# Characterization of the secretion and anchoring domains of *Caulobacter crescentus* SapA metalloprotease

by

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**ABSTRACT**

The *Caulobacter crescentus* type I secretion system can be used to display foreign peptides at high density on the bacterium's surface as part of the S-layer. Certain recombinant proteins, however, are subject to proteolytic cleavage by SapA, a unique S-layer associated metalloprotease. SapA (71 kDa) is an unstable protein that breaks down to a 45-kDa product when over-expressed. It needs to be secreted before it can become an active enzyme that anchors to the cell. A point mutation adjacent to the protease's active site reduced SapA processing, indicating that SapA is self-processing. The last 10 and 50 amino acids were removed and prevented secretion, indicating SapA was a type I secreted protein. Further, SapA secretion was blocked in an S-layer type I secretion deficient strain. Lack of secretion prevents this protease from becoming an active enzyme evidenced by the type I defective clones, which are not processed at all. The last 100 amino acids of the protease are sufficient for anchoring, as determined by immunofluorescence. Interestingly, SapA could be detected on the cell surface by immunofluorescence only in an S-layer negative, O-antigen deficient strain. This suggests that SapA is localized on the cell membrane, beneath the S-layer and is hidden by smooth LPS. A fusion protein, containing a 242 amino acid protein G peptide attached to the last 238 amino acids of SapA secreted and anchored to the cell surface of *C. crescentus*. This fusion was detectable an anti-IgG antibody. SapA is the first identified self-processing protease that uses its C-terminus for both type I secretion and anchoring.

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| | |
|---|---|
| aa | Amino acid |
| ABC | ATP binding cassette |
| Amp | Ampicillin |
| $Amp^r$ | Ampicillin resistant |
| ATP | Adenosine triphosphate |
| BAC | Replication genes (repB, repA, repC) |
| BCA | Bicinchoninic acid assay |
| Cm | Chloramphenicol |
| $Cm^r$ | Chloramphenicol resistance |
| C-terminus | Carboxy terminus |
| DNA | Deoxyribonucleic acid |
| DNase I | Deoxyribonuclease |
| EDTA | Ethylene diamine tetracetic acid |
| EGTA | Ethylene glycol tetraacetic acid |
| EtBr | Ethidium bromide |
| EPS | Exopolysaccharide |
| GB1 | Immunoglobulin-binding domain B1 of streptococcal protein G |
| GSP | General secretory pathway |
| IgG | Immunoglobulin G |
| IM | Inner membrane |
| kDa | Kilodalton |
| Km | Kanamycin |
| $Km^r$ | Kanamycin resistance |
| LPS | Lipopolysaccharide |
| MCS | Multiple cloning site |
| MFP | Membrane fusion protein |
| mRNA | Messenger RNA |
| Ni-NTA | Nickel-nitrilotriacetic acid |
| N-terminus | Amino terminus |
| $OD_{600}$ | Optical density at 600nm |
| OM | Outer membrane |
| OMP | Outer membrane protein |
| PCR | Polymerase chain reaction |
| PYE | Peptone yeast extract |
| RNA | Ribonucleic acid |
| RNase A | Ribonuclease |
| RTX | Repeat in toxin |
| Sm | Streptomycin |
| S-layer | Surface layer |
| SLPS | Smooth lipolysaccharide |
| Tris | Trishydroxymethylaminomethane |

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# 1 INTRODUCTION

## 1.1 *Caulobacter crescentus*

*Caulobacter crescentus* is a crescent-shaped, Gram-negative, non-pathogenic, aerobic bacterium that is ubiquitous in most aquatic communities and in many soils (67). This bacterium has a dimorphic developmental lifestyle where it is able to switch from a motile swarmer phase to a stalked phase (17, 38, 66). Swarmer cells have a flagellum, pili and holdfast whereas stalk cells lose their flagellum and form a stalk from the cell envelope. When stalked cells divide, they produce new swarmer cells with the flagellum on the opposite pole of the stalked cell (Figure 1-1). Both are able to attach to solid surfaces by means of their holdfast, a material secreted at the flagellated or stalked end of *C. crescentus* cells. Rosettes of multiple cells often form between *C. crescentus* by binding at their holdfast. Although many labs study the life cycle of *C. crescentus*, our lab has focused on the surface layer (S-layer) of this bacterium. *C. crescentus* possess an S-layer composed of a single protein RsaA, which covers the entire cell surface throughout its life cycle (70, 38).

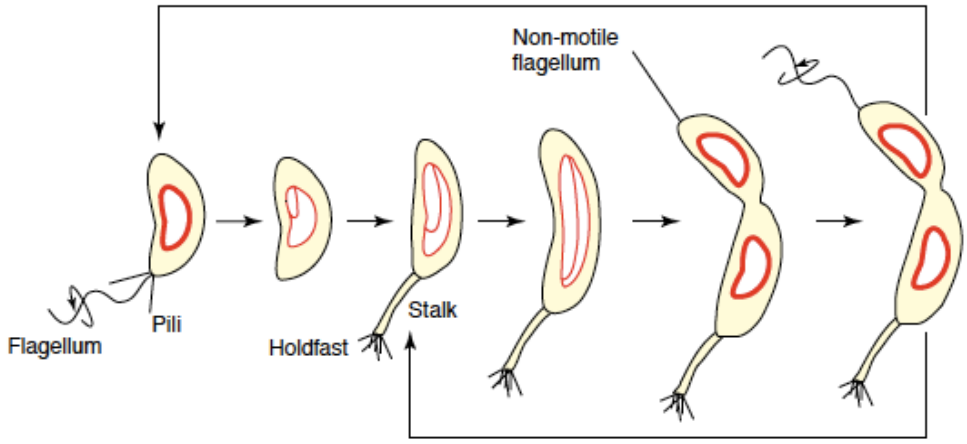



Figure 1-1. Cartoon representation of *C. crescentus* life cycle (figure from Quardokis and Brun, 2003).

## 1.2 S-layers of bacteria

S-layers are found on the surface of many microorganisms such as Gram-positive bacteria, Gram-negative bacteria, Archeabacteria, and Eubacteria. These two-dimensional crystalline arrays composed of proteinaceous subunits can be found in over 400 different microorganisms (7, 75). Surface proteins are typically one of the most abundant proteins in the cell and can account for up to 10-15% of the total cellular protein in the bacterium (13, 21). There is, however little similarity between the primary sequences of S-layer proteins (13). S-layers are thought to have varying functions, the most common of which is to serve as a protective coating (75). However, they have also been shown to act as molecular sieves and ion traps (16, 74, 79), attachment sites for exoprotein (30, 46), extracellular virulence factors (26, 29, 79), as well as promoting adhesion and surface recognition, or simply providing rigidity and maintaining cell shape (6).

The S-layer is usually composed of thousands of copies of a single protein or glycoprotein 40-200 kDa in size that self-assembles into a crystalline-like lattice. The lattice of protein subunits is arranged in a tetragonal or hexagonal lattice with pore sizes ranging from 2-8 nm (21, 13). The S-layer is bound to the surface of peptidoglycan in Gram-positive bacteria whereas its attachment involves lipopolysaccharide (LPS) in the outer membrane for Gram-negative bacteria. The majority of S-layers from Gram-negative bacteria are secreted through the signal peptide Sec-dependent general secretory pathway (GSP), most notably the type II secretion system (7, 21). The type II secretion system transports S-layer protein through the inner membrane (IM) and the outer membrane (OM) using two separate processes that involve many accessory proteins. Alternatively, the S-layer can also be secreted by the type I Sec-independent secretion

system found in bacteria such as *C. crescentus*, *Campylobacter fetus*, and *Serratia marcescens* (88, 2, 43).

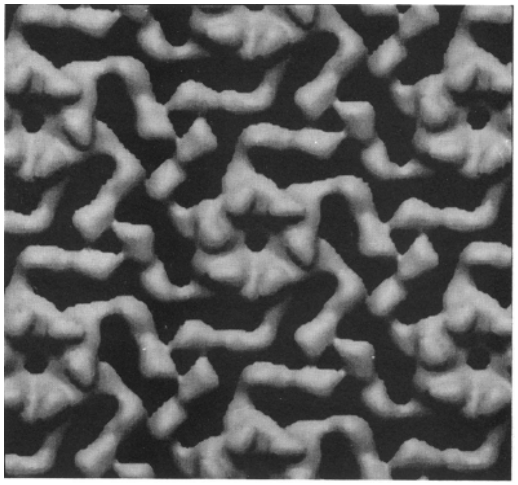


Figure 1-2. 3-D hexagonal array of the S-layer (figure from Smit *et al*, 1992).

### 1.3 S-layer of *Caulobacter crescentus*

The S-layer of *C. crescentus* is made up of a single 98-kDa protein, RsaA that constitutes 10-12% of total cellular protein (2, 81). RsaA is secreted at high levels and covers the entire cell surface by approximately 40,000 inter-linked copies to form the hexagonal crystalline array of the S-layer (Figure 1-2) (82). Once RsaA is secreted, it is anchored to the cell membrane by smooth lipopolysaccharide (SLPS) (32). The RsaA N-terminal anchoring region lies in the first 225 amino acids and binds to the O side chain of SLPS (11, 32). Strains deficient in O side chain biogenesis shed their S-layer and are identifiable by the formation of a halo around their colonies (3, 95). Proper crystallization and correct hexagonal pattern formation of the S-layer requires $Ca^{2+}$ that binds to RsaA's glycine-rich (RTX) motifs (GGXGXD) (5, 11, 28).

The S-layer of *C. crescentus* is thought to function as a protective coat against bacteriophage, proteases, and parasites such as *Bdellovibrio*-like organisms (7, 45). *Bdellovibrio*, which are Gram-negative, obligate aerobic bacteria, are known for their

ability to parasitize other Gram-negative bacteria, such as *C. crescentus*, *C. fetus*, and *Aeromonas salmonicida*. They prey on bacteria by first breaking through the outer membrane, and then entering into their periplasm and using host proteins and nucleic acids as a source of nutrition (71, 79). The S-layer may block these organisms from attacking the cell; however, the complete role the S-layer plays in nature has yet to be determined.

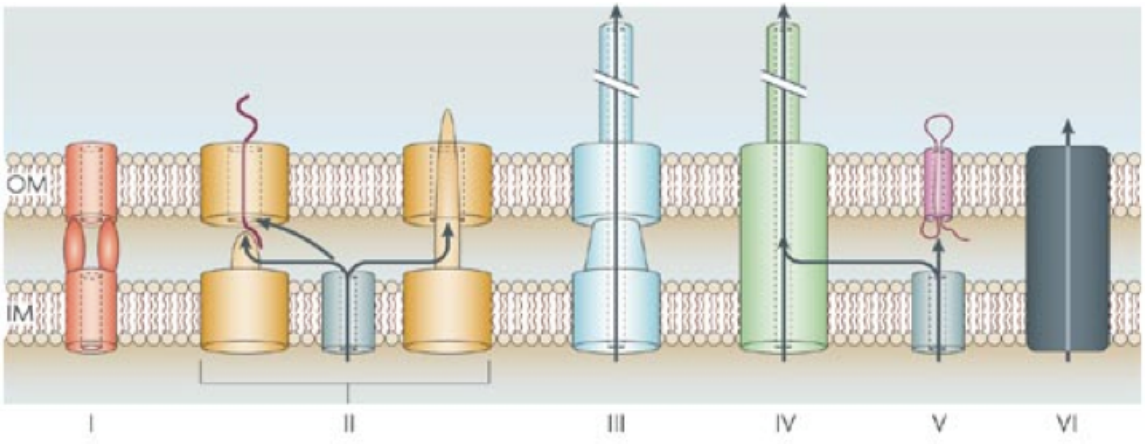



Figure 1-3. Cartoon depiction of the type I through type VI transport systems (figure from Abdallah *et al*, 2007).

## 1.4 Transport systems

The secretion of proteins to the extracellular space or a host cell is complex in Gram-negative bacteria since these proteins have to pass through two separate membranes and the periplasmic space in between. To date, there are six different classes of secretion systems in Gram-negative bacteria; type I to type VI (Figure 1-3) (1). The type I secretion system, which is used to secrete RsaA in *C. crescentus*, will be discussed in detail in sections 1.5 and 1.6.

The type II secretion system is the main transport system used in Gram-negative bacteria. This system requires the Sec-dependent general secretory pathway (GSP) for

transport of unfolded proteins. An N-terminal secretion signal is required to translocate proteins from the cytoplasm through the IM to the periplasm. Upon entry into the periplasm, the N-terminal signal sequence is cleaved and proteins are folded before transport through the OM (68, 87). The OM machinery known as the secreton can then recognize proteins for secretion to the extracellular space. The secreton is made up of a conserved multimeric complex of secretin proteins forming an OM pore and, a pilus-like structure in the IM believed to act as a piston that pushes proteins through the pore. The type II system is similar to the Gram-negative type IV pili (1).

The type III secretion system does not use GSP machinery and is therefore known as a Sec-independent secretion system. The type III system is thought to have evolved from the flagellum apparatus and, is closely associated with bacterial pathogenesis in *Salmonella, Shigella, Yersinia* and *Vibrio* (65). Proteins are translocated from the cytoplasm into eukaryotic host cells or the extracellular environment using a complex needle-like structure called an injectisome. The injectisome is a composed of more than 20 different structural proteins, which form a channel connecting the entire cell envelope and extends to directly contact host cells (1). In *Yersinia pestis*, the gate of the type III secretion system is opened by low $Ca^{2+}$ concentrations in the cytoplasm detected by the lcrV (low calcium response) antigen (72).

The type IV secretion system is similar to the type III system in that it is able to transport DNA or proteins directly into host cells. However, the process of transporting DNA between bacteria or proteins from bacteria to eukaryotic cells in the type IV system is homologous to bacterial conjugation (18). An example of the type IV secretion is the VirB system in *Agrobacterium tumefaciens*, which requires at least 10 proteins for

transport via complex *trans*-envelope structures and a pilus-like structure at the bacterial surface (1, 20). In most cases, type IV utilizes a Sec-dependent transport system for translocation across the IM, however recent evidence indicates that transport of substrates can also take place via a one-step mechanism directly from the cytoplasm to the host (1).

The type V secretion system, or autotransporter, is a Sec-dependent system that translocates proteins in a simple two-step fashion (41). The type V system is considered a self-sufficient pathway because it does not require ATP and all the information necessary for secretion, aside for the Sec machinery, is present on the protein itself (86). The three basic functional domains of the protein are an N-terminal signal peptide, the passenger domain, and the C-terminal translocator domain. The C-terminal translocator domain has been proposed to form a β-barrel pore in the OM to transport the passenger domain to the extracellular surface (1). The β-barrel domain can be cleaved to release the passenger domain or, remain uncleaved and act as a display system (86). A diverse range of proteins is transported using the type V system including toxins, adhesins, proteases and invasins (41).

The type VI secretion system was recently identified in *Vibrio cholera* and *Pseudomonas aeruginosa* for the secretion of certain virulence factors into the target host cell (59, 69). There are several components involved in this system (1). Based on genomic data, the type VI system is thought exist in bacteria that come into close contact with eukaryotic cells such as plant and animal pathogens (19). Although not much is known about type VI, this system is considered a Sec-independent system since proteins secreted by this pathway do not contain an N-terminal signal leader. It has been

suggested that this system may secrete cell material via the budding of vesicles from the OM with the aid of periplasmic cargo proteins (1).

### 1.5 Type I protein secretion

One of the major advantages of the type I secretion system is its simplicity. This Sec-independent system requires only three proteins for transport across the IM and OM (2, 11). The type I system is found in many Gram-negative bacteria such as *Pseudomonas fluorescens, P. aeruginosa, Escherichia coli*, *S. marcescens* and *Erwinia chrysanthemi* for the transport of several types of proteins including lipases, toxins, proteases, and S-layers (28, 87).

The three components that constitute the transport apparatus are an ATP binding cassette (ABC) transporter in the inner membrane, a membrane fusion protein (MFP) and an outer membrane protein (OMP). Although the genes encoding the ABC transporter and the MFP are usually clustered with the target protein, the OMP gene can be found clustered together with the target protein or elsewhere on the chromosome (87). These three components form a direct channel from the cytoplasm to the extracellular space, preventing proteins from entering the periplasm. The ABC transporter hydrolyzes ATP to provide energy for this process after recognition of an uncleaved C-terminal type I secretion signal on the protein (64). The secretion signal is often 30-60 amino acids in length. Upstream of the signal, there are typically glycine-rich repeats (RTX motifs or repeat in toxin motifs) that bind calcium and have been suggested to allow correct presentation of the secretion signal to the inner membrane secretion machinery (10, 23, 28, 49). Although RTX motifs are present in these proteins, they are not absolutely necessary for secretion as has been shown by the secretion of truncated versions of *E.*

*chrysanthemi* protease B lacking the RTX region (35). Further, proteins secreted by this pathway often lack cysteine residues to eliminate the potential formation of disulfide bonds (10).

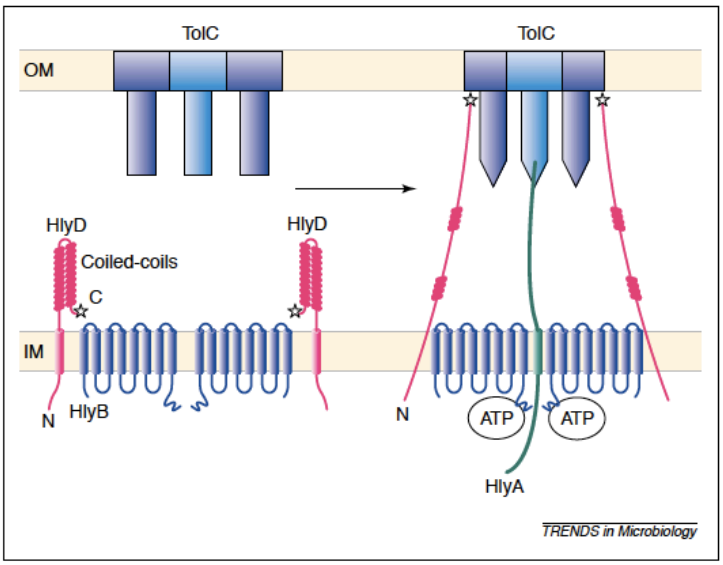



Figure 1-4. Cartoon depiction of type I hemolysin secretion (figure from Gentschev *et al*, 2002).

The most well known and best characterized type I secretion system is the *E. coli* α-hemolysin (HlyA) secretion system (34, 84). HlyA accounts for 2-3% of total cellular protein whose transport is mediated by three components: HlyB (inner membrane ABC transporter), HlyD (MFP attached to the IM) and TolC (OMP) (4, 34, 85). The *hlyA* gene is clustered together with *hlyB* and *hlyD* and, transcription of the latter two genes occurs as a read-through product of the *hlyA* promoter. The OMP gene, *tolC* is located elsewhere on the chromosome and is transcribed separately (4, 85). Interestingly, TolC is involved in at least four different export systems (34).

Before transport of HlyA occurs, homodimeric HlyB embedded in the IM forms a stable complex with homotrimeric HlyD (MFP) spanning the periplasm (4, 85). Once HlyA binds to the HlyB-C complex, this induces contact to TolC, via the HlyD trimer, to

form a trans-periplasmic export channel (Figure 1-4) (34). HlyA is transported in an unfolded state through the channel in one step into the extracellular space (4, 64, 85). As HlyA emerges from the outer membrane, the concentration of $Ca^{2+}$ in the extracellular medium or on the bacterial cell surface is important for the correct folding of the protein. A lack of extracellular $Ca^{2+}$ required to interact with HlyA's RTX motifs can adversely affect the folding of HlyA (64).

### 1.6 Secretion of RsaA

Similar to HlyA secretion, RsaA is secreted by a type I secretion system comprised of three components. The first is an ABC transporter, RsaD, that is embedded in the IM and is able to recognize an uncleaved C-terminal secretion signal on RsaA located on the last 82 amino acids of the protein (2, 12). Upstream of the C-terminal secretion signal are six RTX motifs (36). The second component is a MFP, RsaE, which is anchored to the IM and spans the periplasm (25). Finally, an OMP interacts with the MFP to form a channel to the outside of the cell (2). The two identified outer membrane transporter genes in *C. crescentus* are *rsaFa* and *rsaFb* (32). The genes, *rsaD* and *rsaE*, are transcribed by a separate promoter immediately downstream of *rsaA* (91). Whereas *rsaFa* is located further downstream of *rsaE* and *rsaFb* and, is located 322 kbp downstream of *rsaA* (92). Once RsaA is secreted it is anchored to the cell membrane by SLPS and forms an S-layer around the entire surface of the cell (32).

### 1.7 Biotechnology applications of the RsaA Type I secretion system

The S-layer in *C. crescentus* can be used for several biotechnological and research applications. RsaA proteins can either be displayed on the cell surface or secreted, and easily recovered and purified as aggregates from the culture supernatant with 90-95%

purity (61). Since RsaA is secreted by a type I secretion system whose sole export requirement is an uncleaved C-terminal secretion signal, this system can allow for the secretion of many types of proteins (2, 10). As such, RsaA can accommodate large foreign polypeptides (>100kDa), unlike the outer membrane proteins of other Gram-negative bacteria or, in the sortase-mediated display of some Gram-positive bacteria (53, 94). Further, the S-layer system has the potential to be used for the production of cellular adsorbents, whole-cell vaccines, tumor suppressors, high-density peptide display libraries and screening of antibody libraries (8, 33, 61). Finally, *C. crescentus* can be grown with relative ease, thus the S-layer protein secretion system is an attractive option for efficient low-cost, high purity protein production (12).

An example of peptide display that is relevant to this project is the display of *P. aeruginosa* type IV pilin adhesintope on the surface of *C. crescentus*. The pilus of *P. aeruginosa* is involved in receptor-mediated binding to the epithelial cell surface of humans and has the potential to be used as an anti-adhesive vaccine for this pathogen (10). The adhesintope, which is solely exposed at the tip of the pilus, is a useful target for display because it mediates the initial binding of the pathogen (42, 63, 99). Since antibodies can be produced against the adhesintope, the S-layer system provides a fast and economical method of displaying the epitope (TSDQDEQFIPKG) to generate an antibody response. Antibodies may prevent this opportunistic bacterium from infecting host cells of immunocompromised individuals or the lungs of cystic fibrosis patients causing chronic lung infections. When compared to other anti-adhesion vaccines against *P. aeruginosa*, RsaA/adhesintope fusion proteins generate a 1000-fold greater antibody response against the pilin. Despite these drastic improvements in antibody titer, however,

none of the fusion proteins significantly protect mice infected with *P. aeruginosa* (94). Nevertheless, this example shows the utility of the S-layer for cheaply producing large amounts of fusion proteins that are able to stimulate a substantial amount of antibody production.

An example of S-layer peptide display that is useful in research applications is the display of IgG-binding protein G. IgG-binding protein G is typically found on the surface of streptococcal cells and acts as a bacterial Fc receptor. Thus, IgG-binding protein G is frequently used for immunoprecipitation or immunoadsorption-based assays. One of the limitations of using streptococcal protein G is its cost because it needs to be conjugated to Sepharose beads. A more affordable alternative is whole cell display of *Staphylococcus aureus* protein A however, protein A does not bind to as broad a spectrum of host species IgG. The solution to all these setbacks is the use of the *C. crescentus* S-layer system to display protein G IgG-binding domains which binds more IgG than protein A and is cheaper to use than protein G-Sepharose beads (60).

Protein G is composed of 3 domains, one of which is GB1. In the *C. crescentus* S-layer system, three GB1 domains (54 amino acids each) flanked by Muc1 peptides (20 amino acids each) are inserted into RsaA, secreted at wild type levels, and displayed on the cell surface. These whole *Caulobacter* cells display densely packed GB1 domains that can bind rabbit, goat and mouse Ig. In fact, they bind twice as much rabbit IgG per cell as compared to *S. aureus* and perform at a level comparable to protein G-Sepharose beads (60). Since *C. crescentus* can secrete up to 250 mg/litre of protein, 1 mg of cells can bind a theoretical maximum of 14 ug of IgG (12, 60). Furthermore, *C. crescentus* produces relatively small amounts of lipopolysaccharide endotoxin and the lipid A on its

LPS has low endotoxicity, which would otherwise interfere with immune-based applications. These are key considerations for the production of recombinant protein from any Gram-negative bacteria (7, 60, 61). As such, whole *Caulobacter* cells that display protein G IgG-binding domains are undoubtedly useful to research applications.

### 1.8 SapA metalloprotease

A limitation of the surface display system is that some of these heterologous proteins are subject to proteolytic cleavage by SapA, a 658 amino acid S-layer associated metalloprotease (93). Based on previous experiments in our lab, cleavage by SapA had been solely linked to proteins transported by the type I pathway. In the case of the pilin peptide insertions, 9 of the 11 peptide insertion sites on RsaA results in some form of proteolytic cleavage. In some cases, a single cleavage occurs within the pilin peptide depending on where it is inserted in RsaA (10). Another type of cleavage that always occurs at a site distant from the peptide insertion yields a surface anchored 26 kDa N-terminal fragment and a released C-terminal cleavage product carrying the pilin peptide (10, 93). Thus, the introduction of a foreign peptide in certain locations affects the native conformation of RsaA and exposes a proteolytic cleavage site on the polypeptide that would otherwise not be accessible. Based on these results, it can be inferred that the native function of SapA is to cleave misfolded or environmentally damaged RsaA to maintain the integrity of the S-layer (32). Further, since SapA targets only some recombinant proteins, it may be a site-specific protease. Yet, based on previously N-terminally sequenced recombinant RsaA proteins, so far there was no direct evidence of site-specificity, as cleavages were found between methionine and serine residues and between phenylalanine and isoleucine residues (10, 11).

## 1.9 Zinc metalloproteases

Proteases are essential in maintaining homeostatic control of cells, playing physiological roles in the life cycle of organisms and, in pathogenic bacteria, they can act as toxic factors to host cells (57). Several metalloproteases that contain a zinc (II) ion in their catalytic site are considered toxic proteases. One example is in *P. aeruginosa*, which produces two metalloproteases that digest host plasma proteins required for coagulation or complement action and structural proteins of the cornea and basement membrane (100).

There are four groups of zinc-containing metalloproteases. Those with a consensus sequence of HEXXH are from the zincins superfamily, where the histidine residues act as the first and second zinc ligands. Within the zincins superfamily, bacterial metalloproteases fall into three families: thermolysin (e.g. *Bacillus thermoproteolytics*), neurotoxin (e.g. *Clostridium botulinum*), and serralysin (e.g. *Serratia marcescens*) (57).

The thermolysin family uses three amino acid residues and one water molecule to bind to a zinc (II) ion. Aside from the two histidine molecules, a glutamic acid residue, which is 25 residues downstream of the motif, acts as the third zinc ligand (55). The neurotoxin family is not believed to use a third zinc ligand. These enzymes are known for their role in inhibiting the release of the neurotransmitter acetylcholine (58).

The serralysin family has an extended zinc-binding motif of HEXXHXUGUXH, where the third histidine and a water molecule act as the third and fourth zinc binding ligands, and U are bulky hydrophobic residues (44, 54). There may also be a possible fifth ligand, a tyrosine at position 41. Two examples of zinc-metalloproteases that share this motif are *S. marcesens* serralysin and *P. aeruginosa* alkaline protease (AprA) (54,

78). Members of the serralysin family are important virulence factors in pathogenic bacteria. These metalloproteases target a variety of substrates such as host immunoglobulins, complement proteins, and cell matrix and cytoskeletal proteins.

Serralysin-like metalloproteases also have a characteristic C-terminal domain that forms a single-stranded right-handed beta-helix. This region contains RTX motifs that bind calcium between the turns of the helix. More specifically, these repeats fold into a parallel β-roll where calcium binds within the turns that connect the β-strands. *P. aeruginosa* AprA, for example, possesses six RTX repeats (50). As such serralysin-like metalloproteases are also known as RTX toxins, that can possess between ~6 to 45 tandem repeats of a glycine-rich nine-residue motif whose consensus sequence is GGXGXDX(L/I/F)X. Interestingly, α-hemolysin, which is not a member of this family, also possesses the same sequence motif (56). The activity of serralysin-like metalloproteases is inhibited in the presence of EDTA or EGTA (15, 50).

Members of the serralysin family of metalloproteases include *P. aeruginosa* alkaline protease (AprA), *S. marcescens* serralysin, *Erwinia chrysanthemi* proteases A (PrtA), B (PrtB), C (PrtC) or G (PrtG), and *Pseudomonas sp.* psychrophilic alkaline protease (83, 97, 98). All of these proteases have been shown to be secreted by a type I secretion mechanism. They possess gycine-rich repeat motifs (RTX toxins) close to their C-terminus; they are secreted using similar membrane transporters, two inner membrane proteins and one outer membrane protein; the transport components have a significant degree of homology and some can be used interchangeably; the inner membrane component is a conserved ATP binding cassette; and their secretion signal is located in their C-terminus (15, 22, 47, 51, 101).

The secretion system components of the serralysin-like metalloproteases are closely related to the HlyA system. AprA for instance, requires proteins AprD, AprE, and AprF for secretion (27). These three proteins are necessary components for type I secretion and, are homologous to HlyB, HlyD and TolC from the type I secretion system of *E. coli* α-hemolysin (85). Similarly, PrtB and PrtC require homologs PrtD, PrtE and PrtF for type I secretion. For the above examples, AprF, TolC and PtrF are the ABC transporters. Although there is substantial homology between the components of these pathways, the C-terminal secretion signal sequence of these proteins are quite different (23).

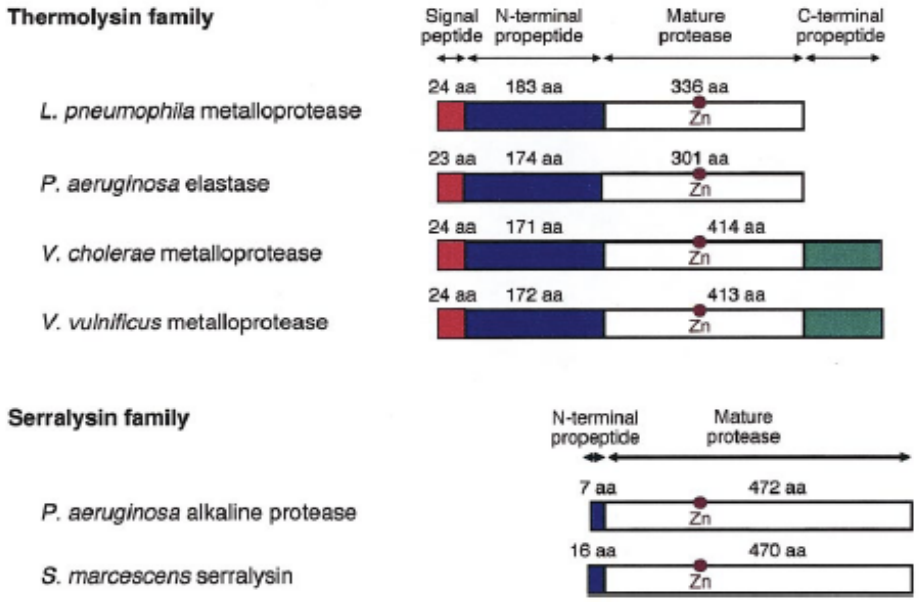



Figure 1-5. Cartoon representation of precursors of bacterial zinc metalloproteases (figure from Miyoshi and Shinoda, 2000).

## 1.10 Processing of extracellular proteases

Bacterial extracellular proteases are transcribed as inactive precursors or zymogens with an additional polypeptide sequence (the propeptide) that is not present on

the mature secreted protein. These propeptides, which come in various lengths and locations, are thought to help keep the proteases in an inactive state, promote correct folding, alter the protease's specificity, act as a membrane anchor or, serve as a secretion signal (97).

Zinc-metalloproteases from the thermolysin family are synthesized as inactive precursors and subsequently undergo several processing stages. Serralysin zinc-metalloproteases on the other hand, do not contain N-terminal signal peptides (57). Instead, these proteases have their first few N-terminal amino acids cleaved after transmembrane translocation (Figure 1-5). The N-terminal propeptide has been suggested to play a role in folding of the proenzyme or it may temporarily anchor the protease to the outer membrane (39). Additionally, metalloproteases of the serralysin family do not contain cysteine residues to eliminate the potential for disulfide bond formation (10).

Type I secreted zinc-metalloproteases from the serralysin family are usually secreted as zymogens that require N-terminal processing and $Ca^{2+}$ binding from the environment to be active (50, 56). Two examples of zymogens are PrtB and PrtC from *E. chrysanthemi* that have a short amino-terminal propeptide; 15 amino acids for PrtB and 17 amino acids for PrtC. Both proteases are processed in the external medium after secretion. This prevents any unwanted activity of the proteases prior to secretion (22, 97). Based on studies of PrtB and PrtC in *E. coli*, it has been shown that these proteases accumulate as zymogens within the *E. coli* cells, which are two kDa larger than the mature enzymes purified in *E. chrysanthemi*. Thus, the proteases need to be expressed in their native host for removal of the propeptide.

## 1.11 Potential secretion and anchoring of SapA

SapA negative strains with point mutations in its active site have been generated that eliminate the negative effects of SapA on RsaA fusion protein surface display. However, little is understood about how SapA is secreted and anchored to the cell surface in order to target some recombinant proteins. It was previously published that SapA is an intracellular protease because it lacks C-terminal sequence homology to RsaA C-terminus (93). However, the C-terminal signal of type I secretion varies widely (10). Further, there exist type I secretion systems that are able to transport more than one type of protein. For instance, the *S. marcescens* lipase (LipA) secretion system, which consists of LipB, LipC and LipD, is also able to secrete the *S. marcescens* S-layer protein and the metalloprotease PrtSm (24, 43).

One difficulty with defining the C-terminal secretion signal of proteins secreted by type I systems is their lack of a high degree of primary sequence homology (9). For example, the C-terminus of AprA protease in *P. aeruginosa* shows even less sequence homology to the RsaA C-terminus but it is still secreted by the RsaA type I transporter (32).

The C-terminus of SapA shows sequence homology to the N-terminus of RsaA that anchors to SLPS (e value = 2e-19), thus SapA may similarly anchor to SLPS. Alternatively, SapA may anchor to RsaA monomers via subunit-subunit interactions in the same way that individual RsaA monomers attach to one another to form a hexagonal array (32). Examining SapA anchoring in RsaA and SLPS negative strains can address this question. Interestingly, SapA possesses RTX motifs between amino acids 347 to 389 that bind $Ca^{2+}$ and there is little to no intracellular $Ca^{2+}$ present in *C. crescentus* (93).

Since $Ca^{2+}$ is required for S-layer formation, this may explain the presence of RTX motifs on SapA. Nevertheless, the process of secretion and anchoring in Gram-negative bacteria is not well characterized and SapA provides an opportunity to examine both in a single protein.

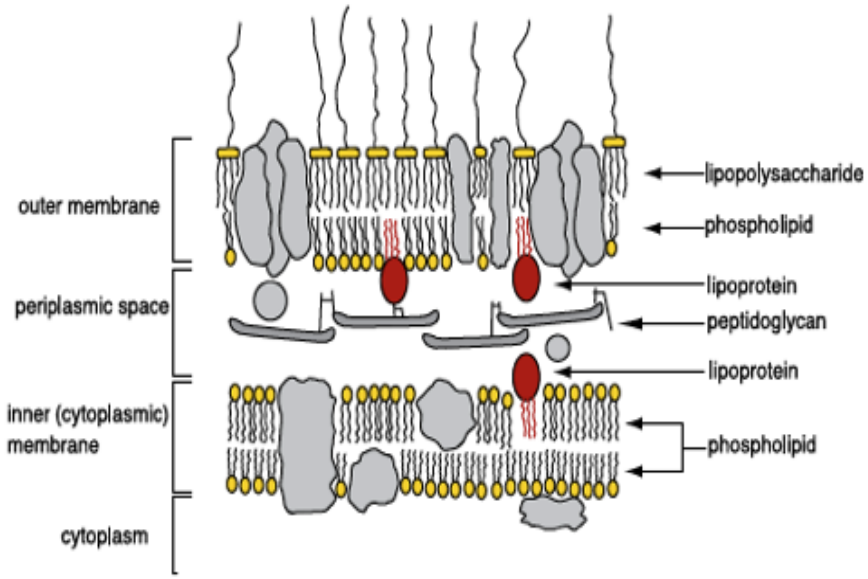



Figure 1-6. Gram-negative *E. coli* cell envelope structure. Lipoproteins (in red) are typically anchored to the periplasmic side of the inner or outer membrane (figure from Tokuda and Matsuyama, 2004).

## 1.12 Sorting and anchoring of lipoproteins

A second possibility of SapA secretion and anchoring is that it is secreted to the cell surface as a lipoprotein (Figure 1-6). One of the most well studied lipoproteins is Braun's lipoprotein (BLP or Murein lipoprotein), which is 7.2 kDa in size (40, 76). Braun's lipoprotein is an abundant membrane protein present in Gram-negative bacteria. This particular lipoprotein is embedded in the inner leaflet of the outer membrane using its N-terminal cysteine. A lysine on its C-terminal end is covalently attached to the diaminopimelic acid moieties of the peptidoglycan layer. The role of Braun's lipoprotein

is to link the outer membrane and peptidoglycan layer tightly and to provide structural integrity to the outer membrane (76).

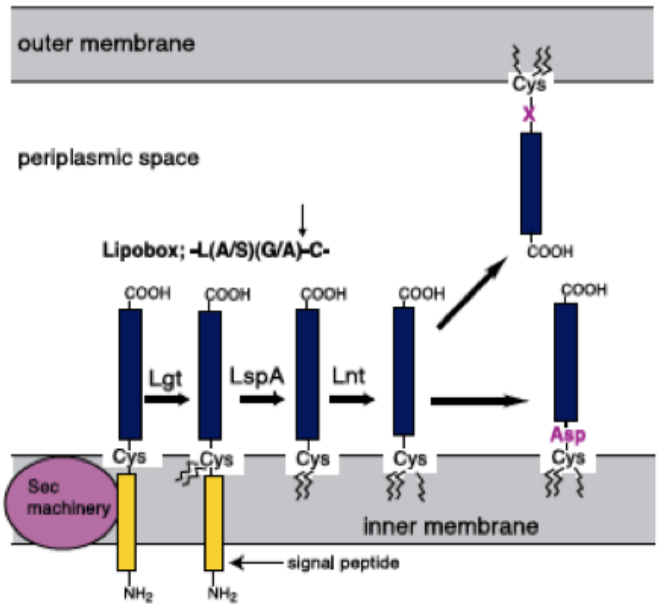



Figure 1-7. Biogenesis of lipoproteins (figure from Tokuda and Matsuyama, 2004).

Bacterial lipoproteins are attached to the membrane via an N-terminal *N*-acyl-diacylglyceride-cysteine (glycerylcysteine containing two ester-linked fatty acids and one amide-linked fatty acid) (40, 17). These proteins are translocated across the inner membrane via the Sec-dependent pathway after which lipidation and folding takes place in the periplasm (17). Protein secreted by a Sec-dependent secretion pathway contains an N-terminal secretion signal. In the case of lipoproteins, the C-terminal region of the signal sequence contains a consensus sequence typically Leu-Ala(Ser)-Gly(Ala)-Cys (Figure 1-7) (40). Before cleavage of the signal sequence or lipobox within the periplasm, an enzyme Lgt transfers a diacylglycerol group from phosphatidylglycerol to the sulfhydryl group of the cysteine that is always present at the +1 position of the protein relative to the processing site (17, 90). At that point, the diacylglycerylprolipoprotein is

processed by a dedicated signal peptidase, signal peptidase II or LspA, after which the amino group on the cysteine is acylated by the enzyme Lnt (phospholipid/apolipoprotein transacylase) yielding the mature lipoprotein (17, 90).

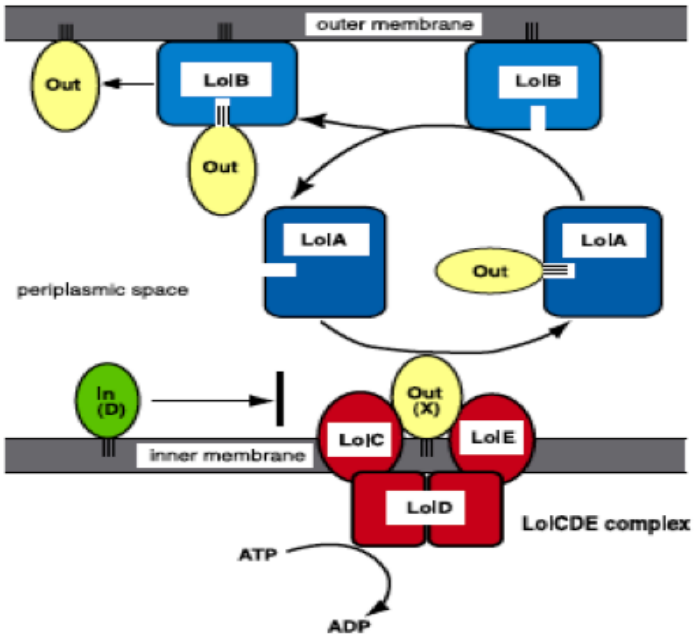



Figure 1-8. Sorting and outer membrane localization of lipoproteins by the Lol system (figure from Tokuda and Matsuyama, 2004).

In order to determine whether lipoproteins are sorted to the IM or the OM, one must examine the amino acids flanking the lipidated cysteine in the mature protein. Lipoproteins that do not possess an IM retention signal, typically an aspartate at the +2 position, are transported to the OM by the Lol system (Figure 1-8) (17). Serine is an example of a common amino acid at position +2 that targets the mature lipoprotein to the OM (90). Lipoproteins destined to the OM, are initially bound to the ABC transporter LolCDE in the inner membrane and are then passed onto a periplasmic protein LolA that interacts with an outer membrane receptor LolB. Once transferred to LolB, the

lipoprotein inserts into the OM where further transport to the outer leaflet occurs by unknown mechanisms (17, 90).

Evidence that SapA may be a lipoprotein is the amino acid composition of the protease at its extreme N-terminus. The second residue in SapA is a cysteine (Cys), a criterion for most lipoproteins in Gram-negative bacteria. Such proteins can be anchored to the OM or IM through the lipid moiety attached to the N-terminal Cys. The third residue in SapA is a serine (Ser); when the residue at this position is anything other than aspartate (Asp), the lipoprotein gets anchored to the outer membrane. Further, bioinformatics data indicates that the *C. crescentus* (CB15) genome contains a homolog of LolD, the ATP-binding protein, and LolC/E, the transmembrane proteins, as part of a lipoprotein releasing system at between coordinates 2129568-2128288. Typically this system is found in Sec-dependent systems such as type II secretion system; however, SapA could be the first known example of a lipoprotein secreted extracellularly by a Sec-independent pathway.

### 1.13 Thesis objectives

I propose three different hypotheses for SapA anchoring in *C. crescentus*, and one hypothesis for SapA secretion. I also hypothesize that SapA is a self-processing enzyme and requires its active site for cleavage. To test these hypotheses, I will employ genetic analysis along with direct and functional assays. In this project, I will use immunofluoresence and infrared westerns for direct detection of SapA, use mutagenesis for detection of functional domains.

For my first anchoring hypothesis, I predict that SapA uses its C-terminus to anchor to the SLPS in the outer membrane in a similar fashion as RsaA. This hypothesis

was developed by observing the sequence similarity between SapA (amino acids 451-650) and the RsaA N-terminus (amino acids 23-242), with an e-value = 2e-19 (% ID = 33), as well as previous findings regarding the involvement of the N-terminus of RsaA in mediating S-layer anchoring to the cell surface of *C. crescentus* (32). Thus I predict that strains negative for SLPS will test negative for SapA surface anchoring. I will also make various sized C-terminal clones of *sapA* to determine whether SapA uses its C-terminus for anchoring. The protein produced by these clones is expected to no longer attach to *C. crescentus* in an SLPS negative strain.

In my second hypothesis, I propose an alternative mechanism for SapA anchoring. Here I predict that SapA is lipid-linked to the outer membrane of *C. crescentus*. To test this hypothesis I will attempt to demonstrate that replacing the first cysteine residue with an alanine in SapA by site-directed mutagenesis (PCR) will disrupt anchoring on the cell surface.

For my third anchoring hypothesis, SapA will be tested for anchoring to RsaFa/RsaFb, the outer membrane components of the S-layer type I secretion system, using a SapA reattachment assay. Reattachment of SapA will be examined via immunofluorescence and infrared western analysis.

For my secretion hypothesis, I predict that SapA is secreted in *C. crescentus* by the S-layer type I secretion system. It will be determined whether SapA possesses a secretion signal in its C-terminus. In order to determine if SapA uses its C-terminus for secretion by a type I secretion system, a clone with a deletion of the last 10 and 50 amino acids of SapA (*sapA*Δ10C/*sapA*Δ50C) will be tested to see if secretion is prevented. It will also be determined if a plasmid-based clone of SapA can be secreted in a strain null

for RsaFa and RsaFb, the OM components of the S-layer type I secretion system. Finally it will be determined if the last 100 and 208 amino acids of SapA are sufficient for secretion. The 100 amino acid segment will also contain a C-terminal his6 tag, while the 208 amino acid segment will contain an N-terminal c-myc tag, to determine if the type I system can accommodate insertions of 6-10 amino acids attached to SapA's C-terminus. It will also be determined whether *C. crescentus* can secrete and anchor an N-terminal protein G (MGMGMGM) peptide fused to the last 238 amino acids of SapA.

Finally, it will be determined if SapA is a self-processing enzyme or that this protease is able to cleave another nearby SapA protease at a poorly recognized site. I will examine the processing of SapAΔP6, which contains a point mutation at amino acid 188 beside the protease's active site from a valine to a lysine.

# 2 MATERIALS AND METHODS

## 2.1 Bacterial strains, plasmids, and growth conditions

All of the strains and plasmids used in this study are listed in Tables 2-1 and 2-2, respectively. *E. coli* DH5α was used for most *E. coli* cloning manipulations and was grown at 37°C in Luria broth (1% tryptone, 0.5% NaCl, 0.5% yeast extract) with 1.3% agar for plates. *C. crescentus* strains were grown at 30°C in PYE medium (0.2% peptone, 0.1% yeast extract, 0.01% $CaCl_2$, 0.02% $MgSO_4$) with 1.2% agar for plates. Ampicillin (Amp), kanamycin (Km), and streptomycin (Sm) were used at 50 ug/ml, and chloramphenicol (Cm) was used at 20 ug/ml for *E. coli* and 2 ug/ml for *C. crescentus.*

## 2.2 Plasmid and DNA manipulations

Standard methods of DNA manipulation were used (73). Isolation of plasmid DNA was performed using the Qiaprep spin mini prep system (Qiagen) eluting in buffer or water. Restriction enzyme digestions were performed with Invitrogen or New England Biolabs Inc. enzymes by eletrophoresis on 0.9% TBE agarose gels with 0.5 ul/ml EtBr running at 120V. DNA fragments were isolated and excised from gels to be purified using the Qiaex II gel extraction kit (Qiagen) following the manufacturer's protocol. Ligations were done with T4 DNA ligase from Invitrogen according to the manufacturer's protocol. Electroporation of *C. crescentus* was performed as previously described (37). PCR products were generated using Platinum *Pfx* DNA polymerase (Invitrogen), Taq DNA polymerase, or Phusion polymerase (New England Biolabs Inc.). PCR primers used in this study are listed in Table 2-3.

**Table 2-1. Bacterial strains used**

| Strain | Relevant characteristic(s) | Reference or source |
|---|---|---|
| ***C. crescentus* strains** | | |
| CB15A aka NA1000 | Ap$^r$ *syn*-1000; variant of wild-type strain CB15 | 31 |
| CB15CA5BAC (JS1019) | Calcium independent strain, S-layer shedding strain with BAC replication genes | J. Lau, manuscript in preparation |
| CB15ACA5BAC353øß (JS1024) | CB15CA5BAC RsaA- strain | J. Lau, unpublished work |
| CB15AΔsap-RBAC353øß (JS1023) | CB15ABAC RsaA-, SapAΔR- strain | J. Nomellini, unpublished work |
| CB15AΔsap-RBAC353øßΔ471 (JS1025) | CB15ABAC RsaA-, SapAΔR-, exopolysaccharide negative (EPS-) strain | J. Nomellini, unpublished work |
| CB2AB5 (JS4011) | Spontaneous RsaA- mutant strain of CB2 | 62 |
| CB2AB5BAC (JS4019) | CB2AB5 with BAC replication genes. | 94 |
| CB2AΔP6 (JS4015) | SapAΔP6, UV-nitrosoguanidine-induced point mutation | 32 |
| CB15ΔrsaA | CB15 with *rsaA* gene knocked out deleting *rsaA* promoter and portion of the *rsaA* gene | 91 |
| CB15ΔrsaABAC (JS2003) | CB15ΔrsaA with BAC replication genes | J. Nomellini, manuscript in preparation |
| CB15ΔrsaAΔ973FaΔ1984FbBAC (JS2007) | CB15ΔrsaABAC with *rsaFa* and *rsaFb* internal deletions | J. Lau, manuscript in preparation |
| CB15ΔrsaAΔsap(1-658) (JS2008) | CB15ΔrsaA with complete *sapA* knockout | This study |
| CB15ΔrsaAΔsap(1-658)BAC (JS2009) | CB15ΔrsaABAC with complete *sapA* knockout | This study |
| CB15ΔrsaAΔsap(1-658)BAC pk*mobsacB*ManBΔNΔC (JS2011) | CB15ΔrsaAΔsap(1-658)BAC with *manB* internal deletion;Km$^r$ | This study |
| CB15ΔrsaABAC pk*mobsacB*ManBΔNΔC (JS2012) | CB15ΔrsaABAC with *manB* internal deletion;Km$^r$ | This study |
| CB15ΔrsaAΔ973FaΔ1984FbBAC pk*mobsacB*ManBΔNΔC (JS2013) | CB15ΔrsaAΔ973FaΔ1984FbBAC with *manB* internal deletion;Km$^r$ | This study |

| Strain | Relevant characteristic(s) | Reference or source |
|---|---|---|
| DH5α | λ-φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*($r_k$- $m_k^-$) *supE44 thi-1 gyrA relA1 dam-3 dcm-6 metB1 galK2 galT22 his-4 thi-1 tonA31 tsx-78 mtl-1 supE44* | Invitrogen |
| Top10 | F-*mcr*A Δ(*mrr-has*RMS-*mcr*BC)φ80*lacZ*Δ*M15*Δ*lacX74 rec*A1 *ara*D139Δ(*araleu*)7696 *gal*U *gal*J *rps*L (StrR) *end*A1 *nup*G | Invitrogen |
| BL21DE3 pET21b(+)-Sap | BL21DE3 with plasmid containing *sapA* | John Nomellini, this study |

**Table 2-2. Plasmids used**

| Plasmid | Relevant characteristic(s)[a] | Reference or source |
|---|---|---|
| pk18*mobsacB* | *oriT sacB*; *E. coli*-based suicide vector; $Km^r$ | Schafer 1994 |
| pK18*mobsacB*:*sap*FU/FD | pk*mobsacB* with upstream (FU) and downstream (FD) flanking regions of *sapA*;$Km^r$ | J. Nomellini, this study |
| pK18*mobsacB*:*xylx*X2BACΔES | pk*mobsacB* with BAC genes cloned into the middle of the *xly*X gene;$Km^r$ | 32 |
| pK18*mobsacB*:*manB*ΔNΔC | pk*mobsacB* with upstream and downstream regions of *manB* missing;$Km^r$ | 32 |
| pwB9 | pKT215-derived expression vector incorporating the *rsaA* promoter; $Cm^r$, $Sm^r$ | 11 |
| pwB9M13(450)PE3Δ | pwB9:*rsaA*ΔP with pilin insert at aa 450; $Cm^r$ | 10 |
| pBSKII | ColE1 cloning vector, lacZ; $Amp^r$ | Stratagene |
| pBSKIIEEH | Modified pBKSII cloning vector with modified MSC: EcoRI-EcoRV-HindIII restriction sites;$Amp^r$ | 92 |
| pUC8 N cvx0.690ϕP (pn336C) | Vector with NdeI and HindIII restriction sites containing last 336 aa of RsaA; $Cm^r$ | J. Nomellini, unpublished work |
| pn*sap* | *sapA*-over-expression plasmid with a *lac* promoter; $Cm^r$ | This study |

| Plasmid | Relevant characteristic(s)[a] | Reference or source |
|---|---|---|
| pn*sap*his6N | pn*sap* with an N-terminal his6 tag; $Cm^r$ | This study |
| pn*sap*Δ10C | *sap*Δ10C-over-expression plasmid; $Cm^r$ | This study |
| pn*sap*Δ50C | *sap*Δ50C-over-expression plasmid; $Cm^r$ | This study |
| pnC1A*sap* | C1A*sap*-over-expression plasmid; $Cm^r$ | This study |
| pn*sap*100C-his6C | *sap*100C-over-expression plasmid with an C-terminal his6 tag; $Cm^r$ | This study |
| pn*sap*188C | *sap*188C-over-expression plasmid ; $Cm^r$ | This study |
| pn*sap*208C-cmyc | *sap*208C-over-expression plasmid with an N-terminal cmyc tag; $Cm^r$ | This study |
| pn*sap*238C | *sap*238C-over-expression plasmid; $Cm^r$ | This study |
| pn*sap*268C | *sap*268C-over-expression plasmid; $Cm^r$ | This study |
| p4A | pUC8-type vector containing *oriV* with a modified *rsaA* promoter region; $Cm^r$ | 60 |
| p4B | Derivative of p4A with a modified *rsaA* promoter region followed by an EcoRI site; $Cm^r$ | J. Lau, manuscript in preparation |
| p4A723ΔMGMGMGM | p4A containing *rsaA*ΔP with a MGMGMGM inserted at BamHI linker site corresponding to aa 723 of RsaA. MGMGMGM is a 242 aa peptide of 3 protein G domains (GB1) with Muc1 antigen spacers; $Cm^r$ | 60 |
| pNMGMGMGM*sap*238C | *sap*238C-over-expression plasmid with N-terminal insert of MGMGMGM; $Cm^r$ | This study |

[a] aa, amino acid.

**Table 2-3. List of primers used**

| Primer name | Sequence |
|---|---|
| FUSapf | 5'-GCC TGG GAC CTG CAG CAC AAA CGC GC-3' |
| FDSapr | 5'-AGC GTC GCT CAT TCG GCG TCC TGA AC-3' |
| TN5 Kan R F | 5'-GTG GAG AGG CTA TTC GGC TAT GAC TG-3' |
| TN5 Kan Rv | 5'-CTT CAG CAA TAT CAC GGG TAG CCA AC-3' |
| JNBAC-1 | 5-GAC AGG GGC GGC ATG GGT GGA GCT GGC-3' |
| JNBAC-2 | 5'-CCG GGC AAT CTG CCC CCG AAG TTC ACC-3' |
| pN336Cf | 5'-GGC TTT ACA CTT TAT GCT TCC GGC-3' |
| pN336Cr | 5'-GTA CGG GAG TGA CGG GCA CTG-3' |
| Sap-n-f | 5'-CAT ATG TGT AGT CAG TGC GAG CGG TAT G-3' |
| Sap-his6-N-f | 5'-CCC CAT ATG CAC CAC CAC CAT CAC CAT TGT AGT CAG TGC GAG CGG TAT GGA CTG AAC CTC-3' |
| Sap-h-r-2 | 5'-CCC AAG CTT TCA GAT GAG GTT GTA TTC CGG CTT GGC-3' |
| Sap-his6-H-r2 | 5'-CCC AAG CTT TCA GTG GTG GTG GTG GTG GAT GAG GTT GTA TTC CGG CTT GGC GTA GAC-3' |
| Fs-H-648-r | 5'-CCC AAG CTT TCA GCC GAT CAG ATC CAC GCC ATA GAC ATT CTT-3' |
| Sap-h-50-r2 | 5'-CCC AAG CTT TCA CAT GGC CGC CTT GGT TCC CTG GCC-3' |
| Sap-n-ala-f | 5'-CAT ATG GCT AGT CAG TGC GAG CGG TAT-3' |
| Sap188c-N-f | 5'-CCC CAT ATG AGC CAT AGC GAC GCC ATC GGC CAG GTG-3' |
| Sap-208c-cmyc-N-f | 5'-CGC CAT ATG GAG CAG AAG CTG ATC TCG GAA GAG GAC CTC TTC AGC GCC TCG GCC GAA CCG CTG TCC-3' |
| Sap-238c-N-f | 5'-CCC CAT ATG ATC GAG TTC CTG GCC TTT ACC GAT CGG-3' |
| Sap-268c-N-f | 5'-CGC CAT ATG TAC GGC AAC TAC ACC CTG ACC GCC GCC-3' |
| MG-NPNh-r | 5'-CCG CAT ATG GCC TGC AGC GCT AGC GGT GCT-3' |
| MG-NBS-f | 5'-GCC CAT ATG AGA TCT ACT AGT CCG CCC GCC-3' |

## 2.3 *Caulobacter crescentus* expression vectors

pUC8 N cvx0.690ϕP (pn336C)

This 3955 bp construct was made by Dr. John Nomellini (University of British Columbia). N=*Nde*I (the plasmid has an altered MCS which starts with an *Nde*I site). ϕP=*Pst*I to *Pst*I on the plasmid backbone has been deleted. This is a continuation of the pUC 8 CVX vector (60), which has had the first 7 aa of LacZ (including the translation

start ATG) replaced with an *Nde*I site which bears an internal translation start site (CAT ATG). It was generated using the oligonucleotide primers 8NDE1 5'-G GAA TTC *CAT ATG* TTC GCC TGT AAA ACC GCC AAT GGT ACC-3' and 8NDE2 5'-G GAA TTC *CAT ATG* TGT TTC CTG TGT GAA ATT GTT ATC CGC-3' (*Nde*I site underlined) in an inverse PCR reaction. For template DNA a pUC8 vector containing a 495 bp *E. coli* FimH gene was used from another project. The resulting PCR product was then digested with *Nde*I and self-ligated to produce pUC8-N FimH N14 T3. The FimH gene was removed by digesting with *Nde*I and *Hind*III and was replaced with two annealed oligos that resulted in a small multiple cloning site containing an *Nde*I, *EcoR*I, *Sma*I, *BamH*I and *Hind*III site. The oligos used for this were JN ESBH-1 5' T ATG ACG AAT TCC CGG GGA TCC CCA 3' and JN ESBH-2 5' A GCT TGG CCA TCC CCG GGA ATT CGT CA 3'. The chloramphenical and oriV backbone of the plasmid came from digesting pUC 8 CVX with *Sap*I and *Hind*III (the *Sap*I is upstream of the promoter and this is most of the vector) and replacing the *Sap*I – *Hind*III of pUC 8 N (J. Nomellini, unpublished work). The clone of *sapA* and its recombinant counter parts that contain an *Nde*I upstream of the gene (contains built in ATG start codon) and a *Hind*III after the stop codon, can then be placed into pUC8 N cvx0.690ϕP digested with *Nde*I and *Hind*III.

### 2.4 Construction of plasmid used for gene knockout of *sapA*

pK18*mobsacB*:*sap*FU/FD

This construct was made by Dr. John Nomellini (University of British Columbia) to make a complete knock out of *sapA* in *C. crescentus*. The 1111 bp region upstream of *sapA* (FU) was PCR amplified using primers FU-f (5'-GGC GTG GGA GTC GGC TCG AGC GGC GGA-3') and FU-KB-r (5'-CGG *GGT ACC* CCG **GGA TCC** CGA CGC

GCT CCA CTC ACC TGA AAG GAG TAT-3) from wild type *C. crescentus* which would add a *Bam*HI site (shown in bold) and a *Kpn*I site (shown in italics) at the end of the PCR product. This PCR product was cloned into pBSKIIEEH digested with *Eco*RV creating pBSKIIEEH:FU. The 1032 bp region downstream of *sapA* (FD) was similarly PCR amplified using primers FD-B-f (5'-CGC **GGA TCC** CCG TTC GAA GGG CGC GGC GAC AAA GGT-3') and FD-K-r (5'-CCG *GGT ACC* AGT TGC CCA GGG GGT TCA TGG TCC AGG-3') from wild type *C. crescentus* which would add a *Bam*HI site (shown in bold) at the front of the PCR product and add a *Kpn*I site (shown in italics) at the end of the PCR product. This PCR product was cloned into pBSKIIEEH digested with *Eco*RV creating pBSKIIEEH:FD. Both pBSKIIEEH:FU and pBKSIIEEH:FD were digested with *Bam*HI and *Kpn*I. The released FD product was then ligated into the back end of pBSKIIEEH:FU using these sites creating pBKSIIEEH:FU/FD. The new pBKSIIEEH:FU/FD was digested with *EcoR*I/*Hind*III releasing FU/FD and ligated into pk18*mobsabB* digested with *EcoR*I/*Hind*III creating pk18*mobsabB*:*sap*FU/FD.

### 2.5 Construction of plasmid used for gene disruption of *manB*

pk18*mobsacB*:*manB*ΔNΔC

The *manB* gene which is required for phosphomannomutase production can be disrupted by pk18*mobsacB*:*manB*ΔNΔC however, $Km^r$ is needed for the plasmid to be maintained in *C. crescentus* (32). This plasmid was constructed by Matt Ford (University of British Columbia) to make an internal deletion in the *manB* gene required for mannose production. Knocking out this gene affects the synthesis of perosamine required for the O-antigen on SLPS, the production of L-fucose required to make exopolysaccharide, the production D-rhamnose that is a component of LPS and EPS in Gram-negative bacteria

and, the production of 6-deoxy-D-talose, a rare deoxyhexose that is a constituent of cell wall and capsule structures. A PCR product of *manB* with its N and C termini was made using NA1000 chromosomal DNA with primers ManB 169 (5'-CCT GGG TCT GGG AAC CTA TAT CC-3') and IManB 1202 (5'-CAG TGC GGG CTC ATG GTC AG-3'). The *manB*ΔNΔC PCR product was then blunt-end ligated into pBSKIIEEH digested with *EcoRV*. The new pBSKIIEEH:ManBΔNΔC was then cut with *Eco*RI/*Hind*III to release the PCR product and ligated into pK18mobsacB cut with *Eco*RI/*Hind*III creating pK18*mobsacB*:*manB*ΔNΔC.

### 2.6 Construction of plasmid used to introduce BAC genes

pK18*mobsacB*:*xylx*X2BACΔES

This construct was made by Louis Lam (University of British Columbia) to insert the BAC replication genes into *C. crescentus* at the *xly*X gene that is needed for xylose utilization. Within the *xly*X there are unique *Pst*I and *Eco*RI sites. The *xly*X region with 1kb flanking sequence at each end were amplified using the primers LLxyX2F (5'-ACG ACG TCG TTG GTG TTG GAC GGG-3') and LLxlyX2R (5'-**GCG GAT CCG** GCA TTC GCC GGG GAG GTC GG-3') from wild type *C. crescentus* which would add a *Bam*HI site (shown in bold) at the end of the PCR product. The PCR product was amplified using Taq polymerase (NEB) and was cloned into pTOPO using the TA cloning method (Invitrogen). The PCR product was then excised as a *Hind*III and *Bam*HI fragment and closed into the same site in the MCS of pBSKII to make pBKSII:*xyl*X2. To isolate the BACΔES replication genes from pKT215, which also has the OriV genes, the plasmid was linearized with *Pst*I and fused to pBSKII and selected on LB/Amp/Sm plates. This fusion plasmid was then cut with *Eco*0109 and re-ligated to excise out the

2745 bp of pKT215 that held the OriV, while leaving the 5023 bp of the BAC genes in pBSKII creating pBSKII:BAC. The BAC genes from pBSKII:BAC were then ligated into pUC18 as a *Kpn*I and *Pst*I fragment to add *Hind*III and *Eco*RI flanking sites to the BAC genes. The BAC genes were then cut from pUC18 first with *Hind*III, which was filled with Klenow followed by a digest with *Eco*RI. This resulted in the BAC genes cloned into the middle of the *xly*X gene while also removing a small portion of the *xly*X. The *xly*X2 BAC fusion segment was then ligated into pK18*mobsacB* as a *Hind*III and *Bam*HI fragment.

### 2.7 Construction of plasmids for *sapA* and *sapA* variants for over-expression

pn*sap*

PCR product of *sapA*, with restriction sites *Nde*I at the start and *Hind*III at the end of the gene, was produced using from DH5α pET21b(+)-Sap, an *E. coli* strain containing *sapA* as a template. The *sapA* gene was amplified using primers Sap-n-f (5'-**CAT ATG** TGT AGT CAG TGC GAG CGG TAT G-3') and Sap-h-r-2 (5'-CCC *AAG CTT* TCA GAT GAG GTT GTA TTC CGG CTT GGC-3') which would add a *Nde*I site (shown in bold) at the front of the PCR product and add a *Hind*III site (shown in italics) at the end of the PCR product. This product was ligated into pBSKIIEEH cleaved by *Eco*RV, electroporated into DH5α cells and plated on LB/Amp plates with X-gal. The plates were screened for white colonies, which were grown up 5 ml LB/Amp. Plasmid isolation was performed using GeneJet Plasmid Mini-Prep kit from Fermentas. The plasmid with the correct insert and pn336C were cleaved by *Hind*III and *Nde*I and run on agarose gel along with their respective controls; the expected size for the *sapA* insert is 1986 bp and the expected size of the pn336C plasmid is ~2500 bp. The *sapA* insert and pn336C insert

were ligated, electroporated into Top10 cells and plated on LB/Cm plates. Cm resistant colonies were tested for the *sapA* insert by PCR. The plasmid can now be expressed in *C. crescentus*.

pn*sap*Δ10C and pn*sap*Δ50C

In order to determine if SapA used its C-terminus for secretion by a type I secretion system in *C. crescentus*, two clones (pn*sap*Δ10C and pn*sap*Δ50C) were made with a deletion in last 10 amino acids of SapA. These clones were made the same way as pn*sap* except the reverse primer created annealed either 30 or 50 bases upstream of the end of the gene to remove the last 10 or 50 amino acids and pn*sap* was used as template DNA. The reverse primer used for SapAΔ10C was Fs-H-648-r (5'-CCC *AAG CTT* TCA GCC GAT CAG ATC CAC GCC ATA GAC ATT CTT-3') with a *Hind*III site (shown in italics) at the end of the PCR product and the reverse primer used for SapAΔ50C was Sap-h-50-r2 (5'-CCC *AAG CTT* TCA CAT GGC CGC CTT GGT TCC CTG GCC-3') with a *Hind*III site (shown in italics) at the end of the PCR product.

pnC1A*sap*

To test the hypothesis that SapA is lipid-linked to the outer membrane of *C. crescentus* and demonstrate that replacing the first cysteine (Cys) residue in SapA would disrupt anchoring, PCR was used for site-directed mutagenesis in order to change the first amino acid in Sap from Cys to Ala (alanine). This clone was made the same way as pn*sap* except the forward primer encoded an alanine as the first amino acid instead of a cysteine and pn*sap* was used as template DNA. The forward primer used was Sap-n-ala-f (5'-*CAT ATG* GCT AGT CAG TGC GAG CGG TAT-3') with an *NdeI* site (shown in italics) at the end of the PCR product.

pnsapΔP6

This clone was made in order to test whether SapA was able to self-process itself in *C. crescentus*. This clone was made in the same way as pn*sap* except I did PCR on a *sapA* mutant, *sap*ΔP6 from JS4015 that possesses a lysine instead of a valine at amino acid 188 next to the active site. This mutation results in an almost complete loss of proteolytic activity of SapA against recombinant S-layer proteins.

pn*sap*his6N

In order to purify SapA by a Ni-NTA column, an N-terminal his6 tag was added. This clone was made the same way as pn*sap* except the forward primer encoded six histidines right after the start codon and pn*sap* was used as template DNA. The forward primer used was Sap-his6-N-f (5'-CCC *CAT ATG* CAC CAC CAC CAT CAC CAT TGT AGT CAG TGC GAG CGG TAT GGA CTG AAC CTC-3') with an *NdeI* site (shown in italics) at the end of the PCR product.

pn*sap*100C-his6C

This clone was made in order to test whether SapA required its last 100 amino acids for secretion and anchoring in *C. crescentus*, to determine if a histidine tag inserted at the extreme C-terminus inhibited secretion, and to try to purify the C-terminal clone by a Ni-NTA column. This clone was made the same way as pn*sap* except the reverse primer encoded six histidines right before the stop codon, the forward primer annealed at amino acid 558 and, pn*sap* was used as template DNA. The reverse primer used was Sap-his6-H-r2 (5'-CCC **AAG CTT** TCA GTG GTG GTG GTG GTG GAT GAG GTT GTA TTC CGG CTT GGC GTA GAC-3') with a *Hind*III site (shown in bold) at the end of the PCR product. The forward primer used was FS-B-100c-f (5'-CGC *GGA TCC* AGC CTG

TTT GAC GCC ACG CGC AAG GCC-3') with a *Bam*HI site (shown in italics) at the start of the PCR product.

pn*sap*208C-cmyc

This clone was made in order to test whether SapA required its last 208 amino acids for secretion and anchoring in *C. crescentus* and to test if SapA could tolerate a secretion and anchoring with a c-myc tag (N-EQKLISEEDL-C) inserted at the N-terminus of the C-terminal clone. This clone was made the same way as pn*sap* except the forward primer annealed at amino acid 450 and added an N-terminal c-myc tag and, pn*sap* was used as template DNA. The forward primer used was Sap-208c-cmyc-N-f (5'-CGC *CAT ATG* GAG CAG AAG CTG ATC TCG GAA GAG GAC CTC TTC AGC GCC TCG GCC GAA CCG CTG TCC-3') with an *NdeI* site (shown in italics) at the end of the PCR product.

pn*sap*188C, pn*sap*238C, and pn*sap*268C

These clones were made in order to test whether SapA required its C-terminus for secretion and anchoring in *C. crescentus*. These clones were made the same way as pn*sap* except the forward primer annealed at either amino acid 470 (SapA188C), amino acid 420 (SapA238C) or amino acid 390 (SapA268C) and, pn*sap* was used as template DNA. The forward primer used for SapA188C was Sap188c-N-f (5'-CCC *CAT ATG* AGC CAT AGC GAC GCC ATC GGC CAG GTG-3') with an *NdeI* site (shown in italics) at the end of the PCR product. The forward primer used for SapA 238C was Sap-238c-N-f (5'-CCC CAT ATG ATC GAG TTC CTG GCC TTT ACC GAT CGG-3') with an *NdeI* site (shown in italics) at the end of the PCR product. The forward primer used for SapA268C

was Sap-268c-N-f (5'-CGC CAT ATG TAC GGC AAC TAC ACC CTG ACC GCC GCC-3') with an *NdeI* site (shown in italics) at the end of the PCR product.

pNMGMGMGM*sap*238C

This clone was made to test whether the last 238 amino acids of SapA fused C-terminally to MGMGMGM, which is a 242 amino acid peptide of 3 protein G domains (GB1) with Muc1 antigen spacers in between, could still be secreted and anchored in *C. crescentus*. In order to make this clone, a PCR product of the MGMGMGM clone was made using primers MG-NBS-f (5'-GCC *CAT ATG* AGA TCT ACT AGT CCG CCC GCC-3') and MG-NPNh-r (5'-CCG *CAT ATG* GCC TGC AGC GCT AGC GGT GCT-3'), with an *Nde*I site (shown in italics) at the start and end of the PCR product. The PCR product was then ligated into pn*sap*238C digested with *Nde*I, electroporated into Top10 cells and plated on LB/Cm plates. Since the MGMGMGM clone could be inserted into *Nde*I digested pn*sap*238C in both directions, colonies were grown up and tested by PCR for the correct direction of insertion using primers MG-NBS-f and Sap-h-r-2. The plasmid in the proper direction can now be expressed in *C. crescentus.*

## 2.8 Construction of strains with gene disruptions

JS2008

The S-layer deficient *Caulobacter* strain used to knock out *sapA* was CB15Δ*rsaA*. Electrocompetent CB15Δ*rsaA* were made by standard methods, electroporated with pk*mobsacB* FU/FD and plated on PYE/Km plates. The 1$^{st}$ cross was confirmed on Km$^{r}$ colonies with PCR using the Sap FU/FD region specific oligos which anneal upstream and downstream of the *sapA* respectively: FUSapf (5'-GCC TGG GAC CTG CAG CAC AAA CGC GC-3') and FDSapr (5'-AGC GTC GCT CAT TCG GCG TCC TGA AC-3').

Integration of pk*mobsacB* FU/FD into CB15Δ*rsaA* can be determined by the production of two PCR products, named PCR product 1 (2148 bp) and PCR product 2 (514 bp). However, after many trials of PCR, PCR product 1 could not be produced. Thus strains with PCR product 2 were also tested for the presence of the TN5 $Km^r$ gene cassette present on pk*mobsacB* FU/FD using the following primers: TN5 Kan R F (5'-GTG GAG AGG CTA TTC GGC TAT GAC TG-3') and TN5 Kan Rv (5'-CTT CAG CAA TAT CAC GGG TAG CCA AC-3'). Strains with both $Km^r$ and PCR product 2 (CB15Δ*rsaA* pk*mobsacB* FU/FD) were grown in 10 ml PYE/Km. Four outgrowths in PYE were performed and dilutions of outgrowth #4 were plated on 3% sucrose PYE plates. There are two possible $Km^s$ strains; CB15Δ*rsaA* where pk*mobsacB* FU/FD recombined out to produce the original strain and, JS2008 where pk*mobsacB* FU/FD recombined out and removed the entire *sapA* gene. $Km^s$ colonies were screened for and grown in 5 ml PYE. PCR was performed to test for *sapA* knockout strains and compared to the original CB15Δ*rsaA* strain and strains with pk*mobsacB* FU/FD plasmid insert.

JS2011, JS2012 and JS2013

The strains used to disrupt the *manB* gene were JS2009, JS2003, and JS2007 and electroporated each with pk*mobsacB*:*manB*ΔNΔC and selected on PYE/Km plates. These cells are a little clumpy and spin down really well.

### 2.9 Construction of strains with BAC genes

JS2009

The plasmid pK18*mobsacB*:*xylx*X2BACΔES was electroporated into JS2008 cells and selected on PYE/Km plates. The 1st cross was confirmed on $Km^r$ colonies with PCR using the BAC specific oligos: JNBAC-1 (5'- GAC AGG GGC GGC ATG GGT GGA

GCT GGC –3') and JNBAC-2 (5'- CCG GGC AAT CTG CCC CCG AAG TTC ACC – 3'). The PCR positive, $Km^r$ colonies were made electrocompetent and pn336C (Cm) was electroporated in, after the 2 hour outgrowth 100 ul was plated on PYE/Cm to see if colonies would come up. A single colony was selected from PYE/Cm plate and did 4 subsequent outgrowths in PYE (10mls) and Cm at 2ug/ml. Dilutions were plated on PYE/Cm sucrose plates. Colonies were picked for replica plating on PYE/Km and PYE/Cm plates, to look for Km sensitive clones. Colonies that only grew on PYE/Cm were kept and 4X 10ml outgrowths in PYE alone were performed on them. Dilutions were plated on PYE plates. Colonies were picked for replica plating on PYE and PYE/Cm to look for Cm sensitive clones. Once again the clones were tested for the presence of the BAC genes by PCR.

### 2.10 SapA antibody production

Antibodies used to detect SapA were prepared by Dr. John Nomellini and Sadeem Fayed using *E. coli* strain BL21DE3 pET21b(+)-Sap, which produces SapA with an N-terminal his6 tag. SapAhis6N was purified using a Ni-NTA column under denaturing conditions. The protein was only produced in the form of inclusion bodies, thus the protein was extracted by inclusion body preparations. Samples were dialyzed (30,000 MW dialysis tubing) in $dH_2O$ to remove traces of urea. Samples were subsequently injected into a New Zealand white rabbit and rabbit serum was collected and processed using standard protocols (73).

## 2.11 Protein techniques

Low pH extraction

The S-layer of *C. crescentus* was extracted by low pH extraction as previously described using 100 mM HEPES pH 2 solution (96). Cells were grown to log phase and normalized to $OD_{600}$ for protein extraction. Equal amounts of extracted protein samples were loaded onto SDS PAGE gels for analysis.

Whole culture protein preparations

Equal volumes of cell culture grown to log phase and normalized by spectrometry at $OD_{600}$ were collected and lysozyme (300 ug/ml) was added to the cells and incubated for 30 minutes at 37°C. RNase A (60 ug/ml), DNase I (6 ug/ml) and $MgCl_2$ (3 ul of 1M solution) were added and incubated for 1 hour at 37°C. Powdered urea was added to a final concentration of 3M urea. Equal amounts of whole culture protein preparations were loaded onto protein gels.

Culture supernatant prepartions

Equal volumes of culture media (between 50 ml to 500 ml) from different strains of *C. crescentus* grown to log phase, normalized to the same $OD_{600}$ and centrifuged for 20 min at 13 000K. The supernatant was recovered and concentrated to 500 ul to 1 ml using Centricon Plus-20 centrifugal filter devices from Millipore. Supernatant was run on SDS-PAGE and equal amounts were loaded onto protein gels.

SDS-PAGE and Western blot analysis

SDS-PAGE using 5% stacking and 7.5%, 12% or 15% separating gels were run at 200 Volts. Coomassie staining of gels and western immunoblotting were done following standard methods (73). Protein gels were transferred onto 0.2 um BioTrace NT

nitrocellulose membranes (Pall Biosciences) and blocked by 3% skim milk, 0.9% NaCl, and 20 mM Tris-HCl pH 8. Western blots were probed with primary rabbit polyclonal antibodies and used at 1/30 000 (J Smit and J Nomellini, University of British Columbia). Infrared secondary antibody, AlexaFluor 680 goat anti-rabbit IgG (Invitrogen), used at 1/50,000 dilutions, detected and quantified by Odyssey 2.0 on the Licor Odyssey system.

Fractionation of Cell Membrane Proteins by Ultracentrifugation

Cell membrane proteins were concentrated beyond their maximum concentrations in the whole cell preparations by isolating the cell membranes with ultracentrifugation. Cell cultures grown up in 50 ml PYE were normalized to 1.0 $OD_{600}$ and centrifuged for 10 min at 8500 rpm. Cell pellets were washed once with 25 ml Tris HCl pH 7.5 buffer, and re-suspended in 10 ml Tris HCl pH 7.5 buffer. Cells were lysed by sonication with a medium probe for a total of 2 minutes (split into 4 x 30 second intervals). Unlysed cells were removed by centrifugation for 10 min at 8500 rpm and supernatants (lysed cells) were collected and transferred to 8 ml ultracentrifugation tubes. Lysed cells were ultracentrifuged at 40 000 rpm for 1 hour at 10°C and the supernatants containing cytoplasmic proteins were discarded. The pellets of ultracentrifugation containing the cell membranes were resuspended in Tris HCl pH 7.5 buffer with 1% Triton-X 100 or an alternate detergent by brief sonication with a microprobe, in a volume appropriate to the pellet size (less than 400 ul). This solution was allowed to sit on ice for at least 30 minutes to encourage solubilization of proteins by Triton-X 100. Resuspended pellet solutions were transferred to microfuge tubes and spun for 10 minutes at 13 000 rpm/4°C to separate the Triton-X 100 soluble and insoluble proteins. The Triton-X 100 soluble protein fractions (supernatants) were transferred to new microfuge tubes and the Triton-X

100 insoluble protein fractions (pellets) were washed once with 1 mL Tris HCl pH 7.5 buffer and 1% Triton-X 100 and then resuspended in Tris HCl pH 7.5 buffer in a volume similar to the pellet volume. At all steps, the samples were kept at less than 10°C, either on ice or refrigerated, to minimize protein degradation. Samples were stored at -80°C and loaded onto SDS-PAGE gels after boiling for 1 minute in at least 1X SDS-PAGE sample buffer (4XSDS-SB: 192 mM Tris pH 6.8, 3.8% SDS, 38.5% glycerol, 3.8% B-Mercaptoethanol, 0.25% Bromophenol Blue).

### 2.12 Test for loss of proteolytic activity of SapA in JS2008

Strains used for this study were NA1000, CB15Δ*rsaA*, and JS2008. NA1000 was a positive control for low pH extraction. CB15Δ*rsaA* and JS2008 were electroporated with pwB9M13(450)ΔPE3. Cm resistant colonies were grown up in 10 ml PYE/Cm and, NA1000 was grown up in 10 ml PYE at the same time. The next day, the $OD_{600}$ of the strains were be taken and then normalized to the lowest OD. Low pH extraction were performed on the samples, and run on 7.5% SDS PAGE for subsequent infrared western analysis using anti-rsaA 1° antibodies.

### 2.13 Proteolytic activity tests of concentrated supernatant fractions

Strains used in this study were CB15Δ*rsaA* pwB9M13(450)ΔPE3 and JS2008 pwB9M13(450)ΔPE3. The 1 ml aliquots of 10 ml JS2008 pwB9M13(450)ΔPE3 and 1 ml CB15Δ*rsaA* pwB9M13(450)ΔPE3 were transfered to microfuge tubes and the cells were spun down. The supernatant was removed and the cell pellets were washed in 1 ml PYE. Cells were spun again to remove the supernatant. Then 100 ul of the supernatant to be tested was added to a pellet of JS2008 pwB9M13(450)ΔPE3; nine different supernatants could be tested. Also, 1 ml 10 mM TrisHCl was added to a pellet of JS2008

pwB9M13(450)ΔPE3 and the CB15Δ*rsaA* pwB9M13(450)ΔPE3 pellet as negative and positive controls, respectively. All of the samples were incubated for 4 hr at 30°C on a rotary shaker. The cultures were pelleted for low pH extraction. The samples were run on 7.5% SDS PAGE gel and an infrared western was performed using polyclonal anti-188/784 RsaA antibodies.

### 2.14 Proteolytic activity tests of protein purified by low pH extraction

Strains used in this study were JS2009, JS2003 pn*sap* and JS2009 pn*sap*ΔP6. Cells were normalized to $OD_{600}$ = 1.0 in 10 ml and low pH extractions was performed on the cells a final volume of 100 ul. Then 5 ml JS2008 pwB9M13(450)ΔPE3 and 1ml CB15Δ*rsaA* pwB9M13(450)ΔPE3 were grown up and normalized to $OD_{600}$ = 1.0 in 1 ml. Four test tubes were aliquotted with 1 ml JS2008 pwB9M13(450)ΔPE3 and, the fifth test tube was aliquotted with 1 ml CB15Δ*rsaA* pwB9M13(450)ΔPE3. Then 100 ul of low pH'd JS2009 protein was added to test tube 1 (negative control), 100 ul of low pH'd JS2003 pn*sap* protein was added to test tube 2, 100 ul of low pH'd JS2009 pn*sap*ΔP6 protein was added to test tube 3, 100 ul of 10 mM TrisHCl was added to test tube 4 (negative control), and 100 ul of 10 mM TrisHCl was added to test tube 5 (positive control). The volume of each test tube was brought up to 300 ul for incubation on a rotary shaker for 4 hrs at 30°C. Low pH extraction was performed on the samples, which were run on 10% SDS PAGE for subsequent an infrared western analysis using polyclonal anti-188/784 RsaA antibodies.

### 2.15 SapA surface localization and detection by immunofluoresence

*C. crescentus* cells were grown up overnight and normalized to $OD_{600}$ = 0.8 in 50 ul. The supernatant was removed and the cells were resuspended in 150 ul cold PYE.

Then 1 ul of α-Sap 1° antibody was added and incubated on ice for 30 min. The mixture was washed with 1 ml cold PYE and resuspended in 150 ul cold PYE. Then 1.0 ul of goat anti-rabbit Alexa 448 2° antibody was added and incubated on ice for 30 min. The mixture was again washed with 1 ml cold PYE, the cells were resuspende in 5 ul mounting media, and 1 ul was used to visualize them under the microscope. Pictures of the cells were taken using a 2 second exposure on camera attached to microscope.

### 2.16 Reattachment assay of SapAΔP6 purified by low pH extraction

Strains used in this study were JS2009 pns*ap*ΔP6, JS2009, JS2011, JS2007, and JS2013. After two days of growth, the $OD_{600}$ of 80 ml JS2009 pn*sap*ΔP6 was checked and the culture was divided into 10 ml aliquots for low pH extraction with a final volume of 100 ul. At the same time, JS2009, JS2011, JS2007, and JS2013 were grown up and normalized to $OD_{600}$ = 1.2 in a final volume of 1.5 ml. The cultures were spun down to remove the supernatant. To each pellet, two aliquots of SapAΔP6 (total volume 200 ul) were added and incubated for 3-4 hr on a rotary shaker at 30°C. After incubation, low pH extraction was performed on the samples which were ran on 12% SDS PAGE and analyzed by infrared western using anti-sap 1° antibodies.

### 2.17 Purification of SapA for Micro BCA protein assay

DH5α pn*sap*his6N was purified by growing up 5 ml LB/Cm with Top10F' pn*sap*his6N overnight. In the morning, 0.5 ml of the culture was added to 4.5 ml LB/Cm and incubated for 1 hr at 37°C. Then 4.5 ul 1M IPTG was added and incubated for 2hr. The culture was checked for inclusion bodies under the microscope. Then the culture was spun down and, to each pellet 300 ul lysis buffer pH 8 (50 mM $NaH_2PO_4$, 300 mM NaCl, 0.5% Tween, 10 mM Imidazole, 1 mM PMSF) and 20 ul lysozyme (50 mg/ml) were

added. The mixture was incubated for 30 min at 37°C. Then 10 ul of DNase I (1 mg/ml), RNase A (10 mg/ml) and 1 M $MgCl_2$ were added and incubated for 1 hr at 37°C. Next 50 ul 10% SDS was added and boiled for 2 min. The mixture sat on ice for 10 min. Then the cells were sonicated and 50 ul 8M urea was added to sit for 10 min. The mixture was spun down for 15 min at 13 000 rpm to remove cell debris. To the supernatant 500 ul of 50% Ni-NTA agarose (Qiagen) was added and mixed rotary shaker for 1 hr. A column was made by adding cheesecloth to the end of a syringe to prevent beads from flowing out of the column. The lysate-NiNTA mixture was loaded into the column and the flow through was collected. The column was washed twice with 1 ml wash buffer pH 8 (50 mM $NaH_2PO_4$, 300 mM NaCl, 0.5% Tween, 20 mM Imidazole) and the washes were collected. Then the protein was eluted at least four times using 1 ml elution buffer pH 8.0 (50 mM $NaH_2PO_4$, 300 mM NaCl, 0.5% Tween, 250 mM Imidazole) and the elutions were collected. The elutions were combined and dialyzed overnight the sample in 8 L 10 mM TrisHCl pH 8 using a dialysis membrane (VWR Scientific Inc.) with a molecular weight cut off of 14,000. The sample containing the purified *E.coli* SapA was collected and a Micro BCA Protein Assay Kit (Pierce) was used to quantify SapA. The amount SapA was then equated by infrared quantification provided by Odyssey 2.0 on the Licor Odyssey system.

### 2.18 Far western for detection of SapA-binding proteins

Performed cell membrane preparations on 50 ml of JS2009 cells, separating the soluble and insoluble fractions after treatment with the following detergents: 2% Triton X-100/CHAPSO, 2% Triton X-100, 2% Zwittergent TM314, 2% *n*-octyl-β-D-glucoside, 2% sodium deoxycholate, 2% sodium-lauroyl sarcosinate, and 2% sodium dodecyl

sulfate. Ran samples on a 15% SDS PAGE gel and performed a western transfer onto a 0.2 um BioTrace NT nitrocellulose membrane. Blocked the membrane with blotto for 1 hr. Removed the blotto and added 15 ml Tween-Tris buffer with 1-2 ml of the SapAΔP6 (isolated from low pH fractions). Incubated on the shaker for 2 hr. Removed the Tween-Tris buffer mixture and added 15 ml fresh Tris buffer with 5 ul anti-sap 1° antibody for 1 hr. Finally, removed the Tris buffer mixture and added 15 ml fresh Tris buffer with 0.3 ul goat anti-rabbit Alexa Fluor 680 2° antibody. Detected the SapA-binding protein using Odyssey 2.0 on the Licor Odyssey system.

# 3 RESULTS

## 3.1 Loss of proteolytic activity of SapA

Whenever processing of recombinant RsaA occurred, a protease that was not well understood was thought to be the reason. In 2002, it was determined that SapA, a zinc-metalloprotease that shares some sequence homology to RsaA, was responsible for this processing (93). Three different mutants of SapA over the years have since been made: SapAΔPst1 which contains a 342 amino acid internal deletion, SapAΔRsa1 which contains a 71 amino acid internal deletion, and SapAΔP6 which contains a point mutation from a valine to a lysine at amino acid 188 near the predicted active site. All of these mutants significantly knocked down the processing of recombinant RsaA. However, a complete knockout of SapA had never been made and without one, localizing SapA would not be possible. This is because SapA is still visible on the cell's surface of all of the previous SapA deletions made. In this study, a complete knockout of SapA was made and the loss of proteolytic activity was confirmed (Figure 3-1, lane 4). The processing of recombinant RsaA with a pilin epitope insertion (TSDQDEQFIPKG) at amino acid 450 expressed in a SapA positive strain and SapA knockout strain of *C. crescentus* was examined for this purpose. Recombinant RsaA was cleaved in the SapA positive *C. crescentus* strain but was not cleaved in the SapA knockout *C. crescentus* strain.

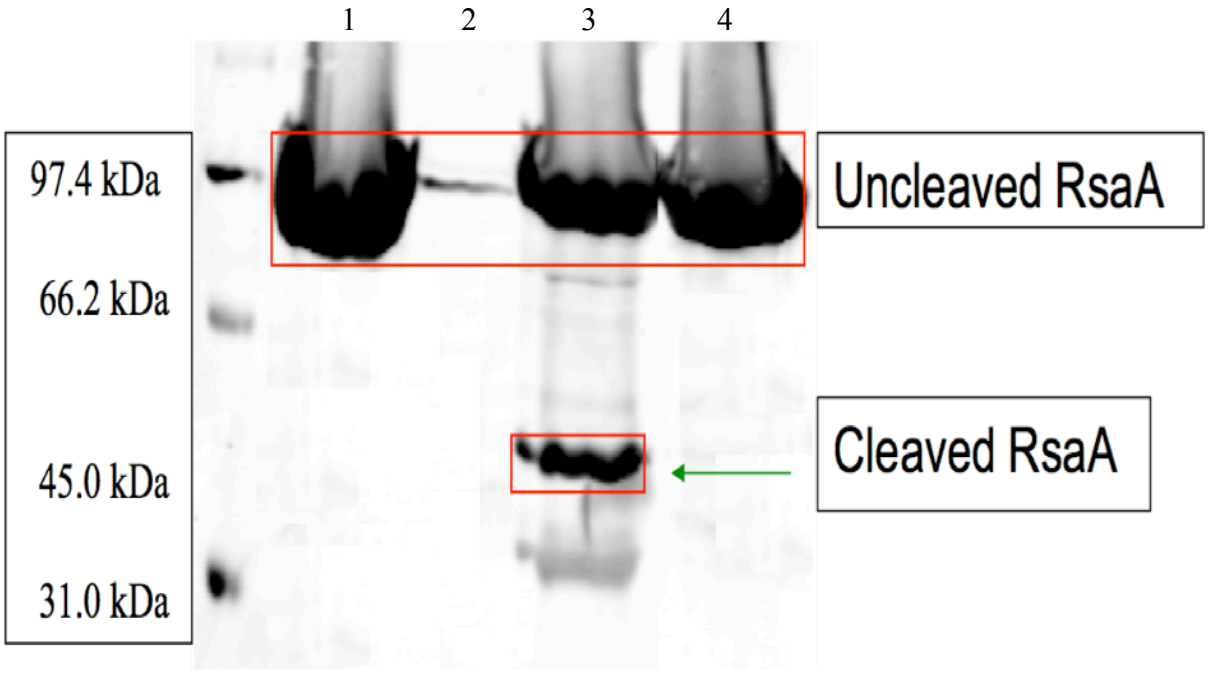



**Figure 3-1. Cleavage of pilin epitope insertion at amino acid 450 of RsaA by SapA**

Infrared western blot with polyclonal anti-188/784 RsaA antibodies of low pH preparations. Lane 1, Wild type NA1000 cells (*sapA*$^{+ve}$, *rsaA*$^{+ve}$); Lane 2, JS2008 cells (*sapA*$^{-ve}$, *rsaA*$^{-ve}$); Lane 3, CB15ΔrsaA pwB9M13(450)PE3Δ cells (*rsaA*$^{+ve}$ with pilin insertion at aa 450, *sapA*$^{+ve}$); Lane 4, JS2008 pwB9M13(450)PE3Δ cells (*rsaA*$^{+ve}$ with pilin insertion at aa 450, *sapA*$^{-ve}$). Pilin epitope = TSDQDEQFIPKG.

## 3.2 Detection and over-expression SapA

Attempts to study SapA in *C. crescentus* have been challenging, as the protease was expressed as such low levels that it could not be detected from infrared westerns of cell membrane preparations or whole cell culture preparations (Figure 3-2). Being unable to detect the SapA from these preparations under wild type conditions makes it difficult to study the secretion and anchoring of this protease. To solve this problem, SapA was over-expressed using a lac promoter on a multi-copy plasmid (pn*sap*).

Under wild type conditions, SapA could be detected at very low levels from low pH extraction with an infrared western (Figure 3-3A, lane 2). Based on low pH extraction, over-expression of SapA resulted in a ~8.8 fold increase in SapA expression compared to wild type (Figure 3-3B). Interestingly, the expected size of SapA based on its sequence is 71 kDa, however SapA was detected as 67-kDa and 45-kDa bands.

Low pH extraction experiments, which isolate protein from the cell surface, confirmed that SapA is attached to the cell surface. In order to determine how effective low pH extraction was at removing SapA from the cell surface, a whole culture preparation was performed on JS2003 pn*sap* before and after low pH extraction. The results indicate that low pH was effective at removing most of SapA from the cell surface (Figure 3-4). Notice in lane 2 how the whole culture prep was degraded into a 45-kDa band and in lane 3, SapA from low pH extraction produced three bands.

Under wild type conditions SapA could not be detected by immunofluorescence in the presence of SLPS, however in the absence of SLPS, *C. crescentus* exhibited a faint spotty fluorescence of SapA (Figure 3-5 B and D). Visualization by immunofluorescence of the protease on the cell surface once over-expressed was significant but only in SLPS

negative strains (Figure 3-5 C and E). Based on the immunofluorescence data, SapA over-expression in the absence of SLPS was a spotty label covering the entire cell.

The processing was also visible from concentrated supernatant preparations of over-expressed SapA from JS2003 pn*sap*. Here supernatant was concentrated ~100 fold. There were numerous processed bands detected in the supernatant however, the major band was 45-kDa in size (Figure 3-6, Lane 3). SapA could not be detected under native expression conditions in the supernatant even after concentration (Figure 3-6, Lane 2).

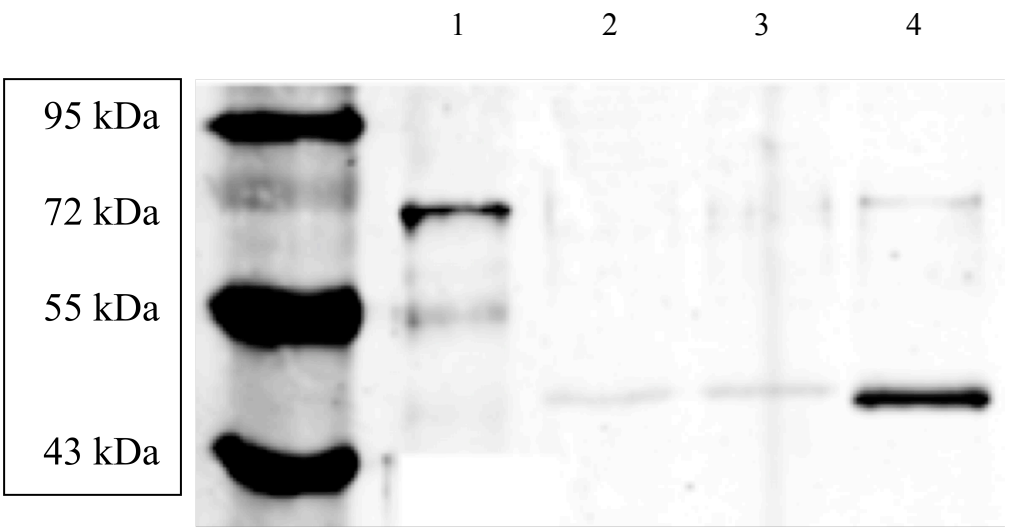



**Figure 3-2. Whole culture expression of wild type versus over-expressed SapA**

Infrared western blot with anti-sap antibodies of whole culture protein preparations normalized to $OD_{600}$ = 1. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, JS2009 cells (*sapA*$^{-ve}$); Lane 3, JS2003 cells (*sapA*$^{+ve}$); Lane 4, JS2003 pn*sap* cells (*sapA*$^{++ve}$).

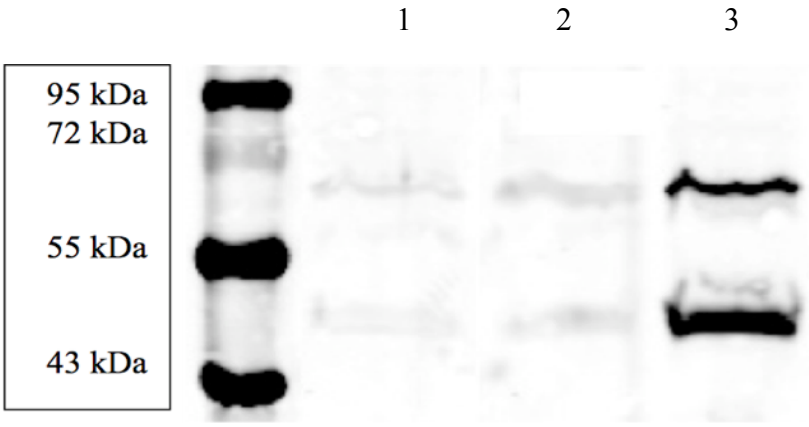



**Figure 3-3A. Expression of SapA under native and over-expressed conditions**

Infrared western blot with anti-sap antibodies of low pH preparations normalized to $OD_{600}$ = 1. Lane 1, JS2009 cells (*sapA*$^{-ve}$); Lane 2, JS2003 cells (*sapA*$^{+ve}$); Lane 3, JS2003 pn*sap* cells (*sapA*$^{++ve}$).

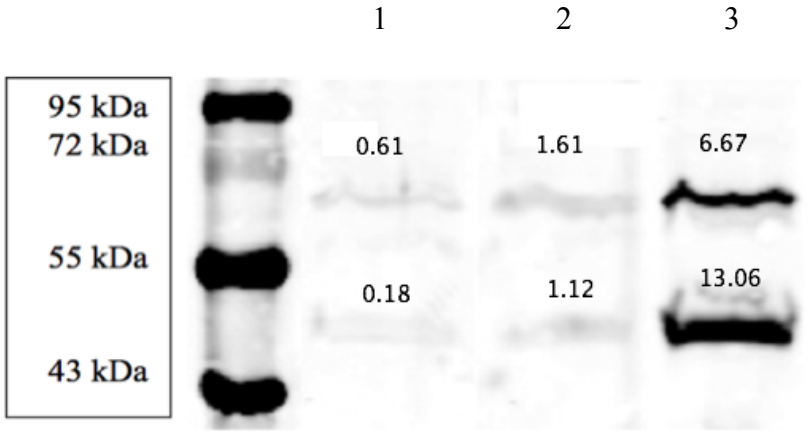



**Figure 3-3B. Quantified expression of SapA under native and over-expressed conditions**

Infrared western blot with anti-sap antibodies of low pH preparations normalized to $OD_{600}$ = 1. Lane 1, JS2009 cells (*sapA*$^{-ve}$); Lane 2, JS2003 cells (*sapA*$^{+ve}$); Lane 3, JS2003 pn*sap* cells (*sapA*$^{++ve}$). There is an 8.8 fold increase in the amount of SapA produced under over-expression conditions using a lac promoter on a multi-copy plasmid.

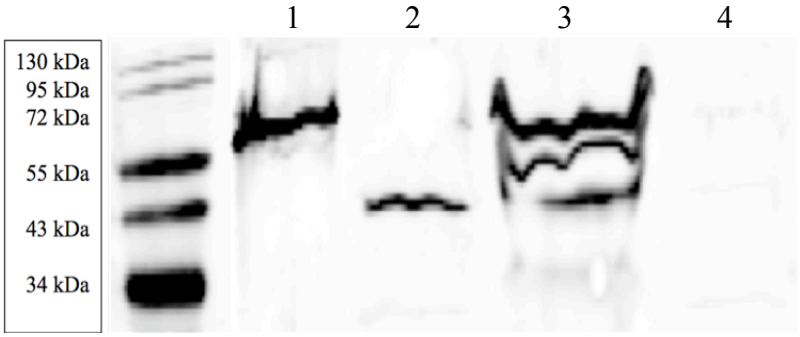



**Figure 3-4. Effectiveness of low pH extraction of SapA from the cell surface**

Infrared western blot with anti-sap antibodies of low pH preparations and whole culture preparations of *C. crescentus*. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, Whole culture prep of JS2003 pn*sap* cells ($sapA^{+ve}$) before low pH extraction; Lane 3, Low pH extraction of JS2003 pn*sap* cells ($sapA^{++ve}$); Lane 4, Whole culture prep of JS2003 pn*sap* cells ($sapA^{+ve}$) after low pH extraction.

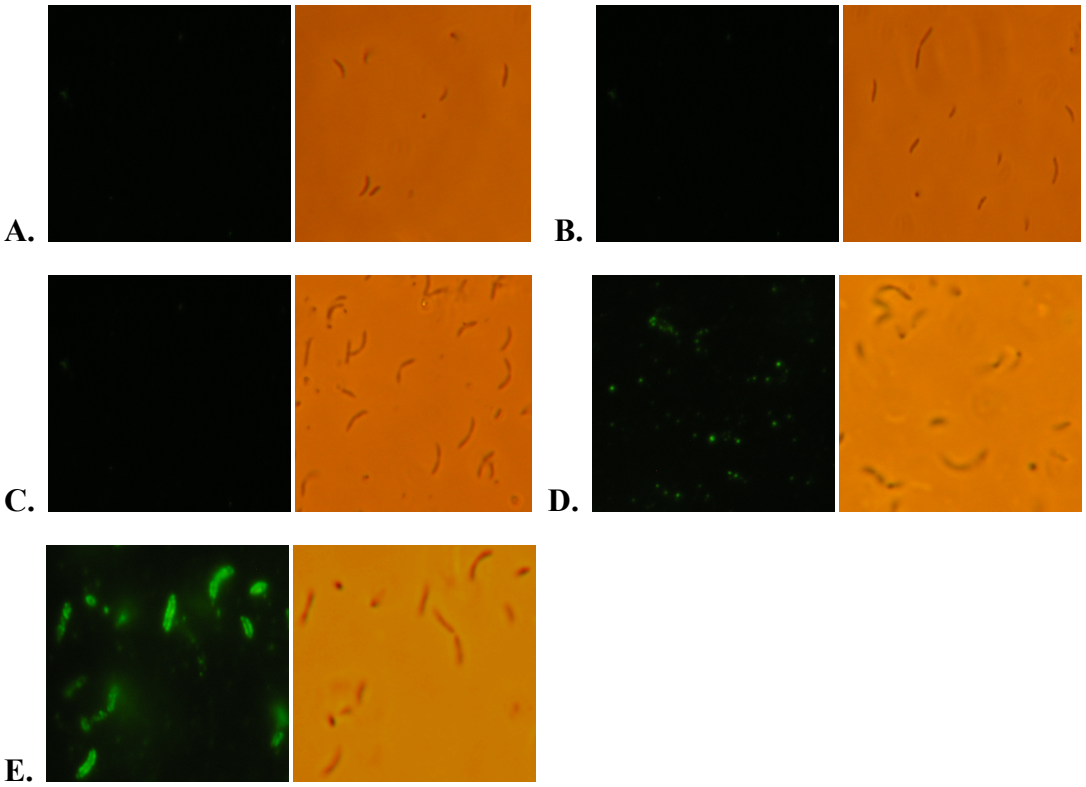



**Figure 3-5. Visualization of SapA on the cell surface through over-expression**

Immunofluorescence pictures of *C. crescentus* using 1° SapA antibody and goat anti-rabbit Alexa Fluor 448 2° antibody. A. JS2009 cells ($sapA^{-ve}$); B. JS2003 cells ($sapA^{+ve}$); C. JS2003 pn*sap* cells ($sapA^{++ve}$); D. JS2012 cells ($sapA^{+ve}$, $manB^{-}$); E. JS2011 pn*sap* cells ($sapA^{++ve}$, $manB^{-}$).

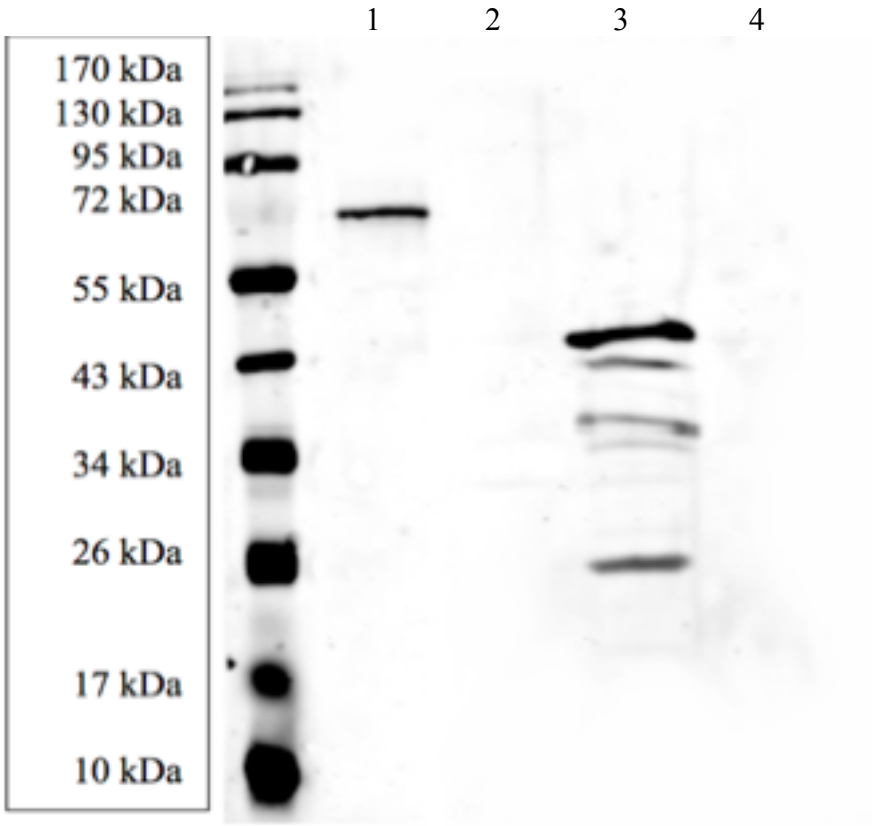



**Figure 3-6. Concentrated supernatant of SapA under native and over-expressed conditions**

Infrared western blot with anti-sap antibodies of supernatant from 150 ml *C. crescentus* cells concentrated using Centricon Plus-20 centrifugal filter devices from Millipore normalized to $OD_{600}$ = 1. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, JS2003 supernatant (*sapA*$^{+ve}$); Lane 3, JS2003 pn*sap* (*sapA*$^{++ve}$) supernatant; Lane 4, JS2009 supernatant (*sapA*$^{-ve}$).

### 3.3 SapA is expressed at low levels under over-expression conditions in *C. crescentus*

In order to determine how much SapA is expressed in *C. crescentus* and what fraction of SapA was released into the supernatant during over-expression, a BCA assay was performed on *E. coli* SapAhis6N purified from a Ni-NTA column dialyzed in dH2O. *E. coli* SapAhis6N concentration was determined to be ~164.29 ug/ml. Then, 328.58 ng (2 ul) of the *E. coli* SapAhis6N control was loaded on a gel with 15 ul of *C. crescentus* whole culture preparations, concentrated supernatants, or low pH extraction samples. Infrared westerns were performed and SapA from each sample was quantified. Based on infrared analysis, it was determined that JS2003 pn*sap* produces 74.0 ng/ul of a whole culture preparation (Figure 3-7A). Based on low pH extraction it was determined that over-expressed SapA has 41.6 ug/ml of low pH extracted protein per microlitre (Figure 3-7B, lane 3). The total amount of SapA in the supernatant was calculated to be 0.24 ul/ml per microlitre of supernatant from JS2003 pn*sap* (Figure 3-7B, lane 4). When total SapA protein present from whole culture preparations was compared to the amount present in the supernatant, the 45 kDa processed band constituted only ~0.6% of total SapA under over-expression conditions.

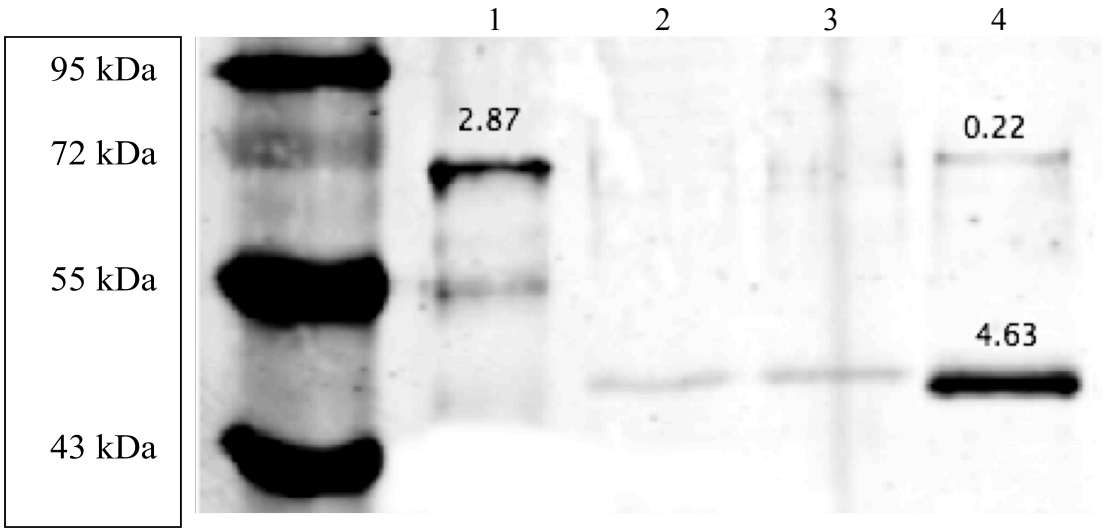



**Figure 3-7A. Quantified over-expressed SapA protein from whole culture preparations**

Infrared western blot with anti-sap antibodies of whole culture protein preparations normalized to $OD_{600}$ = 1. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, JS2009 cells (*sapA*$^{-ve}$); Lane 3, JS2003 cells (*sapA*$^{+ve}$); Lane 4, JS2003 pn*sap* cells (*sapA*$^{++ve}$).

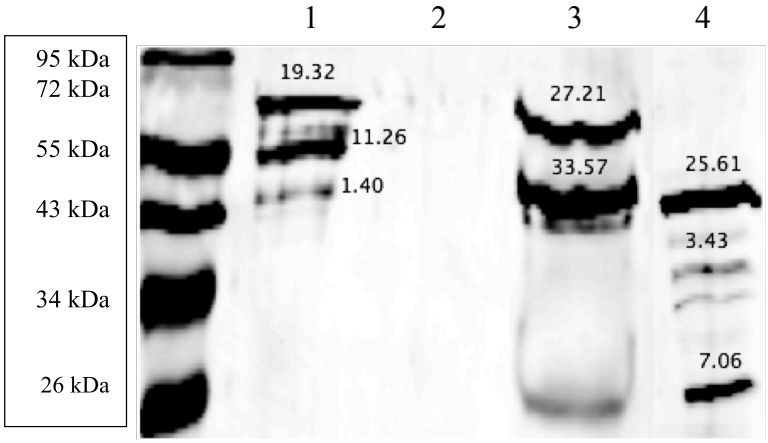



**Figure 3-7B. Quantified over-expressed SapA protein from low pH extraction and concentrated supernatant**

Infrared western blot with anti-sap antibodies of low pH preparations and supernatant of *C. crescentus* cells grown up in 150 ml, normalized to $OD_{600}$ = 1 and concentrated using Centricon Plus-20 centrifugal filter devices from Millipore. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, JS2009 cells (*sapA*$^{-ve}$); Lane 3, JS2003 pn*sap* cells (*sapA*$^{++ve}$); Lane 4, JS2003 pn*sap* supernatant (*sapA*$^{++ve}$).

### 3.4 Mass spectrometry of the secreted SapA 45-kDa processed band

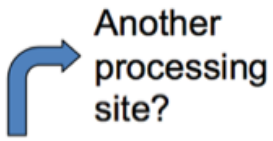
When the purified band of 45-kDa from the concentrated supernatant samples was submitted for mass spectrometry, it was established that it was the N-terminal 428 amino acids of SapA (Figure 3-8). From the mass spectrometry data, it was shown that the first 44 amino acids are not part of the 45-kDa protein. Interestingly, attempts to purify SapA with an N-terminal his6 tag when grown in *C. crescentus* were unsuccessful.

Proteolytic activity tests of SapA were performed using the supernatant fractions of over-expressed SapA and the SapA complete knockout (Figure 3-9). The supernatant fraction of over-expressed SapA cleaved recombinant RsaA, containing a pilin insertion at position 450, into a 64-kDa product. Conversely, the supernatant fraction of the SapA knockout did not cleave the recombinant RsaA.

One possible explanation for the production of the 45-kDa band was that SapA may contain an internal start site that created the smaller protein product. As such, the amino acid sequence of SapA was tested using BPROM, a database that predicts possible promoter regions of proteins with probable -10 and -35 boxes (http://linux1.softberry.com/berry.phtml). There were no significant hits found within the *sapA* coding sequence. This provides evidence that the shortened products of SapA are not as a consequence of an intern al start site in the protease.

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **23%**

Matched peptides shown in **Bold Red**
Peptides highlighted were too large to be detected




```
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 51 AALQLLRSEP GWSNQFLVPA TVTYAFRATA PASMPSDTGG FSQFNAAQIL
101 QAEKALQAWS DVANITFVRV GQGTSGEAAY SDNASILFAN FSTGSEGSAG
151 FAYYPGNPAA SSRSGDVWIK STAGYNTNPT GSNYGGMVLV HELGHAIGIA
201 HPSEYNASAD DTLTYAVNAT YYQDSRQYTV MSYFSEANTG GSFGGAYASS
251 PLLDDIAAAQ LAYGANMTTR TGDTVYGFNS TAGREWFAAT SSSTRLVFAV
301 WDAGGVDTLD FSGYRVAQTI DLRAGYFSSV GGLKGNVTIA MNAVIENAIG
351 GSAADTINGN AVDNRLTGGA GADILDGGRG VDTAVFSGAY GNYTLTAATN
401 GAWSVLDRVG TDATDTLANI EFLAFTDRTV TLVDSRVATA ISNILRLQTF
451 SASAEPLSKS LAASMAAGAS HSDAIGQVSK TALSTSGVAV LAYQFFTGKT
501 PTAAGMDYLV NPDGVNANNL NSAYYQSFNL ENRYINFAVN LGKIGEGATK
551 FLADYGGLSL FDATRKAYAT IFGLTPTDDK VRALIDGRTD YFAAYGQDGP
601 NGQGTKAAMV GWLMAEAGKA DIGVYAKSAG AFFADQATKN VYGVDLIGVY
651 AKPEYNLI
```

**Figure 3-8A. Mass spectrometry of 45-kDa SapA product using MASCOT database**


**Figure 3-8B. N-terminal processing of Serralysin-like metalloproteases** (57)

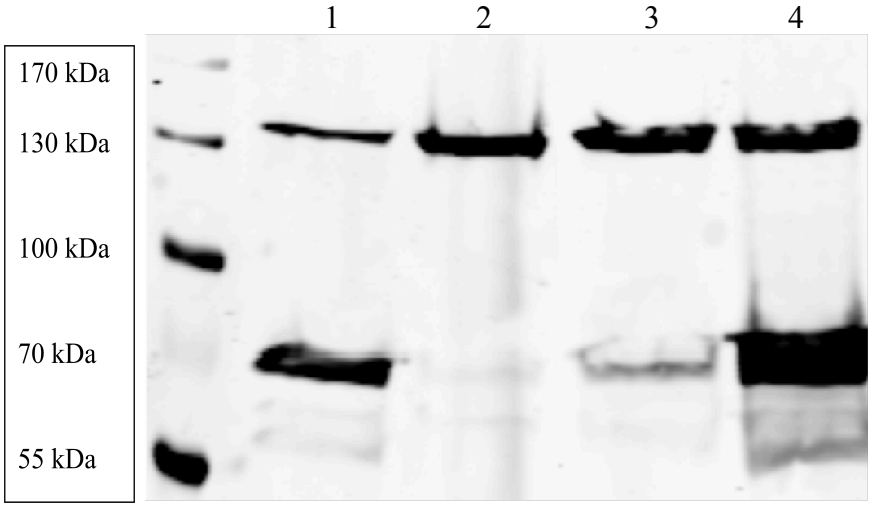


**Figure 3-9. Proteolytic activity tests from the supernatant of SapA**

Infrared western blot with polyclonal anti-188/784 RsaA antibodies of low pH preparations. Lane 1, CB15ΔrsaA pwB9M13(450)PE3Δ cells (*rsaA*$^{+ve}$ with pilin insertion at aa 450, *sapA*$^{+ve}$) positive control; Lane 2, JS2008 pwB9M13(450)PE3Δ cells (*rsaA*$^{+ve}$ with pilin insertion at aa 450, *sapA*$^{-ve}$) negative control; Lane 3, JS2009 supernatant (*sapA*$^{-ve}$, *rsaA*$^{-ve}$) incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells; Lane 4, JS2003 pn*sap* (*sapA*$^{++ve}$, *rsaA*$^{-ve}$) supernatant incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells.

### 3.5 SapA is a self-processing enzyme

In order to determine whether SapA was responsible for all the processing events that were observed, SapAΔP6 was over-expressed in JS2009. SapAΔP6 contains a point mutation at amino acid 188 near protease's active site (from a valine to a lysine). Interestingly, when JS2009 pn*sap*ΔP6 cells were examined under a light microscope, the cells tended to aggregate. Also when these cells were centrifuged, their pellet was diffuse, unlike JS2003 pn*sap* cells, which formed a tight circular pellet. Although the proteolytic activity of the protease was reduced by this mutation as was demonstrated (see below), the SapAΔP6 was still secreted to the cell surface and visible by immunofluorescence (Figure 3-10). When it was secreted into the supernatant, there was almost a complete elimination of processing (Figure 3-11). Further, SapAΔP6 isolated from the cell surface by low pH was not processed (Figure 3-12A). Similarly, whole culture preparations of SapAΔP6 exhibited a great reduction in processing (Figure 3-12B).

One observation from the SapAΔP6 data was that although this protein (67 kDa) was not being processed into the 45-kDa band as seen by over-expressed SapA, it was still ~ 4-kDa smaller than the *E. coli* SapAhis6N control (71 kDa) (Figure 3-11 and 3-12A). The his6 tag alone (0.8 kDa) could not account for the change in size observed.

In order to confirm the loss of proteolytic activity of SapAΔP6 the concentrated supernatant was incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells, which contain a pilin insertion at amino acid 450 of RsaA. There was a significant reduction in the proteolytic activity of SapAΔP6 (Figure 3-13A). Additionally, SapA and SapAΔP6 purified by low pH extraction confirmed that while SapA maintained its activity after extraction, SapAΔP6 did not (Figure 3-13B).

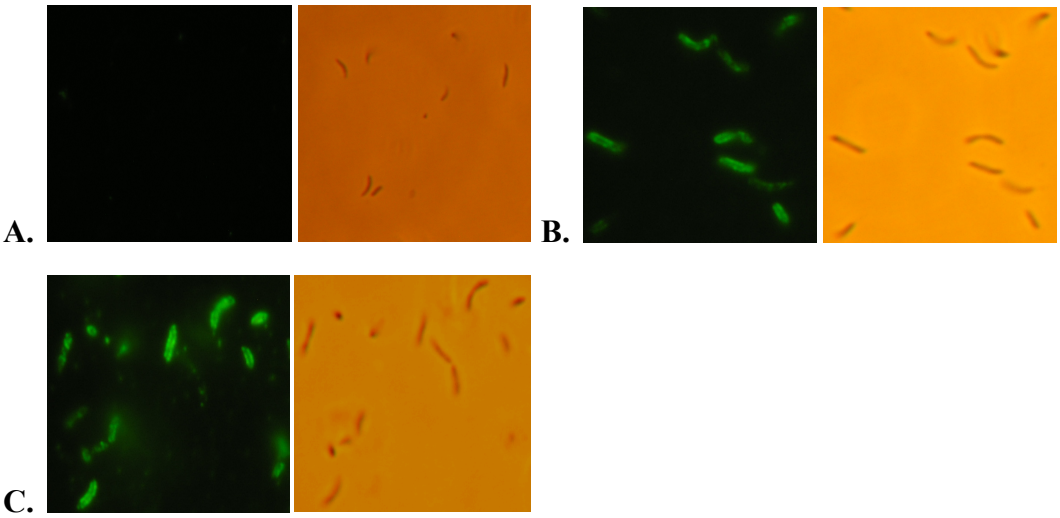


**Figure 3-10. Visualization of SapAΔP6 on the cell surface of *C. crescentus***

Immunofluorescence pictures of *C. crescentus* using 1° SapA antibody and goat anti-rabbit Alexa Fluor 448 2° antibody. A. JS2011 cells (*sapA*$^{-ve}$, *rsaA*$^{-ve}$, *manB*$^{-ve}$); B. JS2011 pn*sap*ΔP6 cells (*sapA*$^{-ve}$, *rsaA*$^{-ve}$, *manB*$^{-ve}$); C. JS2011 pn*sap* cells (*sapA*$^{++ve}$, *rsaA*$^{-ve}$, *manB*$^{-ve}$);.

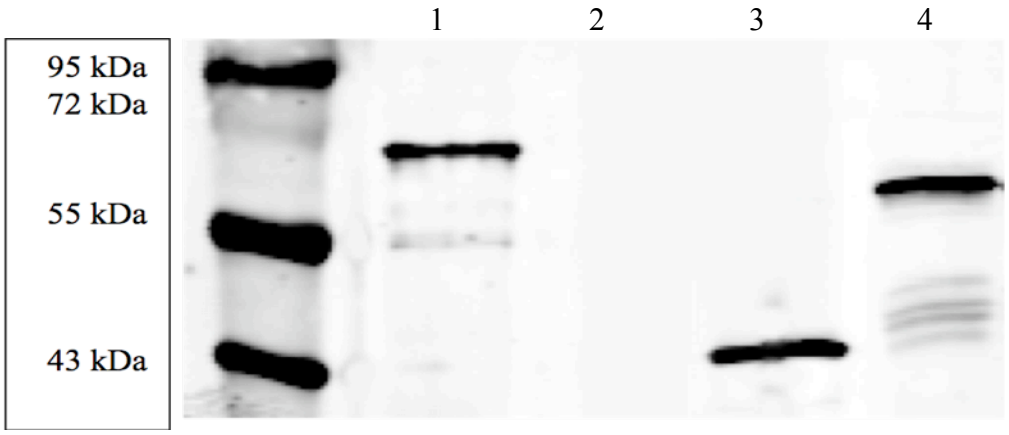


**Figure 3-11. Size difference in secreted SapA and SapAΔP6**

Infrared western blot with anti-sap antibodies of supernatant of *C. crescentus* cells grown up in 50 ml, normalized to $OD_{600}$ = 1 and concentrated using Centricon Plus-20 centrifugal filter devices from Millipore. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, JS2009 supernatant ($sapA^{-ve}$); Lane 3, JS2003 pn*sap* ($sapA^{++ve}$) supernatant; Lane 4, JS2009 pn*sap*ΔP6 supernatant ($sapA\Delta P6^{++ve}$).

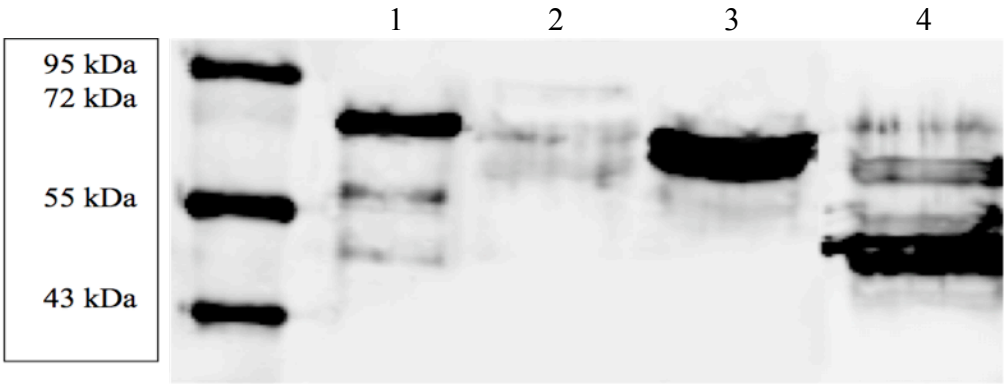


**Figure 3-12A. Attachment of SapA and SapAΔP6 on the cell surface of *C. crescentus***

Infrared western blot with anti-sap antibodies of low pH preparations normalized to $OD_{600}$ = 1.2. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, JS2009 cells (*sapA*$^{-ve}$); Lane 3, JS2009 pn*sap*ΔP6 cells (*sapA*ΔP6$^{++ve}$); Lane 3, JS2003 pn*sap* cells (*sapA*$^{++ve}$).

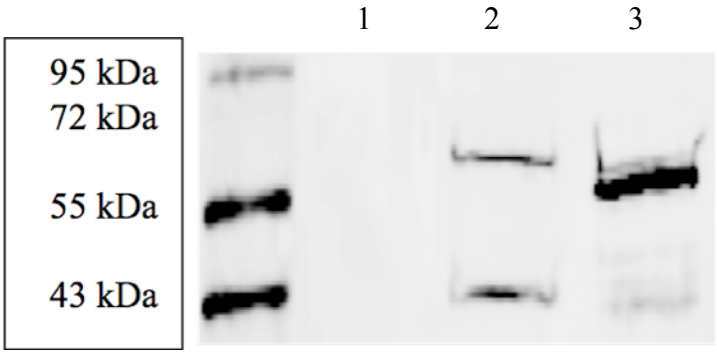


**Figure 3-12B. Reduction in processing of SapAΔP6**

Infrared western blot with anti-sap antibodies of whole culture protein preparations, normalized to $OD_{600}$ = 1.0. Lane 1, JS2009 cells (*sapA*$^{-ve}$); Lane 2, JS2003 pn*sap* cells (*sapA*$^{++ve}$); Lane 3, JS2009 pn*sap*ΔP6 cells (*sapA*ΔP6$^{++ve}$).

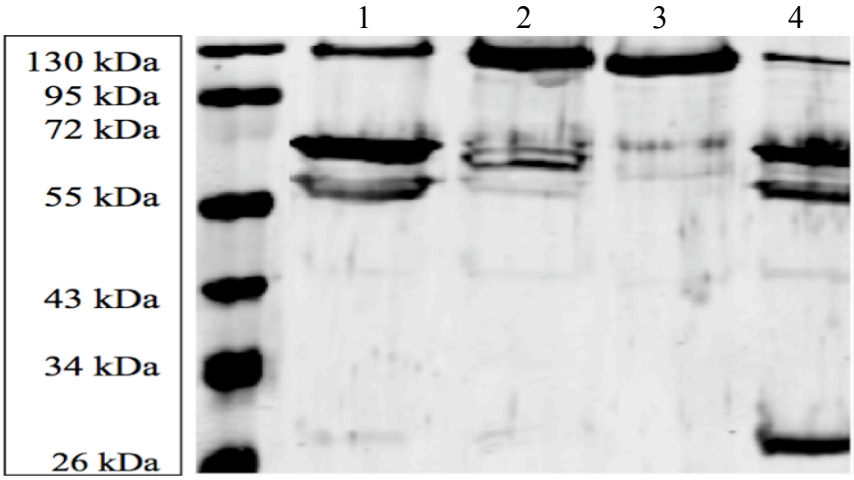


**Figure 3-13A. Reduction in proteolytic activity of SapAΔP6 from concentrated supernatant**

Infrared western blot with polyclonal anti-188/784 RsaA antibodies of low pH preparations. Lane 1, JS2003 pn*sap* supernatant (*sapA*$^{++}$) incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells; Lane 2, JS2009 pn*sap*ΔP6 supernatant (*sapAΔP6*$^{++}$) incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells; Lane 3, JS2008 pwB9M13(450)PE3Δ cells negative control; Lane 4, CB15ΔrsaA pwB9M13(450)PE3Δ cells positive control.

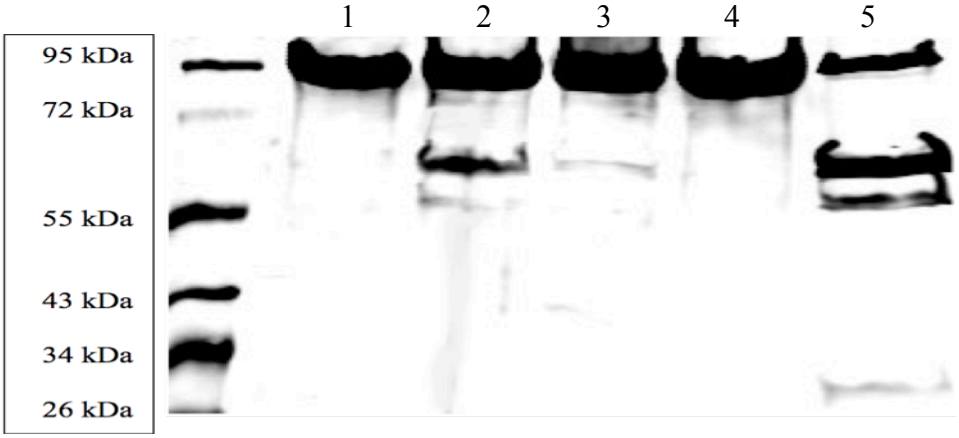


**Figure 3-13B. Reduction in proteolytic activity of SapAΔP6 isolated from low pH extraction**

Infrared western blot with polyclonal anti-188/784 RsaA antibodies of low pH preparations, normalized to $OD_{600}$ = 1.0. Lane 1, JS2009 low pH'd protein (*sapA*$^{-ve}$) incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells; Lane 2, JS2003 pn*sap* low pH'd protein (*sapA*$^{++ve}$) incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells; Lane 3, JS2009 pn*sap*ΔP6 low pH'd protein (*sapA*ΔP6$^{++ve}$) incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells; Lane 4, JS2008 pwB9M13(450)PE3Δ cells negative control; Lane 5, CB15ΔrsaA pwB9M13(450)PE3Δ cells positive control.

## 3.6 SapA is not a lipoprotein that anchors to the outer membrane by an aminoacylated N-terminal cysteine

PCR was used for site-directed mutagenesis of SapA's second N-terminal amino acid from a cysteine to an alanine in order to determine whether SapA is lipid-linked to the outer membrane of *C. crescentus*. It was expected that if SapA was a lipoprotein, lack of the second N-terminal cysteine would prevent the protease from being recognized by the Lol secretion system and it would therefore not be secreted. Immunofluorescence data proved this hypothesis to be false as C1ASapA was still visible on the cell surface of *C. crescentus* (Figure 3-14). From whole culture preparations, it became clear that there is no change in the expression or processing of C1ASapA compared to SapA (Figure 3-15A and 15B). Similarly, there was no change in the attachment of protein to the cell surface as confirmed by low pH extraction (Figure 3-16A and 16B). Finally, the same 45-kDa processed product was found in the supernatant of *C. crescentus* over-expressing C1ASapA (Figure 3-17). Thus, substituting the second amino acid, cysteine, for an alanine did not prevent secretion or anchoring of the protease.

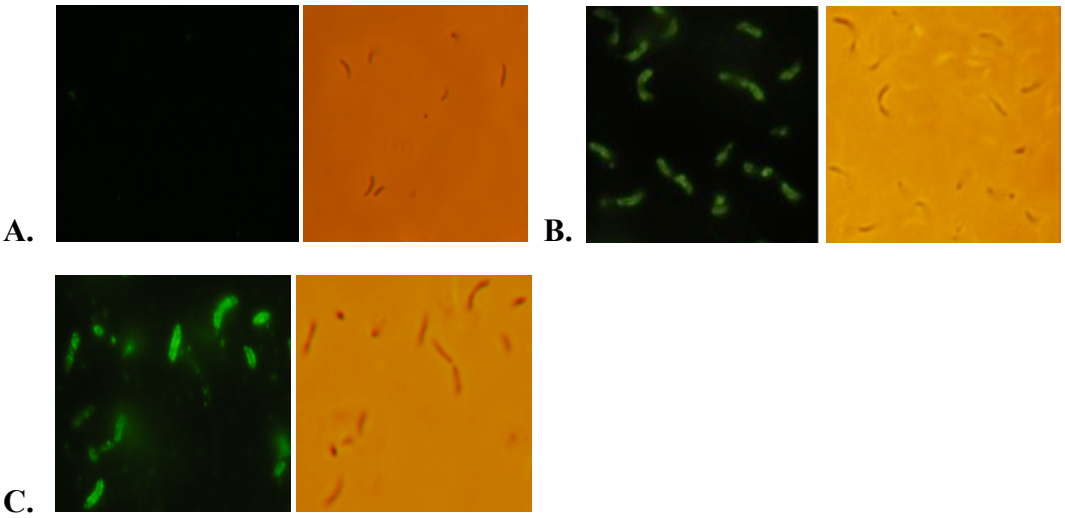


**Figure 3-14. Visualization of C1ASapA on the cell surface of *C. crescentus***

Immunofluorescence pictures of *C. crescentus* using 1° SapA antibody and goat anti-rabbit Alexa Fluor 448 2° antibody. A. JS2011 cells (*sapA*$^{-ve}$, *rsaA*$^{-ve}$, *manB*$^{-ve}$); B. JS2011 pnC1A*sap* cells (C1A*sapA*$^{++ve}$, *rsaA*$^{-ve}$, *manB*$^{-ve}$); C. JS2011 pn*sap* cells (*sapA*$^{++ve}$, *rsaA*$^{-ve}$, *manB*$^{-ve}$).

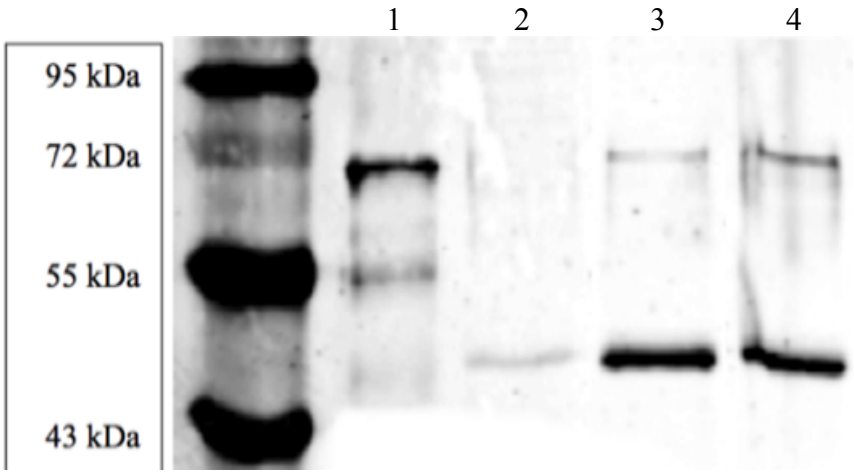


**Figure 3-15A. Expression of SapA and C1ASapA**

Infrared western blot with anti-sap antibodies of whole culture protein preparations normalized to $OD_{600}$ = 1. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, JS2009 cells (*sapA*$^{-ve}$); Lane 3, JS2003 pn*sap* cells (*sapA*$^{++ve}$); Lane 4, JS2009 pnC1A*sap* cells (C1A*sapA*$^{++ve}$).

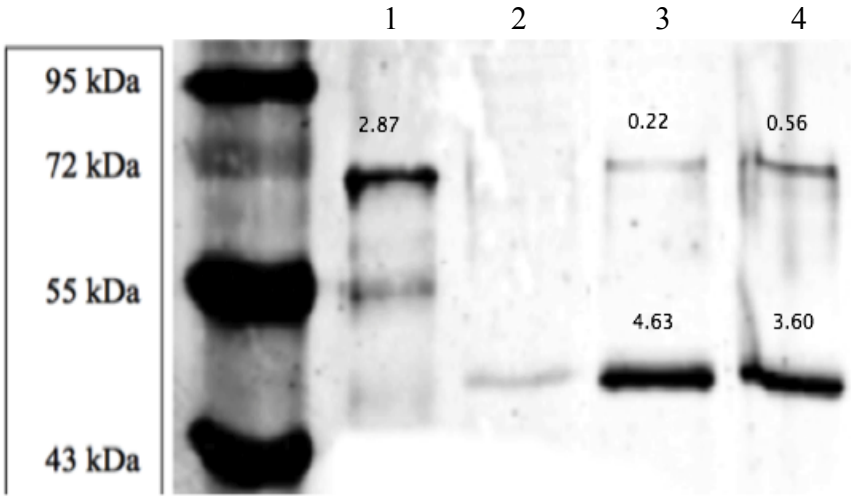


**Figure 3-15B. Quantified expression of SapA and C1ASapA**

Infrared western blot with anti-sap antibodies of whole culture protein preparations normalized to $OD_{600}$ = 1. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, JS2009 cells (*sapA*$^{-ve}$); Lane 3, JS2003 pn*sap* cells (*sapA*$^{++ve}$); Lane 4, JS2009 pnC1A*sap* cells (C1A*sapA*$^{++ve}$).

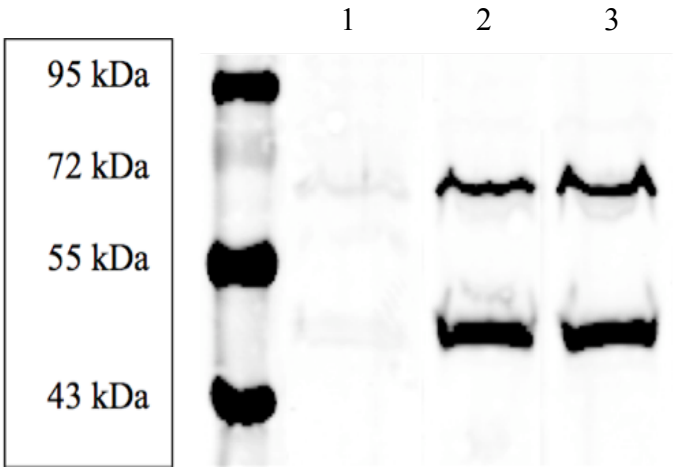


**Figure 3-16A. Attachment of C1ASapA on the cell surface of *C. crescentus***

Infrared western blot with anti-sap antibodies of low pH preparations normalized to $OD_{600}$ = 1. Lane 1, JS2009 cells (*sapA*$^{-ve}$); Lane 2. JS2003 pn*sap* cells (*sapA*$^{++ve}$); Lane 3, JS2009 pnC1A*sap* cells (C1A*sapA*$^{++ve}$).

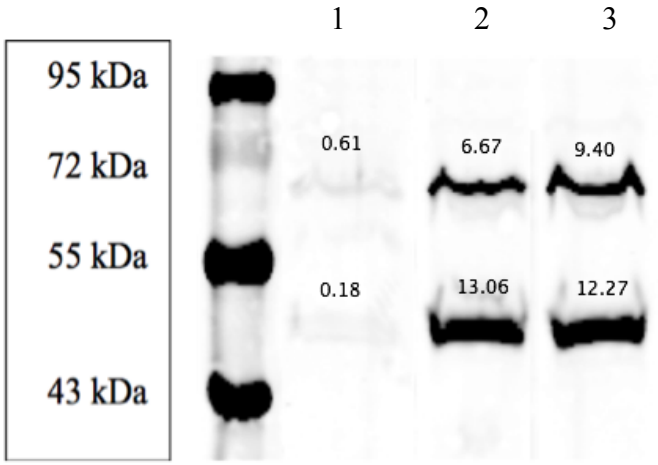


**Figure 3-16B. Quantified attachment of C1ASapA on the cell surface of *C. crescentus***

Infrared western blot with anti-sap antibodies of low pH preparations normalized to $OD_{600}$ = 1. Lane 1, JS2009 cells (*sapA*$^{-ve}$); Lane 2. JS2003 pn*sap* cells (*sapA*$^{++ve}$); Lane 3, JS2009 pnC1A*sap* cells (C1A*sapA*$^{++ve}$).

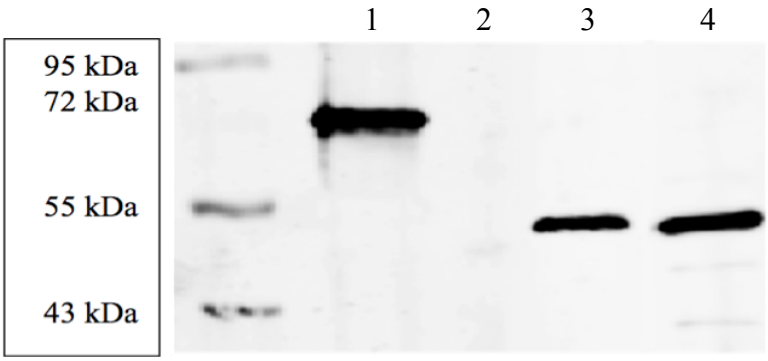


**Figure 3-17. Secretion of SapA and C1ASapA**

Infrared western blot with anti-sap antibodies of supernatant of *C. crescentus* cells grown up in 50 ml, normalized to $OD_{600}$ = 1 and concentrated using Centricon Plus-20 centrifugal filter devices from Millipore. Lane 1. *E.coli* Saphis6N control purified by Ni-NTA, Lane 2. JS2009 cells, Lane 3. JS2003 pn*sap* cells, Lane 4. JS2009 pnC1A*sap* cells. There is no change in the processing of C1ASapA compared to SapA.

### 3.7 SapA uses the Sec-independent S-layer type I secretion system

When the first 70 amino acids of SapA was examined by SignalP 3.0 an N-terminal secretion signal database for Sec-dependent proteins, SapA was found to not contain a signal peptide secretion signal (Figure 3-18) (103). This suggested that a Sec-independent secretion pathway secretes SapA.

Since type I secreted proteins use their C-terminus as a secretion signal, the last 10 or 50 amino acids of SapA were removed and, the truncated proteins were tested for secretion. The supernatant proteins of SapA, SapAΔ10C or SapAΔ50C over-expressing strains were compared by concentrating 150 ml of culture to 500 ul and detected by infrared western. It was determined that SapA does not get released into the supernatant when the last 10 or 50 amino acids were removed (Figure 3-19). Only wild-type SapA was secreted into the supernatant. Also, low pH extraction of SapA from the cell surface of *C. crescentus* showed that the truncated clones did not attach to the cell surface (Figure 3-20, lanes 4 and 5). The same result was observed on the cell surface where *C. crescentus* over-expressing either SapAΔ10C or SapAΔ50C did not fluoresce (Figure 3-21, B and C). Finally, whole culture preparations of SapAΔ10C and SapAΔ50C showed that both truncated clones were not processed (Figure 3-22, lanes 5 and 6).

To determine whether SapA uses the S-layer type I secretion system, SapA was over-expressed in JS007, an *rsaA*-, *rsaFa*- and *rsaFb*- strain. RsaFa and RsaFb are the outer membrane components of the *C. crescentus* type I S-layer secretion system and are thus required for RsaA secretion (92). Over-expression of SapA showed that the protease was not secreted into the supernatant of the *rsaFa*-/*rsaFb*- minus strain (Figure 3-23). Further, low pH extraction confirmed that SapA is not attached to the cell surface in an

*rsaFa*/*Fb* mutant (Figure 3-20, lane 6). Similarly, SapA did not fluoresce in an *rsaFa*/*Fb* mutant (Figure 3-21 D). Finally, without these two outer membrane proteins present, SapA was not processed at all in *C. crescentus* (Figure 3-22, lane 7).

### 3.8 SapA does not anchor to S-layer type I secretion system outer membrane proteins RsaFa or RsaFb

In order to determine whether SapA attaches to the S-layer type I secretion system outer membrane proteins RsaFa or RsaFb, reattachment assays in an *rsaFa/Fb*- strain and an *rsaFa/Fb*-, *manB*- strain of *C. crescentus* were performed using SapAΔP6 isolated by low pH extractions. SapAΔP6 was able to reattach to *C. crescentus* with a *manB* mutation, to *C. crescentus* with an *rsaFa/Fb* mutation, and to *C. crescentus* with a *manB* and *rsaFa/Fb* mutation (Figure 3-24). Reattached protein was also detected on the cell surface of these strains by immunofluorescence.

**>SapA amino acid sequence first 70 aa**

**SignalP-NN result:**

# data

>SapA length = 70

| # Measure | Position | Value | Cutoff | signal peptide? |
|---|---|---|---|---|
| max. C | 21 | 0.063 | 0.52 | NO |
| max. Y | 15 | 0.047 | 0.33 | NO |
| max. S | 4 | 0.343 | 0.92 | NO |
| mean S | 1-14 | 0.107 | 0.49 | NO |
| D | 1-14 | 0.077 | 0.44 | NO |

**SignalP-HMM result:**

# data

>Sap

Prediction: Non-secretory protein

Signal peptide probability: 0.000

Max cleavage site probability: 0.000 between pos. -1 and 0

**Figure 3-18. Signal P result to test whether SapA has characteristics of a protein secreted by the general secretory pathway**

SapA is predicted to not be a protein secreted by the Sec-dependent pathway (103).

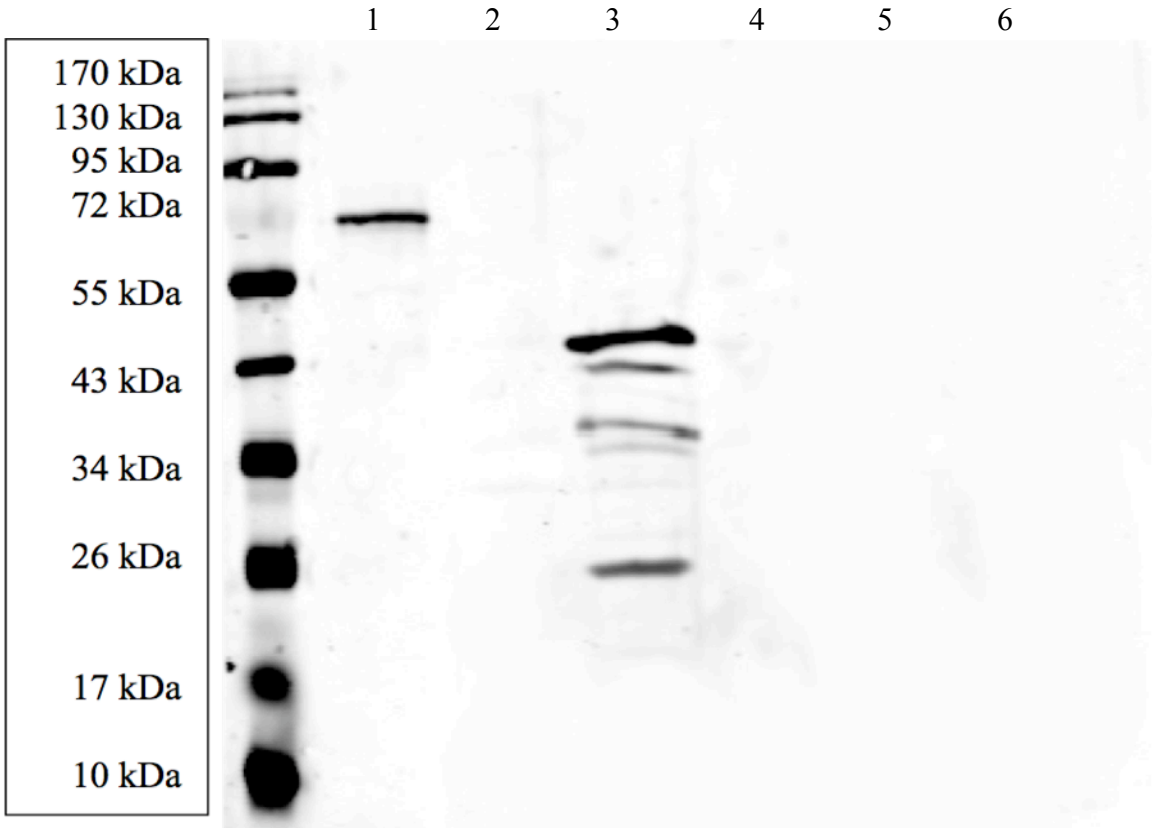


**Figure 3-19. Concentrated supernatant from over-expresssed SapA and truncated SapA in *C. crescentus***

Infrared western blot with anti-sap antibodies of supernatant of *C. crescentus* cells grown up in 150 ml and concentrated using Centricon Plus-20 centrifugal filter devices from Millipore normalized to $OD_{600}$ = 1. Lane 1. *E.coli* Saphis6N control purified by Ni-NTA, Lane 2. JS2003 supernatant ($sapA^{+ve}$), Lane 3. JS2003 pn*sap* supernatant ($sapA^{++ve}$), Lane 4. JS2009 supernatant ($sapA^{-ve}$), Lane 5. JS2009 pn*sap*Δ10C supernatant ($sapA\Delta10C^{++ve}$), Lane 6. JS2009 pn*sap*Δ50C supernatant ($sapA\Delta50C^{++ve}$).

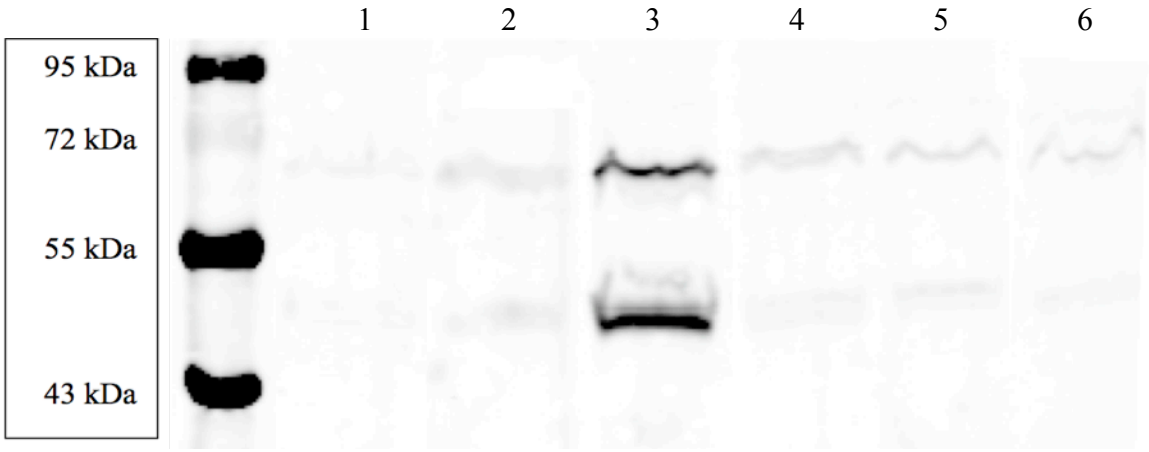


**Figure 3-20. Cell surface attachment of type I secretion deficient strains**

Infrared western blot with anti-sap antibodies of low pH preparations normalized to $OD_{600}$ = 1. Lane 1. JS2009 cells (*sapA*$^{-ve}$), Lane 2. JS2003 cells (*sapA*$^{+ve}$), Lane 3. JS2003 pn*sap* cells (*sapA*$^{++ve}$), Lane 4. JS2009 pn*sap*Δ10C cells (*sapA*Δ10C$^{++ve}$), Lane 5. JS2009 pn*sap*Δ50C cells (*sapA*Δ50C$^{++ve}$), Lane 6. JS2007 pn*sap* cells (*sapA*$^{++ve}$, *rsaFa*$^{-ve}$, *rsaFb*$^{-ve}$).

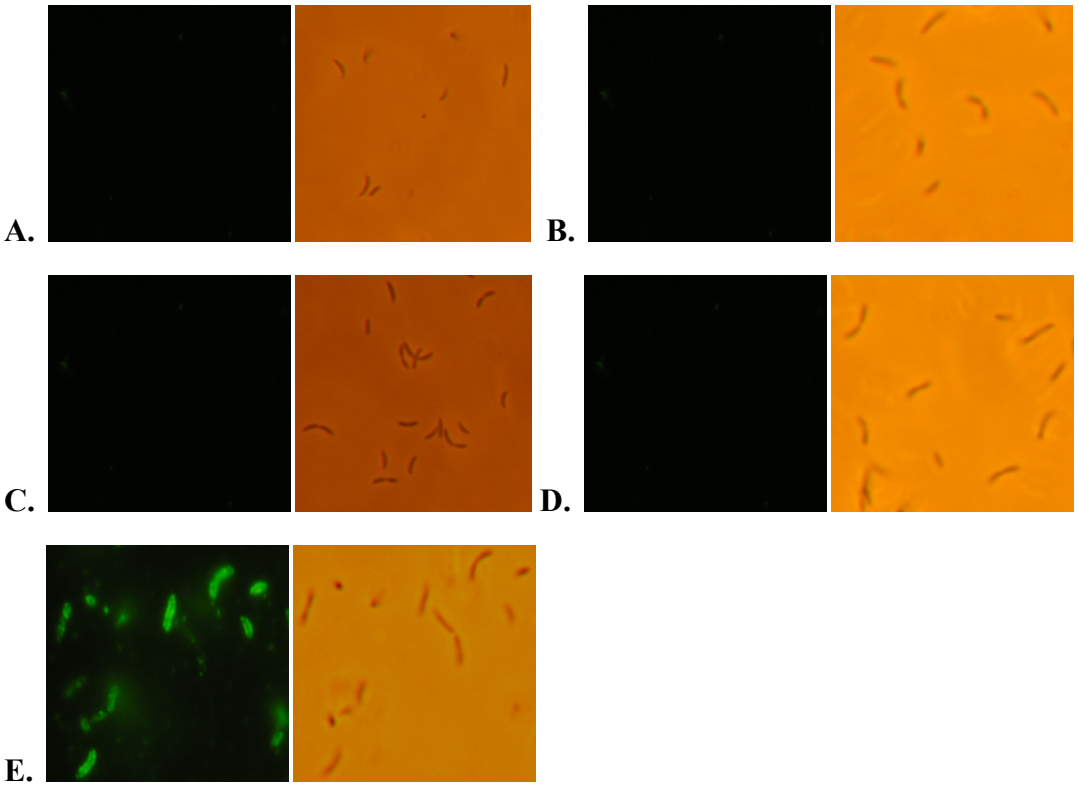


**Figure 3-21. Visualization of SapA on the cell surface of type I defective mutants**

Immunofluorescence pictures of *C. crescentus* using 1° SapA antibody and goat anti-rabbit Alexa Fluor 448 2° antibody. A. JS2011 cells (*sapA*$^{-ve}$, *rsaA*$^{-ve}$, *manB*$^{-ve}$); B. JS2011 pn*sap*Δ10C cells (*sapA*Δ10C$^{++ve}$, *rsaA*$^{-ve}$, *manB*$^{-ve}$); C. JS2011 pn*sap*Δ50C cells (*sapA*Δ50C$^{++ve}$, *rsaA*$^{-ve}$, *manB*$^{-ve}$); D. JS2013 pn*sap* cells (*sapA*$^{++ve}$, *rsaFa*$^{-ve}$, *rsaFb*$^{-ve}$); E. JS2011 pn*sap* cells (*sapA*$^{++ve}$, *rsaA*$^{-ve}$, *manB*$^{-ve}$).

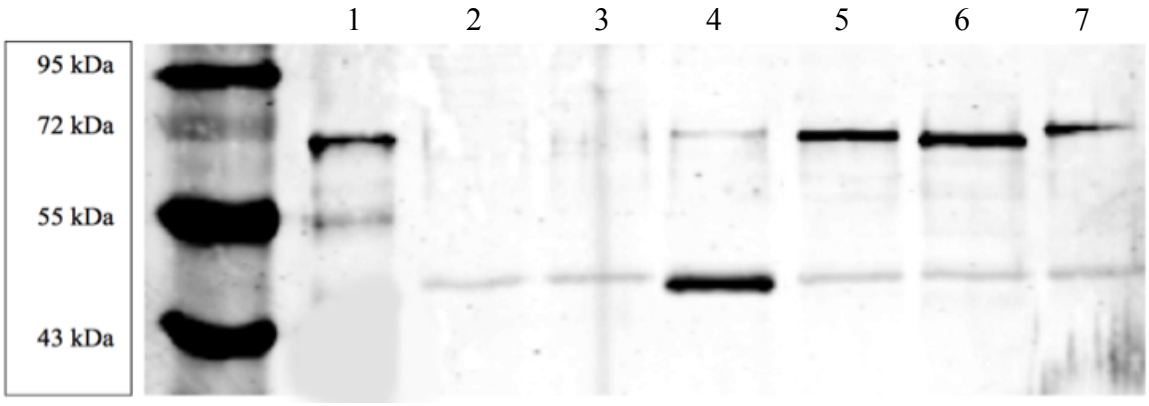


**Figure 3-22. Expression of SapA in an RsaFa$^{-}$/RsaFb$^{-}$ mutant**

Infrared western blot with anti-sap antibodies of whole culture protein preparations normalized to $OD_{600}$ = 1. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, JS2009 cells (*sapA*$^{-ve}$); Lane 3, JS2003 cells (*sapA*$^{+ve}$); Lane 4, JS2003 pn*sap* cells (*sapA*$^{++ve}$); Lane 5, JS2009 pn*sap*Δ10C cells (*sapA*Δ10C$^{++ve}$); Lane 6, JS2009 pn*sap*Δ50C cells (*sapA*Δ50C$^{++ve}$);
Lane 7, JS2007 pn*sap* cells (*sapA*$^{++ve}$, *rsaFa*$^{-ve}$, *rsaFb*$^{-ve}$).

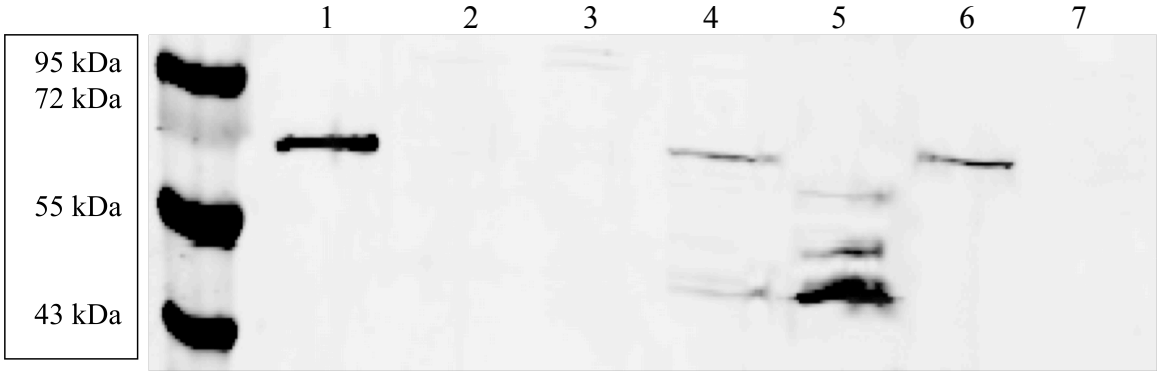


**Figure 3-23. Expression and secretion of SapA in a *C. crescentus* strain deficient in type I secretion (RsaFa$^{-}$/RsaFb$^{-}$)**

Infrared western blot with anti-sap antibodies of whole culture protein preparations and, supernatant of *C. crescentus* cells grown up in 50 ml and concentrated using Centricon Plus-20 centrifugal filter devices from Millipore. Lane 1. *E.coli* Saphis6N control purified by Ni-NTA, Lane 2. JS2009 cells (*sapA*$^{-ve}$), Lane 3. JS2009 supernatant (*sapA*$^{-ve}$), Lane 4. JS2003 pn*sap* cells (*sapA*$^{+ve}$), Lane 5. JS2003 pn*sap* supernatant (*sapA*$^{-ve}$), Lane 6. JS2007 pn*sap* cells (*sapA*$^{++ve}$, *rsaFa*$^{-ve}$, *rsaFb*$^{-ve}$), Lane 7. JS2007 pn*sap* supernatant (*sapA*$^{++ve}$, *rsaFa*$^{-ve}$, *rsaFb*$^{-ve}$).

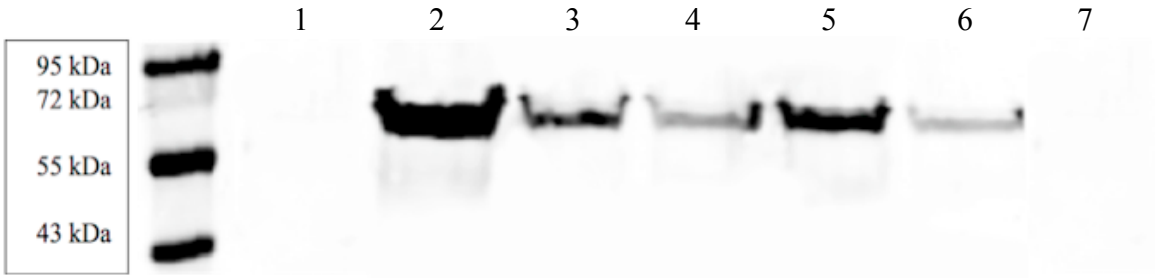


**Figure 3-24A. Reattachment assay of SapAΔP6, isolated from low pH, to *C. crescentus* strains deficient in *manB, rsaFa/Fb*, or both**

Infrared western blot with anti-sap antibodies of low pH preparations normalized to $OD_{600}$ = 1.2. Lane 1. JS2009 (*sapA*$^{-ve}$) negative control before reattachment, Lane 2. JS2009 pn*sap*ΔP6 (*sapA*ΔP6$^{++ve}$) positive control before reattachment, Lane 3. JS2009 cells with reattached SapAΔP6, Lane 4. JS2011 cells (*sapA*$^{-ve}$, *manB*$^{-ve}$) with reattached SapAΔP6, Lane 5. JS2007 cells (*sapA*$^{+ve}$, *rsaFa*$^{-ve}$, *rsaFb*$^{-ve}$ ) with reattached SapAΔP6, Lane 6. JS2013 cells with reattached SapAΔP6, Lane 7. JS2009 negative control after reattachment. (note: SapA protein is unable to reattach to cells because it gets processed into a 45-kDa band that is no longer able to reattach)

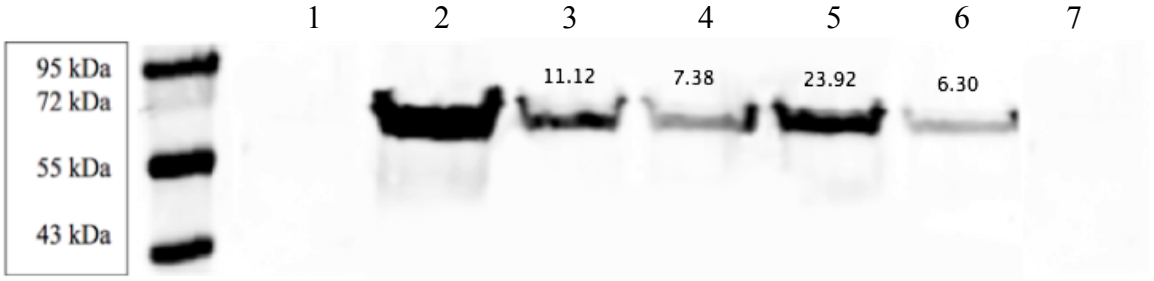


**Figure 3-24B. Quantified reattachment assay of SapAΔP6, isolated from low pH, to *C. crescentus* strains deficient in *manB, rsaFa/Fb*, or both**

Infrared western blot with anti-sap antibodies of low pH preparations normalized to $OD_{600}$ = 1.2. Lane 1. JS2009 (*sapA*$^{-ve}$) negative control before reattachment, Lane 2. JS2009 pn*sap*ΔP6 (*sapA*ΔP6$^{++ve}$) positive control before reattachment, Lane 3. JS2009 cells with reattached SapAΔP6, Lane 4. JS2011 cells (*sapA*$^{-ve}$, *manB*$^{-ve}$) with reattached SapAΔP6, Lane 5. JS2007 cells (*sapA*$^{+ve}$, *rsaFa*$^{-ve}$, *rsaFb*$^{-ve}$ ) with reattached SapAΔP6, Lane 6. JS2013 cells with reattached SapAΔP6, Lane 7. JS2009 negative control after reattachment.

### 3.9 Bioinformatics data confirm that SapA is part of the type I secreted serralysin-like subfamily of zinc-metalloproteases

In a sequence search using Blast for the conserved domains present in SapA, the protease was found to be part of the zinc-dependent metalloprotease sub-family and possess similarity to zinc-dependent metalloproteases of the serralysin_like subfamily. This group of proteins is secreted into the medium via a type I secretion mechanism found in Gram-negative bacteria, that does not require N-terminal signal sequences. Also this class of proteins has calcium-binding domains C-terminal to the metalloprotease domain, which contain multiple tandem repeats of a nine-residue motif including the pattern GGXGXD (RTX regions). These motifs form a parallel beta roll that may be involved in the translocation mechanism and/or substrate binding. Interestingly, SapA has four RTX regions downstream of its active site. The second conserved domain in SapA is to the peptidase M10 serralysin C-terminus. This C-terminal domain forms a corkscrew and is thought to be important for secretion of the protein through the bacterial cell wall. Also, this domain contains the calcium ion-binding domain. According to protein blast analysis, SapA possesses the conserved serralysin-like C-terminal domain between amino acids 69 to 264 (9e-37) (106).

The motifs database Prosite 20.41, detected a zinc-binding region signature of neutral zinc metallopeptidases in SapA. This zinc-binding site (VLVHELGHAI) is the active site of the protease. The predicted active site of *C. crescentus* SapA metalloprotease starting at amino acid 188 to 205 is VLVHELGHAIGIAHPSEY (105). As far as neutral zinc metallopeptidase families go, SapA appears to be classifiable under the M10A family whose other members include serralysin from *S. marcescens*, alkaline

protease AprA from *P. aeruginosa*, and type I secreted proteases A, B, C and G from *Erwinia chrysanthemi*.

Further evidence of SapA's similarity to serralysin-like zinc metalloproteases came from the Psipred database, which obtained the predicted secondary structure of SapA from its amino acid sequence. The most interesting piece of information gathered from the results was the presence of a potential beta-roll in the central part of the protease (~ amino acids 265 to 440). Within this region, SapA's four RTX motifs are each on a coiled region, surrounded on both sides by beta-strands, in a repeating manner. This finding is similar to published results of the parallel beta-roll present in alkaline protease AprA in *P. aeruginosa*. In AprA, the first six residues of each RTX motif form a turn, which binds calcium and the remaining three residues build a short beta-strand (50). This is the exact pattern seen in SapA from the Psipred results (107). Also, in AprA the consecutive beta-strands are connected in such a way that a right-handed helix of parallel beta-strands is formed. One turn of this helix consists of two consecutive nine-residue motifs.

Additional evidence to support SapA's relationship to these type I secreted proteins was provided by the protein fold recognition server Phyre 2.0, which is able to predict the 3-dimensional structure of a protein based on its amino acid sequence. The closest related proteins to SapA were indeed AprA, Serralysin, protease C (PrtC) and, the beta-roll (single-stranded right-handed beta-helix) from the C-terminal domain of Serralysin-like metalloproteases (30% - 39% i.d.) (102). Based on PSORTb 2.0, SapA was predicted to be an extracellular protein with homology to the outer membrane alkaline metalloprotease precursor (AP) in *Pseudomonas aeruginosa* (104).

SapA was aligned with some of these homologous proteins using Blast. When SapA (658 amino acids) was aligned with type I secreted alkaline metalloproteinase AprA from *P.aeruginosa* (479 amino acids) using bl2seq, amino acids 30 to 382 of SapA align with amino acids 21 to 374 of AprA (expect value = 2e-83). When SapA was aligned with type I secreted zinc-metalloproteinase Serralysin from *S. marcescens* (487 amino acids) using bl2seq, amino acids 40 to 375 of SapA align with amino acids 40 to 374 of Serralysin (expect value = 5e-66) (106).

When SapA (658 amino acids) was aligned with type I secreted RsaA (1026 amino acids) using bl2seq, two noteworthy alignments are produced. The first is amino acids 451 to 650 of SapA, which aligns with amino acids 23 to 242 of RsaA (expect value = 2e-19). Thus the C-terminus of SapA has homology to the N-terminus of RsaA. The second is amino acids 67 to 515 of SapA, which aligns with amino acids 608 to 1004 of RsaA (expect value = 0.022) (106). Although the e-value is low, this finding provides evidence that the C-terminus of RsaA and SapA have some similarities.

### 3.10 SapA uses its C-terminus to anchor to the cell surface of *C. crescentus*

In order to show that the C-terminus possesses all the information necessary for secretion and anchoring of SapA, various sized C-terminal clones were engineered (Figure 3-25B). The five C-terminal clones which were expressed in *C. crescentus* were the last 268 amino acids (SapA268c), the last 238 amino acids (SapA238c), the last 208 amino acids with an N-terminal c-myc tag (SapA208c-cmyc), the last 188 amino acids (SapA188c) and the last 100 amino acids with a C-terminal his6 tag (SapA100c-his6C). Figure 3-25A shows whole culture preparations of *C. crescentus* expressing all of the C-terminal clones, except SapA188c. All of the C-terminal clones engineered produced protein that was detectable by immunofluorescence on the cell surface of SLPS$^-$ *C. crescentus* cells (Figure 3-26).

SapA268C, SapA238C and SapA208-cmyc were detected in the supernatant of *C. crescentus* over-expressing these clones (Figure 3-27A). SapA208C-cmyc that possessed a foreign N-terminal 10 amino acid c-myc peptide (EQLISEEDL) was further confirmed to be sufficient for secretion using the S-layer type I secretion system as it was detected in the supernatant of an RsaFa/Fb wild type strain whereas, SapA208C-cmyc was no longer secreted in an RsaFa/Fb mutant (Figure 3-27B).

Additional confirmation of the use of SapA's C-terminus for anchoring was accomplished by low pH, which showed low levels of SapA286C and SapA238C were able to attach to the cell surface of *C. crescentus* at reduced levels compared to native SapA (Figure 3-28).

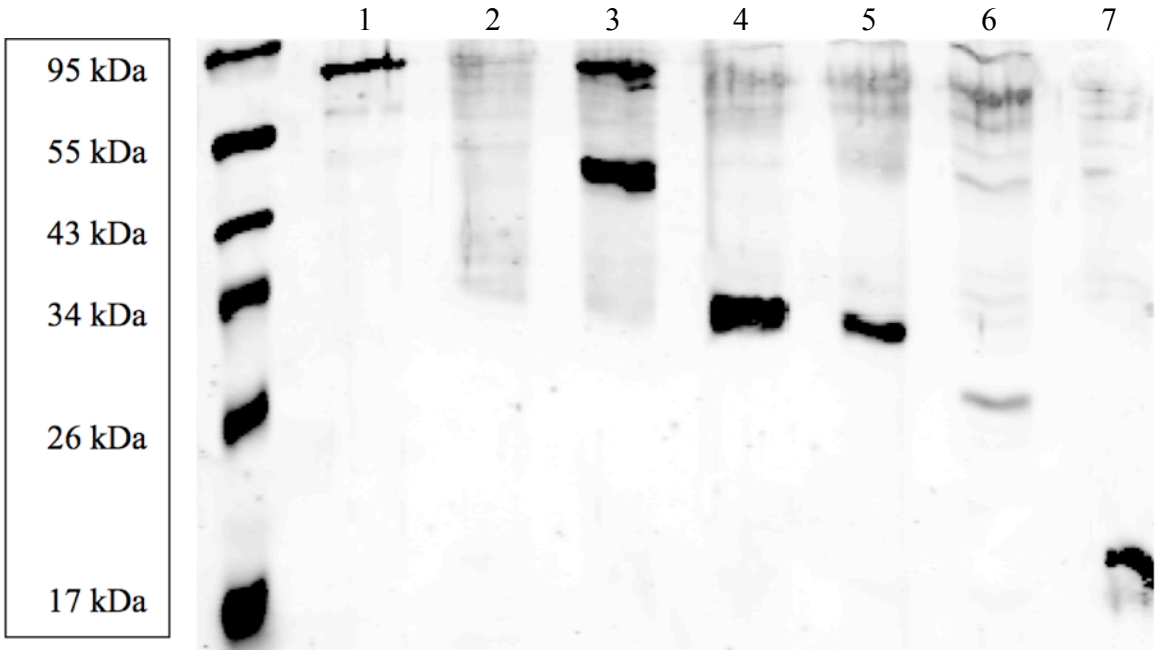


**Figure 3-25A. Expression of various sized C-terminal SapA clones**

Infrared western blot with anti-sap antibodies of whole culture protein preparations and normalized to $OD_{600}$ = 1.0. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, JS2009 cells (*sapA*$^{-ve}$); Lane 3, JS2003 pn*sap* cells (*sapA*$^{+ve}$); Lane 4, JS2009 pn*sap*268C cells (*sapA*268C$^{++ve}$); Lane 5, JS2011 pn*sap*238C cells (*sapA*238C$^{++ve}$); Lane 6, JS2009 pn*sap*208C-cmyc cells (*sapA*208C-cmyc$^{++ve}$); Lane 7, JS2009 pn*sap*100C-his6C cells (*sapA*100C-his6C$^{++ve}$).

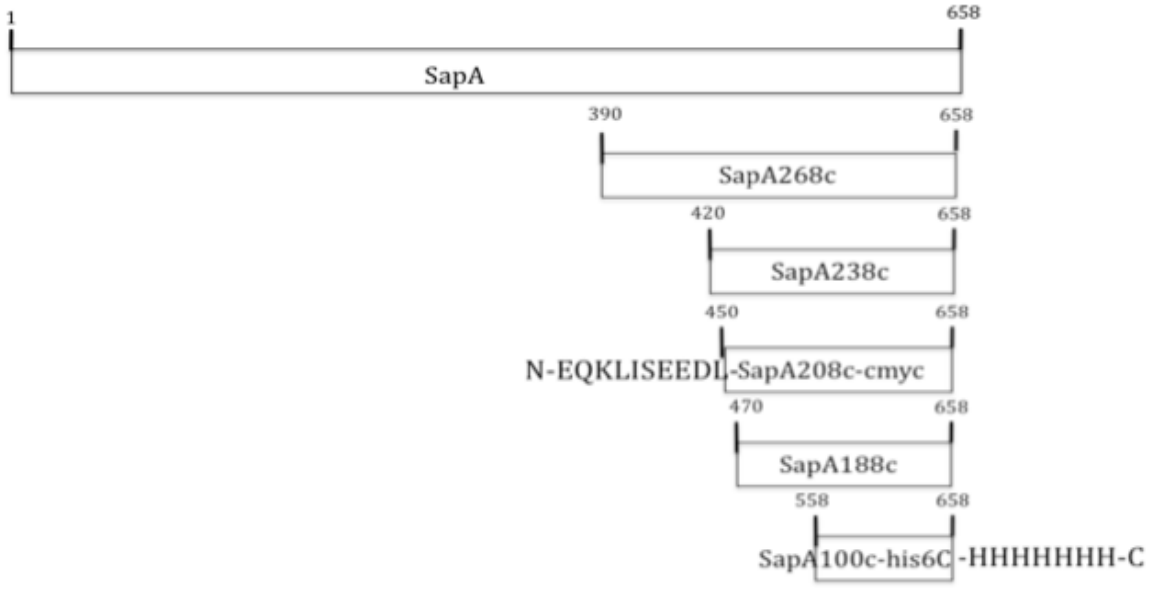


**Figure 3-25B. Schematic representation of SapA C-terminal clones**

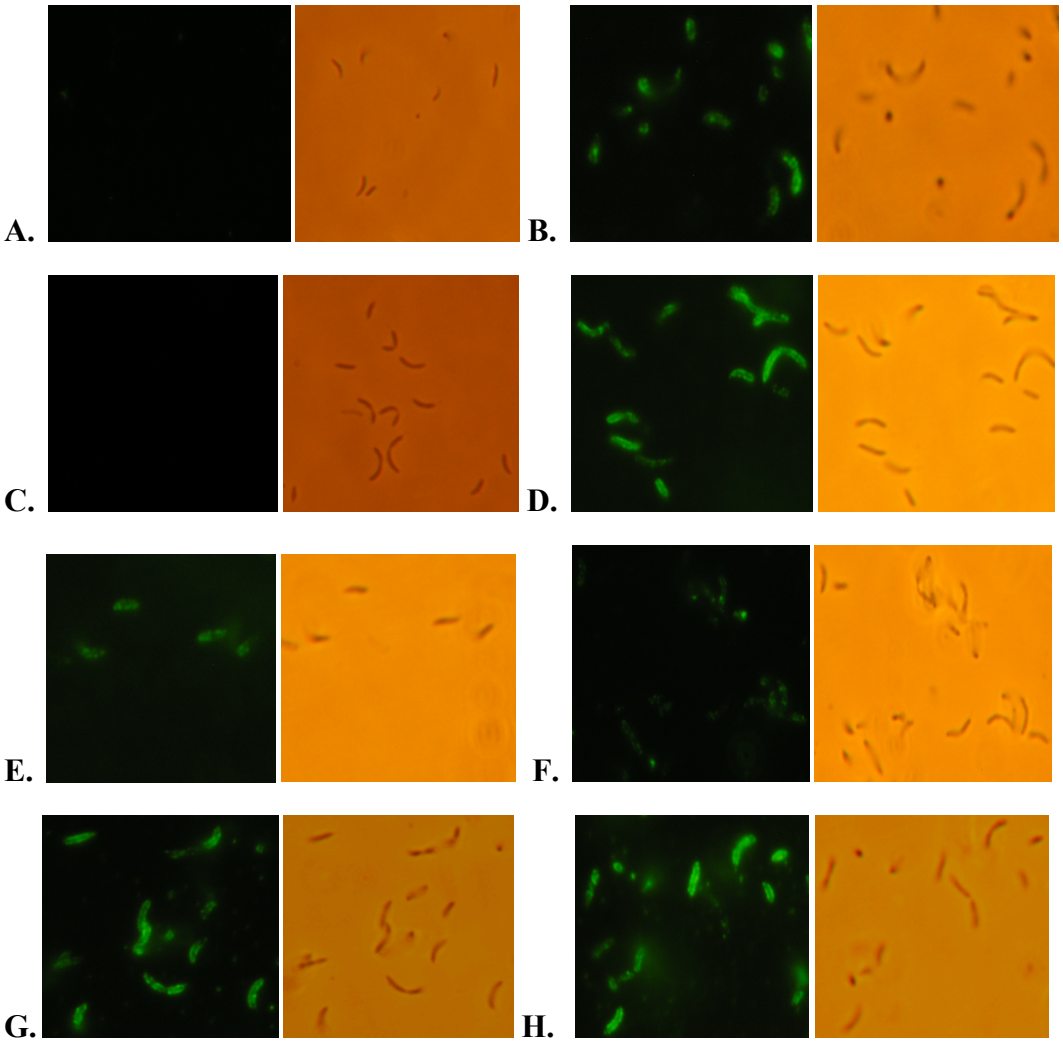


**Figure 3-26. Visualization of SapA on the cell surface of C-terminal clones**

Immunofluorescence pictures of *C. crescentus* using 1° SapA antibody and goat anti-rabbit Alexa Fluor 448 2° antibody. A. JS2011 cells (*sapA*$^{-ve}$, *manB*$^{-ve}$); B. JS2011 pn*sap*268C cells (*sapA*268C$^{++ve}$, *manB*$^{-ve}$); C. JS2013 pn*sap*268C cells (*sapA*268C$^{++ve}$, *manB*$^{-ve}$, *rsaFa*$^{-ve}$, *rsaFb*$^{-ve}$); D. JS2011 pn*sap*238C cells (*sapA*238C$^{++ve}$, *manB*$^{-ve}$); E. JS2011 pn*sap*208C-cmyc cells (*sapA*208C-cmyc$^{++ve}$, *manB*$^{-ve}$); F. JS2011 pn*sap*188C cells (*sapA*188C$^{++ve}$, *manB*$^{-ve}$); G. JS2011 pn*sap*100C-his6C cells (*sapA*100C-his6C$^{++ve}$, *manB*$^{-ve}$); H. JS2011 pn*sap* cells (*sapA*$^{++ve}$, *manB*$^{-ve}$).

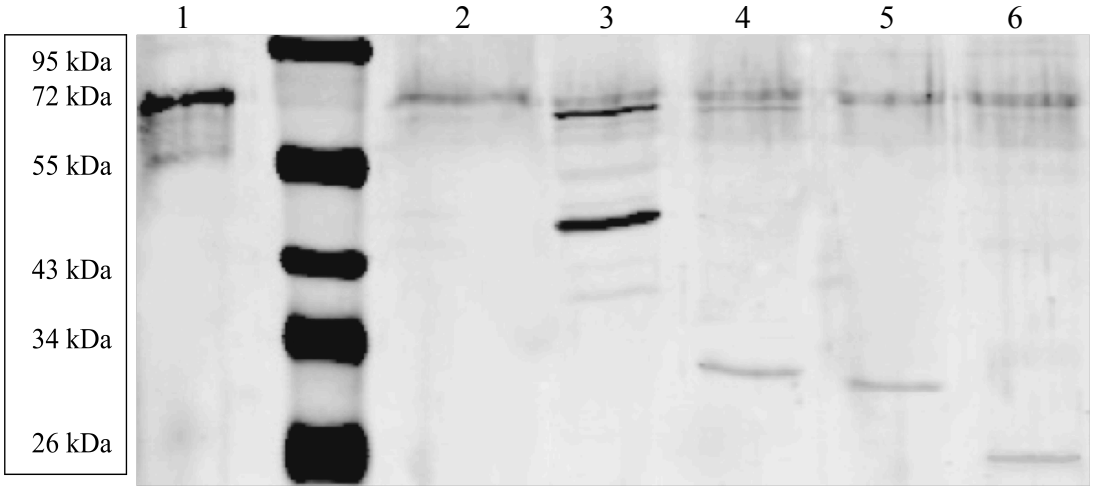


**Figure 3-27A. Secretion of SapA C-terminal clones**

Infrared western blot with anti-sap antibodies of supernatant of *C. crescentus* cells grown up in 50 ml, normalized to $OD_{600}$= 1.0 and concentrated using Centricon Plus-20 centrifugal filter devices from Millipore. Lane 1. *E.coli* Saphis6N control purified by Ni-NTA, Lane 2, JS2009 supernatant (*sapA*$^{-ve}$); Lane 3, JS2003 pn*sap* supernatant (*sapA*$^{++ve}$); Lane 4, JS2009 pn*sap*268C supernatant (*sapA*268C$^{++ve}$); Lane 5, JS2011 pn*sap*238C supernatant (*sapA*238C$^{++ve}$); Lane 6, JS2009 pn*sap*208C-cmyc supernatant (*sapA*208C-cmyc$^{++ve}$).

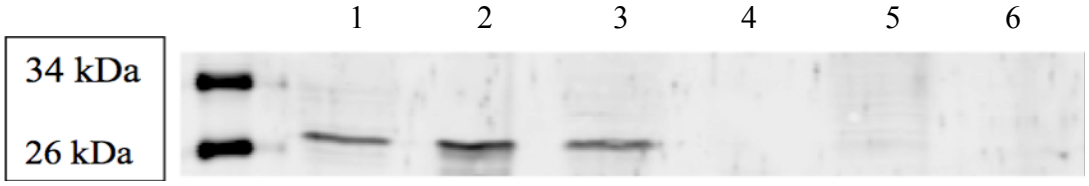


**Figure 3-27B. Expression and secretion of SapA208C-cmyc**

Infrared western blot with anti-sap antibodies of whole culture protein preparations and, supernatant of *C. crescentus* cells grown up in 50 ml, normalized to $OD_{600}$= 1.0 and concentrated using Centricon Plus-20 centrifugal filter devices from Millipore. Lane 1, JS2009 pn*sap*208C-cmyc cells (*sapA*208C-cmyc$^{++ve}$); Lane 2, JS2009 pn*sap*208C-cmyc supernatant (*sapA*208C-cmyc$^{++ve}$); Lane 3, JS2007 pn*sap*208C-cmyc cells (*sapA*208C-cmyc$^{++ve}$, *rsaFa*$^{-ve}$, *rsaFb*$^{-ve}$); Lane 4, JS2007 pn*sap*208C-cmyc supernatant (*sapA*208C-cmyc$^{++ve}$, *rsaFa*$^{-ve}$, *rsaFb*$^{-ve}$); Lane 5, JS2009 cells (*sapA*$^{-ve}$); Lane 6, JS2009 supernatant (*sapA*$^{-ve}$). c-myc tag = EQKLISEEDL.

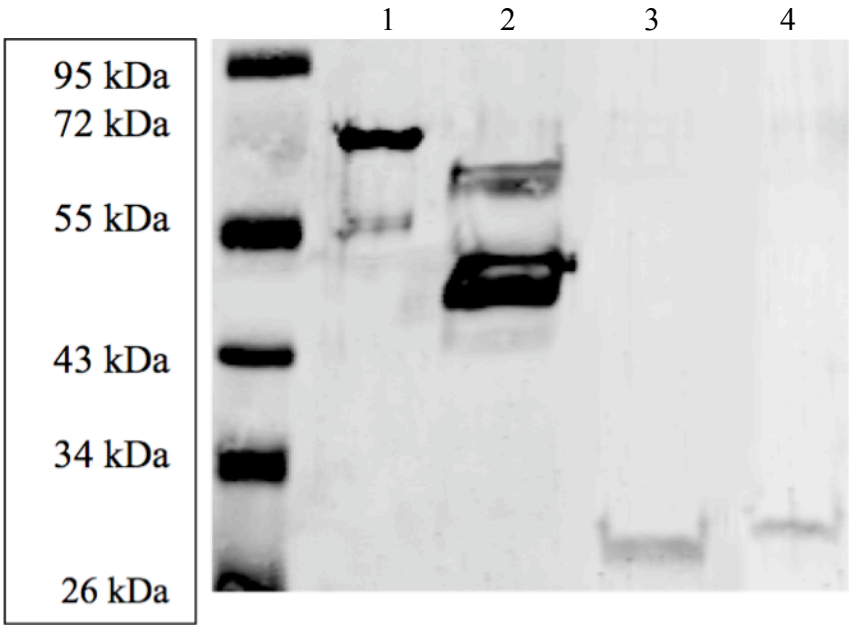


**Figure 3-28. Attachment of SapA and the last 238 and 268 amino acids of SapA**

Infrared western blot with anti-sap antibodies of low pH preparations, normalized to $OD_{600}$ = 0.8. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, JS2003 pn*sap* cells (*sapA*$^{++ve}$); Lane 3, JS2009 pn*sap*238C cells (*sapA*238C$^{++ve}$); Lane 4, JS2009 pn*sap*268C cells (*sapA*268C$^{++ve}$).

### 3.11 An N-terminal protein G (MGMGMGM) peptide fused to the last 238 amino acids of SapA is able to anchor to the cell surface of *C. crescentus*

A protein G fusion protein (242 amino acids) containing 3 GB1 (54 amino acids each) domains each flanked by Muc1 (20 amino acids) spacers, has been previously displayed on the cell surface of *C. crescentus* using the S-layer display type I system in RsaA at amino acid 723. High levels of the recombinant protein were displayed on the cell surface of *C. crescentus* (60). In order to determine if SapA could also display the protein G peptide, a recombinant clone containing the 242 amino acid protein G peptide was fused to the last 238 amino acids of SapA and tested for secretion and anchoring.

Faint fluorescence of JS2011 pnMGMGMGM*sap*238C and JS1024 pnMGMGMGM*sap*238C was observed whereas fluorescence of JS4019 p4A723ΔMGMGMGM displayed an intense fluorescence of *C. crescentus* cells (Figure 3-29). Pictures of JS2011 pnMGMGMGM*sap*238C and JS1024 pnMGMGMGM*sap*238C cells were taken using a longer 8 second exposure.

Unfortunately, standard low pH extractions using 10 ml of *C. crescentus* pnMGMGMGM*sap*238C clones were undetectable. In order to increase protein concentration, 50 ml low pH extractions were performed to concentrate the proteins and MGMGMGMSapA238C was detected using only the Alexa Fluor 680 2° antibody via infrared western (Figure 3-30).

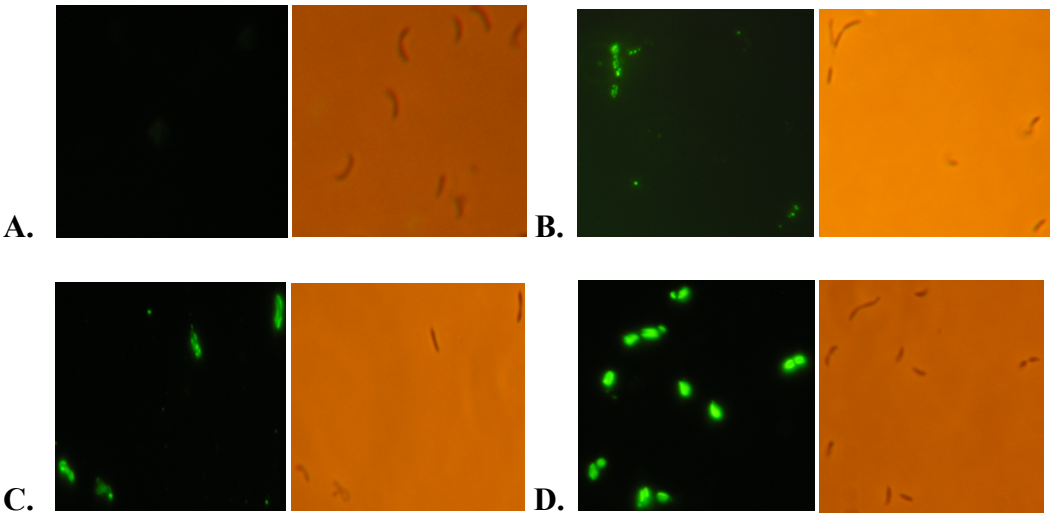


**Figure 3-29. Immunofluorescence of the MGMGMGM peptide in *C. crescentus***

Immunofluorescence pictures of *C. crescentus* using goat anti-rabbit Alexa Fluor 448 2° antibody. A. JS2009 pnMGMGMGM*sap*238C cells (*sapA*$^{-ve}$, *MGMGMGMsap238C*$^{+ve}$, *manB*$^{+ve}$, O-antigen$^{+ve}$); B. JS2011 pnMGMGMGM*sap*238C cells(*sapA*$^{-ve}$, *MGMGMGMsap238C*$^{+ve}$, *manB*$^{-ve}$); C. JS1024 pnMGMGMGM*sap*238C cells (O-antigen$^{-ve}$, *MGMGMGMsap238C*$^{+ve}$); D. JS4019 p4A723ΔMGMGMGM cells (*manB*$^{+ve}$, O-antigen$^{+ve}$).

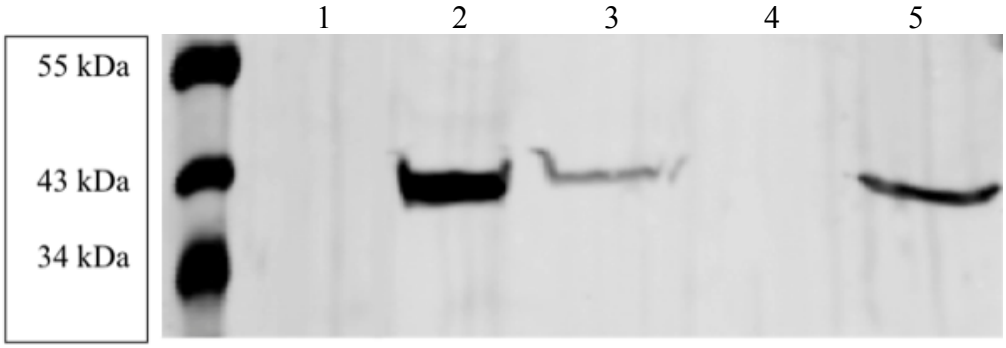


**Figure 3-30. Attachment of MGMGMGMSapA238c fusion to *C. crescentus* cell surface**

Infrared western blot with anti-Alexa Fluor 2° 680 antibodies of 50 ml low pH preparations normalized to $OD_{600}$ = 0.7. Lane 1, JS2009 cells (*sapA*$^{-ve}$, *manB*$^{+ve}$, O-antigen$^{+ve}$); Lane 2, JS2009 pnMGMGMGM*sap*238c cells (*sapA*$^{-ve}$, *MGMGMGMsap238C*$^{+ve}$, *manB*$^{+ve}$, O-antigen$^{+ve}$); Lane 3, JS2011 pnMGMGMGM*sap*238c cells (*sapA*$^{-ve}$, *MGMGMGMsap238C*$^{+ve}$, *manB*$^{-ve}$); Lane 4, JS1024 cells (O-antigen$^{-ve}$); Lane 5, JS1024 pnMGMGMGM*sap*238c cells (*sapA*$^{-ve}$, *MGMGMGMsap238C*$^{+ve}$, O-antigen$^{-ve}$).

### 3.12 SapA over-expressed in a *manB* mutant strain is not processed

As previously described, SapA could only be detected on the cell surface on a *manB* mutant or an O-antigen (Ca5) mutant (Figure 3-31). The *manB* mutant (JS2011) prevents the formation of the O-antigen of SLPS and the formation of exopolysaccharide (EPS), whereas a Ca5 (JS1019) mutant solely prevents the formation of the O-antigen of SLPS. JS1025 on the other hand is believed to be an EPS mutant. JS2011 pn*sap* cells appear very clumpy and their pellet is very spread out, while JS1019 pn*sap* cells are not clumpy and their pellet is not spread out, similar to wild type *C. crescentus*. Similar to JS2003 pn*sap*, the concentrated supernatant of JS1019 pn*sap* produced a processed product 45-kDa in size, however, in JS2011 pn*sap*, SapA was not processed to the same extent (Figure 3-32A). The EPS mutant, JS1025 pn*sap* was also processed into a 45-kDa product in the supernatant (Figure 3-32B). Whereas SapA isolated from the cell surface of a *manB* mutant of *C. crescentus* was not processed (Figure 3-33A).

In order to determine if the activity of SapA on recombinant RsaA was affected by these mutations, proteolytic activity tests were performed on their concentrated supernatants. Interestingly, JS1019 pn*sap* concentrated supernatant maintained the proteolytic activity of SapA (Figure 3-34A). However, SapA from JS2011 pn*sap* concentrated supernatant lost its proteolytic activity (Figure 3-34B).

Finally, the last change that was observed between *manB* mutants and non-*manB* mutant strains of *C. crescentus* was the level of protein that was isolated from their cell surface by low pH extraction. There was 0.56-fold reduction of protein isolated from *manB* mutant JS2011 pn*sap* compared to JS2003 pn*sap* (Figure 3-33 B).

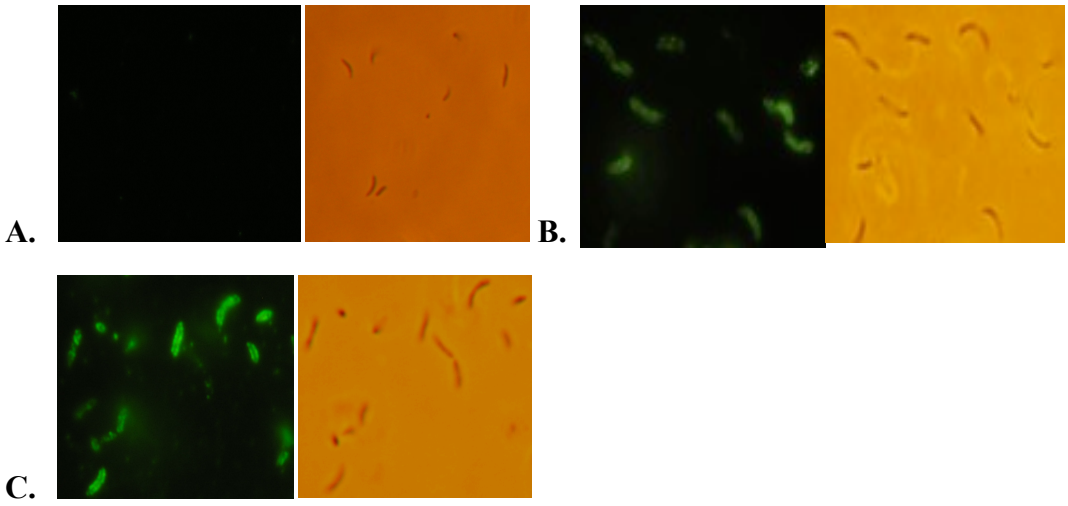


**Figure 3-31. Visualization of SapA in O-antigen mutant vs *manB* mutant on the cell surface of *C. crescentus***

Immunofluorescence pictures of *C. crescentus* using 1° SapA antibody and goat anti-rabbit Alexa 448 2° antibody. A. JS2011 cells ($sapA^{-ve}$,$manB^{-ve}$); B. JS1019 pn*sap* cells ($sapA^{++ve}$, O-antigen$^{-ve}$); C. JS2011 pn*sap* cells ($sapA^{++ve}$,$manB^{-ve}$).

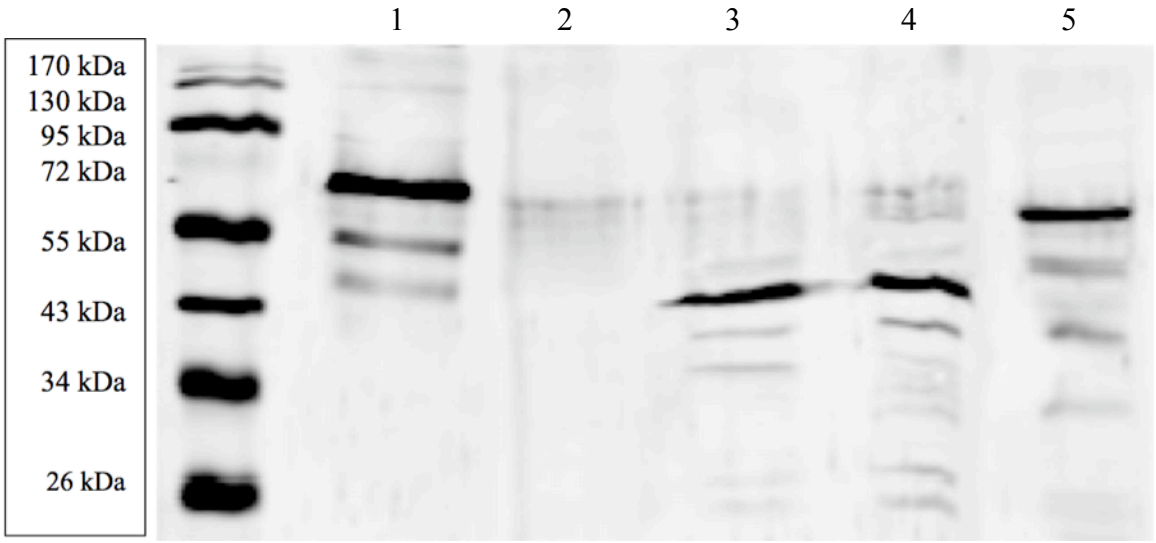


**Figure 3-32A. Processing of SapA in the supernatant**

Infrared western blot with anti-sap antibodies of supernatant of *C. crescentus* cells grown up in 50 ml, normalized to $OD_{600}$ = 1.0 and concentrated using Centricon Plus-20 centrifugal filter devices from Millipore. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, JS2009 supernatant (*sapA*$^{-ve}$); Lane 3, JS2003 pn*sap* supernatant (*sapA*$^{++ve}$); Lane 4, JS1019 pn*sap* supernatant (*sapA*$^{++ve}$, O-antigen$^{-ve}$); Lane 5, JS2011 pn*sap* supernatant (*sapA*$^{++ve}$, *manB*$^{-ve}$).

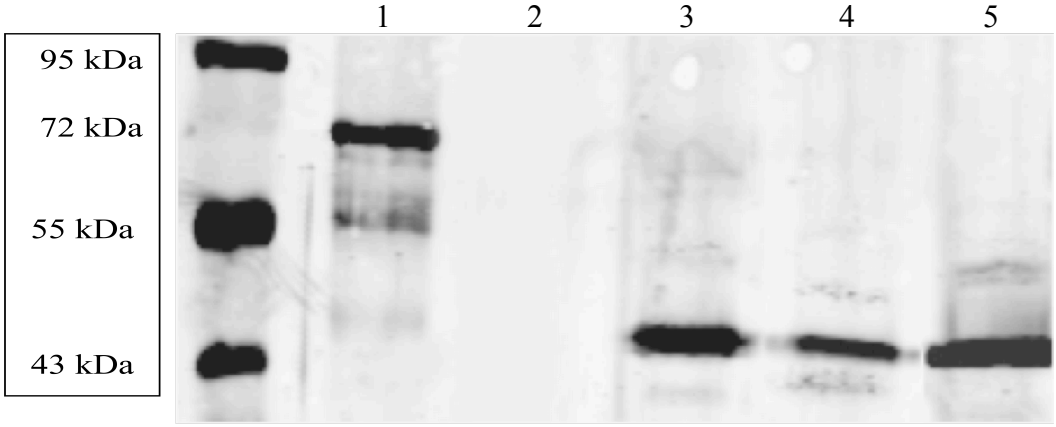


**Figure 3-32B. Processing of SapA in the supernatant of an EPS mutant**

Infrared western blot with anti-sap antibodies of supernatant of *C. crescentus* cells grown up in 50 ml, normalized to $OD_{600}$ = 1.0 and concentrated using Centricon Plus-20 centrifugal filter devices from Millipore. Lane 1, *E.coli* Saphis6N control purified by Ni-NTA; Lane 2, JS2003 supernatant (*sapA*$^{+ve}$); Lane 3, JS2003 pn*sap* supernatant (*sapA*$^{++ve}$); Lane 4, JS1019 pn*sap* supernatant (*sapA*$^{++ve}$, O-antigen$^{-ve}$); Lane 5, JS1025 pn*sap* supernatant (*sapA*$^{++ve}$, EPS$^{-ve}$).

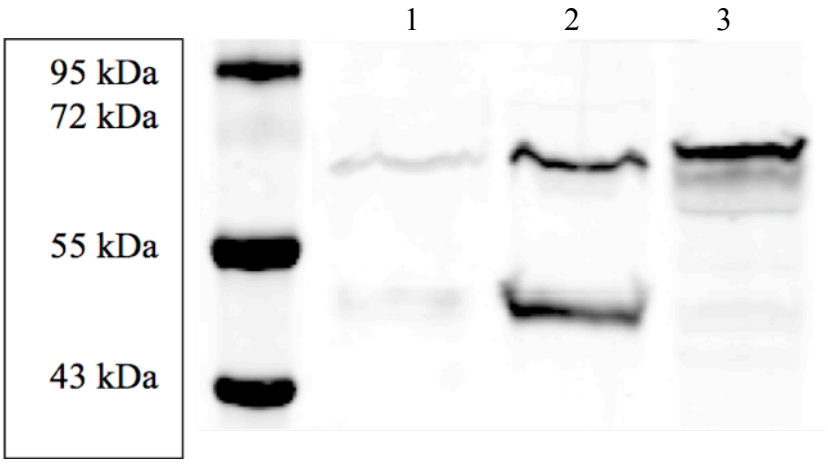


**Figure 3-33A. Attachment of SapA in a *manB* deficient strain**

Infrared western blot with anti-sap antibodies of low pH preparations normalized to $OD_{600}$ = 1. Lane 1, JS2009 cells (*sapA*$^{-ve}$); Lane 2, JS2003 pn*sap* cells (*sapA*$^{+ve}$); Lane 3, JS2011 pn*sap* cells (*sapA*$^{++ve}$).

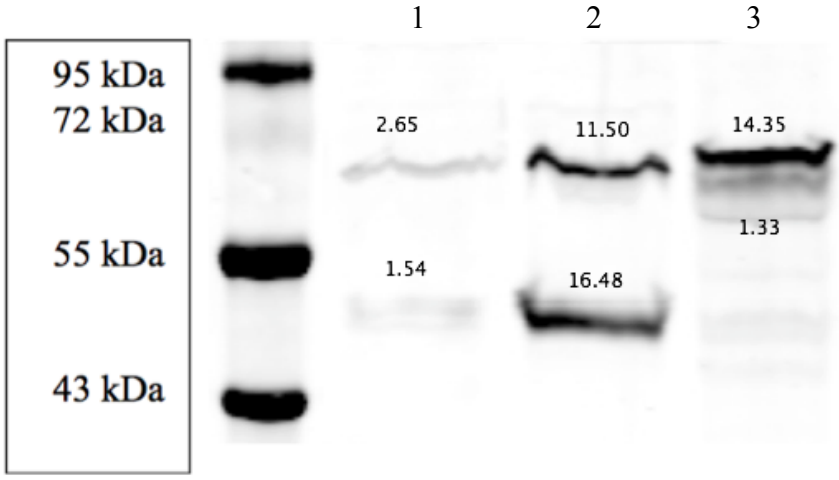


**Figure 3-33B. Quantified attachment of SapA in a *manB* deficient strain**

Infrared western blot with anti-sap antibodies of low pH preparations normalized to $OD_{600}$ = 1. Lane 1, JS2009 cells (*sapA*$^{-ve}$); Lane 2, JS2003 pn*sap* cells (*sapA*$^{+ve}$); Lane 3, JS2011 pn*sap* cells (*sapA*$^{++ve}$). Notice the reduction by half (0.56) of the amount of protein produced by the *manB* mutant.

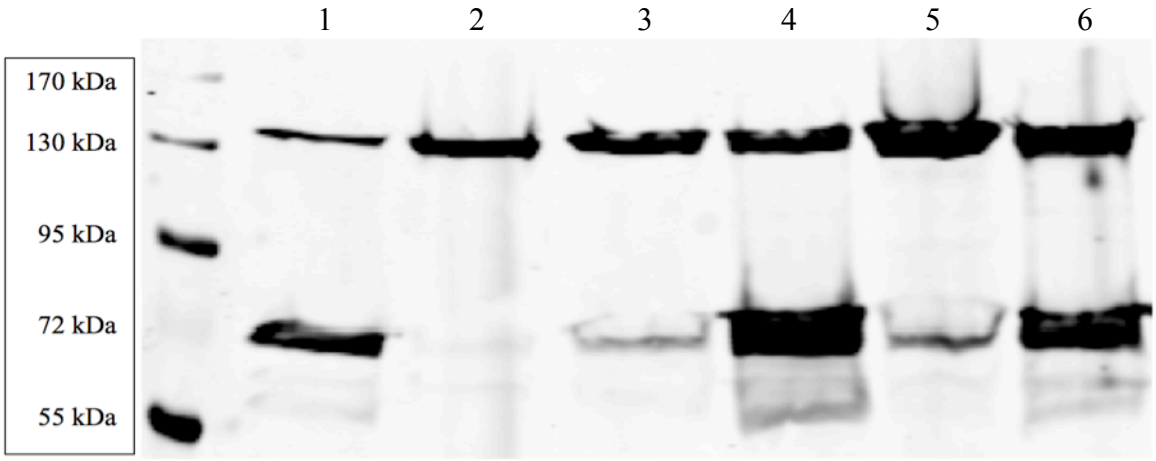


**Figure 3-34A. Proteolytic activity tests from the supernatant of SapA in JS1019**

Infrared western blot with polyclonal anti-188/784 RsaA antibodies of low pH preparations. Lane 1, CB15ΔrsaA pwB9M13(450)PE3Δ cells positive control; Lane 2, JS2008 pwB9M13(450)PE3Δ cells negative control; Lane 3, JS2009 supernatant (*sapA*$^{-ve}$) incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells; Lane 4, JS2003 pn*sap* supernatant (*sapA*$^{++ve}$) incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells; Lane 5, JS1019 (*sapA*$^{+ve}$, O-antigen-) supernatant incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells; Lane 6, JS1019 pn*sap* (*sapA*$^{++ve}$, O-antigen$^{-ve}$) supernatant incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells.

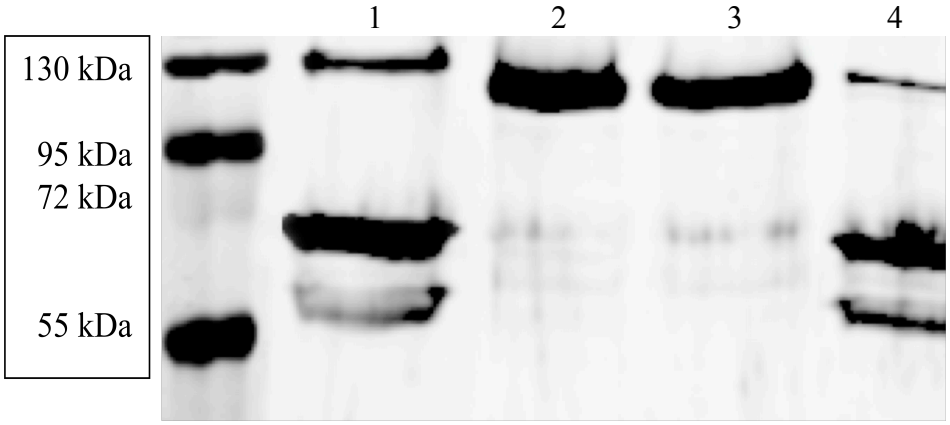


**Figure 3-34B. Proteolytic activity tests from the supernatant of SapA in JS2011**

Infrared western blot with polyclonal anti-188/784 RsaA antibodies of low pH preparations. Lane 1, JS2003 pn*sap* supernatant (*sapA*$^{++ve}$) incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells; Lane 2, JS2011 pn*sap* (*sapA*$^{++ve}$, *manB*$^{-ve}$) supernatant incubated with CB15ΔrsaA pwB9M13(450)PE3Δ cells; Lane 3, JS2008 pwB9M13(450)PE3Δ cells negative control; Lane 4, CB15ΔrsaA pwB9M13(450)PE3Δ cells positive control.

### 3.13. SapA may bind to a 27-kDa molecule on far western from cell membrane preparations of JS2009

To attempt to identify the protein or molecule that SapA anchors to on the cell surface of *C. crescentus*, far westerns using SapAΔP6 isolated from low pH extraction were performed on cell membrane preparations of JS2009. The cell membrane preparations were treated with various detergents to separate the soluble and insoluble proteins present: 2% TritonX-100/CHAPSO, 2% TritonX-100, 2% Zwittergent TM314, 2% *n*-octyl-β-D-glucoside**,** 2% sodium deoxycholate, 2% sodium-lauroyl sarcosinate, and 2% sodium dodecyl sulfate. SapAΔP6 bound a unique 27-kDa band in the soluble fraction of the cell membrane preparations most notable in those treated with 2% Zwittergent TM314, 2% sodium deoxycholate, 2% sodium-lauroyl sarcosinate, and 2% sodium dodecyl sulfate. No significant bands were detected from the insoluble cell membrane fractions. Unfortunately, other attempts to purify cell membrane preparations treated with these detergents could not reproduce this result. Further, PBS/EDTA extracted proteins and a modified cell membrane preparation with an extra ultracentrifugation spin could not detect the 27-kDa band.

# 4 DISCUSSION AND CONCLUSION

Over the past decade, little was known about the metalloprotease SapA, which is responsible for the unwanted outcome of cleaving certain recombinant RsaA proteins. In this study, we determined that SapA is a self-processing enzyme that uses its C-terminus for type I secretion by the S-layer secretion system and for anchoring to the cell surface of *C. crescentus*. Further, we were able to use SapA's C-terminus for secretion and display of protein G IgG binding domains. Unlike RsaA, SapA can display proteins in SLPS mutants of *C. crescentus*. This work provides evidence that SapA may be used as a display system, which solely requires its C-terminus for both secretion and anchoring.

## 4.1 SapA is a zinc-dependent metalloprotease

Prior to this work, SapA was not detectable and its characterization was mainly based on bioinformatics data and preliminary experiments involving examining loss of proteolytic cleavage of the recombinant RsaA proteins. In fact, in a paper published in 2002, Umelo-Njaka *et al.* suggested that SapA is likely an internal protease that cleaves recombinant proteins prior to secretion (93). This was determined based on bioinformatic analysis, which could not find a secretion signal for SapA. This notion was later contested by Ford *et al.* who indirectly showed that SapA might be secreted because purified recombinant RsaA protein incubated with *C. crescentus* cells could only have been cleaved by SapA if the protease was present extracellularly (32). In this study, we were able to characterize SapA as part of the zinc-dependent metalloproteases of the serralysin_like subfamily (amino acids 69 to 264 of SapA). This group of proteins is secreted into the medium via a mechanism found in Gram-negative bacteria that does not require N-terminal signal sequences. SapA can be further classified under the M10A

family whose other members include Serralysin from *S. marcescens*, alkaline protease AprA from *P. aeruginosa*, and type I secreted proteases PrtA, PrtB, PrtC and PrtG from *Erwinia chrysanthemi*. All these proteins are secreted by a type I secretion mechanism. Although there is substantial homology between the components of these pathways, the C-terminal secretion signal sequences of these proteins are quite different (23). Furthermore, these proteases that are homologous to SapA are released into the supernatant and do not anchor to the cell surface. This makes SapA a unique serralysin-like metalloprotease that is able to remain attached to the cell surface of *C. crescentus*.

### 4.2 A complete knockout of *sapA* can no longer process recombinant recombinant RsaA

Another setback to understanding SapA function was the lack of a complete knockout of the protein. Mutants of SapA made in the past significantly knocked down or inhibited the processing of recombinant RsaA. However, SapA continued to be expressed in these mutants and SapA was still detectable on the surface of *C. crescentus*. Thus a proper negative control was never established to study the protease. In this study, a complete knockout of SapA was constructed. Knocking out *sapA* resulted in a loss of processing of recombinant RsaA proteins such as RsaA with a pilin epitope insertion (TSDQDEQFIPKG) at amino acid 450 of RsaA.

### 4.3 SapA is processed into 67-kDa and 45-kDa bands

Since SapA was undetected under wild type conditions, the protease needed to be over-expressed. Based on examination of the DNA and amino acid sequences of SapA, the expected size of the protease was 71 kDa. However, over-expression of SapA in *C. crescentus* resulted in the production of 67-kDa and 45-kDa products detectable by

infrared western analysis. The same 45-kDa secretion product of over-expressed SapA was found in both S-layer positive and S-layer negative strains of *C. crescentus*. An explanation for the processing is that since an excess amount of SapA is produced by over-expression, the protein tends to aggregate together and cut adjacent SapA molecules. Immunofluorescence data supports this conclusion since the protein aggregation created the distinct spotty fluorescence that was detected. Furthermore, over-expression of SapA with active site mutations resulted in very clumpy cells that aggregate to each other.

During immunofluorescence studies, there was often some particulate label found unassociated with the cells. This spotty fluorescence is likely processed SapA (~45 kDa) released into the supernatant at low levels (0.6%). Since under native conditions in *C. crescentus*, SapA is expressed at almost undetectable levels, it is likely that there are fewer sites available for SapA to attach when over-expressed. Moreover, because there are limited locations on the cell surface for the protein to bind, a small portion may get released into the supernatant.

Mass spectrometry analysis of the 45-kDa cleaved protein from the concentrated supernatant determined that this product constituted the N-terminal portion of the protease. Based on this data, the predicted cleavage site of the protease is in the C-terminal region of SapA at approximately amino acid 433, 45 kDa after the start of the protein. Interestingly, the C-terminal region of SapA (amino acids 451 to 650) is homologous to the N-terminus of RsaA (amino acids 23-242), which is also subject to proteolytic cleavage of the protease when RsaA is produced as a recombinant protein

(93). Thus it is possible that the sequence recognized by SapA in recombinant RsaA is similar to the sequence recognized by SapA for self-processing.

Proteolytic activity tests of *C. crescentus* over-expressing SapA demonstrated that the concentrated supernatant whose major component contains the 45-kDa processed product was able to maintain its activity. This confirms that it is the N-terminal portion of the protease since the active site is located N-terminally. However, when the proteolytic activity of SapA isolated from *E. coli* was examined, no activity was found. Thus, this suggests that SapA needs to be expressed in its native host, *C. crescentus* to become a functionally active protease.

### 4.4 SapA is a self-processing enzyme

Self-processing of SapA was confirmed by the almost complete loss of processing in the proteolytically deficient version of the protease SapAΔP6, which contains a point mutation beside the protease's active site. One observation from the SapAΔP6 (67 kDa) is that although this protein is not being processed into the 45-kDa band, it was still ~ 4 kDa smaller than the *E. coli* SapAhis6N control (71 kDa). The his6 tag alone cannot account for the change in size observed. It became apparent that there must be another processing event that SapA undergoes. From the mass spectrometry data, the first 44 amino acids were not detected which suggested that they were cleaved during or after secretion of the protease. Thus, SapAΔP6 remained in this intermediate state of 67 kDa in size, where it was unable to self-process. This helps explain the difference in size observed between the SapAhis6N *E. coli* control and SapAΔP6 isolated from low pH extractions or concentrated supernatant preparations of *C. crescentus*. Another protease is

likely responsible for the N-terminal processing since the loss of proteolytic activity of SapA does not prevent it from occurring.

It is unlikely that it is the C-terminal end of SapA that is processed since the C-terminus is required for anchoring and secretion. An N-terminal processing event would help explain why purification of SapAhis6N in *C. crescentus* was not possible by Ni-NTA. This N-terminal processing is thought to be specific to *C. crescentus*, as SapA purified from *E. coli* does not get processed. The same N-terminal processing can be found in SapA homologs such as *P. aeruginosa* AprA protease, *S. marcescens* Serralysin protease, and *E. chrysanthemi* PrtB and PrtC proteases (22, 57). These serralysin zinc-metalloproteases have the first N-terminal amino acids cleaved after transmembrane translocation. These proteases do not anchor to the cell surface; instead they are released into the extracellular space after secretion and N-terminal processing. The N-terminal propeptide of zinc-metalloproteases has been suggested to play a role in folding of the proenzyme or, it may temporarily anchor the protease to the outer membrane (39). N-terminal sequencing of SapA is currently under way to determine where the exact N-terminal processing site in SapA is located.

### 4.5 SapA is secreted using the S-layer type I secretion system

Mutating the cysteine to an alanine disproved the idea that SapA uses its first cysteine for secretion and anchoring as a lipoprotein. This point mutation does not prevent secretion or anchoring of the protease. Instead, it was determined that SapA uses its C-terminus for secretion via the S-layer type I secretion system. It was shown that the C-terminus of SapA, whether it is the last 10 or last 50 amino acids of the protease, is required for secretion into the supernatant. Furthermore, SapA requires RsaFa and RsaFb

to be released into the supernatant. This suggests that the S-layer type I secretion system outer membrane proteins RsaFa and RsaFb play a role in SapA secretion.

An interesting finding through examination of the SapA type I secretion deficient clones (ie. SapA in an RsaFa/Fb mutant of *C. crescentus*, SapAΔ10C, and SapAΔ50C) was that they are not processed at all, unlike SapA in a wild type strain of *C. crescentus*. Even the extreme N-terminal portion of the protease appeared to not be processed as these clones ran at their expected size. Thus, SapA needs to be secreted before it can be processed N-terminally, folded correctly and assume its proteolytic activity. Two examples of zymogens that are known to undergo N-terminal processing after secretion into the external medium are Prt B and Prt C from *E. chrysanthemi* have a short amino-terminal propeptide; 15 amino acids for PrtB and 17 amino acids for PrtC. This prevents any unwanted activity of the proteases prior to secretion (22, 97).

The lack of observed processing may explain why SapA purified from inclusion bodies of *E. coli* does not have any proteolytic activity. Based on studies of PrtB and PrtC in *E. coli*, it has been shown that these proteases accumulate as zymogens within the *E. coli* cells, which are two kDa larger than the mature enzymes purified in *E. chrysanthemi* (97). Thus, these proteases need to be expressed in their native host for removal of the propeptide. Furthermore, SapA is clearly dependent on the S-layer type I secretion system in *C. crescentus* and, as was demonstrated, interruption of secretion by this system blocks the protein's proteolytic and self-processing capabilities.

One possible explanation for why SapA is not active until it is secreted is due to its requirement for $Ca^{2+}$ binding which can only be accessed extracellularly. SapA has four RTX motifs and $Ca^{2+}$ is known to bind to the RTX regions for proper folding, a

phenomenon that has been observed in other RTX containing proteins (50, 93). Thus, SapA from the RsaFa/Fb mutant as well as SapAΔ10C and SapΔ50C were unable to fold correctly and assume proteolytic activity since they could no longer get secreted into the supernatant and interact with $Ca^{2+}$. As a result no N-terminal processing or self-cleavage was observed. A similar result was found for HlyA from *E. coli*, where lack of extracellular $Ca^{2+}$ adversely affected the folding and activity of HlyA (64).

Immunofluorescence data of SapA determined that the protease was only visible on the cell surface in an SLPS negative, RsaA negative strain of *C. crescentus*. Thus, it appears that SapA is localized on the cell surface beneath both the SLPS and RsaA. Furthermore, SapA consistently exhibited a spotty fluorescence on the cell surface, which seems to indicate that the protease bound to specific areas on the cell surface. A possible location was attachment to RsaFa or RsaFb, because these were the last components of the S-layer type I secretion system with which SapA interacted on the outer membrane. However, anchoring of SapA to RsaFa/Fb was determined not to be the case by a SapA reattachment assay to an *rsaFa-/rsaFb- C. crescentus* strain. This was accomplished by incubating purified SapAΔP6 protein from low pH extractions with *C. crescentus* strains. Here it was shown that SapAΔP6 could reattach to the cell surface of *C. crescentus* in strains negative for *rsaFa-/rsaFb-*, *manB* or both *rsaFa-/rsaFb-* and *manB*. Since SapA was able to reattach to an *rsaFa/rsaFb-*, *manB- C. crescentus* strain this supported immunofluorescence data that SapA does not require SLPS for attachment.

### 4.6 SapA uses its C-terminus for anchoring to the cell surface of *C. crescentus*

The C-terminus of SapA was found to be required for the anchoring of the protease to the cell surface of *C. crescentus*. In an *rsaFa-/rsaFb-* strain of *C. crescentus,*

the C-terminal clones were not secreted as was demonstrated by the clone of the last 208 amino acids of SapA with an N-terminal c-myc tag (EQLISEEDL). This provided further evidence that the C-terminus is required for type I secretion through the S-layer system. The clone containing the last 100 amino acids of SapA with a C-terminal his6 tag displayed a similar intensity during immunofluorescence studies as full-length SapA. Thus not only did the last 100 amino acids contain all the necessary information for secretion, but it was also found to be sufficient for anchoring of SapA to the cell surface of *C. crescentus*. Furthermore, this construct did not contain any RTX motifs, yet it was still able to get secreted. A similar effect was seen in *E. chrysanthemi* PrtB, where truncated versions lacking the RTX region were still secreted (35).

The his6 tag located at the extreme C-terminus of the last 100 amino acids of SapA did not disrupt recognition and transport by the S-layer type I secretion system of *C. crescentus*. This shows that the his6 tag does not disrupt the structural presentation of the C-terminal secretion system to RsaD, the inner membrane component of the S-layer type I secretion system. Similarly, the S-layer type I secretion system was able to withstand secretion of the SapA208 that possessed a foreign N-terminal 10 amino acid c-myc peptide. Thus short peptides can be added to either the C-terminus or the N-terminus of a SapA C-terminal clone without disruption of secretion and anchoring. This is useful as tags can be added to help purify recombinant SapA proteins.

### 4.7 SapA can secrete and anchor a 242 amino acid protein G peptide

The effectiveness of using the C-terminus of SapA for secreting and anchoring foreign peptides was tested further by creating a fusion protein containing a N-terminal protein G-muc1 peptide (242 amino acids) was fused to the last 238 amino acids of SapA.

Unlike RsaA, which requires two separate domains for secretion and attachment, the C-terminus and N-terminus respectively, SapA was determined to contain both its secretion and attachment information solely in the C-terminus. Low levels of recombinant MGMGMGMSapA238C were detected by immunofluorescence in SLPS and *manB*-strains of *C. crescentus* and, protein was isolated from low pH extraction scaled up 5X. Thus, *C. crescentus* was able to secrete and anchor a 242 amino acid foreign protein G peptide fused to the last 238 amino acids of SapA. Additionally, while it is not the case for RsaA, SapA could display recombinant proteins in SLPS and *manB* mutants of *C. crescentus*.

### 4.8 SapA is not processed in a *manB* mutant

SLPS is a macromolecule associated with the outer membrane of Gram-negative bacteria. It is composed of three main components; the lipid A region which contains the hydrophobic membrane anchoring region of LPS, the core polysaccharide region which contains the unusual sugars 2-keto-3-deoxyoctonoic acid (KDO) that are used for detection in endotoxin assays and, the O-antigen which is attached to the core polysaccharide and is the hydrophilic domain of the LPS molecule (89). The SLPS mutants used for this study are of two types: the O-antigen mutant where O-antigen was no longer produced and the second was a *manB* mutant where O-antigen and exopolysaccharide were not produced. Phosphomannomutase, the enzyme that is encoded by *manB*, is part of a specific family of isomerases that transfers phosphate groups within in a molecule. In this case, phophomannomutase reversibly converts α-D-mannose-1-phosphate to D-mannose-6-phosphate (80). These molecules are involved mainly in the following pathways: the production of the nucleotide sugar perosamine required to form

the O-antigen in SLPS, the production of GDP-L-fucose required to form exopolysaccharide (EPS), the production D-rhamnose that is a component of LPS and EPS in Gram-negative bacteria and, the production of 6-deoxy-D-talose, a rare deoxyhexose that is a constituent of cell wall and capsule structures (52).

Both SLPS mutants in this study, the *manB* mutant and the O-antigen mutant were able to expose SapA on the cell surface of *C. crescentus*. However, the difference arose in the processing of SapA in these two strains. Upon examination of the supernatant, over-expressed SapA continued to get processed in the O-antigen mutant whereas SapA was not processed at all in the *manB* mutant. Based on this data, it seems that the absence of the O-antigen alone does not affect the typical processing or release of SapA into the supernatant. However, there are other downstream molecules that *manB* affects that do appear to play a role in the processing of SapA.

The *manB* mutant also affects the production of GDP-L-fucose and thereby the formation of EPS, as such a strain of *C. crescentus* whose GDP-L-fucose synthase was knocked out was tested for SapA self-processing. The EPS mutant continued to process SapA as was evidenced by the production of the 45-kDa product in the supernatant. Thus it is likely that the lack of self-processing in the *manB* mutant is due to one of the other two genes affected by the knockout: D-rhamnose or 6-deoxy-D-talose.

Future experiments to locate where SapA anchors on the cell surface of *C. crescentus* are necessary. Other potential regions of localization that may be examined in the future are carbohydrates or another surface protein. Unfortunately, far westerns could not reproducibly identify to what molecule SapA anchors. It is clear that SapA is anchored to the cell surface and that it can reattach to *C. crescentus* using a reattachment

assay. Additional cell membrane preparations and a reliable pull down assay may help isolate the molecule to which SapA anchors. Since SapA can be removed by low pH extraction, it is unlikely to be a covalently bound protein or a lipid-linked protein. Point mutations in the C-terminus directly upstream of the type I secretion signal of SapA may help identify specifically which amino acids are involved in anchoring. Additionally, performing further experiments to explain why SapA is not processed in a *manB* mutant may aid in explaining how this gene plays a role in the folding of the protease. The next genes to consider are those that encode for either D-rhamnose or 6-deoxy-D-talose.

Identifying where SapA self-processes and whether its proteolytic activity towards RsaA recombinant proteins is site-specific has several biotechnological implications. Recombinant RsaA proteins can be engineered such that SapA cleaves RsaA between the secretion signal and the foreign peptide. This would produce completely purified foreign peptide that does not require additional processing. Discovering where SapA anchors on the cell surface of *C. crescentus* and how it folds into an active protease can increase the potential for SapA to be used as a site-specific protease or as an alternate display system.

In summary, we determined that SapA is a unique serralysin-like metalloprotease that cleaves certain recombinant RsaA proteins. SapA is also a self-processing enzyme that uses its C-terminus for type I secretion by the S-layer secretion system and for anchoring to the cell surface of *C. crescentus*. We were able to use SapA's C-terminus for secretion and display of a foreign 242 amino acid protein G IgG binding domain. Further, the use of SapA for display differs from RsaA because SapA can display proteins on the cell surface of *C. crescentus* in SLPS mutants.

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