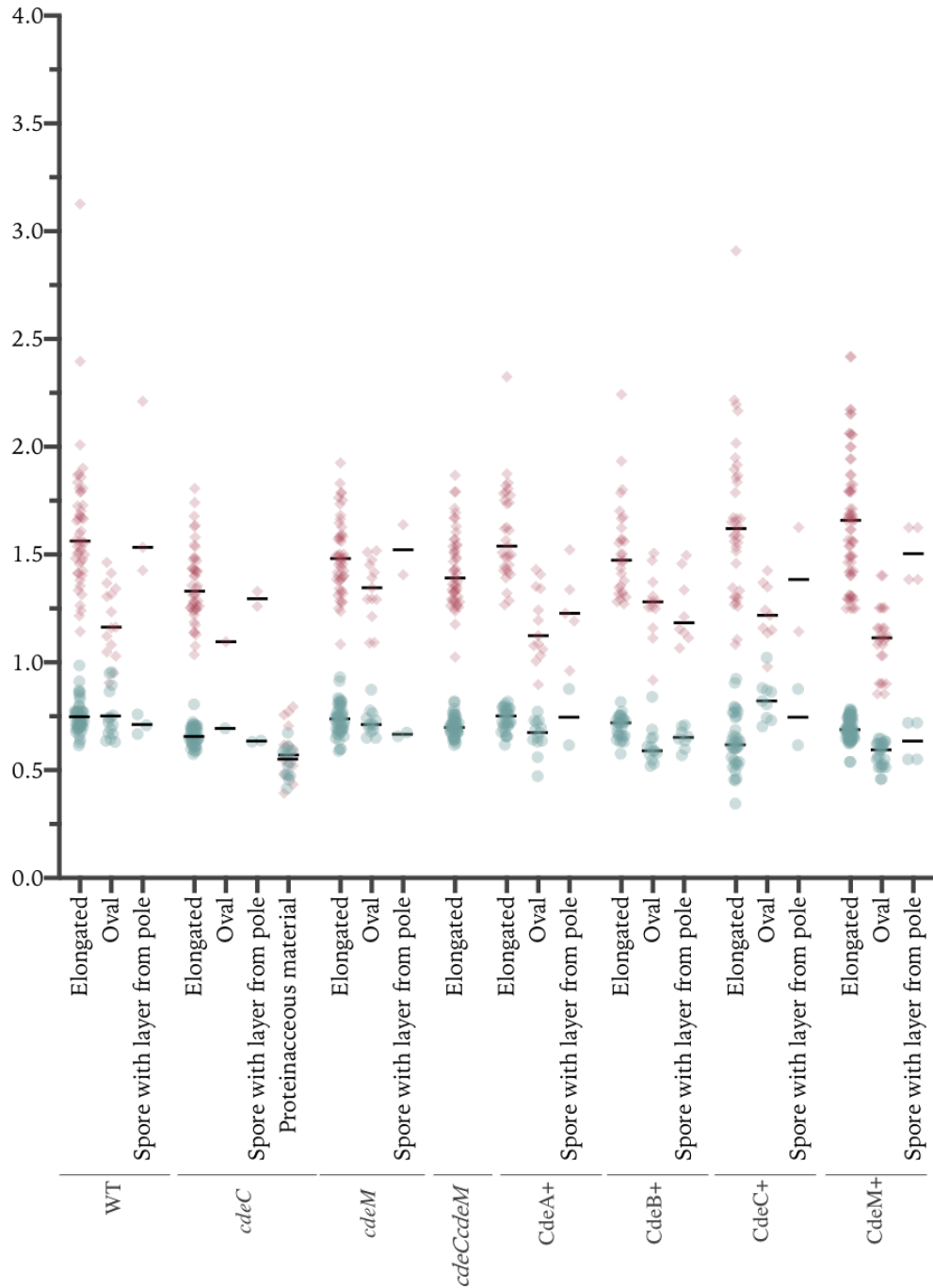


Figure 6.12 Width and length analysis of WT and mutant spores

Scatter plot for width (green) and length (pink) measurements for different phenotypes; elongated, oval, spore with layer, proteinaceous mass. ~50 spore for each spore type was used. The bars display the mean and standard deviation for each phenotype. Low discrepancy observed across width measurements, primary difference observed across length of elongated phenotypes. A subpopulation is the proteinaceous mass which is clearly much smaller in length compared to spores.

6.3.5.3 Para-crystalline layer in mutants

Para-crystalline layers similar to WT ‘exosporium-like layers’ were identified across all spores apart from CdeB+ and $\Delta cdeC\Delta cdeM$ spores (Table 6.3). In $\Delta cdeM$ (Figure 6.13A, B), $\Delta cdeC$ (Figure 6.13C-D) and CdeM+ (Figure 6.14E, F), the para-crystalline layer was found to extend from the poles of the spore. Projection maps were generated by merging three independent images respectively for *cdeM* and CdeM+ (Figure 6.15A, B). Only a single example of the crystalline layer was identified in $\Delta cdeC$, the projection map is displayed in Figure 6.15C. For CdeA+ and CdeC+ these layers were observed detached from the spores in the spore prep (Figure 6.15A, C). The projection maps from these layers are displayed in Figure 6.15D and E.

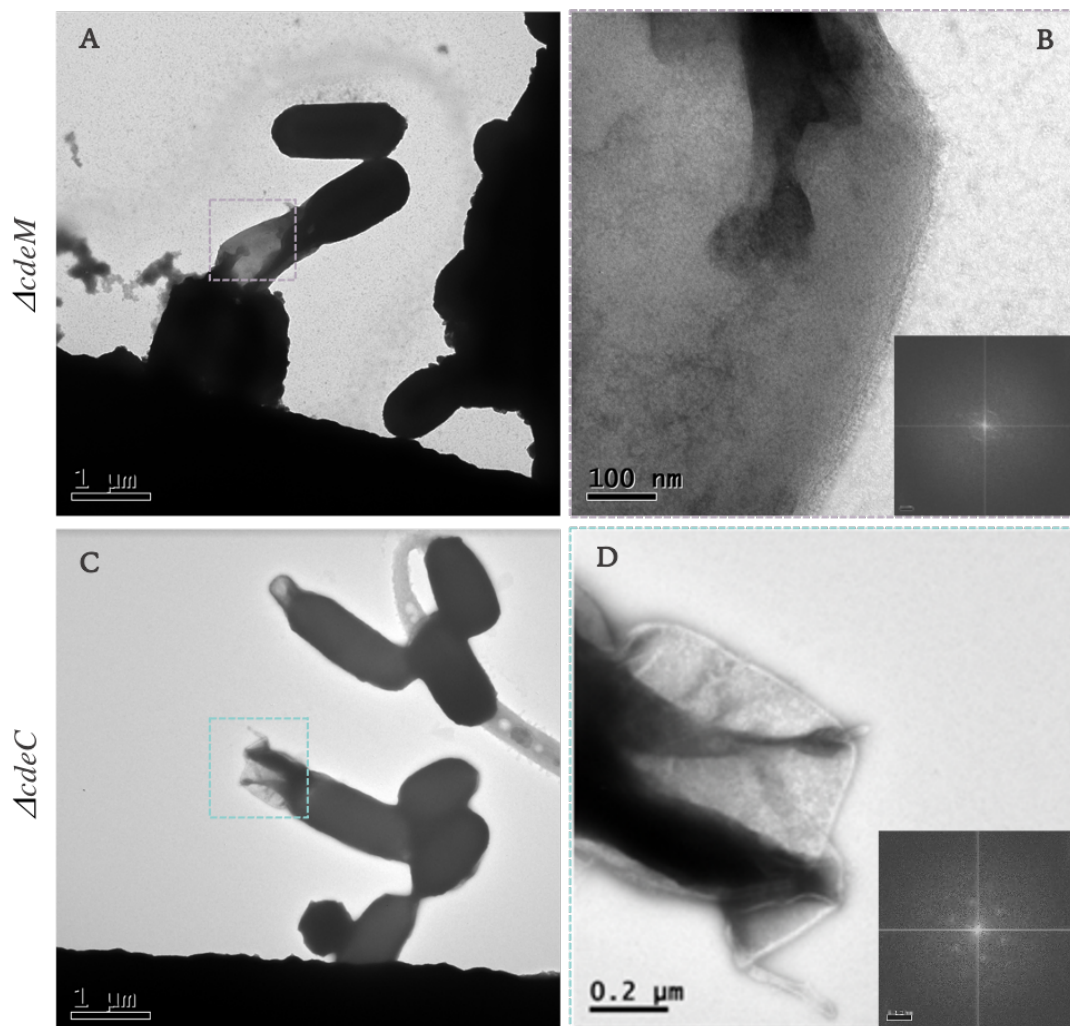


Figure 6.13 Para-crystalline layer in *C. difficile* mutants

TEM micrographs (LHS) and high magnification images with FFT (inset, scale bar = 0.1nm⁻¹) displaying crystallinity (RHS) A,B) $\Delta cdeM$, C,D) $\Delta cdeC$

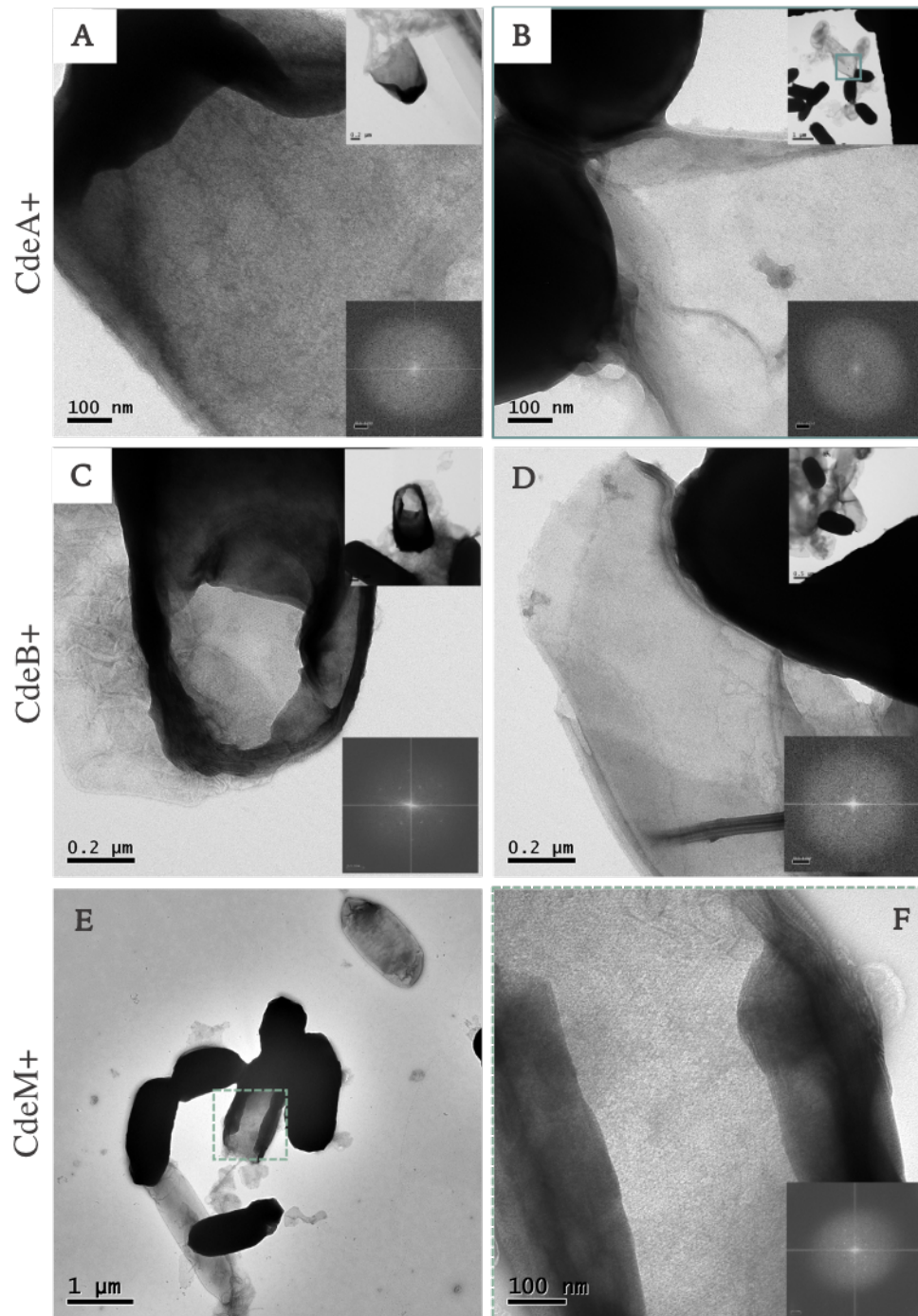


Figure 6.14 Para-crystalline layers in *C. difficile* spores overexpressing Cde proteins

High magnification images with insets of low magnification and FFTS of layer A,B) CdeA+ C+D) CdeC+ E,F) CdeM+. Crystalline layer in A,C, E,F similar unit cell parameter as WT exosporium. B,D crystalline layer with different unit cell parameters from WT exosporium.

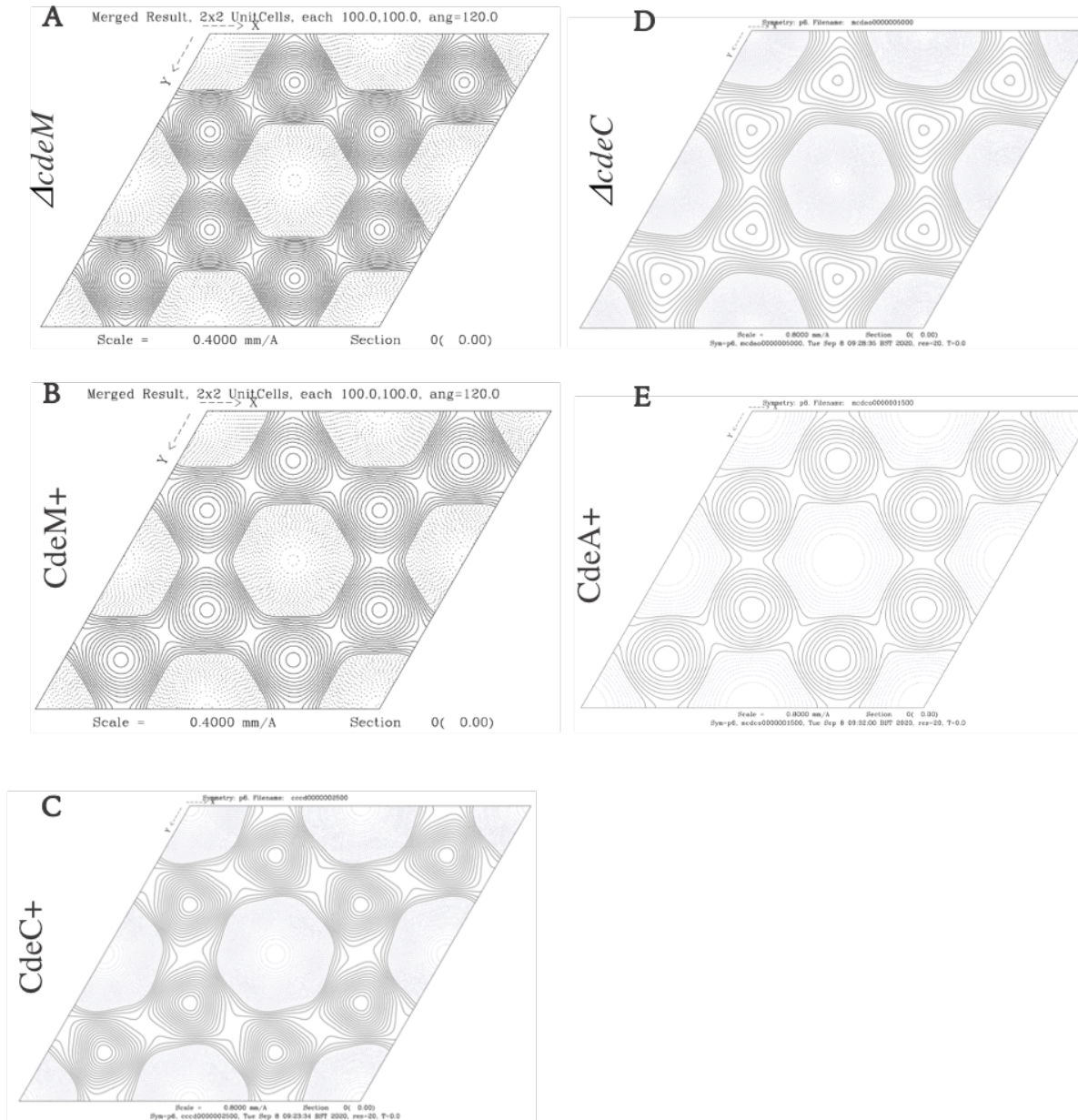


Figure 6.15 Projection maps of para-crystalline layers in *C. difficile* mutants ($p6$ symmetry applied)

A,B) Merge maps generated from 3 images A) $\Delta cdeM$ unit cell size $a=b=95.9\text{\AA} \pm 3.7\text{\AA}$, B) CdeM+ unit cell size $a=b=95.5\text{\AA} \pm 3.2\text{\AA}$ C-E) Map generated from one image C) $\Delta cdeC$ unit cell size $a=b=96.7\text{\AA}$. D) CdeA+ unit cell $a=b=96\text{\AA}$ E) CdeC+ unit cell $a=b=96.5\text{\AA}$

The projection maps of these para-crystalline layers possess an apparent sixfold symmetry and unit cell size suggests no observable difference between the crystalline layer in the mutants and the WT. Any differences observed in these projection maps are not significant due to the limited resolution from negative stain sample preparation and low sample number.

Another crystal type was observed in CdeA+ and CdeC+ (Figure 6.14B,D), appearing to be associated with a spore in CdeA+ and detached from the spore in CdeC+ (Table 6.3). A projection map from this crystal in CdeC+ is displayed in Figure 6.16. It seems to possess a similar overall structure with $p6$ symmetry, although the unit cell length is $a \sim b$ 120Å. Further data are required to confirm this. 2D map generated from a single image of CdeC+ unit cell $a=b=120.4\text{\AA}$

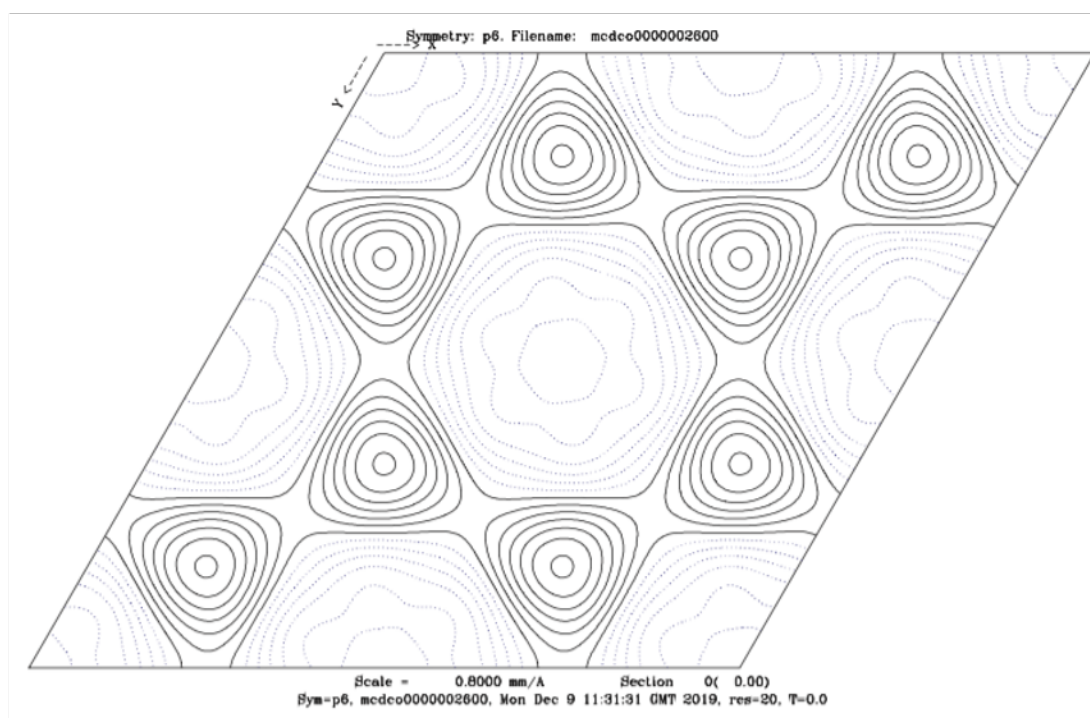


Figure 6.16 Projection map of a different para-crystalline layer observed in CdeA+ and CdeC+ *C. difficile* spores ($p6$ symmetry applied)

6.3.6 Immunogold labelling

Immunogold labelling was employed to identify the relative position of the Cde proteins in the spores, with the hope of gaining insight into the composition of the crystalline layer (Terry *et al.*, 2011).

Antibodies were available against CdeC and CdeM. The spores were first incubated with an anti

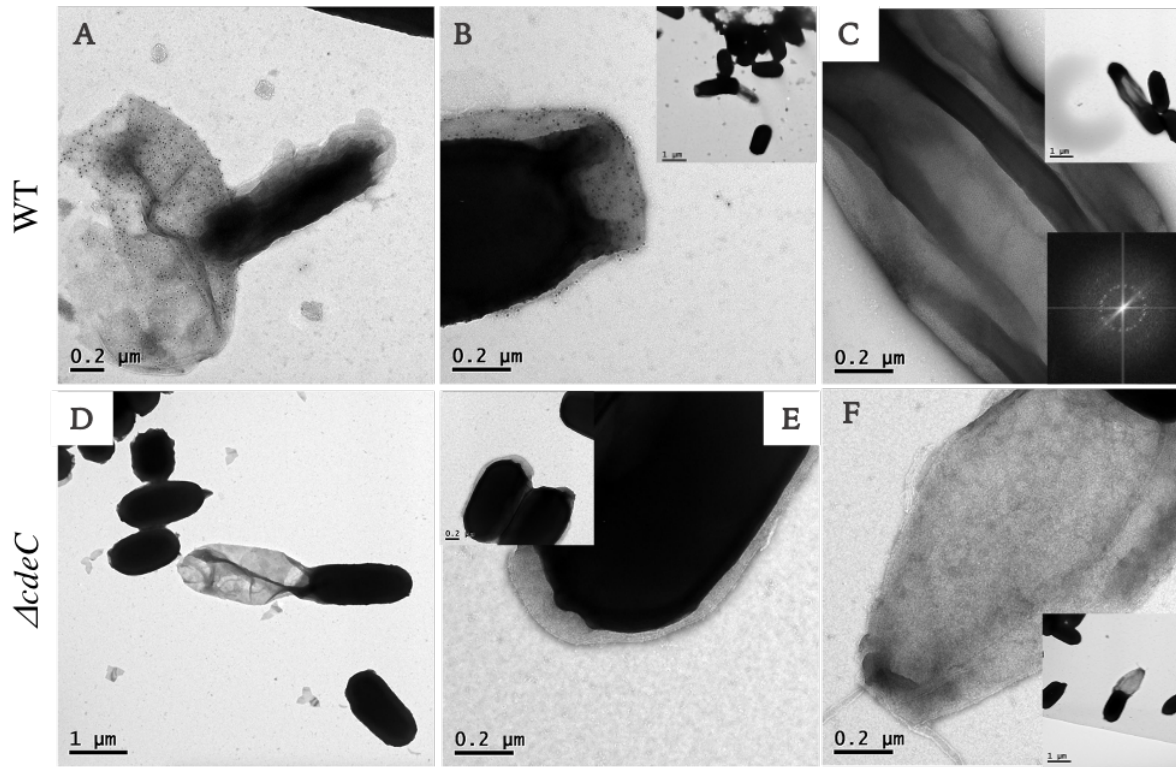


Figure 6.17 Immunogold labelling of CdeC

A-C) WT spores: Gold labelling present on non-crystalline layers (A) and on layers close to spore (B) but not present on crystalline layers (C). D-F) $\Delta cdeC$ spores (control): no gold labelling on layers

CdeX primary antibody, produced in rabbit. Then an anti-rabbit with conjugate gold was used to label the antibodies, visible as dark spots in the EM.

6.3.6.1 CdeC labelling

CdeC labelling was observed on non-crystalline layers both around the spore body and on layers that protruded from the spore pole (Figure 6.17A,B), with no labelling observed on crystalline material (Figure 6.17C). As a control, $\Delta cdeC$ spores were treated with CdeC labelling, which displayed no labelling on any layers.

6.3.6.2 CdeM labelling

No CdeM labelling was observed on either crystalline or non-crystalline layers of WT (Figure 6.18A-C) or $\Delta cdeM$ spores (Figure 6.18G-I). However, low levels of CdeM labelling were observed in $\Delta cdeC$ (Figure 6.18D-F) and CdeM+ (Figure 6.18L) spores, on the non-crystalline layers.

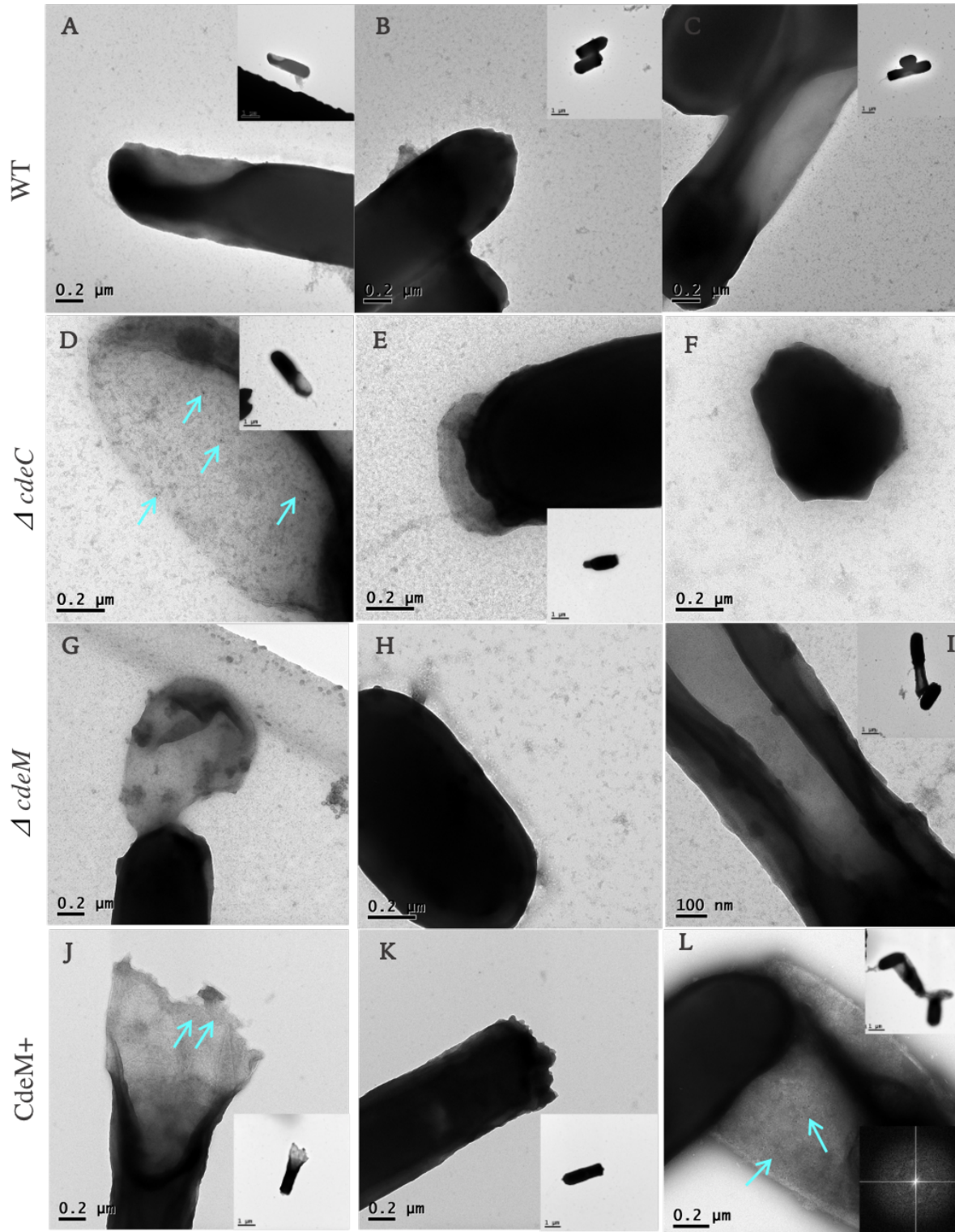


Figure 6.18 Immunogold labelling CdeM

A-C) WT spores, D-F) $\Delta cdeC$ spores, G-I) $\Delta cdeM$ spores J-L) CdeM+ spores. No labelling on: both crystalline (C, I) and non-crystalline (A, G) and layers close to spore (B, E, H). Low labelling on non-crystalline layers in $\Delta cdeC$ (D) and CdeM+ (J) spores. High labelling on non-crystalline patch on crystalline layer in CdeM+ spores (L)

6.3.7 Recombinant expression of Cde proteins in *E. coli*

Cde proteins were expressed both individually and as a combination with other Cde proteins by the lab group in Lisbon. This is demonstrated by the SDS-PAGE and Western blot (Figure 6.19), provided by the Lisbon group. Each sample was studied by screening both thin sections and cell lysate by TEM. The thin section work was conducted by the Lisbon group. The results from screening samples by TEM are summarised in the table below:

Table 6.5 Summary of results from expressing different combinations of recombinant Cde proteins in *E. coli*. *EDB: electron dense body

Protein expressed in <i>E. coli</i>	EDB* + no laminations	EDB* + with laminations
CdeA		
CdeB	✓	
CdeC		✓
CdeM	✓	
CdeA+B		
CdeA+M		
CdeA+C		✓
CdeB+M	✓	
CdeB+C		✓
CdeC+M		✓
CdeA+C+M		✓
CdeB+C+M		✓
CdeA+B+C		✓
CdeA+B+C+M		✓

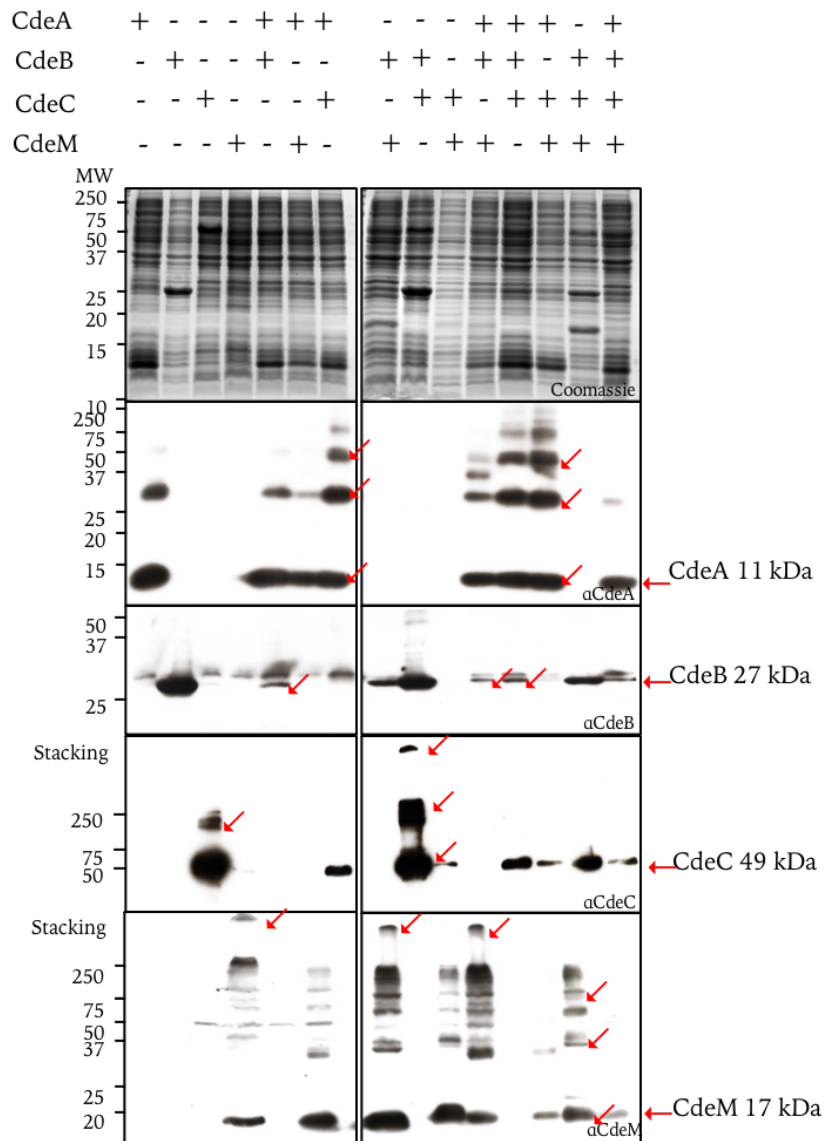


Figure 6.19 SDS-PAGE and Western Blot of Cde expression in *E. coli*

Credit to Eleonora Marini in ITQB Lisbon

Individual Cde protein expression in *E. coli* is displayed in Figure 6.20 and 6.21. As a control, uninduced *E. coli* cells were screened; these displayed no observable mass or laminations in the thin-sections or crystalline sheets in the cell lysate (Figure 6.20G,H). Thin sections indicate that CdeA, CdeB and CdeM possessed an electron dense body (EDB) but no laminations observed (Figure 6.20A,D,F) and CdeC expression possessed EDB with laminations (Figure 6.21 - arrows). None of these samples yielded crystalline layers in the cell lysate (Figure 6.20 and 6.21).

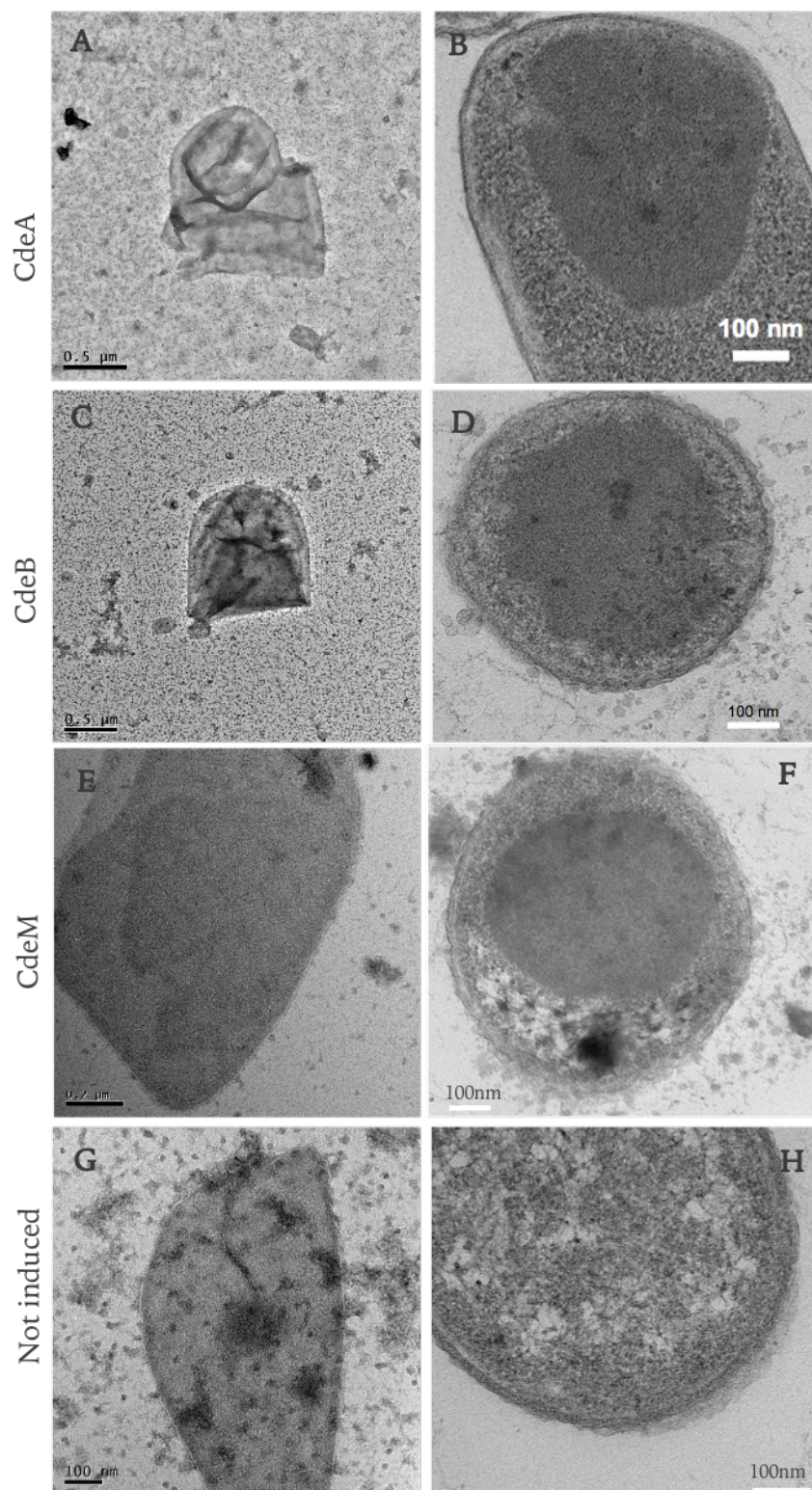


Figure 6.20 Expression of individual Cde proteins in *E. coli*

Thin sections of cell (RHS), images collected by Eleonora Marini in ITQB Lisbon. B,H) No Mass observed within cell, D,F) Mass observed within cells. TEM of lysed *E.coli* cell material (LHS), all possess non-crystalline layers.

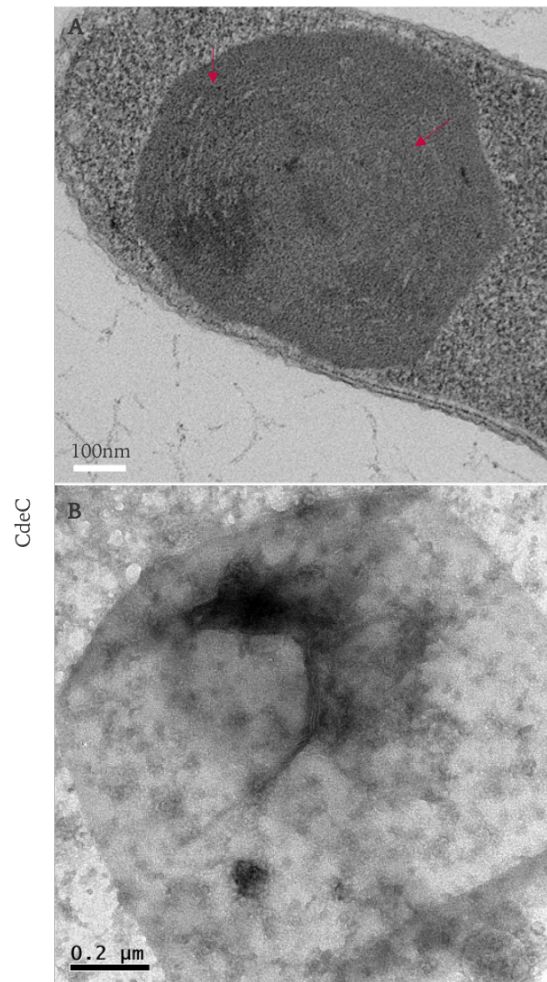


Figure 6.21 Expression of CdeC in *E. coli*

A) Thin sections of cell (image collected by Eleonora Marini in ITQB Lisbon) displays a electron dense mass (EDB) with concentric laminations (arrows) suggesting self-assembly of material. B)TEM of lysed *E.coli* material, non-crystalline layers found in the sample.

Figure 6.22 presents all the combinations of Cde proteins expressed in *E. coli*. Thin sections show that combinations with CdeC yield EDB with laminations (Figure 6.22 F, J, K, N, P, R, T). In the absence of CdeC no mass or laminations were observed (Figure 6.22B, D, H). Interestingly, a combination that included A+C+M assembled into large laminations that spanned the whole cell (Figure 6.22N, T). No crystalline material was observed after screening all the cell lysates across all combinations.

Attempts were made to immunogold label the CdeC assemblies in cell lysate, in order to identify whether non-crystalline assemblies were being assembled containing CdeC. No labelling was observed.

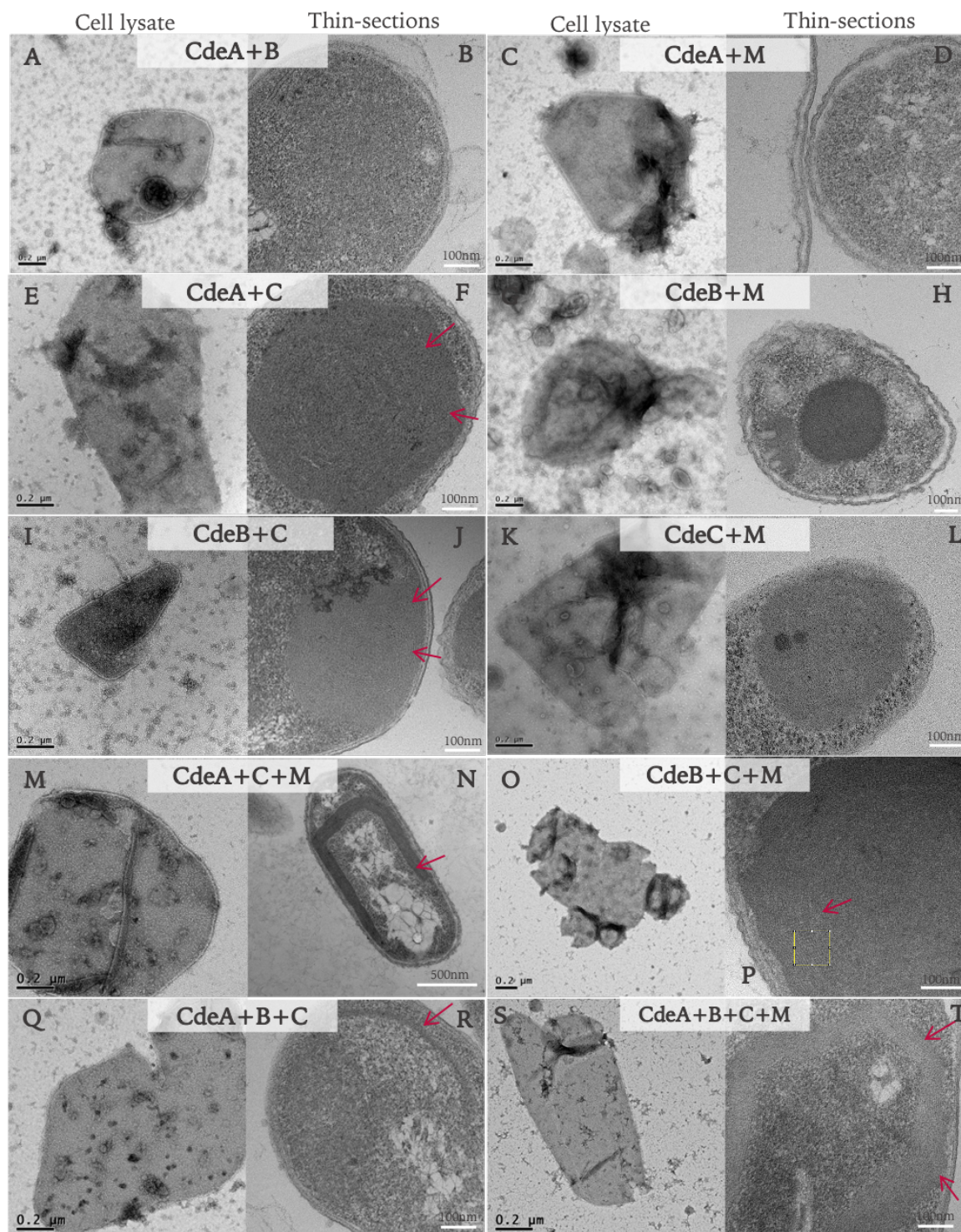


Figure 6.22 Co-expression of Cde proteins in *E. coli*

Thin section images taken by Eleonora Marini (ITQB). Cell lysates display no crystalline layer. Thin sections of cells demonstrate different phenotypes depending on proteins expressed with some laminations observed (maroon arrows)

A+B, A+M: No observed change

B+M: Mass observed without laminations

A+C, B+C, B+C+M: Some laminations like CdeC

A+B+C: Small level of laminations within cell

A+B+C+M, A+C+M: Larger laminations within cell

6.4 Discussion

6.4.1 Diversity in spores and importance

C. difficile spores possess a highly diverse phenotypical population. Negatively stained whole WT 630 Δ erm spores were screened by TEM for the first time and revealed three main morphologies (Figure 6.23A): elongated spores, oval spores and spores with layers extending from the spore pole. These morphologies are analogous to the different morphotypes described in Antunes *et al.* (2018). We propose that the oval and elongated spores resemble ‘morphotypes A+B’ respectively (Figure 6.23), with differing lengths of appendage.

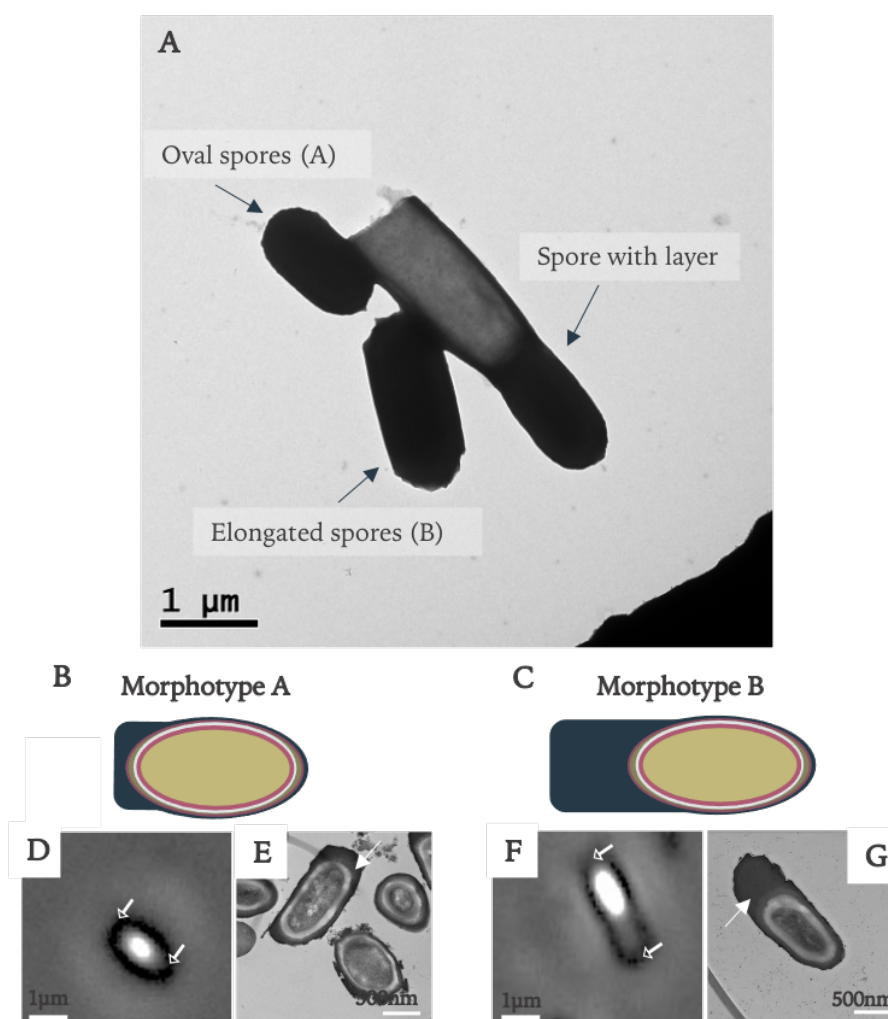


Figure 6.23 WT *C. difficile* 630 Δ erm endospore morphotypes

A) TEM micrograph showing different morphologies: oval spores (morphotype A) and elongated spores (morphotype B)
 B,C) Schematic diagram of morphotypes D-G) Data from * showing spores in phase bright (D,F) and thin sections TEM (E,G) for morphotype A (D,E) and morphotype B (F,G): display spores with long and short/absent appendage (arrows)

Figures B, C, D, F adapted from Antunes, *et al.*, (2018). E,G: image from Eleonora Marini (ITQB, Lisbon)

The third phenotype, spores with layers extending from the spore pole, have never before been described. They appear in very low abundance and in some cases the extended layers are para-crystalline, could this be the *C. difficile* exosporium? This will be discussed in the following sections.

6.4.2 Does *C. difficile* possess an exosporium comparable to that of other spore formers?

Endospores from *B. cereus* sensu lato group (Henriques and Moran, 2007; Stewart, 2015), *C. sporogenes* (Brunt *et al.*, 2015; Janganan *et al.*, 2020; 2016; Warren *et al.*, 1994) and *C. sordelii* (Rabi *et al.*, 2018) possess a ‘classical exosporium’; a baggy continuous assembly that covers the whole spore and extends from one pole with an interspace region, providing a gap from the coat. The basal layer of the exosporium, across different organisms, consists of a para-crystalline layer made up of hexagonal arrays (Ball *et al.*, 2008; Janganan *et al.*, 2020; Terry *et al.*, 2017). However, the proteins that compose the exosporium bear no sequence homology although they have a cysteine-rich content in common.

B. subtilis differs from these spores, in that it does not possess a ‘classical exosporium’ but a ‘crust’ which is assembled close to the main spore body with no interspace. The major protein component of this layer are cysteine-rich proteins, which can self-assemble into hexagonal arrays (Jiang *et al.*, 2015) - analogous to the ‘classical exosporium’.

Previous work on *C. difficile* spores has focussed on thin sections by TEM (Calderón-Romero *et al.*, 2018; Paredes-Sabja *et al.*, 2014; Permpoonpattana *et al.*, 2011), which revealed little distinction between the exosporium and the coat. Thus, the exosporium labelling in the literature has been inconsistent. Permpoottanan *et al.* (2011) described it as a heavily stained patch that was partially attached to the spore. Whereas others label the outermost visible layer, as the exosporium, present on all spores (Calderón-Romero *et al.*, 2018; Paredes-Sabja *et al.*, 2014). With no structural information, it remains unclear as to whether *C. difficile* spores possess an exosporium at all.

Whole spore screening by TEM identified a morphotype of spores with layers extending from a spore pole (Figure 6.23A). The apparent morphology of these layers, extending from a single pole and

having, in some cases, para-crystalline properties, hints at resemblance to the ‘classical exosporium’ found in *Bacillus* and some *Clostridia*. If they were similar to the ‘classical exosporium’ the layers would also cover the whole spore; unfortunately due to the highly electron dense main spore body, whole spore TEM was not able to confirm this.

High magnification micrographs of the crystalline layers were used to gain structural insights by electron crystallography. Data collection was challenging due to the low abundance of crystals, meaning that high levels of searching was required. Additionally, the crystals were very patchy with varied quality, requiring masking for processing. Due to these reasons, the sample was not amenable for Cryo-EM investigation. The difficulty of collecting and processing data from these crystals was exacerbated when moving on to 3D analysis. Greater angle sampling is achieved by having the sample in the centre of the grid square and closer to the centre of the grid, reducing the chances of grid bars blocking higher tilts. Low sample concentration meant that this was not possible for most of the data collected. Due to the highly patchy nature of the crystals, tilted data were very difficult to process as high-quality patches became smaller at higher tilts.

The final 3D reconstruction includes two data sets collected from -30° to $+40^\circ$ at 10° intervals. The reconstruction is low resolution due to low crystal sampling and the limited angular range for the data collected. Nevertheless, the model provides insight into the 3D structure of this crystalline layer, as a porous assembly with sixfold symmetry (Figure 6.10), which resembles exosporium layers from *Bacillus* and *Clostridium sporogenes*. The central pore has an apparent diameter of $\sim 75\text{\AA}$, thus the crystalline layer appears more permeable than exosporium from other spore formers studied (Ball *et al.*, 2008; Kailas *et al.*, 2011; Terry *et al.*, 2017).

Figure 6.24 displays 9 unit cells in the 3D model, with a tentative unit circled in maroon. The model has a total volume of $4.944 \times 10^6 \text{\AA}^3$ across 32 units, which we can use to estimate the molecular weight of its protein composition. Based on the threefold linker and higher resolution structural information obtained from *C. sporogenes* exosporium, discussed in Chapter 4, I suspected that each unit was formed of 3 subunits which cannot be distinguished here due to resolution limitations. Each of these

smaller subunits possess an estimated volume of $51,500\text{\AA}^3 = 5.51 \times 10^{-20}\text{cm}^3$. If we assume that the average protein is 1.35g/cm^3 , this protein is $4.08 \times 10^{-20}\text{g}$, multiplied by Avogadro's number = 24569.76g/mol . If $1\text{Da} = 1\text{g/mol}$, the estimated size of this protein is $\sim 24.5\text{kDa}$.

Spores with layers extending from the pole were not identified by screening thin sections, likely due to the low abundance of the morphotype. However, thin sections of elongated spores revealed crystalline patches on the edge of spores, which appear to have hexagonal arrangement (Figure 6.5). This suggests that elongated spores have an 'exosporium-like layer' assembled close to the main spore body with no interspace. Due to the electron dense nature of the spore, para-crystalline layers assembled close to the spore body would not be observed by whole spore TEM. This would explain why such a low percentage of layers were observed.

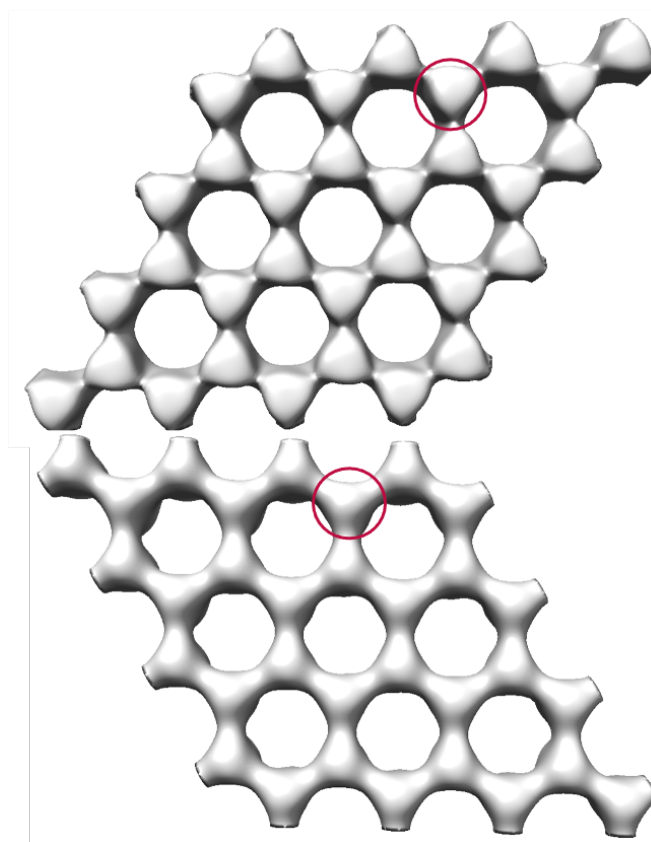


Figure 6.24 Estimate molecular weight of protein in 3D reconstruction of *C. difficile* para-crystalline layer
Views from both sides of the 3D reconstruction with tentative unit circled in pink possibly formed of 3 subunits.

Measurements of the crystal patches in the thin sections revealed a unit cell length $\sim 85\text{\AA}$, $\sim 10\%$ shorter than the unit cell length of the crystalline layer observed at the poles of the spores. Sample shrinkage due to chemical fixation treatment is a well-known phenomenon; other sample preparation approaches have been developed including HPF combined with freeze-substitution or CEMOVIS in effort to overcome shrinkage. Thus, the impact of sample shrinkage on the spores should be considered. These include fixatives such as glutaraldehyde (Lee *et al.*, 1982), organic solvents used for dehydration (Mollenhauer, 1993) and the resin (Lickfeld *et al.*, 1977; Shah *et al.*, 2015). In this case, EPON resin was used, which can have up to 20-30% shrinkage depending on the combination of fixatives used (Lickfeld *et al.*, 1977; Shah *et al.*, 2015). Thus, any of the factors listed could account for $\sim 10\%$ shrinkage in crystal unit cell.

Another crystalline layer was also observed in the spore prep of CdeA⁺ and CdeC⁺, which possessed a hexagonal structure but, at $a \sim b \sim 120\text{\AA}$, differed in unit cell length. Based on the shrinkage levels described in the literature, a 30% shrinkage could also correspond to the crystal layer observed in thin sections. As this crystal has not been seen on any other spore, WT or mutants, apart from CdeA⁺ and CdeC⁺ in low abundance, we propose that it is unlikely to be forming the para-crystalline layer observed on elongated spores. Alternatively, this layer may correspond to the coat or a para-sporal layer.

Based on this evidence it appears that the most common morphotype of *C. difficile* spores, elongated spores, possess a crystalline layer closely associated with the main spore body similar to *Bacillus subtilis* crust. Whether this layer corresponds to the crystalline layer observed at the poles of spores with layers remains unclear. Proposed further work could include screening thin sections of HPF and freeze-substitution spores, as was done with *C. sporogenes* spores in Chapter 5. This approach was demonstrated to preserve the structural integrity of the exosporium, thus could confirm a possible connection between crystal types.

6.4.3 Composition and assembly of outer spore layers in *C. difficile*

Across spore formers, outer spore layers form honeycomb-like assemblies composed of cysteine-rich proteins. These have been found to self-assemble upon heterologous expression in *E. coli* (Janganan *et al.*, 2020; Jiang *et al.*, 2015; Terry *et al.*, 2017).

In *C. difficile* cysteine-rich proteins such as CdeA, CdeC and CdeM have been identified (Díaz-González *et al.*, 2015) and we have revealed crystalline layers with hexagonal arrays associated with spores. Thus, it is tempting to speculate that *C. difficile* may also possess an ‘exosporium-like’ layer composed of cysteine-rich proteins as in other spore formers. In particular we would hypothesize that whichever protein makes up the crystalline layer is also capable of self-assembly upon heterologous expression in *E. coli*.

The most likely candidates for the composition of the ‘exosporium-like’ layer are cysteine-rich proteins: CdeA, CdeC and CdeM (Díaz-González *et al.*, 2015). Based on a lower cysteine content CdeB is a less likely candidate (chapter 1.3.3.6.4). These proteins share no sequence homology between each other (Figure 6.25) and other exosporium proteins or predicted secondary structure based on amino acid sequence.

The expected molecular weight (MW) for a subunit in the crystalline layer identified associated with the spores is ~24.5kDa. If we consider the expected MW for each Cde protein, CdeC at 49kDa is too large thus unlikely to compose this layer. The MW of the other Cde proteins are much smaller, thus could theoretically compose the subunit. At 27kDa, CdeB is the closest in size to the expected subunit. However, CdeA at 11kDa could also be present in a dimeric form. CdeM is 17kDa, which is smaller than the expected 24.5kDa but too big to form a dimeric complex in this assembly. As the MW is based on estimated values from a low resolution reconstruction, none of these proteins can be discarded and further work must be conducted to greater understand their roles.

CdeA	-----MKNNNLNCAATNCAYN-----	16
CdeB	-----	0
CdeC	-----	0
CdeM	MENKKCYSEDWYERGESTAKWFQNDREEYERAAYDEDRRRGSNCGCSDSGGNRPRNCER	60
CdeA	-----TSGYCYAG-----SIKVDGMQATTENTYCASFEDKYTSGITSRS-	56
CdeB	-----	0
CdeC	-----MQDYKKNK-----RRMMNQPMSTMNEEEVYTDEINSEDMRGFKS--	40
CdeM	FRREAEIREREAREAFCESEKKKEALAYCEARKLWKEAEKYWDEYSKYNQIGIEYLAE	120
CdeA	-----NETNQVDTDN-IHCE-----AVCKYKNKNECKAEK-----VHIN	90
CdeB	-----	0
CdeC	-----HHHNGCNTDNKCECHDDCNPCNPCKPNPCNPCKPNPCDDNCGCHD	88
CdeM	AARLFDEGMECEARRNGNNGGNNNNCCHK-----CHKC---NCNCCRK-----	160
CdeA	ERNASCETFFEMK-----	102
CdeB	-----MKGNIQY-----NFADIEEEVYSLDYAIAWNTN-----	29
CdeC	NCKCDCEPCEMDSDECENKCGPECNPISPRNFVSNAVPFAIEANRIFDTMQFQTFTD	148
CdeM	-----	160
CdeA	-----	102
CdeB	--EENVNIIPFTN-----KFCESIESFCLGKINNFEILNEGFVENHHYVHL	75
CdeC	ATGPNGEPLTFETEVVEVFGSVPSAGQASVTIEKICLSN-----DGIVIDTGMTTL	199
CdeM	-----	160
CdeA	-----	102
CdeB	DKM-----ISVPKK-----KVNLYVQQDTHGYLLRDDNDNLI	107
CdeC	EDFDLDPLGDIVGRNCETTFEFAVCGERNSECCRQKGKSVAYKQRLTVAVRNL---VL	256
CdeM	-----	160
CdeA	-----	102
CdeB	PAKITSEQSKSISSKMELFCAGEKCLINI-----LLKAD-----PSY	144
CdeC	ELRGRCCGCTEFVALAFPAVRAGGCKRRVDYVEFTFNTLSAPICLPADGRAVTLRQEYQT	316
CdeM	-----	160
CdeA	-----	102
CdeB	ILDVDSIKDKNILNLGY-ESIDRYKEYNFDDDKILIFFINKKRYSV-----IMKKTNNS	197
CdeC	NLTVDIG-KSILKLECNECCPEFYELIIPNDIDLVLCLQETVSTLISEQIVVLASPNPI	375
CdeM	-----	160
CdeA	-----	102
CdeB	DNDLVSRRNAIKELFTNK-----AGNLN---	220
CdeC	QPRLVDTFSKVCD-FSQCGPNHGSCKPSCHR	405
CdeM	-----	160

Figure 6.25 Sequence alignment of four cysteine-rich proteins present on *C. difficile* endospore surface

No sequence homology found across the proteins. All cysteines are highlighted in yellow.

To gain further insight into their involvement in *C. difficile* spore assemblies, a combination of mutant screening by thin section and negative stain EM, immunogold labelling and heterologous expression was employed. Most of the work focused on CdeC and CdeM, which are clearly extremely important in the assembly of the endospore outer layers (Antunes *et al.*, 2018; Calderón-Romero *et al.*, 2018). This was apparent in the double CdeC and CdeM mutant which yielded a low number of spores. The results obtained thus far did not confirm one the Cde proteins as the primary composer of the crystalline layer but do suggest the involvement of some of the Cde proteins in other assemblies.

6.4.3.1 Heterologous expression of Cde proteins in *E. coli*

Cysteine rich proteins from *Bacillus* and *Clostridia* self-assemble into crystalline sheets when expressed in *E. coli* (Janganan *et al.*, 2020; Jiang *et al.*, 2015; Terry *et al.*, 2017). After expressing individual and combinations of Cde proteins in *E. coli*, no crystalline assemblies were observed. However, thin sections revealed that expression of CdeB and CdeM, both separately and as a combination, generated EDBs.

Upon expression of CdeC, these EDBs also possessed laminations. This suggests that CdeC is able to self-assemble but not into crystalline layer, thus could it be that a combination of proteins are required for this? To answer this, various combinations of the Cde proteins were expressed. The combination of A+C+M appeared to generate more elaborate assemblies in *E. coli*, although not crystalline in nature. This suggests that CdeC interacts with both CdeA and CdeM, although the significance of this remains unclear.

The inability of CdeC to form these crystalline layers (Figure 6.21) also suggests that it is not the component of *C. difficile*'s para-crystalline layer. However, it is possible that CdeC requires post-translational modifications for crystal assembly, that are not performed in *E. coli*. As an example, the SlpA protein in the *C. difficile* S-Layer in its vegetative cell state, is post-translationally cleaved prior to assembly (Fagan and Fairweather, 2014; Fagan *et al.*, 2009), further discussed in chapter 7.

6.4.3.2 CdeA and CdeB

The results for CdeA and CdeB are restricted to overexpression of both proteins in *C. difficile* spores and heterologous expression in *E. coli*, as neither mutants nor reliable antibodies were available. The Lisbon group did have monoclonal antibodies against both CdeA and CdeB used for Western blot analysis. Successful immunogold labelling with monoclonal antibodies was deemed unlikely due to the specificity for single epitopes that may not be exposed *in vivo*. Immunogold labelling with polyclonal antibodies would be desirable to increase likelihood of epitope recognition. Due to the timescales this was beyond this project but will be considered for further work.

As already established, no crystalline layers were observed upon heterologous expression in *E. coli*. Screening whole spores and thin sections of CdeA+ spores suggested no apparent difference from WT endospores. Thin sections of CdeB+ spores however demonstrated increased number of laminations in the spore coat, which could suggest CdeB involvement in coat layers.

6.4.3.3 CdeC

In the literature, CdeC has been proposed to form the exosporium in *C. difficile* spores (Barra-Carrasco *et al.*, 2013). This is due to the high abundance of CdeC on the spore surface (Díaz-González *et al.*, 2015) and the properties that mutant CdeC spores possess including reduced adherence to mammalian cell lines and increased sensitivity to ethanol, heat, macrophages and lysozyme treatment (Calderón-Romero *et al.*, 2018). The exosporium is believed to be involved in adherence to surfaces and confer resilience to harsh conditions, the properties of the CdeC mutant described above are indicative of spores that lack an exosporium layer.

The data presented here suggest that CdeC is not the primary component of the ‘exosporium-like’ para-crystalline layer associated with spores. Based on the MW of CdeC it is too large for the 3D reconstruction, which suggests a protein around 24.5kDa. The screening of mutant CdeC spores also identified, albeit at low levels, crystalline material of identical unit cell size (Figure 19C-D). This eliminates CdeC as a likely candidate for making up the crystalline layer, as it suggests that this low abundance crystalline layer in the CdeC mutant arises from some other protein.

Further evidence for CdeC not being a component of the crystalline layer comes from immunogold labelling of whole spores; this demonstrated no CdeC labelling of crystalline layers, yet non-crystalline layers were consistently labelled (Figure 23). This suggests that the epitope is either inaccessible in the crystalline zones of the spore surface, or most likely because the crystal does not contain CdeC. It is possible that the non-crystalline layer is a kinetically trapped ‘pre-crystalline layer’, with CdeC in its unassembled form and that the epitopes become hidden in the assembled form. However, the high symmetry of other exosporium proteins has been found to drive the

assembly of crystalline layers (Terry *et al.*, 2017), making a ‘pre-crystalline layer’ an energetically unfavourable state even though it would be entropically more favourable.



~42% sequence identity found between both proteins. Symbols indicate the degree of similarity between the sequences: * (asterix) indicates a single fully conserved residue, : (colon) indicates a group with conserved strongly similar properties (>0.5 in Gonnet PAM 250 matrix), . (full stop) indicates a group with weakly conserved properties (= <0.5 Gonnet PAM 250 matrix).

Heterologous expression in *E. coli* revealed its involvement in assemblies with laminations, also observed in CdeC+ spore thin sections. CdeC mutant spores lacked polar appendages and possessed low levels of crystalline layers. CdeC mutant spores also possessed dense material associated with the spores. A proposed source of this material is the appendage, which is unable to assemble in this

mutant, but it still expressed. The double CdeC and CdeM mutant yielded spores with no appendage and no dense material associated with this spore. This further indicates that the material could correspond to mis-assembled CdeM although further research, such as mass spectrometry of the dense material, is required to confirm it.

6.4.3.4 CdeM

CdeM is associated with adherence (Antunes *et al.*, 2018; Janoir *et al.*, 2013; Pizarro-Guajardo *et al.*, 2020), germination and virulence (Antunes *et al.*, 2018). Based on immunofluorescence data it is believed to form the appendage (Antunes *et al.*, 2018). The findings presented here further confirm the link between CdeM and the appendage. $\Delta cdeM$ spores lacked a polar appendage, whereas CdeM overexpression in spores yielded polar appendages with increased length; these findings were apparent in both whole spore EM screens and thin sections.

Due to its possible involvement in the *appendage*, CdeM is unlikely the candidate for the composition of the ‘exosporium-like’ layer. This was confirmed in our findings as $\Delta cdeM$ spores possessed crystals similar to WT ‘exosporium-like’ layers. Immunogold labelling of CdeM also revealed no labelling on crystalline layers, only low levels in CdeC mutants and CdeM+ on non-crystalline layers. This could correspond to exposed CdeM due to CdeC absence, affecting its assembly, or due to excess CdeM.

6.4.4 Proposed model for *C. difficile* spore outer layers

Antunes *et al.* (2019) found that by mechanically disrupting *C. difficile* spores under reducing conditions, shells were released; these were proposed to contain the outermost layers of the spores (Antunes *et al.*, 2018). Immunoblot analysis of the shells revealed high levels of CdeM and multimeric forms of CdeC and CdeM- establishing that both proteins interact *in vivo* (Antunes *et al.*, 2018). Interestingly, these shells possessed low levels of CdeC suggesting that CdeC localization was more internal, likely to be more tightly cross-linked closer to the coat of the spore. Analysis of CdeM mutants further demonstrated that CdeC was localised in a more internal assembly, as CdeM

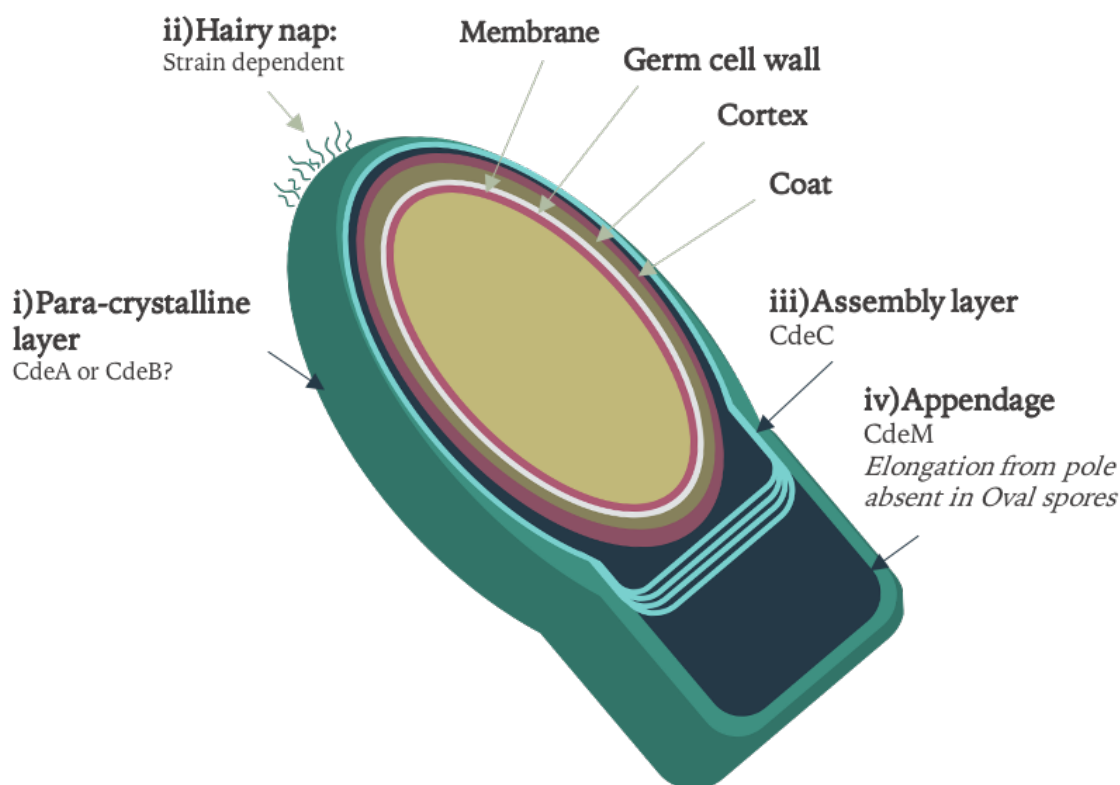


Figure 6.27 Model for the assembly of *C. difficile* spore layers and proposed composition of outer layers

Outermost layer is the i) para-crystalline layer proposed to cover the spore body and closely fitted to appendage layer. Unknown protein forms the para-crystalline layer—could be CdeA or CdeB? ii) Hairy nap covers the surface of some *C. difficile* spores, likely to be strain dependent. iii) Assembly layer: a non-crystalline layer forms laminations within spore, suggested for assembly of para-crystalline layer and appendage. CdeC is proposed to make up this layer. iv) Appendage: CdeM is believed to be a component of the appendage, which encapsulated the whole spore and elongates out of one pole. Spores with layers extended from the pole, likely correspond to spores with para-crystalline layer detached from the spore possibly due to handling of spores.

disruption, which is localised more externally, had no impact on CdeC levels and increased antibody access to CdeC (Antunes *et al.*, 2018).

Based on our findings and those found in Antunes *et al.* (2018), we propose a different role for CdeC as the component of an assembly layer also described in the literature as the sac layer (Henriques and Moran, 2007). This is a non-crystalline layer involved in the assembly of the para-crystalline layer and the appendage (Figure 6.27), consistent with the low levels of crystalline layers and lack of appendage observed in $\Delta cdeC$ spores, in addition to immunogold labelling of non-crystalline layers in WT spores. CdeC+ spores revealed an increase level in laminations observed close to the coat, suggesting that it forms an assembly localised closer to the coat, as suggested from the data of the shells (Antunes *et al.*, 2018). CdeC has also previously been identified associated with the outermost

layers of the spore (Barra-Carrasco *et al.*, 2013), thus it is likely to be important for both the assembly of layers closer to the coat and outermost layers including the para-crystalline layer.

Previous research found that the appendage is composed of CdeM (Antunes *et al.*, 2018; Pizarro-Guajardo *et al.*, 2020). The findings discussed here visually demonstrate the involvement of CdeM on the appendage, as $\Delta cdeM$ spores have no appendage and CdeM+ spores possess an extended pole. As depicted in Figure 6.27 (dark grey) we propose that the appendage material is present around the whole spore but extends furthest from one pole.

The *C. difficile* spore population possesses two morphotypes based on the length of the appendage (Chapter 1.3.3.7). Morphotype B possesses an extended appendage, composed of CdeM, a thick proteinaceous layer which reduces permeability to germinants, thus reducing germination rates and plays an important role in persistence *C. difficile* spores (Antunes *et al.*, 2018). Oval spores, morphotype A, have no appendage and thus can respond faster to germinants, important in establishing the infection (Antunes *et al.*, 2018). The mechanism of germination from morphotypes remains unclear, but AFM data identified a region of reduced rigidity along the axis of elongated spores (Antunes *et al.*, 2018). This could function as an aperture for the release of the spore from the thick proteinaceous encasing (Antunes *et al.*, 2018).

Thin sections of WT elongated spores have demonstrated that a para-crystalline layer is present on the surface of the spores, depicted in green (Figure 6.24). Further research is required to establish whether the crystalline layer identified in this work by negative stain EM is equivalent to that observed in thin sections.

Two possible remaining candidates for composing the para-crystalline layer are CdeA and CdeB. Based on size, both could fit into the 3D reconstruction either as a dimer or a monomer, respectively. CdeB is the least likely candidate due to the low percentage of cysteines. However, CdeB cannot be discarded solely based on this as para-crystalline layers in *C. difficile* may differ slightly from the known structures. An assembly with reduced cysteines could be more susceptible to degradation

which would explain the low crystal abundance. Nevertheless, CdeB+ spores demonstrated increased levels of laminations in thin sections suggesting a more likely involvement in the coat. This would mean that CdeA is the most likely candidate, especially as it has a higher cysteine abundance.

As has been previously discussed (Chapter 1.3.3.5.1), cysteine rich proteins from the exosporium/crust in *Bacillus*, and now confirmed in *Clostridia* (Chapter 4), form crystalline sheets upon heterologous expression in *E. coli* (Janganan *et al.*, 2020; Jiang *et al.*, 2015; Terry *et al.*, 2017). This appears to be a conserved feature across spore formers, however the lack of crystalline material in *E. coli* after heterologously expressing the Cde proteins, suggests *C. difficile* may have a different mechanism for the assembly of its outermost layers. Further work is required to get a better understanding of the ultrastructure of *C. difficile* spores and the composition of its layers.

Chapter 7 – Discussion

7.1 Introduction

Many Firmicutes, which include the classes Bacilli and Clostridia, are spore formers found in different environments around the world. Some spores are important for human and animal health and support the gut microbiota (Browne *et al.*, 2017; Schroeder and Bäckhed, 2016); others include significant pathogens that can cause life-threatening disease (Logan, 2011; Peck *et al.*, 2011; Rupnik *et al.*, 2009). This thesis has focused on two members of the Clostridia class - *Clostridium sporogenes* and *Clostridioides difficile*. *C. sporogenes* is an important model system for studying group I *C. botulinum* (Bradbury *et al.*, 2012; Carter and Peck, 2015; Peck *et al.*, 2011) – the causative agent of botulism and classified under schedule 5 in the UK Anti-terrorism, crime and security act 2001. *C. difficile* is the causative agent of CDI – one of the main nosocomial antibiotic associated diseases and a global health concern (Rupnik *et al.*, 2009).

These pathogens utilise their highly resilient spore state for the transmission and persistence of infection, which poses a major concern within the healthcare environment for disease treatment and prevention. The outermost spore layers are the first point of contact between the spores and their environments. For pathogens, such as *C. botulinum* and *C. difficile*, the outermost spore layers dictate their interaction with host cells including the immune system and gut epithelial cells (Antunes *et al.*, 2018; Calderón-Romero *et al.*, 2018; Panessa-Warren *et al.*, 2007; Paredes-Sabja and Sarker, 2012; Pizarro-Guajardo *et al.*, 2020; Stewart, 2015). The outermost layers present attractive targets for therapeutics against CDI and botulism, including potential epitopes for vaccine development.

The highly resistant nature of spores has also made them candidates of interest for drug delivery systems including vaccines, anti-cancer drugs and even as vectors for gene therapy (Cutting *et al.*, 2009; Farjadian *et al.*, 2018; Minton, 2003). Their properties would allow intact bypass of the gastric environment, thus providing a platform for oral drug delivery - a more accessible approach to administering drugs.

Due to their importance in food safety, pathogenesis and potential for drug development, understanding bacterial spore ultrastructure and their properties is of vital importance. Yet, there are major unknowns when it comes to our knowledge of spores, especially in Clostridia.

The metabolically dormant spore state can be maintained for years and yet the spores are able to respond within minutes to hours of encountering environmental cues for germination. The mechanisms by which spores change from a dormant state to germination within a short time remains unclear, to understand this, we require insight into the spore structure at a molecular level and the work in this thesis attempts to address some of this. Additionally, our understanding of remodelling the spore state to form the vegetative cell is unknown. This thesis does not address this explicitly but provides the groundwork by studying 3D structures in the spore state for further work to be conducted.

The involvement of multiple layers in conferring resilient properties to the spores has previously been established. Yet, an understanding of the structure of these layers, their spatial assembly, and the correlation to function remains unclear. Our understanding of spore ultrastructure across Bacilli and Clostridia is limited as it is based mostly on chemically fixed thin spore sections, including those discussed for *C. sporogenes* (chapter 5) and *C. difficile* spores (chapter 6), which provides insight into the global architecture of the different layers (Chapter 1 – Figure 1.13) but little definition of the substructures present. Additionally, thin-sections (~70-90nm) only provides a small sampling of the 3D volume, thus features become less discernible and may be missed which limit our 3D understanding of spore ultrastructure.

The exosporium has been extensively studied across the *B. cereus* sensu lato group (chapter 1.3.3.5.1) (Ball *et al.*, 2008; Kailas *et al.*, 2011; Terry *et al.*, 2017), however our understanding of the exosporium in Clostridia is very limited. Additionally, our current structural understanding of the exosporium basal layer is capped at low resolutions (Kailas *et al.*, 2011; Terry *et al.*, 2017), thus further research is required to gain high-resolution reconstructions.

The research presented here is an attempt to start to address this gap in understanding and develop the structural tools required for interrogation of the spore assembly and germination processes- in addition to gaining comparative insight of two relatively distantly related Clostridia, *C. difficile* and *C. sporogenes*. The focus in this thesis was on employing different EM tools and technologies, including cryo-EC and ET, to gain novel insights into spore ultrastructure and outer spore layers. Due to the similarity with spore assemblies in Bacilli, these findings also provide a greater understanding of assemblies across spore formers.

7.2 Model for *C. sporogenes* ultrastructure

A model for *C. sporogenes* spore ultrastructure, including tentative assignment of the outermost layers is presented in Figure 7.1. This model is based on the research presented in this thesis including mutant screening (chapter 3), EC (chapter 3 and 4), analysis of optimised spore sections and electron tomography (chapter 5).

The basal layer of *C. sporogenes* is composed of CsxA, confirmed by screening $\Delta csxA$ (chapter 3) and heterologous expression of CsxA in *E. coli* (chapter 4). The type I crystal form observed in $\Delta csxB$ and $\Delta csxC$ strains of *C. sporogenes* corresponds to WT basal layer. In chapter 4, a Cryo-EM 3D reconstruction of the basal layer is presented at 9Å resolution laterally and 20Å axially. Thin sections prepared by HPF + freeze-substitution revealed periodicity in the outermost layers (Chapter 5 – Figure 5.21) with an average of ~79Å - likely corresponding to the distance between crystal planes.

HPF + freeze-substitution sections provided superior sample preservation and contrast of features to obtain remarkable detail of spore assemblies. In combination with ET, a network of parasporal layers were revealed in the interspace of *C. sporogenes* spores. The layers are distinct from the exosporium basal layer, which is more densely stained (Chapter 5 – Figure 5.6), the periodicity within these layers is also shorter in length at ~48Å. This periodicity suggests the crystalline layer may correspond to type III or type IV crystal observed in $\Delta csxB$ and $\Delta csxC$ spores, if the periodicity corresponds to the unit cell length. Due to the mode of sectioning the periodicity is more likely to reveal the distance between crystal planes thus could correspond to type II crystals observed in $\Delta csxA$ spores. Type II

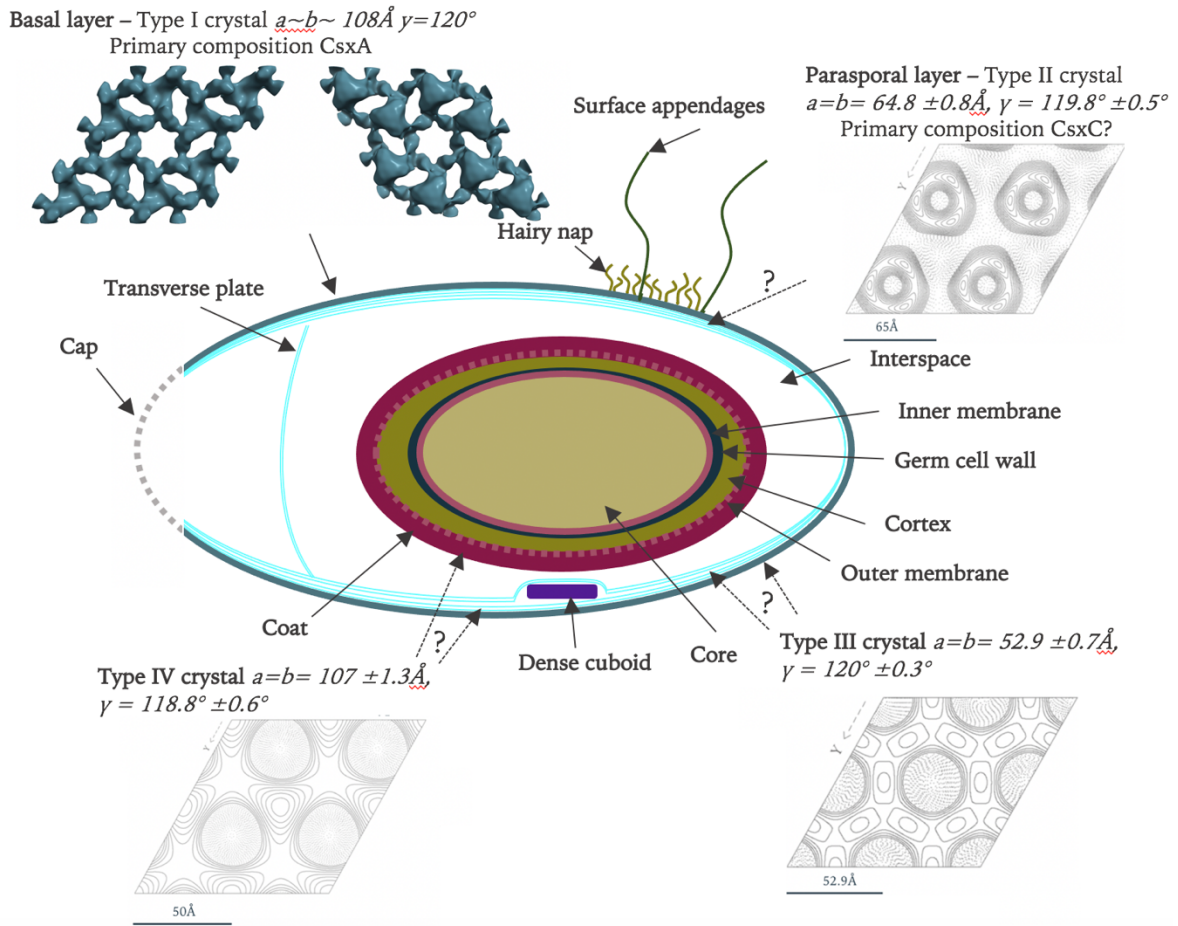


Figure 7.1 Schematic diagram of *C. sporogenes* spore crystal types and their possible location

Spore assemblies labelled include features described in this research: parasporal layers, transverse plate, dense cuboid and putative assignment of a cap at the elongated exosporium pole. Four crystal types observed across mutants: Type I: corresponds to the basal layer of the exosporium and primarily composed of CsxA. Type II: observed in $\Delta csxA$, tentatively assigned as the parasporal layer. Type III: observed in $\Delta csxB$ unclear could correspond to an outer layer or the parasporal layer. Type IV: from $\Delta csxC$ a possible parasporal layer or coat assembly.

crystals are also a likely candidate for parasporal layers as they span the length of the spores and compose of multiple sheets that could correspond to the laminations observed in thin sections. Further work is required to identify the composition of this layer, including sectioning knock out mutants to see the effect of on the layers.

Another two crystal types were observed in $\Delta csxB$ and $\Delta csxC$ spores, type III and type IV. The position of type III, observed in $\Delta csxB$ spores, remains unclear and further work is required to establish its involvement in spore assemblies. However, the presence of hairy nap on these crystals could suggest a crystal type present on the surface (Figure 7.1) which has not yet been accounted for in WT spores or inaccessible for EC studies. Type IV crystal was observed in $\Delta csxC$ spores, does not

reveal a hairy nap thus likely corresponds to the parasporal layer or an outer spore coat layer (Figure 7.1). The periodicity of the parasporal layers may correspond to the unit cell length, thus type III and type IV, with a unit cell length of $a=b= 52.9 \pm 0.7\text{\AA}$ and $a=b= 49 \pm 1.2\text{\AA}$ respectively, cannot be discounted. Alternatively, the parasporal layers may be formed of multiple crystal types.

7.3 Conserved structure of outer spore layers

The outermost layer of *C. sporogenes* spores is the exosporium, a baggy para-crystalline layer with an interspace region comparable to the ‘classical exosporium’ morphology. A similar morphology is present across other Clostridia including *C. sordellii* (Rabi *et al.*, 2018), *C. acetobutylicum* (NCIB 8052), *C. tyrobutyricum* (NCDO 1756), *C. puniceum* (BL70/20), and *C. pasteurianum* (NCDO 1845) (Janganan *et al.*, 2020) and *Bacillus* such as *B. thuringiensis*, *B. cereus* and *B. anthracis* (Ball *et al.*, 2008; Kailas *et al.*, 2011; Terry *et al.*, 2017). EC studies have revealed a conserved basal layer with hexagonal para-crystalline assemblies across the *Bacillus* group (chapter 1.3.3.5.1) (Ball *et al.*, 2008; Jiang *et al.*, 2015; Kailas *et al.*, 2011; Terry *et al.*, 2017), Clostridia (Janganan *et al.*, 2020) and now confirmed in *C. sporogenes*.

TEM and thin sections of *C. difficile* spores did not reveal a ‘classical exosporium’, a baggy loosely fitted exosporium layer with an interspace region, but a layer assembled close to the spore body. Thin sections revealed patches of honeycomb lattice along the edges of the spores, which suggests that *C. difficile* possesses an outermost layer that resembles the crust in *B. subtilis*. A crystalline assembly was identified on whole spores by TEM, a 3D model of this layer revealed a hexagonal lattice interconnected by threefold density (Figure 2B), which we proposed to correspond to the lattice observed in thin sections. Further efforts should be done to prepare *C. difficile* spore sections by HPF + freeze-substitution, which better preserves biological material, to get a more representative measurement of the lattice unit cell on the spore – this would establish a link between the EC model and the spore assemblies. The EC model generated (Figure 7.2B) was at low resolution due to the low number of crystals, sampling in z and was limited by negative stain. To improve the reconstruction

more tilted EC data need to be collected, including from frozen hydrated samples to obtain high resolution structural information.

7.3.1 Basal layer

Composition of the basal layer

The exosporium basal layer in the *Bacillus cereus* group is composed of cysteine rich proteins, ExsY and CotY (Johnson *et al.*, 2006; Terry *et al.*, 2017). The primary composition of exosporium basal layer in *C. sporogenes* has now been established as CsxA, another cysteine rich protein. Despite none of the proteins composing these layers being homologous, they all share the ability to self-assemble into crystals upon heterologous expression in *E. coli* with the same native basal layer structure (Janganan *et al.*, 2020; 2016; Jiang *et al.*, 2015; Terry *et al.*, 2017). The ability of CsxA to self-assemble into para-crystalline layers upon heterologous expression in *E. coli* meant it was suitable for cryo-EC studies, and in Chapter 4 the highest resolution 3D reconstruction of an exosporium basal layer to date is presented.

The composition of the crystalline layer observed in *C. difficile* remains unclear. As discussed in Chapters 1.3.3.5.3 and 6, four cysteine-rich proteins were identified CdeA, CdeB, CdeC, CdeM. In the literature the outermost layer visible on *C. difficile* spores, which is assembled without an interspace, is labelled the exosporium (Permpoonpattana *et al.*, 2011) and CdeC has previously been proposed to assemble this layer (Barra-Carrasco *et al.*, 2013; Calderón-Romero *et al.*, 2018). Our findings suggested that the crystal observed by TEM was still able to assemble in *ΔcdeC* spores but at low abundance. Additionally, unlike cysteine-rich proteins from other bacteria, upon heterologous expression CdeC appears unable to self-assemble into crystalline sheets. This suggests that the outermost layer, which is likely to be para-crystalline, differs from the exosporium in other spore formers. Either the *C. difficile* para-crystalline layer is not formed of cysteine-rich proteins or the cysteine-rich proteins require additional machinery for assembly thus are not able to self-assemble upon expression in *E. coli*.

Structure of the basal layer

A common honeycomb lattice assembly is observed across the basal layer of Bacilli and Clostridia outermost layers of exosporium or crust. This suggests that the hexameric nature of these layers is of high importance.

The different molecular weight of the protein compositions is reflected in the varying unit cell sizes for the basal layer crystals. For *B. cereus* ExsY is 19.4kDa and forms a crystal (Figure 7.2C) with a unit cell length of $a = b = 80\text{-}85\text{\AA}$ (Terry *et al.*, 2017). Whereas *C. sporogenes* basal layer is primarily formed of a 33.4kDa protein, CsxA, which forms a crystal with a unit cell length of $a=b=105\pm 3\text{\AA}$.

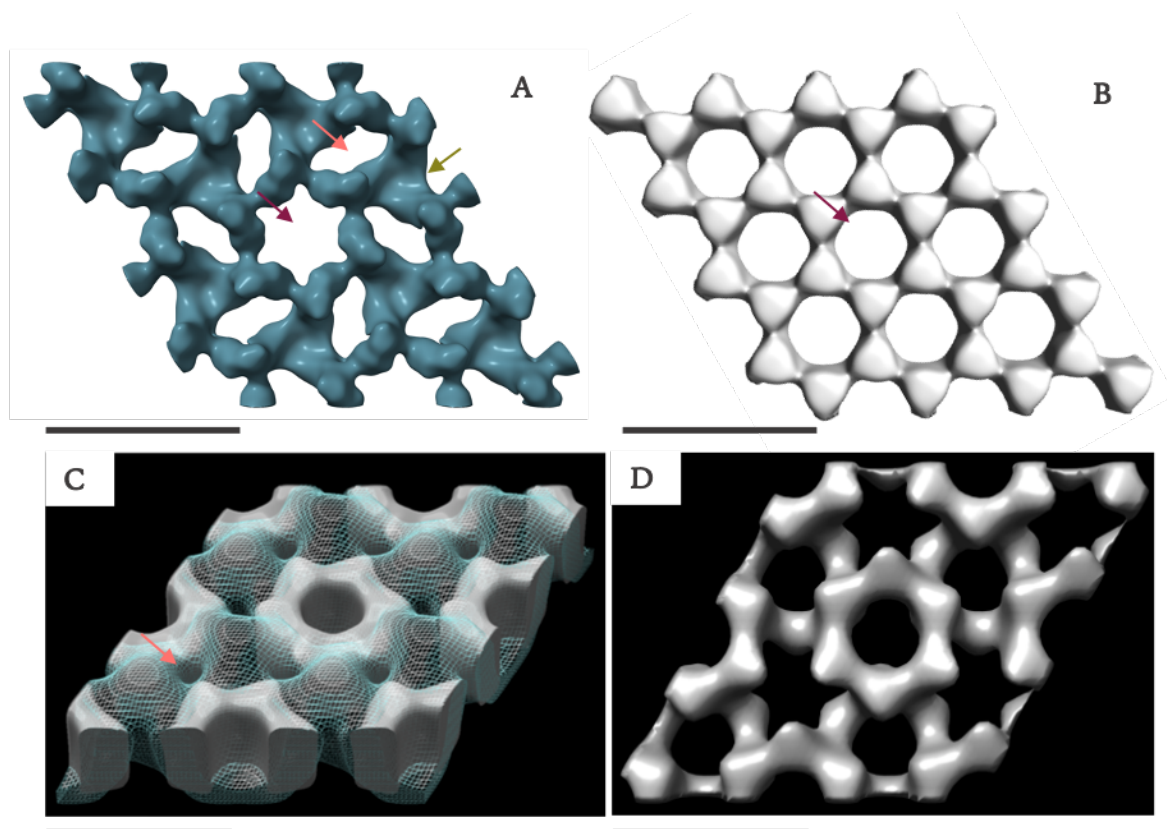


Figure 7.2 Structure of outermost layers of *Bacillus* and *Clostridia*

A) Cryo CsxA crystal reconstruction reveal basal layer of *C. sporogenes*/ *C. botulinum* exosporium B) *C. difficile* outermost layer C) Negative stain reconstruction of ExsY (grey) and native exosporium basal layer (blue mesh) of *B. cereus** D) Negative stain reconstruction of CotY revealing *B. subtilis* crust**. Red arrow highlight the central pore. Pink arrow indicated the peripheral pores. Green arrow corresponds to 'canyon' structures in 3-fold linkers of CsxA crystal.

Figure from:

*Terry, *et al.*, (2017) **Jiang, *et al.*, (2015).

The *C. difficile* crystal possesses a unit cell length of $a=b = 97\text{\AA}$, although its composition remains unknown.

Reconstructions of outermost layers in *C. sporogenes* and *C. difficile* reveal a cog-like ring with central pores (Figure 7.2 – red arrows) with a potentially plugged assembly that forms cup-like structures. Additionally, peripheral pores were observed in *C. sporogenes* (Figure 7.2 – pink arrows) described as ‘craters’ in the reconstruction which may generate an additional cup-like structure within the layer. No peripheral pores were observed in *C. difficile* (Figure 7.2B) although this may be due to limited resolution and not a true representation of the assembly. In *C. sporogenes* the central pore is $56\text{\AA}$ at its widest and $40\text{\AA}$ at its narrowest point, if unplugged the diameters for diffusion through the pores are likely to be narrower (Dechadilok and Deen, 2006). Thus, if the exosporium is sealed around the spore, this layer would still confer molecular sieve properties, preventing insults such as enzymes and antibodies from reaching the spore core. The cryo 3D reconstruction of CsxA, combined with AFM of hydrated CsxA crystals, demonstrate a conformational change with response to changes in hydration (Chapter 4 – Figure 4.22). The resulting crystal possesses reduced central and peripheral pore sizes in its hydrated form (Chapter 4 – Figure 4.13). The host gut is a hydrated environment, where many molecules including biological insults or germinants are present. Thus, the conformational change due to hydration may reveal a mechanism to prevent the entry of larger molecules, including enzymes or antibodies present in the host, from reaching the spore body yet still allowing small molecules including germinants to pass through. This conformational change might also facilitate the release of the exosporium ‘cap’ for vegetative cell outgrowth.

The *C. difficile* reconstruction suggests a pore size of $\sim 75\text{\AA}$, which is much more permeable than other exosporium layers. Even though the diffusion diameter through the pore is likely to be narrower (Dechadilok and Deen, 2006), the size may be wide enough to permit the passage of larger molecules. However, this measurement is based on a model with limited data and negative staining patterns on the sample can provide misleading information of the protein density. Thus further EC data is required to gain more representative insight, including AFM visualisation to consolidate the features observed by EC. Further research should also include cryo EC to see if a conformational change to *C.*

difficile crystal is observed in a hydrated state including the narrowing of the central pore as was observed in CsxA.

As presented in Chapter 1.3.3.5.4, additional proteins are assembled to the main exosporium basal layer crystal to plug the central ring and form the ‘cup’. The enclosed central ring may be an evolutionary adaptation to prevent the entry of insults via the pore. The ‘cup’ like structure may also correspond to a possible matrix for sequestering proteins or enzymes associated with the exosporium, such as arginases or alanine racemase (Kailas *et al.*, 2011; Terry *et al.*, 2017). In *C. sporogenes* ‘cup’ like features could correspond to the crater on the outward facing side (Figure 7.2 – green arrow) but also the central ring if it is confirmed as ‘plugged’ which might provide an analogous function, in sequestering molecules, to the ‘cups’ in the *B. cereus*

7.3.2 Hairy nap and surface appendages

In the *B. cereus* group, collagen-like proteins, including BclA and BclB, form the hairy nap (Sylvestre *et al.*, 2002). The threefold linkers are outwards facing in *B. cereus* group, they are formed of other proteins including ExsFA/ExsFB proteins required for the assembly of BclA (Kailas *et al.*, 2011; Terry *et al.*, 2017).

In this project, *C. sporogenes* ATCC15579 strain was screened by TEM and revealed a hairy nap associated with the exosporium which was previously documented on *C. sporogenes* NCIMB 701792 strain (Janganan *et al.*, 2016). Two collagen-like proteins were identified in *C. sporogenes* (BclA and BclB) (Janganan *et al.*, 2016) which are likely candidates for forming the hairy nap although further work, including screening BclA and BclB knockouts, is required to confirm this. The assembly of the hairy nap in *C. sporogenes* will differ from *B. cereus* group as the CsxA appears able to bind BclA directly (Janganan *et al.*, 2016), without the need of additional proteins such as ExsFA/ExsFB in *B. cereus* group (Kailas *et al.*, 2011; Terry *et al.*, 2017). As the threefold linkers are likely inwards facing, the assembly point of the hairy nap will also differ from *Bacillus*. The exact point of assembly remains unclear and due to the high flexibility of these proteins it is not something that can be resolved by EC.

In *C. difficile* the hairy nap is strain dependent, R20291 strains possess a hairy nap but 630 does not (Díaz-González *et al.*, 2015), which was confirmed in this work as no hairy nap was observed in either thin sections or whole spore TEM screening of the 630 strain. Three genes have been identified in *C. difficile* with collagen-like repeats - BclA1, BclA2 and BclA3, which have no shared homology to *Bacillus* apart from their collagen-like repeats (Díaz-González *et al.*, 2015). Mass spectrometry analysis of *C. difficile* 630 strain spores' outer layers identified BclA1 but not BclA2 or BclA3 (Díaz-González *et al.*, 2015). As no hairy nap is observed on the 630 strain, it is unclear what assembly BclA1 forms on the 630 strain spores (Díaz-González *et al.*, 2015). With our current understanding, this is also no reason to suggest that the hairy nap has any association with the crystal 3D reconstruction revealed.

Larger surface appendages and fibrils are also observed on spores from both Bacilli and Clostridia (Driks and Eichenberger, 2016; Panessa-Warren *et al.*, 2007; Pradhan *et al.*, 2020). These surface appendages have been observed on *C. sporogenes*, including NCIMB 701792 strain (Chapter 1 - Figure 7.3) (Janganan *et al.*, 2016) and ATCC3584 (Panessa-Warren *et al.*, 2007), and *C. difficile* ATCC43594 (Panessa-Warren *et al.*, 2007). However, they were not observed in *C. sporogenes* ATCC15579 and *C. difficile* 630 strains which were analysed in the current work.

Surface appendages are involved in attachment to different surfaces including agar, glass and human cells such as the Caco-2 colon cell line for *C. difficile* and fibroblast cells for *C. sporogenes* (Panessa-Warren *et al.*, 2007). These surface appendages are more pronounced on spores during the early stages of germination, maintaining adherence of spores to human cells thus pivotal for colonisation and infection (Panessa-Warren *et al.*, 2007). This suggests that the appendages may have a role in the spore life cycle and therefore, may not be apparent on dormant spores, such as those imaged in this study. However, both *C. sporogenes* ATCC15579, in the current analysis, and NCIMB 701792 (Janganan *et al.*, 2016) were imaged in similar conditions, with no germinants or nutrient rich media previously shown to trigger surface appendages to become more pronounced (Panessa-Warren *et al.*, 2007), suggesting that no surface appendages are associated with the ATCC15579 strain. This is consistent with thin sections and SEM of germinating *C. sporogenes* ATCC15579 strain, which did

not appear to have these surface appendages (Brunt *et al.*, 2015). This suggests that the surface appendages may be strain specific, and possibly more pronounced on virulent strains, such as *C. difficile* ATCC43594, as they aid colonisation and infection (Panessa-Warren *et al.*, 2007) – further work is required to understand this.

7.3.3 Cap assembly

In Chapter 1.3.3.5.4, the different sections of the *B. cereus* exosporium, consisting of a ‘cap’ region and the rest of the exosporium, were discussed. The cap is the location of initial exosporium assembly at the forespore pole and is likely composed of ExsY and CotY with only BclA forming the hairy nap (Johnson *et al.*, 2006; Thompson *et al.*, 2012). The rest of the exosporium is likely only composed of ExsY with both BclA and BclB forming the hairy nap (Johnson *et al.*, 2006; Thompson *et al.*, 2012). The different regions were visible upon screening *exsY* and *cotY*; the exosporium was only localized to the pole in *exsY* and encapsulated the whole spore in *cotY* although close to the spore body (Johnson *et al.*, 2006).

As discussed in section 2, thin sections and ET of *C. sporogenes* revealed an ill-defined pole which could correspond to a ‘cap’ like region in the exosporium. This region may possess a different composition, as is observed in the *B. cereus* group, which is more fragile and forms the aperture previously observed by TEM and SEM (Brunt *et al.*, 2015; Warren *et al.*, 1994). Based on the structural similarity and composition of cysteine rich proteins in the *B. cereus* group, if there was an analogous ‘cap-like’ assembly in *C. sporogenes* it would likely be assembled from one of the cysteine-rich proteins (CsxA, CsxB or CsxC). In $\Delta csxA$ spores the exosporium basal layer was absent, instead other crystal types were observed across the whole spore that may correspond to the parasporal layer. The lack of crystal type similar to exosporium in $\Delta csxA$ spores suggests that neither CsxB nor CsxC are able to assemble into type I crystal in a similar way to ExsY and CotY in *B. cereus*. Thus, if there is a cap like structure in the *C. sporogenes* exosporium, what is it composed of and how is it structured? To answer this further work should be conducted including sub tomogram averaging to determine whether there is structure to this region. Additionally, immunogold labelling

of the different cysteine rich proteins could be done to see if they are associated with any particular part of the exosporium.

In *C. difficile* the ‘exosporium-like’ layer is assembled close to the main spore body, any changes in crystal structure on the mutants were not readily visible. Therefore, no ‘cap’ like region could be distinguished in *C. difficile*.

7.3.4 Resilience of exosporium

The highly resilient properties of the exosporium were discussed in chapter 1.3.3.5.5; heterologously expressed ExsY and CotY crystals from *Bacillus* demonstrated high resilient properties, requiring a combination of 8M Urea, 2M DTT and >90°C temperature to disassemble the crystals (Jiang *et al.*, 2015; Terry *et al.*, 2017), reflecting their native role in conferring spore resistance. These properties have also been revealed for exosporium from *C. botulinum* (Takumi *et al.*, 1979). The current work has established that the resilience is also observed in *C. sporogenes* (Chapter 4). A combination of both 95°C heat and 2M DTT was required to disintegrate the crystals. The exosporium basal layer crystals appear to have universally conserved robust properties across distantly related spore formers and likely conferring resilience to the spores.

This property has not yet been confirmed for any of the *C. difficile* outermost layers. Isolation of *C. difficile* para-crystalline layers and treatment with harsh conditions would establish whether they possess robust properties. However, the low abundance of crystals observed in spore samples may correspond to the increased fragility of these layers; further research is required to establish this.

7.3.5 Exosporium assembly

In section 1.3.3.5.4 a proposed mechanism of exosporium assembly in *Bacillus* spores was discussed, where monomers of cysteine-rich proteins self-assemble into highly symmetrical packing structures (Terry *et al.*, 2017). Both the cytosol of the native mother cell and *E. coli* are generally considered reducing environments where intracellular disulphide bond crosslinking is rare. The close packing and

high symmetry effectively raise the local concentration of cysteine residues, which favours disulphide bond formation (Chau and Nelson, 1992).

Based on the cryo 3D reconstruction of CsxA, the likely positions for disulphide bond crosslinking between neighbouring rings are the threefold linkers, as distances between subunits of the central ring are too large. Treatment of CsxA crystals with 2M DTT revealed that central ring structure remained whereas the integrity of the crystal was compromised, likely corresponding to the breakdown of the disulphide bonds at the threefold linkers.

In *Bacillus* additional proteins are assembled including ExsFA or ExsFB and the hairy nap (Terry *et al.*, 2017). The density corresponding to these additional proteins are observed in the 3D reconstruction of native exosporium. However, in *C. sporogenes* no additional density is observed in the WT exosporium compared to the CsxA crystal structure, suggesting that it is unlikely that other major proteins form the basal layer. If additional proteins are present on the WT exosporium, then they are likely flexible and thus averaged out.

The composition of the ‘exosporium-like’ layer in *C. difficile* remains unclear, although it is likely to be composed of the cysteine-rich proteins identified on the spore surface. As discussed in chapter 6, none of the cysteine-rich proteins appear to self-assemble into crystalline sheets upon heterologous expression in *E. coli*. CdeC is believed to be important for the assembly of a layer that is not crystalline but aids the assembly of the ‘exosporium-like’ layer, yet no crystals were observed, even when combinations of cysteine rich proteins with CdeC were expressed in *E. coli*. This suggests that the mechanism of ‘exosporium-like’ assembly in *C. difficile*, differs from other studied spore formers, and may require additional machinery for the formation of the exosporium which are not present in *E. coli*. This may include post-translational processes such as those involved in the *C. difficile* S-Layer crystal protein SlpA, which is post-translationally cleaved prior to assembly (Fagan and Fairweather, 2014; Fagan *et al.*, 2009). There is currently no published mass spectrometry data to our knowledge that tests this. Further work is required to investigate if any post-translational processes are required for the assembly of the ‘exosporium-like’ layer in *C. difficile*.

7.4 Parasporal layers

Bacteria with a ‘classical exosporium’ possess an interspace between the exosporium and the coat layers. As discussed in section 1.3.3.6, layers have been described in the interspace region across spores of different species including *B. megaterium* (Beaman *et al.*, 1972) and *B. thuringiensis* (Ebersold *et al.*, 1981; Wehrli *et al.*, 1980) where they assemble into laminations (Chapter 5 – Figure 5.16). Freeze-fracture studies revealed a crystalline assembly (Beaman *et al.*, 1972; Ebersold *et al.*, 1981; Wehrli *et al.*, 1980), which was characterised by EC for *B. thuringiensis* and termed the ‘parasporal’ layer (Ball *et al.*, 2008; Kailas *et al.*, 2011).

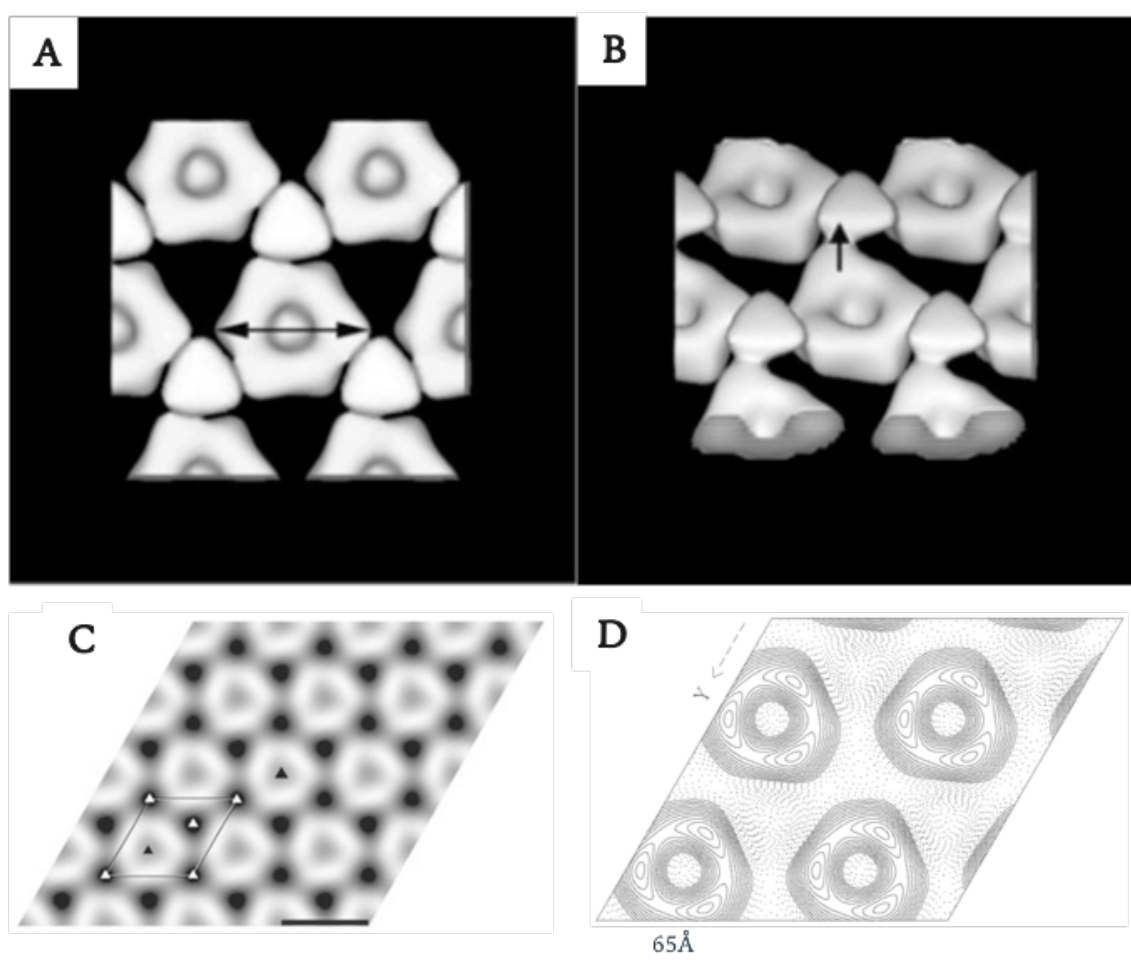


Figure 7.3 Parasporal crystals from *B. thuringiensis* and Type II crystal from *C. sporogenes*

A-C) Type I crystal from *B. thuringiensis* suggested to correspond to parasporal layer. Unit cell length ~65Å with p3 symmetry* A, B) 3D reconstruction C) 2D projection map of type I crystal from *B. thuringiensis* * D) Type II crystal from *C. sporogenes*. Scale bar: unit cell length ~65Å with p3 symmetry

From Ball *et al.*,(2008).

The research presented in chapter 5 revealed that similar layers are also present in *C. sporogenes*, however they tend to be more extensive, travelling across the near full length of the spore, enclosing the main spore body, and assembling into laminations of 2-7 layers. ET revealed that a never before described dense cuboid was associated with these layers. The sections of *C. sporogenes* by HPF + freeze-substitution revealed periodicity measured at $\sim 48\text{\AA}$, indicating that the parasporal layers are composed on para-crystalline assemblies. As discussed in 2.3, the parasporal layers may correspond to type II crystal (unit cell length $a=b=64.8\pm 0.8\text{\AA}$), although type III (unit cell length $a=b=52.9\pm 0.7\text{\AA}$) and IV (unit cell length $a=b=49\pm 1.2\text{\AA}$) cannot be discounted, possibly even all three crystal types. Sub tomogram averaging of the layers in the tomograms would allow the crystal parameters to be determined (Bharat *et al.*, 2017; Kugelgen *et al.*, 2020).

The structure of type II crystal is remarkably similar to the parasporal crystal type I from *B. thuringiensis* (Figure 7.3) (Ball *et al.*, 2008; Kailas *et al.*, 2011). Both crystal type II from *C. sporogenes* and parasporal crystal from *B. thuringiensis* possess a central density with phases consistent with $p3$ symmetry and similar unit cell lengths at $a=b\sim 65\text{\AA}$. A 3D reconstruction of type II crystal is required for a more definitive comparison between the crystal types. Based on the similarities observed in the structure of exosporium basal layers, it would be plausible that other assemblies, including the parasporal layers, are also alike in structure.

Our understanding of the parasporal layers and their function remains unknown. As the parasporal layers run the whole length of the exosporium, they may be involved in maintaining the interspace region by providing mechanical restraint. The parasporal layer could also present an additional molecular sieve layer that further filters out any larger molecules from reaching the spore core. This is particularly attractive in *C. sporogenes* as the parasporal layer envelopes the main spore body and possesses a transverse plate at the spore pole where an aperture is observed, which would provide an opening for insults to reach the spore. Additionally, the parasporal layers may retain proteins important for germination or spore resilience, similar to the coat and exosporium (reviewed in (Henriques and Moran, 2007; Stewart, 2015)).

Laminations in the interspace region have been observed across different spore formers in Bacilli (Ebersold *et al.*, 1981; Wehrli *et al.*, 1980) and now described in Clostridia. We propose that these assemblies are distinct from the exosporium and should be termed the parasporal layers. Chemically fixed *C. difficile* spores did not reveal distinguishable assemblies under the ‘exosporium-like’ layer, thus it remains unclear as to whether *C. difficile* spores possess parasporal layers. HPF and freeze-substitution sample preparation approaches should be considered for improved definition to gain insight into this.

7.5 Adaptation of spore assemblies to changes in hydration

Bacterial spores have apparently conflicting demands, with the need for dormancy and resilience versus the need to respond rapidly to environmental triggers. Environmental states that spores encounter include desiccation and hydration, two polar opposite conditions that require adaptability of the spores for their survival.

For pathogens such as *C. botulinum* and *C. difficile* the host gut is the target environment for colonisation. The host gut presents a hydrated environment with biological insults and germinants, in addition to attachment sites on the host cell surfaces (Panessa-Warren *et al.*, 2007; Swick *et al.*, 2016). Thus, for colonisation of the host, spores must be able to adapt to this environment to establish an infection.

Structural features of spore assemblies, including the cortex and the coat, have previously been studied and associated with the spores’ ability to adapt to environmental conditions including hydration. The cortex possesses a crosslinking gradient of the PG, with progressively more highly crosslinked PG towards the outermost layers of the spore, that prevents the turgor pressure from the core - an important feature for maintaining the dehydrated state of the core when in a hydrated environment (Meador-Parton and Popham, 2000).

Coat layers in *B. subtilis* appear to have an undulating structure in section (Sahin *et al.*, 2012), which confers elasticity providing rapid response of spore to changes in hydration (Sahin *et al.*, 2012).

Similar undulations were also observed in the inner coat of *C. sporogenes* studied by ET (Chapter 5 – Figure 5.14), although the involvement of the undulations in hydration response need to be confirmed by using sample preparation techniques including CEMOVIS.

As discussed in chapter 4, the exosporium basal layer from *C. sporogenes* revealed a conformational change upon imaging in hydrated and dehydrated states, which represent the native structural state of the exosporium in different environments such as the host gut and desiccated surroundings.

The 3D cryo CsxA reconstruction provides structural insight into how the outermost layer changes in response to hydration, likely a significant mechanism for colonisation and establishing infection. In the hydrated state, the basal layer appears to have a central pore that is $\sim 67\text{\AA}$ at its widest and $60\text{\AA}$ at its narrowest. This pore size may be due to reduced necessity of barrier to environmental insults although the actual diffusion diameter of these pores remains unclear. Additionally, the ‘craters’ at the threefold linkers (Figure 7.2 – green arrow) are smaller in the dehydrated state; in section 3.2 we speculate that the ‘craters’ correspond to a matrix for sequestering proteins and possibly ‘trapping’ environmental factors such as germinants by providing increased surface area for adsorption. For the dehydrated state, these ‘craters’ may prevent the adsorption of germinants that would trigger germination in unfavourable environments.

The cryo-EC and AFM in water (Chapter 4 – Figure 4.22) represents the hydrated state of the basal layer, with increased swelling of the central ring causing a ‘crown-like’ or ‘petalling effect’ on the outward facing layer. It has previously been documented that hydration increases adherence of spores to surfaces (Panessa-Warren *et al.*, 1997; 2007; Warren *et al.*, 1994). SEM of *C. sporogenes* spores revealed increased hairy nap and surface appendage decoration upon hydration (Panessa-Warren *et al.*, 1997; 2007; Warren *et al.*, 1994). The conformational change of the basal layer may alter the surface properties and affect the hairy nap or surface appendages to increase adherence in favourable conditions including the host gut - important for colonisation (Panessa-Warren *et al.*, 1997; 2007; Warren *et al.*, 1994). As the hairy nap is averaged out in EC, other techniques need to be employed to

visualize this assembly such as sub tomogram averaging, which would provide insight into changes of surface decorations in response to conformational changes of the basal layer.

The rotational twist of subunits in the central ring of the basal layer affects the other two domains in the subunit including the threefold linkers and the domain connecting to the central ring. This effect causes a narrowing of the peripheral pores which could increase the selectivity of molecules able to pass through the basal layer in the hydrated state. The effect on the central pore remains unclear, as discussed in Chapter 4 further work is required to confirm whether the pore is in fact plugged in the hydrated state. Either way, the central pore does appear to reduce in size in response to hydration. Swelling of threefold linkers on the inner facing side effects the ‘craters’ on the outward facing side, resulting in larger ‘cups’(Figure 7.2 – green arrow) which may have an involvement in adsorption.

The basal layer across Bacilli and Clostridia share structural similarities in their honeycomb-like lattices, formed of cysteine-rich proteins that bear no homology. Based on the structural similarities, it is possible that the conformational change in response to hydration is conserved in the outermost layers of *Bacillus* and possible *C. difficile*, although 3D structures by cryo-EC would be required to confirm this.

7.6 Further work

7.6.1 *C. sporogenes*

7.6.1.1 Improve resolution of CsxA reconstruction

Cryo EC of CsxA crystals was used to generate the highest resolution 3D reconstruction of the exosporium basal layer from any species that we have to date. Yet, the resolution levels are still too low to identify secondary protein structure or model the amino acid sequence. Therefore, efforts should focus on pushing the resolution limits of this reconstruction by improving the sample in terms of crystallinity and flatness on the grids, as described in chapter 4.3.1. Another potential is to investigate new SPA techniques as was conducted for the Mlok1 channel previously (Righetto *et al.*, 2019), however this approach has not been widely tested and the CsxA sample, as a double layered crystal, would not currently be suitable.

A higher resolution structure would allow for the definitive assignment of the subunits in the crystal, their domains and the deduction of any flexible regions that are not visible by EC. A detailed structural understanding of the conformational change and the molecular mechanisms involved in response to hydration could also be attained from a reconstruction at higher resolution.

7.6.1.2 Identify role of CsxB and CsxC

Three cysteine rich proteins were identified in *C. sporogenes* exosporium (Janganan *et al.*, 2016); in collaboration with J. Brunt I have screened mutants to identify their involvement in spore assemblies. We have established the role of CsxA in the basal layer of the exosporium, however the roles of CsxB and CsxC remain unclear. As mutant screening in this research used whole spore TEM, the effect of such mutants on assemblies including parasporal layers or the coat remain unclear. Based on the attainable detail obtained for WT *C. sporogenes* spores by HPF combined with freeze-substitution these sample preparation approaches should be used on the Csx mutants. This will provide insight into assemblies not accessible by whole spore TEM. We hypothesize that if the Csx proteins are involved in the parasporal layers, the laminations will be absent from mutant spores.

Based on their cysteine-rich content, they are likely involved in crystal assembly thus further work should include heterologous expression in *E. coli* to see if they are capable of self-assembly and whether they are similar to the crystal types observed in $\Delta csxA$, $\Delta csxB$ and $\Delta csxC$ spores.

Additionally, immunogold labelling anti-CsxA, -CsxB and -CsxC on both WT and mutant spores could be employed to identify the composition of the different crystal types across the spore.

The possibility that multiple proteins are involved in assemblies such as the parasporal layers was discussed in Chapter 5. Thus further mutant analysis, involving double and triple mutants including $\Delta csxA\Delta csxB$, $\Delta csxB\Delta csxC$, $\Delta csxA\Delta csxC$ and $\Delta csxA\Delta csxB\Delta csxC$ would provide insight into what proteins make up these layers. A combination of $\Delta csxA\Delta csxC$ and immunogold labelling of anti-CsxC on $\Delta csxA$ spores would be particularly interesting for confirming a possible link between type II crystal and CsxC.

Further work should also investigate the biological effect of the Csx mutants on germination, spore resilience and adherence to surfaces. This would provide insight into the effect of certain spore assemblies on properties that are critical for colonisation and establishing infection. We hypothesize that, as CsxA is the primary composition of the exosporium basal layer, it will affect all three properties, as all of them have previously been established as important roles of the exosporium (reviewed in (Stewart, 2015)). If CsxB, CsxC or both are involved in parasporal layer assemblies, screening the properties of the mutants would also give insight into the role of the parasporal layers.

7.6.2 *C. difficile*

A question to be addressed is that of the link between the outermost spore layers observed in chemical fixation thin sections with the crystal structure resolved in this study (Figure 7.2B). The effect of sample handling on outer layers needs to be considered as it may affect the abundance of the ‘exosporium-like’ layer observed by TEM. Thus, alternative more gentle spore purification methods need to be employed and screened by whole spore TEM to see if any difference is observed. Additionally, HPF and freeze-substitution techniques should be employed to provide more representative parameters of the surface crystal observed in sections. These sections are also likely to increase the visible detail of spore assemblies and thus by combining with ET, outer spore layers could be characterized using techniques such as sub tomogram averaging. These findings can then be compared with the 3D model (Figure 7.2B) for a more accurate insight.

The composition of the crystalline layer remains unclear, immunogold labelling studies of anti-CdeA and anti-CdeB could provide some insight into the composition of the crystals observed. Further efforts should also try to isolate these crystals, possibly by using perturbation methods as described in Janganan (2016) and mass spectrometry to identify their composition. If the crystals can be harvested from the spores, then further structural work can be conducted which would allow the improvement of the current 3D model but also cryo EC. A Cryo-EM map of this crystal would provide the hydrated state of this layer, revealing any conformational changes it may undergo similar to that seen for CsxA crystals.

This would provide insight into whether the outermost layers are able to structurally adapt to changes in the environment, especially those that resemble the host gut. It would also establish if the conformational change to hydration is a universally conserved mechanism across spore formers.

7.6.3 Tomography of spores

As observed in chapter 5, HPF and freeze-substitution improved the visualisation of layers in *C. sporogenes* spores. However, freeze-substitution samples are still treated with harsh chemical fixatives, dehydrated and embedded in plastic thus may not represent the spore in its fully hydrated state, a biologically relevant environment as it mirrors the environment in the gut. Further studies should consider imaging of CEMOVIS/FIB milling sections, to provide the hydrated state of the spore, and compare with the current data from chemical fixation and HPF + freeze-substitution-in attempt to visualize biologically relevant changes in spore ultrastructure in response to hydration.

These methods should be used to study spores of *C. sporogenes* and *C. difficile* with response to hydration and at different stages of germination, to further understand the structural changes that occur during colonisation. Previous research revealed changes in surface properties with response to germination, including the formation of surface appendages (Panessa-Warren *et al.*, 1997; 2007; Warren *et al.*, 1994). These techniques may allow the visualisation and characterisation of these features in response to hydration or germination, which would be particularly interesting for spore strains that do not appear to have any surface appendages in their dormant state such as those used throughout this research. For *C. sporogenes* spores these techniques may also provide insight into the structural changes to exosporium basal layers or parasporal layers during germination and establish the involvement of the cap in outgrowth of vegetative cells. Whereas for *C. difficile*, the implications of the appendage in germination can be studied, in addition to other assemblies such as the coat laminations and the ‘exosporium-like’ layer.

Appendix

8.1 Microcompartments

As part of a collaboration with Dr Paul Davidson (PD) and Dr Craig MacGregor-Chatwin (CM-C) (Prof. Neil Hunter's laboratory), we looked at heterologously expressed microcompartment (MC) proteins from *Citrobacter freundii* expressed in *Ralstonia eutropha*. The MCs in *C. freundii* are used for compartmentalisation of diol dehydratase activity (Parsons *et al.*, 2010). Previous work had been conducted expressing proteins in *E. coli* but no structural information was obtained making it inconclusive (Parsons *et al.*, 2010). The aim of the project was to express MC proteins from *C. freundii* in *Ralstonia*, a species frequently used for biotechnological purposes, and target proteins to the MCs. If targeted, these MCs could provide a useful platform for compartmentalisation of reactions in the biotechnology industry.

A six gene plasmid construct was used by PD to express the Pdu proteins: PduA, PduB, PduJ, PduK, PduT, PduU, in *Ralstonia*. Previous research in the laboratory suggested that only 3 of the genes would be required to form MCs, thus a 3 gene construct was also used to express PduA, PduB and PduU. PduA was m-cherry labelled to aid with isolation and visualisation by fluorescence microscopy. The MCs cores were targeted with GFP in both six gene and three gene constructs. A list of all samples used are listed in the table below.

Table 8.1 *Ralstonia* strains and constructs

Strain	Plasmid	Source/Reference	Selectivity	Notes
<i>Ralstonia eutropha</i> H16 (ATCC 17699)		Dr Paul Davidson (Prof. Neil Hunter's lab, University of Sheffield)		WT strain
<i>Ralstonia eutropha</i> H16 (ATCC 17699)	pLO11-pBADmCherry-pduABJKNU	Dr Paul Davidson (Prof. Neil Hunter's lab, University of Sheffield)	Tet ^R	6 gene construct
<i>Ralstonia eutropha</i> H16 (ATCC 17699)	pLO11-pBADmCherry-pduABU	Dr Paul Davidson (Prof. Neil Hunter's lab, University of Sheffield)	Tet ^R	3 gene construct

<i>Ralstonia eutropha</i> H16 (ATCC 17699)	pLO11-pBADmCherry-pduABJKNU:pBADpdu-GFP.	Dr Paul Davidson (Prof. Neil Hunter's lab, University of Sheffield)	Tet ^R	6 gene construct with GFP
<i>Ralstonia eutropha</i> H16 (ATCC 17699)	pLO11-pBADmCherry-pduABU:pBADpdu-GFP	Dr Paul Davidson (Prof. Neil Hunter's lab, University of Sheffield)	Tet ^R	3 gene construct with GFP

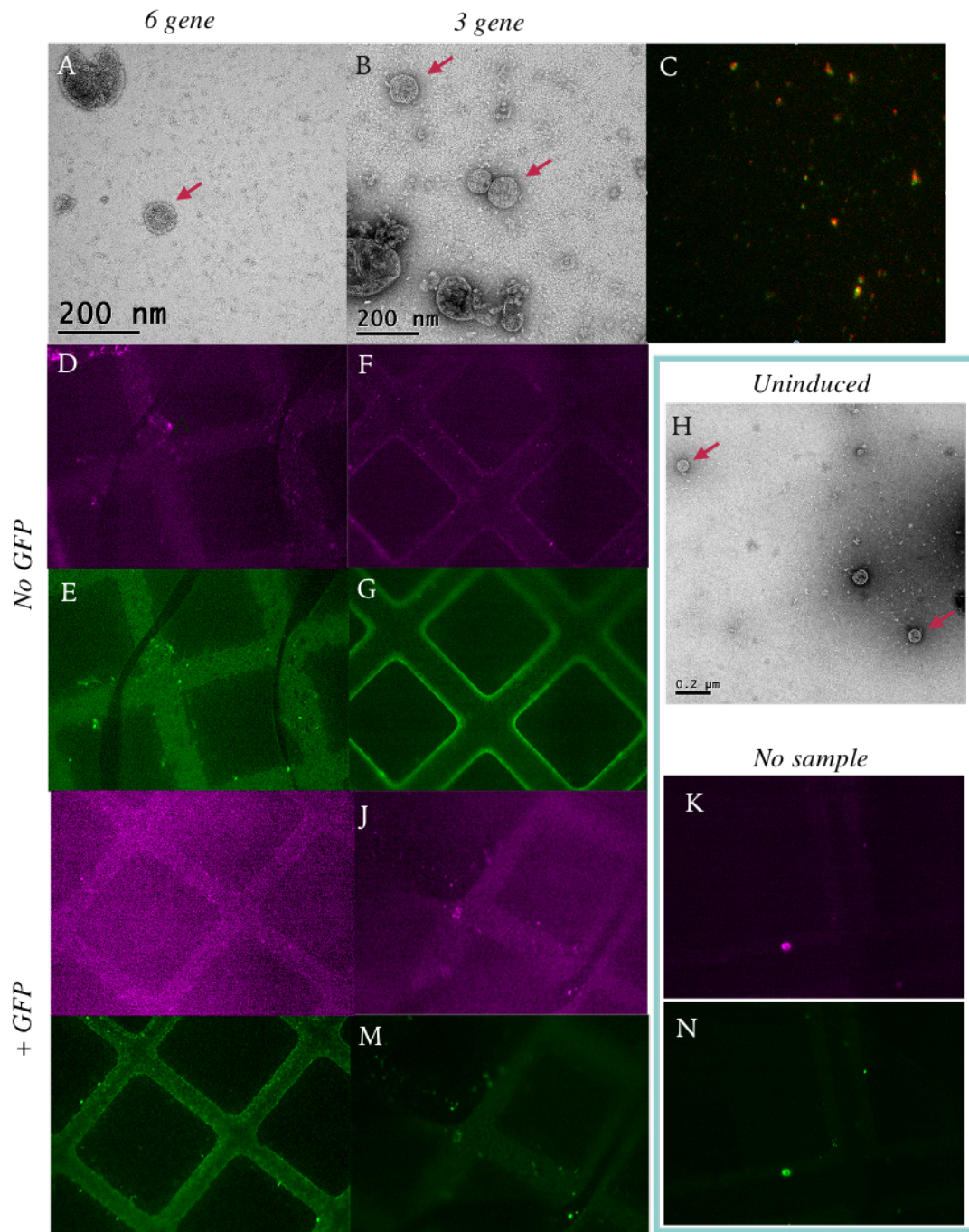
Tet^R = Chloramphenicol selective resistance

My role was to screen the samples using EM with hopes of visualising the MCs for structural analysis using either ET or SPA. Samples were screened using negative stain on the CM100 as described in chapter 2.5.1. MC-like objects were observed in both 6 gene (Appendix 1A) and 3 gene (Appendix 1B) expression preparations; however, the abundance was very low. MC-like objects were also observed in uninduced cell samples (Appendix 1C), and so it was unclear whether these objects corresponded to Pdu MCs or assemblies from *Ralstonia*. Thus, we employed correlative light and electron microscopy (CLEM) aimed at localisation of the MCs using fluorescence and then imaging with TEM.

CM-C used the confocal microscope 3D-SIM microscope (GE Healthcare) to screen MC samples, including the 3 gene MC sample with GFP target which revealed colocalization of m-Cherry and the GFP signal (Appendix 1C). These findings suggested that MCs were assembling and being targeted with GFP, providing promising results for further CLEM work.

The CLEM results (Appendix 1) revealed speckles of signal were observed in DAPI channels corresponding to m-cherry for all samples, primarily localised on or close to the grid bars.

Additionally, signal was detected in GFP channels when no GFP was present in the sample, including the 6 gene construct expression (Appendix 1D+E). This suggested non-specific signal from the background, thus grids with no sample were loaded onto the microscope which were also observed to give signal in both GFP and DAPI channels (Appendix 1K+N). Due to time constraints, further exploration of MC-like structures by CLEM and TEM were not conducted.



Appendix 1 Microcompartments

Negative stain TEM of: A) 6 gene expression, B) 3 gene expression and H) uninduced cell lysate with MC-like features labelled with red arrows. C) Confocal microscopy image of 3 gene construct + GFP: co-localization of m-cherry and GFP signal* D-G, I-N) Images from the CLEM in DAPI (purple) and GFP (green) channels: D+E) 6 gene construct with no GFP: speckles of signal observed in both DAPI and GFP channel F+G) 3 gene construct with no GFP: speckles observed in DAPI channel and low level of co-localisation signal in GFP channel I+L) 6 gene construct + GFP: speckles of signal co-localised in both DAPI and GFP channels J+M) 3 gene construct and GFP: signal detected in both channels that co-localise K+N) No sample: signal detected from just the grid in both DAPI and GFP channels that co-localise.

*Image provided by Craig MacGregor-Chatwin (Neil Hunter's lab, University of Sheffield) on 3D-SIM microscope (GE Healthcare) microscope.

8.2 Proteorhodopsin

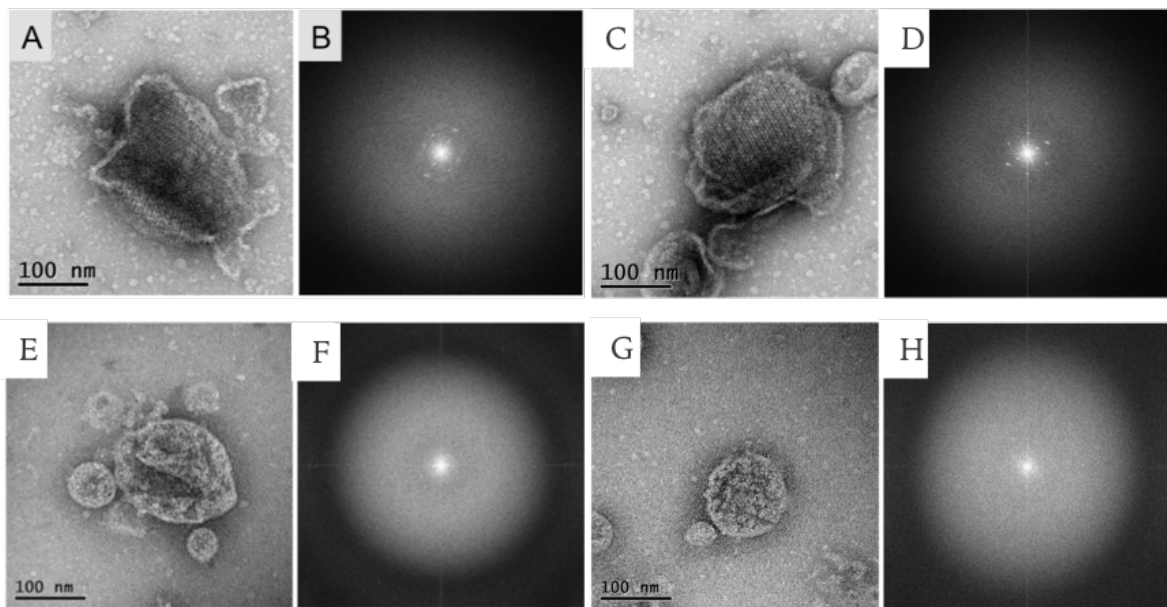
Proteorhodopsin (pR) is a rhodopsin protein that is present in 80% of marine bacteria (Moran and Miller, 2007). Studies of pR abundance on a single SAR86 proteobacteria suggested 24,000 pR copies (Béjà *et al.*, 2001), which are likely to pack into ordered assemblies within the cells. Work conducted at by Dr. Michael Carton (Prof. Neil Hunter's laboratory) found that pR assembled into 2D crystals on isolated membranes from *E. coli* (Appendix 2 – A-D). The aims of this project were to collect more images of 2D crystals of pR on membranes, expressed in *E. coli* (table 2), by negative stain TEM and optimisation for Cryo-EM studies including cryo-EC or cryo-ET.

Table 8.2 *E. coli* strain and Proteorhodopsin plasmid construct

Strain	Plasmid	Source/Reference	Selectivity
<i>Escherichia coli</i> BL21 DE3	pBARpR plasmid	Dr Paul Davidson (Prof. Neil Hunter's lab, University of Sheffield)	Amp ^R

Amp^R = Ampicillin selective resistance

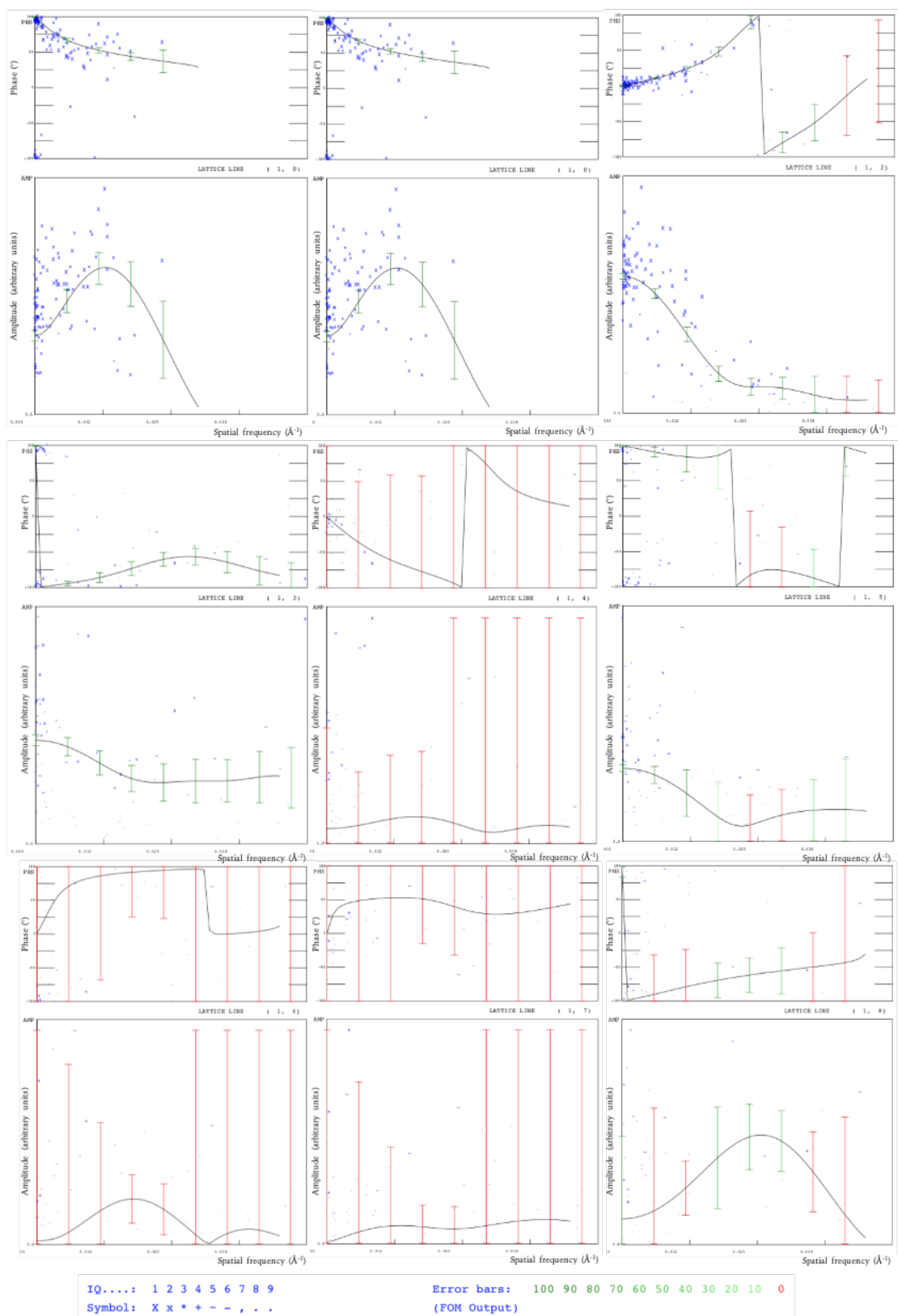
Unfortunately, after months of repeating and optimising the protocol provided by Michael Carton, no pR 2D crystals were observed by TEM. After several months of repeating the crystals were irreproducible.



Appendix 2 Proteorhodopsin expression in *E. coli*

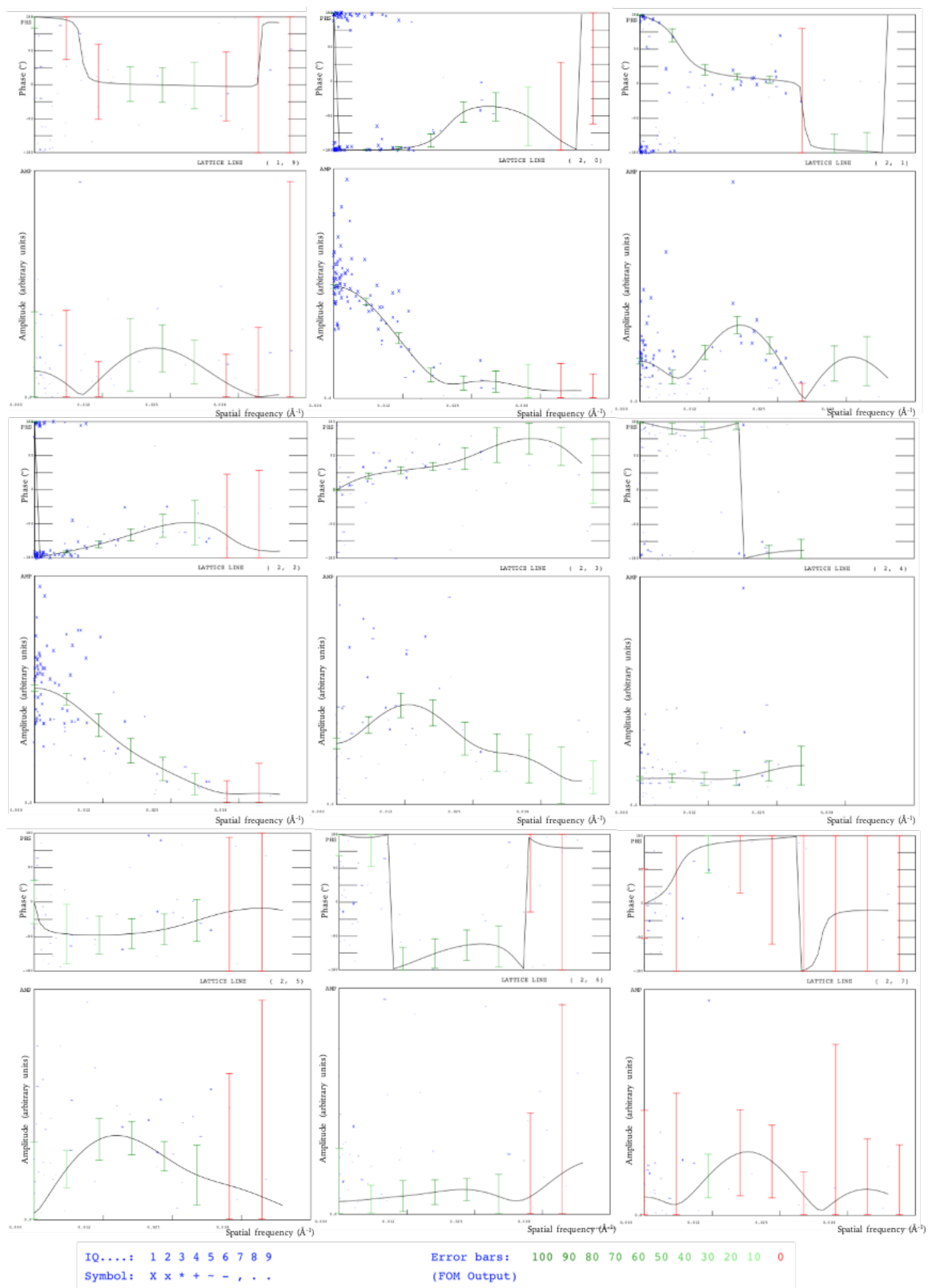
TEM micrographs and respective FFT A-D) Membranes with crystalline arrays, diffraction pattern observed in FFT* E-H) Isolated membrane patches from pR expression in *E. coli* – no 2D crystals observed.

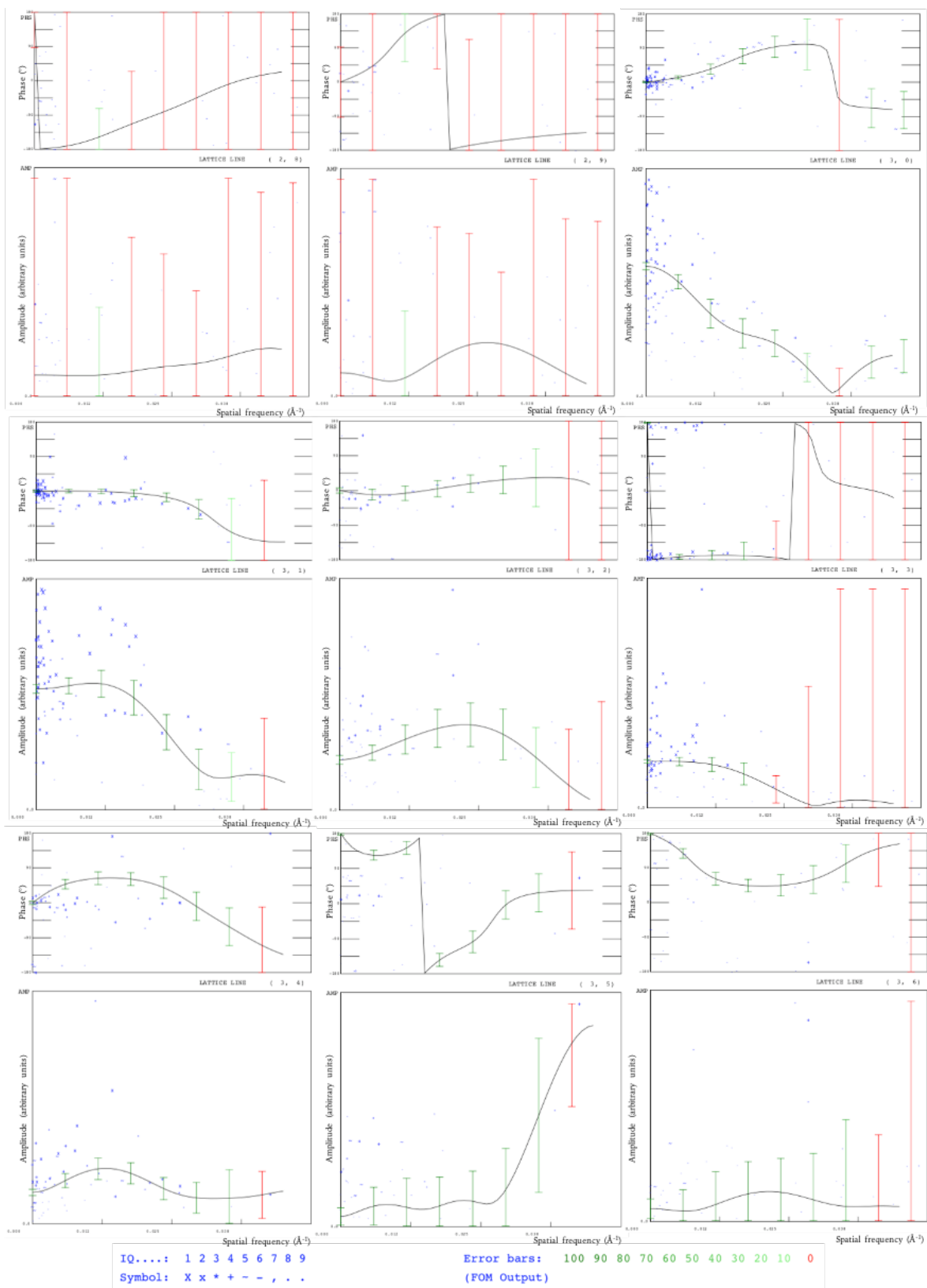
*Images provided by Michael Carton (Neil Hunter's lab, University of Sheffield) from negative stain TEM screening on CM100

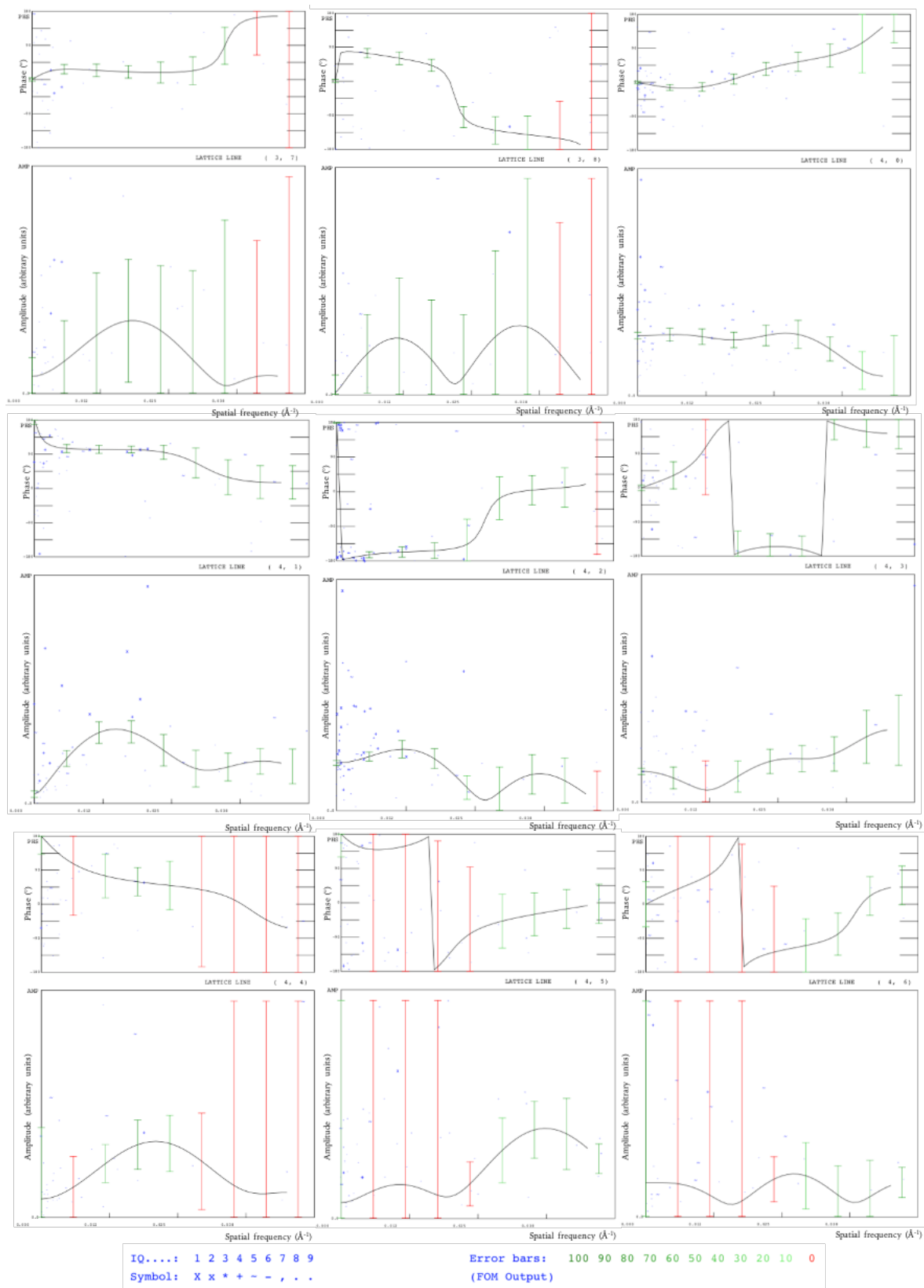


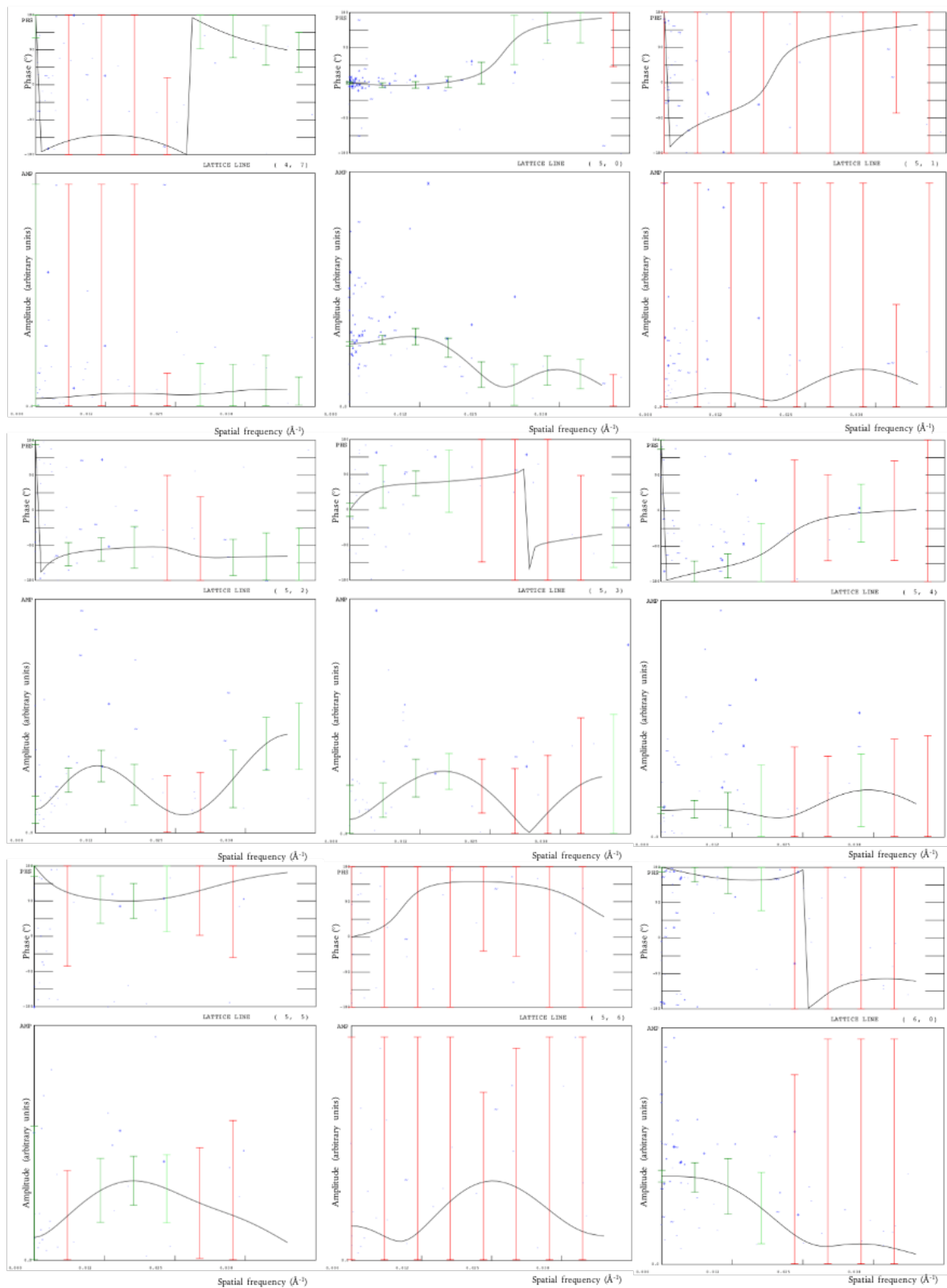
Appendix 3 Cryo-EC CsxA latlines from 3D merging

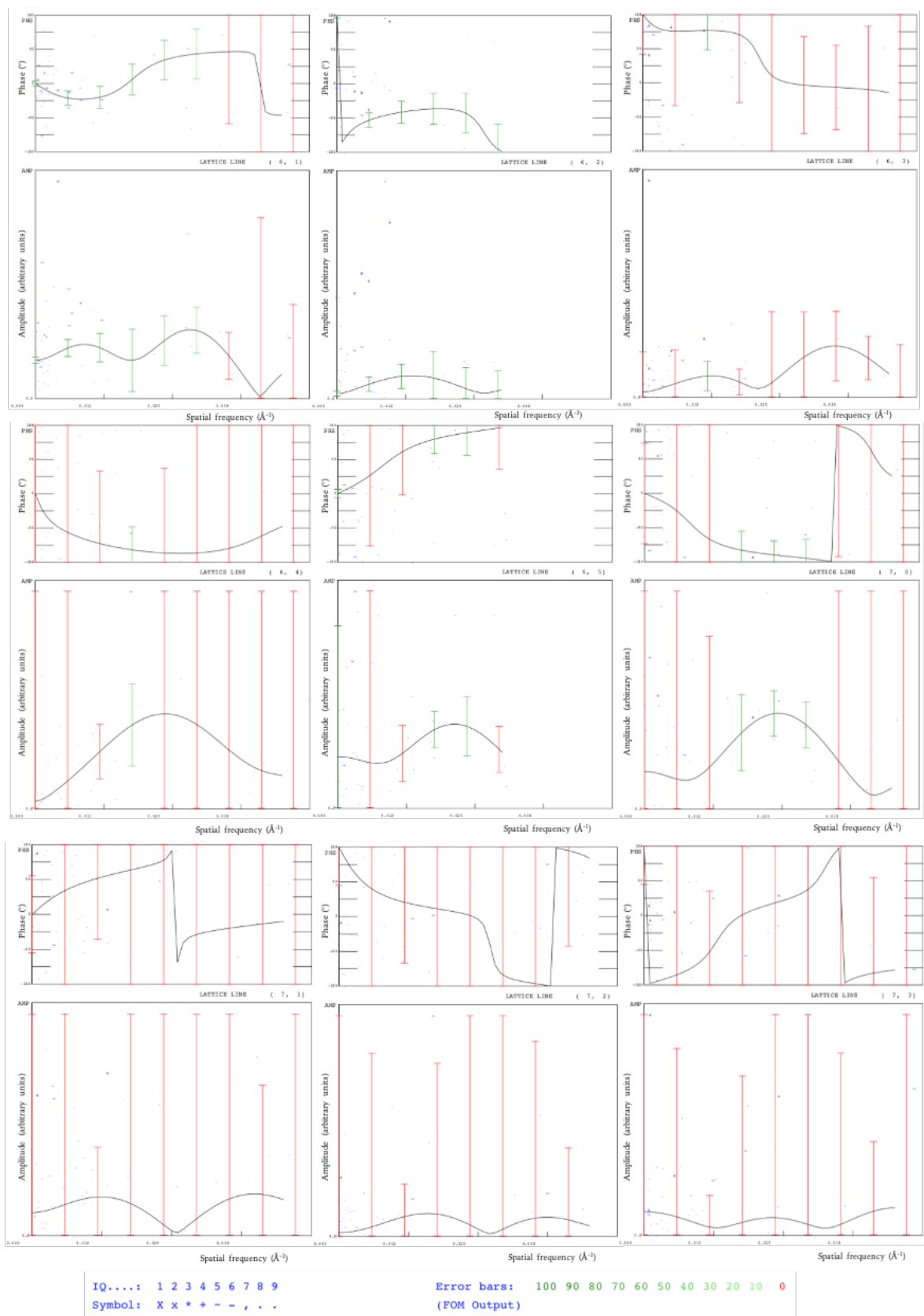
Two graphs provided for each h, k indexed lines: PHS -describes phase variability in degrees, and AMP - describes amplitude variation. Horizontal line displays increments in z^* values ($\text{\AA}^{-1}$) from x^* , y^* plane. Error bars are present in green, darker the green the lower the error, with red indicating high error in fitting. These are observed for second order line, expected as the data is from negative stain thus has limited resolution. The blue symbols indicates the IQ values for experimental spots as described by the table above.

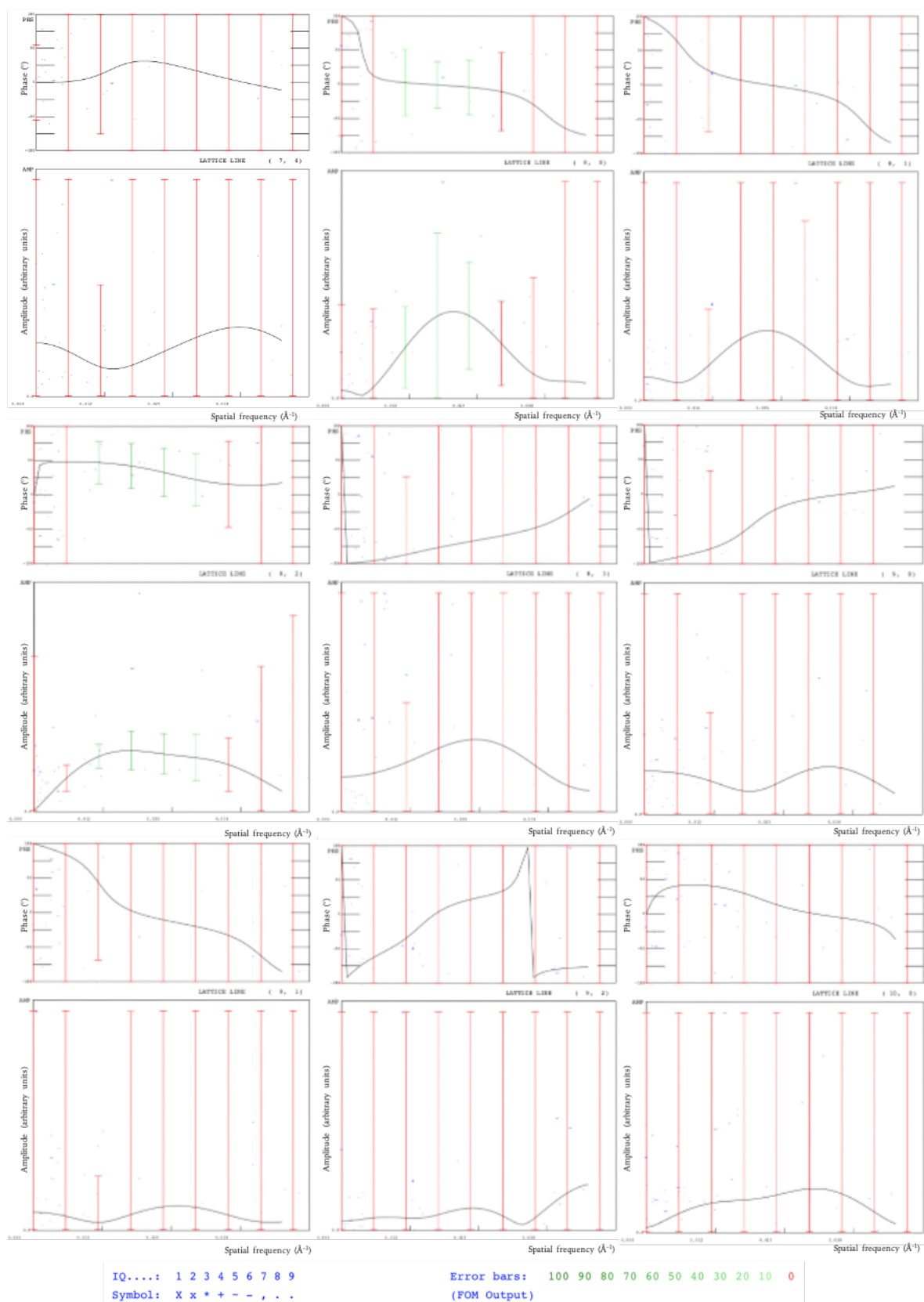


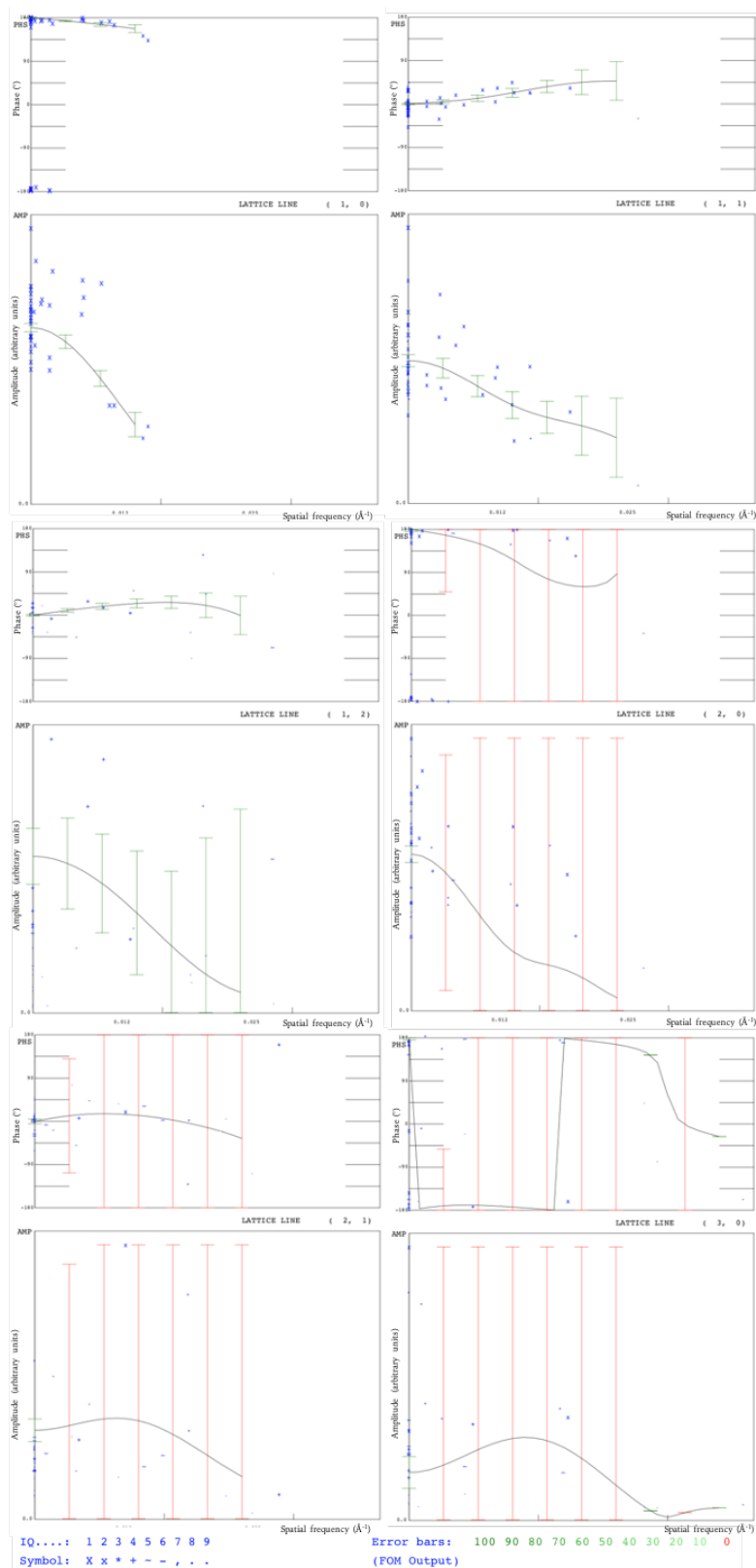












Appendix 4 *C. difficile* para-crystalline layer latlines from 3D merging

Two graphs provided for each h, k indexed lines: PHS - describes phase variability in degrees, and AMP - describes amplitude variation in $|F|$ the magnification of amplitude. Horizontal line displays increment in z^* values (Å⁻¹) from x^* , y^* plane. Error bars are present in green, darker the green the lower the error, with red indicating high error in fitting. These are observed for second order line, expected as the data is from negative stain thus has limited resolution. The blue symbols indicate the IQ values for experimental spots as described by the table above.

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