

Principles of Surface Layer Biogenesis in
Caulobacter crescentus



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Submitted in partial fulfilment of the requirements for the Degree of

Doctor of Philosophy

Certificate of Original Authorship

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Publications Pertaining to this Thesis

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Abbreviations

AMP	Ampicillin
BF	Brightfield
CM	Cytoplasmic membrane
Cryo-EM	Cryoelectron microscopy
Cryo-ET	Cryoelectron tomography
EDTA	Ethylenediaminetetraacetic
FDAA	Fluorescent D-amino acid
FP	Fluorescent protein
FSC	Fourier shell correlation
FT	Fourier transform
IPTG	Isopropyl β -D-1-thiogalactopyranoside
KAN	Kanamycin
LPS	Lipopolysaccharide
LWXD	Long wavelength X-ray diffraction
OD	Optical density
OM	Outer membrane
OMP	Outer membrane protein
OPS	O-specific polysaccharide
PBP	Penicillin binding protein
PCC	Pearson's correlation coefficient
PDB	Protein database
pI	Isoelectric point
RCF	Relative centrifugal force
RT	Room temperature
SC	SpyCatcher
SDS	Sodium dodecyl sulphate
WBP	Weighted back projection
SIRT	Simultaneous iterative reconstruction technique
SLP	Surface layer protein
ST	SpyTag
TISS	Type I secretion system
WT	Wildtype

Units

In addition to SI units, the following units are referenced:

bp	Base pair
Da	Dalton
hrs	Hours
kb	Kilobase
kDa	Kilodalton
mins	Minutes
ns	Nanoseconds
pH	$-\log_{10} c$, where c is $[H^+]$ in mol/L
rpm	Revolutions per minute
s	Seconds
x g	g-force or relative centrifugal force (RCF)

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Abstract

Surface layers, or S-layers, are a proteinaceous component of the prokaryotic cell envelope, found in almost all archaea and most bacteria. They constitute a two-dimensional, paracrystalline lattice that encompasses the entire cell, representing the outermost component of the cell envelope. Since their identification in the 1950s, S-layers have gained attention for their unique but diverse structural properties, symmetry, self-assembly, and range of biological functions. The projects described within this thesis seek to expand on our understanding of some of the most fundamental S-layer properties, including metal-binding, self-assembly, subcellular localisation, and integration of recently secreted surface layer proteins (SLPs) into the expanding S-layer.

Using a combination of light microscopy, structural, and cryoelectron microscopy techniques, this thesis provides insight into how the S-layer producing, Gram-negative bacterium *Caulobacter crescentus* utilises extracellular Ca^{2+} to facilitate S-layer biogenesis. Using an engineered strain of *C. crescentus* expressing a SpyTag peptide on the constituent SLP, RsaA, exposed to the extracellular milieu, we observed a strong dependence of RsaA integration into the S-layer on Ca^{2+} availability, characterised by loss or gain of fluorescence from a SpyCatcher-FP conjugate. Further to light microscopy, cryoelectron tomography of *C. crescentus* cells shows Ca^{2+} depletion results in aggregation and detachment of the S-layer, showing Ca^{2+} by RsaA binding stabilising S-layer proteins and architecture, facilitating oligomerisation, and enabling attachment to the LPS. This binding is further contextualised by mapping the exact locations of Ca^{2+} ions within the structure of RsaA_{CTD} crystals using long wavelength X-ray diffraction. Ca^{2+} ions can be accurately mapped at consistent locations across the RsaA_{CTD} structure, particularly at the central pore and between β sheets that make up the interlocking arms of the hexamer, the latter of which is consistent of toxin-related RTX domains.

Interestingly, two canonical Ca^{2+} binding positions in RsaA_{CTD} show an absence of any cations or a loosely bound K^+ , suggesting flexibility in some binding sites.

The fluorescent labelling of RsaA was further exploited to *expand* on the spatiotemporal localisation of S-layer biogenesis. A growing number of studies are revealing potential localisation of SLP integration to the midcell and cell poles, with similar spatiotemporal profiles to proteins typically associated with cell growth, development, and division. Using pulse-chase labelling, this study shows that flagellated swarmer cells, known to be divisionally redundant, exhibit polar labelling of new S-layer material. Due to the asymmetric nature of *C. crescentus* cell development, one pole will give rise to a stalk (also labelled as being covered in new S-layer) before new RsaA integration is localised to the midcell. This re-localisation is consistent with the redistribution of cell growth and the production of new cellular material to the pre-empted division site. This localisation pattern is unperturbed by induced filamentation of *C. crescentus* cells using sublethal concentrations of cephalixin but can be made highly irregular or completely displaced using A22, which inhibits cell elongation and the cytoskeletal protein MreB. Additionally, when co-labelled with the fluorescent D-amino acid HADA, a marker for highest levels of peptidoglycan maturation in the cell (in this case, the site of division or stalk), there is observable colocalisation of S-layer biogenesis and cell wall turnover.

Finally, this thesis explores the potential applications of cryogenic correlative light and electron microscopy (cryo-CLEM) to investigate S-layer biogenesis. Vitri-fied, dual-labelled *C. crescentus* cells were imaged under liquid nitrogen using widefield light microscopy, followed by data collection via cryoelectron tomography (cryo-ET) on regions of new S-layer insertion. Using cross-correlation techniques and the previously resolved RsaA hexamer structure, resolved by cryo-EM, to create a map along the S-layer

to identify faults in lattice. In several locations, possible events of RsaA insertion were identified at regions of high curvature and membrane distortion, particularly at the base of the stalk, previously identified as a major point for S-layer expansion.

CHAPTER 1. Introduction

1.1. Introduction to S-layers

1.1.1. The Prokaryotic Cell Envelope

Cells, the finite and fundamental unit of life, are encompassed by an envelope that enables not only compartmentalisation of the cell, but acts as the primary boundary to the cell's environment (Ribatti, 2018). Since our origins, compartmentalisation of biological processes using boundaries has been essential to the success of living organisms (Deamer, 1997). Fascinatingly, and somewhat counterintuitively, the most intricate cell envelopes are found amongst the Prokaryotes, a domain comprised of the Bacteria and Archaea (Kapli et al., 2020), whose sophisticated, multi-tiered casing seems to exceed in complexity their Eukaryotic relatives. This complexity may be unsurprising, given that these microscopic organisms, which predate the Eukarya by billions of years, occupy a huge diversity of environments, and engage in huge variety of biochemical activities. Most attempts to classify prokaryotic organisms also utilise differences in the composition of the cell envelope; diderm and monoderm prokaryotes, possessing two or one membrane, respectively. This designation is more inclusive of Gram stain-intractable prokaryotes, such as the Mycobacteriaceae, whose dual-enveloped structure is distinct from Gram negative bacteria, and the non-bacterial Archaea, whose envelope structure have their own distinct organisations and diversity.

The composition of the prokaryotic cell envelope can be described by the presence of the following structures. The first and innermost layer, the cytoplasmic membrane, comprised of a phospholipid bilayer, surrounds and contains the enveloped cytoplasm, is present in practically all cell types (Strahl and Errington, 2017). In some prokaryotes, most famously *Mycoplasma* species of the class *Mollicutes*, the cytoplasmic membrane is the sole component of the cell envelope (Razin and Hayflick, 2010). More commonly,

prokaryotes produce a large polymeric structure known as the cell wall, which encompasses the cell and provides a layer of protection as well as structural integrity (von K  gelgen et al., 2023). The molecular composition of the cell wall can vary across species, but in bacteria, most cell walls are comprised of peptidoglycan, a complex macromolecule composed of polysaccharides crosslinked by peptide fragments. Beyond the cell wall, many species possess an additional layer, known as the outer membrane, consisting of a lipopolysaccharide bilayer structure or, in mycobacteria, an even more complex structure arabinogalactan and mycolic acids.

Despite tremendous diversity among prokaryotic cell envelopes, the Surface layer, or S-layer, is arguably the most ubiquitous (Sleytr and Beveridge, 1999, Sara and Sleytr, 2000, Albers and Meyer, 2011, Fagan and Fairweather, 2014, Rodrigues-Oliveira et al., 2017, Bharat et al., 2021), and is gaining recognition as a major element of the prokaryotic cell envelope. First reported in *Spirillum spp.* over 70 years ago (Houwink and Le Poole, 1952), S-layers are a proteinaceous, two-dimensional lattice that constitute the outermost component of the cell envelope, identified by their striking, regular symmetry (Sleytr, 1978, Messner and Sleytr, 1992). Half a century on, S-layers have been identified across all major prokaryotic clades and appear to be universally distributed across archaea, including in some of the most phylogenetically deep-branching species (Sleytr et al., 2014, Bharat et al., 2021, von K  gelgen et al., 2022) (Figure 1.1). S-layer proteins (SLPs) adopt a variety of symmetries, including oblique (p1, p2), square (p4), and hexagonal (p3, p6) symmetry. Center-to-center spacing of the symmetrical units typically varies between 2.5 and 35 nm (Sara and Sleytr, 2000, Sleytr and Beveridge, 1999). The isoelectric points (pIs) of SLPs is generally low, falling into the acidic pH range, owing to the high content of acidic and hydrophobic amino acids (Messner, 1996). SLPs, particularly of Gram-negative bacteria, are often enriched in glycine and aspartate

residues, potentially due to the commonly observed calcium ion binding modalities and outer membrane binding (Gilchrist et al., 1992).

Despite their biological and biotechnological utility, SLPs have garnered limited attention by the scientific community, in part due to their absence the bacteriological model organisms, such as *Escherichia coli* and *Bacillus subtilis*. In addition, SLPs intractability to common research methods and molecular manipulation (Bingle et al., 1997a). Despite this, S-layers appear to be the standard for prokaryotes, rather than the exception, and in species producing them, their constituent SLPs can account for 15-30 % of the total protein content on the cell (Sleytr, 1996, Lau et al., 2010a). Clearly, there is great need for more research into S-layer biology, and thanks to recent advances in structural and biological techniques are helping unravel the mysteries of these beautiful complexes.

1.1.2. Evolution and Diversity of S-layer Proteins

Closely related SLPs often show some degree of sequence similarity, typically limited to their respective binding domains that mediate attachment and retention on the cell surface (Sleytr and Beveridge, 1999)(Engelhardt and Peters, 1998). Cell-binding and retention is perhaps one of the best studied qualities of S-layers, second only to their ability to form spontaneously. SLPs bind to their underlying cells via a variety of mechanisms, ranging from direct embedding into or attachment to the cytoplasmic membrane (particularly common in Archaeal species) {Albers, 2011 #9}, binding accessory peptides in the cell wall such as teichoic acids {Navarre, 1999 #323}, or non-covalent binding of the O-antigen in Gram-negative bacteria {von Kugelgen, 2020 #425} (Figure 1.2). Beyond this, there is limited sequence homogeneity among S-layer proteins, despite observable common features and behaviours, such as bipartite structural

organisation or metal binding propensity (Bharat et al., 2021). This may suggest multiple evolutionary origins for SLPs across bacteria and archaea, or that the S-layer is a site of significant selection pressure, which is unsurprising given its position as the first line of interaction between the cell and the environment. Unfortunately, even as our understanding of S-layer structure, biochemistry, and function have grown, we still know remarkably little about their evolutionary origin and relationship with their cells.

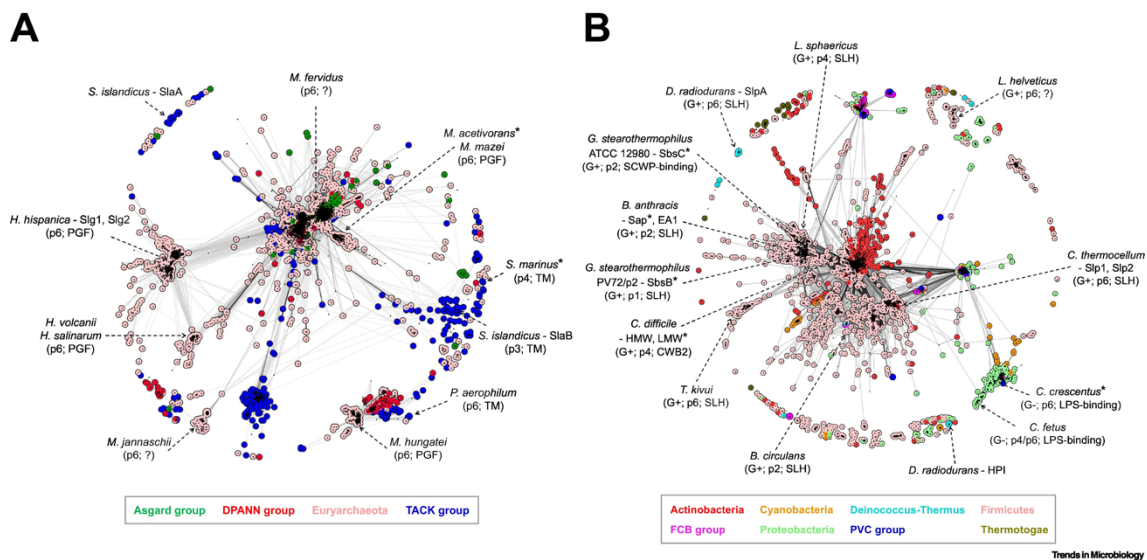


Figure 1.1. Cluster maps of S-layer proteins.

(A) Archaeal and (B) bacterial SLPs gathered using BLAST searches and clustered using the CLANS software based on their all-against-all pairwise similarities as measured by BLAST P values. Dots represent SLP sequence, while line colour reflects BLAST P values (darker lines correspond to lower P values). Colours denote major archaeal and bacterial groups, and some well-studied species are highlighted (species marked with * have published data on their constituent SLP structure). Adapted from Bharat *et al.*, 2021.

SLPs from closely related species do share some sequence homology (Figure 1.1), particularly among their cell-binding domains (Engelhardt and Peters, 1998), and can share some structural homogeneity. However, between distantly related species, there is

virtually no overlap in structure, with the exception of relative enrichment in β -strand secondary structure, particularly within the oligomerisation domains (Figure 1.3).

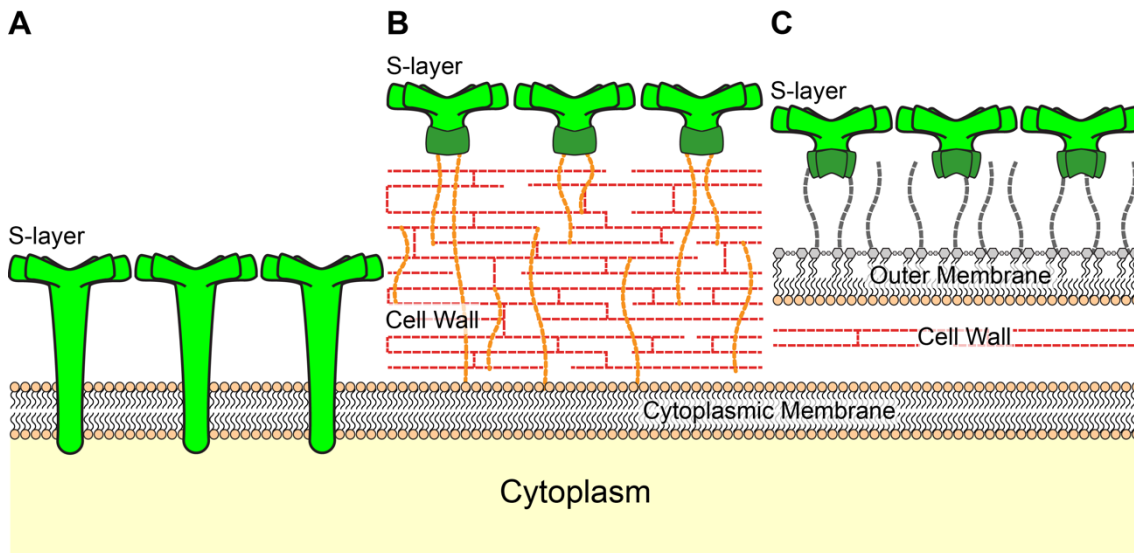


Figure 1.2. Diversity of envelope composition in SLP-producing prokaryotes.

Schematic showing different envelope compositions and S-layer binding locations in (A) archaea (showing SLP binding directly to the cytoplasmic membrane), (B) Gram-positive bacteria (showing SLP binding directly to accessory peptides in the cell wall), and (C) Gram-negative bacteria (showing SLP binding directly to the O-antigen). SLPs coloured green, peptidoglycan red, accessory peptides orange, and O-antigen grey.

It is tempting to speculate that S-layers may represent one of the earliest forms of a cell wall or bacterial exoskeleton, particularly given their presence in even the most ancient lineages of Archaea and their ability to generate by self-oligomerisation (Imachi et al., 2020, Pum et al., 2013). Several authors have also drawn the intriguing comparison between SLPs and viral capsid proteins, which share a degree of structural homology (Arbing et al., 2012). Additionally, viral envelope proteins demonstrate a similar capacity for lattice formation and spontaneous organisation (Valbuena et al., 2020). However, without strong evidence, the origins of S-layers can only be hypothesised, as of yet.

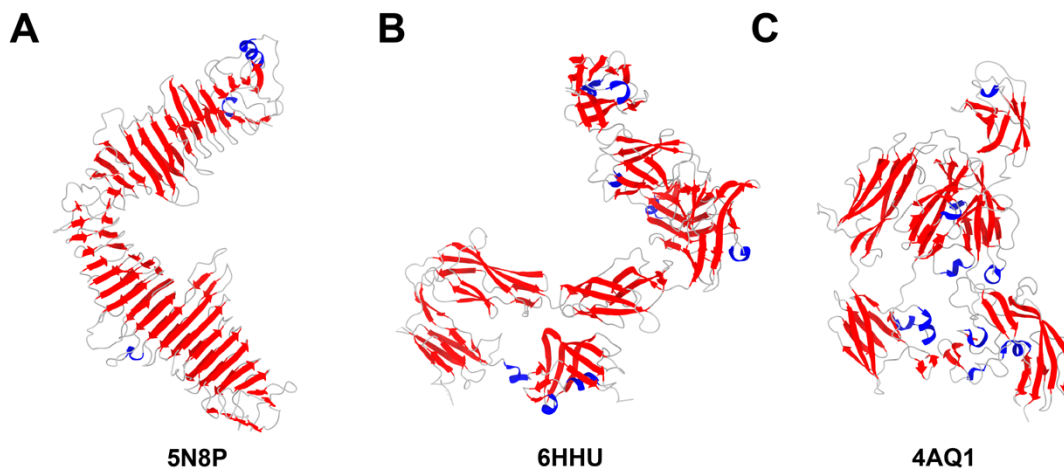


Figure 1.3. S-layer domains have highly variable structure and composition.

(A) RsaCTD from *Caulobacter crescentus* (PDB 5N8P), (B) SAP from *Bacillus anthracis* (PDB 6HHU), (C) SbsB from *Geobacillus stearothermophilus* (PDB 4AQ1). Proteins are coloured as follows: coils, grey; β -strands, red; α -helices, blue.

1.1.3. The Many Roles of S-layers

Explanations seeking to provide a universal function for the presence of S-layers and why they are apparently dispensable in some species, such as *C. crescentus* (Marks et al., 2010), while being essential in others, like *Clostridioides difficile* (Oatley et al., 2020), has been unsuccessful. The essentiality of SLPs in certain species is a barrier to their study and many lab-based approaches fail to reconstitute the environments that necessitated the maintenance of a burdensome and encompassing protein chassis, owing to the longstanding question of “microbial dark matter” and limitations of lab-based culturing methods (Kapinusova et al., 2023). Nevertheless, tremendous progress has been made to understand the function of various S-layers, and much like their composition, their roles appear to be equally as diverse. Many of these functions appear to be direct adaptations owing to the species’ environment. An illustrated summary of several reported S-layer functions can be found in Figure 1.4.

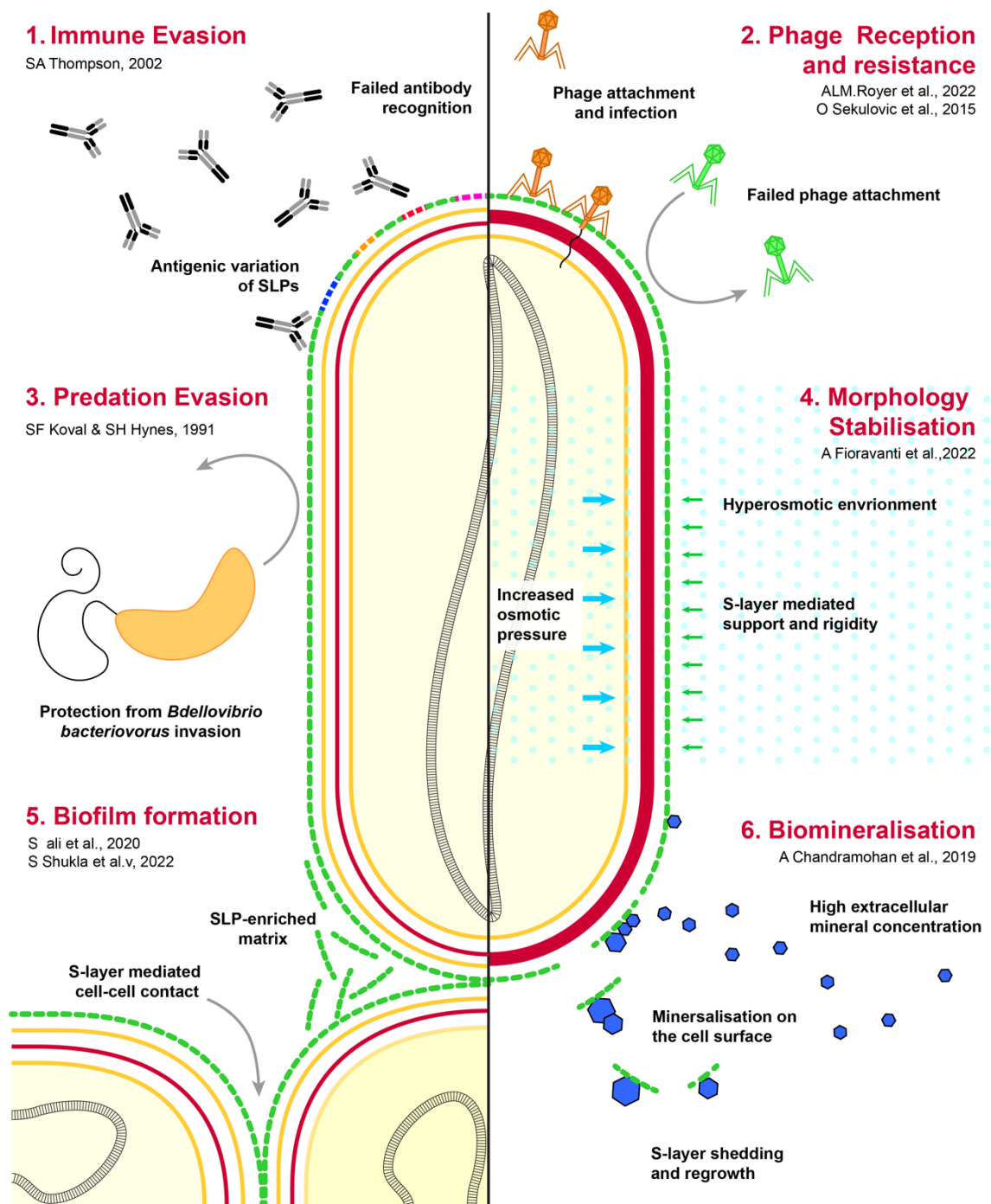


Figure 1.4. The many roles of S-layers.

An illustrated collection of S-layer functions. The functions described are not exhaustive and include (1) immune evasion, (2) Phage reception and resistance, (3) evading predation, (4) stabilising the cell envelope in response to osmotic stress, (5) aiding in biofilm development and formation, and (6) biomineralisation. Relevant citations to supporting literature are provided for each example.

1.1.3.1. S-layers as a Functional Exoskeleton

One of the most compelling and earliest descriptions of S-layers likened them to a molecular exoskeleton. In its simplest terms, an exoskeleton is a defensive and supportive structure that exists outside of the underlying organisms, providing structural integrity for the organism or the cell. The peptidoglycan cell wall of bacteria is analogous to exoskeletons in many ways, providing both protection for the bacterium and rigidifying the cell envelope, maintaining cell turgor and shape (Typas et al., 2012). Even small aberrations or ruptures in the cell wall can result in cell lysis and so cells invest significant energy in its maintenance (Yadav et al., 2018, Mueller and Levin, 2020). In Gram-negative cells, the outer membrane is also heavily implicated in the maintenance of cell morphology and stability (Rojas et al., 2018).

As with many aspects of SLP biology, S-layers function as an exoskeleton in some species but appear to be irrelevant as a supporting structure in others. *Bacillus anthracis*, a highly pathogenic Gram-positive bacterium, produces a relatively complex S-layer comprised of two major structural proteins, EA1 and SAP (Mignot et al., 2002). The S-layer is considered a major virulence factor during *B. anthracis* infection (Missiakas and Schneewind, 2017), but has recently been reported to confer rigidity to the cell envelope during periods of hyperosmotic stress (Fioravanti et al., 2022). In Archaea, S-layers appear to be a universal feature of the cell envelope, (Rodrigues-Oliveira et al., 2017, Albers and Meyer, 2011). The fascinating and perplexing geometric shapes Archaeal cells adopt, as well as their morphological plasticity, appear to be at least partially dependent on their S-layers (Sleytr and Sára, 1997), especially as in many archaeal species such as *Haloferax volcanii* and *Halobacterium salinarum*, the S-layer is the only major structural component of the cell envelope besides the cytoplasmic membrane (Rodrigues-Oliveira et al., 2019, Mescher and Strominger, 1976).

Conversely, several SLP-expressing species show no link between their cell-shape or stability and their S-layer. *C. crescentus* adopts a curved cell shape and produce a stalk during sessile stages of cell development (Shapiro, 1976). Throughout the cell cycle, the S-layer, composed of the hexameric SLP RsaA, completely envelops the *C. crescentus* cell and stalk (Bharat et al., 2017). However, given certain mutations or repeated growth cycling under lab conditions, *C. crescentus* can stop producing its S-layer, which results in no observable morphological defects to the stalk or cell shape (Marks et al., 2010). This is unsurprising given that the curved cell shape of *C. crescentus* is the result of the intermediate-filament homolog, Crescentin, which localises to the negatively curved cytoplasmic membrane (Charbon et al., 2009, Cabeen et al., 2009). Additionally, stalk biogenesis is subject to a sophisticated network of regulatory proteins (Billini et al., 2019, Sundararajan and Goley, 2017), with which the S-layer production machinery has no known interaction. It may be that the S-layers of species like *C. crescentus* only behave as structural exoskeletons under certain stressors or environments, or perhaps some S-layers are redundant in their role as load-bearing exoskeletons, as more sophisticated mechanisms of cell morphology take over.

1.1.3.2. S-layers and Disease; SLPs are a major virulence factor in key pathogens

Several species of pathogenic bacteria produce S-layers that are essential for or greatly increase virulence, by aiding in immune evasion (Thompson, 2002), enhancing cell attachment (Allievi et al., 2014), or suppressing the immune response (Sekot et al., 2011). *C. difficile* and *Bacillus anthracis*, two species of highly pathogenic, Gram-positive bacteria, can attribute their virulence at least partially to their S-layer (Vaz et al., 2019, Missiakas and Schneewind, 2017). *C. difficile* strains treated with a modified bacteriocin have downregulated virulence as a result of aberrations or loss of their

associated S-layer machinery (Kirk et al., 2017a). This demonstrates not only a major role for the S-layer in virulence, but that the S-layer is, at least partially, dispensable, as S-layer-compromised strains were still able to colonise and persist within the rat gut, albeit with lesser success than WT strains (Ormsby et al., 2023, Kirk et al., 2017b). While the major utility of the *C. difficile* S-layer appears to be colonisation, compromised S-layer synthesis also resulted in compromised motility, reduced toxin production, and even disrupted sporulation (Vaz et al., 2019).

The *B. anthracis* S-layer is comprised primarily of two structural proteins, SAP and EA1, which are at their highest concentration in the S-layer in either the vegetative or the encapsulated stages of the *Bacillus* life cycle, respectively (Mignot et al., 2002). While neither are identified individually as virulence factors, EA1 expression is linked to the production of toxins via AtxA, a master regulator of both capsule and virulence factor production (Mignot et al., 2004). In addition, the adhesion protein BslA, while not an SLP, is an S-layer associated virulence protein (Kern and Schneewind, 2010). BslA is essential in mediating adherence of vegetative *B. anthracis* cells and host tissues, potentially binding host laminin (Wang et al., 2016).

To move to Gram-negative bacteria, *Campylobacter* species utilise their S-layers in an ingenious way. Possessed by two species, *Campylobacter fetus* and *Campylobacter rectus*, the *Campylobacter* S-layer can aid in the cell's immune evasion (Thompson, 2002). Firstly, both S-layers prevent the binding of the complement factor C3b, conferring resistance to complement-mediated immunity (Blaser et al., 1988, Okuda et al., 1997). While still vulnerable to other immune pathways, including opsonisation by SLP-directed antibodies. However, *C. fetus* utilises changes in SLP gene expression to achieve antigenic variation, allowing the S-layer to escape detection by antibodies (Thompson, 2002, Boot and Pouwels, 1996). *C. rectus* produces a more typical S-layer of

uniform composition and hexameric symmetry (Lai et al., 1981). While antigenic variation has been noted between strains, the *C. rectus* S-layer does not display the same capacity for antigenic variation as *C. fetus* (Kobayashi et al., 1993). However, its S-layer appears to play a role in modulating host immune cytokines, potentially in a way that favours *C. rectus* infection (Wang et al., 2000).

1.1.4. S-layer Biogenesis

1.1.4.1. Synthesis, Secretion, and Modification

A major difference in the composition of S-layers between species is not only the sequences of their constituent SLPs, but also the varying number of SLPs present in each species. For example, *H. volcanii*, an archaeon, and *C. crescentus*, a Gram-negative bacterium, produce a monomolecular S-layer (Rodrigues-Oliveira et al., 2019, Bharat et al., 2017), as is most common in S-layer producing species (Messner and Sleytr, 1992). However, many species express S-layers comprised of numerous proteins. *C. difficile*, for example, is reported to produce 29 S-layer proteins, identified based on homology of their cell wall binding domain (Fagan and Fairweather, 2014).

SLPs often undergo modification before their eventual recruitment into the S-layer. This can include glycosylation, as many S-layers are composed of glycoproteins, or other forms of modification. The major structural component of the *C. difficile* S-layer, SlpA, is post-translationally cleaved by Cwp84 into a high and low molecular weight SLP, both of which are essential for S-layer biogenesis (Lanzoni-Mangutchi et al., 2022). In other cases, the cell envelope itself may be in some way modified to enable SLP binding and S-layer biogenesis.

SLP secretion is, as expected, governed by a variety of systems, which are generally SLP-specific. Many homologs of well-established secretion pathways, such as

the Sec secretion system in *C. difficile* and *B. anthracis* (Sara and Sleytr, 2000, Fagan and Fairweather, 2011, Nguyen-Mau et al., 2012). Both species also utilise the accessory ATPase, SecA2, to recruit the SLP peptide from the cytoplasm, prior to secretion by its partner SecYEG complex (or SecY2EG for *B. anthracis*) (Fagan and Fairweather, 2011, Nguyen-Mau et al., 2012). In *C. crescentus* the SLP is secreted using a canonical T1SS, RsaDEF (Awram and Smit, 1998). This appears to be common among S-layer producing Gram-negative species, including *C. fetus* and *Serratia spp.* (Thompson et al., 1998, Riedel et al., 2001, Agarwal et al., 2018, Kawai et al., 1998), as the T1SS provides an envelope spanning complex that enables convenient passage of the nascent SLP peptide through both membranes, avoiding the periplasm (Kanonenberg et al., 2018).

While Sec and T1SS systems appear most ubiquitous, there are alternatives. For example, *Tannerella forsythia* secretes its constituent SLP using a type IX secretion system (Tomek et al., 2014). *Aeromonas salmonicida* uses a type II secretion system (Noonan and Trust, 1995), wherein the SLP is first secreted by the Sec pathway into the periplasm where it undergoes partial folding before being transported across the OM by an complex analogous to the type IV pilin assembly machinery (Korotkov et al., 2012).

1.1.4.2. Localisation of S-layer Biogenesis

S-layer biogenesis remains a fascinating but elusive aspect of SLP biology. Prokaryotic cells have demonstrated a tremendous ability to localise the synthesis of large macromolecular structures in both space and time. This is best demonstrated by the cell wall, which, in many species, is under the control sophisticated protein-regulated networks to help ensure localised peptidoglycan remodelling and cell wall expansion (Kleanthous et al., 2015, Surovtsev and Jacobs-Wagner, 2018). It is reasonable to suggest that S-layers may be subject to a similar kind of spatiotemporal regulation. Temporal

regulation has already been established in *B. anthracis*, where the S-layer is composed of one of two its major SLPs, EA1 and Sap, during exponential and stationary growth phase, respectively (Fioravanti et al., 2019). However, this delineation of S-layer composition in time may be rare.

The colocalisation of SLP secretion, modification, and integration into the growing S-layer is supported by evidence provided by the archaeon *H. volcanii*. The constituent SLP of *H. volcanii*, Csg, appears to colocalise with both FtsZ, a key component of the prokaryotic divisome, and a lipid-modification protein, ArtA. The latter protein enables Csg binding to the cytoplasmic membrane, ensuring the S-layer is retained by the cell following Sec-mediated secretion (Abdul-Halim et al., 2020). However, this data appears to conflict with S-layer producing bacteria, *C. crescentus* and *C. difficile*, which are Gram-negative and Gram-positive bacterial species respectively. Comerci *et al.*, 2019, suggests that S-layer biogenesis in *C. crescentus* is mediated largely biophysically. Following secretion, monomeric RsaA, the sole SLP in this species, diffuses beneath the S-layer until it finds a gap in the S-layer to insert itself into. Additionally, in *C. difficile*, the SecA2 machinery responsible for the secretion of SlpA, the major structural component of the S-layer, is heterogeneously distributed along the cell surface (Oatley et al., 2020). Despite the localisation of SecA2 and the diffuse nature of RsaA, both species routinely show incorporation of S-layer material at the cell poles and midcell, similar to *H. volcanii* (Oatley et al., 2020, Abdul-Halim et al., 2020, Comerci et al., 2019). This may be a unifying behaviour of SLP-producing species, suggesting a potential robustness in the localisation of S-layer biogenesis despite such vast differences in their sequence and structure.

1.1.4.3. Metal Binding and *in vitro* Polymerisation of SLPs

A feature of S-layer biology that was identified shortly after their discovery is the propensity for SLPs to bind metals (Inouye et al., 1979, Garduno et al., 1995). This has been observed in S-layers across domains, including Archaeal (Cohen et al., 1991, Kessel et al., 1988), Gram-positive (Baranova et al., 2012, Lupas et al., 1994), and Gram-negative species (Bharat et al., 2017, Herrmann et al., 2020). Intriguingly, the metal-binding capacity of S-layers appears to be intimately linked with their biogenesis, as is evident by the rapid oligomerisation of SLPs in the presence of divalent cations (Garduno et al., 1995, Sleytr, 1996, Nomellini et al., 1997).

As a result of their metal-binding proficiencies, S-layers are promising tools for biological remediation. SLPs, both in their cell-bound state and when recombinantly expressed, eagerly incorporate metal ions into the structure (Vogel et al., 2017), act as a nucleation point for biomineralisation (Kish et al., 2016, Chandramohan et al., 2018), and can additionally bind dangerous heavy metals (Velasquez and Dussan, 2009, Xu et al., 2010). S-layers are even capable of acting as a sink for uranium waste, representing powerful potential in bioremediation (Pollmann et al., 2006).

1.1.5. **Functionalisation of the S-layer**

S-layers have garnered significant attention for their potential as synthetic biology platforms. (Ben-Sasson et al., 2021). The exploitation of natural systems for engineering purposes offers tremendous opportunities (Nguyen et al., 2018) and S-layers represent ideal candidates for such adaptations (Pum et al., 2004). In particular, the self-organising, self-healing nature of S-layers and their regular symmetry and ability to form *in vitro* with or without an underlying scaffold (Nomellini et al., 1997, Sleytr and Sára, 1997), provides an ideal framework for the design of a high-density biological interaction (Pum et al.,

2004, Molinari et al., 2022, Charrier et al., 2019). The heterogeneity of S-layers and their varying properties also provides intriguing diversity that may inform design. SbpA of *Bacillus sphaericus*, for example, can form single or double layered S-layers depending on the relative hydrophobicity of its underlying support (Moreno-Flores et al., 2008).

C. crescentus S-layers have already been modified for protein display systems (Bingle et al., 1997a, Nomellini et al., 2007, Nomellini et al., 2010) and as scaffold for the design of customisable, living materials (Molinari et al., 2022, Charrier et al., 2019). The ease of which its constituent RsaA SLP can be purified (Nomellini et al., 2007, Xu et al., 2010) and well characterised lifecycle (Ely, 1991) makes *C. crescentus* an attractive candidate for synthetic biology development.

1.2. *Caulobacter crescentus*; A Model Organism for Studying S-layer Biology

1.2.1. *C. crescentus* Cell Biology and RsaA

C. crescentus (formally *Caulobacter vibrioides*) is a Gram-negative, aquatic bacterium, is the type species for the Caulobacteraceae family (Abraham et al., 2014, Poindexter and Lewis, 1966). Having been originally isolated from freshwater environments, *C. crescentus* is a well-recognised bacterium, owing to its unique cell morphology, asymmetric lifecycle, and genetic tractability (Poindexter, 1981, Poindexter, 1964). Featuring in a spate of publications and offering valuable insight into fundamental biological processes, including cell cycle regulation, cell differentiation, and bacterial development, *C. crescentus* has become a popular model organism for microbiologists (Barrows and Goley, 2023, Govers and Jacobs-Wagner, 2020).

One of the most fascinating aspects of *C. crescentus* biology is its dimorphic lifecycle, which includes two morphologically distinct developmental cell stages; swarmer cells and stalked cells (Figure 1.5A). Juvenile *C. crescentus* cells start their lives off as highly

motile swarmer cells, with a single polar flagellum and one or two type IV tight adherence (tad) pili, ideal for dispersal (Shapiro, 1976, Sommer and Newton, 1988). *C. crescentus* motility is impressive by bacterial standards, owing to the incredibly low torque rate of its flagella motor, which is likely an adaption to nutrient-poor, aquatic environments (Li and Tang, 2006). Following surface contact and engagement of the pilin and, most likely, the flagella (Snyder et al., 2020, Bodenmiller et al., 2004, Hershey et al., 2021), swarmer cells undergo a rapid developmental transition, resulting in loss of the flagella and retraction of the pilus (Figure 1.5B) (Shapiro, 1976).

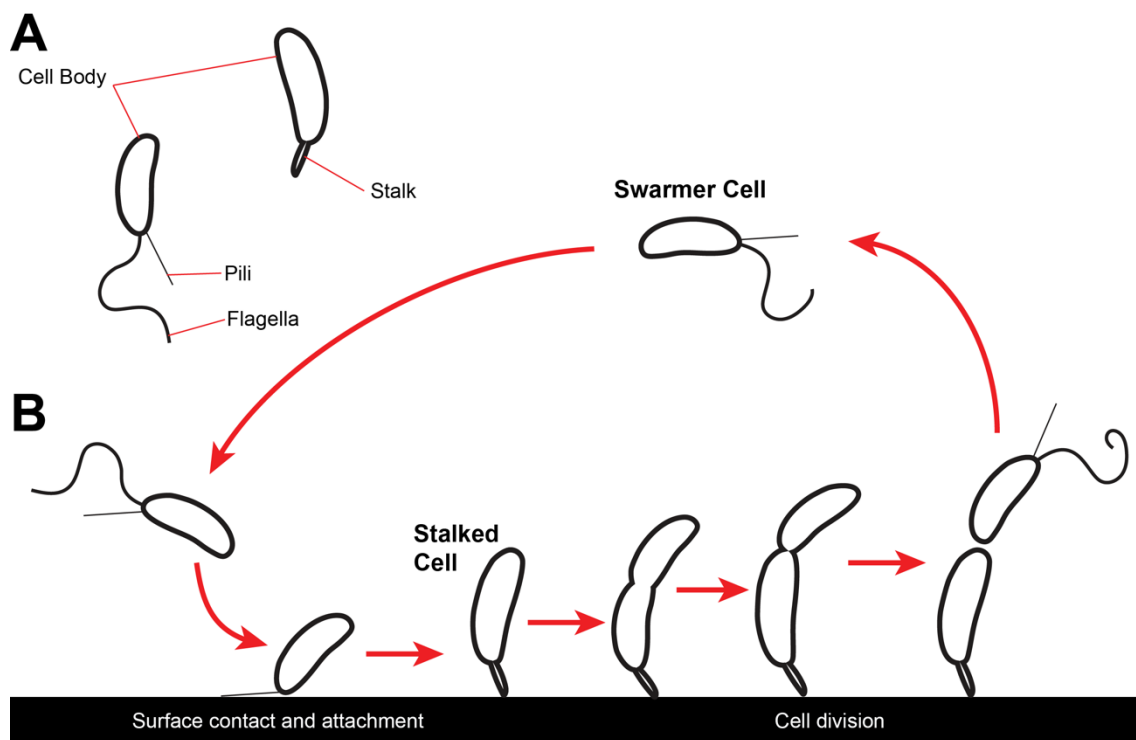


Figure 1.5. Overview of the cell cycle of *Caulobacter crescentus*.

(A) *C. crescentus* possesses a dimorphic lifecycle of morphologically distinct swarmer and stalked cells. Motile swarmer cells express a polar flagella and type IV pili. (B) Upon surface sensing, flagella and type IV pili are disposed, replaced by a cell stalk at the same pole, which facilitates surface adhesion. As stalked cells mature, cell division progresses asymmetrically, resulting in both a single swarmer and single stalked progeny.

Following the loss of the flagella and pilus, the pole quickly becomes a site of intense biosynthetic activity as the cell produces a cylindrical appendage known as a stalk. An extension of the cell, the stalk is composed of a thin tube of cytoplasmic material encompassed by the cell envelope. At the tip of the stalk lies the holdfast; a complex, multi-layered structure that mediates attachment of *C. crescentus* to the surface, secured by the holdfast anchor complex (Sulkowski et al., 2019). The holdfast binds a remarkable variety of substrates and possesses the highest tensile strength of any known adhesive, biological or synthetic, and demonstrates remarkable versatility in substrate binding (Hernando-Pérez et al., 2018). The holdfast is a complex, multi-layered structure comprised of a mixture of molecules, including polysaccharides, proteins, and DNA (Sulkowski et al., 2019).

The growth of the stalk and attachment via the holdfast anchor complex represent a key change in *C. crescentus* development, beyond just the transition from motile to sessile. Stalked cells, unlike swarmer cells, are capable of DNA replication and cell division (although stalk biogenesis is not strictly necessary for either (Fukuda et al., 1977)) (Hung et al., 1999, Shapiro, 1976). This transition, while appearing straightforward, is a result of the combined activity of four regulators, controlling over 200 genes. These are DnaA (Hottes et al., 2005), GcrA (Crosson et al., 2004), CtrA (Laub et al., 2002), and CcrM (Gonzalez et al., 2014). Together, facilitate the progression of the cell cycle, including chromosome replication, cell elongation, and cell division. Cell division proceeds asymmetrically (Skerker and Laub, 2004); *Caulobacter* cells do not form a typical septum, instead constricting the cell, with distinct constriction and fission events for the inner and outer membranes (Judd et al., 2005). The final result is two progeny cells: a stalked, sessile, division-competent cell and a mobile, flagellate, swarmer cell (Figure 1.5AB).

It may seem odd to have control of major cell cycle events coupled so closely with polar development, but it actually serves an ingenious purpose. That is, while swarmer cells are in transit, their energy is dedicated largely to motility. Once it comes into contact with a suitable surface, likely one that provides the necessary resources, it can undergo differentiation into a sessile cell and focus its energy on producing far-reaching progeny. Many prokaryotes achieve this type of differentiation across many different cells, for example *Streptomyces* spp. differentiate their cells into vegetative or aerial hyphae, respectively responsible for nutrient acquisition and dispersal (Bush et al., 2015). *Caulobacter* appears to have mastered differentiation in a single cell cycle through an incredibly robust and, in many ways, simple group of mechanisms.

1.2.2. The *Caulobacter crescentus* S-layer

1.2.2.1. RsaA; a hexameric S-layer protein

As mentioned above, the *C. crescentus* S-layer is composed of a single 1,026-amino acid residue SLP called RsaA (Smit et al., 1992). RsaA is one of the best characterised SLPs, having been the subject of numerous attempts at generating functionalised S-layers (Charrier et al., 2019, Nomellini et al., 2007, Bingle et al., 1997b, Bingle et al., 1997a) and featuring in a spate of structural studies of the S-layer (Bharat et al., 2017, von Kugelgen et al., 2020, Herdman et al., 2022). It encompasses the entire cell, creating a continuous, porous mesh across the surface, adapting to the underlying membrane curvature (Figure 1.6A). The atomic structure and organisation of the S-layer lattice is well characterised; lying approximately 180 Å from the outer membrane, the S-layer is composed of two distinct, repeating densities, reflecting RsaA's canonical bipartite arrangement (Figure 1.6B-C). The outermost, more continuous density is comprised of the lattice-forming C-terminal domain, RsaA_{CTD}, which consists of residues

251–1,026. The underlying densities, regularly spaced at intervals of approximately 220 Å (Figure 1.6B-C), are the O-antigen binding N-terminal domains, RsaA_{NTD}, comprised of residues 1–250 (Figure 1.6B-C). The former and latter domains, which are respectively composed of majority β -strands and α -helices, are joined by a flexible linker region to form a single RsaA monomer (Figure 1.7A-B), six of which oligomerise to form a hexameric unit (Figure 1.7C-D), tessellated to form the S-layer, bound to the O-antigen of the bacterial LPS via RsaA_{NTD} (Bharat et al., 2017, Smit et al., 1992). The central pore is around 20 Å wide, with additional gaps between RsaA monomers approximately 27 Å in length. These spaces, while larger than those reported for more compact S-layer architectures, such as in *C. difficile* (Lanzoni-Mangutchi et al., 2022), would suitably occlude phage and a range of macromolecules from reaching the cell surface.

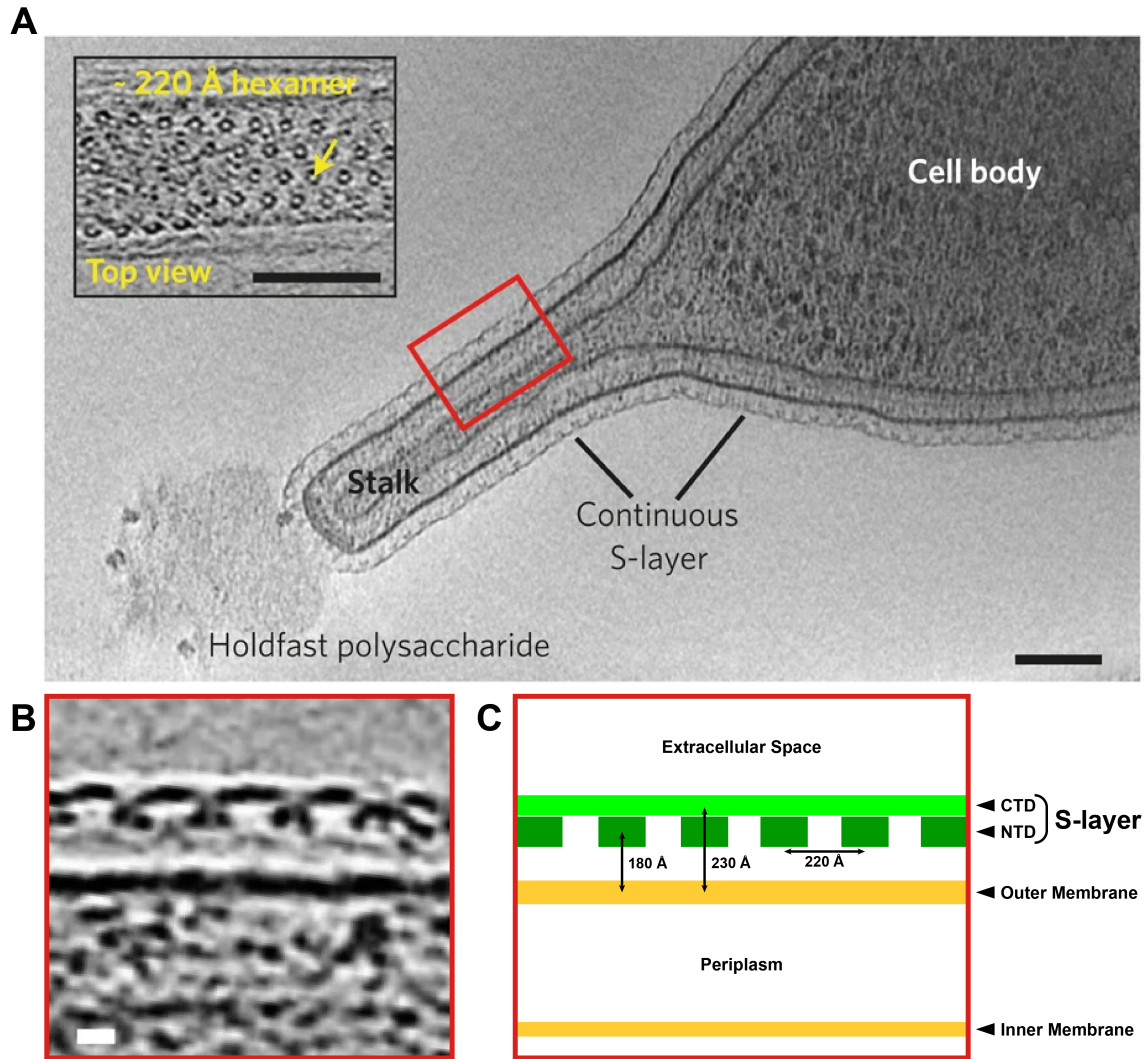


Figure 1.6. Architecture of the cell-bound *C. crescentus* S-layer.

(A) Slice through a tomogram of a *C. crescentus* cell. The S-layer is highlighted, representing a confluent layer encompassing the cell body and stalk. The inset shows a magnified tomographic slice along the top of the stalk, showing the hexameric symmetry of the S-layer. Distance between each hexameric pore is ~ 220 Å. Scale bar = 100 nm. (B) Side view of the cell envelope; the bipartite structure of the S-layer is clearly visible. Scale bar = 10 nm. (C) Schematic representation of the envelope architecture; Rsa^{ANTD} domains are ~ 220 Å apart from each other and ~ 180 Å from the outer membrane. Rsa^{ACTD} is ~ 230 Å from the outer membrane.

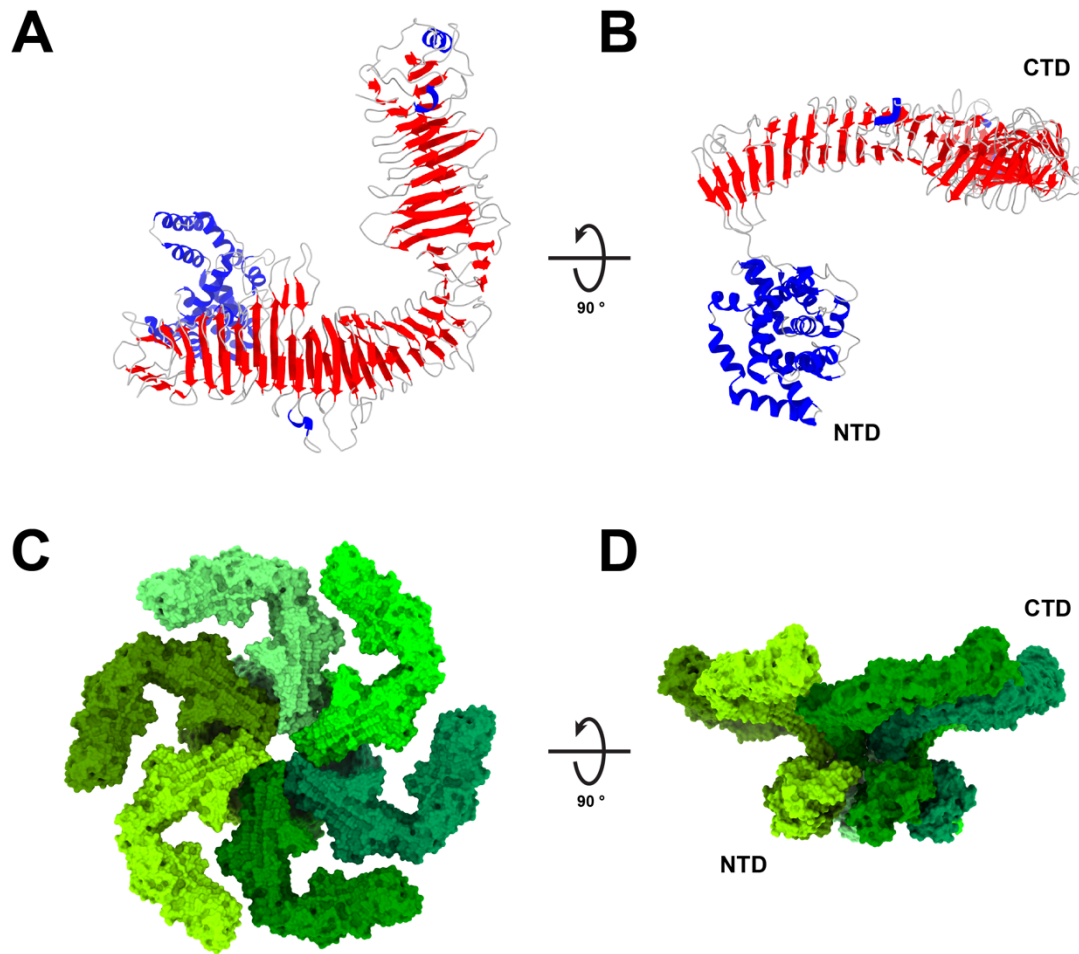


Figure 1.7. RsaA, the constituent protein of the *C. crescentus* S-layer, forms a bipartite hexamer.

(A-B) Top and side views of an RsaA monomer, displayed as a ribbon diagram (PDB 6Z7P). Secondary structure is coloured as follows: coils, grey; β -strands, red; α -helices, blue. (C-D) Top and side views of the RsaA hexamer, displayed as density diagrams, coloured in green-scale. CTD and NTD are labelled in B and D.

1.2.2.2. RsaADEF

The S-layer biosynthetic machinery in *C. crescentus*, which, as mentioned above, is composed of a single SLP, RsaA, and an envelope-spanning TISS, RsaDEF (Awram and Smit, 1998) (Figure 1.8A). Secretion of RsaA to the cell surface is dependent on an uncleaved, C-terminal secretion signal (Bingle et al., 2000). The operon, spanning

CCNA_01069 to CCNA_01067 (Marks et al., 2010), encodes RsaD, an ABC transporter protein, RsaE, a membrane fusion protein, and RsaFa, a TolC-like outer membrane protein, are encoded downstream of RsaA (Marks et al., 2010). While RsaA is the primary cargo of the secretion system, it is also responsible for the export of SapA, or S-layer-associated protease, a secreted protease implicated in S-layer maintenance and repair (Ford et al., 2007). The RsaADEF operon additionally encodes five proteins between RsaE and RsaFa (Figure 1.8A), three of which have been identified as Gmd1 (a GDP-mannose 4,6 dehydratase), PerB (a UDP-perosamine 4-acetyl transferase), and PerA (a perosamine synthetase) (Herr et al., 2018). The remaining proteins, CCNA_01065 and CCNA_01066, are putative glycotransferases, predicted to be involved in O-antigen synthesis (Herr et al., 2018). Together these proteins likely facilitate the synthesis of LPS and O-antigens necessary for RsaA binding. This is evidenced by mutations in Gmd1 results in S-layer shedding and increased resistance to the SLP-binding CR30 phage, likely due to compromised LPS synthesis (Herr et al., 2018).

The protein composition of RsaDEF and their predicted structures are similar to that of other TISS proteins (Figure 1.8B-D). However, *C. crescentus* encodes an additional TolC-like protein, RsaFb, further downstream on its chromosome (Toporowski et al., 2004). While structurally similar to RsaFa (Figure 1.8D-E), RsaFb share only ~40% peptide sequence similarity (using BLOSUM and PAM comparison matrices) .

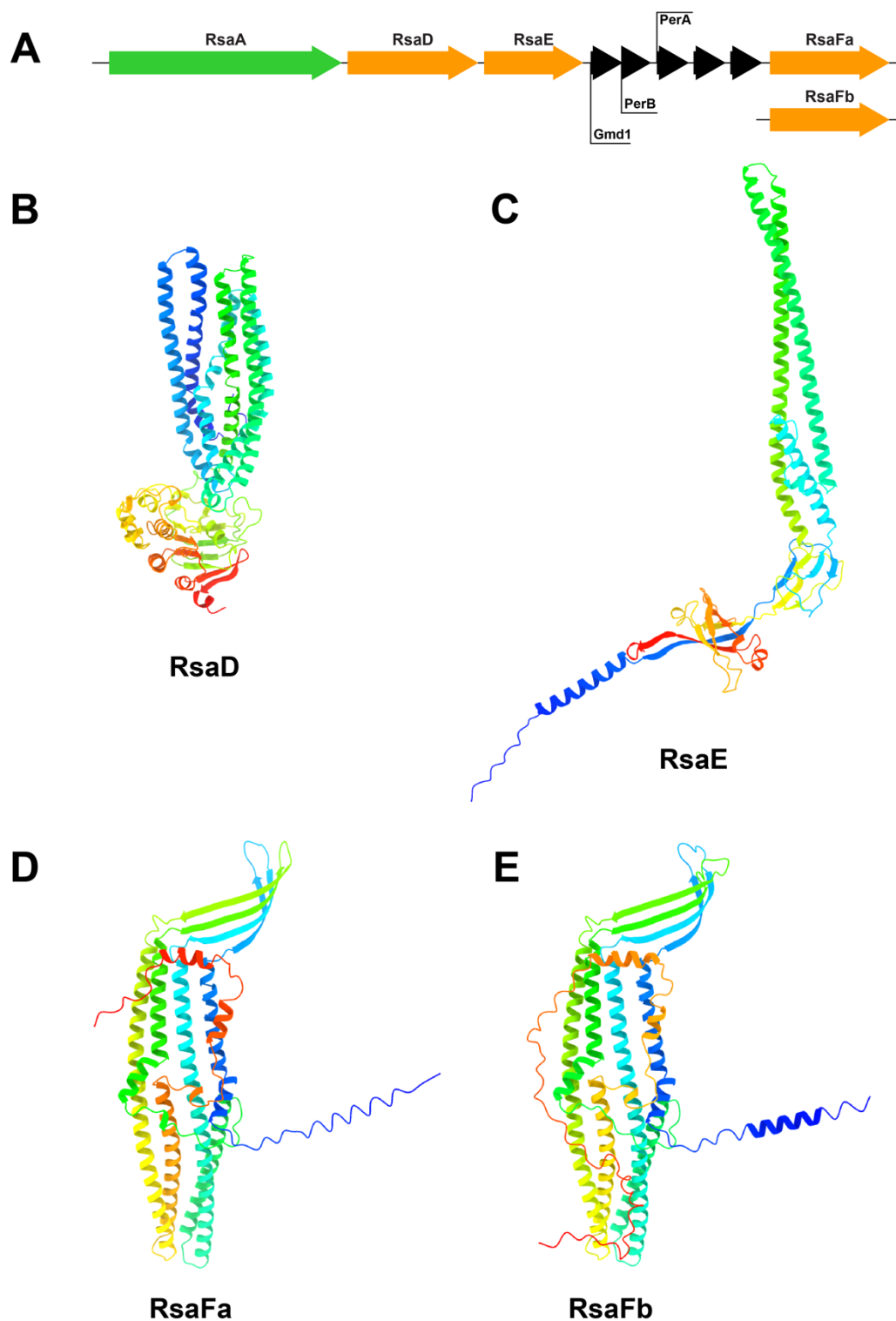


Figure 1.8. Genetic composition of the RsaADEF secretion machinery.

(A) Composition of the RsaADEF operon. Components of the TISS are coloured orange. RsaFa and RsaFb are encoded on distant loci. (B-E) AlphaFold models of constituent TISS proteins, (B) RsaD, (C) RsaE, (D) RsaFa, and (E) RsaFb. Proteins are displayed as rainbow diagrams, coloured rainbow (blue C-terminus to red N-terminus).

Both RsaFa and RsaFb are required for optimal RsaA secretion; deletion of either one results in disrupted S-layer biogenesis (Overton et al., 2016). As well as being disruptive to S-layer biogenesis, RsaFa/RsaFb deletion results in growth defects in *C. crescentus*, as well as increased activity of protein degradation pathways, due to the accumulation of RsaA in the cytoplasm (Overton et al., 2016). The composition of RsaF *in vivo* is unknown, i.e. if the TolC-like barrel, which is typically composed of three TolC monomers (Koronakis et al., 2004), exists as a hetero-oligomer of RsaFa or RsaFb, or if the two proteins exist in independent homo-trimers. AlphaFold multimer prediction suggests that RsaFa and RsaFb are capable of forming a heterotrimeric porin (Figure 1.10A-B), with high pLDDT suggesting stable complex formation (Figure 1.10C).

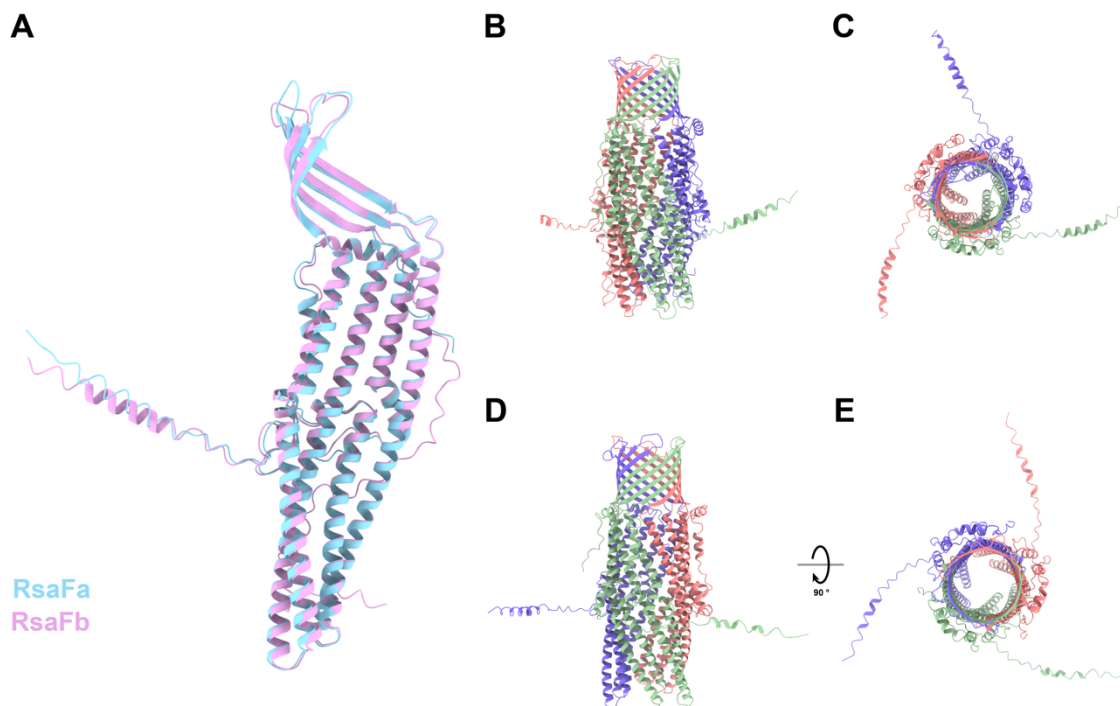


Figure 1.9. RsaFa and RsaFb are structurally homologous, TolC-like proteins.

(A) Superimposed ribbon diagrams of for RsaFa (blue) and RsaFb (pink). (B-C) Side and top views of a TolC-like, outer membrane trimeric pore complex composed of RsaFa. (D-E) Side and top views of a TolC-like, outer membrane trimeric pore complex composed of RsaFb. All protein structures shown, including oligomers, were predicted using AlphaFold.

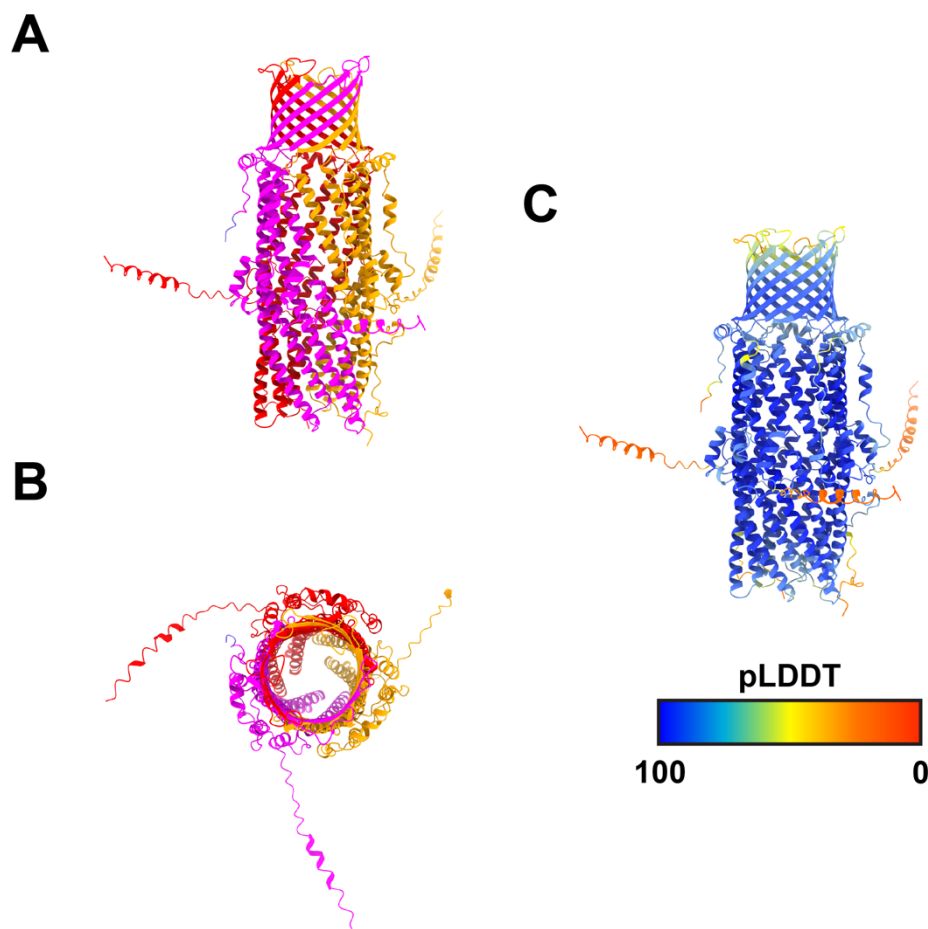


Figure 1.10. AlphaFold predicts stable heterotrimer formation between RsaFa and RsaFb.

(A-B) Side and top views of a TolC-like trimer, displayed as a ribbon diagram, composed of two copies of RsaFa (red and orange red) and one copy of RsaFb. (C) RsaF trimer coloured by AlphaFold prediction confidence; colour scale provided below corresponding to pLDDT score.

1.2.2.3. The SpyCatcher-SpyTag System in *C. crescentus*

As previously mentioned, S-layers have been subject to several attempts at creating functionalised, biological chassis composed of repeating biological units (Pum et al., 2004). Unfortunately, attempts at modifying the SLPs without disrupting S-layer biogenesis has proved difficult (Bingle et al., 1997a). However, a recent progression in SLP engineering was carried out by Charrier et al., 2019, has combined the S-layer with the SpyCatcher-SpyTag conjugation system. Utilising an engineered strain of *C.*

crenscentus (CB15N), deficient in the S-layer modifying SapA metalloprotease, Charrier et al. introduced a small peptide sequence called a SpyTag at several positions along the RsaA_{CTD} domain. Originating from the CnaB2 domain of the FbaB protein in *Streptococcus pyogenes*, the SpyTag (ST) and SpyCatcher (SC) system conjugate through the formation of an isopeptide bond between their respective Aspartic acid and Lysine residues (Figure 1.11AB) (Zakeri et al., 2012, Li et al., 2014). The SpyTag peptide component of the conjugation system, 13 amino acids in length, can be integrated into the RsaA structure seamlessly, without any observed defects to secretion, retention by the LPS, or oligomerisation (Charrier et al., 2019). This allows for any number of modified SpyCatcher proteins to stably bind the *C. crescentus* S-layer (Figure 1.11C).

Not only does this provide a promising new platform capable of using the sophisticated self-organisation offered by S-layers, which is already being explored (Molinari et al., 2022), it presents an incredible opportunity to study S-layer biogenesis in living *C. crescentus* cells. Previous studies of S-layer biogenesis in *C. crescentus* have focused on the addition of exogenous RsaA molecules to S-layer deficient strains or used in vitro formed RsaA sheets as a proxy for the S-layer (Comerci et al., 2019, Nomellini et al., 1997). Now, through the implementation of the SC-ST system into *C. crescentus*, the S-layer can be further studied in its biological context.

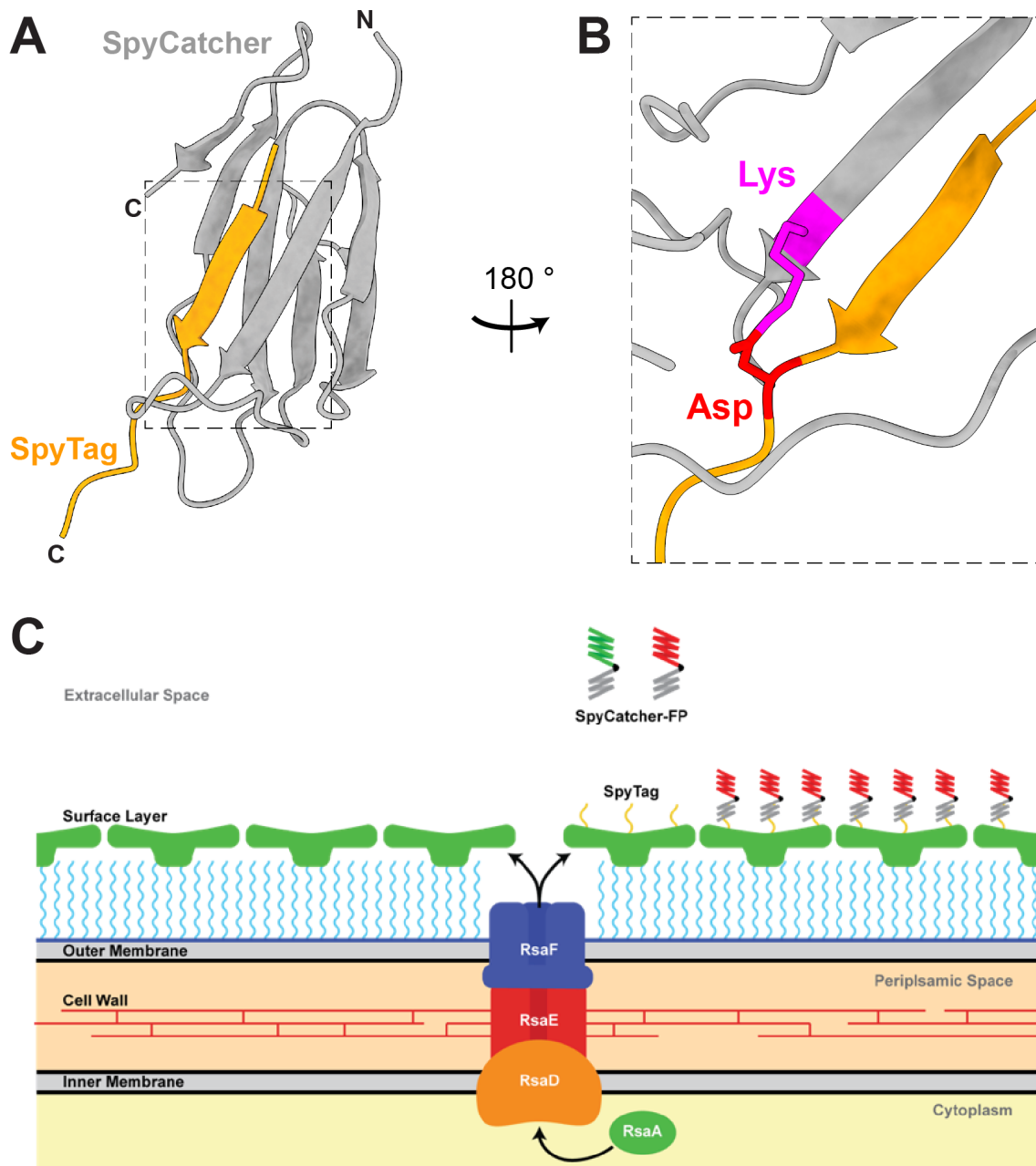


Figure 1.11. SpyCatcher/SpyTag-based Labelling of the *C. crescentus* S-layer

The recombinant protein conjugation system, SpyCatcher/SpyTag, is an engineered form of the CnaB2 domain of the FbaB protein from *Streptococcus pyogenes*. (A) Crystal structure of the SpyCatcher/SpyTag complex, (PDB: 4MLI), consisting of a 12.3 kDa SpyCatcher protein (grey) and a 13-aa SpyTag peptide (orange). (B) Stable conjugation of the SpyCatcher and SpyTag components are mediated by an isopeptide bond between their respective lysine and Aspartic acid residues. (C) Schematic representation of an engineered strain of *C. crescentus* expressing RsaA-SpyTag for S-layer labelling. SpyCatcher proteins modified with N-terminal fluorescent proteins (SpyCatcher-FP). SpyCatcher-FP proteins stably bind RsaA hexamers in the S-layer.

CHAPTER 2. Materials and Methods

2.1. Strains and Growth Conditions

Strains and plasmids used in this study are listed in Table 2.1. *C. crescentus* strains were grown in PYE (Poindexter, 1964) containing no antibiotics at 30 °C (with shaking for liquid cultures). Where specified, *C. crescentus* liquid cultures were also grown in defined M2G media (Johnson and Ely, 1977). *E. coli* BL21 (DE3) (ThermoFisher) and LMG194 (ATCC 47090) were grown on LB agar at 37 °C with 100 mg/mL ampicillin following transformation. *C. crescentus* and *E. coli* strains were preserved for long term at -80 °C in 10% DMSO or 20% glycerol respectively.

Table 2.1. Bacterial strains and plasmids.

Description of bacterial strains and plasmids used in this study .

Strain or Plasmid	Description	Source or Reference
<i>Escherichia coli</i>		
BL21 (DE3) (200131)	Chemically competent BL21 (DE3) strain for protein over-expression	Agilent
LMG194	Chemically competent strain for protein over-expression	Caroline Ajo-Franklin (Charrier et al., 2019)
<i>Caulobacter crescentus</i>		
CB15N	Wildtype	ATCC
<i>C. crescentus</i> CB15N $\Delta sapA$ <i>rsaA467::SpyTag</i>	Engineered strain; RsaA467-SpyTag for SC-ST conjugation labelling	Caroline Ajo-Franklin (Charrier et al., 2019)
<i>C. crescentus</i> CB15N <i>rsaA</i> _{TEV250}		
pDEST14-SpyCatcher-sfGFP	SC-sfGFP overexpression strain	
pBAD-SpyCatcher-mRFP1	SC-mRFP1 overexpression strain	
pBAD-SpyCatcher-mTurquoise	SC-mTurquoise overexpression strain	

2.2. SpyCatcher Purification

His-tagged SpyCatcher conjugates were purified as previously described using nickel-affinity chromatography (Charrier et al., 2019). Plasmids pDEST14-SpyCatcher-sfGFP (Keeble et al., 2019) and pBAD-SpyCatcher-mRFP1 (Charrier et al., 2019) were transformed into chemical competent cells *E. coli* BL21 (DE3) and LMG194 cells respectively, and grown on LB agar with 100 µg/mL Ampicillin (LB-Amp). A single colony of each strain was inoculated into 6 L of LB-Amp media and incubated at 37 °C with shaking until cells had reached mid-log growth phase. Cells were induced with 0.2% (w/v) arabinose (LMG194) or 0.4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (BL21) and incubated at 20 °C for 16 h. Induced cultures were harvested by centrifugation, resuspended in lysis buffer (30 mM Tris/HCl pH 8.0, 500 mM NaCl, 1 mM MgCl₂, 50 µg/mL DNase, 300 µg/mL lysozyme, and 1x cOmplete Protease Inhibitor), and lysed by five passes through the homogeniser at 15,000 psi (pounds per square inch) pressure. Cell debris were pelleted and the supernatant filtered using a 0.22 µm syringe filter. SpyCatcher proteins were then bound to a 5 mL HisTrap HP column (GE Healthcare) using an ÄKTA pure 25 M system (GE Healthcare) and eluted against the same buffer including 500 mM imidazole over 10 column volumes. Eluates were dialysed overnight with 1:100 (w/w) His₆-TEV protease at 4 °C against 2 L of MilliQ H₂O. The dialysates were further purified via size exclusion chromatography using a HiLoad Superdex S200 16/600 (prep grade) column; final proteins were eluted in HEPES buffer (25 mM HEPES/NaOH pH 7.5, 150 mM NaCl), and flash frozen in liquid nitrogen and stored at –80 °C.

2.3. SpyCather and HADA Labelling

C. crescentus RsaA467-ST cells were grown in M2G (6.1 mM Na₂HPO₄, 3.9 mM KH₂PO₄, 9.3 mM NH₄Cl, 0.5 mM MgSO₄, 0.01 mM FeSO₄, 0.2% (w/v) Glucose) media, supplemented with 0–500 µM CaCl₂, at 30 °C with aeration by shaking to mid-log growth phase (~OD₆₀₀ = 0.6). Cells were resuspended to an OD₆₀₀ of 0.1 in the same media supplemented with 10 µM of the appropriate SC-FP conjugate. All labelling steps were carried out at 4 °C for 16 h, after which point cells were harvested by centrifugation and washed three times in their respective M2G media. Where indicated, cells were resuspended in the appropriate M2G media and incubated at 30 °C for 3 h with 10 µM of a contrasting SC-FP to promote growth and S-layer labelling. After labelling, cells were harvested, washed as previously and resuspended.

For cell wall labelling, samples were supplemented with 500 µM of the fluorescent D-amino acid HADA(Peters et al., 2019) (Cambridge Biosciences) for the last 10 minutes of the chase-labelling incubation. Cells were then harvested and washed as described above. All labelling steps were carried out with the specimen protected from light-exposure.

2.4. Sample Preparation for Light Microscopy

3-5 µL of labelled cell suspensions were spotted onto agarose pads (1% (w/v) agarose in distilled water) enclosed by a 15 mm × 16 mm Gene Frame (ThermoFisher) on a glass slide and sealed with a glass coverslip. Samples were protected from light prior to imaging.

2.5. Light Microscopy

For widefield microscopy, samples were imaged using a 100× objective lens using a Zeiss AxioImager M2 widefield microscope (Carl Zeiss). For high resolution microscopy in Chapter 4 and 5, cells were imaged using an Olympus SoRa spinning disc confocal microscope, equipped with Olympus IX-83 inverted frame, Yokogawa SoRa super-resolution spinning disc module, and Prime BSI camera. Slides were kept at room temperature and imaged using the 60x (1.5 NA) lens, with excitation at 488 nm (sfGFP) and 561 nm (mRFP1) (solid state lasers), 200 ms exposure. Z-stacks were taken at 0.26 μm intervals and a super resolution filter applied to the entire stack using Olympus CellSens software, followed by deconvolution using a maximum likelihood algorithm (5 iterations).

For HADA-labelled samples, samples were imaged with an Olympus Fluoview FV1200 equipped with GaAsP detectors. Images were acquired using a 100 x (1.4 NA) lens with excitation via solid state 405 nm, 559 nm, and 488 nm lasers. Scanning progressed left to right over a 1024 x 1024 pixel area. A 2-iteration Kalman filter was applied for all image collections. Z-stacks were not collected for sample imaged in this way.

2.6. Light Microscopy Image Processing and Analysis

All Images were background-subtracted and filtered using a 1-pixel Gaussian blur unless stated otherwise. Z-stacks, when collected, were condensed using a maximum Z-projection of frames containing the cell of interest (± 1 frame on the upper and lower Z-axis), unless otherwise stated. Cell debris, overlapping cells, or cells on the edge of the micrographs were excluded from all analyses. Using the MicrobeJ plugin for ImageJ

(Ducret et al., 2016), cells were segmented and the average pixel intensity value extracted for quantification of the SC-FP labelling efficiency.

Demographs and cell profile analyses were generated using the MicrobeJ for ImageJ (Ducret et al., 2016). Fluorescence profiles are normalised unless otherwise stated. For cell-profile analysis, a 3-pixel line was drawn along the long-axis of the cell through the cell body, straightened, and the pixel values extracted. Pixel intensity was displayed at a relative scale to show localisation of the fluorescence signal along the cell body. For colocalisation studies, masks were developed for individual cells in ImageJ and colocalisation measured using Pearson's Colocalisation Coefficient (PCC) in the Coloc2 plugin. Colocalisation was then measured between the indicated channels and reported as an R-score.

2.7. RsaA Purification by Low pH Exposure

RsaA^{CTD} was purified as described by Bharat et al., 2017. Briefly, *C. crescentus* CB15N (NA1000) cells were grown in PYE (Poindexter, 1964) medium for 24 h at 25 °C with shaking. The culture was centrifuged (5000 rcf, 4°C, 30 min) and the cell pellet was re-suspended in chilled, 50 mM HEPES/HCl (pH 2.0). The suspension was placed on ice for 10 minutes with vigorous shaking, then centrifuged (16000 rcf, 4 °C, 30 minutes). The supernatant was retained and pH adjusted to 7.0 using 5 M NaOH, then syringe filtered. The filtrate was loaded onto a 5 mL HiTrap SP HP column (GE Healthcare) at a rate of 1 mL/min; the flow-through from the column was collected, transferred to 10'000 MWCO dialysis tubing, and dialysed against 10 mM Tris/HCl pH 8.0 overnight at 4 °C. The dialysed solution was loaded onto a 5 mL HiTrap Q HP column (GE Healthcare) (flow rate 2 mL/min throughout), washed with 20 mM Tris/HCl pH 8.0, and eluted using an increasing concentration of NaCl. RsaA-containing fractions were

collected and dialyzed against 20 mM Tricine/NaOH (pH 8.0) and then concentrated to ≥ 25 mg/mL. Aliquots were flash frozen prior to storage at -80 °C

2.8. RsaA Crystallisation

RsaA_{CTD} protein was crystallised as described previously (Bharat et al., 2017), following previous optimisation in the MRC Laboratory of Molecular Biology's inhouse robotic nano-litre crystallisation facility (Stock et al., 2005). Prior to crystallisation, the protein solution was supplemented with 5 mM CaCl₂ prior to crystallisation. Crystals were grown at 19 °C by sitting-drop vapour-diffusion in a drop composed of 100 nL of reservoir solution (0.07 M KSCN, 24% (w/v) PEG 8000, 0.075 M TAPS/ NaOH pH 8.5) and 100 nL of protein solution at 30 mg/mL. Plate-like crystals appeared in 3–10 days and continued growing to a final size of 300 x 300 x 30 μm^3 . Crystals were flash-cooled with liquid nitrogen for data collection using an additional 25% (v/v) PEG 200 as cryo-protectant.

2.9. Cryo-EM Grid Preparation

For cryo-EM grid preparation, *C. crescentus* cells resuspended in PBS supplemented with 0.1 mM CaCl₂ and 0.1 mM CaCl₂ or 1 X M2G salts to a final OD₆₀₀ of 0.5. 2.5 μL was then applied to a freshly glow discharged Quantifoil R2/2 Cu/Rh 200 mesh grid, adsorbed for 10 s, blotted for 2.5-4 s and plunge-frozen into liquid ethane in a Vitrobot Mark IV (ThermoFisher), while the blotting chamber maintained 100% humidity at 10°C. 10 nm protein-A gold (CMC Utrecht) was additionally added to the cell suspension prior to blotting (ratio 1 Au : 12 sample).

2.10. Cryo-ET Data Collection

Initial cryo-EM assessment of samples was conducted on an FEI Talos Arctica 200-kV cryo-transmission electron microscope (cryo-TEM) fitted with a Falcon 3 direct electron detector. Cryo-ET data collection was performed on Titan Krios microscopes running at 300 kV (FEI/ThermoFisher), each fitted with a Quantum energy filter (slit width, 20 eV) and a K2 direct electron detector (Gatan) running in counting mode with a dose rate of approximately 8 electrons/pixel/s at the camera level. Tilt series were collected between $\pm 60^\circ$ in two directions with a 1° increment using SerialEM (Mastronarde, 2005) as previously described (Sulkowski et al., 2019). A total dose of 100-121 $e^-/\text{\AA}^2$ was applied, and data were sampled at pixel sizes of 5.3 $\text{\AA}$ for all other tomograms.

2.11. Tomogram Reconstruction

Following data collection, contrast transfer function (CTF) parameters for each micrograph in the tilt series were estimated using CTFFIND4 (Mindell and Grigorieff, 2003). Tilt series alignment was performed using gold fiducial markers using IMOD (Kremer et al., 1996). Gold fiducial densities were erased before CTF compensation within IMOD, followed by tomographic reconstruction using the Simultaneous Iterative Reconstruction Technique (SIRT) algorithm implemented within TOMO3D (Fernandez et al., 2018, Agulleiro and Fernandez, 2015). Tomograms were analysed in IMOD and binned 4 x (x and y axis) prior to inclusion in all figures, unless otherwise stated.

2.12. Molecular Dynamics Simulations

Atomistic simulations were run in triplicate for 100 ns using the CHARMM36m forcefield. Simulations were performed at 310 K using the velocity-rescaling temperature

coupling algorithm (Bussi and Parrinello, 2007), with a time constant of 0.1 ps and Parrinello-Rahman isotropic pressure coupling of 1 bar with a time constant of 2 ps (Parrinello and Rahman, 1981). Electrostatics were handled using the Particle-Mesh-Ewald method (Darden et al., 1993), and a force-switch modifier was applied to the van der Waals forces. Dispersion corrections were turned off. The parameters for the O-antigen were generated using the CHARMM-GUI (Kim et al., 2017, Lee et al., 2016). All simulations were run using Gromacs 2019 (Pronk et al., 2013).

2.13. Particle Induced X-ray Emission using a microfocused beam (microPIXE)

Calcium stoichiometry in purified protein was measured using μ beam Particle Induced X-ray Emission (μ PIXE), an established technique for such analyses (Garman & Grime, 2005). For μ PIXE measurements of proteins, sulphur acts as an internal standard, since the number of methionines and cysteines is known from the primary sequence. Measurements were carried out at the Ion Beam Centre, University of Surrey, UK (Grime, Dawson, Marsh, McArthur, & Watt, 1991). Characteristic X-ray emission was induced using a 2.0 μ m diameter 2.5-MeV proton beam incident on dried protein droplets (volume per droplet approx. 0.1 μ l) under vacuum. Emitted X-rays were detected in a high energy resolution solid state lithium drifted silicon detector. Through scanning the proton beam in x and y over the dried sample, spatial maps were obtained of all elements heavier than magnesium present, and 3- or 4-point spectra were collected from each droplet. GUPIX (Maxwell, Teesdale, & Campbell, 1995) within DAN32 (Grime, 1996) was used to analyse these spectra to extract the relative amount of each element per protein molecule, particularly calcium, in the sample.

2.14. Long Wavelength X-ray Diffraction

X-ray diffraction data were collected at beamline I23, Diamond Light Source (Wagner et al., 2016), equipped with a Pilatus 12M (Dectris AG, Switzerland) detector, at four energies, 4.1, 3.95, 3.7 and 3.55 keV using the inverse beam method with 20° wedges. Data were processed using XDS and half-datasets were merged using XSCALE. Data were collected from several crystals; however, non-isomorphism forbade using multi-crystal averaging to improve the signal and the datasets were treated separately. The structures were solved by molecular replacement using the deposited model (PDB ID 5N97) with removed metal ions. From the structure factors deposited in the PDB, native electron density maps were generated in Phenix (Adams et al., 2011). The anomalous difference maps from the long-wavelength data were generated using ANODE (Thorn and Sheldrick, 2011a). The positions of anomalous peaks higher than 5.0 σ from datasets both above and below the calcium and potassium K-edge were inspected in COOT (Emsley and Cowtan, 2004) and compared with the difference peaks in the native omit maps. Ca^{2+} and K^{+} ions were modelled in the positions where positive omit difference density peaks ($>3.5 \sigma$) overlapped with peaks in the anomalous difference map from data collected above the respective absorption edge while absent in the corresponding map below the edge.

2.15. Additional Quantification and Statistical Analysis

Quantification of micrographs from light microscopy experiments were carried out using MicrobeJ and analysed using GraphPad PRISM. For all figures where appropriate, the number of measurements performed, along with mean and/or median and standard deviations are reported within the figure or the figure legends.

Table 2.2. Software and Algorithms.

A list of software and algorithms used in this thesis.

Software	Reference	Availability
ANODE	(Thorn and Sheldrick, 2011b)	https://shelx.uni-goettingen.de/
CCP-EM suite	(Burnley et al., 2017)	https://www.ccpem.ac.uk/
CCP4	Collaborative Computational Project, Number 4, 1994	https://www.ccp4.ac.uk/
ChimeraX	(Goddard et al., 2018)	https://www.rbvi.ucsf.edu/chimerax/
Coot 0.8.9.1	(Emsley and Cowtan, 2004)	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/cool/
CTFFIND4	(Rohou and Grigorieff, 2015)	https://grigoriefflab.umassmed.edu/ctf_estimation_ctffind_ctfilt
Excel 16	Microsoft	https://www.office.com
Gromacs 2019	(Pronk et al., 2013)	https://manual.gromacs.org/documentation/2019/index.html
GUIXWIN	(Maxwell et al., 1995)	https://www.physics.uoguelph.ca/about-gupix-and-gupixwin
ImageJ/Fiji	(Schindelin et al., 2012)	https://www.fiji.sc/
IMOD	(Mastronarde and Held, 2017)	https://bio3d.colorado.edu/imod/
MicrobeJ	(Ducret et al., 2016)	https://www.microbej.com/
MotionCor2	(Zheng et al., 2017)	https://emcore.ucsf.edu/
PHENIX	(Adams et al., 2011)	http://www.phenix-online.org/
Prism 9	GraphPad	https://www.graphpad.com/
Python		
OMDAQ-3	Oxford Microbeams Ltd	http://www.microbeams.co.uk/
Refmac5	(Murshudov et al., 2011)	https://www.ccp4.ac.uk/
Relion 3.0	(Zivanov et al., 2018)	
SerialEM	(Mastronarde, 2005)	https://bio3d.colorado.edu/SerialEM/
Tomo3D	(Agulleiro and Fernandez, 2015)	
UCSF Chimera	(Pettersen et al., 2004, Goddard et al., 2018)	https://www.cgl.ucsf.edu/chimera/
XDS	(Kabsch, 2010)	https://xds.mr.mpg.de/
XSCALE	(Kabsch, 2010)	https://xds.mr.mpg.de/

CHAPTER 3. Calcium-dependent S-layer Biogenesis in *C. crescentus*

3.1. Overview

This project sought to evaluate the role of calcium in S-layer biogenesis in *C. crescentus*. SLPs, including RsaA, form high-order structures and S-layers *in vitro* following the addition of divalent cations (Herrmann et al., 2020, Farci et al., 2018, Baranova et al., 2012, Smit et al., 1992). Additionally, *C. crescentus* has an unusually high demand for calcium for optimal growth. Partnered with previous observations that a Ca^{2+} depletion results in S-layer defects (Herrmann et al., 2017), it is clear that calcium plays a crucial role in S-layer maintenance. .

Tagging of RsaA or its component secretion system, the T1SS RsaDEF, often results in defective S-layer biogenesis, meaning the system is intractable to common genetic tagging methods. An engineered strain of *C. crescentus* encoding a SpyTag presenting RsaA[†] enables stable labelling of surface-bound RsaA through SC-ST conjugation (Charrier et al., 2019, Zakeri et al., 2012, Keeble et al., 2019), enabling observation of S-layer dynamics under a variety of conditions. Using a combination of light microscopy, cryo-ET and biochemical analyses, this study provides insight into the importance of Ca^{2+} ions in S-layer oligomerisation and binding to the OPS.

3.2. Acknowledgments

The work in this chapter was performed by the candidate with the following exceptions. Initial crystallisation of RsaA (reported previously) was performed by Tanmay Bharat. X-ray diffraction measurements on the long-wavelength beamline I23

[†] For simplicity, strain *C. crescentus* CB15N $\Delta sapA$ *rsaA467:SpyTag* will be referred to as *C. crescentus* RsaA467-ST here forth.

were performed by Armin Wagner, Ramona Duman and Kamel El-Omari. MD simulations were performed in collaboration with Phillip Stansfeld. MicroPIXE measurements were performed in collaboration with Elspeth Garman. Cryo-ET data collection was performed with the guidance of Tanmay Bharat and Andriko von K  gelgen.

3.3. Aims

- (i) Labelling of the *C. crescentus* S-layer using fluorescent SC-ST conjugation to observe the effects of calcium depletion on S-layer biogenesis and retention, using light microscopy to quantify.
- (ii) Directly observe *C. crescentus* cells using cryo-ET to investigate S-layer composition and stability, providing further context to observations made in light microscopy studies.
- (iii) Use molecular dynamics simulations to parse the importance of predicted metal-binding residues in the RsaA structure.
- (iv) Map the locations and identities of bound divalent ions, particularly Ca^{2+} , to high resolution using LWXD of RsaA_{CTD} crystals.

A portion of results relevant to this chapter are published in Herdman *et al.*, *Structure*, February 2022.

3.4. RESULTS

3.4.1. Reduction in CaCl₂ Concentrations in *C. crescentus* Growth Medium Inhibits SpyCatcher-led Labelling of the S-layer.

C. crescentus requires an unusually high concentrations of CaCl₂ (500 µM) in its medium to achieve optimal growth (Ely, 1991, Johnson and Ely, 1977). This demand has typically been attributed to the constituent SLP of *C. crescentus*, RsaA, as its attachment to the cell and assembly into the S-layer appears to be Ca²⁺ dependent (Nomellini et al., 1997, Smit et al., 1992). In calcium-starved conditions, RsaA has been shown to either shed from the cell or adopt an aggregated form (Rodrigues-Oliveira et al., 2019). Additionally, S-layer deficient *C. crescentus* mutants show an increased sensitivity to calcium starvation that can be overcome by further mutations in the LPS-biosynthetic pathway, adapting the cell to low-calcium conditions (Walker et al., 1994).

To understand the relationship between Ca²⁺ availability and S-layer biogenesis, we utilised an engineered strain of *C. crescentus* RsaA467-ST to directly label cell-bound RsaA. Cells were grown to mid-log phase in M2G defined medium (Johnson and Ely, 1977), supplemented with increasing concentrations of CaCl₂. Cells were incubated with SC-mRFP1 (10 µM) for 16 h at 4 °C to achieve the maximum labelling of surface-bound RsaA467-ST peptides. *C. crescentus* growth was negatively impacted by reduced concentrations of Ca²⁺ ions, and, in CaCl₂-depleted media, lag phases were often in excess of 24 h. We observed that cells grown in M2G medium containing optimal (500 µM) CaCl₂ showed an intense fluorescent signal (Figure 3.1A), suggesting efficient conjugation of cell-bound RsaA467-ST with SC-mRFP1. Lower CaCl₂ concentrations also resulted in cells with an observable fluorescent signal (Figure 3.1B-C) however the signal was less intense when compared to that of cells grown in optimal CaCl₂. Cells with faint fluorescent signals appear indistinguishable from the unlabelled control cells (Figure

3.1F) when images were contrasted under the same calibration, due to the overwhelming fluorescence signal of cells grown with optimal calcium.

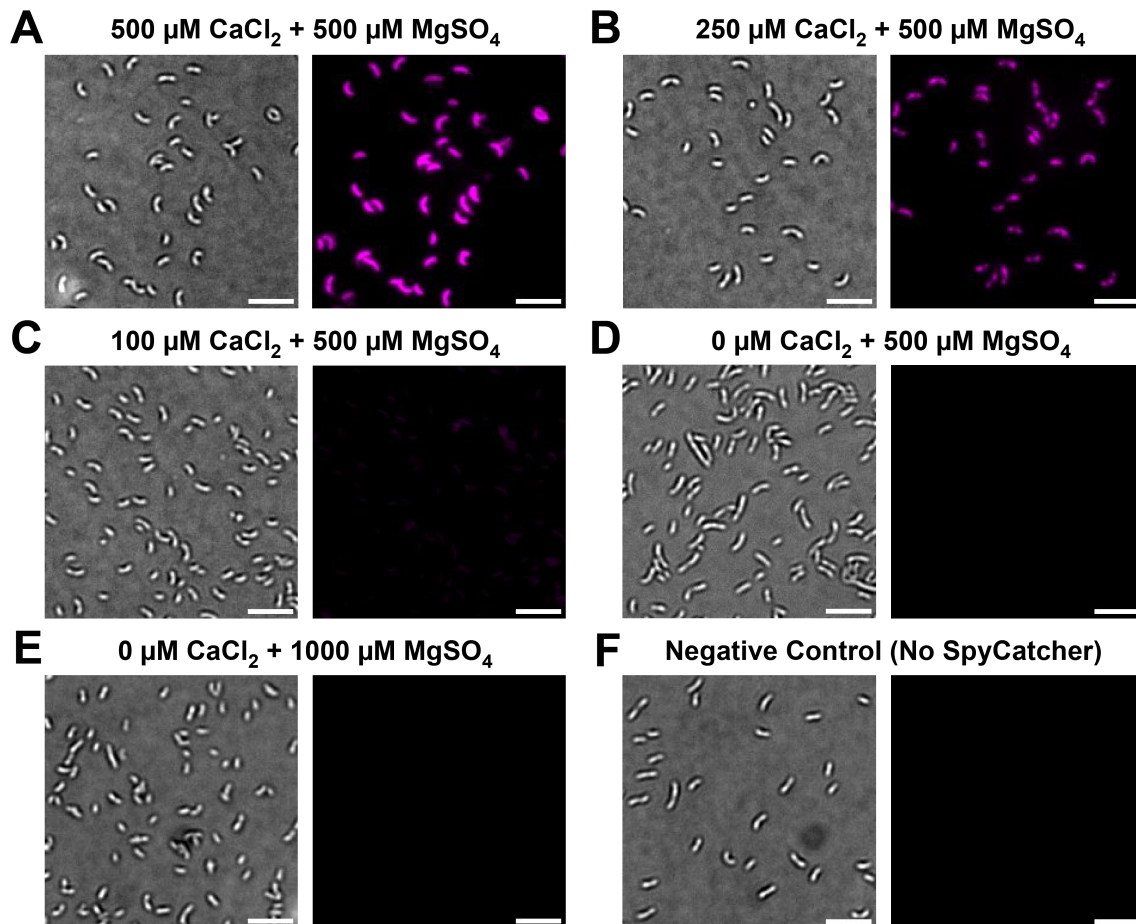


Figure 3.1. Reduction of CaCl_2 results in attenuated SC-mRFP1 labelling of cell-bound RsaA467-ST.

C. crescentus RsaA467-ST cells labelled with SC-mRFP1 in M2G media supplemented with (A) 500 μM (B) 250 μM (C) 100 μM (D) 0 μM CaCl_2 , or (E) M2G media without CaCl_2 and 1000 μM MgSO_4 (a two-fold increase from other media conditions). (F) Cells grown in M2G containing 500 μM CaCl_2 (as A) and without SC-mRFP1 serve as a negative control. Scale bar = 10 μm .

To determine if the changes in fluorescence signals were specific to Ca^{2+} ions, *C. crescentus* RsaA467-ST cells were also grown in M2G containing 0 μM CaCl_2 and 1,000 μM MgSO_4 (Figure 3.1E) – a 2-fold increase in Mg^{2+} ions over standard media. The

fluorescence from these cells was indistinguishable from the unlabelled control (Figure 3.1F), Mg^{2+} is not capable of restore SC-mRFP1 binding by RsaA467-ST. Unfortunately, cells grown in media containing reduced $MgSO_4$ failed to grow, so it is unclear what effect a loss of Mg^{2+} ions may have on labelling.

To quantify the changes in fluorescence signal between samples, cells were segmented from using ImageJ and their pixel values extracted in MicrobeJ (Ducret et al., 2016). As expected, cells grown in 500 μM $CaCl_2$ had the highest average fluorescence intensity (Figure 3.2). As $CaCl_2$ concentrations were reduced, an almost exponential decline in average fluorescence was observed. At 0 μM $CaCl_2$, the average fluorescence intensity was indistinguishable from unlabelled cells, even when supplemented with excess $MgCl_2$, as determined by one-way ANOVA. As SpyCatcher proteins form a spontaneous isopeptide bond with their partner SpyTag peptide across variable temperature, buffer, and pH conditions (Zakeri et al., 2012) independently of either Ca^{2+} or Mg^{2+} ions (Zakeri et al., 2012), the media compositions used in this study, including the variable concentration of $CaCl_2$, is unlikely to have affected SC-ST conjugation. Together, these results clearly suggest the labelling of the S-layer of *C. crescentus* using ST-SC conjugation requires the presence of Ca^{2+} and any reduction in $CaCl_2$ has a direct effect on labelling efficiency.

Cell shape appears to be largely unaffected by $CaCl_2$ variation; cells had comparable distributions of length and area across media compositions (Figure 3.3A-B). However, in Therefore, the observed difference in SC-mRFP1 binding is likely due to the composition of the S-layer on the cell surface, either being shed or aggregated (and therefore unavailable for labelling via SC-ST conjugation) as seen in previous studies. Additionally, the inability of excess Mg^{2+} to restore labelling may be attributed to the

reduced ability of Mg^{2+} to facilitate S-layer oligomerisation, as previously reported (Bharat et al., 2017).

These observations support the hypothesis that the formation of a viable S-layer is strongly dependent on the concentration of Ca^{2+} in the growth medium and shows that even a small reduction in the available CaCl_2 can significantly affect the retention and display of the S-layer on cells, in agreement with other reports (Herrmann et al., 2017). This metal-dependent S-layer biogenesis may be specific to Ca^{2+} , as these results seem to suggest, as Mg^{2+} is unable to fulfil the same function. Interestingly, the increase of MgSO_4 did appear to overcome the extended lag phases seen in calcium-starved cells, despite being unable to facilitate S-layer formation. Future experiments may seek to determine if other divalent cations may fulfil a similar role to Ca^{2+} , as several studies have shown alternative ions may mediate S-layer formation in vitro, including Mg^{2+} , albeit with varying success (Engelhardt and Peters, 1998, Engelhardt, 2007).

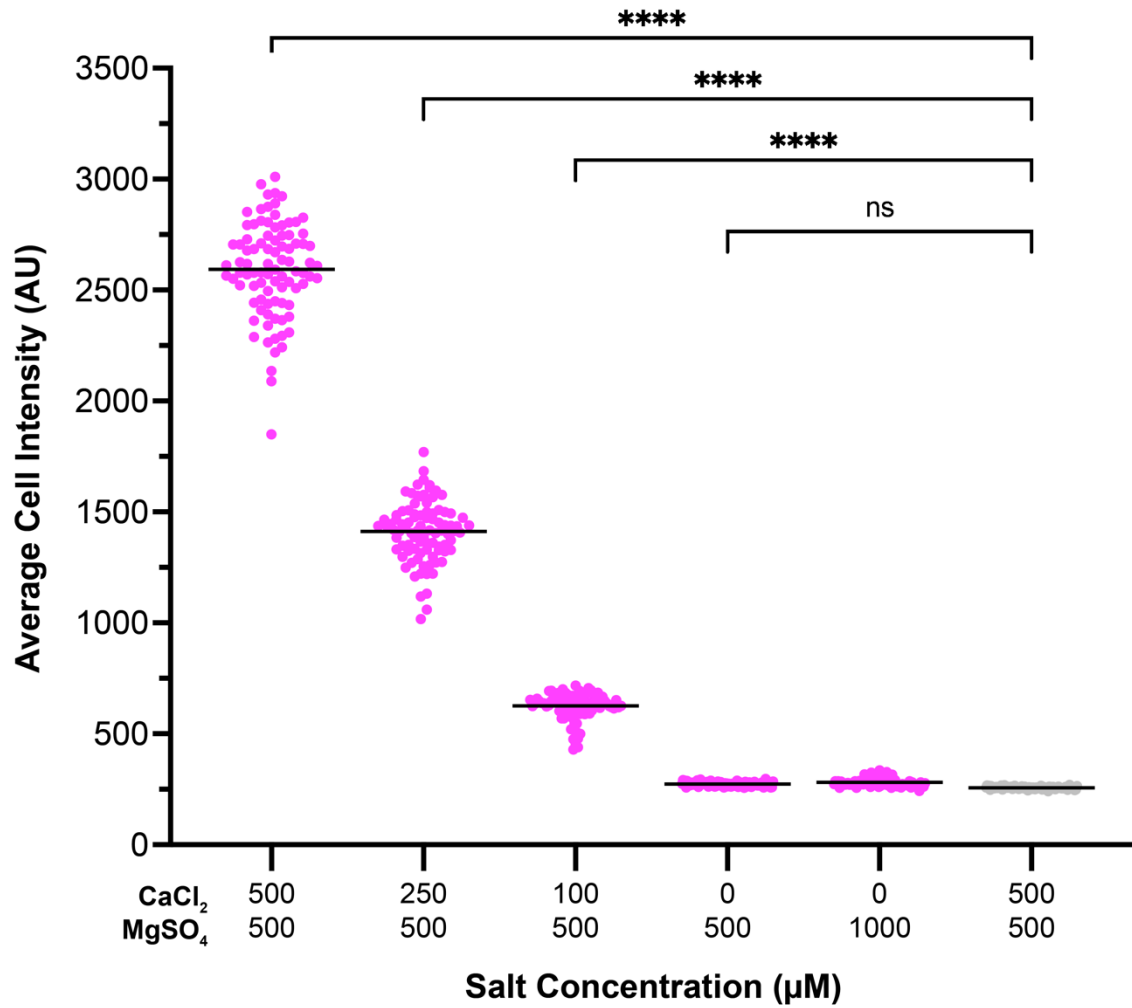


Figure 3.2. Quantification of fluorescence reduction following CaCl₂ depletion.

Average cell intensities (● for SC-mRFP1 labelled cells) were quantified for each condition using the MicrobeJ plugin for ImageJ (Ducret et al., 2016). One-way ANOVA shows significantly different means for cells labelled in the presence of CaCl₂ compared to the negative control containing no SC-mRFP1 (●). Cells grown in media containing no CaCl₂ showed no significant difference from the negative control, even with additional MgSO₄. Significance is indicated by ns (not significant) or an asterisk (****, $p < 0.0005$). Black line shows average.

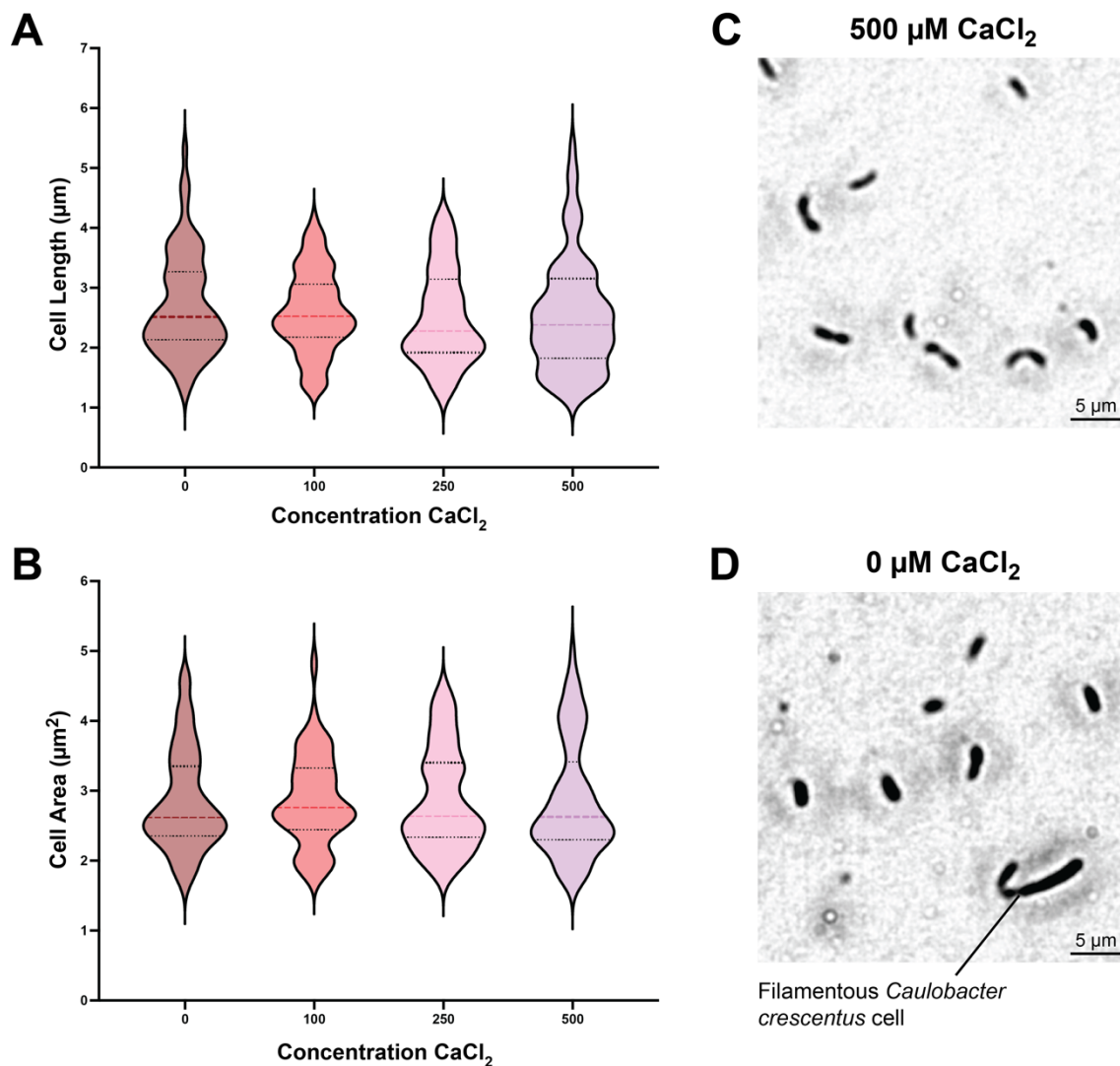


Figure 3.3. Morphological comparisons between *C. crescentus* cells grown in varying concentrations of CaCl_2 .

Cell (A) lengths and (B) areas were extracted and analysed to compare potential variations in cell size and morphology across different CaCl_2 concentrations (cell $n \geq 50$). One-way ANOVA (not shown) indicated there was no significant difference in either cell length or cell area across all analysed conditions ($P > 0.5$). Subjective evaluation of cell morphology using widefield microscopy showed broadly comparable cell morphologies across media conditions, even between cells grown in (C) 500 μM and (D) 0 μM CaCl_2 . However, the presence of filamentous cells was only observed in calcium-depleted conditions, albeit at a relatively low frequency ($< 1/100$ cells).

3.4.2. Varying CaCl_2 Concentrations during Cell Growth Impedes the Incorporation of new S-layer.

As previously noted, several studies, including one exploiting exogenously labelled RsaA (Comerci et al., 2019), has shown SLPs are integrated at discrete locations along the cell surface. To further probe the role of Ca^{2+} mediated S-layer assembly, the SC-based labelling methodology was adapted to investigate continuous S-layer biogenesis. As it is now evident that calcium is essential for RsaA oligomerisation and retention, it is unclear if previously formed S-layer present on the surface is sufficient in itself to allow for continued biogenesis of the array, even following calcium-reduction.

C. crescentus RsaA467-ST cells were grown in optimal M2G medium containing 500 μM CaCl_2 (hereafter known as M2G-500) and labelled with 10 μM SC-sfGFP to saturate RsaA-ST on the cell surface (described in section 3.4.1). Excess SC-sfGFP was removed, and cells chased labelled with SC-mRFP1 in M2G-500 or M2G media supplemented with 100 μM CaCl_2 (hereafter known as M2G-100). The lower limit was set as it was sufficient to achieve a reduced level of fluorescence that would be evident in light microscopy (Figure 3.1), while still allowing the growth necessary for chase labelling. Pulse-chase labelling resulted in dual-labelled cells, showing distinct regions of old (SC-sfGFP) and new (SC-mRFP1) S-layer (Figure 3.4). Cells grown in M2G-500 showed localised SC-sfGFP along the cell body and SC-mRFP1 at mid-cell and cell poles (Figure 3.4A). However, cells chased using M2G-100 only showed strong fluorescence for SC-sfGFP (Figure 3.4B). There are distinct gaps in the sfGFP signal along the cell body, which localise with the regions we would expect to see labelling of newly integrated RsaA-ST into the S-layer, were it successful.

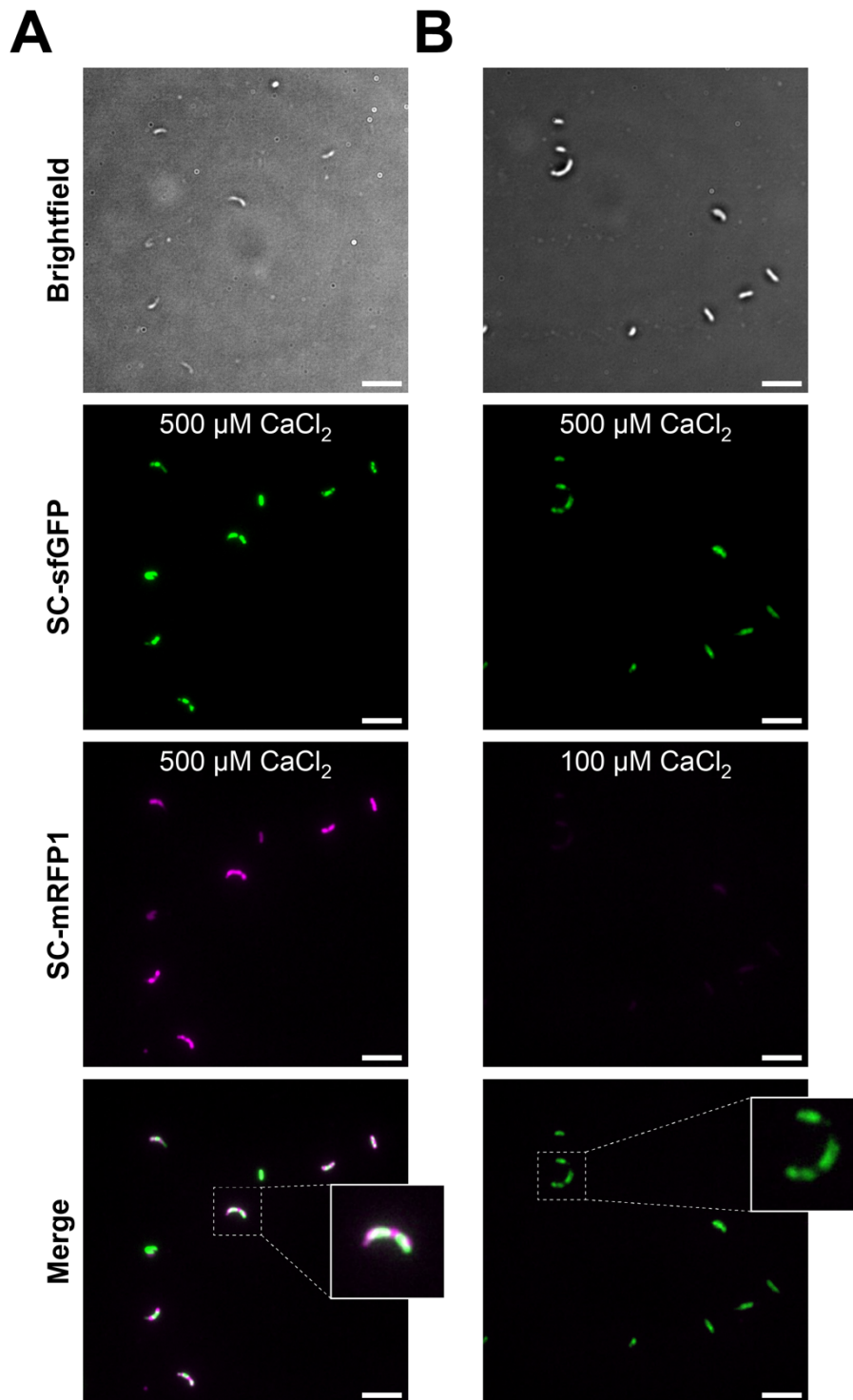


Figure 3.4. RsaA requires a constant supply of CaCl_2 for efficient S-layer biogenesis.

C. crescentus cells pulse labelled with SC-sfGFP in M2G-500, followed by chase labelling with SC-mRFP1 in either (A) M2G-500 or (B) M2G-100. Each channel is contrasted individually for consistency between labelling conditions. A zoomed in view of a representative cell is provided with each merged micrograph as an inset. A sharp reduction in chase-fluorescence is observed in cells grown in the presence of 100 μM CaCl_2 . Scale bar = 10 μm .

Fluorescence profiles of cells from the two different conditions were extracted, showing a stark contrast between labelling conditions (Fig. 2.5). The consistently strong sfGFP in both labelling conditions suggests previously integrated RsaA467-ST is retained by cells, even when subjected to CaCl₂ reduction during growth. However, it appears the presence of previously formed S-layer is in itself insufficient for integration of new RsaA467-ST, and additional Ca²⁺ ions are still required for proper assembly of the new S-layer.

An alternative labelling approach was implemented using a modified same pulse-chase methodology, whereby cells are moved from M2G-100 to the more optimal media, M2G-500. This resulted in cells with low levels of pulse (or SC-sfGFP) labelling (Figure 3.6A-B), as expected for a low calcium environment. Chase labelling resulted in SC-mRFP1 puncta distributed across the cell, with no obvious localisation to midcell or polar regions. Unfortunately, cells labelled under these conditions showed unusual morphology and inconsistent labelling, with many cells being completely non-fluorescent in both fluorescent channels (Figure 3.6C-D). These morphological changes (which included cell elongation, loss of curvature, and multiple points of constriction) may be a response to the sudden change in osmolarity brought on by the sudden addition of high concentrations of CaCl₂ into the media. The observed elongated cell phenotype absent of the classic *C. crescentus* curvature was previously noted in the calcium-limiting experiments described earlier (Figure 3.3). To give the cells time to adapt, the cells were pre-incubated in M2-500 at room temperature. Unfortunately, this was unsuccessful, and cells retained a labelling pattern and morphology as seen in Figure 3.6.

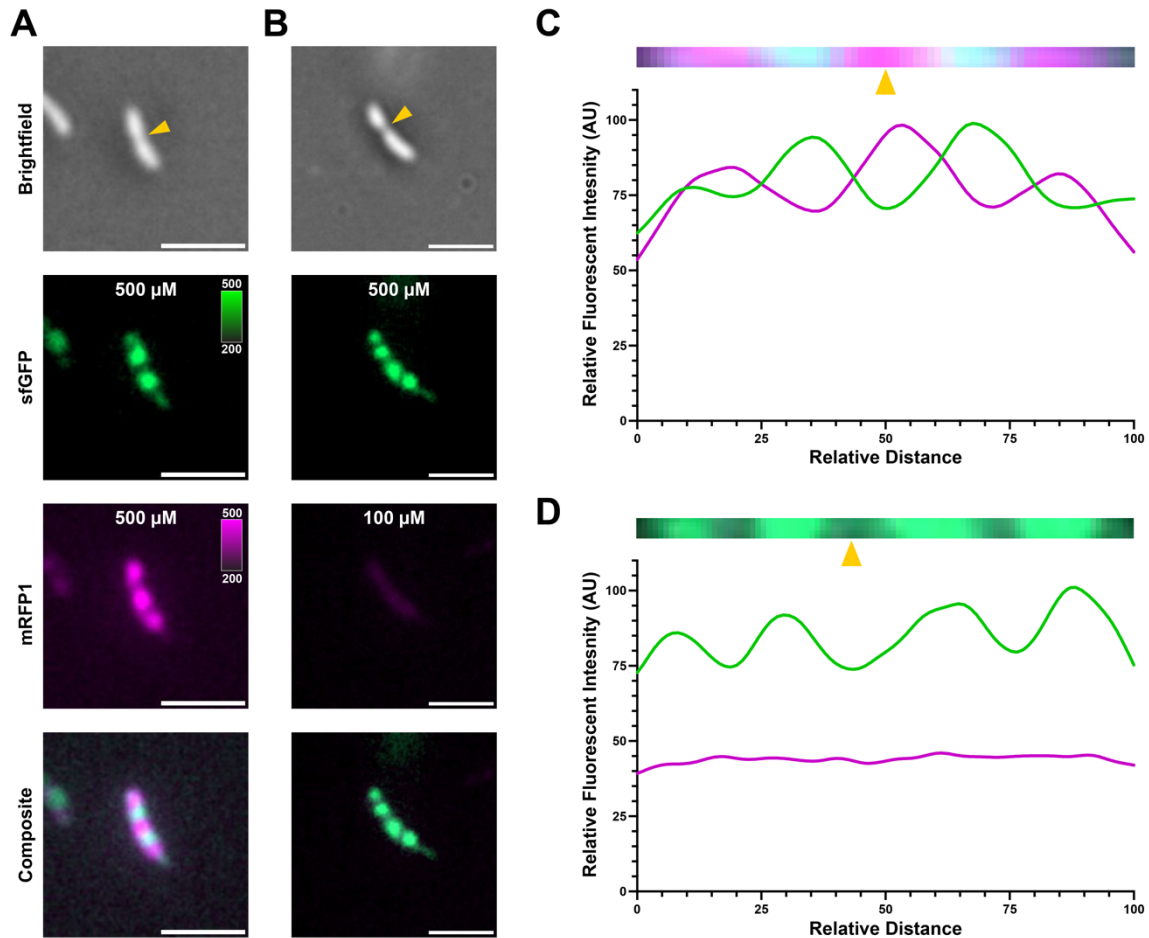


Figure 3.5. Correct integration of RsaA into the S-layer of growing *C. crescentus* requires a constant supply of Ca^{2+} ions.

(A) *C. crescentus* Rsa467-ST pulse-chase labelled using SC-mRFP1 and SC-sfGFP in the M2G-500 throughout results in distinct regions of sfGFP and mRFP1 labelling, suggesting spatial separation of old and new S-layer. (B) Cells pulse-labelled in M2G-500, but chase-labelled in M2G-100 produced cells with prominent sfGFP-fluorescent regions, but reduced mRFP1 fluorescence. Calibration bars are provided for their respective channels in A. Brightfield images show highlighted midcell (yellow arrow). Scale bars = 5 μ m. (C-D) Quantification of relative fluorescence along the medial axis of each cell, plotted against relative cell length. The fluorescence profile of the merged channels of both cells are given above the representative graphs, with the midcell indicated by a yellow arrow, as in A and B (C) Cell A shows a clear separation of strong mRFP1 and sfGFP signals, owing to differential labelling along the S-layer. (D) Cell B retains a strong sfGFP signal with reduced mRFP1 fluorescence, the latter also showing no localisation pattern.

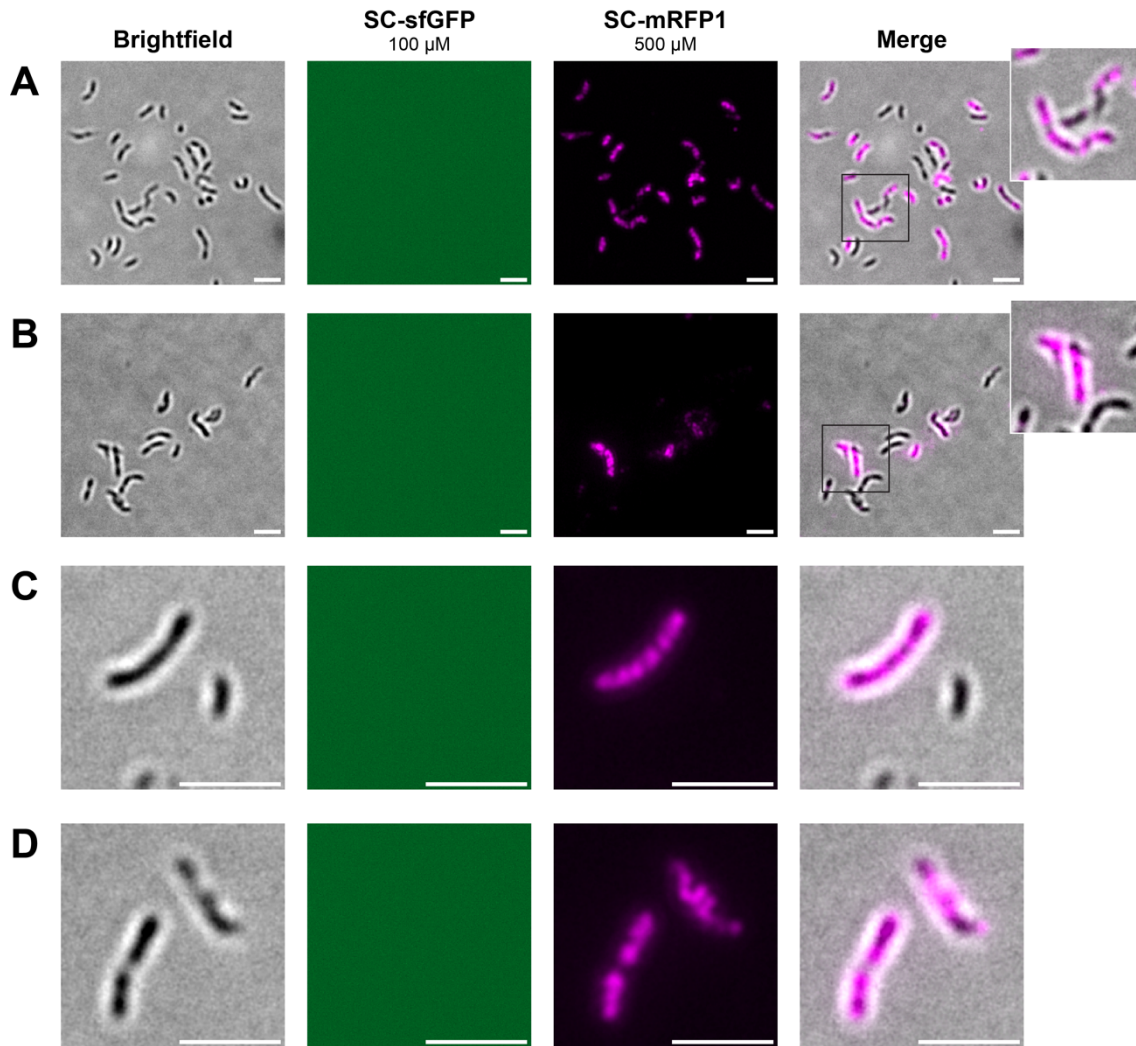


Figure 3.6. Pulse-chase labelling the S-layer when moving from low to high CaCl_2 results in a patchy fluorescence and disrupted cell morphology.

(A-B) Pulse-chase labelled *C. crescentus* cells grown in M2G media containing increasing concentrations of CaCl_2 . Labelling protein and calcium concentrations are provided above the relevant micrographs. Pulse labelling, SC-sfGFP, was carried out in 100 μM CaCl_2 , before being moved to chase labelling media containing SC-mRFP1 and 500 μM CaCl_2 . Micrographs represent images collected from two repeats of the labelling experiments. Merged images shows SC-mRFP1 imposed on the brightfield micrographs. Zoomed images have been provided for both micrographs as an inset. (C-D) Examples of morphologically aberrant *C. crescentus* cells following pulse-chase labelling. Scale bars = 5 μm .

3.4.3. Cryo-ET of *C. crescentus* S-layers under Varying CaCl_2 Concentrations.

Fluorescent labelling of the S-layer and widefield microscopy of *C. crescentus* cells has clearly demonstrated calcium-dependent nature of S-layer biogenesis (Figure

3.1 and Figure 3.4). However, it is unclear the observations are due to loss of the S-layer from the cell surface, aggregation of RsaA (rendering the SpyTag peptide inaccessible by SpyCatcher proteins), or a combination of both. It has been previously reported that following low-calcium exposure, surface-bound RsaA can become irreversibly aggregated (Herrmann et al., 2017). Cryo-ET of vitrified *C. crescentus* cells allows for direct examination of the cell surface to examine the presence (or lack thereof) and condition of the S-layer.

C. crescentus grown in M2G media supplemented with either 500 μM CaCl_2 (M2G₅₀₀) or 100 μM CaCl_2 (M2G₁₀₀). Cells were subsequently vitrified and imaged for tomogram collection. Following tomogram reconstruction, cells grown in M2G500 possess a confluent S-layer bound to the OPS, as expected (Figure 3.7A). In addition, the constituent RsaA hexamers have adopted their typical bipartite arrangement, i.e. an underlying RsaA_{NTD} domain closer to the OM, covered by an external RsaA_{CTD} (Figure 3.7B). In contrast, cells grown in M2G100 have a patchy S-layer, with inconsistent SLP retention across the surface. RsaA, when present (identity determined based on its proximity to the OM), appears heavily aggregated and malformed (Figure 3.7C). This is additionally apparent by the loss of the bipartite arrangement of the S-layer (Figure 3.7D).

These cryo-ET observations are consistent with previous literature (Herrmann et al., 2017) and support the findings of the S-layer labelling studies reported in 3.4.1 and 3.4.2. Together, they support the suggestion that in media containing low concentrations of CaCl_2 , the S-layer becomes aggregated and may even shed from the *C. crescentus* surface.

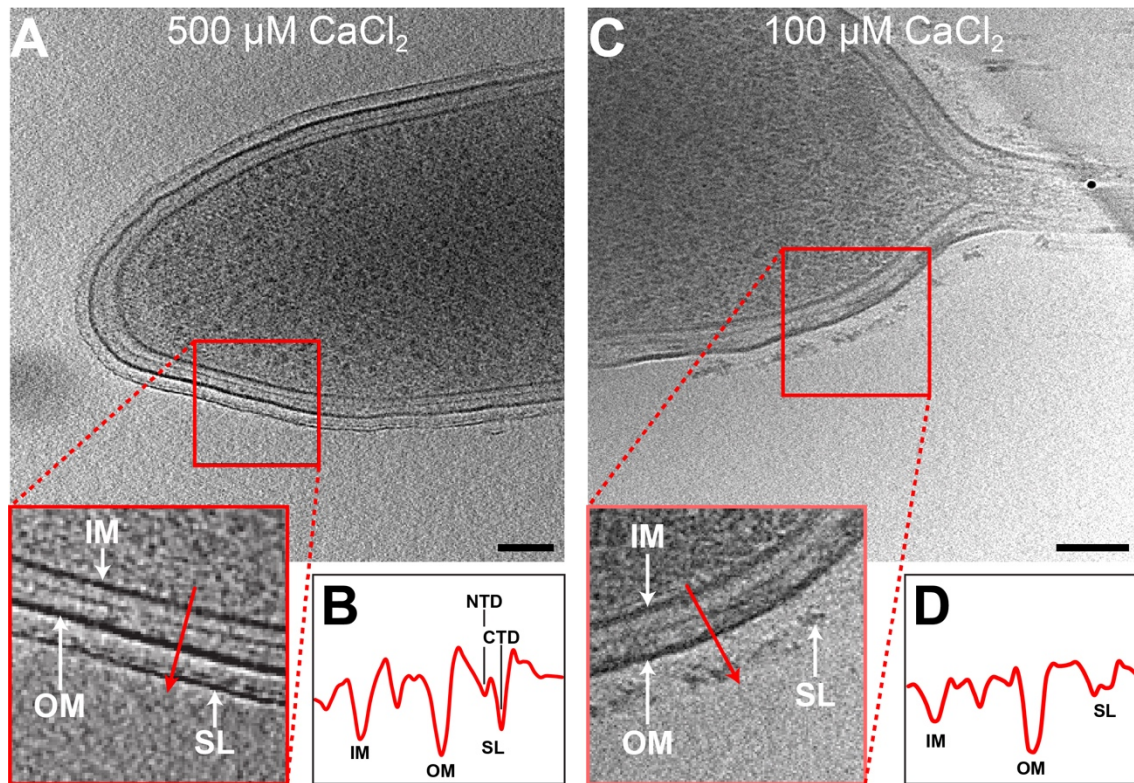


Figure 3.7. Cryo-ET shows S-layer disruption as a result of low-calcium media.

Cryo-ET slices through *C. crescentus* cells grown in (A) M2G₅₀₀ or (C) M2G₁₀₀ media. Scale bars = 100 nm. Inner membranes (IM), outer membranes (OM), and S-layers (SL) are labelled. Cells grown in M2G₅₀₀ (A) possess a confluent S-layer. Cells grown in M2G₁₀₀ (C) have a patchy, aggregated S-layer. (B, D) Grey values along the red line indicated in the tomogram inset, showing loss of characteristic RsaA_{NTD} and RsaA_{CTD} peaks.

3.4.4. Molecular Dynamics Simulations Suggest Alternative Binding Affinities for Canonical Ca²⁺ Binding Sites in RsaA

RsaA has been suggested to contain 22 putative metal binding sites, normally predicted to be Ca²⁺. The exact identities of the bound ions remained unknown and were located based on unidentified densities seen in structural studies of RsaA (von Kugelgen et al., 2020, Bharat et al., 2017). Surprisingly, RsaA lacks the canonical calcium-binding motifs, such as the EF-hand (Lewit-Bentley and Réty, 2000), but calcium is a ubiquitous metal with highly variable binding sequences as Ca²⁺ coordination is most often achieved by multiple secondary structure elements, often separated by stretches of amino acid

sequence (Bindreither and Lackner, 2009). However, the unidentified densities were often found in close proximity to negatively charged residues which are often indicative of Ca^{2+} binding sites (Dudev and Lim, 2003, Linse et al., 1988). MD simulations can help inform multiple different aspects of protein biology (Kumari et al., 2017, Adcock and McCammon, 2006), including the identities of metal ions bound to a protein structure (Vidossich and Magistrato, 2014). Using an MD simulation framework of a full-length RsaA hexamer bound to the OPS configured into a hexagonal-prism-shaped unit cell (Figure 3.8A), allows for the simulation of all inter- and intra-hexameric protein-protein interactions found in the S-layer (Figure 3.8B).

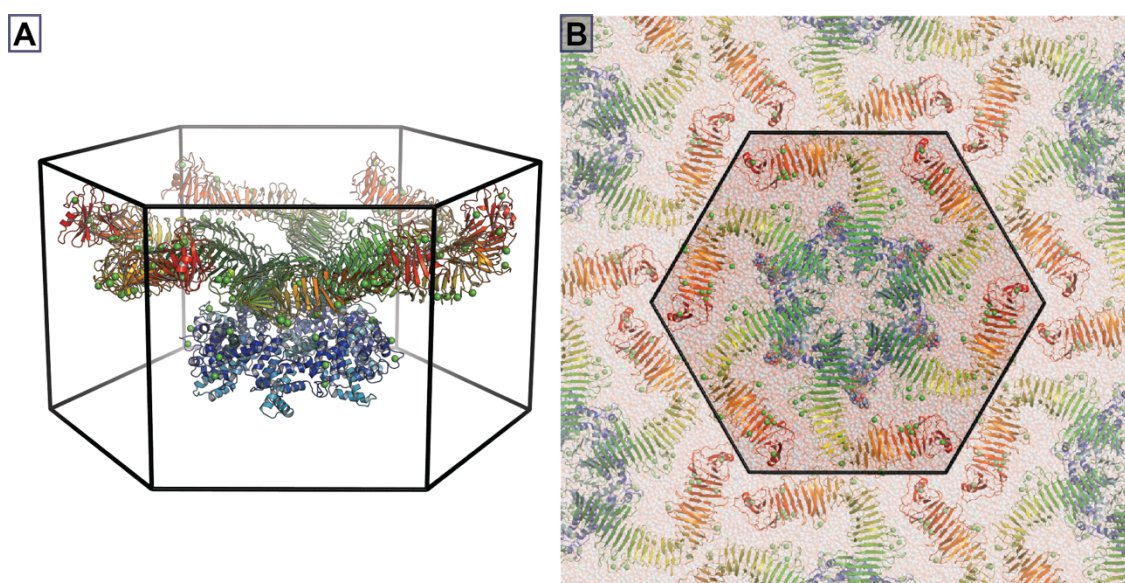


Figure 3.8. Set up of molecular dynamics simulations using full-length RsaA.

(A) Full-length RsaA positioned in a prism-like box to simulate the S-layer of *C. crescentus*. (B) The box dimensions allowed for the placement of lattice contacts in a hexagonal arrangement, consistent with the cellular S-layer arrangement. The RsaA protein was solvated and bound to the O-antigen (not shown) at its binding site, as described by von K  gelgen et al., 2020. Ion locations are displayed as green spheres.

When Ca^{2+} is placed at each of the 22 putative metal binding sites in the MD simulation framework over 100 ns, 20 of the 22 positions are able to stably bind Ca^{2+} (Figure 3.9A-B). Positions 17 and 18 showed large RMSF, suggesting poor binding propensity of these positions for Ca^{2+} . As the surrounding residues of the putative positions were negative, K^+ and Mg^{2+} were substituted for Ca^{2+} in positions 17 and 18, respectively, to gauge the suitability of alternative metal ions for binding. Position 18 was able to bind Mg^{2+} , achieving low RMSF, with the exception of two outliers in the simulation (Figure 3.9C-D). In contrast, placing K^+ at position 17 resulted in high RMSF, exceeding that even of Ca^{2+} at this position.

Looking past the ions themselves, the inclusion of different metals in the MD framework resulted in changes in the RMSF of protein residues (Figure 3.10). The presence of Ca^{2+} ions greatly stabilise the RsaA secondary structure (Figure 3.10A) compared to simulations without any metal ions (Figure 3.10C). When binding positions 17 and 18 are replaced in simulations by K^+ and Mg^{2+} respectively, RMSF remained largely comparable to simulations using Ca^{2+} in all positions (Figure 3.10B). This suggests that, despite K^+ ionic RMSF remaining high when simulated at position 17 (Figure 3.9C-D), the presence of alternative ions in positions 17 and 18 are able to stabilise the structure of RsaA and can be retained within the S-layer array.

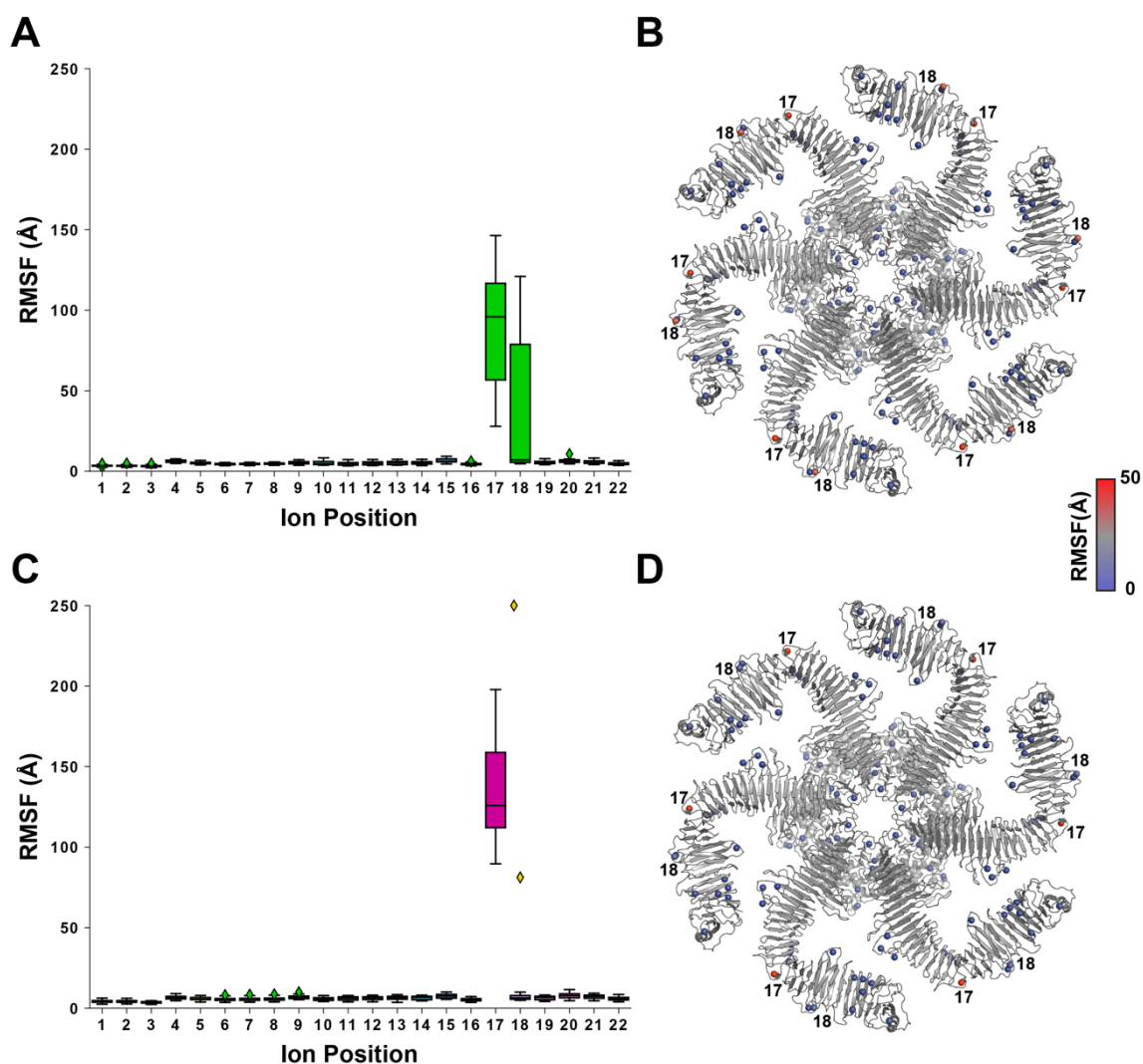


Figure 3.9. Ca²⁺ is stably retained in a majority of putative binding sites in MD simulations.

RMSF values of metal ions in positions 1 to 22 from MD simulations. (A) Ca²⁺ ions (green) are stably bound at a majority of putative binding positions, with the exception of positions 17 and 18. (C) Following replacement of Ca²⁺ in positions 17 and 18 with K⁺ (purple) and Mg²⁺ (yellow) respectively (all other locations are occupied by Ca²⁺), Mg²⁺ is able to be stably bound by RsaA, as indicated by low RMSF. Outliers are represented by diamonds. (B, D) Putative binding sites are presented within the RsaA protein hexamer (ribbon diagram, grey), representing simulations with (B) only Ca²⁺ and (D) Ca²⁺ in all positions, excepting K⁺ in position 17 and Mg²⁺ in position 18. Ions are coloured from low to high RMSF (Å) according to the given calibration bar on the right.

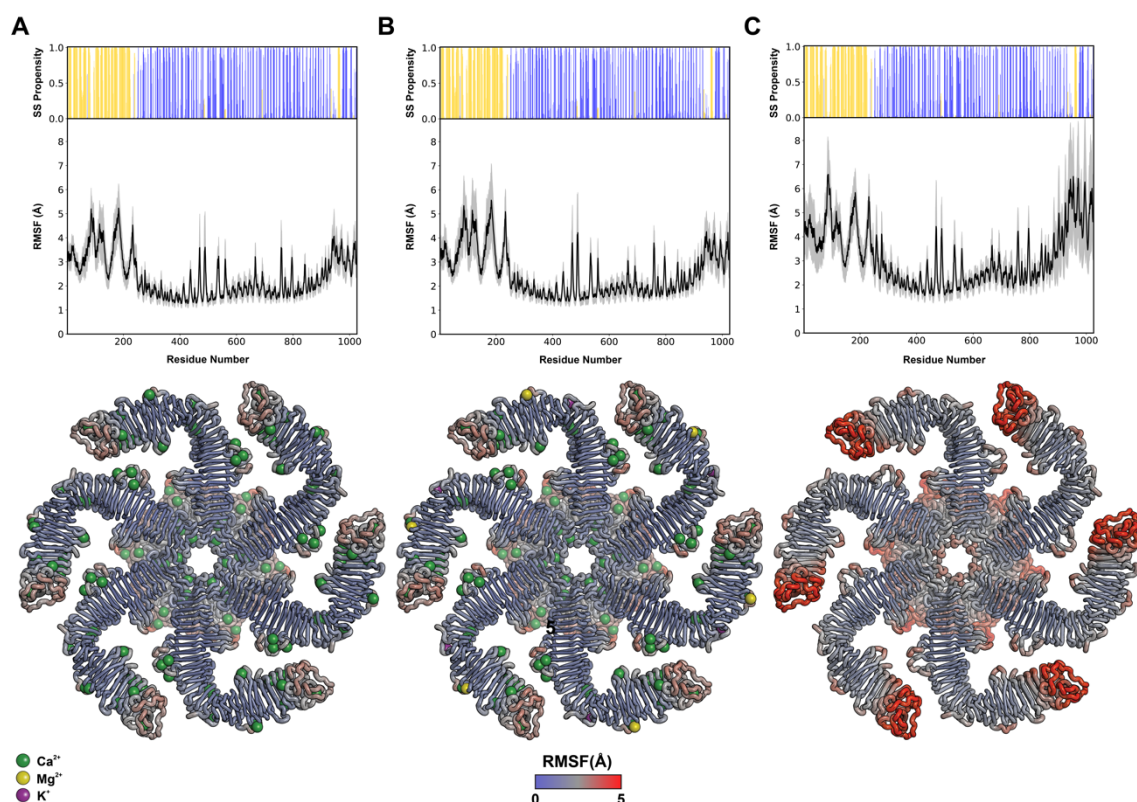


Figure 3.10. MD demonstrates stabilisation of the RsaA S-layer array by bound metal ions.

Secondary structure propensity (SSP) and RMSF of the backbone carbons of RsaA are shown along the RsaA sequence. Alpha-helical regions are shown in yellow and beta strands are shown in blue. RMSF data was plotted onto the RsaA hexamer protein structure, provided below each graph (corresponding RMSF colour calibration bar below). Simulations cover RsaA hexamers containing: (A) Ca^{2+} in ion positions 1-22; (B) K^{+} in position 17, Mg^{2+} in position 18, and Ca^{2+} in all remaining positions; (C) no ions in any position. Ions are represented by coloured spheres, with the corresponding colour key provided in the lower left.

3.4.5. MicroPIXE Analysis Shows Monomeric RsaA Binds Calcium

To confirm the binding and determine the stoichiometry of calcium by RsaA, the elemental composition of RsaA was examined using particle-induced X-ray emission with a microfocused beam (microPIXE) (Grime, 2010). The characteristic X-rays emitted by metal atoms following particle bombardment can be used to quantify the metal content of a protein sample, liquid or crystalline (Garman and Grime, 2005, Johansson, 1989).

The number of calcium atoms bound to the protein can be determined relative to the number of sulphur atoms in the sample (m_z/m_s , or the mass of the unknown metal over the mass of sulphur). Sulphur content serves as an internal standard, determined by amino acid composition of the subject protein, specifically the number of methionine and cysteines (4 and 0, respectively, in RsaA).

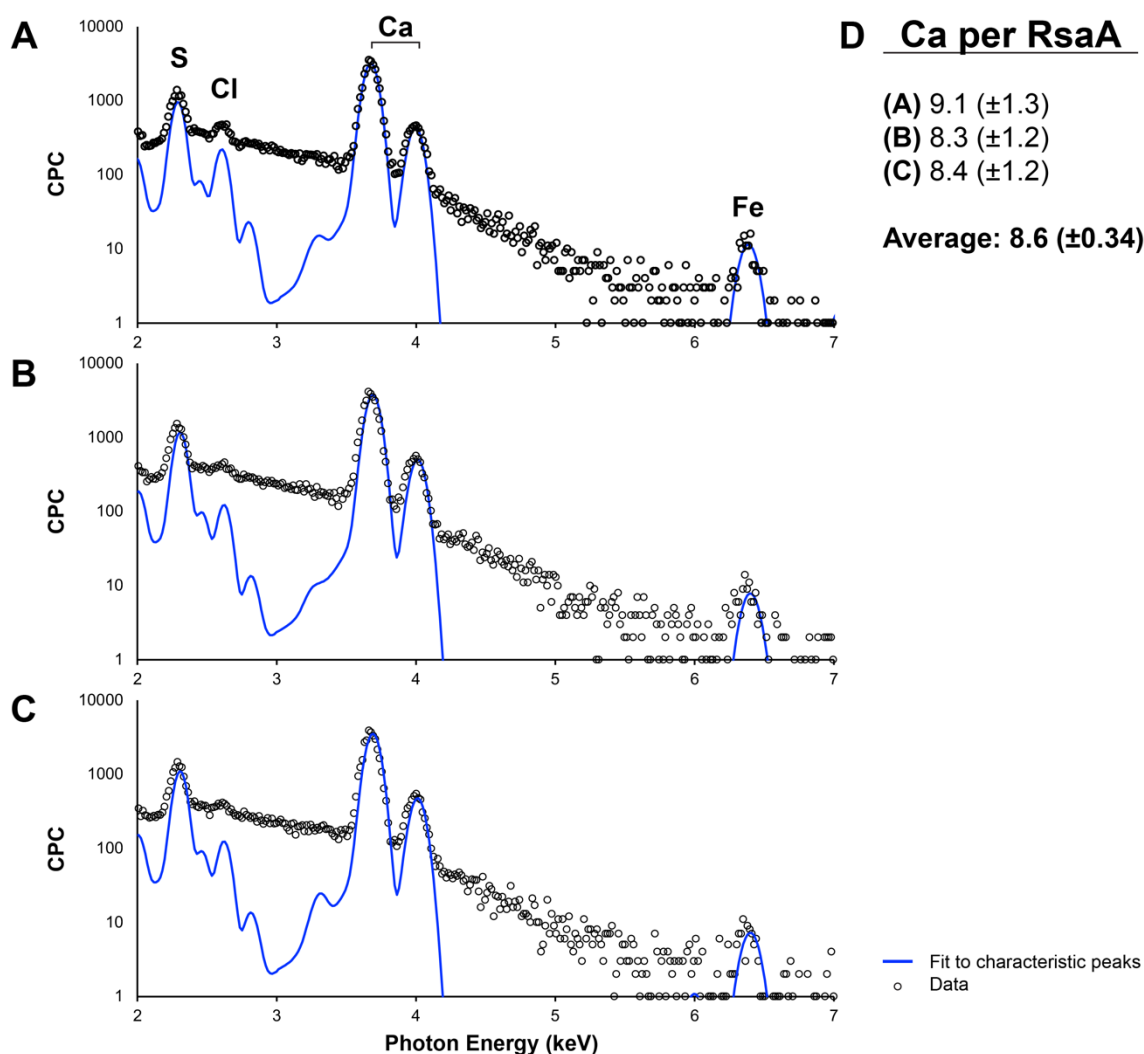


Figure 3.11. MicroPIXE analysis of monomeric RsaA reveals bound calcium atoms.

(A-C) Results of three microPIXE experiments using purified RsaA. Note the $\log^{10}$ scale of the y axis. Data points are indicated by $\bullet$; black line shows fit to characteristic peaks following background subtraction. Characteristic peaks for sulphur (S) atoms, chlorine (Cl), calcium (Ca), and iron (Fe) are highlighted. (D) Calculated calcium atoms per RsaA monomer across each experiment, resulting in an average of 8.6 (± 0.34).

MicroPIXE analysis suggests that full-length, non-oligomerised RsaA binds on average 8.6 calcium atoms per monomer (Figure 3.11), meaning as many as 13 of the 22 putative calcium binding sites are unoccupied or bind alternative metals. However, under these conditions, RsaA is largely monomeric and unstable (Bharat et al., 2017), and RsaA may bind more calcium atoms (or other metals) in its oligomeric state, explaining the inconsistency between the number of stably bound Ca^{2+} ions proposed by MD simulations (Figure 3.9).

3.4.6. Long Wavelength X-ray Diffraction Reveals Locations and Identities of Metal Ions in the RsaA_{CTD} Crystals

Due to the 2D crystalline nature of S-layers, the macromolecular structure of purified SLP crystals is often closely related to their organisation on the cell surface (Lanzoni-Mangutchi et al., 2022, Bharat et al., 2017). The crystal structure of the RsaA_{CTD} has been resolved using X-ray crystallography and cryo-EM techniques (Bharat et al., 2017); however, while putative ion binding sites were discovered using the electron density, the identities of the occupying metals remained undetermined. MD simulations suggest at least two of the putative calcium-binding sites in RsaA, positions 17 and 18, bind Ca^{2+} ineffectively or have preference for alternative ions (Figure 3.10A-B). To confirm the locations and identities of the bound ions, crystals composed of RsaA_{CTD} were produced for analysis by LWXD, which allows for measurements below and above the X-ray absorption edges of both calcium and potassium (4.0381 keV/3.0704 Å and 3,6074 keV/3.4369 Å, respectively) (Wagner et al., 2016).

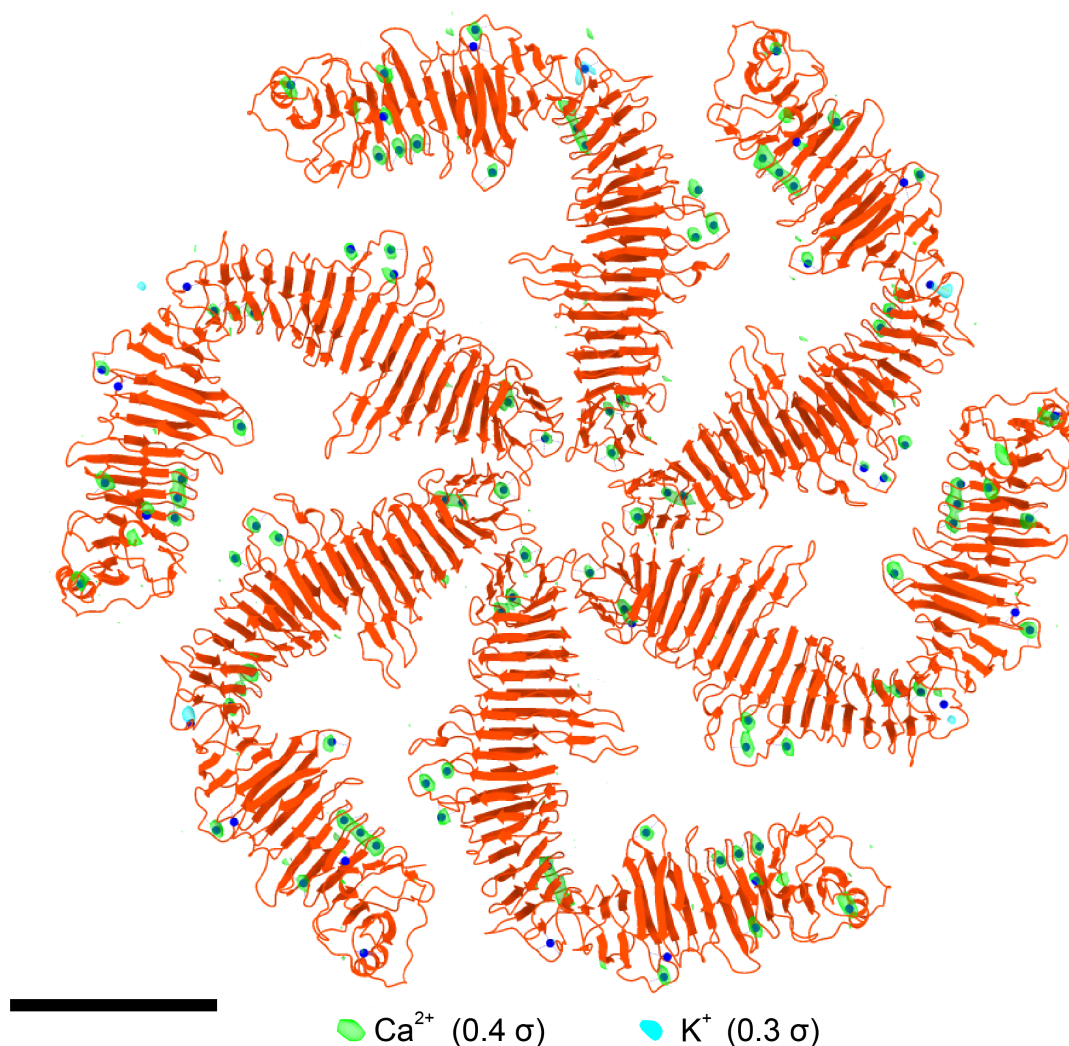


Figure 3.12. High resolution mapping of metal ions in RsaA_{CTD} crystals using Long Wavelength X-ray Diffraction.

Ca²⁺ (green) and K⁺ (cyan) densities produced using anomalous X-ray diffraction maps overlaid onto the RsaA_{CTD} structure (red) (PDB 6P5T). Ion densities are closely associated with putative calcium-binding sites (blue sphere). Scale bar = 50 Å.

The identities and position of ions in the RsaA_{CTD} hexameric structure was determined using the anomalous diffraction maps generated by these analyses (Figure 3.12). As expected, Ca²⁺ was seen throughout the hexameric structure in close proximity to the predicted calcium-binding positions. Surprisingly, K⁺ densities were observed at position 17 in several of the constituent RsaA monomers comprising the hexamer (Figure

3.12). This finding is somewhat unexpected given that MD simulations suggested K^+ was unstably bound at position 17 (Figure 3.9C-D). However, upon closer inspection, K^+ densities are not present in each monomer (Figure 3.12), and when present, show signs of poor-retention, i.e. irregularly shaped or small densities (Figure 3.13). Unexpectedly, no ion appeared to be retained at position 18 (Figure 3.12 and Figure 3.13), despite being able to stably bind Mg^{2+} in the MD simulations (Figure 3.9C-D). A summary of densities observed in the RsaACTD anomalous diffraction maps is summarised in Table 3.1.

Table 3.1. Metal ions in RsaACTD as identified by anomalous diffraction.

A summary of ion densities observed in the RsaACTD crystal structure, following identification using LWXD. Chain IDs, α to ζ , correspond to annotations in Figure 3.13. Ca^{2+} -corresponding densities are listed as “Ca”, K^+ as “K”, and no ion density as “–”.

Binding Position	Chain ID					
	α	β	γ	δ	ϵ	ζ
1	Ca	Ca	Ca	Ca	Ca	Ca
2	Ca	Ca	Ca	Ca	Ca	Ca
3	Ca	Ca	Ca	Ca	Ca	Ca
4	Ca	Ca	Ca	Ca	Ca	Ca
5	Ca	Ca	Ca	Ca	Ca	Ca
6	Ca	Ca	Ca	Ca	Ca	Ca
7	Ca	Ca	Ca	Ca	Ca	Ca
8	Ca	Ca	Ca	Ca	Ca	Ca
9	Ca	Ca	Ca	Ca	Ca	Ca
10	Ca	Ca	Ca	Ca	Ca	Ca
11	Ca	Ca	Ca	Ca	Ca	Ca
12	Ca	Ca	Ca	Ca	Ca	Ca
13	Ca	Ca	Ca	Ca	Ca	Ca
14	Ca	Ca	Ca	Ca	Ca	Ca
15	Ca	Ca	Ca	Ca	Ca	Ca
16	Ca	Ca	Ca	Ca	Ca	Ca
17	K	K	–	–	K	–
18	–	–	–	–	–	–
19	Ca	Ca	Ca	Ca	Ca	Ca

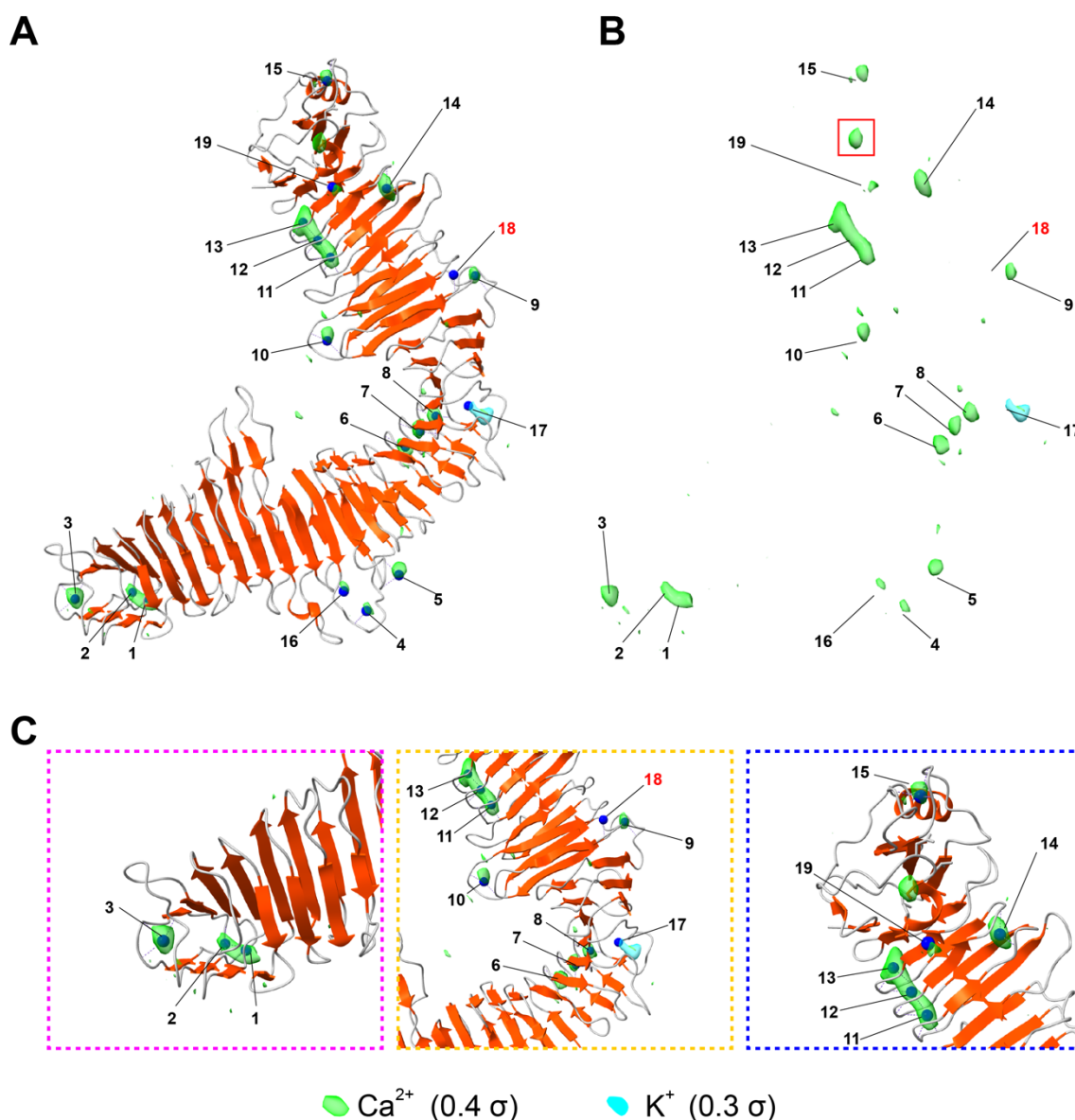


Figure 3.13. Identification of metal ions bound to a RsaACTD monomer reveals unbound and alternative binding partners for putative Ca^{2+} binding sites.

(A) An RsaACTD (PDB 5N97) monomer displayed in red/grey, putative metal binding locations indicated by blue spheres, numbered 1 to 18. Ca^{2+} (0.4 σ) and K^{+} (0.3 σ) densities (0.4 σ) are displayed in green and cyan, respectively. (B) Densities displayed without the associated RsaACTD monomer. Position 17 has an irregularly shaped K^{+} density associated, while position 18 is unoccupied. All other positions, 1 to 16, have an associated Ca^{2+} density. A red box highlights a Ca^{2+} density in close proximity to an α -helix at toward the C-terminus. (C) Close up views of core (pink), hinge (orange), and C-terminal (blue) regions.

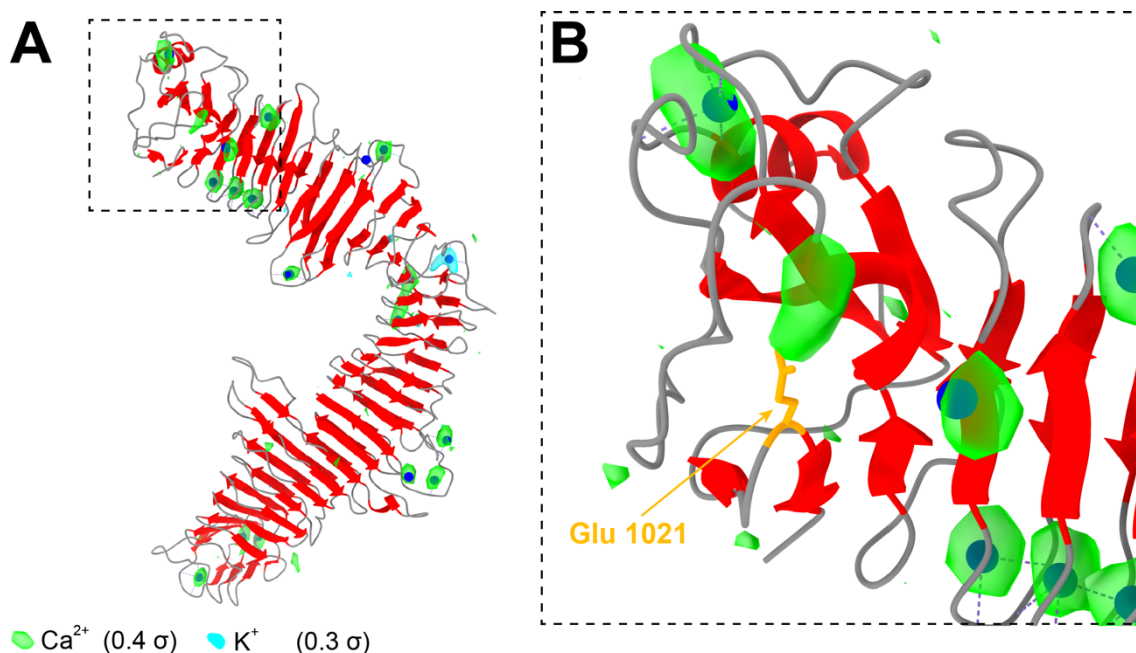


Figure 3.14. Identification of a previously unidentified calcium-binding site in RsaA_{CTD} crystals.

C-terminal region of RsaA (PDB 5N97). Ca^{2+} densities (0.4 σ) confirmed by LWXD are displayed in green and putative metal binding locations are indicated by blue spheres (A) Monomer and (B) CTD zoomed views are provided. A majority of the densities in the provide view are in close spatial association with a putative binding location, with the exception of a prominent density found in proximity to a Glu residue (orange, highlighted).

Intriguingly, in several of the constituent RsaA monomers, there was an additional Ca^{2+} density in the near the C-terminus (Figure 3.13 and Figure 3.14), suggesting the presence of a previously unidentified binding site. It may have been overlooked in previous structural studies due to its absence in several subunits of the RsaA hexamer lacking the associated density. The density is found in close proximity to a short-length loop to α -helix region, aa's 933-939 (Val-Gly-Iso-Ser-Thr-Asn-Gly-Ala). While the most common residues observed in calcium binding sites are Asp and Glu, Asn, Lys, and Ser sidechains have also been implicated in the binding of Ca^{2+} (Choi et al., 2012, Bindreither and Lackner, 2009). Upon closer inspection, the negatively charged sidechain of a downstream Glu (aa 1021), a common calcium-binding residue (Vavrusova and Skibsted,

2013), extends toward the Ca^{2+} density (Figure 3.14). Together, this suggest the presence of a new Ca^{2+} binding site in the RsaA hexamer, which may be structurally important C-terminal region of RsaA. The relatively high RMSF of the far C-terminal regions of RsaA hexamers in MD simulations (Figure 3.10), even with all other ion binding sites occupied with Ca^{2+} , may be reduced through rigidifying of the RsaA secondary structures through coordination with this new Ca^{2+} ion. Given that the newly identified binding site is occupied in at least four of the six RsaA_{CTD} monomers, it is likely there is a Ca^{2+} at this position and its presence is important for RsaA stabilisation.

3.5. Discussion

The metal-binding propensities of SLPs has been recognised for the many decades of ongoing S-layer research (Engelhardt, 2007), and has been observed in species across domains, from archaea (Cohen et al., 1991, Kessel et al., 1988), to diderm bacteria (von Kugelgen et al., 2020, Bharat et al., 2017, Herrmann et al., 2017), to monoderm bacteria (Baranova et al., 2012, Lupas et al., 1994). Together, the studies presented in this thesis shed light on the importance of calcium-binding in *C. crescentus* S-layer biogenesis, demonstrating Ca^{2+} ions play a major role in RsaA oligomerisation and binding to the cell surface (Figure 3.15).

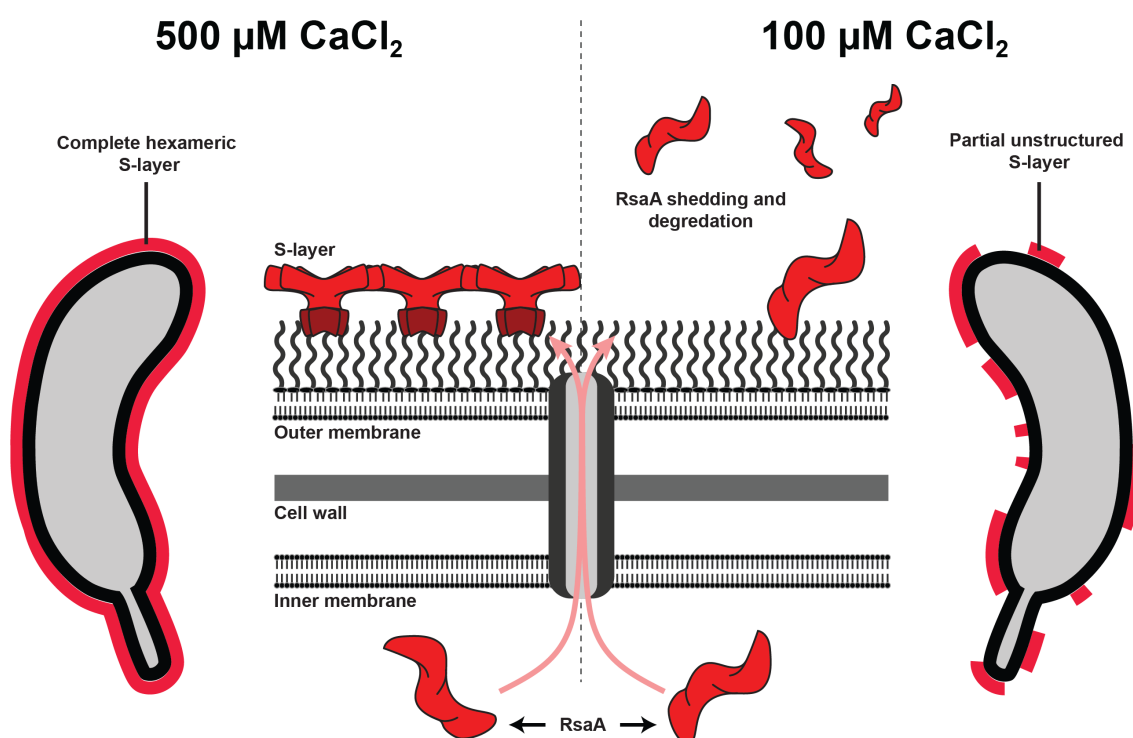


Figure 3.15. Cell-surface Retention and Oligomerisation of RsaA Requires CaCl_2 .

Schematic representation of calcium-dependent S-layer biogenesis in *C. crescentus*. RsaA, prior to secretion, is localised to the cytoplasm and retained in an unfolded state due to the lack of available Ca^{2+} ions. Following secretion by RsaDEF, RsaA in high-calcium environments forms a hexameric array, bound to the OPS, as confirmed by light microscopy, LWXD, cryo-ET, and cryo-EM. In low calcium-media, S-layer formation is diminished by lack of available Ca^{2+} ions, resulting in patches unfolded or aggregated SLP along the cell surface.

CaCl₂ is added to *C. crescentus* cell media in surprising excess compared to other bacterial species, even including other fresh water bacterial species (Andersen et al., 2005, Andersen, 2005). A reduction in the availability of Ca²⁺ has an immediate effect on S-layer biogenesis, as evidenced by light microscopy studies of RsaA-binding fluorescent proteins, and a depletion of CaCl₂ can eliminate S-layer from the cell surface completely (Figure 3.1). This high demand for available Ca²⁺ is present throughout the cell cycle, and previously crystallised S-layer is not in itself sufficient to allow for further SLP oligomerisation or retention (Figure 3.4). The observations of light microscopy are strengthened by cryo-ET of vitrified *C. crescentus* cells. The direct examination of the S-layer confirms that reduction of calcium in the growth medium results in significant deterioration of the S-layer where only patches of unstructured RsaA remain, in stark contrast to the characteristic well-structured S-layer (Figure 3.7).

Previous studies of the metal-binding SLPs have established that numerous different divalent cations, including Mg²⁺, Mn²⁺, Sr²⁺ and Co²⁺ (Nomellini et al., 1997), can facilitate the formation of S-layers in vitro. Interestingly, this appears to be untrue for S-layer biogenesis on the cell surface. A major contributing factor here is likely the necessity of Ca²⁺ ions in mediating OPS-RsaA binding, allowing for S-layer retention to the cell surface. Therefore, while excess MgSO₄ in the growth medium may still drive S-layer oligomerisation in solution, this still results in loss of the RsaA from the surface of *C. crescentus* (Figure 3.1E).

However, there is still a clear flexibility in the preferred ions for specific metal binding sites in RsaA. Positions 17 and 18 were significantly unwilling to bind Ca²⁺ in MD simulations (Figure 3.9A), which was further substantiated by direct identification of bound metal ions in the RsaA crystal structure. Intriguingly, position 17, which was suggested to unsuccessfully bind K⁺ ions in MD simulations (Figure 3.9B), was occupied

(albeit loosely) in LWXD experiments, and position 18 was consistently empty of any bound ion (Figure 3.13). While potentially artefactual and dependent on the crystallisation process, these positions may represent a deviation from the expected Ca^{2+} binding activity of RsaA. In combination, anomalous scattering of biologically relevant elements in LWXD using RsaA_{CTD} and single particle cryo-EM of RsaA_{NTD} have peeled back the ambiguity of the metal-binding landscape of the *C. crescentus* S-layer. Of the 22 putative metal-binding sites, 19 have been confirmed to successfully bind Ca^{2+} (Figure 3.16), and a new potential site has even been identified in the far C-terminal region (Figure 3.14). An additional experiment, not reported in this thesis, confirmed the binding of a Ca^{2+} ion at position 21 in the RsaA_{NTD}, closely associated with the OPS-binding pocket (Figure 3.16) (Herdman et al., 2022). MicroPIXE data suggests a lower calcium-burden for unfolded RsaA (Figure 3.11), affirming a model in which Ca^{2+} enables successive folding and oligomerisation.

Whether the calcium requirement of *C. crescentus* is exclusively dependent on the S-layer's binding demand is difficult to ascertain. Due to *C. crescentus*' position as the type species for the Caulobacteraceae (Poindexter and Lewis, 1966, Poindexter, 1964), its nutrient demand was set as the benchmark for closely related species. Closely related, such as *Caulobacter segnis* and *Asticcacaulis excentricus*, lack genes associated with S-layer biogenesis machinery, and have additionally never been reported to contain an S-layer (Patel et al., 2015, Poindexter, 2015). However, both are routinely cultured in PYE media containing 500 μM CaCl_2 as standard, (Abraham et al., 2014, Jiang et al., 2014, Sulkowski et al., 2019, Santin et al., 2023). It is unknown if the reduction of CaCl_2 will negatively impact the growth of these SLP deficient species, and this may represent an area of further research.

Much of the recent research on SLP-metal binding interactions have been preoccupied by the potential applications in bioremediation and biomaterial synthesis (Pollmann and Matys, 2007, Velasquez and Dussan, 2009, Fahmy et al., 2006). Understanding the metal binding properties of S-layers not only sheds light on adaptive strategies employed by prokaryotes in metal-rich environment, but also opens avenues for biotechnological applications, including the development of novel materials for metal ion sensing, remediation, and recovery (Park and Taffet, 2019, Xu et al., 2010).

However, the metal-binding activities of SLPs is not often related back to S-layer assembly. Despite such large variation in SLP sequence (Bharat et al., 2021), metal binding and ion-induced S-layer assembly may be a widespread solution to the difficulties presented by the assembly of a regularly structured, proteinaceous layer beyond the cell surface. The S-layer, as well as the OM in Gram-negative bacteria, is energetically inert in that It is uncoupled from the electron transport chain. Relying on environmentally available metal ions such as Ca^{2+} serves a dual purpose; Ca^{2+} ions are in poor availability in the cytoplasm due to careful intracellular regulation of free calcium (Naseem et al., 2008, Holland et al., 1999), meaning RsaA remains unfolded prior to secretion, avoiding the potential build-up of a large crystal beneath the surface. A comparable environment-induced folding mechanism seen across RTX family proteins (Linhartová et al., 2010), a family of T1SS-associated protein (Kanonenberg et al., 2018), many of which are toxins that share structural homology with the C-terminal Ca^{2+} binding regions of RsaA. RTX toxins are retained in the producing cell in an unfolded state, and fold into their toxin-active state following secretion and Ca^{2+} binding. Similarly, RsaA requires no further energetic input from the cell beyond translation and export, after which the binding to the OPS and oligomerisation is orchestrated exclusively by available Ca^{2+} (Figure 3.15).

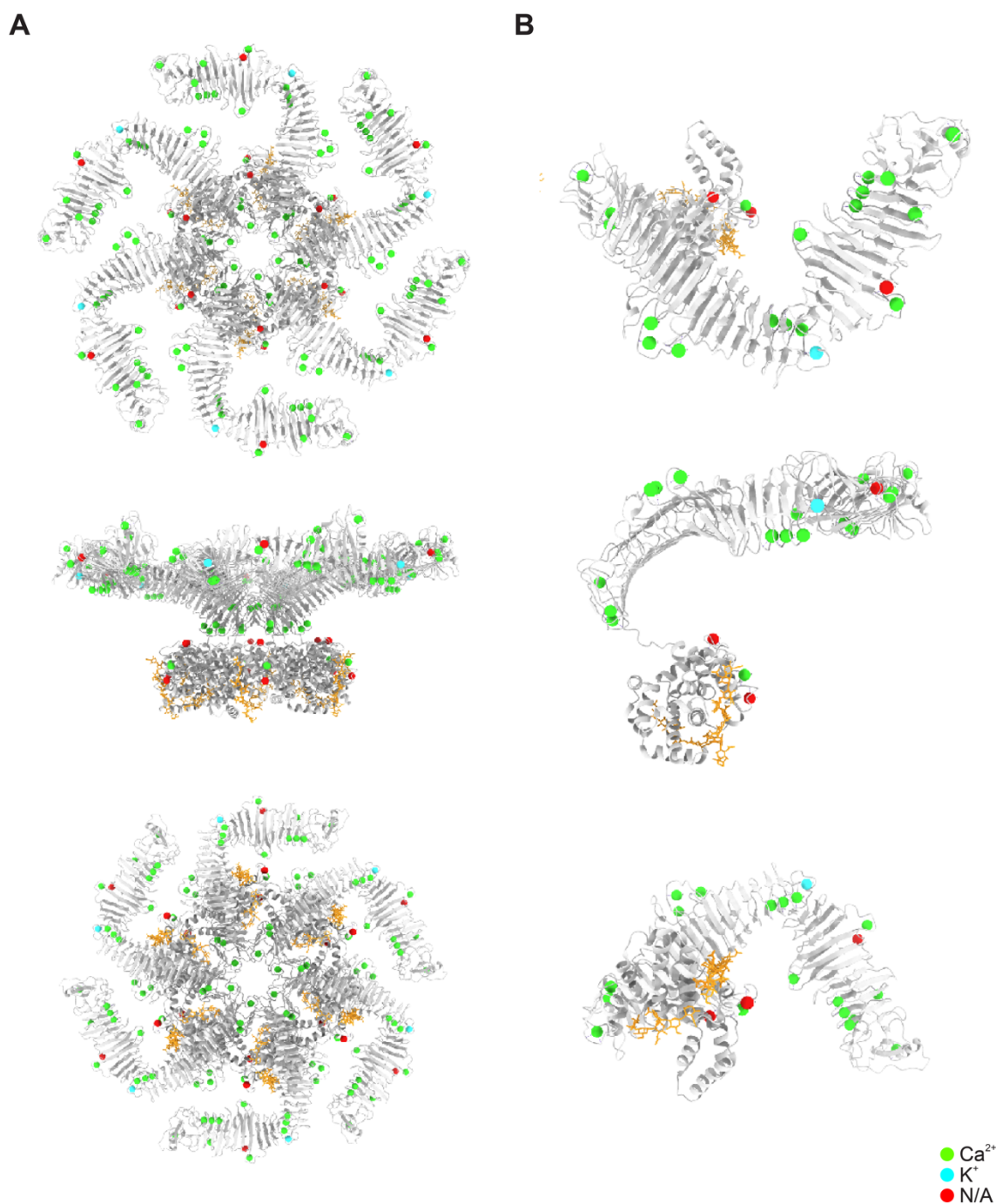


Figure 3.16. Locations and identities of confirmed protein-bound metal ions in hexameric RsaA.

Ribbon diagrams of (A) hexameric and (B) monomeric, full-length RsaA. OPS is bound to the NTD, coloured in orange. The putative Ca^{2+} binding sites are colour-coded according to their experimental identification. Ca^{2+} ions identified by LWXD (in the CTD) and cryo-EM (in the NTD) (von Kugelgen et al., 2020) are coloured green, K^{+} ions (confirmed by LWXD, in the CTD) are coloured cyan. Unoccupied binding sites, or binding sites with currently unconfirmed binding partners, are red (as determined through LWXD and cryo-EM experiments).

CHAPTER 4. Spatiotemporal Localisation of S-layer Expansion

4.1. Overview

S-layers biogenesis represents a significant material and energetic investment by the prokaryotic cell. Given the demand imposed by high levels of protein synthesis and secretion (Lau et al., 2010b), it is reasonable to assume that S-layer producing species will have evolved ways to mitigate the cost and improve the efficiency of S-layer biogenesis. An example of one such process, discussed in the previous chapters, involves utilising environmentally available ions to facilitate SLP retention and oligomerisation along the cell surface, requiring no additional energetic input from the cell beyond secretion (Herdman et al., 2022). Additional energy-conserving systems include transcriptional regulation of SLP biosynthetic operons (Toporowski et al., 2005), limiting the production of S-layer material, or post-translational regulation of SLP synthesis, which provides the additional benefit of preventing cytoplasmic crowding by unfolded SLP (Toporowski et al., 2004, Fernández-Herrero et al., 1997).

Intriguingly, several S-layers show localisation of newly secreted SLP to the midcell or cell poles, potentially representing a common method of organising S-layer biogenesis (Oatley et al., 2020, Comerci et al., 2019, Abdul-Halim et al., 2020, Bharat et al., 2021). This is especially interesting, as these localisation patterns are consistent with the biogenesis of other macromolecular components of the cell envelope, in particular the peptidoglycan cell wall, the divisome, and the elongasome (van Teeseling, 2021, Goley et al., 2011, Lutkenhaus and Addinall, 1997, Divakaruni et al., 2007). This chapter seeks to examine the spatiotemporal localisation of S-layer biogenesis in *C. crescentus*, using the SpyCatcher labelling methodology previously described in thesis.

4.2. Acknowledgments

The work in this chapter was performed by the candidate.

4.3. Aims

Briefly, the project aims were as follows:

- (i) Confirm the presence of discrete regions composed of old and new S-layer material along the cell surface using SpyCatcher-based labelling of live *C. crescentus* cells.
- (ii) Determine if the localisation of S-layer biogenesis is regulated spatially or temporally, i.e. if the localisation pattern of new and old S-layer changes over the course of the cell cycle.
- (iii) Investigate the potential link between the localisation of S-layer biogenesis and cell wall expansion in *C. crescentus*, using fluorescent probes to label peptidoglycan turnover.

A portion of results relevant to this chapter are published in Herdman *et al.*, *bioRxiv*, June 2023, and under revision in the journal *Nature Communications*.

4.4. Results

4.4.1. Time-lapse imaging of cell-bound S-layers.

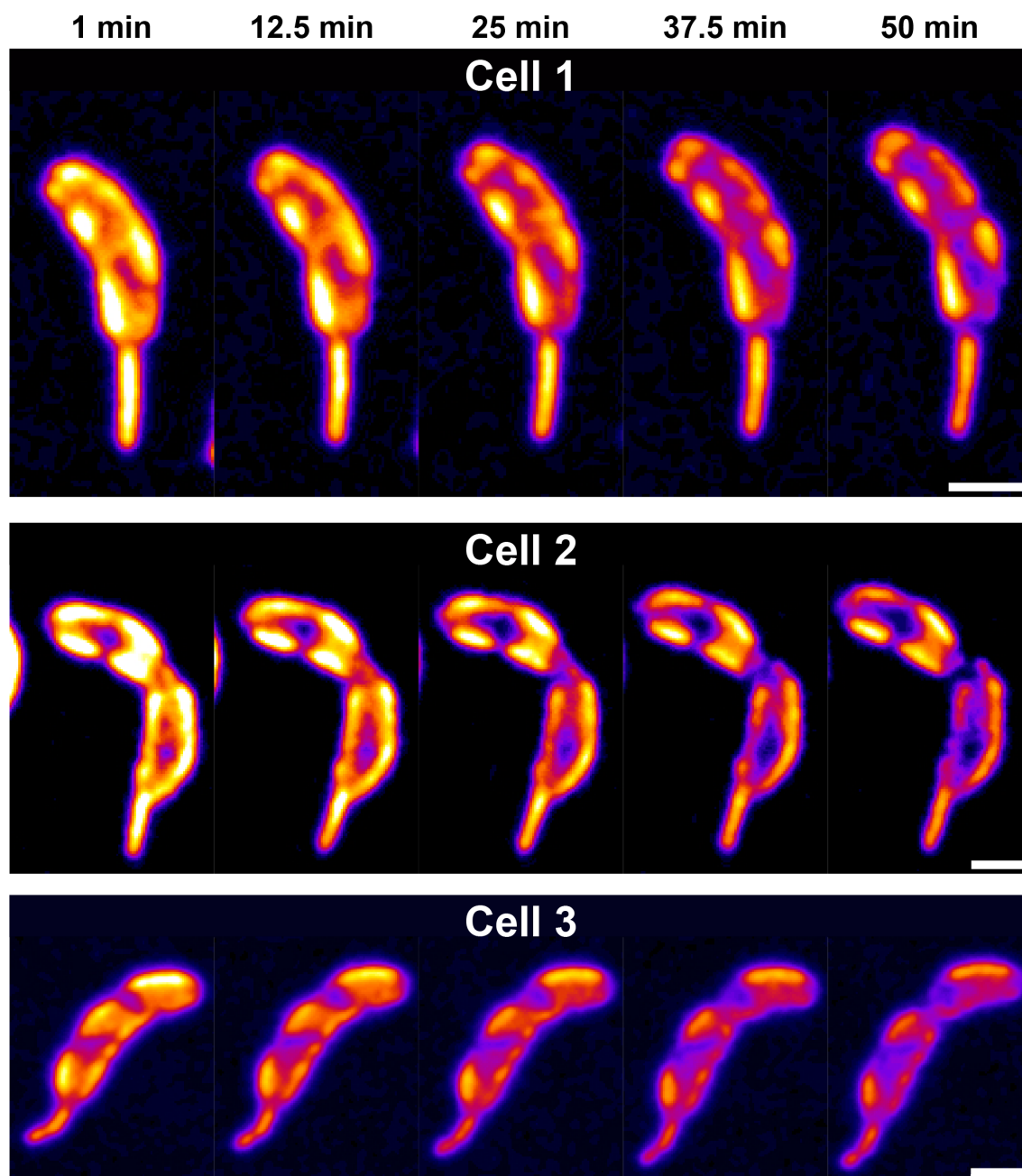


Figure 4.1. Timelapse microscopy shows non-diffusible nature of cell-bound *C. crescentus* S-layer.

C. crescentus cells labelled using SC-mRFP1, grown on PYE-agar at 30 °C. Cells were imaged over every 30 second over 1 hours. SC-mRFP1 fluorescence is presented as a fire heatmap. Five snapshots across 50 minutes are presented for each cell, showing continued cell growth and static regions of SC-mRFP1 labelling. Scale bars = 1 μm .

To identify regions of S-layer biogenesis, cells were first saturated in a single 10 μ M SC-mRFP1 before being placed onto a PYE agar pad and imaged at 30 °C. Cells were imaged over 1 hour, but often failed to grow. Therefore, regions where signs of S-layer separation (indicating successful cell elongation following labelling) were deliberately selected. The resulting timelapses demonstrate the non-diffusible nature of the cell-bound S-layer relative to the underlying cell. This is evident given the fluorescent regions appear static relative to overall cell size during growth (Figure 4.1). The fluorescently labelled regions, which are confined largely to the cell body, i.e. regions containing old S-layer, remain broadly consistent in size throughout the imaging. In addition, the non-fluorescent regions found at the cell poles (particularly at the base of the stalk) and midcell, appear to expand. This is particularly evident in kymograph images where fluorescent regions have a uniform size throughout, but with loss of fluorescence due to bleaching (Figure 4.2).

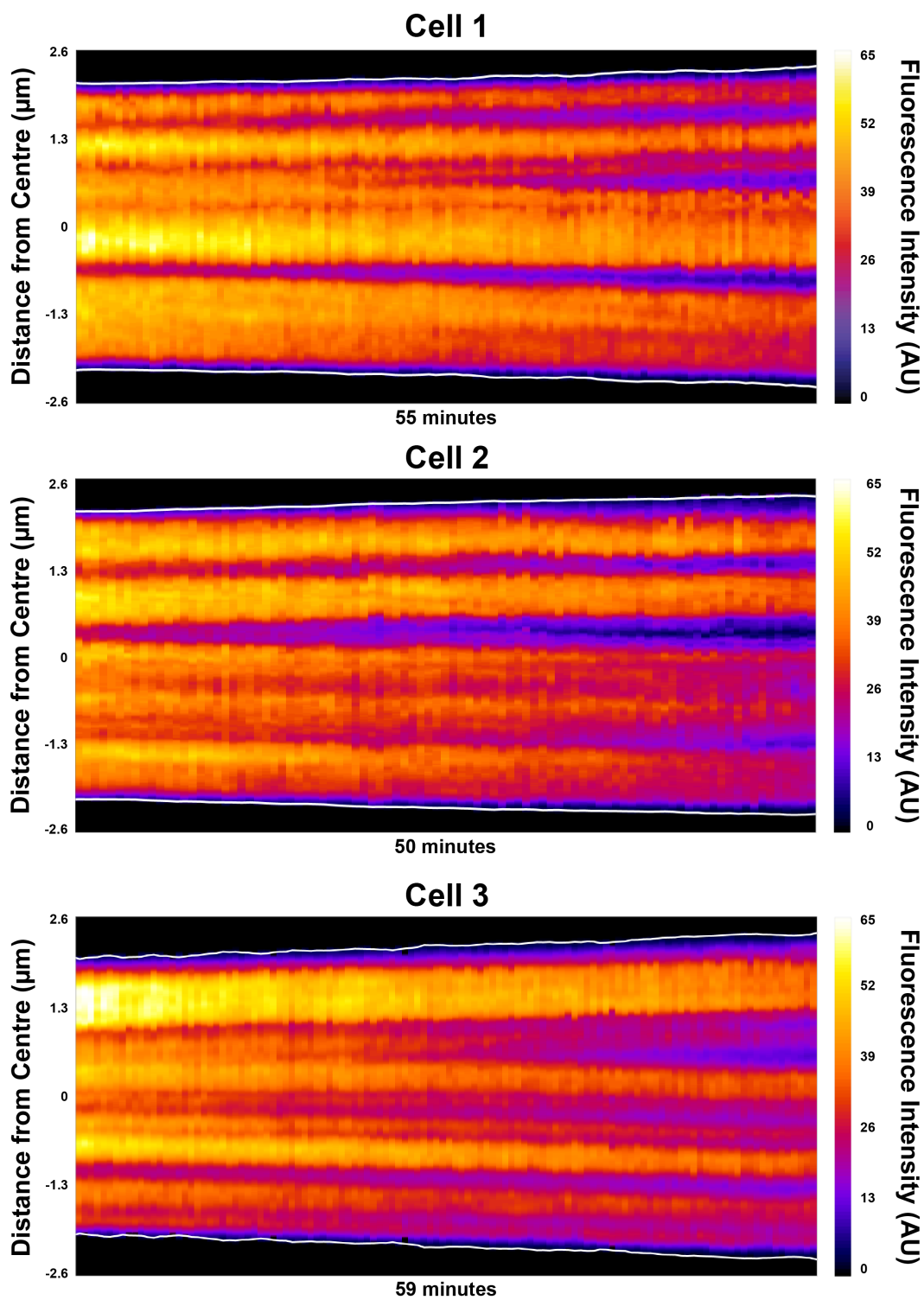


Figure 4.2. Kymographs of growing *C. crescentus* cells following S-layer labelling.

Compiled kymographs for each cell shown in Figure. 4.7. Kymographs represent fluorescence measurements along the medial axis; snapshots are ordered chronologically. Fluorescent regions remain uniform in size throughout the labelling, while non-fluorescent regions appear to expand. SC-mRFP1 fluorescence is presented as a fire heatmap.

4.4.2. Integration of the S-layer in *C. crescentus* is localised to the mid-cell and cell poles

In order to better examine the localisation of SLP insertion into the *C. crescentus* S-layer, a dual-labelling approach was adopted, as previously described in the exploration of calcium binding by RsaA (Figure 3.4) (Herdman et al., 2022). A pulse-chase technique using alternating fluorescent proteins permits the visible differentiation of old and new regions of S-layer (described earlier in this thesis). Growing (exponential phase) *C. crescentus* cells expressing RsaA-467-SpyTag were incubated with SC-mRFP1 to saturate the surface, followed subsequently by thorough washing and chase labelling with SC-sfGFP at 30 °C, to encourage cell growth. Cells were permitted to grow for 1h, approximately 67% of the standard doubling time of WT *C. crescentus* in PYE (Hottes et al., 2004), after which the cells exhibited distinct fluorescent regions of mRFP1 and sfGFP, identified respectively as old and new S-layer (Figure 4.3. A).

Cells generally displayed new S-layer (SC-sfGFP) at the poles and midcell, with older S-layer covering the remainder of the cell. Notably, the cell populations displayed asynchrony, and the fluorescence profiles showcased notable variations contingent upon the cell size and specific stage of the cell cycle. Intriguingly, non-dividing cells exhibited limited or polar SC-sfGFP labelling (Figure 4.3. B), while pre-divisional cells displayed a prominent midcell SC-sfGFP signal (Figure 4.3. C). The localisation of new S-layer content to the midcell and poles has been previously reported, albeit with exogenously added RsaA-fluorescence conjugates or under varying growth conditions. (Comerci et al., 2019, Herdman et al., 2022).

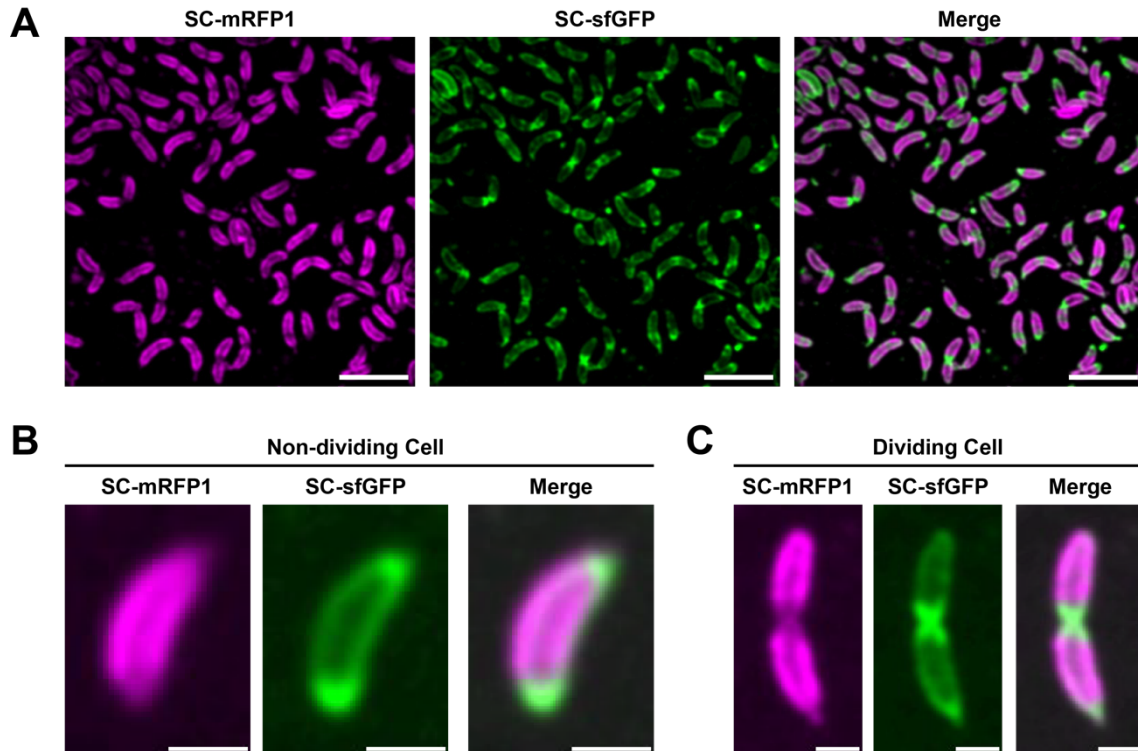


Figure 4.3. Dual-labelling of the *C. crescentus* S-layer reveals distinct regions of old and new S-layer at the midcell and cell poles.

(A) Micrographs of *C. crescentus* RsaA-467-SpyTag cells pulse-chase labelled using SC- mRFP1 (top), SC-sfGFP (middle). Merged channels (bottom) show distinct localisation of the two SC-conjugates along the cell surface. Micrographs were gaussian filtered to remove noise. Scale bar = 5 μ m. (B-C) Micrographs of a representative non-dividing and dividing *C. crescentus* cell. Scale bar = 1 μ m. Polar or mid-cell localisation of newly inserted S-layer being more prominent in non-dividing and dividing cell populations, respectively

Cell cultures were briefly synchronised by density centrifugation using 50% Percoll, resulting in two distinct bands containing predominantly larger, pre-divisional cells (top band) and shorter swarmer cells (bottom band) (Figure 4.4A-C) (Schrader and Shapiro, 2015). However, even following density centrifugation, there remained an observable overlap in the size distributions of cells separated into the top and bottom band (Figure 4.4D). This may be an indication of potential cross-contamination between the two bands, or indicative of only partially successful cell separation. Separation of the cell populations was successful enough that swarmer cells were collected from the bottom

band and released into PYE media, then subjected to the pulse-chase labelling as described above. Cell samples were taken 30 minutes post chase and 80 minutes post chase labelling, resulting in a majority of either swarmer or dividing cells, respectively, with clear dual labelling (Figure 4.5). Labelling patterns between synchronised and unsynchronised cell samples were broadly comparable, except polar labelling was observed in almost all cells. Midcell labelling was once again enriched in larger, pre-divisional cells (Figure 4.5B), confirming there is indeed a redistribution of SLP insertion from the cell poles to the midcell as the cell progresses through development.

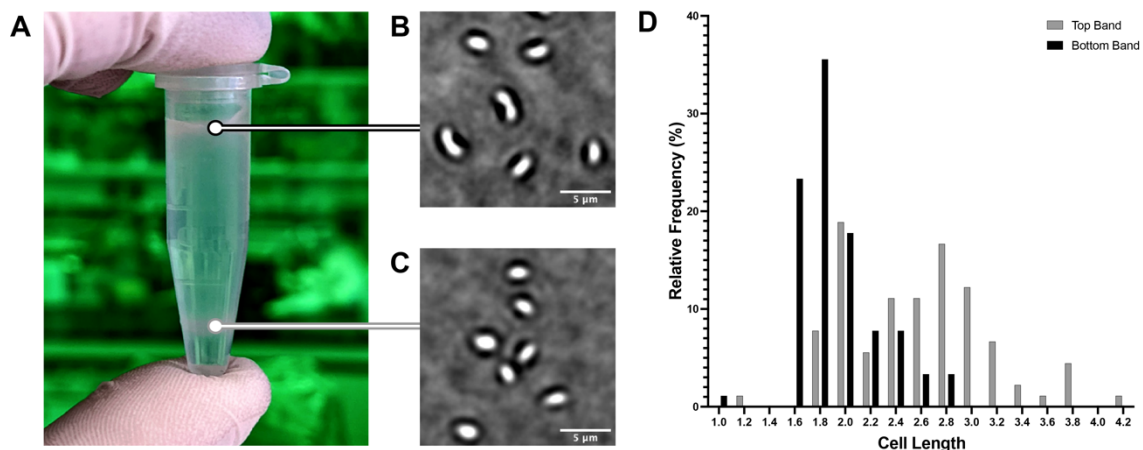


Figure 4.4. Separation of stalked and swarmer *C. crescentus* cell populations by density centrifugation.

(A) Density centrifugation of *C. crescentus* culture suspended in 50% Percoll segregates cells by buoyancy into two bands. Widefield microscopy confirms the top band (A) contains mostly larger, pre-divisional cells. The lower band (C) is composed mostly of smaller cells, presumed to be swarmer cells. At this resolution, stalks are indiscernible, so cell length was examined to confirm the separation of cell populations. (D) The bottom band contains a higher proportion (%) of small cells $< 2.5 \mu\text{m}$ in length compared to the top band. There is still a significant level of overlap between the two distributions, suggesting a potential limitation of the centrifugation-based separation method.

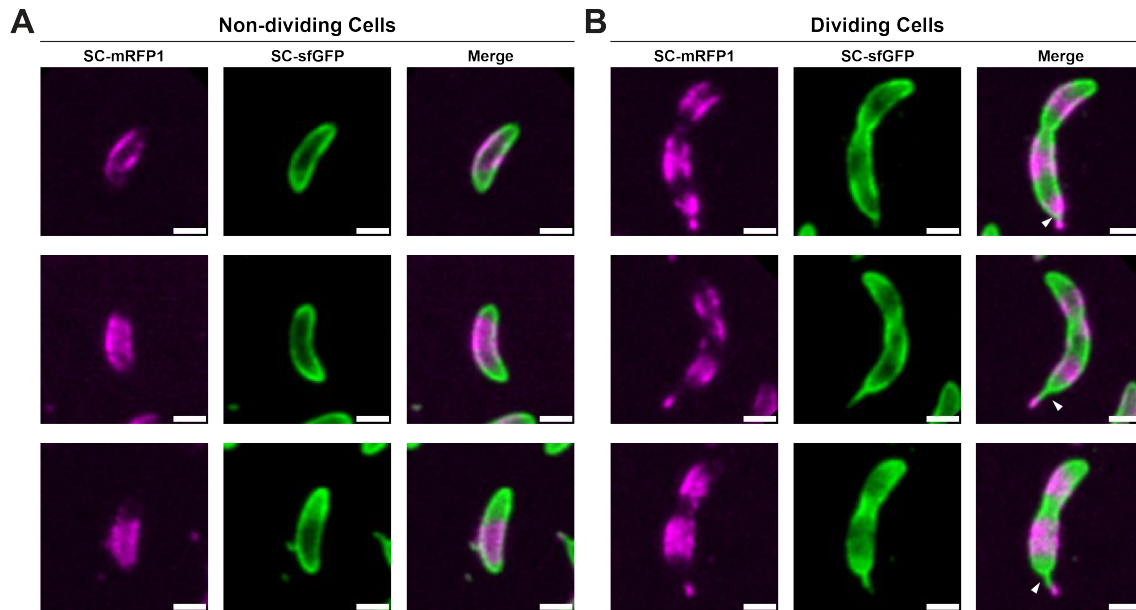


Figure 4.5. Dual-labelled *C. crescentus* cells retain polar and midcell labelling after synchronisation.

Following synchronisation using density centrifugation and pulse-chase labelling using alternative SC-FP conjugates, dual-labelled *C. crescentus* cells retain polar and mid-cell localisation of the new S-layer (SC-sfGFP). As previously observed, (A) non-dividing cells exhibit largely polar localisation of new S-layer material, while in (B) dividing cells show polar and midcell localisation. Merged micrographs of show the presence of dividing cells with dual-labelled stalks. Scale bars = 1 μ m.

Remarkably, many cells at late stages of development had clearly distinguishable stalks which often exhibited dual-coloured labelling (Figure 4.5B). This was marked by localisation of new (sfGFP) S-layer material at the base of the stalk, and older (mRFP1) SLP at the stalk tip. Stalk biogenesis is driven by significant cell wall reconstruction and production at the base of the new prosthecae, leading to the stalk growing outwards from the cell body, rather than being synthesised from the tip (Billini et al., 2019, Kuru et al., 2012). The labelling patterns of the cell stalks seemed to depend on whether the stalks were present during the pulse labelling or generated during the chase-labelling. The stalk denotes a rapid transition from swarmer to sessile cell type in *C. crescentus*, with the latter representing the dividing population. This labelling pattern, along with the

accumulation on new SLP at the midcell during the onset of division, suggests a co-localisation of S-layer biogenesis with macromolecular complexes typically associated with cell elongation, division, and stalk biogenesis.

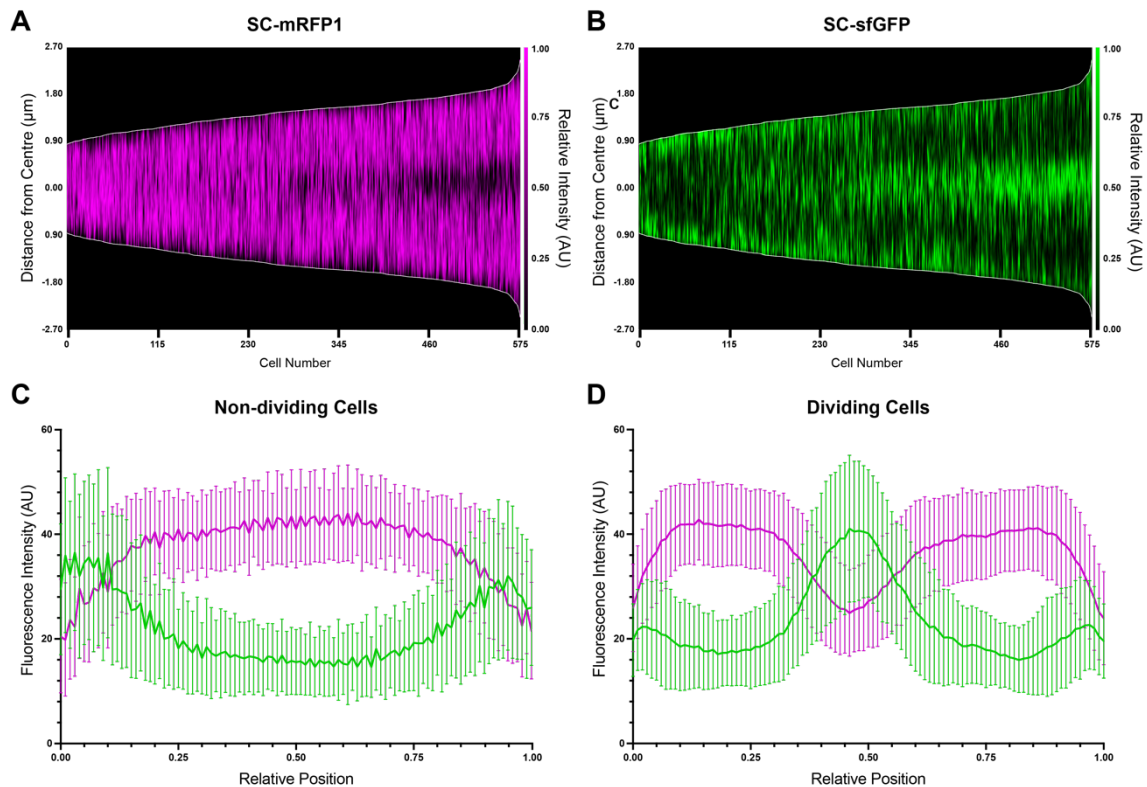


Figure 4.6. Polar and midcell localisation of chase-labelled S-layer is dependent on cell length.

Cells were segmented and demographs depicting fluorescent profiles along the medial axis of each cell ($n = 575$) were produced, which were then organised by ascending cell length, showing the localisation of (A) old S-layer (SC-mRFP1) and (B) new S-layer (SC-sfGFP). Fluorescence profiles were normalised. From the same dataset, the fluorescent profiles along the medial axis of (C) non-dividing and (D) dividing cells (selected based on cell length and the absence/presence of both a cell stalk and mid-cell invagination) were averaged for both channels and plotted against relative cell length ($n = 100$ for both cell types). SC-mRFP1 labelling is shown in magenta, SC-sfGFP is shown in green, the bold line indicating mean intensity. error bars show standard deviation.

To assess the relationship between S-layer localization and cell size, cell profiles were ordered according to cell length using MicrobeJ (Ducret et al., 2016). Consistent with visual inspection, the assembled demographs confirmed a clear progression of new S-layer labelling away from the poles and toward the midcell (Figure 4.6A-B). To further quantify the relationship between the cell cycle stage and the labelling pattern, normalized fluorescence profiles of non-dividing swarmer cells and dividing cells were plotted against relative cell length (Figure 4.6C-D). As anticipated, non-dividing cells exhibited a stronger normalized SC-sfGFP signal at their poles, while dividing cells displayed a much stronger signal at their mid-cell.

While a vast majority of cells show restriction of SC-sfGFP labelling to the midcell or cell poles, depending on position along their cell cycle, several cells appeared to have additional sites of new S-layer material (Figure 4.7). These cells were all in the late stages of division, as indicated by their length and the prominent invagination site at the midcell. In addition, they all displayed the now-familiar polar and mid-cell labelling seen in similarly aged cells. However, there are points along the cell body, in one or more locations between both the cell poles and midcell, where there appears to be new S-layer material (Figure 4.7). Extracting the fluorescent profiles along the contours of the cell perimeter, these regions are found to be small in comparison to the dominant midcell fluorescence (Figure 4.8). However, they still correspond to a reduction in SC-mRFP1 labelling and appear to encompass the cell in a ring-like orientation. While intriguing, these represent a minority of cells reported, and are often so faint they are difficult to distinguish from the surrounding SC-mRFP1 signal.

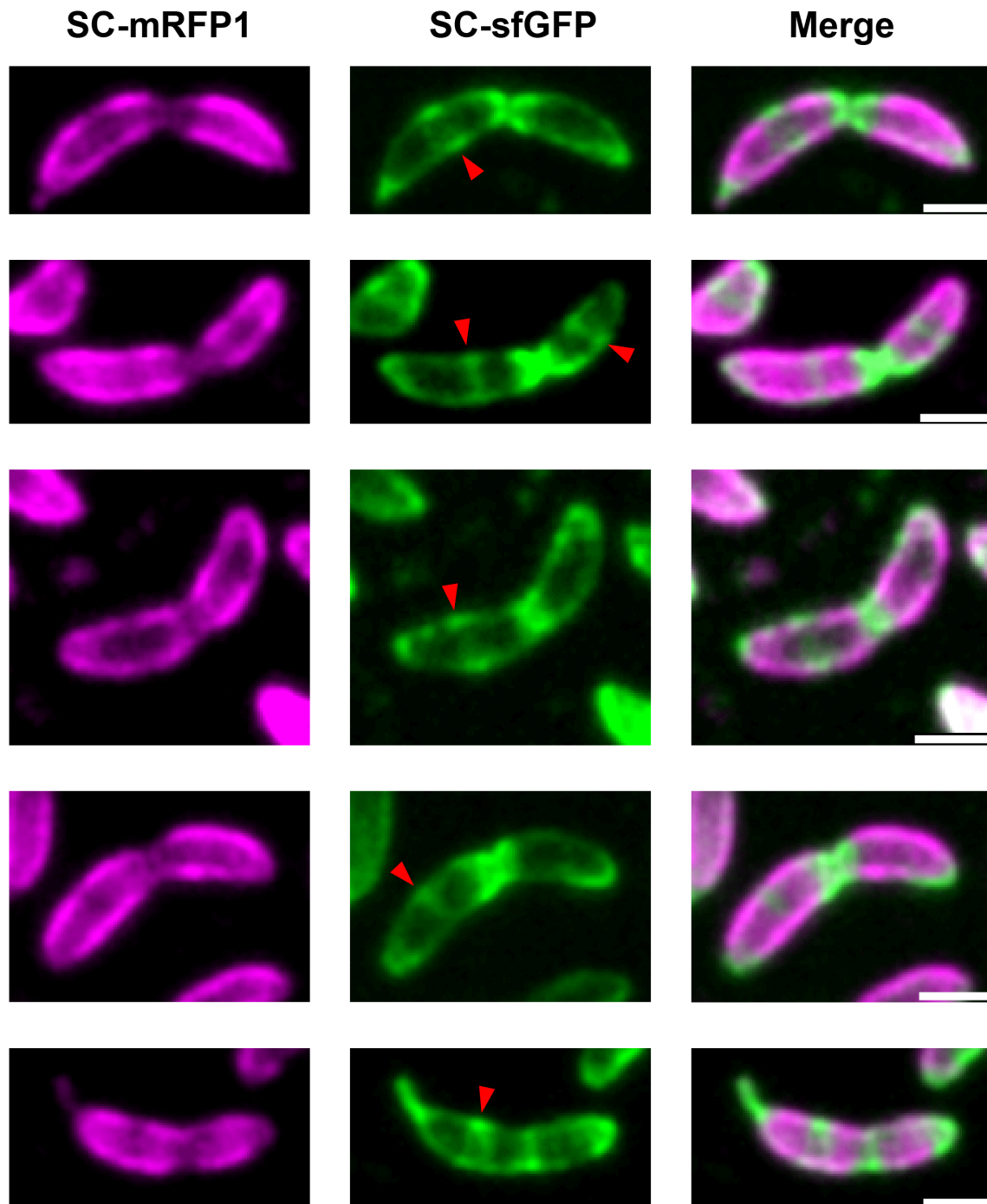


Figure 4.7. Additional S-layer insertion sites in *C. crescentus*, between the cell pole and midcell.

C. crescentus cells pulse-chase labelled in SC-mRFP1 and SC-sfGFP, as described in Figure 4.3. Cells show midcell and polar chase labelling, consistent with previous observations, but have additional insertion sites at an intermediate position along the cell body (indicated by red arrows). Merged images are provided to show anticorrelation of SC-mRFP1 and SC-sfGFP labelling, corresponding to old and new S-layer material respectively. Scale bar = 1 μm .

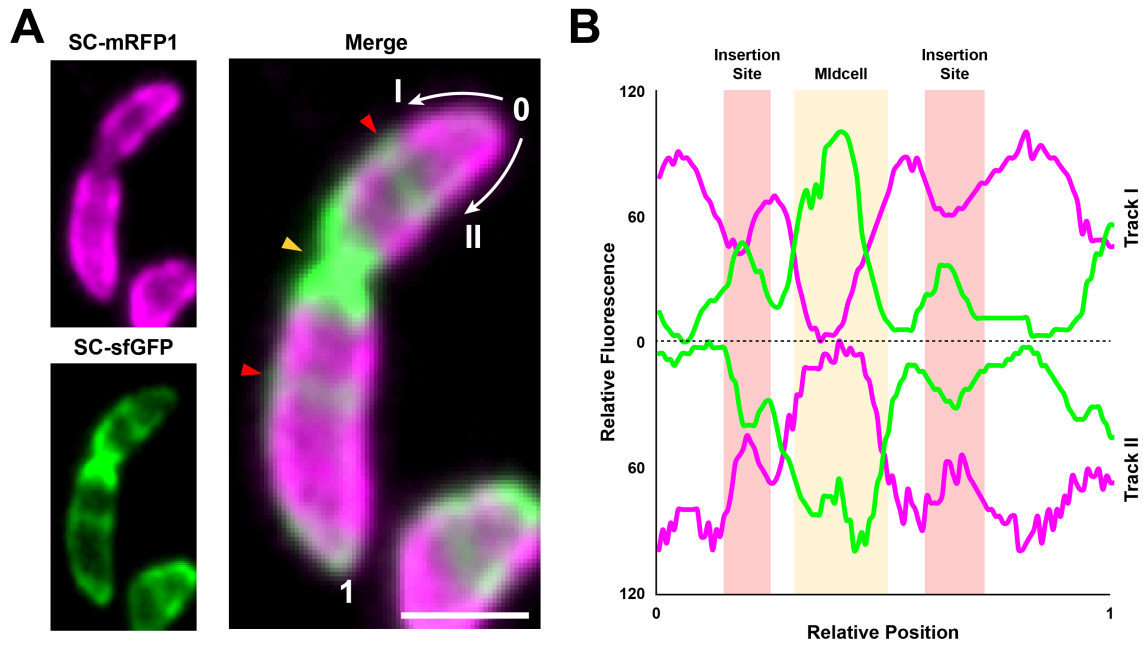


Figure 4.8. Presence of multiple points of new S-layer insertion in a sub-population of cells.

(A) *C. crescentus* cells pulse-chase labelled in SC-mRFP1 and SC-sfGFP, as described in Figure 4.3. The midcell is indicated by a yellow arrow and the intermediate insertion sites are indicated by red arrows. Scale bar = 1 μm . (B) Relative fluorescence profiles of Tracks I and Tracks II, corresponding to I and II in A, beginning at cell tip 0 and proceeding to cell tip 1. Fluorescence readings are extracted along the cell boundary and plotted relative to cell length (Relative Position). Regions of new S-layer insertion are clearly identifiable by an increase in relative SC-sfGFP fluorescence – highlighted in yellow for midcell or red for new insertion sites.

4.4.3. Cell wall turnover precedes S-layer insertion

Previous studies on the S-layer of *C. difficile* have proposed a link between cell wall turnover and S-layer biogenesis (Oatley et al., 2020). In Gram-positive monoderm bacteria, the S-layer is bound directly to the thick peptidoglycan cell wall via secondary cell wall polymers (Willing et al., 2015). To explore potential coordination of the S-layer and cell wall biogenesis in the Gram-negative diderm bacterium *C. crescentus*, the SC-ST labelling strategy was adapted to include the use of fluorescent D-amino acids (FDAA). FDAAs are incorporated into the bacterial cell walls at regions of peptidoglycan

synthesis or recycling, such as the septum, by PBPs (Kuru et al., 2012). The addition of HADA, a blue-fluorescent FDAA (Peters et al., 2019), into the labelling strategy permits simultaneous localisation of regions with high cell wall turnover and S-layer biogenesis, supporting an analysis of both processes.

In initial HADA labelling (for 10 minutes), fixed and unfixed samples resulted in comparable fluorescence intensities emanating from the cells. Therefore, cell samples were left unfixed to maintain consistency with previous labelling experiments presented in this and other chapters. The HADA fluorescence was relatively faint compared to the SC signal, and an alternative microscope equipped with GASSP detectors was used to maximise signal detection. *C. crescentus* cells were labelled with SpyCatcher using the previously described pulse-chase method, with HADA was added for the last 5 minutes of the chase labelling stage, followed by thorough washing. As expected, this resulted in cells with dual-labelled S-layers showing clear regions of old and new S-layer (Figure 4.9. A-C). HADA labelled cells showed heterogenous labelling of the whole cell, with the strongest fluorescence present at the midcell and cell poles (Figure 4.9. D). When arranged by ascending length, the fluorescence pattern appears to be localised in a similar cell-age dependent pattern to that observed using SpyCatcher labelling (Figure 4.9. E). The spatiotemporal localisation of HADA fluorescence is consistent with previous studies (Peters et al., 2019, Billini et al., 2019) and expected given redistribution of cell wall turnover to the cell stalk and midcell (Sundararajan and Goley, 2017, Goley et al., 2011, Takacs et al., 2010).

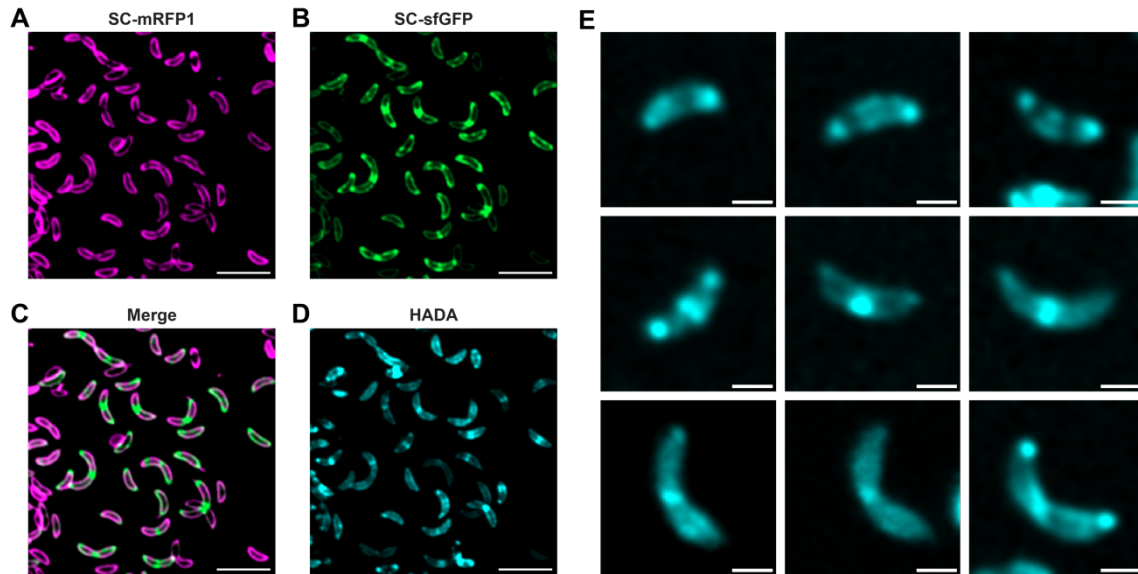


Figure 4.9. Cell wall and S-layer have similar patterns of biogenesis localisation.

C. crescentus cells pulse labelled using (A) SC-mRFP1 and chased with (B) SC-sfGFP. (C) Merged micrograph shows distinct regions of SC-mRFP1 and SC-sfGFP S-layer. (D) Fluorescent labelling using HADA, incorporated into the last five minutes of the SC-sfGFP chase labelling. Scale bar = 5 μm . (E) Gallery of individual HADA-labelled *C. crescentus* cells arranged according to cell length, beginning top left and ending bottom right. Scale bar = 1 μm .

While HADA appears to label the entire cell to some degree, in pre-divisional cells, the strongest signal is found at the midcell. In many cells, there is a clearly observable overlap of SC-sfGFP signal, corresponding to new S-layer material, and HADA signal localisations (Figure 4.10). However, this is not apparent in every cell. In addition, several cells which show the expected polar S-layer labelling corresponding to stalk biogenesis often lack any associated HADA signal, despite the known increase in cell wall turnover that drives stalk biogenesis (Billini et al., 2019, Kuru et al., 2012).

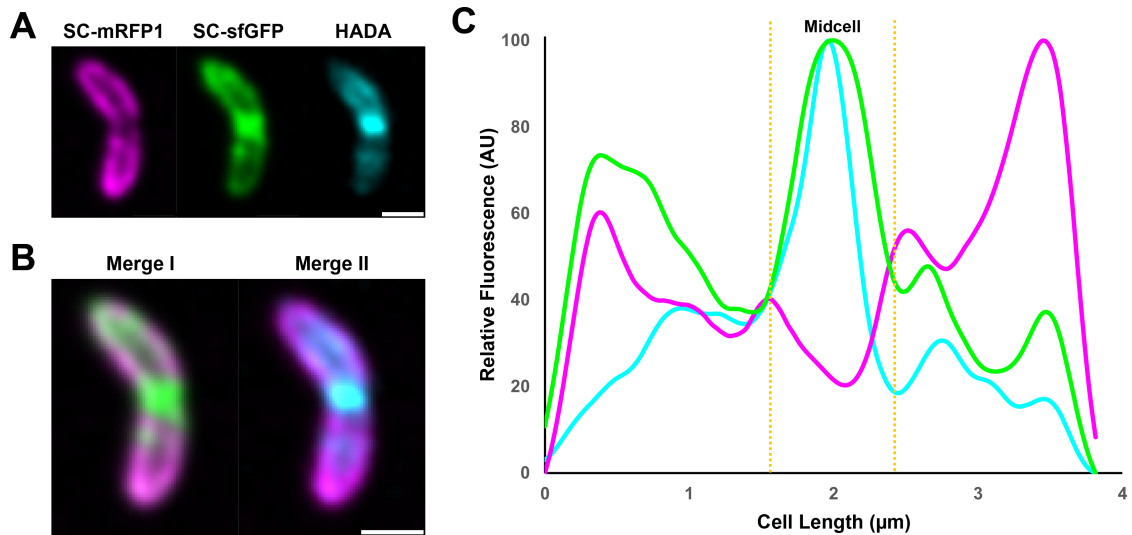


Figure 4.10. Colocalisation of new S-layer material with cell wall turnover.

(A) *C. crescentus* cells pulse-chase labelled in SC-mRFP1 and SC-sfGFP, as described previously. Peptidoglycan was briefly labelled (5 min) with HADA at the end of the chase-labelling phase. (B) Merged images showing anti-colocalisation of SC-mRFP1 with SC-sfGFP (Merge I) and SC-mRFP1 with HADA (Merge II). Scale bars = 1 μm. (C) Extracted fluorescence profiles along the medial axis of the cell shows a colocalisation of midcell (dashed yellow line) SC-sfGFP and HADA signals.

The discrepancy in localisation observed in some cells may be due to the exact timeframes in which the labelling of different envelope components was performed. Chase labelling with SC-sfGFP was carried out over 1 hour to label newly inserted RsaA, while cell wall turnover was only briefly labelled using HADA for 5 minutes, at the very end of the chase. This explains why polar fluorescence associated with stalk biogenesis is preserved predominantly in SC-sfGFP, as this irreversible labelling is representative of the entire chase. By the time HADA is introduced, the accumulation of the peptidoglycan rebuilding machinery necessary for stalk biogenesis has likely concluded its job, meaning it is no longer active or present to incorporate HADA. Polar HADA signal is still present in some late stage dividing cells, showing that some latent cell wall turnover is still occurring at these regions.

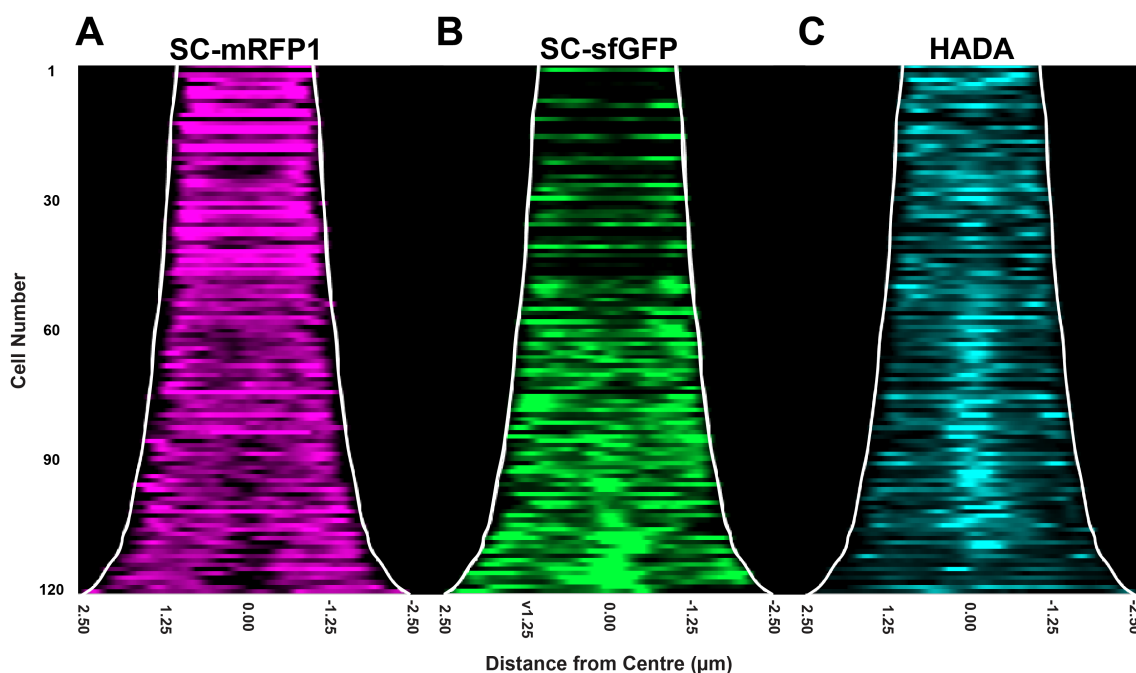


Figure 4.11. Cell wall turnover precedes S-layer biogenesis.

Demographs comprised of segmented *C. crescentus* cells pulse-chase labelled in SC-mRFP1 (A), SC-sfGFP (B), and HADA (C). Cells are arranged in ascending length beginning at the top of each demograph and fluorescence intensity has been normalised.

To better evaluate the timing of cell wall and S-layer biogenesis, kymographs were compiled in a similar way to those presented in Figure 4.6. This presented challenges as HADA signal was much weaker and more heterogenous than either fluorescent protein used in S-layer labelling. In addition, the resolution afforded by the microscope used in HADA-labelling experiments is lower than that used for dual-labelling experiments. Nevertheless, the kymographs were able to replicate the loss of SC-mRFP1 localisation at the midcell as cells increase in length, corresponding to the local increase of SC-sfGFP fluorescence in the same regions (Figure 4.11A-B). The kymograph compiled using HADA fluorescence showed a similar trend, but the localisation of cell wall turnover to the midcell occurred at shorter cell lengths than seen for SC-sfGFP (Figure 4.11C). This suggests that cell wall turnover in these regions precedes the localisation of S-layer biogenesis.

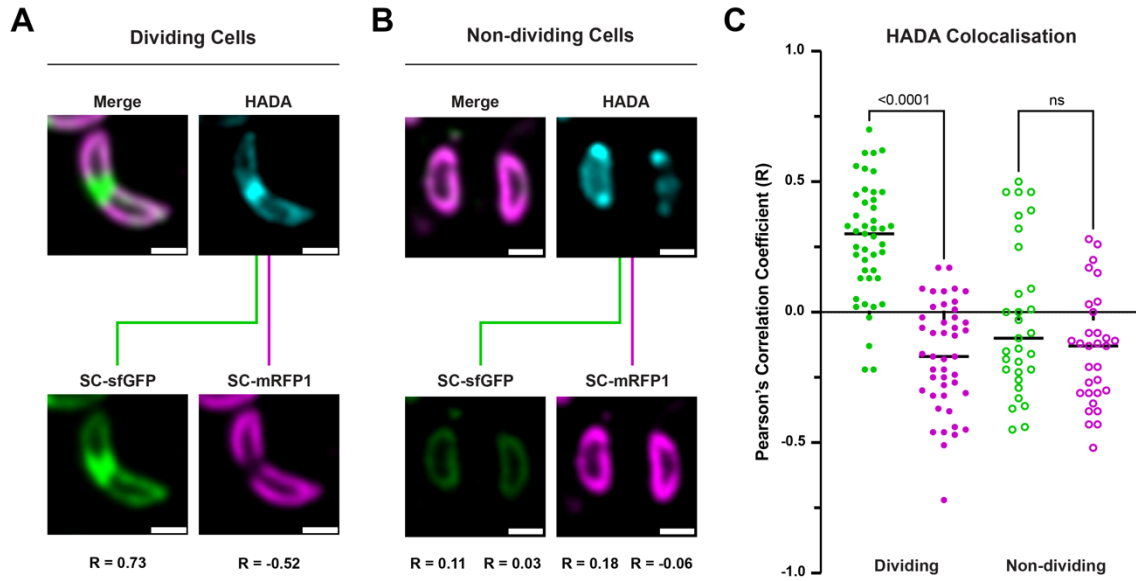


Figure 4.12. Cell wall turnover co-localises with new S-layer in dividing cells.

Fluorescently labelled cells were used for co-localisation analysis; the HADA fluorescence signal was compared to both SC-sfGFP and SC-mRFP1. Representative dividing (A) and non-dividing (B) are shown, with colocalisation pairing indicated by the green and magenta lines. The respective PCC R-values are provided below each cell. Scale bar = 1 μm . (C) PCC scores (R-values) between HADA and sfGFP or mRFP1 channels for dividing ($n = 45$) and non-dividing ($n = 31$) cells measuring colocalisation. Dividing cells show a significantly higher R value for colocalisation between HADA and sfGFP (●) compared to mRFP1 (●), suggesting stronger colocalisation. Conversely, non-dividing cells return a negative average R-value for correlation between HADA and SC-sfGFP (○) or HADA and SC-mRFP1 (○), with nonsignificant difference between the two. Significance was determined using Student's t-test.

Having confirmed that the biogenesis of the cell wall and S-layer is spatially localised, the extent of the colocalisation between HADA and SC-sfGFP was quantified using Pearson's Correlation Coefficient (PCC) (McDonald and Dunn, 2013, Manders et al., 1993). PCC measures the linear correlation between two datasets, i.e. two fluorescent channels, reported as an R-value. A PCC R-value > 0 suggests that there is colocalisation between the two fluorescent signals, whereas an R-value < 0 shows anticorrelation (Schober et al., 2018, Taylor, 1990, Hemphill, 2003, McDonald and Dunn, 2013).

Dividing and non-dividing cells were individually segmented, masked, and HADA colocalisation with both SC-mRFP1 and SC-sfGFP measured (Figure 4.12A-B).

Dividing cells, as expected from visual inspection of the data and measurement of their fluorescence profiles, showed an on-average position colocalisation of HADA with SC-sfGFP ($R = 0.30$), and were anticorrelated with SC-mRFP1 ($R = -0.15$). In non-dividing cells, the colocalisation of HADA with both SC-mRFP1 and SC-sfGFP was broadly indistinguishable and confirmed to be not statistically different by Student's *t*-test (Figure 4.12C). Together with the kymographs of HADA and SC labelled cells in Figure 4.11, the data suggests that cell wall turnover and localised growth at the midcell precedes S-layer localisation at the same area. Therefore, it is possible that expansion of the underlying cell envelope, as driven by cell wall biogenesis, support the expansion of the S-layer, mediating the attachment and integration of new RsaA at these areas.

4.5. Discussion

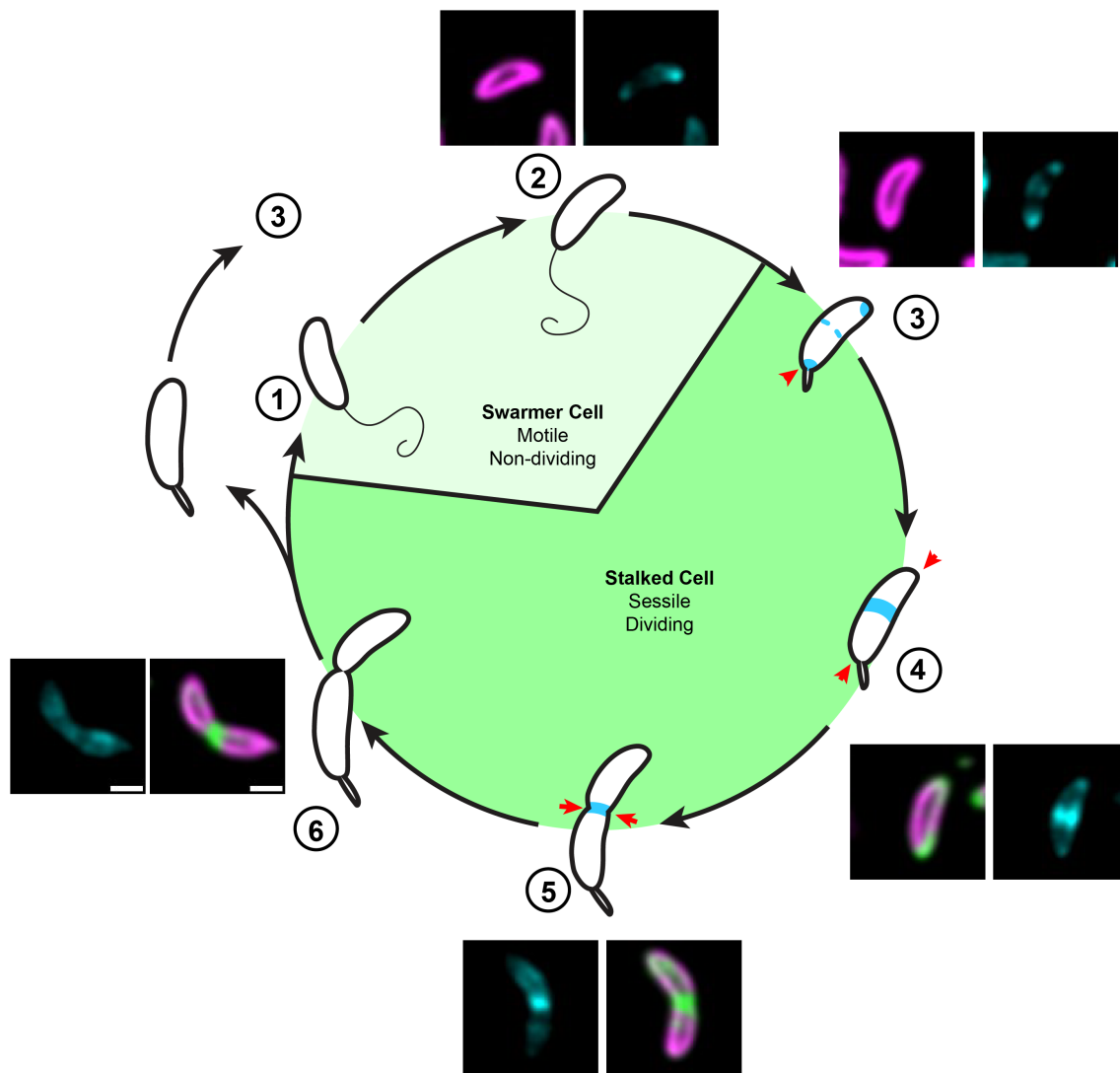


Figure 4.13. Spatial localisation of cell wall turnover and S-layer biogenesis as *C. crescentus* progresses through the cell cycle.

Cell wall turnover and S-layer biogenesis are spatially localised depending on the stage of the *C. crescentus* cell cycle. (1-2) In division-quiescent swarmer cells, HADA labelling is either absent or present at the cell poles, representing the previous division site or the beginnings of the transition to stalked-stages of cell development. (3) Stalk biogenesis results in strong polar labelling at the stalk base, signalling the presence of high cell wall turnover necessary for stalk formation, and the beginning of Z-ring formation can be seen by a HADA-fluorescent ring at the midcell. In this stage, new S-layer may be found at polar regions in some cells. (4) As the divisome matures, HADA signal at the midcell intensifies. Polar S-layer labelling is more prominent following the conclusion of stalk biogenesis. (5) Invagination at the midcell becomes visible. Divisome-associated HADA labelling is still prominent, and S-layer labelling at the midcell becomes visible. (6) Late-stage division is often marked by the loss of HADA fluorescence at the midcell as the divisome dissociates. Midcell S-layer labelling is still clear. Following division, cells are differentiated into swarmer (1) and stalked (3) cells. Scale bar = 1 μm .

Prokaryotes have long been recognised for their ability to localise the biogenesis of cell structures in space and time (Rudner and Losick, 2010, Surovtsev and Jacobs-Wagner, 2018). *C. crescentus* has been particularly integral in uncovering the sophisticated mechanisms that underpin this precise spatiotemporal cell organisation (Govers and Jacobs-Wagner, 2020, Barrows and Goley, 2023). Several studies have suggested that S-layer biogenesis follows no particularly spatiotemporal control and is instead subject to rapid crystallisation mediated by biophysical properties of the SLP (Comerci et al., 2019, Herrmann et al., 2020). Despite this, S-layer biogenesis has been posited to be under a similar level of precise localisation, as would be expected for such a large macromolecular structure encompassing the entire cell. This has been observed independently in multiple species across prokaryotes, including the Gram-positive bacterium *C. difficile* and the Haloarchaeon *H. volcanii* (Oatley et al., 2020, Abdul-Halim et al., 2020). Both species show localisation of new S-layer material to the midcell and cell poles, similar to the observed localisation of RsaA reported here. S-layer labelling experiments with *C. crescentus* has clearly demonstrated that as the cell progresses through the cell cycle, RsaA is integrated into the growing lattice at specific regions of the cell, i.e. the midcell and cell poles.

The localisations of new S-layer material appear to be heavily linked to the cell cycle, with redistribution from polar labelling to the midcell following the transition to dividing cell type (Figure 4.3 and Figure 4.4). Fluorescent co-labelling with RsaA-binding SC proteins and PG-labelling HADA revealed a clear spatial localisation between the biogenesis of two envelope components, despite the lack of any known molecular crosstalk between the two (Figure 4.10). Temporally, while cell wall and S-layer biogenesis appear to be slightly offset, this is perhaps due to the differential approaches to labelling, i.e. direct labelling of PG-turnover at a specific time point vs the longer

timescale of S-layer labelling using fluorescent SC proteins. Despite this ambiguity, this suggests is that cell wall turnover at areas like the midcell and stalk base precede integration of RsaA into the S-layer at those regions, resulting in a temporal offset between the two processes that can be tracked over the cell cycle (Figure 4.13).

Together, these data suggest a model in which local PG-turnover drives local expansion of the cell envelope, which in turn drives the separation of the above S-layer. These gaps can then be filled by underlying, secreted RsaA. Given the high rate of secretion achieved by RsaDEF and the high copy number of RsaA present in the cell, it is reasonable to expect a pool of RsaA beneath the S-layer surface, prepared for integration.

The proposed model of S-layer biogenesis a model would also be consistent with previously proposed functionality of the *C. crescentus* S-layer, which has remained relatively elusive. In the environment, *C. crescentus* would be subject to a number of external pressures, such as biomineralisation. A dispensable, self-healing S-layer could feasibly bear the brunt of such stress, thereby protecting the underlying cell. A pool of unintegrated RsaA beneath the S-layer would allow the cell to simultaneously fill gaps at points of envelope expansion and damage (Chandramohan et al., 2018).

Interestingly, the localisation of S-layer biogenesis in other species has been studied in the context of their partner secretory and biosynthetic machineries. In *C. difficile*, the secretion machinery of SlpA, the major structural SLP, SecA2, was localised using a fluorescently tagged, secretion deficient version of SlpA (Oatley et al., 2020). This study reported localisation of the SecA2 machinery across the cell surface, despite localisation of SlpA to the midcell, consistent with the proposed model of S-layer biogenesis in *C. crescentus* (Figure 4.14). However, these bacterial systems appear to contrast with the *H. volcanii*, whose SLP (referred to as Csg) colocalises with both FtsZ

and lipid modification protein ArtA, which enables direct binding of the S-layer to the cytoplasmic membrane (Abdul-Halim et al., 2020). This suggests that *H. volcanii*, and potentially other archaea, more strictly localise their S-layer secretion and biogenesis. These contrasting mechanisms of S-layer biogenesis may owe to the S-layer being a core cell wall component in *H. volcanii*, requiring more precise control over its synthesis, while *C. crescentus* and *C. difficile* have additional components (i.e. the peptidoglycan cell wall) that make S-layer disruptions less impactful to cell fitness. However, in *C. difficile*, S-layer disruptions can be quite detrimental to cell pathogenesis (Merrigan et al., 2013, Vaz et al., 2019).

The question remains regarding the localisation of the RsaA secretion machinery, RsaDEF, a canonical T1SS (Awram and Smit, 1998, Costa et al., 2015). T1SS activity and the secretion of the cargo has been well characterised, particularly in the case of HlyA toxin secretion (Hubert and Lenders, 2015), but the localisation of T1SS components has been unexplored, largely due to the challenges of labelling a multi-component secretory system (Barlag et al., 2016). Further studies on the localisation of S-layer biogenesis in *C. crescentus*, particularly in the context of RsaDEF, are needed to provide a full explanation of how prokaryotes build such a complex and expansive structure on their cell surface. It will be of particular interest to see how these approaches vary across species and domains.

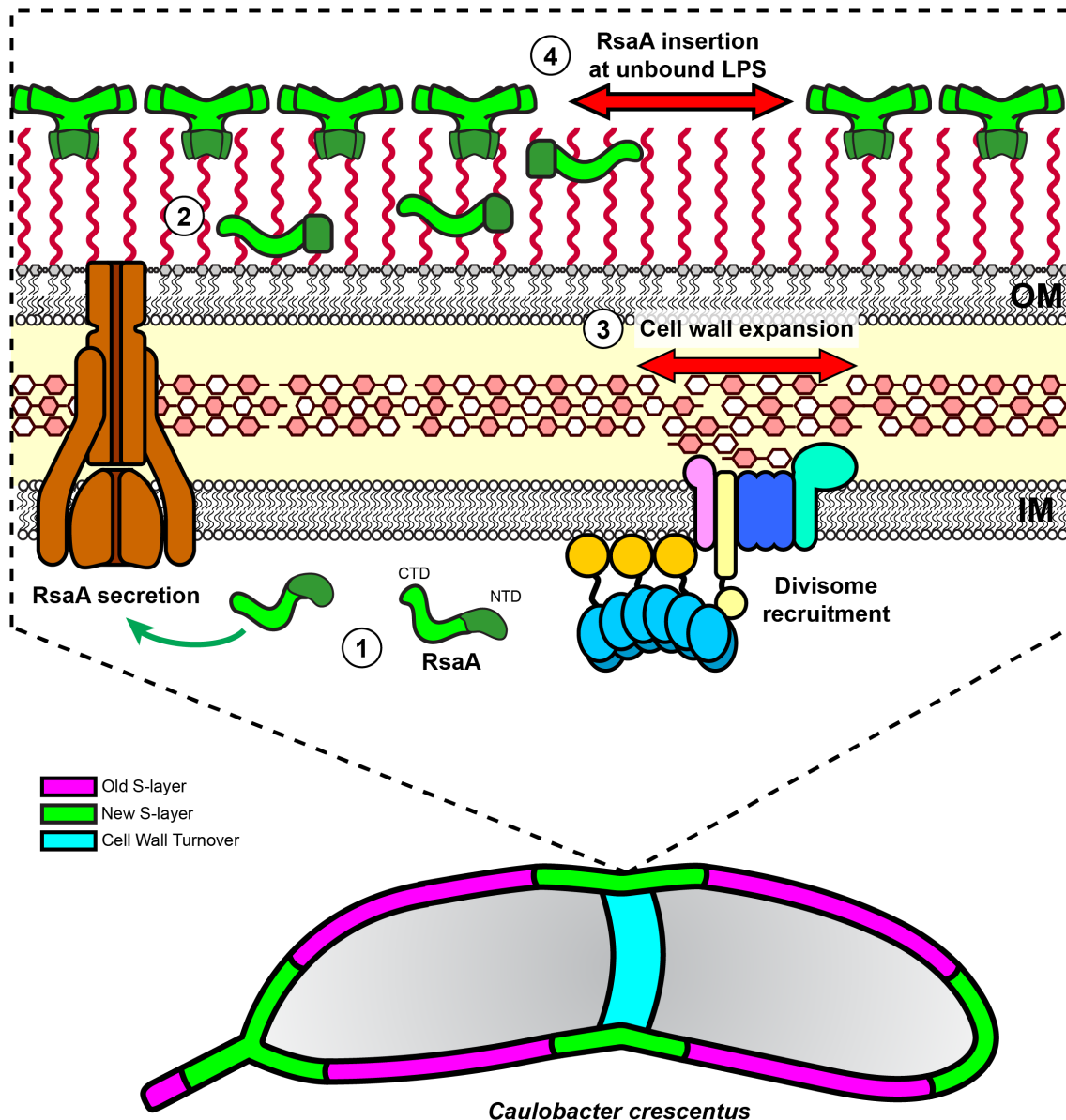


Figure 4.14. Cell wall expansion as a guiding force for the localisation of S-layer biogenesis.

Schematic illustrating the proposed model of localised S-layer biogenesis dependent on PG-turnover and cell envelope expansion, (1) Unfolded, monomeric RsaA (represented in green) in the cytoplasm is secreted by the RsaDEF secretion system. (2) Following secretion and Ca^{2+} binding, RsaA_{NTD} attaches to the OPS. (3) The expanding cell wall, driven by peptidoglycan turnover and remodelling (the divisome provided here as an illustrative example), drives divergence of the overlying S-layer. (4) RsaA diffuses to the tip of the OPS to the gap in the S-layer, where it oligomerises and incorporates into the RsaA.

CHAPTER 5. S-layer Biogenesis Following Cell-cycle Disruption

5.1. Overview

With the knowledge of the spatiotemporal localisation of S-layer biogenesis, its dependence on the cell cycle can be examined. Localisation of biosynthetic machineries in prokaryotes are well studied, particularly in *C. crescentus*, largely owing to its asymmetric life cycle (Govers and Jacobs-Wagner, 2020, Barrows and Goley, 2023). In addition, the careful partition of stalk biogenesis and cell division from the swarmer stage of the *C. crescentus* life cycle is the result of spatiotemporally co-ordinated protein networks (Biondi et al., 2006, Skerker and Laub, 2004, Sundararajan and Goley, 2017). S-layer biogenesis may well be similarly localised, as suggested by its proximity to cell wall turnover, as described in Chapter 4. Using cell cycle disrupting compounds, the cell-cycle dependent nature of S-layer biogenesis may be studied.

Cephalexin, a biostatic, cephalosporin antibiotic, has been shown to inhibit cell division by binding and inhibiting FtsI, or PBP3 (Griffith, 1983, Pogliano et al., 1997). Localised to the cytoplasmic membrane, FtsI is largely periplasmic and, together with PBP1b, catalyses the glycan-crosslinking of peptidoglycan during cell division, interacting with multiple components of the divisome complex (Sauvage et al., 2014, Bertsche et al., 2006). Inhibition of cell division via FtsI using cephalexin results in *C. crescentus* filamentation that retain asymmetric cell organisation (Chen et al., 2011). The spatiotemporal localisation of S-layer biogenesis under exposure to cephalexin has been partly investigated using exogenously labelled RsaA (Comerci et al., 2019), however its effect on endogenous RsaA integration is yet to be explored.

5.2. Acknowledgments

The work in this chapter was performed by the candidate with the following exceptions. Cryo-ET data collection was performed in collaboration with Andriko von K gelgen.

5.3. Aims

Briefly, the project aims were as follows:

- (i) Evaluate the effect of cephalexin on the localisation of S-layer biogenesis in *C. crescentus*.
- (ii) Evaluate the effect of A22 on the localisation of S-layer biogenesis in *C. crescentus*.
- (iii) Examine the organisation of RsaA in drug-treated cells using cryo-ET.

A portion of results relevant to this chapter are published in Herdman *et al.*, *bioRxiv*, June 2023, and under revision in the journal *Nature Communications*.

5.4. Results

5.4.1. *C. crescentus* undergoes distinct morphological changes following exposure to cell cycle disrupting compounds

To confirm activity of cephalixin and A22 in *C. crescentus*, cells were screened using widefield microscopy for morphological changes, as previously described (Koyasu et al., 1981, Takacs et al., 2010). *C. crescentus* has a very identifiable cell morphology, owing to crescent shape and unipolar stalk (Figure 5.1A, B). Its length is also relatively uniform, between 2 and 5 μm , depending on its current stage of the cell cycle (Poindexter, 1981). Cells were initially incubated in PYE media containing 50 $\mu\text{g/mL}$ cephalixin for four hours, resulting in filamentous *C. crescentus* cells, as expected (Figure 5.1A, C). Even at A22 concentrations ≥ 10 $\mu\text{g/mL}$ did not result in any observable morphological changes over four hours (not shown). To compensate, cells were incubated with a low concentration of A22 (3 $\mu\text{g/mL}$) overnight. This resulted in cells with a clear lemon-shaped phenotype, an indication of successful MreB disruption (Figure 5.1A, D). A summary of morphological changes, including reference micrographs, is provided in Figure 5.1.

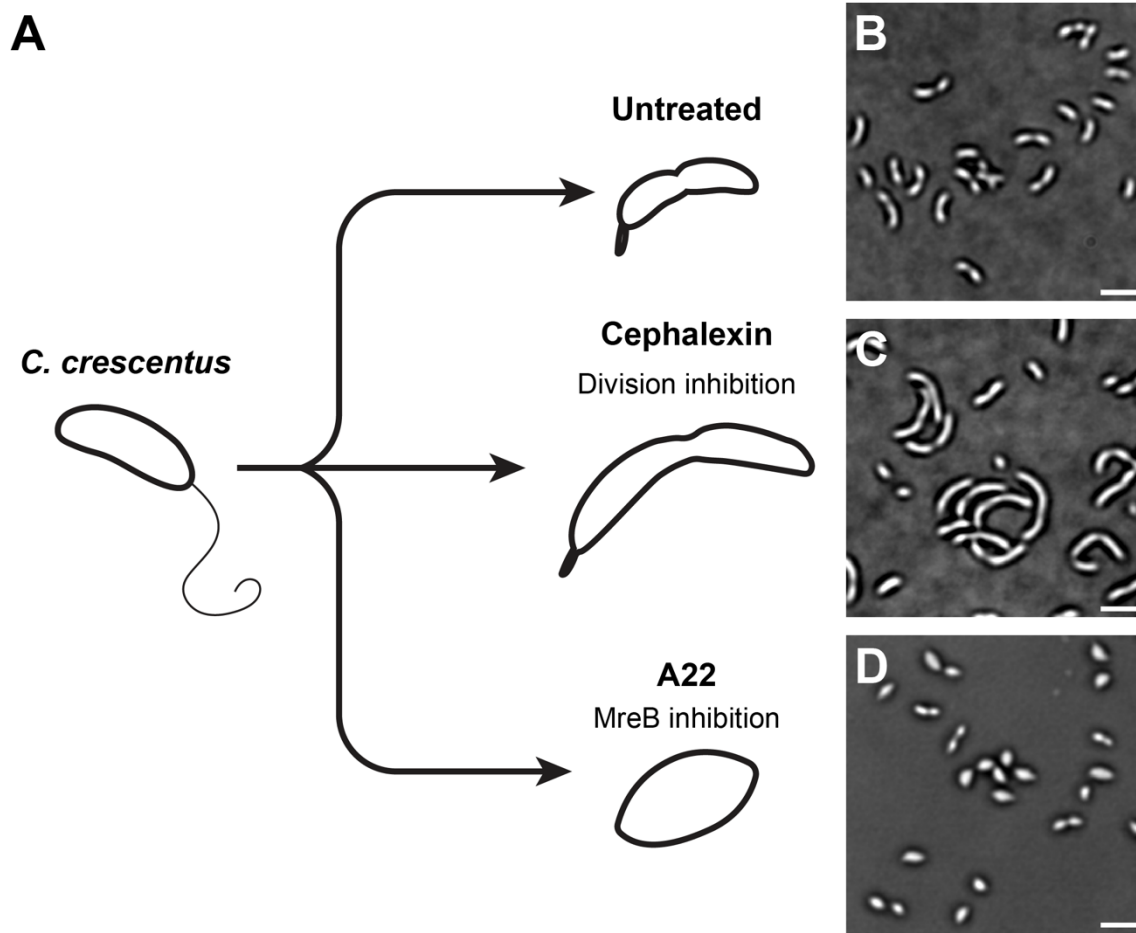


Figure 5.1. Cell-disrupting drugs result in morphological changes in *C. crescentus*.

(A) *C. crescentus* cells become filamentous or lemon shaped when exposed to cephalaxin or A22 respectively. (B-D) Micrographs for untreated, cephalaxin, and A22 treated cells. Cell invagination sites are visible in all conditions. Scale bars = 5 μm .

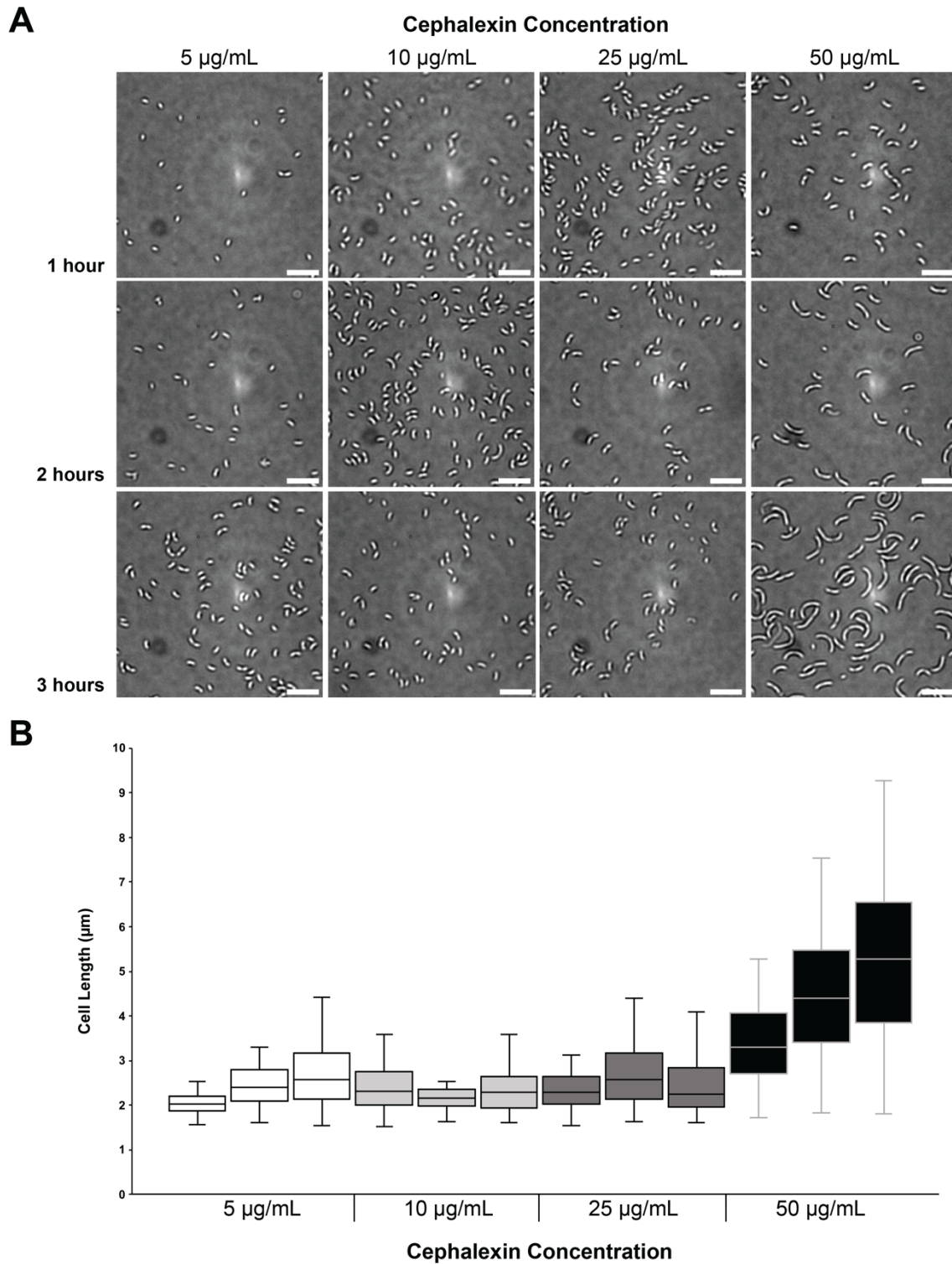


Figure 5.2. Cell elongation of *C. crescentus* following cephalexin exposure.

(A) *C. crescentus* cells grown under increasing concentrations of cephalexin, imaged hourly for three hours. Scale bars = 5 μm . (B) Cell lengths were quantified using MicrobeJ. For each concentration, hours 1, 2, and 3 are presented left to right.

5.4.2. Cephalalexin-treated cells retain spatially distinct S-layer labelling

To optimise SC labelling of cephalalexin-treated filamentous cells, *C. crescentus* cultures were incubated with varying concentrations cephalalexin (Figure 5.2). Three hours exposure to 50 µg/mL cephalalexin resulted in the greatest increase in average cell length (5.22 µm), significantly greater than the next highest concentration (25 µg/mL, 2.7 µm) (Figure 5.2A-B). These conditions were used for all subsequent labelling experiments using cephalalexin.

Cells were prepared for imaging using a slightly modified SC-mRFP1 and SC-sfGFP labelling strategy, adapted for the longer incubation periods necessary for filamentous cells inhibited by cephalalexin. Following overnight saturation of available cell-surface ST peptides with SC-mRFP1 at 4 °C, cells were incubated with 50 µg/mL cephalalexin for 1 hour at 30 °C. Cells were then washed and chase labelled with SC-sfGFP for the remaining two hours. This resulted in filamentous cells with stalks, invagination sites, and striated labelling along the cell body (Figure 5.3).

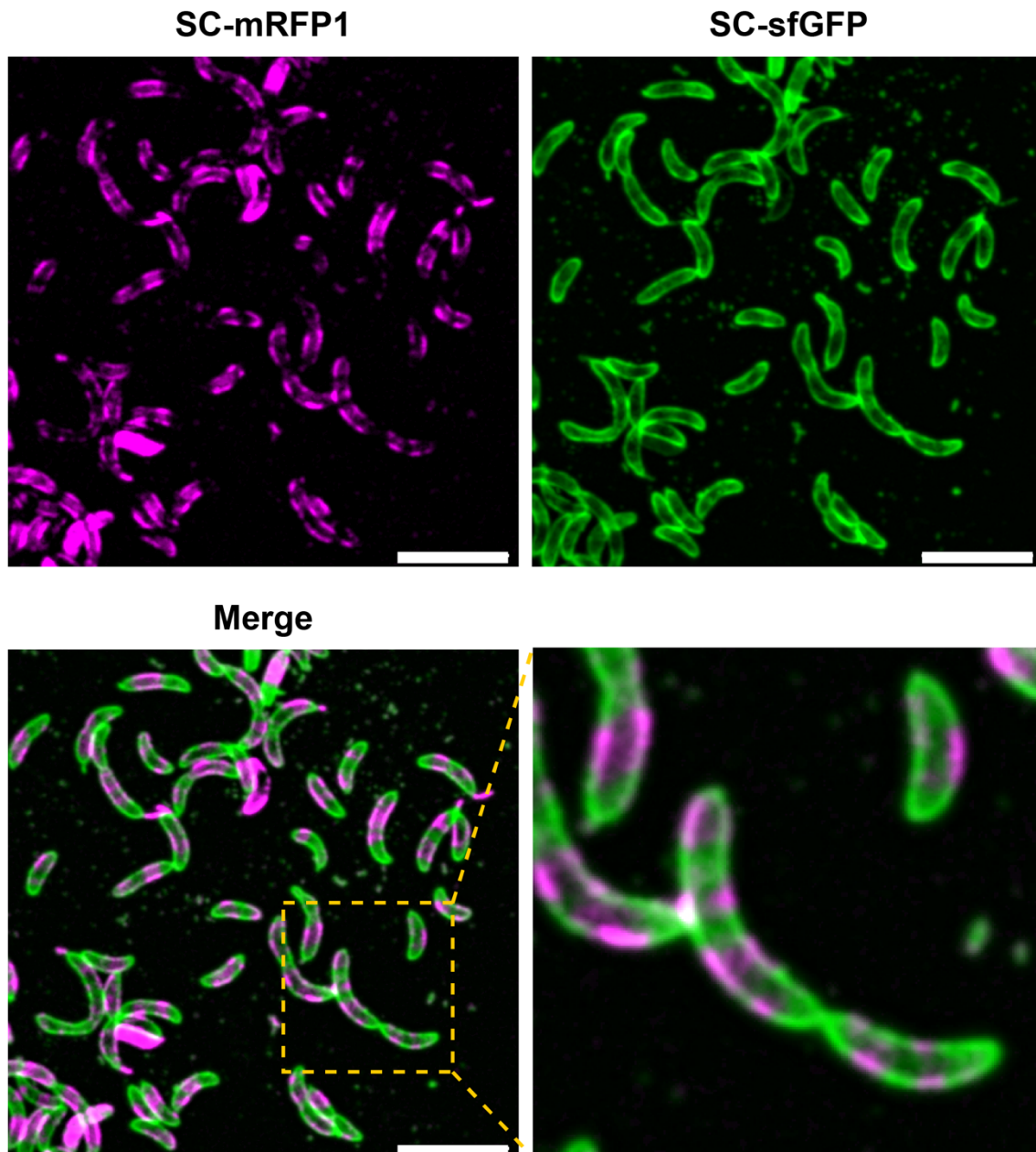


Figure 5.3. Dual-labelled *C. crescentus* cells show distinct regions of old and new S-layer following cephalixin exposure.

Micrographs of *C. crescentus* RsaA-467-SpyTag cells pulse-chase labelled using SC- mRFP1 and SC-sfGFP under cephalixin-treated conditions. Merged channels (bottom) show distinct localisation of the pulse and chase-labelled S-layer. Scale bar = 5 μ m. A zoom of a significantly elongated *C. crescentus* cell is provided in the lower right.

Notably, the invagination sites in both untreated and cephalixin treated samples were prominently SC-sfGFP labelled (Figure 5.4), showing that the S-layer in this region was more recently generated. The presence of invagination sites in cephalixin-treated cells is likely due to attempted but unsuccessful division following inhibition of FtsI. In addition, polar SC-sfGFP labelling was also prominent, meaning the labelling patterns of cephalixin-treated cells are broadly consistent with that seen in untreated cells (Figure 5.4).

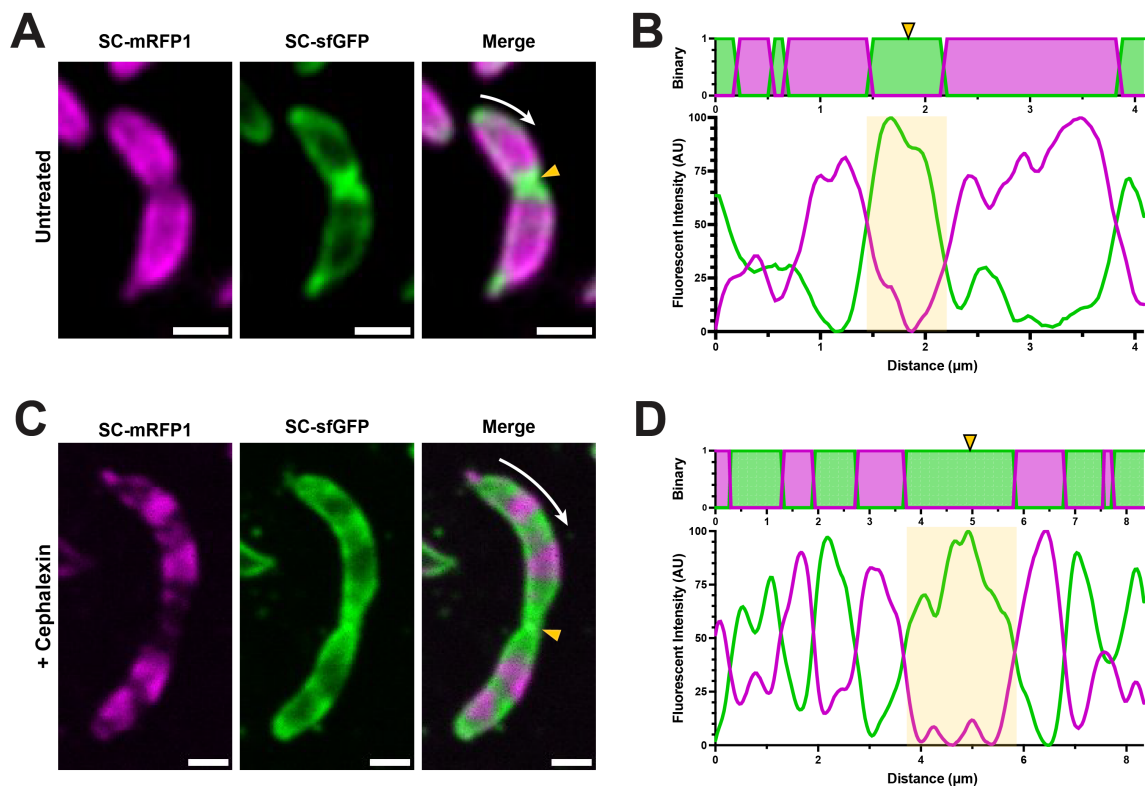


Figure 5.4. Dual-labelled *C. crescentus* cells retain broadly comparable labelling patterns across cephalixin and untreated conditions.

Cell micrographs showing representative, dual-labelled *C. crescentus* cells in (A-B) untreated and (C-D) cephalixin-treated conditions. Scale bars = 1 μm . The white arrows in A, B indicate the direction of the cell profiles used in B, D. Yellow arrows indicate invagination site. Relative fluorescence intensity along the cell profile is plotted against cell length. A binarised version of the cell profiles are provided above.

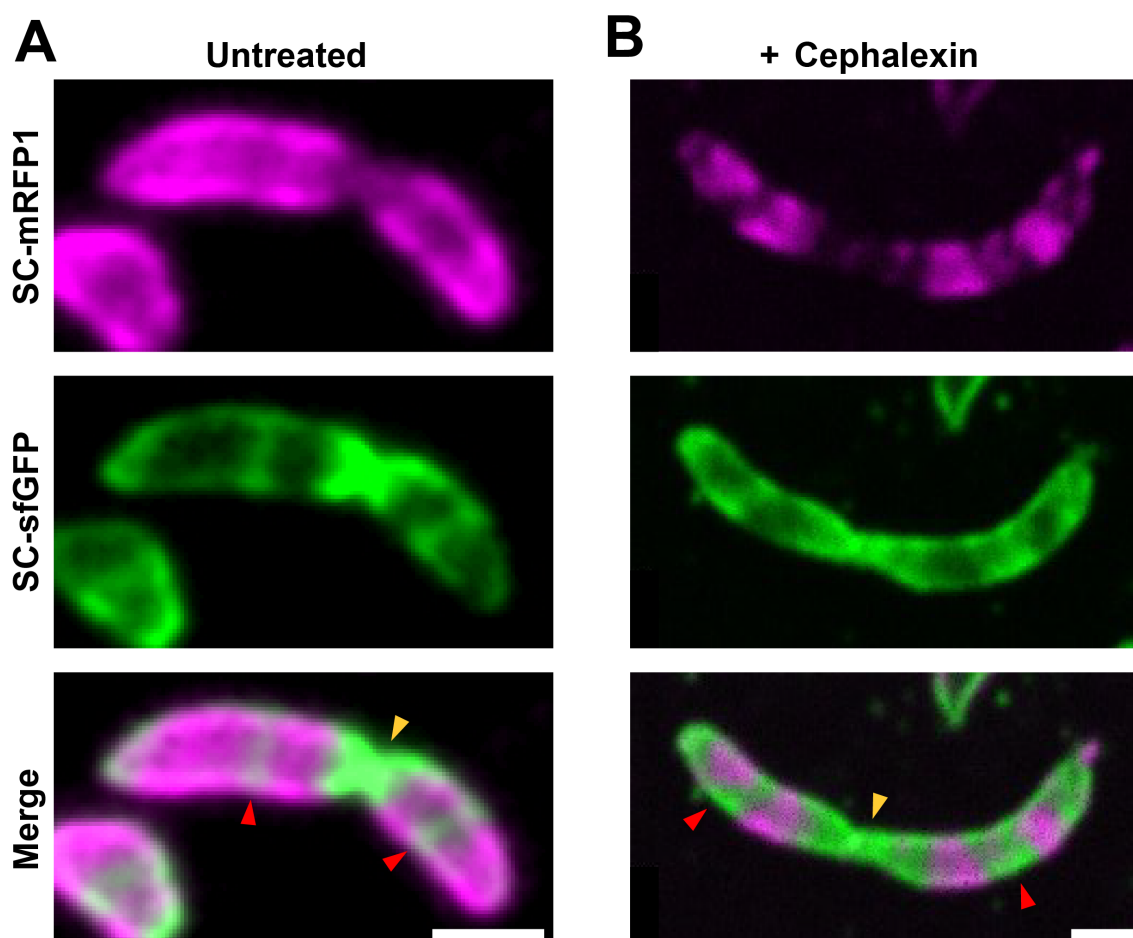


Figure 5.5. Cephalalexin treated and untreated *C. crescentus* cells have regions of new S-layer between the midcell and cell poles.

Cell micrographs showing representative, dual-labelled *C. crescentus* cells under (A) untreated and (B) cephalalexin-treated conditions. Scale bars = 1 μm. Invagination sites are indicated by yellow arrows. Additional insertion sites (non-polar and non-midcell) are indicated by red arrows.

It should be noted that the additional regions of new S-layer sites in cephalalexin treated cells that lie between the invagination site and cell pole are reminiscent to those reported in Figure 4.7. These additional S-layer insertion sites, which are present in only a small number of untreated cells, appear to be more common in cephalalexin treated cells (Figure 5.5), potentially due to local elongation in these regions, enabling more SC-sfGFP to bind. It is difficult to ascertain if these regions are subject to increases in local cell wall turnover, as described in Figure 4.14. Unfortunately, cephalalexin disrupts HADA

incorporation (not shown), meaning both compounds cannot be simultaneously integrated into the same labelling strategy.

5.4.3. A22 treated *C. crescentus* cells lose spatially distinct regions of S-layer labelling

As mentioned above, *C. crescentus* were treated with A22 using a low concentration of 3 µg/mL overnight. The resulting cells were uniformly labelled in SC-mRFP1, lemon-shaped (confirming successful MreB inhibition), stalkless, and relatively slow growing. To compensate for this reduced growth rate, cells were chase-labelled using SC-sfGFP for 2 hours. The resulting dual-labelled cells have lost their characteristic striated labelling and the localisation of new S-layer to the midcell or cell poles (Figure 5.6A-C). Cells could be broadly categorised into two groups; those predominantly labelled in SC-mRFP1 with some punctate SC-sfGFP fluorescence, and cells with patchy fluorescence in both channels (Figure 5.6D). Again, those patchy cells did not appear to show localisation of new S-layer to the midcell or cell poles, but rather shows an uneven distribution of punctate green signal across the cell (Figure 5.7).

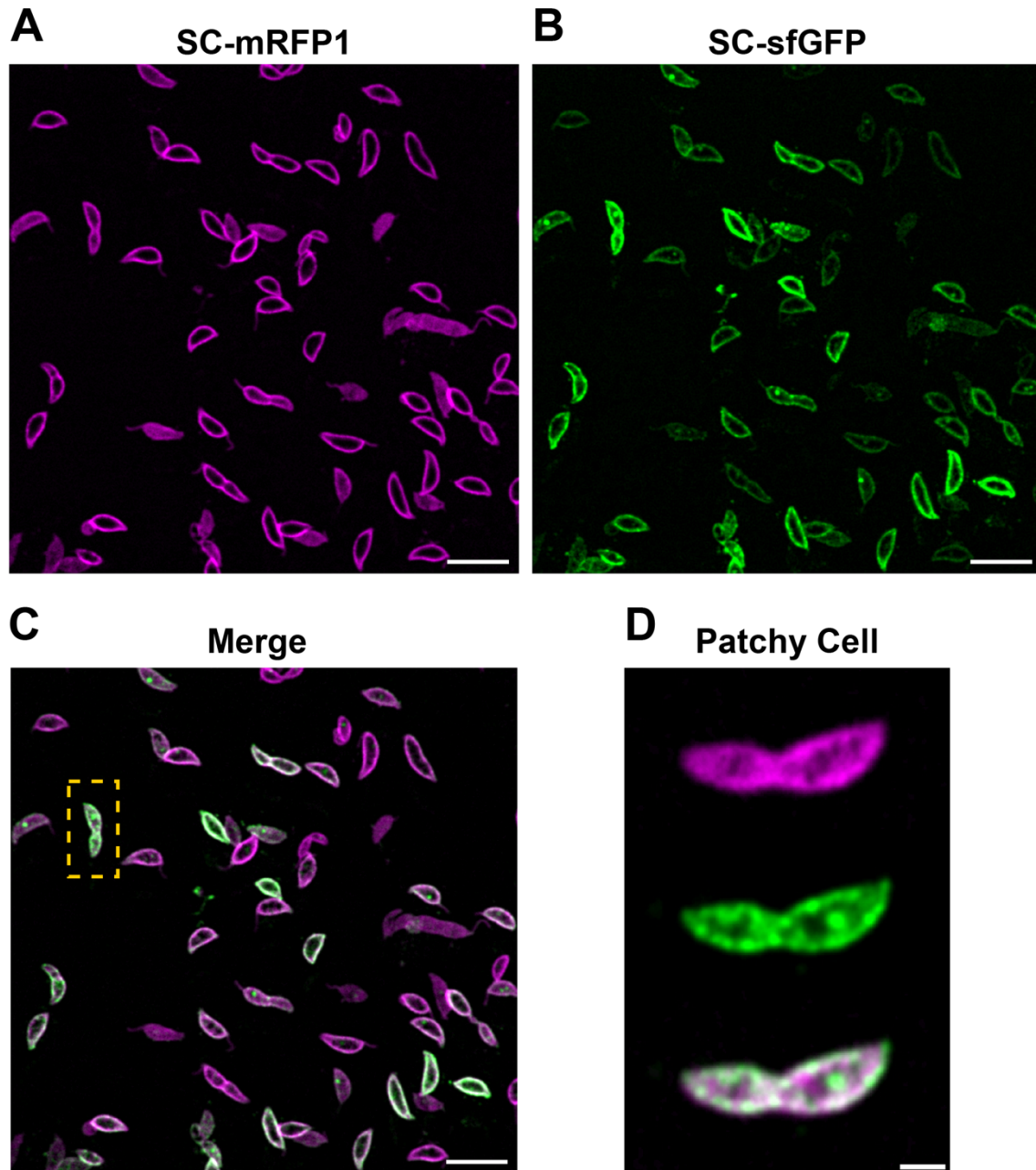


Figure 5.6. Dual-labelled *C. crescentus* cells show loss of spatially distinct old and new S-layer following A22 exposure.

Micrographs of *C. crescentus* RsaA-467-SpyTag cells pulse-chase labelled using (A) SC- mRFP1 and (B) SC-sfGFP under A22-treated conditions. (C) When merged, there appears to be little spatial separation of SC-mRFP1 and SC-sfGFP signals. (D) A representative “patchy” cell, showing heterogeneously distributed SC-sfGFP puncta across the cell. Scale bars (A-C) = 5 μm . Scale bar (D) = 1 μm .

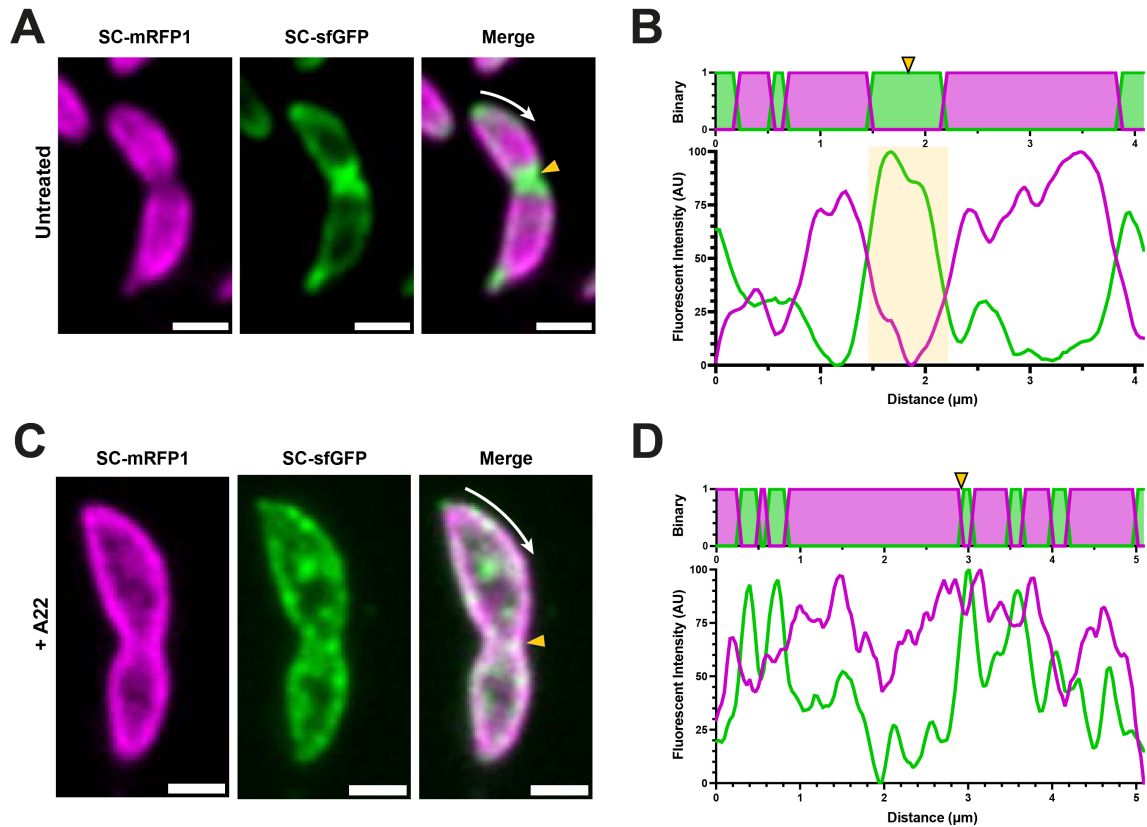


Figure 5.7. Dual-labelled *C. crescentus* cells retain broadly comparable labelling patterns across cephalalexin and untreated conditions.

Cell micrographs showing representative, dual-labelled *C. crescentus* cells in (A-B) untreated and (C-D) A22-treated conditions. Scale bars = 1 μm . The white arrows in A and B indicate the direction of the cell profiles used in B and D. Yellow arrows indicate invagination site. Relative fluorescence intensity along the cell profile is plotted against cell length, with a binarised version provided above.

5.4.4. Quantifying retention of S-layer spatial separation using Pearson's Correlation Coefficient.

To confirm or reject the spatial separation of old and new S-layer regions under different treatment conditions, PCC analysis was applied to the fluorescence images of cells in the same manner as that described for Figure 4.12. In this case, PCC was used to quantify the anticorrelation (or lack thereof) between old and new S-layer regions, i.e. SC-mRFP1 and SC-sfGFP, respectively. Cells with clearly visible sfGFP and mRFP1

fluorescence were selected for analysis to ensure the PCC quantification was not hindered by weak signal in one or more channels. This also avoids cells which failed to grow or grew too slowly during the chase labelling phase. In addition, in cephalixin and A22 treated cell samples, only cells with clear morphological defects were selected, as those represented cells in which inhibition of FtsI and MreB were successful.

Untreated *C. crescentus* cells were used as a control. As expected, following visual examination of the dual-labelled cells, the old and new S-layers were anticorrelated according to PCC, returning an average of -0.46 for R (Figure 5.8A, D). Filamentous, cephalixin-treated cells had a comparable average R of -0.45 following PCC analysis, confirmed to be not significantly different from untreated cells by Student's *t*-test ($p < 0.0001$) (Figure 5.8B, D). Conversely, all A22-treated cells analysed returned positive R values for PCC, with an average R-value of 0.44 (Figure 5.8C, D). This confirms that MreB inhibition using A22 results in a loss of old and new S-layer anticorrelation, while inhibiting cell division through cephalixin appears to allow for continued spatial localisation of S-layer biogenesis.

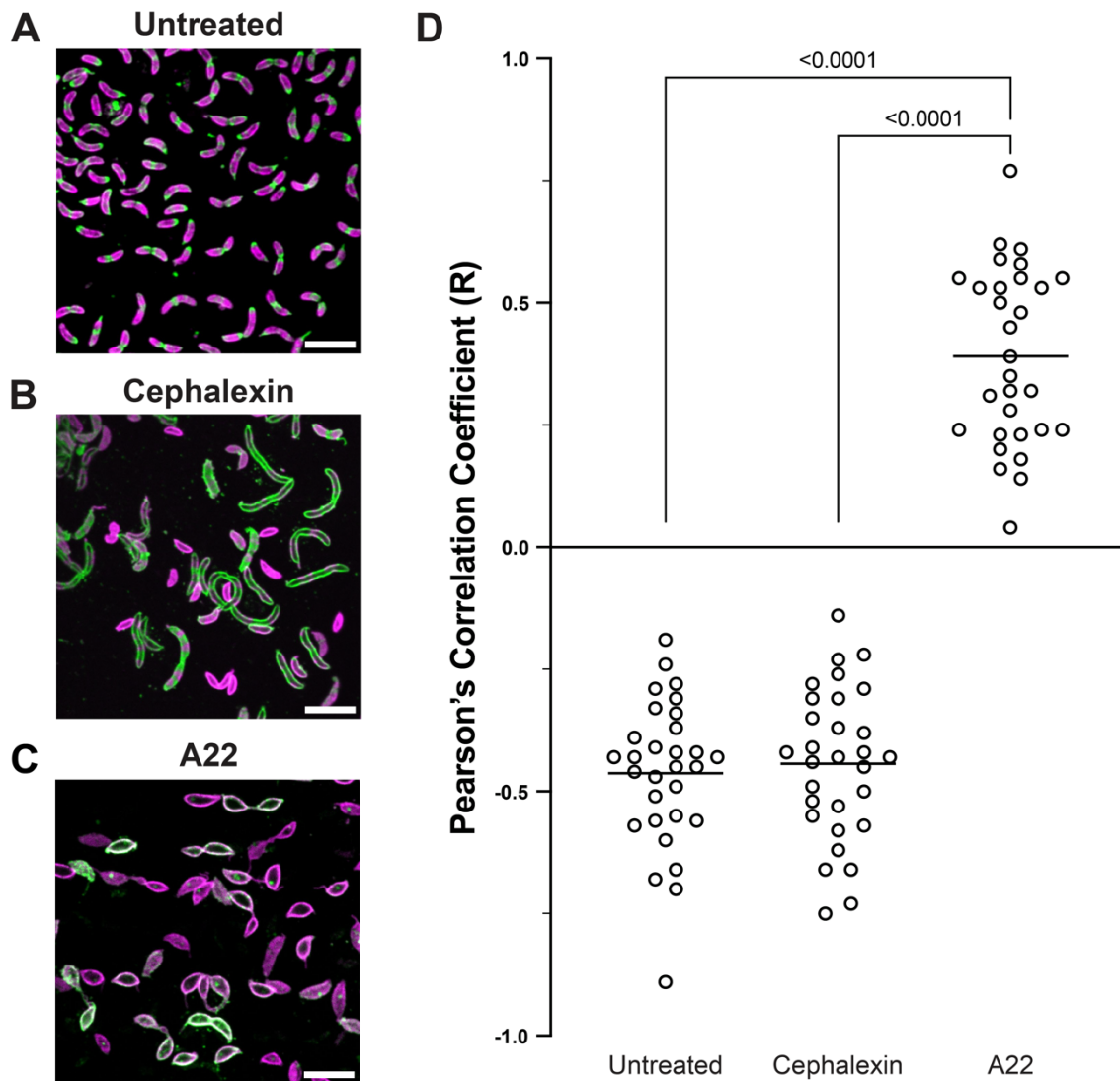


Figure 5.8. A22, but not cephalalexin, disrupts anticorrelation of old and new S-layer.

(A-C) Micrographs of dual-labelled *C. crescentus* RsaA-467-SpyTag cells. Cells were labelled under varying treatment conditions, as described, including (A) no treatment, (B) cells treated with 50 $\mu\text{g/mL}$ cephalalexin, and (C) 3 $\mu\text{g/mL}$ A22. SC-mRFP1 and SC-sfGFP are merged. Scale bars = 5 μm . (D) PCC analysis comparing SC-mRFP1 and SC-sfGFP fluorescence show untreated and cephalalexin treated cells have a negative colocalisation (R value). A22 treated cells return a positive R value and colocalisation between the both channels, suggesting the loss of discrete spatial separation of old and new S-layer. One way ANOVA analysis of the data shows a strong significant difference between A22-treated cells the alternate treatment conditions ($p < 0.0001$), while Student's *t*-test suggests untreated and cephalalexin treated cells have no statistical difference ($p < 0.0001$, not shown). $N=30$ for each treatment condition.

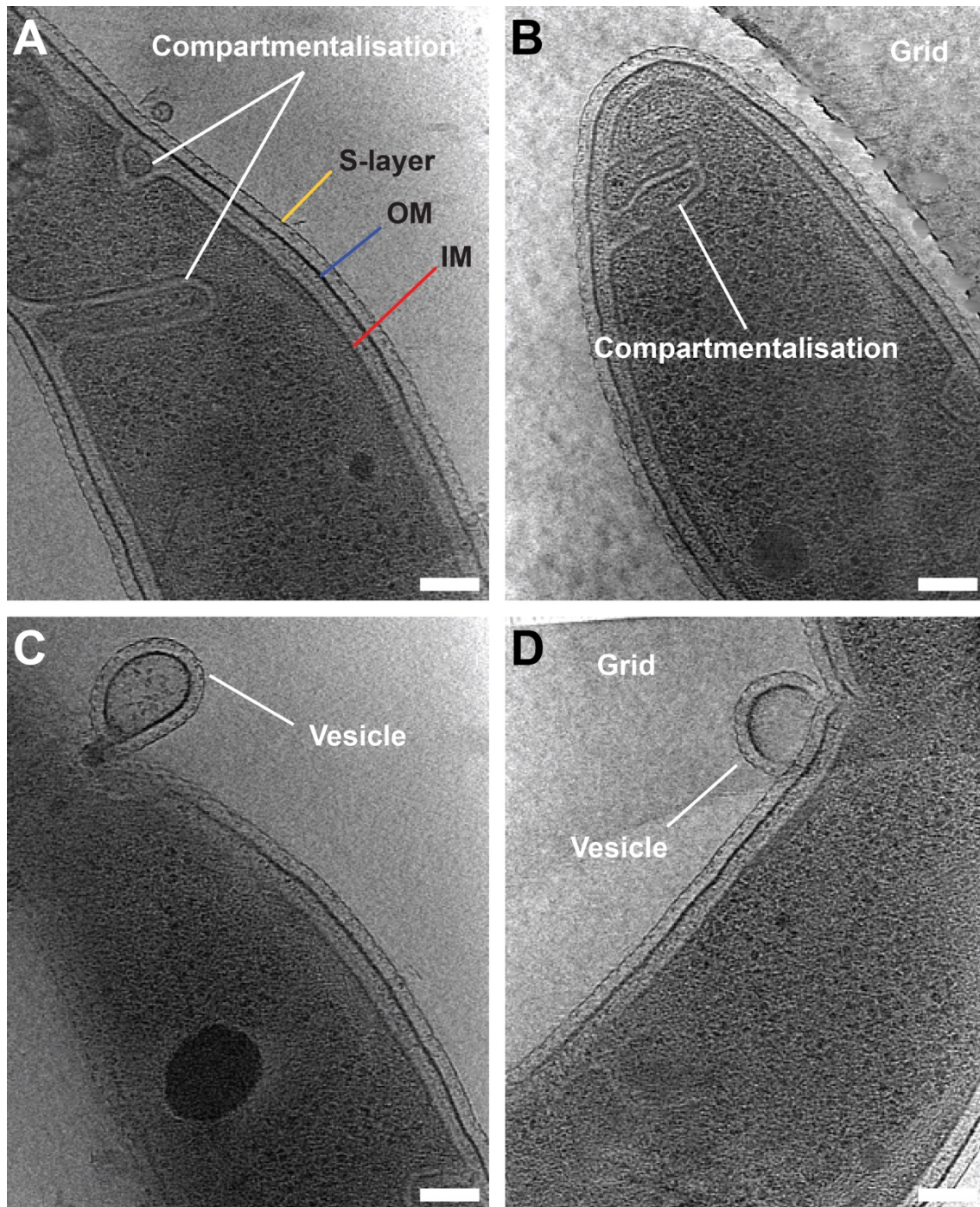


Figure 5.9. Cephalixin treatment results in cell stress, whilst retaining the S-layer

Following cephalixin treatment, *C. crescentus* cells have characteristic traits of cell stress, including (A-B) compartmentalisation of the cytoplasm and (C-D) vesicle formation, most often seen near the site of attempted division. A and C represent slices through the same tomogram and different z heights. Envelope components labelled in A are consistent across all tomograms. The carbon grid has also been labelled where it is visible. Scale bars = 100 nm.

5.4.5. Retention of S-layer ultrastructure following cell cycle disruption.

Given that the cephalixin and A22 treated cells in Figure 5.3 and Figure 5.6 retained complete fluorescent boundaries, cell-bound RsaA was likely retained on the cell surface in a folded state with accessible ST for SC binding. However, direct evaluation of the S-layer, its composition, and its confluence is not possible using light microscopy and may well have been disrupted by treatments with cell-cycle disrupting compounds. To evaluate this possibility, cephalixin and A22 treated cells were vitrified on EM-grids and used for cryo-ET data collection, to examine the state of the S-layer.

To begin, cephalixin-treated cells showed clear signs of stress, including vesicle formation, retraction or folding of the cytoplasmic membrane, and intracellular compartmentalisation (Figure 5.9). Vesicle formation in response to cephalixin exposure is unsurprising, as this is a well-documented defence mechanism implemented by many Gram-negative bacteria to defend against antibiotics (Mozaheb and Mingeot-Leclercq, 2020). Fluorescent micrographs of dual-labelled, cephalixin-treated *C. crescentus* cells, there was also the presence of background, punctate sfGFP signal (Figure 5.3). These may be SC-sfGFP coated vesicles, which, as seen in cryo-ET, retain their S-layer coat (Figure 5.9C-D). The observed compartmentalisation of the cytoplasm may be remnant of unsuccessful division, or an attempt by the cell to sequester cephalixin activity.

The S-layer in cells treated with cephalixin appears identical to that of untreated cells. The cephalixin-treated cells are covered in an unbroken S-layer, consistently spaced 18 nm from the OM. The bipartite structural arrangement of the S-layer, consisting of the outer oligomerisation (RsaA_{CTD}) and inner O-antigen binding (RsaA_{NTD}) domains are clearly visible, the latter being 180 Å away from the OM, consistent with previous data (Bharat et al., 2017) (Figure 5.10A-C). In addition, the hexameric pores are uniformly spaced, 220 Å apart, as would be expected of a characteristic *C. crescentus* S-

layer (Figure 5.10D). Therefore, cephalixin treatment, even at concentrations as high as 50 $\mu\text{g/mL}$, appears to have no effect on the spatiotemporal localisation of S-layer biogenesis (Figure 5.4), as well as no effect on RsaA binding or oligomerisation.

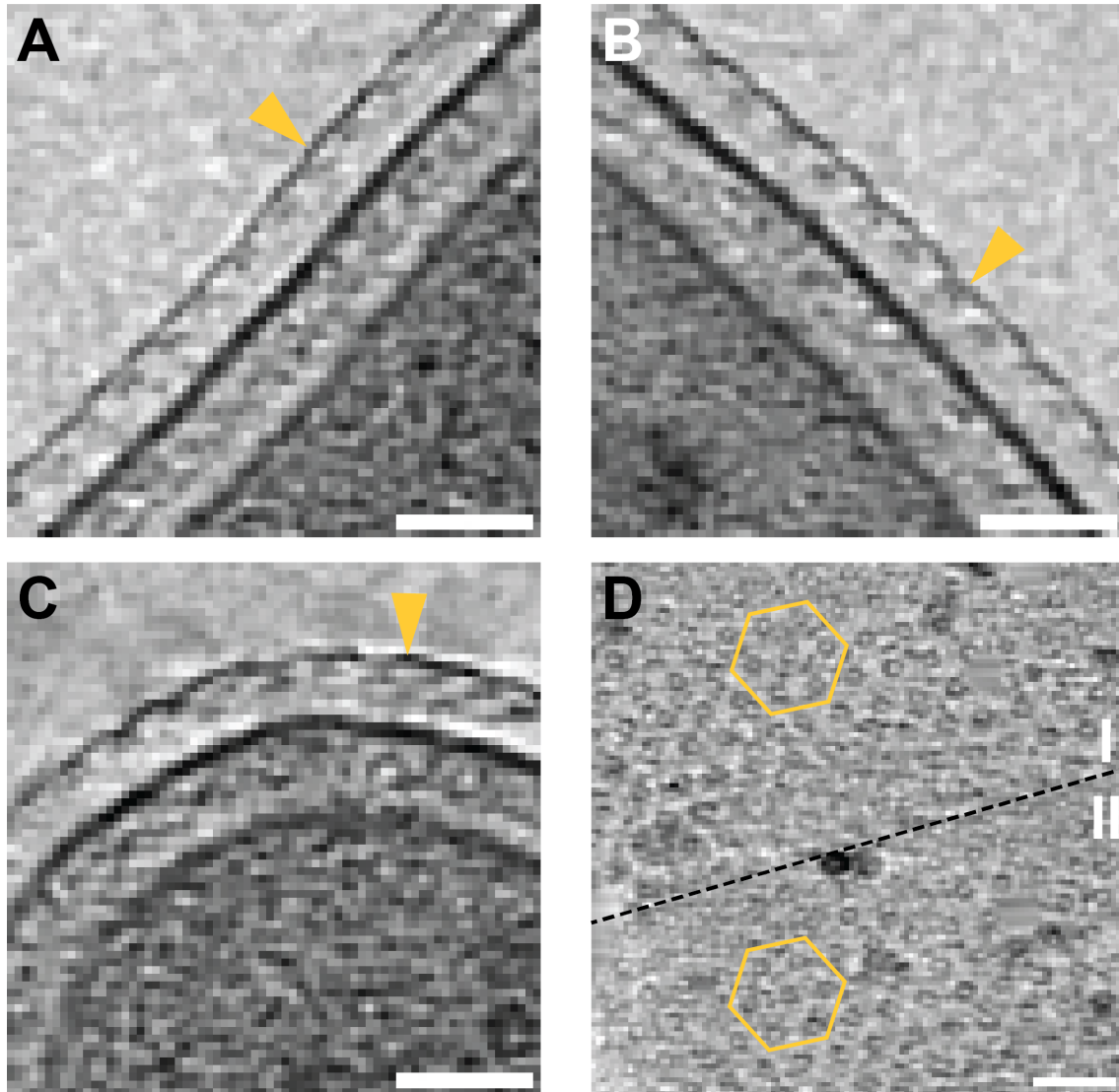


Figure 5.10. Cephalixin treatment has no adverse effects on S-layer architecture.

(A-C) Side-profiles of cell-bound S-layer (yellow arrows) sheets showing characteristic inner and outer domain architecture. (D) Slices through the tomogram at the top of the cell, showing regularly spaced, hexameric S-layer conformation. I and II represent the same region imaged at two different z slices, I higher than II, to bring the S-layer into focus. The hexagon boundaries contain seven RsaA hexamer pores. Distance between central hexamer pores is 220 $\text{\AA}$ and RsaA_{NTD} domain is 180 $\text{\AA}$ from the OM, consistent with previous reports (Bharat et al., 2017). Scale bars = 50 nm.

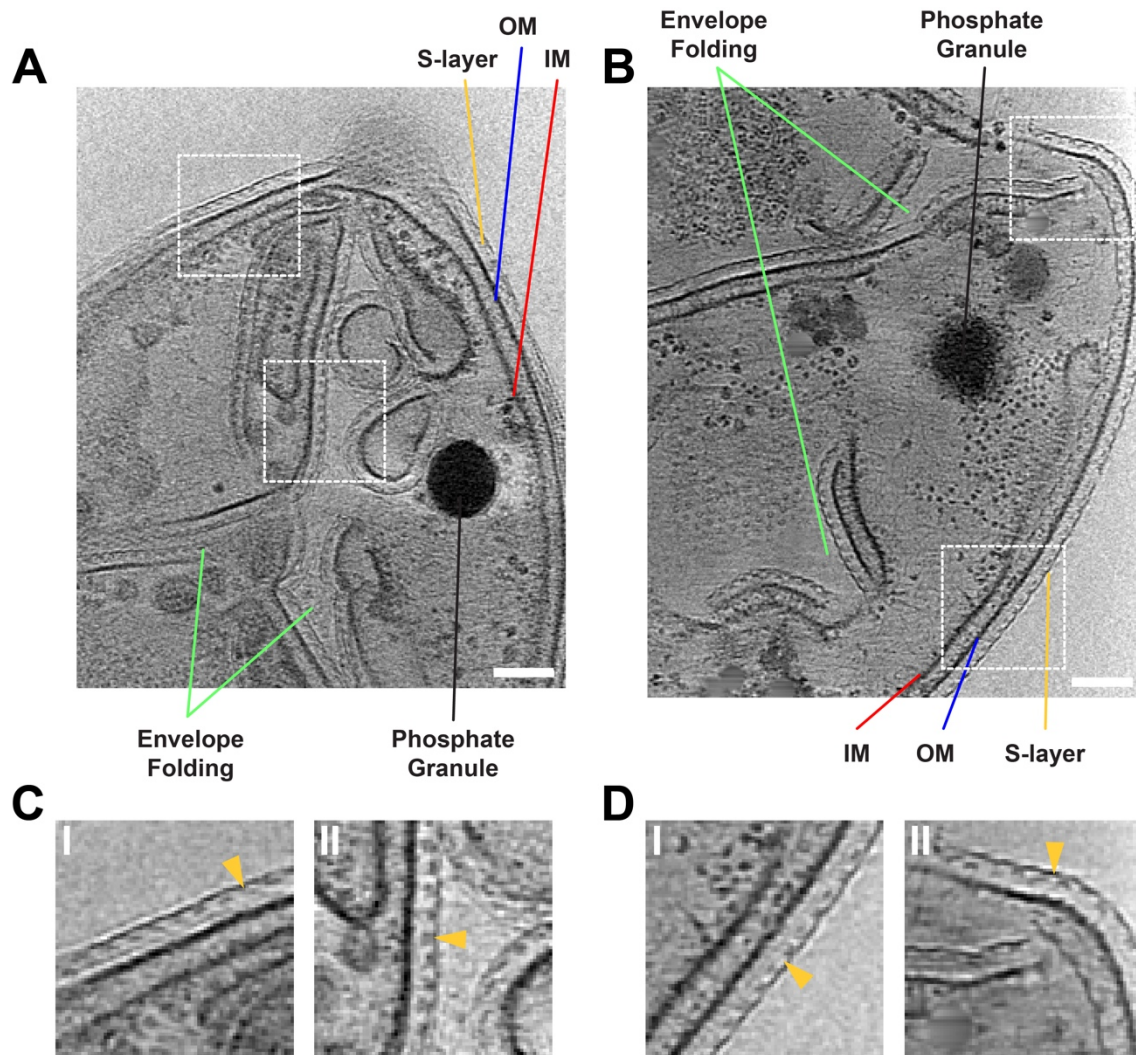


Figure 5.11. A22 treatment induces severe envelope folding in a subpopulation of A22 treated cells.

(A-B) Slices through tomograms of A22-treated *C. crescentus* cells. Cells show severe examples of envelope folding, but still retain envelope features including inner membrane (IM), outer membrane (OM), and the S-layer. Features have been labelled, including the electron dense phosphate storage granule. Scale bars = 100 nm. (C-D) Zooms of the tomogram slices, numbered I and II, indicated in A-B by their respective dashed white square, are provided to show S-layer architecture. The S-layer (yellow arrow) lies above the OM and retains its distinct bipartite structure, i.e. the confluent CTD outer oligomerisation domain and the regularly spaced NTD inner domain, which binds O-antigen.

A22-treated cells were particularly adversely affected, demonstrating highly folded cell envelopes and dysregulated cell morphology (Figure 5.11A-B and Figure 5.12A-B). However, the S-layer, which is conveniently identified by its proximity to the

electron dense OM, bipartite structure, and regular hexameric arrangement, has retained its characteristic organisation. The bipartite structural arrangement of the constituent RsaA hexamers, i.e. the oligomerisation domain and O-antigen binding domains, are clearly visible and consistent with previous data (Bharat et al., 2017) (Figure 5.11C-D). The p6 symmetry of the array is also well maintained, including the 220 Å distance between adjoining RsaA hexamers (Figure 5.12). One benefit of A22 treatment appears to be dramatic flattening of the cells, potentially due to the observed folding, which provides ideal contrast for the S-layer in cryo-ET. This data shows that despite disruption of spatially distinct regions of S-layer biogenesis, as suggested by light microscopy experiments, A22 treatment has no impact in the ability of the S-layer to bind the underlying cell or oligomerise into a confluent 2D array.

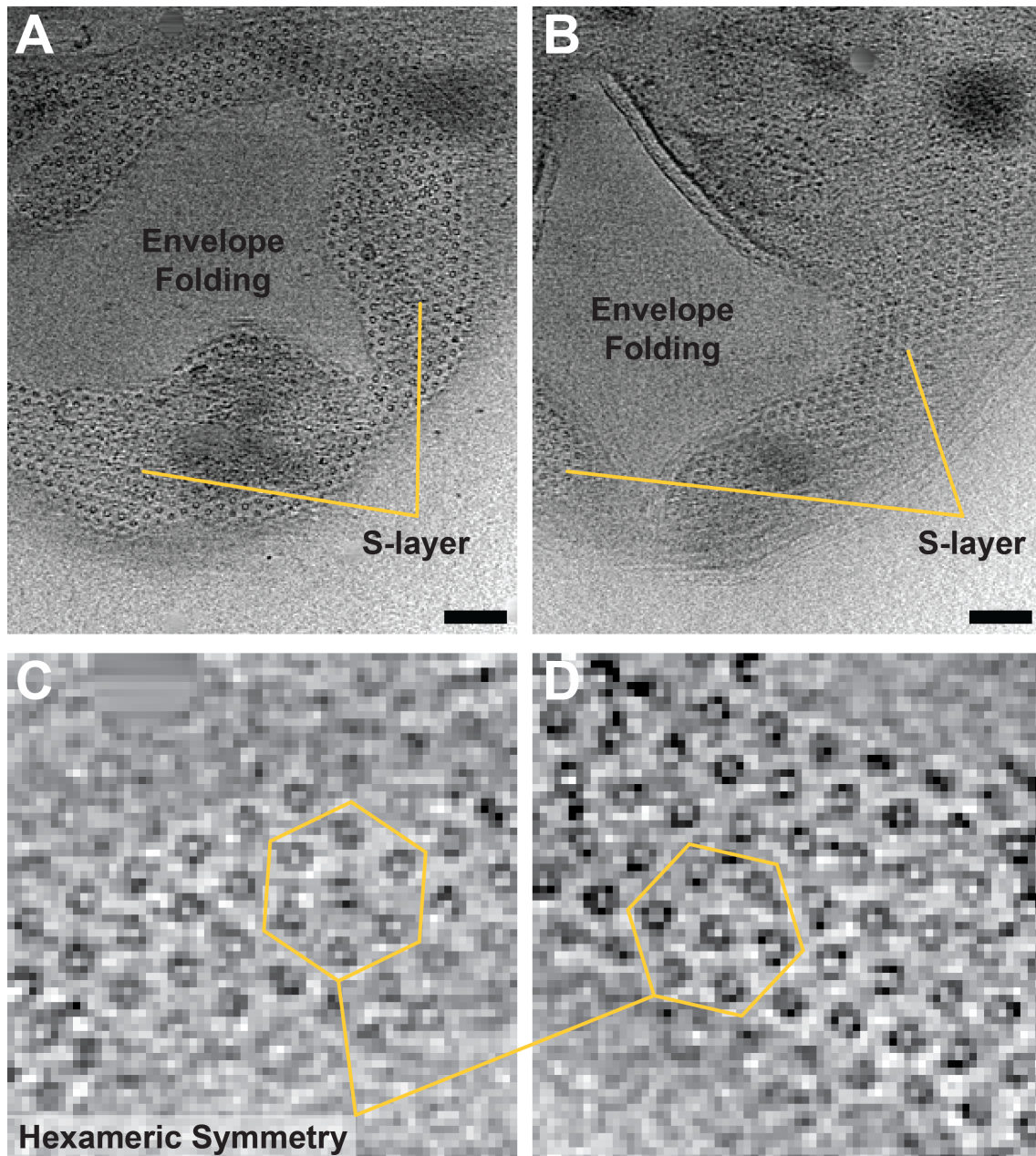


Figure 5.12. A22 treatment has no effect on S-layer attachment or symmetry.

(A) Top and (B) bottom slices of an A22-treated *C. crescentus* cell. Regions of envelope folding are labelled, as well as the surface-bound S-layers. Scale bars = 100 nm. (C-D) Zooms of cell-bound S-layer sheets showing characteristic hexameric symmetry. The hexagon boundaries contain seven RsaA hexamer pores. Distance between central hexamer pores is 220 Å, as previously reported (Bharat et al., 2017).

5.5. Discussion

Having established the localisation of S-layer biogenesis to the cell poles (particularly the stalk base) and the midcell, disrupting cell cycle mediators seemed a tempting direction to explore the underlying mechanisms of S-layer biogenesis. These regions are associated with significant rates of cell wall turnover, as demonstrated by the fluorescence microscopy reported in Chapter 4 and in previously published literature (Billini et al., 2019, van Teeseling, 2021). These sites are also subject to significant spatial and temporal regulation, particularly in *C. crescentus* where cell asymmetry is carefully maintained (Skerker and Laub, 2004). By disrupting two key cell cycle events, cell division and cell elongation, using cephalixin and A22 respectively, the principles which govern localised S-layer biogenesis begin to be unravelled.

Starting with cell division, the ability of cephalixin to inhibit FtsI in *C. crescentus* is limited, due to its encoded metallo- β -lactamase capable of cleaving cephalosporins (Palzkill, 2013). However, this result was ideal for the studies in this chapter, as it allowed for *C. crescentus* to continue growing, even in the presence of high concentrations of cephalixin, making it amenable to the pulse-chase labelling methodology. This allowed cells to reach lengths of almost 10 μm , with the invagination sites, remnants of attempted cell division, still visibly apparent. These invagination sites, as well as the cell poles, were prominently labelled in SC-sfGFP, showing successful localisation of S-layer biogenesis to these regions, despite being challenged by cephalixin (Figure 5.13A). The spatial separation of old and new S-layer, as labelled by SC-mRFP1 and SC-sfGFP respectively, was confirmed by PCC analysis. Both untreated and cephalixin-exposed cells consistently resulted in comparable, negative R values, confirming anticorrelation of the differently labelled regions.

Interestingly, newly integrated RsaA was also labelled at regions roughly equidistant between the cell poles and invagination site, suggesting a new area of S-layer biogenesis. While this may be dependent on the filamentation driven by cephalixin, much smaller, similarly localised regions were also identified in untreated cells (Figure 5.5). It is tempting to suggest these regions have increased levels of localised cell wall turnover, consistent with the model of S-layer biogenesis proposed in Figure 4.14, however without compelling HADA fluorescence data, this is currently speculative.

Given the successful and intense fluorescence observed in cephalixin-treated *C. crescentus*, it is unsurprising the S-layer is in excellent condition. Despite the morphological changes brought on by cephalixin's inhibition of cell division, cryo-ET revealed a regular S-layer that appears to encompass the entire cell, despite its length and signs of cell stress (Figure 5.13B). The RsaA hexamers are characteristic, consistent with previously published data and the S-layers of untreated cells (Figure 5.13C) (Bharat et al., 2017).

MreB, unlike FtsI, is a major cytoskeletal component of *C. crescentus*, mediating cell elongation, stalk biogenesis, and cell polarity (Billini et al., 2019, Dye et al., 2011, Takacs et al., 2010). Therefore, the effects of its dysregulation should be cautiously interpreted, as several processes are being simultaneously disrupted, compared to cephalixin's more narrow action. A22 occupies the nucleotide binding pocket of MreB, preventing ATP attachment, thereby inhibiting the treadmilling activity of MreB cytoskeletal fibres (Bean et al., 2009). The resulting stalkless, lemon shaped cells owe their morphology to the loss of intracellular MreB treadmilling along the cytoplasmic membrane, orchestrating the localisation of cell wall remodelling complexes (Kim et al., 2006). At low concentrations, these cells are still viable, capable of slow growth and stunted division, meaning some cell functions are still ongoing. While A22-treated *C.*

crenscentus cells are near unrecognisable morphologically, their S-layer remains perfectly characteristic. It's bipartite structural arrangement, OM binding, and hexameric symmetry is almost completely unperturbed by A22 action, even in cells where the drug has led to exaggerated folding of the cell envelope (Figure 5.13B).

It is relatively unsurprising that cell cycle disrupting compounds have such a minimal effect on S-layer ultrastructure. RsaA needs no more from the cell than secretion, at which point it can bind the O-antigen and incorporate itself into gaps in the growing S-layer. However, where A22 appears to have a strong effect is the location of this RsaA integration. S-layer biogenesis, which has been shown to be carefully and consistently localised, appears completely delocalised (Figure 5.13A). The clean, distinct regions of SC-sfGFP normally associated with the poles and midcell are now punctate, heterogeneously distributed across the cell. The anticorrelation of old and new S-layer seen in untreated and division-inhibited cells appears to be completely gone (Figure 5.13C-D). Whether this is simply due to the new cell morphology or due to delocalised regions of cell wall expansion is difficult to say.

Together, these studies demonstrate a robustness to S-layer biogenesis; RsaA is not easily disrupted by cell cycle inhibiting compounds and can form its S-layer, despite underlying cell stress. However, localisation appears to be more acutely dependent on the spatial and temporal tuning of the *C. crescentus* cell cycle. While inhibiting cell division through FtsI inactivation resulted in elongated cells, the localisation of S-layer biogenesis is unaltered. When rod morphogenesis and cell asymmetry are lost and the localisation of cell wall remodelling complexes are disrupted by A22, S-layer biogenesis in turn becomes delocalised. The findings of this chapter are summarised in Figure 5.13.

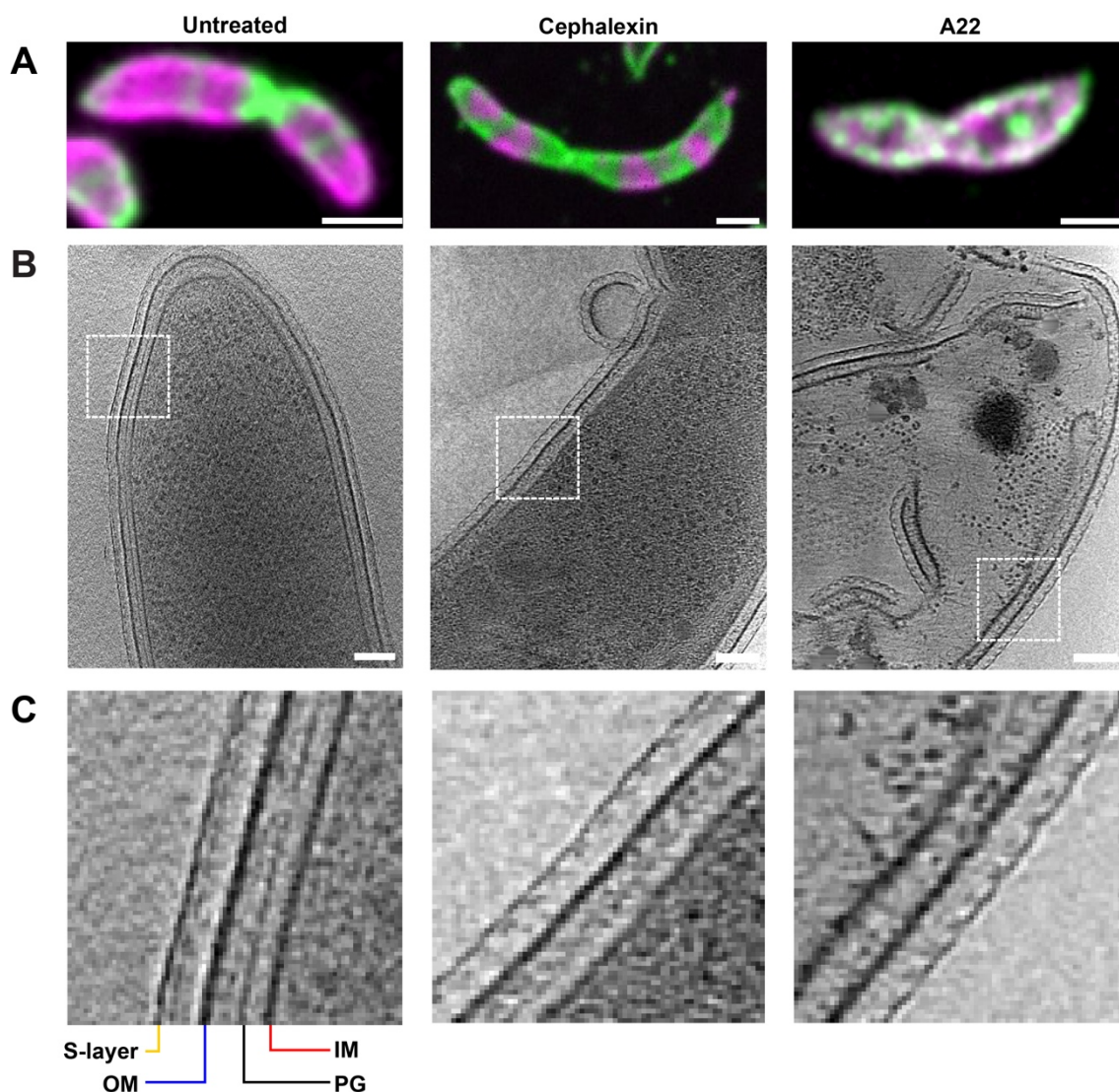


Figure 5.13. S-layer ultrastructure is retained in the presence of cell-cycle disrupting compounds, whilst localised biogenesis may be disrupted.

(A) Representative *C. crescentus* cells pulse-chase labelled using SC-mRFP1 and SC-sfGFP. Cells were labelled in the presence of no drug (untreated), or the cell cycle disrupting compounds cephalalexin and A22. Cephalalexin and A22 induce distinct morphological changes in *C. crescentus*, but only A22 results in a loss of discrete localisation of new S-layer to the midcell and cell poles. (B) Cryo-ET shows that OM-bound S-layers are present in all three conditions, covering the cell, even along sites of major envelope distortion. (C) The S-layer retains its characteristic bipartite structural arrangement, with the regularly spaced, OM-binding domain facing the cell, and the oligomerising domain coating the outer surface.

The compounds used in these studies are relatively well tolerated by *C. crescentus*, making them amenable to the described labelling strategies. More in depth

information on the underlying mechanism driving S-layer localisation will likely require molecular manipulation of the RsaADEF machinery and cell cycle proteins. For example, cephalixin inhibits FtsI, but FtsZ is a key component of the eponymous divisome, recruiting a broad host peptidoglycan remodelling proteins (Goley et al., 2011). Alternatively, *C. crescentus* asynchrony is highly dependent on PopZ, a polar-accumulating protein that orchestrates localised stalk biogenesis (Lasker et al., 2020). FtsZ or PopZ depletion mutant, as well as mutations in several other cytoskeletal components, could potentially shed further light on how and why the S-layer localises in the way it does.

CHAPTER 6. Cryo-CLEM and STA Studies of S-layer Biogenesis.

6.1. Overview

RsaA has been extensively structurally characterised using a range of techniques (Herdman et al., 2022, von Kugelgen et al., 2020, Bharat et al., 2017), which, when combined with the new knowledge regarding localised SLP reported in this thesis, provides the perfect opportunity to explore S-layer biogenesis across multiple resolutions. *C. crescentus* has proven amenable to light and cryoelectron microscopy techniques, offering the ideal candidate applying cryo-CLEM and STA techniques in the study of S-layer biogenesis.

Cryo-correlative light and electron microscopy (cryo-CLEM) integrates the high resolutions afforded by cryo-EM and cryo-ET with the localisation data and functional insights provided by light microscopy (LM) (Zhang, 2013). Cryo-CLEM has already been applied in prokaryotes to investigate cell ultrastructure, yielding exciting results (Koning et al., 2014, Sexton et al., 2022). In addition, STA is greatly applicable to structures with repeating and regular symmetry, such as S-layers. By combining these techniques, it is possible to accurately map the architecture of the S-layer in situ, potentially identifying regions of deformity, overlap, and biogenesis. These studies do have limitations, particularly in the LM resolutions offered by most commercially available cryo-CLEM setups. In addition, the high contrasts needed for cryo-ET are often compromised by cell thickness, meaning samples often require Focused Ion Beam (FIB) milling prior to data collection (Sexton et al., 2022, Wagner et al., 2020).

6.2. Acknowledgments

The work in this chapter was performed by the candidate with the following exceptions. Cryo-ET data collection was performed with the guidance of Tanmay Bharat

and Andriko von K gelgen. Subtomogram averaging was performed with the assistance of Andriko von K gelgen.

6.3. Aims

Briefly, the project aims were as follows:

- (i) Identify and investigate regions of S-layer biogenesis using cyro-CLEM.
- (ii) Examine the S-layer along regions associated with cell growth, i.e. septum and cell poles.
- (iii) Build lattice maps onto tomograms of *C. crescentus* using STA.

A portion of results relevant to this chapter are published in Herdman *et al.*, *bioRxiv*, June 2023, and under revision in the journal *Nature Communications*.

6.4. Results

6.4.1. Investigating regions of S-layer Insertion in *C. crescentus* using cryo-CLEM

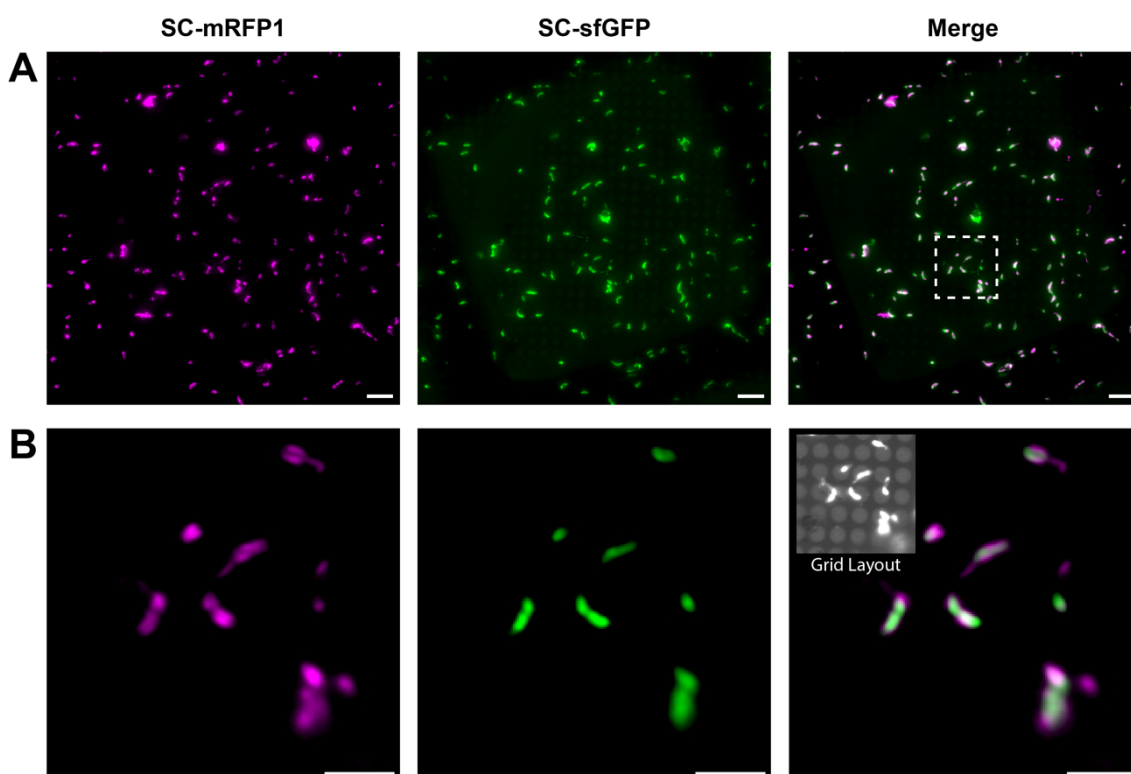


Figure 6.1. Vitriified *C. crescentus* cells retain spatially separated dual labelling in cryo-LM.

(A) Max-projection micrographs of Z-stack through a vitrified, dual-labelled sample of *C. crescentus* cells. Old S-layer is labelled in SC-mRFP1, new S-layer in SC-sfGFP. Merges of both channels are provided. (B) Enlarged view of the region within the white-dashed box in A. The merged image has been grey-scaled in the merge inset to show the grid layout. Scale bars = 5 μm .

For cryo-CLEM studies of *C. crescentus*, cells were labelled as described above before being transferred to grids and vitrified. Grids were transferred to a Linkam CMS196V cryostage mounted to a Zeiss AxioImager M2 widefield microscope (Carl Zeiss) for imaging. Grids were kept to temperature by a constant supply of filtered LN₂; for imaging, grids are mounted on the stage below the LN₂ vapour. Grids were initially mapped before locations were selected for Z-stack collection; stacks were collected at approximately 0.26 μm intervals. Maximal Z-projections were made through the stack to identify cells with clear spatial separation of old and new S-layer (Figure 6.1AB).

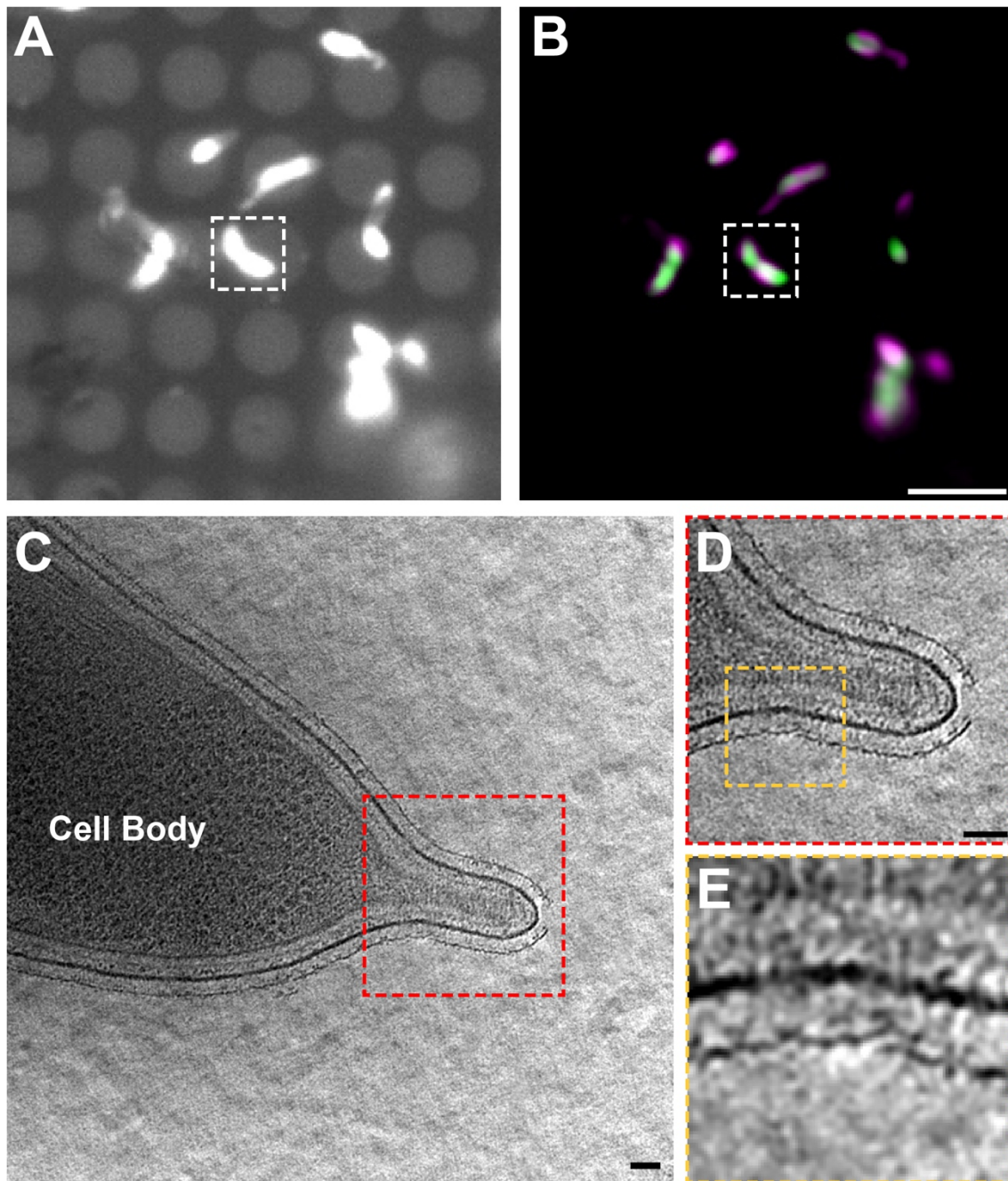


Figure 6.2. Combining cryo-LM and cryo-TM to identify regions of S-layer biogenesis.

(A) Grey-scaled micrograph, showing locations of *C. crescentus* cells in relation to the grid holes. (B) Max-projection of Z-stack through a vitrified, dual-labelled sample of *C. crescentus* cells. For unmerged images, see Figure 6.1. Scale bar = 5 μm . (C) Tomographic slice through the lower pole of the cell highlighted by the white-dashed square in B. Scale bar = 50 nm. (D) Enlarged view of the region highlighted by the red-dashed box in C. (E) Enlarged view of the region highlighted by the yellow-dashed box in D.

Dual-labelled cells were relocated in the Krios for cryo-ET data collection using SerialEM (O'Toole et al., 2017). The resulting tomogram of the lower cell pole, positively labelled for S-layer biogenesis (Figure 6.2AB), shows a small cell stalk (Figure 6.2C); an indication of recent cell wall remodelling activity. Inspection of the S-layer reveals two notable defects: one at the stalk tip, which may indicate the presence or development of the holdfast attachment complex (Sulkowski et al., 2019), and one toward the base of the stalk. This second defect appears to show one hexameric subunit of the S-layer tilted to lie under another – this is surprising given the potential steric hindrance imposed by pushing the S-layer toward the OM, condensing the LPS. It is also unclear as to what the cause of the defect may be. RsaA hexamers are flexible bodies, capable of aligning to a variety of curvatures (Bharat et al., 2017). In addition, it seems unlikely that the S-layers are converging, forcing one to subduct, as this location is most often associated with sporadic cell growth (Billini et al., 2019). Given its localisation to a region of known S-layer biogenesis, it is possible that the defect is a result of an RsaA hexamer incorporating itself into the growing S-layer.

6.4.2. Identifying defects in the S-layer at regions of biogenesis.

As S-layer biogenesis in *C. crescentus* appears to be consistently localised to the septum and cell poles, these areas were targeted for cryo-ET collection without corresponding LM, as previously described (Li et al., 2007, Szwedziak et al., 2014). Cryo-ET revealed sites of distortion in the S-layer at these regions, comprised of gaps in the lattice, loss of confluency, or overlapping RsaA domains. In dividing cells, the septum OM was highlight negatively curved, with S-layers on either side apparently colliding or subducting (Figure 6.3). This may be a result of the septum constriction, wherein the OM is being drawn toward the division plane, pulling the S-layer along with it and breaking

the lattice. This gaps, as the septum continues to close, would provide the necessary space for new RsaA to incorporate into the S-layer.

At cell poles, a similar phenomenon is observed. Distortions in the S-layer can be seen in cell poles both with and without a stalk (Figure 6.4). In the latter, the S-layer appears to overlap slightly at the cell tip. In lower z-slices, a flagellum can be observed protruding from the cell (not shown), suggesting this pole has not been the site of a recent division event. In stalked cells, these gaps appear to localise to the base of the stalk (Figure 6.4B-C). However, gaps can be observed further down the stalk, where curvature is less prominent (Figure 6.4C). Given their localisation and those of gaps seen at the cell septum, and the presence of intact S-layers elsewhere along the cell body, it appears that sites associated with cell growth and RsaA incorporation are more prone to disruption of the S-layer. This may be derived from increased steric hindrance between RsaA hexamers, driven by more significant local envelope curvature. These breaks, when present, may be healed by recovering interaction between two RsaA hexamers, or be filled by new RsaA inserting into the lattice.

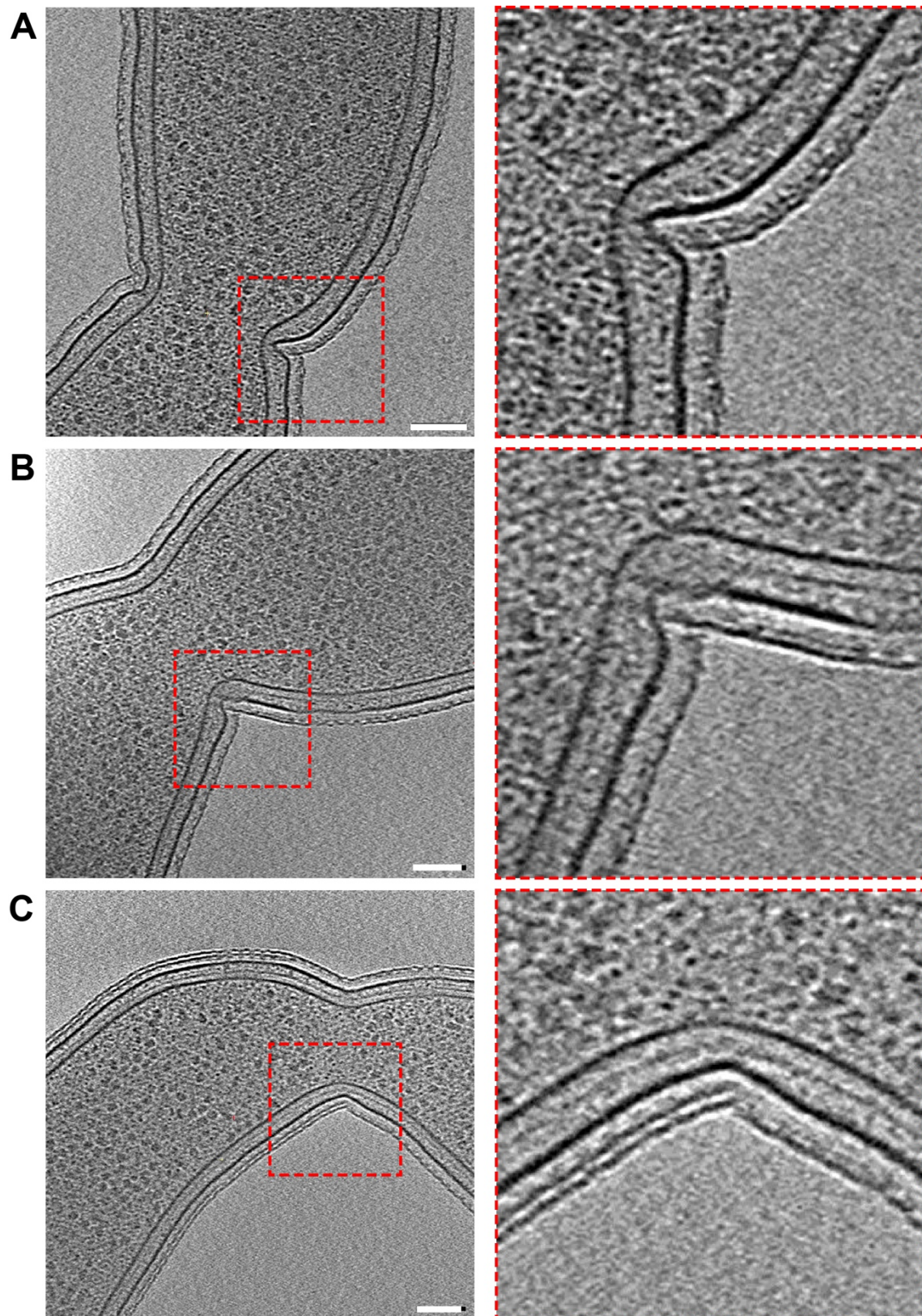


Figure 6.3. S-layer defects at *C. crescentus* cell septa.

(A-C) (A-C) Tomogram slices through *C. crescentus* cell poles. Scale bars = 100 nm. Right panels show enlarged views of the regions highlighted by red-dashed boxes. Envelope components and S-layer distortions are labelled in enlarged views.

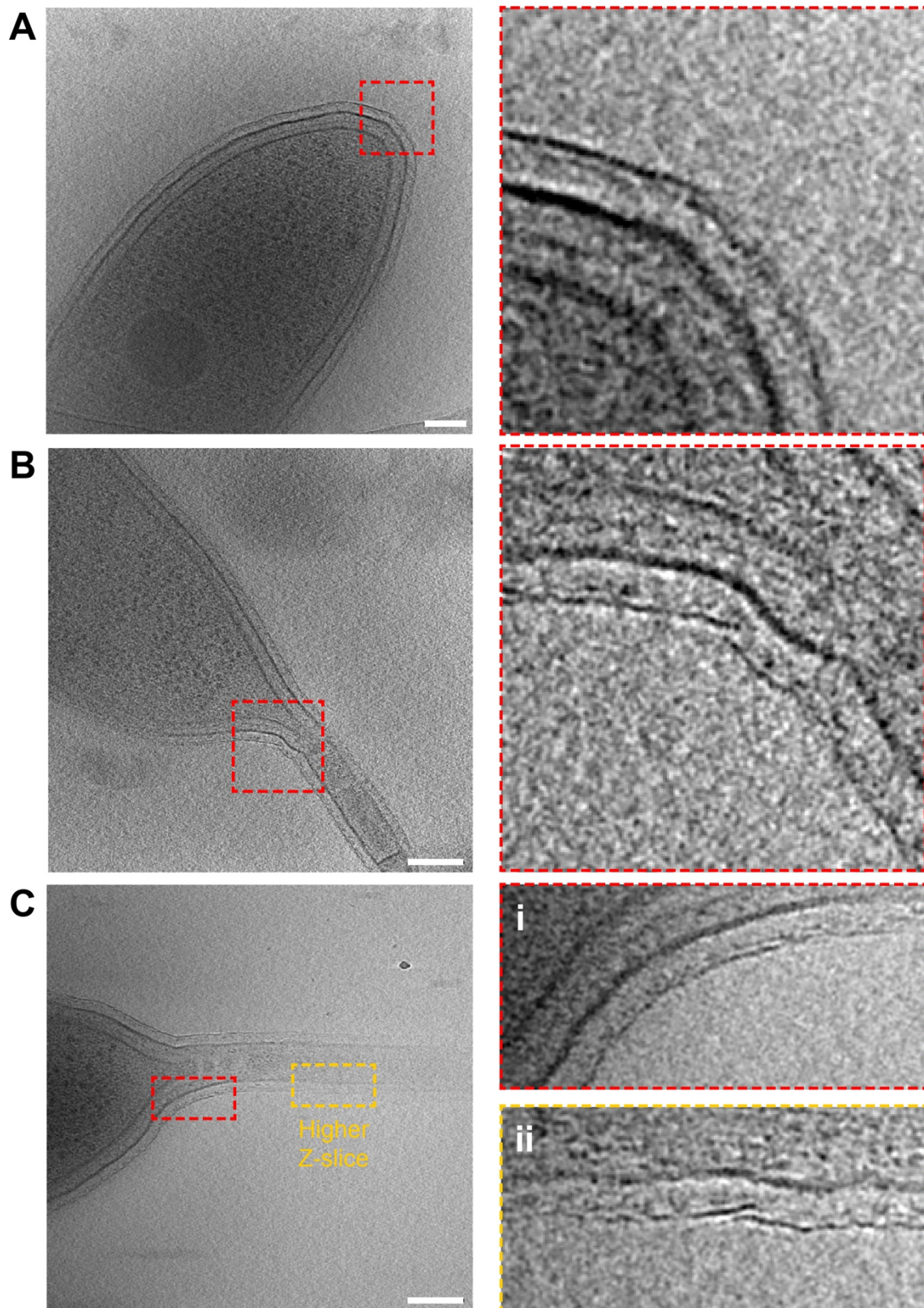


Figure 6.4. S-layer defects at *C. crescentus* cell poles.

(A-C) Tomogram slices through *C. crescentus* cell poles. Scale bars = 100 nm. Right panels show enlarged views of the regions highlighted by red- and yellow-dashed boxes. Where indicated, the height of the Z-slice has been adjusted to bring the S-layer into focus. Envelope components and S-layer distortions are labelled in enlarged images.

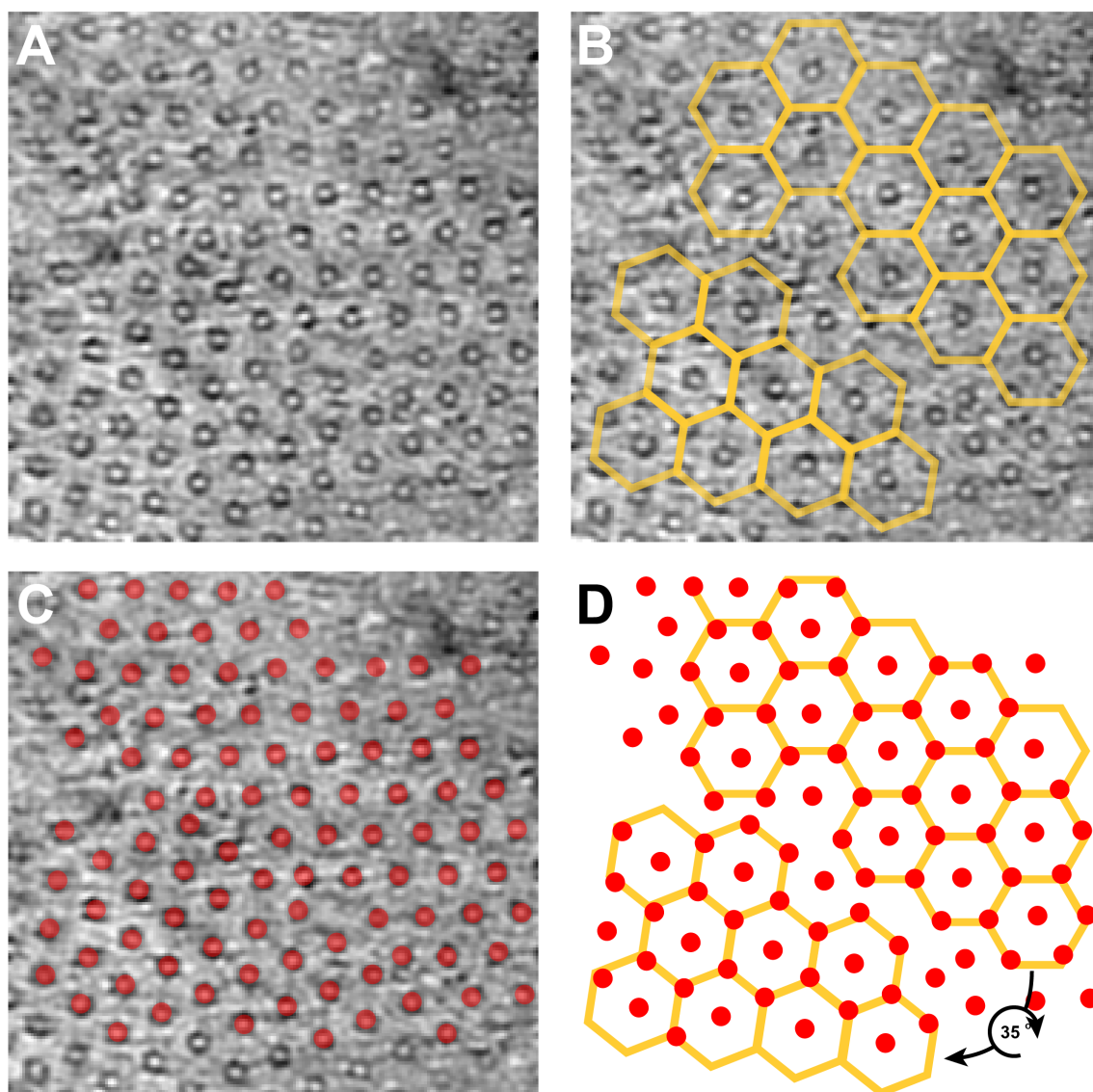


Figure 6.5. Change in the orientation of hexameric symmetry along the S-layer.

(A) Cryo-ET slice along the top of a *C. crescentus* cell, showing the hexameric S-layer. (B) Hexameric symmetry is highlighted by a yellow hexagonal grid. (C) Central hexamer pores are highlighted in red. (D) Overlay of hexagonal symmetry grid and central RsaA pores highlights the offset between adjacent S-layer sheets.

In addition to breaks in the S-layer, top views of cells were analysed to examine the orientation of continuous S-layers. This approach was limited as the body of *C. crescentus* cells are relatively thick, resulting in poor contrast where the S-layer is difficult to discern. A majority of observable S-layers appeared continuously hexameric; however,

one region appeared to show a prominent grain boundary between differently-oriented S-layer sheets (Figure 6.5A-C). The offset between the S-layers is quite significant, with an approximate 35 ° change in symmetry orientation (Figure 6.5D). Unfortunately, it is difficult to distinguish if the RsaA oligomers along this boundary here are adequately tessellated, have gaps, or are composed of less than six RsaA subunits.

6.4.3. Generating lattice maps of cell-bound S-layer using STA

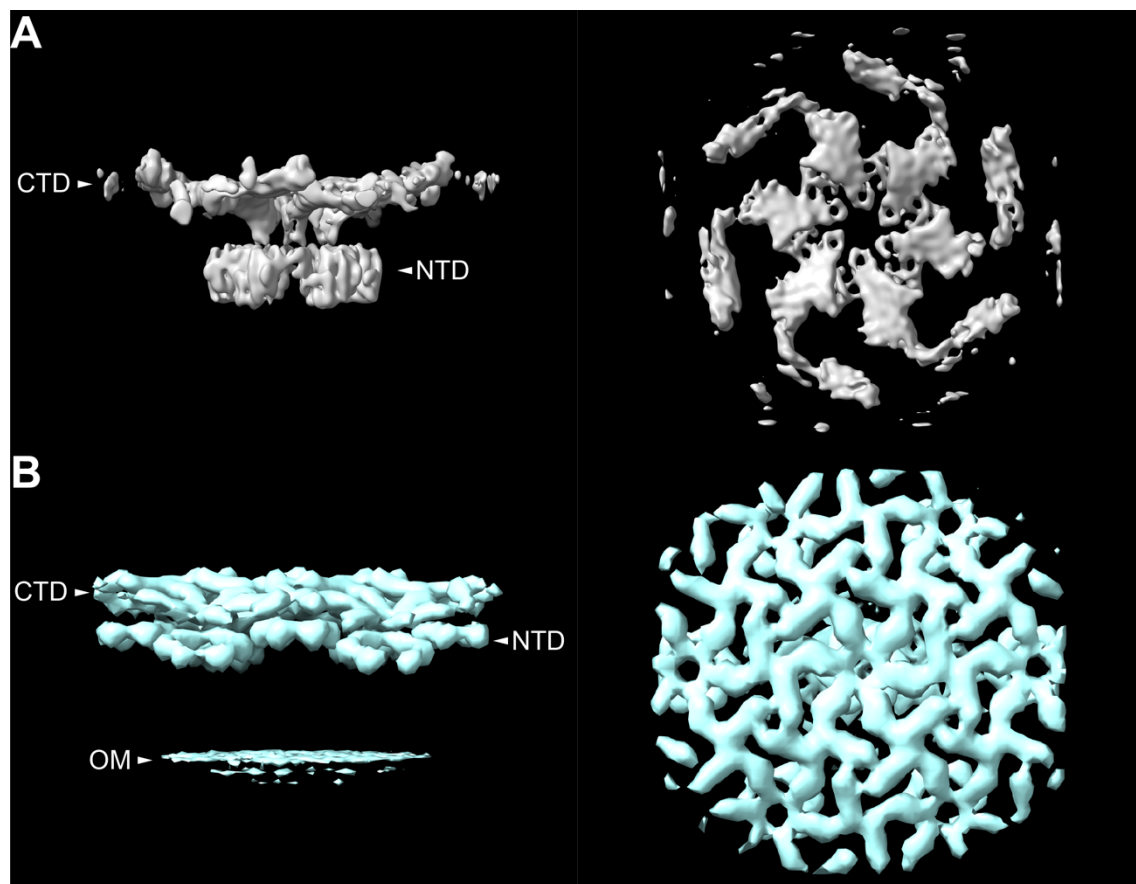


Figure 6.6. RsaA reference maps for STA of S-layers collected by cryo-ET.

(A) Side and top views of RsaA cryo-EM density map (EMD-3604). (B) Low-pass filtered RsaA density map; the map shows six tessellated RsaA hexamers. RsaA_{CTD} and RsaA_{NTD} are labelled in side view for both maps, as is the OM in B.

The cell-bound RsaA hexamer has been structurally resolved using cryo-EM to a resolution of 4 Å (EMD-3604) (Bharat et al., 2017) (Figure 6.6A). The density maps of

this structure, extended to include the surrounding six RsaA hexamers, were low-pass filtered and used as an initial reference map for lattice building in *C. crescentus* tomograms using a subtomogram averaging (STA) based method described previously (von Kügelgen et al., 2021a). The tomograms used for this process were generated using a weighted back projection (WBP) reconstruction algorithm in IMOD (Kremer et al., 1996).

Lattice generation was begun by defining a contour through the centre of the cell in the tomogram – the starting coordinates for STA were then selected along the S-layer. The lattice itself is designed to overestimate the number of RsaA hexamers in the surrounding regions by a factor of six, as many starting positions will likely be lost in successive alignment cycles. Alignment search was limited by a pillar defined through the centre of the reference map, allowing movement in x, y, and z directions. A total of nine successive alignments were made, generating new motive lists for each cycle, with the lowest cross-correlation scoring hexamers removed each time. As a quality check, inverted maps were produced following each iteration, demonstrating a partial loss of RsaA resolution, likely due to the S-layer curvature across the cell and the misalignment of lattice subunits (Figure 6.7). However, RsaA symmetry was broadly retained, as was bipartite structural arrangement of RsaA_{CTD} and RsaA_{NTD}.

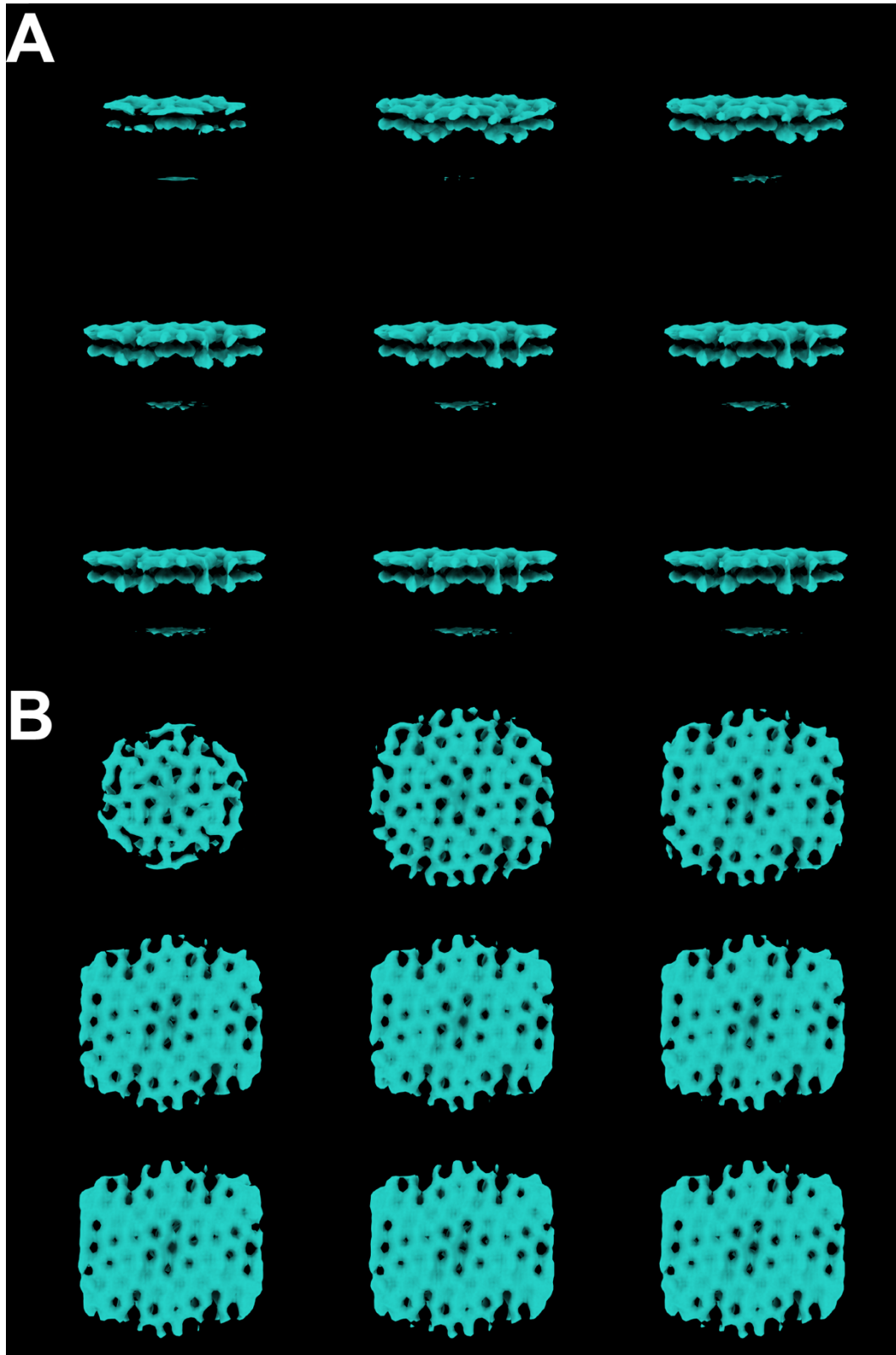


Figure 6.7. Repeated rounds of S-layer subtomogram averaging results in feature loss.

(A-B) Inverted contrast maps of averaged RsaA, progressing from top left to bottom right, following repeated rounds of mapping by cross-correlation and STA. A shows side views, with RsaA_{CTD} and RsaA_{NTD} domains highlighted in the first map. B shows top views.

RsaA hexamers were hidden from view based on low cross-correlation, and overlapping subunits were removed using a centre-to-centre distance-based cleaning script. The resulting lattice maps were considerably patchy, corresponding to only some of the S-layer (Figure 6.8A-B). Lattice generation was most successful along the cell stalks, likely as it is thin enough to provide enhanced contrast (Figure 6.8C). In addition, the starting maps are slightly positively curved (having been generated initially from cell stalks), meaning cross-correlation between the reference maps and cryo-ET densities are improved in this region. In many cases, subunits were erroneously aligned to the outer membrane (Figure 6.9), potentially explaining the significant loss of resolution seen in the inverted maps.

Using this methodology, several regions of *C. crescentus* have well-assigned lattice subunits that tessellate to form a continuous lattice along the S-layer (Figure 6.8C). Subunits appear capable of aligning to a variety of cell curvature, however there is a clear bias toward positively curved S-layers. It should be noted that in cases where there are S-layer distortions (as described in section 0), the lattice appears to either skip over this or attempts to align to the misfit S-layer (Figure 6.8D). With further optimisation, this method may be applicable to detect more defects in the S-layer than can be seen manually.

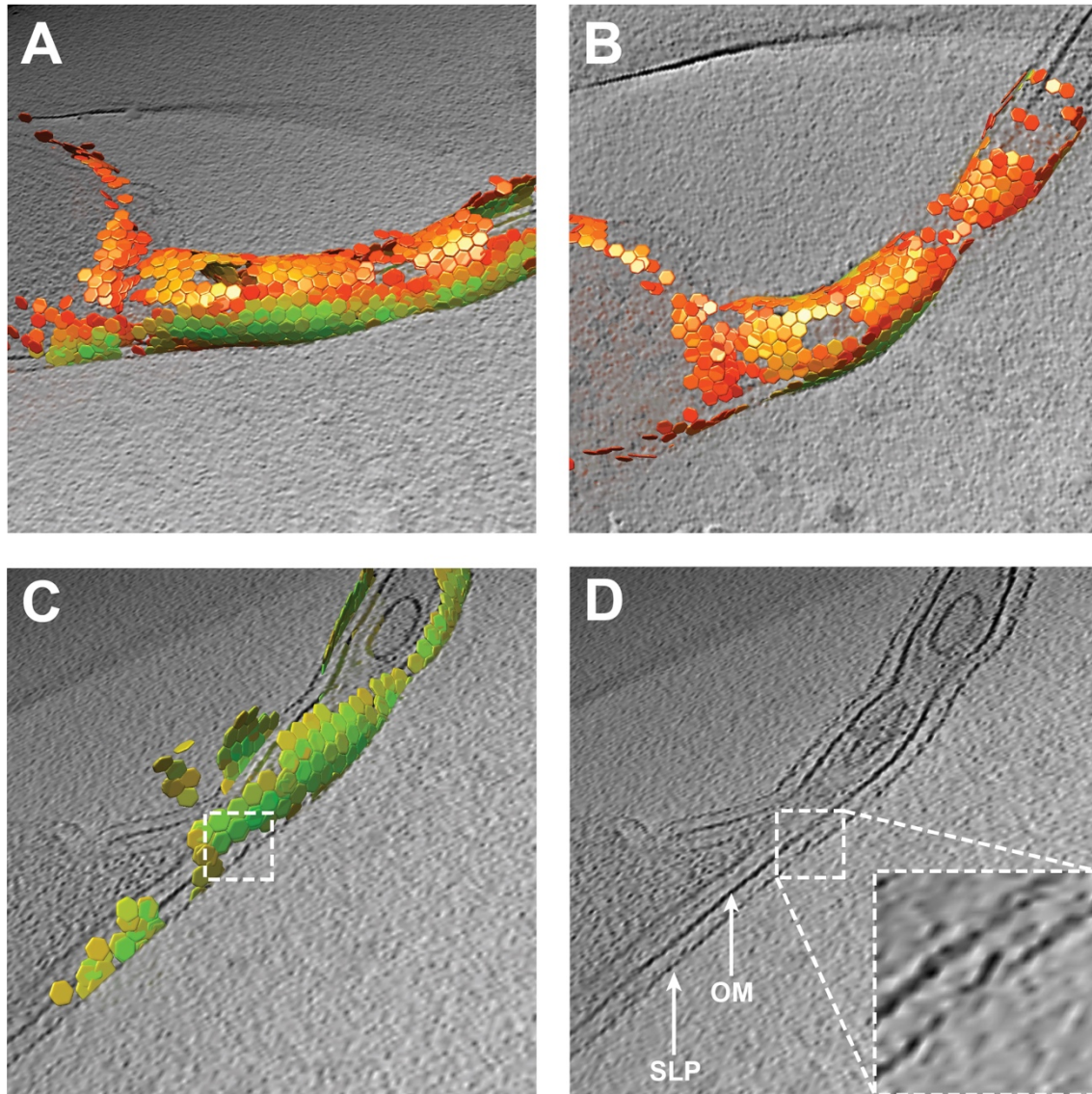


Figure 6.8. STA-generated lattice maps align S-layer along a variety of curvatures.

(A-B) Tomogram slices superimposed with STA-generated lattice maps corresponding to the S-layer of the *C. crescentus* stalk. Hexagons represent lattice subunits, coloured by cross-correlation scores from 1 (green) to 1.75 (red). (C) Reprojected lattice with subunits scoring <1.5 for cross-correlation score removed. (D) Tomogram of *C. crescentus* cells with lattice hidden from view; S-layer (SLP) and outer membrane (OM) are highlighted. Inset; magnified view of a distortion in the S-layer, corresponding to a gap in the lattice map in C.

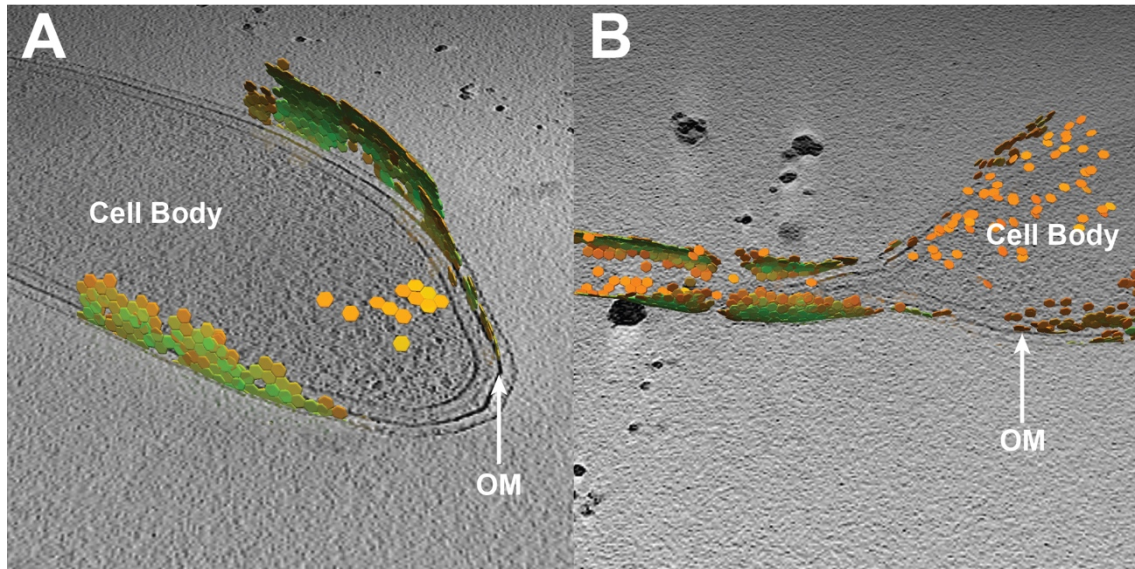


Figure 6.9. STA-generated lattice subunits erroneously align to the outer membrane in successive alignments.

(A-B) Tomogram slices superimposed with STA-generated lattice maps corresponding to the S-layer of the *C. crescentus*. Hexagons represent lattice subunits, coloured by cross-correlation scores from 1 (green) to 1.75 (red). Regions where lattice subunits align to the OM are indicated in each tomogram.

6.5. Discussion

High studies of S-layers have driven the field forward in recent years, spurring new interest in the biology of these fascinating 2D-arrays, resulting in a variety of novel S-layer structures from a diverse range of species (Lanzoni-Mangutchi et al., 2022, von K gelgen et al., 2021b, von K gelgen et al., 2020, Gambelli et al., 2019, Fioravanti et al., 2019, Bharat et al., 2017, Baranova et al., 2012). However, there are few studies published on the S-layer in the context of cell division or growth, leaving a large gap in our understanding of S-layer biology.

The presence of gaps in the S-layer of *C. crescentus* is to be expected; a hexagonal lattice cannot traverse a curved surface without leaving pentameric gaps. What is surprising is the extent and size of the observed distortions, which in some cases appear to provide a direct in-road to the underlying cell membrane. In *H. volcanii*, gaps and distortions are absent, even in areas of significant cell curvature (von K gelgen et al., 2021b). In this case, the gaps in lattice (those expected to arise from hexagonal-insufficiency to completely enwrap the curved cell) are occupied by pentameric SLPs, seemingly composed of the same surface glycoprotein subunits as the hexamers (von K gelgen et al., 2021b). Again, this may reflect differences in S-layer utility between species, given the S-layer is the sole structure-providing component of the *H. volcanii* envelope (Mullakhanbhai and Larsen, 1975), while in *C. crescentus* cell stability is also supported by the cell wall and outer membrane (Agabian and Unger, 1978).

C. crescentus appeared amenable to cryo-CLEM studies, however there are some key limitations. Firstly, the resolutions offered by LM in the cryo-CLEM setup were not particularly impressive. The equipment used allows for mounting of a cryostage to standard widefield microscopes, keeping the grid cold and relatively safe from contamination, but requires large working range between sample and optical equipment.

In the described cryo-CLEM experiments, the resolution was sufficient to distinguish between old and new regions of S-layers on the cell, however this was not the case for every cell, significantly limiting the candidate pool for cryo-ET. In addition, the direct manual handling of grids and repeated transition from storage to sample acquisition inevitably leads to the build-up of non-vitrified ice and contamination, oftentimes obscuring cells by the time they reach the cryoelectron microscope. Nevertheless, using the cryo-CLEM setup as described resulted in a successful attempt at bridging EM and LM data, resulting in identification of potential S-layer insertion events. Advances in cryo-CLEM have been bolstered by significant breakthroughs in the technology required to conduct these experiments (Wolff et al., 2016, Moser et al., 2019), meaning issues of resolution and contamination may become less disruptive. Further studies using cryo-CLEM methods will shed much needed light on the questions surrounding S-layer biology.

Similarly, the STA-studies of S-layers is limited by expected factors, such as sample thickness limiting contrast. While this is generally avoided by using high defocus and dose, this comes at the cost of resolution detail and sample integrity (Schur, 2019, Bohning and Bharat, 2021). The STA-based pipeline for the generation of S-layer lattices described in this thesis can likely be further optimised. For example, using smaller box sizes for the alignment of RsaA reference maps and more rigid searching parameters may prevent misalignment and loss of lattice subunits. However, much of the S-layer will likely be unobtainable due to the size of the cell body and the imposed limitations on sample thickness (Turk and Baumeister, 2020). Much like cryo-CLEM, the success STA-based studies of S-layer will heavily depend on the research question at hand, and the nature of the sample being imaged.

Concluding Remarks and Future Outlook

The projects described in this thesis sought to identify and examine the principles underpinning S-layer Biogenesis in prokaryotes. Importantly, this thesis describes and provides evidence for a model of S-layer biogenesis that can be applied to SLP-producing species across all prokaryotic domains.

To begin, the metal binding properties of RsaA, the sole SLP comprising the S layer of *C. crescentus*, was examined using the SpyCatcher-SpyTag conjugation system. While metal binding has been recognised as a common feature of SLP biology for many decades, the focus within the literature has been on the spontaneous formation and stabilisation of S-layer lattices *in vitro*, driven by metal ions, particularly Ca^{2+} and Mg^{2+} . This has been observed in S-layers of multiple species and across all prokaryotic domains (Baranova et al., 2012, Cohen et al., 1991, Kessel et al., 1988, Rodrigues-Oliveira et al., 2019, Berenguer et al., 1988, Farci et al., 2018, Garduno et al., 1995). This thesis reports a mechanism by which prokaryotes can generate an S-layer without laborious involvement of cell, wherein the SLP, post translation, is exported across the cell envelope. Here, the SLP undergoes folding and oligomerisation upon binding of the divalent cations within the environment; a process which requires tremendous speed and efficient folding. How this process may look in prokaryotes such as *A. salmonicida* whose SLPs undergo a two-step secretion process is unclear (Noonan and Trust, 1995); however, the SLPs of *A. salmonicida* has been shown to form spontaneously form S-layers *in vitro* when in the presence of divalent cations (Garduno et al., 1995), suggesting a commonly derived solution to extracellular folding among unrelated SLPs.

In *C. crescentus*, Mg^{2+} was unable to support S-layer biogenesis *in vivo*, suggesting a potential specificity of RsaA for Ca^{2+} binding. Other SLPs may have more flexibility in their ion binding propensities, as is suggested by their ability to oligomerise

in vitro in the presence of a variety of divalent cations (Yang et al., 1992). High-resolution studies were able to demonstrate that RsaA, while predominantly binding Ca^{2+} , was able to bind alternative K^{+} ions, albeit loosely. It is reassuring that molecular dynamics simulations were so accurately able to recreate the metal-binding behaviours of RsaA, and even correctly predict alternative binding modalities, supporting the argument for an inherent variation RsaA-ion binding interactions. Future studies may seek to adapt the framework reported in this thesis, which spans a range of biological methods and microscopical resolutions, to elucidate this potential ion-binding flexibility.

In addition, this thesis adds much needed context to the question of S-layer biogenesis localisation. While it is possible that S-layer formation *in vivo* mirrors that of *in vitro*, where the SLP oligomerises spontaneously, extending from a point of nucleation, studies from multiple species suggested a more directed biogenesis. In *H. volcanii* (Abdul-Halim et al., 2020), *C. difficile* (Oatley et al., 2020), and *C. crescentus* (Comerci et al., 2019, Herdman et al., 2022), new S-layer material was consistently localised to the midcell and cell poles. This observation points to a potentially universal mechanism of S-layer biogenesis wherein SLP is incorporated at regions of cell growth. Imaging of hundreds of dual-labelled *C. crescentus* cells has revealed repeated and reliable localisation of new S-layer appearing at the midcell and cell poles, in particular the base of the cell stalk, lending tremendous weight to a model whereby S-layer biogenesis is directed by underlying cell growth.

C. crescentus is especially useful for these kinds of studies, not just due to its monomolecular S-layer and amenability to a range of imaging techniques, but also its well characterised asymmetric lifecycle. The localisation of new S-layer material to regions closely associated with cell growth and key checkpoints in cell development was further strengthened by the apparent spatial localisation with HADA-labelled sites of cell

wall remodelling. However, the co-labelling of S-layer biogenesis with cell wall turnover events appears to suggest the localisation of new S-layer is driven by expansion of the underlying envelope. This process is more emergent in origin, but is intriguingly compatible with findings in *C. difficile*, described above. SecA2, the secretion machinery for the *C. difficile* S-layer, appears heterogeneously distributed through the cell, while new S-layer material is found at the cell poles and midcell (Oatley et al., 2020). The authors propose a model similar to the one described in this thesis, wherein SLPs, following secretion, are retained under the preformed S-layer, allowing them to diffuse to gaps in the lattice they can self-incorporate into. This model is also consistent with findings in *C. crescentus*, which demonstrated monomeric RsaA is cell bound but freely diffuse prior to its insertion into the crystallised S-layer (Comerci et al., 2019).

The robustness of *C. crescentus*' ability to localise its S-layer was challenged by the incorporation of cell-cycle disrupting compounds into the labelling strategy. Excitingly, inhibition of cell division, while causing prominent cell filamentation, resulted in a similar localisation of new and old S-layer. Still recruited to the midcell and cell poles, the discrete incorporation of RsaA at these regions remained unchanged, but with new insertion sites equidistantly spaced between pole and septum. Whether these additional sites are also driven by underlying cell wall turnover or instead reflect a new mechanism by which RsaA can localise is difficult to ascertain. However, it is not beyond possibility that there exist in filamentous *C. crescentus* cells multiple regions of growth, as is observed in cells depleted of FtsZ (Meier et al., 2017). Conversely, A22, had a striking impact on the localisation of S-layer biogenesis, resulting in a complete loss of discrete regions of old and new S-layer, as well as drastic distortion of cell morphology and the envelope. However, cells in both conditions were still able to build characteristic, beautifully structured S-layers. A potentially unsurprising observation, given earlier

observations that S-layer oligomerisation and binding is facilitated by little more than secretion and the presence of Ca^{2+} ions in the environment for RsaA to bind. Of course, these studies can be built upon by direct molecular manipulation of the RsaADEF system and key developmental proteins. It would be interesting, for example, to see how S-layer labelling is impacted by inhibiting PopZ, which plays a crucial role in maintaining *C. crescentus* asymmetry, or overexpressing FtsEX, which results in simultaneous cell filamentation and FtsZ ring formation (Meier et al., 2017).

Of course, the question remains regarding the localisation of the RsaA (and other SLP) secretion machinery. While crosstalk between the outer membrane and cell wall has been explored, a mechanism by which RsaA secretion is directly localised to regions of cell wall turnover is unknown. It may be hypothesised that the multiple components of RsaDEF, a TISS, are more stably colocalised at regions of cell wall remodelling. Potentially, the peptidoglycan barrier in these regions is more readily navigated by a multicomponent secretion complex. However, the whereabouts of RsaDEF can really only be addressed by localising the constituent proteins. While both protein and drug efflux via TISSs are well studied (Bohnert et al., 2010, Koronakis et al., 2004), there is remarkably little information on the localisation of the complexes themselves. One study in *Salmonella enterica* sought to achieve this and found outer and inner membrane components of the subject TISS to be differentially localised, proposing that the few sites of colocalisation represented active secretion complexes (Barlag et al., 2016). This highlights the challenges in localising a structure composed of multiple proteins, spanning, and embedding in multiple membranes.

While capable of providing tremendous information on the localisation of fluorescently-tagged cell components, the resolutions permitted in light microscopy still do not compare to those offered by cryo-EM and cryo-ET. However, much of the cellular

context is lost in electron microscopy techniques, without which renders the collection and exploration of cryo-ET data an incredibly laboursome and directionless effort. Combining fluorescent localisation with cryo-ET makes searching for that needle in the biological haystack much more feasible. The cryo-CLEM methods described in this thesis are by no mean high throughput, requiring manual handling and evaluation of each grid, each time imposing the risk of contamination or obscuring that invaluable cell or grid square. Regardless, the cryo-CLEM techniques applied here have tremendous utility and are easily adapted to a range of biological questions, particularly S-layer biogenesis. It is using these methods that these S-layer gaps were identified and placed in their biological context. Cryo-CLEM has seen much interest in the past few years and will likely see more investment as the advantages of combining light and electron microscopy are too big to pass up. It would be exciting to see these methods applied to yet more SLP-producing species, particularly those whose S-layers appearing to lack gaps or grain boundaries.

A recurring motif within this thesis is the universality and robustness of S-layer biology, despite a lack of sequence homogeneity. The *C. crescentus* and *H. volcanii* S-layer, for example, share virtually no similarity in sequence, cell binding mechanism, or fold. It is perfectly reasonable to suggest that they may have completely different evolutionary origins or be so distant related that homology has been thoroughly eroded. However, both produce SLPs that form hexameric lattices, bind metal ions, and incorporate themselves into the S-layer at regions of cell growth. As the field of S-layer biology progresses, it will be interesting to see how commonplace these shared characteristics are. Has the field just chanced across the few species amenable to study that also just so happen to build their S-layers in a similar way? Or, have multiple species, when faced with the question of how to reliably reinforce and protect their cells, arrived at the same conclusion? As a promising candidate for the earliest attempt cells have made

at building cell walls and reinforcing the boundary between life and environment, S-layer biology is a fascinating field with incredible opportunity for future discovery.

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