S-layer biogenesis studies in *Caulobacter crescentus*: RsaA anchoring and the localization of the S-layer-associated protease

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ABSTRACT

Despite the widespread occurrence of bacterial S-layers, little is known about the mechanisms of attachment to the cell surface, especially in the case of Gram-negative organisms. The S-layer of *Caulobacter crescentus* is composed of a single protein, RsaA. After export, RsaA assembles into a hexagonal crystalline array that covers the bacterium. In this array, some RsaA monomers are directly attached to the cell surface, while others are surface anchored only by interacting to other RsaA monomers. Since truncations (9) and mutations (8) in the RsaA N-terminus result in S-layer shedding into the culture medium, we hypothesized that the N-terminus of RsaA anchors the monomer to the cell surface. However, since disruption of the RsaA N-terminus and disruption of the putative RsaA subunit-subunit interaction domain both result in the same phenotype (S-layer shedding), when a particular mutation results in a shedding phenotype, it is difficult to know whether RsaA anchoring or RsaA subunit-subunit interaction has been perturbed. To tease apart these issues, we have developed an assay where small RsaA fragments are incubated with S-layer-negative cells to assess the ability of the fragments to re-attach. In doing so we found that the RsaA anchoring region lies in the first ~225 amino acids, that this RsaA anchoring region requires a smooth lipopolysaccharide molecule found on the outer membrane, and that even minor perturbations within the first ~225 amino acids of RsaA cause loss of anchoring. Mutations that lie outside of the RsaA anchoring region but still result in the shedding phenotype are likely disrupting RsaA-RsaA subunit-subunit interactions rather than directly disrupting RsaA anchoring.

As a by-product of these anchoring studies, we have recent preliminary data that Sap, an S-layer editing protease, is likely to be an extracellular membrane-bound protease, rather than an intracellular protease as previously proposed. Additionally, we have found that Sap is likely to be secreted to the cell surface via the same Type I secretion transporter that the S-layer protein utilizes.

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LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
Amp	Ampicillin
Ap^r	Ampicillin resistance
Cm	Chloramphenicol
Cm^r	Chloramphenicol resistance
C-terminus	carboxy terminus
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
EGTA	β-aminoethyl ether)-N, N, N', N'-tetraacetic acid
GSP	General Secretory Pathway
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Kan	Kanamycin
kb	kilobases
Km^r	Kanamycin resistance
kDa	kilodaltons
µg	microgram
µl	microlitre
mg	milligram
ml	millilitre
min	minute
MCS	Multiple Cloning Site
MFP	Membrane Fusion Protein
N-terminus	amino terminus
OD_{600}	Optical Density at absorbance of 600 nm
OM	Outer membrane
OMP	Outer Membrane Protein
MCC	Multiple Cleavage Cassette
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PGN	Peptidoglycan
PMN	polymorphonuclear leukocytes
PYE	Peptone Yeast Extract
RNase	ribonuclease
RTX	Repeat in Toxin
SDS	Sodium dodecyl sulfate
S-layer	Surface layer
SLH	Surface Layer Homology
SLPS	Smooth Lipopolysaccharide
Sm	Streptomycin
Sm^r	Streptomycin resistance
Suc^s	Sucrose sensitivity
Tris	Tris (hydroxymethyl) methylamine

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1 - INTRODUCTION

The *Caulobacter crescentus* S-layer has been the subject of extensive investigation and manipulation in our lab. Our lab has essentially two major areas of focus: manipulating the S-layer for biotechnological applications, and understanding S-layer biogenesis from a basic science perspective. On the biotechnology front, we have been successful in modifying the S-layer to cause it to shed from the bacterium into the culture medium, by itself or together with fused passenger proteins (9). This shed protein aggregates and is easily retrieved by simple filtration. We have also displayed many heterologous proteins within the S-layer on the bacterial cell surface (7).

In terms of wild-type S-layer biogenesis, we have learned about aspects of RsaA production, secretion, and regulation (1), (71). We have had some indication that the N-terminus of the S-layer monomer, RsaA, mediates RsaA anchoring, because RsaA N-terminal truncations (9) and mutations (8) lead to the shedding phenotype. This thesis aimed to define the RsaA anchoring region more precisely, and to discover which regions or residues within this anchoring domain are involved in cell surface attachment.

Results from this study suggest that indeed the RsaA N-terminus mediates RsaA anchoring, but surprisingly we learned that all minor perturbations constructed thus far within the first ~225 amino acids of RsaA disrupt surface attachment. Through this work, we also clarified some other aspects of S-layer biogenesis, such as the requirement for the cell surface molecule smooth lipopolysaccharide (SLPS) for S-layer attachment.

As a by-product of these S-layer anchoring studies, we have learned more about a *C. crescentus* metalloenzyme, the S-layer-associated protease, Sap. We have recent data

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that Sap, an S-layer editing protease, is likely to be an extracellular membrane-bound protease, rather than an intracellular protease as previously proposed. Additionally, we found that Sap is likely secreted to the cell surface via the same Type I secretion transporter that the S-layer protein utilizes. Current investigation is directed to confirm and extend these hypotheses.

1.1 – General features of S-layer composition

Bacterial surface layers (S-layers) are composed of protein or glycoprotein subunits. Although the S-layers of the Gram-positive bacteria *Clostridium difficile* and *Bacillus anthracis* contain two different S-layer subunits (43, 69), most S-layers are composed of a single protein or glycoprotein species, with molecular masses ranging from 40 to 200 kDa (59). These subunits assemble on the outermost surface of the cell, forming two-dimensional lattices that cover the organism (5). The S-layer is the first contact point between the S-layer-possessing cell and the external environment. For this reason, it has been suggested that differing S-layer compositions among organisms reflects specific adaptations to the environmental and ecological conditions in which these organisms live (4).

1.2 - Frequency of S-layer occurrence in nature

Many bacteria possess S-layers. However, the high frequency of occurrence of S-layer-possessing organisms has only been recognized over the last 25 years. It is now known that S-layers represent an almost universal feature of archaebacteria (65). S-layers have also been found on hundreds of different species of almost every taxonomic

group of eubacteria (57). Even some eukaryotic algae have been reported to have S-layers (52). Given the frequency of occurrence of S-layers-possessing organisms, it seems important to understand various S-layer functions, and how S-layer structures and compositions enable S-layers to perform these functions. Despite the large number of S-layer-possessing organisms and the considerable knowledge that has accumulated regarding S-layer structure, assembly, biochemistry, and genetics, relatively little is known about specific S-layer functions (63). The next several paragraphs describe what is currently known about the functions of S-layers in different bacterial species.

1.3 – Functions of S-layers: Gram-positive bacteria

Efforts have been mounted to understand the functions of S-layers from several Gram-positive bacteria including *Bacillus cereus*, *Staphylococcus aureus*, *Bacillus stearothermophilus*, and *Clostridium thermosulfurogenes* (reviewed in (5)). The S-layers of *B. cereus* and *S. aureus* offer these organisms protection against phagocytosis (5). *B. cereus* is often found in oral infections in humans. To clear these types of infections, phagocytosis is the most important defense mechanism (5). The phagocytic process can be enhanced by the opsonization of the bacteria by host opsonins such as antibodies and complement proteins. However, a shortage of host opsonins might occur as a result of bacterial defense proteases secreted during infection which destroy the opsonins (34, 68). Accordingly, phagocytosis of *B. cereus* in the absence of host opsonins has been a research focus. Studies suggest that cells from young cultures of *B. cereus* are readily ingested by polymorphonucleur leukocytes (PMNs), while cells from aged cultures are not (35). This phenomenon has been attributed to changes in the properties of the *B.*

cereus S-layer during the aging of the culture which result in increased resistance to non-opsonin-mediated phagocytosis (35). This suggests that for *B. cereus*, a mature S-layer offers protection against this form of phagocytosis. S-layers can also play a role to prevent opsonin-mediated phagocytosis. *S. aureus* is an opportunistic pathogen that usually infects immuno-comprimised individuals, and is often found to be the causative agent of hospital-acquired infection. S-layer encapsulation of this bacterium interferes with opsonin-mediated phagocytosis by PMNs by preventing opsonin binding to *S. aureus* (49). Evidently, these S-layers help these pathogens evade host immune responses during infection.

Some Gram-positive S-layers are anchoring points for exo-proteins, as is the case for the S-layers of *B. stearothermophilus* and *C. thermosulfurogenes*. The S-layer of *B. stearothermophilus* acts as an adhesion site for an exo-enzyme (amylase) secreted by the bacterium into the culture medium (18). Similarly, the pullulanase of *C. thermosulfurogenes* remains anchored to the cell surface by binding to the S-layer of *C. thermosulfurogenes* (41). Both the amylase and the pullulanase are surface-anchored by the same mechanism, as will be discussed later. Thus S-layers are acting as platforms for attaching other exo-proteins in these bacteria.

Since the S-layer is often a first contact point between the S-layer-possessing cell and the external environment, it is intuitive that S-layers serve as barriers that protect organisms from harmful substances in their external environments, such as lytic enzymes. Since S-layer lattices possess pores identical in size and diameter in the 2- to 8-nm range, they function as precise molecular sieves, excluding most macromolecules and proteins (59). The S-layer of *Bacillus stearothermophilus* has been shown to act in this way,

excluding molecules with molecular weights greater than 30, 000 Da (58). Since wild-type strains are often outgrown by S-layer-negative strains under optimal growth conditions in the laboratory (64), there must be strong selective pressure to maintain S-layers in competitive environments, further demonstrating that S-layers play a vital role for organisms in their natural environments.

1.4 - Functions of S-layers: Gram-negative bacteria

Although most information regarding S-layer function pertains to Gram-positive organisms, the functionality of some S-layers from Gram-negative organisms is beginning to become understood. The S-layers from the Gram-negative bacteria *Aeromonus salmonicida* and *Campylobacter fetus* are important virulence factors. *A. salmonicida* was originally characterized as a pathogen that infects salmon, but it is now known that many fish species are susceptible to *A. salmonicida* infection (77). *A. salmonicida* possesses an S-layer that assembles into tetragonal arrays on the cell surface (42). It has been shown that S-layer-expressing but not S-layer-deficient *A. salmonicida,* can adhere to, enter, and survive within macrophages (24). This S-layer appears to be critical for the adhesion step of infection, since S-layer-expressing *A. salmonocida* adheres to trout and murine macrophages successfully, while S-layer-deficient *A. salmonicida* cannot adhere to the same macrophages (22, 23).

Another pathogen, *C. fetus,* is a causative agent of extraintestinal infections in humans (11), and of infertility and abortion in sheep and cattle (16). *C. fetus* possesses an S-layer that assembles into a hexagonal array (20). S-layer-deficient *C. fetus* has never been seen in human nor ovine *C. fetus* clinical isolates (5), suggesting the *C. fetus* S-layer

plays a key role in successful pathogenesis. The presence of the *C. fetus* S-layer results in impaired human complement binding, which may explain why S-layer-expressing *C. fetus* is resistant to serum and opsonization (11). *C. fetus* S-layer-containing subunit vaccines offer sheep protection against *C. fetus*-induced ovine abortion (42). For these reasons, the *C. fetus* S-layer is thought to be the predominant virulence factor in ovine and human *C. fetus* infections (48).

S-layers offer protection from predators found in a competitive environment (reviewed in (64). For example, S-layers from the Gram-negative bacteria *Aeromanas salmonocida, Campylobacter fetus, Aquaspirillum serpens,* and *Caulobacter crescentus* protect the cells from attack by bacterial parasites such as *Bdellovibrio bacteriovorus* (5). These S-layer-possessing prey cells are resistant to *Bdellovibrio* predation, whereas isogenic S-layer-deficient prey cells are not (36). Interestingly, some predatory organisms may have adapted to overcome the barrier role that S-layers serve. For example, the *C. crescentus* S-layer acts as a phage receptor for transducing ΦCR30, and S-layer-deficient *C. crescentus* is actually ΦCR30-resistant (17). This type of use of S-layers as phage receptors has been reported in a few other instances (30, 38).

1.5 – Summary of S-layer functions and applied utility of S-layers

Taken together, the evidence to date suggests that S-layers have evolved to perform a variety of functions. S-layers can act as molecular sieves, excluding harmful molecules found in a competitive environment. S-layers also protect organisms from predation from other organisms found in nature. Some S-layers have been shown to aid pathogens to evade host immune responses, while others provide platforms for exo-protein

anchoring. However, since there has been little study on S-layer function in many organisms, this list of S-layer functions is unlikely to be exhaustive.

Additionally, the S-layers of some bacteria (including *C. crescentus*) have been engineered to express foreign peptides or proteins, giving rise to many potential biotechnology applications (reviewed in (44)). Because S-layers occur frequently, are poorly understood, and have promising potential in biotechnological applications, efforts are being made to better understand various aspects in this developing field.

1.6 – *Caulobacter crescentus*

There are many reasons why the S-layer of the Gram-negative bacterium *Caulobacter crescentus* is a good choice for further study. First, *C. crescentus* is a harmless bacterium. Indeed, *C. crescentus* can often be isolated from "clean" drinking water (40). Therefore risk involved with working with this bacterium in the laboratory is minimal. Second, the *Caulobacter* genus of bacteria is very common, and perhaps even abundant in many aquatic and terrestrial environments (40, 50, 51). *Caulobacter* is therefore of general scientific interest if we wish to understand the organisms that are prevalent in our environment. Third, *C. crescentus* has been widely used as a model system for understanding fundamental aspects of cell development and differentiation (14, 28). *Caulobacter* differs from other bacteria in that it exhibits an unusual biphasic lifestyle, alternating between a stalked cell and a non-stalked dispersal (swarmer) cell (61). The switch between these two phases of the *Caulobacter* life cycle has been studied as a differentiation process. Fourth, large amounts of genetic studies have been performed on this bacterium; the sequenced genome is available (www.tigr.org). Fifth, the *C.*

crescentus S-layer has been engineered to display foreign peptides of biotechnological interest, and has also been used to effect the secretion of fusion proteins, ideal for use in antibody production, into the culture medium (46). Therefore many data are available regarding *C. crescentus*, but not in the area of wild-type S-layer biogenesis.

1.7 – *C. crescentus* S-layer function

The only known function of the S-layer of *C. crescentus* is the protective role it plays in the predation by a *Bdellovibrio*-like organism; S-layer-positive *C. crescentus* is resistant to this predation, whereas isogenic S-layer-negative *C. crescentus* is not (36). Since *C. crescentus* forms biofilms in nature, an important role for the *C. crescentus* S-layer may be protection by selective porosity from various predatory assaults that are likely to occur in complex bacterial biofilm communities (5). Difficulty in transforming *C. crescentus* by electroporation may be a reflection of this protective role (27).

1.8 – Composition and assembly of *C. crescentus* S-layer

The S-layer of *C. crescentus* is composed of a single protein, RsaA (26), and a representation of RsaA is illustrated in Fig. 1-1. RsaA is a 1,026 amino acid protein, with a predicted molecular weight of 98 kDa (26). RsaA is not modified post-translation, aside from the cleavage of the initial methionine residue, nor is it glycosylated (26). RsaA assembles on the cell surface into a two-dimensional hexagonal lattice that completely covers the bacterium (67). Proper S-layer assembly is dependent on smooth lipopolysaccharide (SLPS) found on the outer membrane of *C. crescentus*, and mutants

N ——— [868-905 RTX] ——— C

Putative anchoring domain
(First ~225 residues)

Insertion-tolerant region

Type I secretion signal
(last ~80 residues)
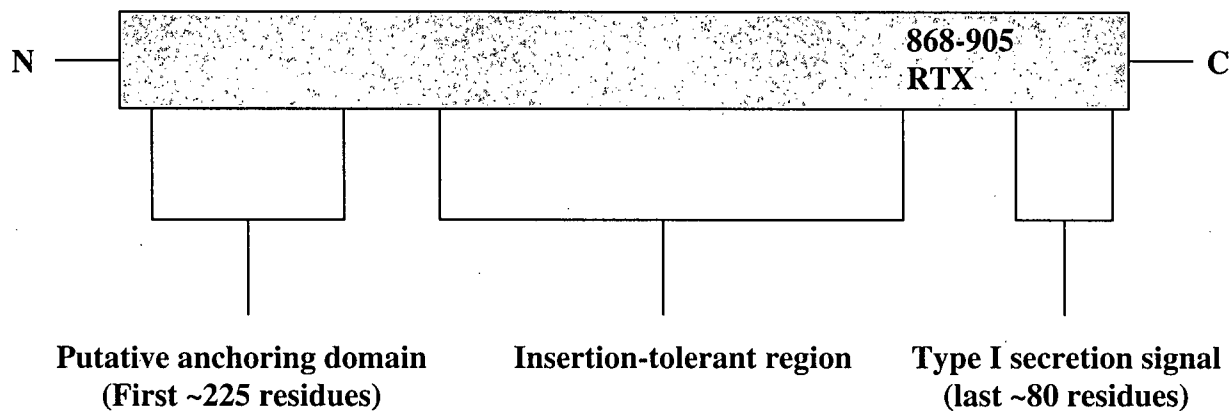
Fig. 1-1. **Box model representation of the C. crescentus S-layer monomer, 1026 amino acid RsaA.**
Mutation of the N-terminus causes S-layer shedding, as does mutation of the Repeat in Toxin (RTX) region, a calcium-binding motif (8). Mutation beyond amino acid 945 causes loss of S-layer secretion (9). Many sites within the middle of RsaA can tolerate insertion of foreign peptides without causing any adverse effects on S-layer anchoring or crystallization (8).

that are S-layer-proficient but SLPS-deficient shed RsaA into the culture medium (2). The prominent morphological unit in the hexagonal array has six RsaA monomers, and is spaced at 22 nm, as determined by electron microscopy and three-dimensional image reconstruction (67). Current research is focused on building correlations between primary RsaA structure and the spatial organization of the S-layer as evidenced by electron microscopy, in order to understand which RsaA regions are mediating the anchoring of RsaA to the cell surface, and which regions are involved in RsaA subunit-subunit interactions. Clearly, a crystal structure at the atomic level for RsaA would aid tremendously in this task, but so far such a structure is not available. In fact, there is no 3-dimensional crystal structure available for any S-layer protein to date. This is probably because the intrinsic nature of S-layers to form 2-dimensional structures likely impedes the formation of 3-dimensional structures that are needed to obtain a crystal structure.

1.9 – S-layer secretion apparatus in *C. crescentus*

C. crescentus employs an ABC transporter (Type I) secretion apparatus to export RsaA monomers to the cell surface (1). In most bacteria, S-layer secretion does not occur via the Type I system, but rather by the general secretory pathway, GSP (12). In contrast to the Type II GSP, the Type I secretion system for Gram-negative bacteria exports substrates to the cell surface without a periplasmic intermediate, and utilizes a non-cleaved C-terminal secretion signal present on secretion substrates to effect export. Two well-described Type I secretion systems are those required for the secretion of *E. coli* hemolysin, HlyA (6), and a *Pseudomonas aeruginosa* alkaline protease, AprA (55). Aside from the S-layer of *C. crescentus*, only two other S-layer proteins are known to be

exported by a Type I secretion apparatus: the *Campylobacter fetus* S-layer protein (70), and the *Serratia marcescens* S-layer protein (33). With the exception of the S-layer proteins from these three organisms, all S-layer proteins sequenced to date are produced with an N-terminal secretion signal peptide that is cleaved during translocation through the plasma membrane (59).

1.10 – Components of the RsaA secretion system

The *C. crescentus* S-layer Type I secretion system has three components: an ABC transporter, its accessory molecule (membrane fusion protein, MFP), and an outer-membrane protein (OMP) (6). The S-layer transporter genes in *C. crescentus* have been cloned and sequenced. The *rsaD, rsaE,* and *rsaF* genes encode the putative ABC transporter, MFP and OMP proteins, respectively. This secretion apparatus is responsible for secreting a large amount of protein (RsaA constitutes 10-12% of total cell protein (1)). It is perhaps not surprising that recent gene deletion studies have found that there are two homologous genes that encode for the OMP (RsaF) component of this transport system, perhaps enabling the cell to continuously export the large amounts of RsaA produced, even in the event that one of these OMP genes becomes non-functional (71). It may also be that *C. crescentus* has developed other mechanisms to ensure that these large amounts of RsaA do not build up intracellularly, should RsaA transport break down (71).

1.11 – RsaA editing: Sap metalloprotease

An editing process occurs during biogenesis of the *C. crescentus* S-layer, which appears to eliminate bad (mutant) copies of RsaA that might otherwise impair S-layer

crystallization and tight packing of the RsaA monomers. A metalloprotease has been identified, which cleaves some mutated versions of RsaA, and has been named Sap (72). The C-terminal of Sap is homologous with the N-terminal of RsaA. Because the Sap sequence does not appear to contain (a) a signal leader peptide at the extreme N-terminal, (b) a Type I C-terminal secretion signal, and (c) obvious transmembrane domains, it was previously suggested that Sap is an intracellular enzyme. Although the native function of Sap is unknown, our previous model of Sap-mediated editing of recombinant S-layer suggested that Sap associates with nascent RsaA monomers inside the cell to "scan" for bad copies of RsaA (72). As an extension of this model, when Sap detects a bad RsaA copy, it cleaves the mutant RsaA, which separates the RsaA C-terminal secretion signal from the portion of RsaA that is N-terminal to the cleavage site, and may also result in the intracellular degradation of the cleavage products (72). Confusingly, however, sometimes both Sap cleavage products ended up on the cell surface, though only one of these products contains the C-terminal secretion signal to effect secretion to the cell surface. Because Sap sequence suggests it is an intracellular enzyme, we hypothesized that RsaA intramolecular forces (such as hydrogen bonding) keep the two Sap cleavage products linked together, which could account for the secretion of both products. However, the current study challenges our previous model of Sap, suggesting that it is in fact an outer membrane-bound enzyme.

1.12 - The "weak spot" in RsaA

I found that Sap was responsible for undesired proteolysis of some RsaA mutants that I constructed. In a particular RsaA mutant that I constructed that contained a collagenase

cleavage site, after treatment with collagenase, an additional Sap-mediated cleavage of RsaA occurred. This Sap cleavage at this particular site has been observed in other RsaA mutants (8). This suggests that there is a weak Sap recognition site only exposed when certain mutations in RsaA alter the folding of the protein. This may be a Sap-mediated strategy employed by *C. crescentus* to release from the cell surface various RsaA mutants that might perturb S-layer assembly. In terms of RsaA anchoring studies, if a particular mutation caused loss of RsaA anchoring, it was difficult to assess whether this loss of anchoring was a result of the mutation perturbing the RsaA anchoring domain itself, or whether the mutation converted the protein into an Sap substrate, and the proteolysis of the RsaA mutant caused the loss of anchoring. Therefore, when investigating the importance of RsaA regions or residues for RsaA attachment or crystallization, after mutating RsaA regions or residues, Sap-negative strains were employed to eliminate this variable. In that way I could test the effects of the particular RsaA mutations and be confident that the phenotype observed (loss of anchoring) was a consequence of the mutation perturbing the RsaA anchoring domain, rather than a consequence of simply exposing or creating a Sap cleavage site on RsaA.

1.13 – Region of RsaA that mediates secretion

The secretion of RsaA to the cell surface is directed by a C-terminal secretion signal, localized to the last 82 amino acids of RsaA (9). This secretion signal can be genetically fused to peptides of choice, creating hybrid proteins that get secreted to the culture medium and aggregate into macroscopic particles that can be recovered by simple filtration (9). The primary RsaA sequence reveals the presence of a calcium binding

motif, the so-called RTX (repeat in toxin) motif, near the C-terminal of RsaA (26). The RTX motif is a tandemly-repeated nine amino acid (L-X-G-G-X-G-(N/D)-D-X) sequence found in Type I secreted proteins, of which the *E. coli* hemolysin protein HlyA is the prototype (reviewed in (76)). Proper cell-surface assembly of RsaA into the native hexagonal array requires calcium (67, 75). The RTX region of RsaA is thought to mediate RsaA crystallization (subunit-subunit interaction) on the cell surface via calcium bridging between RsaA monomers (67). Calcium does not directly bind to the cell surface: attempts to bind radioactively-labeled calcium to the surface of S-layer-deficient cells have been unsuccessful. Therefore we believe that calcium does not directly mediate RsaA attachment to the cell surface. Perturbations in RsaA near or at the RTX motif results in the shedding of RsaA into the culture medium, possibly by damaging RsaA subunit-subunit interaction which may be mediated by calcium bridging at the RTX region.

The sequences of *C. crescentus* RsaA and the *C. fetus* S-layer protein SapA share significant homology in their C-termini. This, and the fact that SapA is also exported to the cell surface by a Type I secretion apparatus (70) suggests a common secretion mechanism for these two S-layers proteins. RsaA and SapA are not, however, homologous in their N-termini, which are believed to mediate anchoring of these proteins, suggesting a different surface anchoring mechanism exists for these proteins.

1.14 – Region of RsaA that mediates cell surface anchoring

The regions of RsaA that mediate S-layer anchoring to the cell surface remain unknown, but since mutations (8) and truncations (9) in the RsaA N-terminus lead to the

shedding phenotype, it is likely that the N-terminus mediates RsaA anchoring. Insight into RsaA anchoring would further the utility of *C. crescentus* as a tool for surface display of peptides or proteins. If the RsaA anchoring regions were determined, then their maintenance when designing heterologous proteins for surface display would facilitate the effective anchoring of these proteins on the *C. crescentus* cell surface. A better understanding of the RsaA anchoring region may aid in manipulating RsaA such that it could anchor to other inert surfaces, opening a door to many applications in biotechnology.

1.15 – Mechanism of S-layer anchoring to the cell surface: Gram-positive bacteria

S-layers from most Gram-positive bacteria are considered to remain surface associated by binding to peptidoglycan (PGN) and/or lipoglycans, two molecules that are abundant in the cell wall of this type of organism (59). Specifically, Gram-positive S-layers are thought to bind to the carbohydrates moieties of these molecules, and therefore S-layers can be thought of as surface-located carbohydrate binding proteins (59). The ability of S-layers to bind to these carbohydrates on the cell surface is often a consequence of the presence of a particular amino acid motif on the S-layer protein, the so-called Surface Layer Homology (SLH) domain (39). The SLH domain, found in many Gram-positive S-layer proteins, is a conserved sequence of approximately 55 amino acids, usually repeated in tandem 3 times (39). The SLH domain is proposed to function as a peptidoglycan (PGN) binding structure (39), or more generally, a carbohydrate binding structure (19). As an example, the S-layer protein of *Thermus thermophilus* has an SLH domain that anchors this S-layer protein to PGN found in the cell wall of this Gram-

positive organism (47).   One or more copies of the SLH domain has also been identified in at least 40 exo-enzymes and exo-proteins (19, 21, 37, 41, 53, 54). SLH domains present on these S-layer-associated proteins are thought to mediate the attachment of the S-layer-associated protein to the S-layer itself (19).   Both the amylase of *B. stearothermophilus* and the pullulanase of *C. thermosulfurogenes* anchor to their respective S-layers via their SLH domains (5).

1.16 – Mechanism of S-layer anchoring to the cell surface: Gram-negative bacteria

Little information is available regarding the cell surface anchoring of S-layers of Gram-negative bacteria. Most evidence suggests the involvement of the N-terminus of the S-layer protein monomers. The S-layer of *A. salmonicida* is thought to be anchored to the cell surface via the N-terminus of the S-layer subunits because the N-termini of these S-layer subunits are inaccessible to trypsin digest when S-layer-proficient *A. salmonicida* cells are treated with this protease (15). However, mutagenesis studies have yet to be performed to confirm this hypothesis. Similarly, the *C. fetus* S-layer is probably anchored to lipopolysaccharide on the cell surface via the N-terminus of the *C. fetus* S-layer monomer, SapA. The evidence for this is that deletions in the SapA N-terminal region result in the loss of SapA anchoring to the cell surface (16), and C-terminal SapA truncations retain the ability to reattach to SapA-deficient *C. fetus* cells (78).   The anchoring of the *C. crescentus* S-layer protein RsaA is perturbed by N-terminal mutations and truncations (8), and is discussed in greater detail below.

1.17 – Tolerated insertions in RsaA

The *C. crescentus* S-layer has been engineered to display foreign peptides inserted within the RsaA sequence (7, 8, 46). Sites in *rsaA* have been prepared for insertion of heterologous material at gene positions corresponding to RsaA amino acids 266, 622, 690, 723, 784, 860, and 944, and heterologous sequences have been displayed at these positions (reviewed in (46)). Since heterologous insertion at the above-mentioned sites does not impair RsaA anchoring or crystallization, it is likely that those insertion-tolerant RsaA regions are not involved in RsaA anchoring or crystallization. Recent RsaA mutagenesis studies suggest that amino acids 229, 316, 450 and 510 can be mutated without causing adverse affects on RsaA attachment or crystallization, and so it is likely that these RsaA regions are also not involved in RsaA attachment or crystallization (7, 8).

1.18 – The N-terminal region of RsaA

The function of the N-terminal region of RsaA may be to anchor RsaA to the *C. crescentus* cell surface, since RsaA N-terminal truncations (9) and mutations (8) result in the shedding of RsaA into the culture medium. Indeed, it is on this premise that proteins of interest can be collected and easily purified from *C. crescentus* cultures: the secreted hybrid proteins (that lack the RsaA N-terminal sequence) aggregate in the culture supernatant, and are not found to be cell surface-associated. Thus it appears that perturbations in the RsaA N-terminal region disrupt RsaA anchoring. However, the exact RsaA N-terminal regions or residues that interact with the cell surface to effect RsaA

anchoring remain unknown. Additionally, it is unknown if there are additional RsaA regions other than the N-terminal region that might mediate RsaA anchoring.

1.19 – Summary of the study

Shedding of the *C. crescentus* S-layer occurs when the putative anchoring domain is removed (RsaA N-terminal truncations shed into the culture medium (9). However, since disruption of the RsaA anchoring domain and disruption of RsaA crystallization (subunit-subunit interaction) domain both result in the same phenotype (S-layer shedding), when a particular mutation results in a shedding phenotype, it is difficult to know whether RsaA anchoring or RsaA subunit-subunit interaction has been perturbed. To focus on RsaA anchoring rather than RsaA crystallization (subunit-subunit interaction), we developed an assay where small RsaA fragments are incubated with S-layer-negative cells to assess the ability of the fragments to re-attach. In this way we investigated the possibility of other regions in RsaA that might contain anchoring information. We found that only the N-terminus of RsaA contained anchoring information, and that the smallest S-layer fragment sufficient for RsaA attachment was 277 amino acids, which is ~1/4 of the size of full-length RsaA. This small RsaA fragment would not be expected to retain its cell surface crystallization (subunit-subunit) capabilities, since it is missing ~3/4 of the native RsaA. Therefore this small RsaA fragment likely directly anchors to the cell surface, rather than being surface-associated by interacting with other RsaA monomers. Consequently, loss of anchoring mutations created in this small RsaA fragment can be attributed to perturbations in the RsaA anchoring domain, rather than perturbations in the RsaA crystallization domain.

My results suggest that the N-terminus of RsaA mediates attachment of the *C. crescentus* S-layer, and even minor perturbations within the first ~220 RsaA residues cause loss of RsaA anchoring. The project also shows that RsaA anchoring requires smooth lipopolysaccharide (SLPS). As a by-product of the S-layer anchoring studies, we refute the previous notion that the S-layer Associated Protease, Sap, is intracellular. Results from this work suggest that Sap is in fact an outer membrane-bound protease, and furthermore, it is probably secreted by the S-layer Type I secretion system.

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 Table 1. *E. coli* DH5α was used for all *E. coli* cloning manipulations. *E. coli* was grown at 37^0C in Luria broth (1% tryptone, 0.5% NaCl, 0.5% yeast extract) with 1.3% agar for plates. *C. crescentus* strains were grown at 30^0C 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, kanamycin, and streptomycin were used at 50 µg/ml, and chloramphenicol was used at 20 µg/ml in *E. coli* cultures. Kanamycin and streptomycin were used at 25 µg/ml and chloramphenicol was used at 2µg/ml in *C. crescentus* cultures, when needed.

2.2 – Plasmid and DNA manipulations

Standard methods of DNA manipulation and isolation were used (56). Electroporation of *C. crescentus* was performed as previously described (27). All PCR products were generated using Platinum *Pfx* DNA polymerase (Invitrogen, Burlington, ON) following the manufacturers suggested protocols.

2.3 – *C. crescentus* expression vectors

pUC8CVXΔSDA19

The peptide display vector pUC8CVXΔSD is a pUC8-based vector that contains a full-length copy of the *rsaA* gene under the control of a modified *rsaA* promoter. It contains an origin of replication, oriV, which allows for replication in both *E. coli* strains and *C.*

Strain or plasmid	Relevant characteristics	Reference
Bacterial strains		
C. crescentus		
NA1000	Ap^r *syn* -1000; variant of wild-type strain CB15 that synchronizes well	ATCC 19089
JS1001	S-LPS mutant of NA1000, sheds S-layer into medium	[17]
JS1003	NA1000 with *rsaA* interrupted by KSAC Km^r cassette	[17]
JS1004	JS 1001 with *rsaA* interrupted by KSAC Km^r cassette	[17]
JS1008	Cmr, NA1000, *rsaFb* -negative	[71]
JS1010	NA1000, *rsaFa* -negative strain	This study
JS1011	Cmr, NA1000, *rsaFa* -negative, *rsaFb* -negative, *rsaA* -negative strain	This study
JS1012	JS1001, Sap-negative, S-layer-negative	This study
JS1013	NA1000, S-layer-negative (amber codon in *rsaA*)	This study
JS1014	NA1000, S-layer-negative (amber codon in *rsaA*) with *manB* interuption rendering this strain SLPS-negative	This study
CB15δrsaA	NA1000, *rsaA* knocked out deleting *rsaA* promoter and portion of *rsaA*	This study
CB15ATn5F9	NA1000 with Tn5 insertion in *manB* rendering this strain SLPS-negative	[2]
CB15ATn5F23	NA1000 with Tn5 insertion in *manB* rendering this strain SLPS-negative	[2]
JS 4000	Spontaneous RsaA-negative mutant of strain CB2 maintained in the laboratory of J. Smit	[66]
JS 4015	Sap-negative UV-NTG mutant of strain JS4000	[72]
JS4022	JS4015, *recA* -negative. *repBAC* -positive	This study
JS4023	JS4000, *rsaFa* -negative strain	This study
JS4024	Kanr, JS 4015 with *manB* interuption rendering this strain SLPS-negative	This study
JS4025	JS 4000, *rsaFa* -negative and *rsaFb* -negative	This study
E. coli		
DH5α	*recA endA*	Invitrogen
Rb404	*F-dam-3, dam-6, metB1, galK2, galT22lacY1, thi-1, tonA31, tsx-78, mtl-1, supE44*	[13]
Plasmids		
pWB9	Cmr, Smr, pKT215-derived expression vector incorporating the rsaA promoter	[8]
pWB9:*rsaA* ΔP	Cm^r, Sm^r, *rsaA* gene and *rsaA* promoter	[6]
pWB9:*rsaA* ΔP:B162MCCΔ	Cmr, Smr, pWB9:rsaAΔP with collagenase site at a.a. 162	This study
pWB9:*rsaA* ΔP:Hps1MCCΔ	Cmr, Smr, pWB9:rsaAΔP with collagenase site at a.a. 277	This study
pWB9:*rsaA* ΔP:Hps4MCCΔ	Cmr, Smr, pWB9:rsaAΔP with collagenase site at a.a. 690	This study
pWB9:*rsaA* ΔP:Hps12MCCΔ	Cmr, Smr, pWB9:rsaAΔP with collagenase site at a.a. 723	This study
pWB9:*rsaA* ΔP:B7Hps1MCCΔ	Cmr, Smr, pWB9:rsaAΔP with BamHI site at a.a. 7 and collagenase site at a.a. 277	This study
pWB9:*rsaA* ΔP:Taq29Hps1MCCΔ	Cmr, Smr, pWB9:rsaAΔP with BamHI linker at a.a. 29 and collagenase site at a.a. 277	This study
pWB9:*rsaA* ΔP:Mps4Hps1MCCΔ	Cmr, Smr, pWB9:rsaAΔP with BamHI linker at a.a. 69 and collagenase site at a.a. 277	This study
pWB9:*rsaA* ΔP:B154Hps1MCCΔ	Cmr, Smr, pWB9:rsaAΔP with BamHI site at a.a. 154 and collagenase site at a.a. 277	This study
pBBR3ΔSD:Taq169Hps1MCCΔ	Cmr, Smr, pWB9:rsaAΔP with BamHI linker at a.a. 169 and collagenase site at a.a. 277	This study
pWB9:*rsaA* ΔP:B222Hps1MCCΔ	Cmr, Smr, pWB9:rsaAΔP with BamHI linker at a.a. 222 and collagenase site at a.a. 277	This study
pBSKII	Apr, ColE1 cloning vector; lacZ Ap^r	Stratagene
pTZ18UCHE:*rsaFb* ΔNΔC	Cmr, cloning vector, rsaFb missing N and C termini	[71]
pAL1	Smr, Sucs, E. coli-based pNPTS138 suicide vector with Δ−rsaA fragment	This study

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pK18mobsacB	Kmr, Sucs, E. coli-based suicide vector	[60]
pK18mobsacB:*rsaFa* ΔKP	Kmr, Sucs, E. coli-based suicide vector with RsaFa internally-deleted between KpnI and Pst I sites	This study
pK18mobsacB:*manB* ΔNΔC	Kmr, Sucs, E. coli-based suicide vector with manB fragment missing N and C termini	This study
pWB9:rsaAΔP(723/VP2CΔ)	Cmr, Smr, pWB9:rsaAΔP with 112 amino acid segment of VP2 glycoprotein of IPNV at RsaA a.a 723	[72]
pUC9CXSMCC	Apr,, Cmr pUC9 vector carrying a modified multiple cloning site; a promotorless Cm-resistance gene from Tn9 is inserted in the *Bgl* II site, and a multiple cleavage cassette is inserted into the XhoI-StuI MCS	This study
pBBR3ΔSD	Smr, *repBAC* -positive, modified *rsaA* promoter	This study
puc8CVXΔSDA19	Cmr, high copy number puc8-based *E.coli/C.crescentu* s shuttle vector with modified *rsaA* promoter and wild type *rsaA* gene	This study
pBSKI+	Apr, LacZ, cloning vector	Stratagene
pK18mobsacB*rsaA* 353ΦB	Kmr, Sucs, E. coli-based suicide vector with *rsaA* with amber codon at a position corresponding to RsaA a.a. 353	This study
pBSkIIESH	Apr, modified pBSkII cloning vector with EcoRI- StuI-, HindIII-modified MCS	[71]
pK18mobsacB*pwb* Δ R	Kmr, Sucs, E. coli-based suicide vector with 200 bp internal deletion of S-layer associated protease gene (*sap*)	This study
pBBR3	Smr, broad host range vector	[31]
pBSkIIEEH	Apr, modified pBSkII cloning vector with EcoRI- EcoRV-, HindIII-modified MCS	[71]

crescentus strains that express the *repBAC* genes (73). It was constructed by Dr. John Nomellini as follows: the oligonucleotides I 1060 5' GAG GCC TAC TCT TCC TTT TTC AAT ATT ATT GAA 3' (*Stu*I underlined) and 1920 5' GAG GCC TAG TAC TCT GTC AGA CCA AGT TTA CTC ATA 3' (*Sca*I site underlined) were used to do inverse PCR on the plasmid pUC 8. This PCR product was digested with *Stu*I and *Sca*I and ligated to *Hpa*I digested chloramphenicol gene called CHE. The resulting plasmid was called pUC 8 CX. The CHE gene was made by using the oligonucleotides JNCHE-1 5' GGA AGA TCT GTT AAC TTT TCA GGA GCT AAG GAA GCT 3' and JNCHE-2 5' GGA AGA TCT GTT AAC ACA ATA ACT GCC TTA AAA AAA TTA 3' (*Hpa*I sites are underlined) to PCR the chloramphenicol gene from the plasmid pMMB206 (44), which does not have an *Eco*RI site in the middle of the gene. The oriV was inserted by first cutting the pUC 8 CX plasmid with *Eco*O109, filling in the recessed ends resulting from this digestion with the Klenow enzyme, recircularizing the plasmid by ligation, then digesting the resulting plasmid with *Hind*III. This *Hind*III/*Hind*III fragment was removed and replaced with the 521bp *Hind*III/*Xmn*I oriV fragment from plasmid pCR2.1 oriV (73), and the resulting plasmid was called pUC 8 CVX. The next step was to remove the lac promoter and replace it with a modified *rsaA* promoter. This was done in the follow manner. The *Eco*RI/*Hind*III fragment from plasmid pSSa49ΔSD (10) containing the modified *rsaA* promoter was cloned into pUC 8 cut with *Eco*RI and *Hind*III, called pUC 8ΔSD. Then both this new pUC 8 plasmid and pUC 8 CVX were cut with *Eco*RI and ligated together and selected on Amp/Cm plates. The correct orientation of the plasmids was determined with an *Nde*I digest. The correct plasmid fusion was then digested with *Sap*I and *Pst*I, filled in with the Klenow enzyme and ligated back together

and selected on Cm plates. This last manipulation removed almost all of the pUC 8 ΔSD plasmid except the modified *rsaA* promoter with is now upstream of the *Eco*RI/*Hind*III multiple cloning site. This plasmid was called pUC 8 CVXΔSD. All previous full length RsaA gene constructs can be easily cloned into this vector as *Eco*RI/*Hind*III fragments and will be transcribed off the modified *rsaA* promoter. The wild type *rsaA* gene was excised from pTZ18UB:*rsaA*ΔP (8) as an *Eco*RI/*Hind*III fragment and cloned into *Eco*RI/*Hind*III cut pUC 8 CVXΔSD, resulting in the plasmid pUC 8 CVXΔSDA19.

pBBR3ΔSD

The pBBR3ΔSD vector (constructed by Dr. John Nomellini) was used as a shuttle *E. coli/C. crescentus* shuttle vector, and does not require the *repBAC* genes to be integrated into the chromosome of the *C. crescentus* host cell in order to replicate, in contrast to the pUC 8 CVXΔSDA19 vector described above. The modified *rsaA* promoter was included in this vector, which results in increased RsaA production (10). To create this vector, the plasmid pUC8CVXΔSDA19 (which contains the modified *rsaA* promoter) was digested with *Nsp*I and the recessed ends were filled in using the Klenow enzyme. The resulting linearized, blunt-ended plasmid was ligated into pBBR3 (31) cut with *Sma*I, creating a ~12 kb plasmid fusion. Correct orientation of the plasmid fusion was confirmed by *Eco*RI digestion, where the desired clone gave a ~6 kb and a 6.7 kb band upon *Eco*RI digestion. The plasmid fusion with the correct orientation was then digested with *Hind*III, releasing most of the pUC8CVXΔSDA19 plasmid, but leaving the modified *rsaA* promoter with the pBBR3 vector. The remaining fragment containing the modified

rsaA promoter with the pBBR3 vector was circularized by ligation, and this resulted in the creation of pBBR3ΔSD.

2.4 – Construction of plasmids carrying *rsaA* with collagenase cleavage sites

pUC9CXSMCC

pUC9CXSMCC was created by ligating the annealed oligonucleotides 5'-TCG AGG CAT GAT CGA GGG TCG CGG CCC GCA CGG TCC CGC CGG CCC GG-3' and 5'-CCG GGC CGG CGG GAC CGT GCG GGC CGC GAC CCT CGA TCA TGC C-3' into the pUC9CXS plasmid (7) cut with *Xho*I-*Stu*I, inserted in the same orientation as the promotorless Cm-resistance gene. These oligonucleotides encode, from 5'-3', a Factor X protease cleavage site, followed by two collagenase cleavage sites in tandem (Multiple Cleavage Cassette, MCC).

pWB9:*rsaA*ΔP:Hps1MCCΔ, pWB9:*rsaA*ΔP:Hps4MCCΔ, and pWB9:*rsaA*ΔP:Hps12MCCΔ

pUC9CXSMCC was cut with *Bam*HI, releasing a *Bam*HI-*Bam*HI fragment that included the MCC + promotorless Cm-resistance gene. The entire *Bam*HI cassette was then ligated into pTZ18UB:*rsaA* (*Hin*PI277*Bam*HI) (7) to place the MCC at the position in *rsaA* corresponding to amino acid 277, pTZ18UB:*rsaA* (*Hin*PI690*Bam*HI) (7) to place the MCC at the position in *rsaA* corresponding to amino acid 690, or pTZ18UB:*rsaA* (*Hin*PI723*Bam*HI) (7) to place the MCC at the position in *rsaA* corresponding to amino acid 723. Proper orientation of the MCC achieved by selecting for clones with Cm-resistance (the Cm gene is driven by the lac promoter in the pTZ plasmid if the Cm-

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resistance cassette was oriented properly). The Cm-resistance genes were then excised

by cutting with *Bgl*II, and subsequent circularization of the plasmids was achieved by

ligation. The *Eco*RI-*Sst*I fragments of the resulting plasmids were excised and ligated

into the pWB9 plasmid that had been cut with *Eco*RI-*Sst*I. This resulted in the creation

of plasmids pWB9:*rsaA*ΔP:Hps1MCCΔ, pWB9:*rsaA*ΔP:Hps4MCCΔ, and

pWB9:*rsaA*ΔP:Hps12MCCΔ.

pWB9:*rsaA*ΔP:B162MCCΔ

The *rsaA Eco*RI/*Cla*I fragment was excised from pTZ18UB:*rsaA* (*Hin*PI723*Bam*HI) (7)

that was harbored by and subsequently derived from RB404 (13), a non-DNA

methylating strain of *E. coli* that allowed the *Cla*I digestion to proceed. This *rsaA*

*Eco*RI/*Cla*I (wild type) fragment was then ligated into pBSKII cut with *Eco*RI and *Cla*I.

To destroy the *Bam*HI site in the MCS of pBSKII, the pBSKII plasmid was cut with

*Bam*HI, and the resulting overhangs were filled in using the Klenow enzyme, then the

blunt ends were ligated to circularize the plasmid. Mutation at RsaA amino acids 162/163

was achieved using the Quickchange method (Stratagene, La Jolla, CA), following the

manufacturer's suggested protocols, using the primers 5'-GTT GGC CTG GCG GCT

GGA TCC AGC CAC GGC GGC CGC-3' and 5'-GCG GCC GCC GTG GCT **GGA**

TCC AGC CGC CAG GCC AAC-3' where the bases that were non-complementary to

the template are shown in bold. This changed RsaA amino acids F162/L163 to

G162/S163, and created a *Bam*HI site at a position corresponding to RsaA amino acids

162/163. The MCC cassette from pUC9CXSMCC was inserted into this *Bam*HI site and

the Cm-resistance gene was removed as described above, yielding

pBSKIIφ*BamHI:rsaAEcoRI-ClaIBamHI162*. The plasmid puc8CVXΔSDA19 (described

above) was cut with *PstI*, releasing a *PstI-PstI* fragment containing two undesired *ClaI*

sites from a non-coding region of the plasmid. The remaining backbone plasmid was

recircularized by ligation, creating puc8CVXΔSDA19φ*PstI*. This plasmid, derived from

E. coli strain RB404 to allow for *ClaI* digestion, was cut with *EcoRI* and *ClaI*, and then

the *rsaA EcoRI/ClaI* fragment with the desired mutation, from digestion of

pBSKIIφ*BamHI:rsaAEcoRI-ClaIBamHI162* with *EcoRI* and *ClaI*, was inserted by

ligation, creating puc8CVXΔSDA19φPstI*BamHI162*. Next, to replace the *PstI-PstI*

fragment (that contained the *ClaI* sites that were now inconsequential, but that also

contained an *SstI* site required for the next step of the cloning strategy), the *AvrII/HindIII*

fragment (containing within it the *PstI-PstI* fragment) was obtained from cutting

puc8CVXΔSDA19 with *AvrII* and *HindIII*, and this fragment was ligated into

puc8CVXΔSDA19φ*PstIBamHI162* cut with *AvrII* and *HindIII*, giving the plasmid

puc8CVXΔSDA19*BamHI162*. Finally, the mutant *rsaA*-encoding *EcoRI/SstI* fragment

was excised from puc8CVXΔSDA19φ*PstIBamHI162* and ligated into the pWB9 plasmid

that had been cut with *EcoRI-SstI*. This resulted in the creation of

pWB9:*rsaA*ΔP:B162MCCΔ.

2.5 – Construction of plasmids carrying *rsaA* with collagenase cleavage at RsaA position 277 with additional mutations in the RsaA N-terminus

pWB9:*rsaA*ΔP:B7Hps1MCCΔ, pWB9:*rsaA*ΔP:B154Hps1MCCΔ, and

pWB9:*rsaA*ΔP:B222Hps1MCCΔ

The pTZ18UB:*rsaA* (*Hin*PI277*Bam*HI) plasmid with the MCC cassette at RsaA position 277 (described above) was used as the template for PCR for site-directed mutagenesis of *rsaA* to achieve the mutation at RsaA position 7, 154, and 222. Mutation at RsaA amino acid 7 was achieved using the Quickchange method (Stratagene, La Jolla, CA), following the manufacturer's suggested protocols. The mutation at amino acid 7 was generated using the primers 5'-GCC TAT ACG ACG GCC **GGA TCC** GTG ACT GCG TAC ACC-3' and 5'-GGT GTA CGC AGT CAC **GGA TCC** GGC CGT CGT ATA GGC-3', where the bases that were non-complementary to the template are shown in bold. This changed RsaA amino acids Q7/L8 to G7/S8. The mutation at amino acid 154 was generated using the primers 5'-GCG ACC GCC GCT GGC **GGA TCC** GTC GCG GCC GCC GTG-3' and 5'-CAC GGC GGC CGC GAC **GGA TCC** GCC AGC GGC GGT CGC-3', where the bases that were non-complementary to the template are shown in bold. This changed RsaA amino acids V154/D155 to G154/S155. The mutation at amino acid 222 was generated using the primers 5'-GCC GCG ATG ATC AAC **GGA TCC** TCG GAC GGC GCC CTG-3' and 5'-CAG GGC GCC GTC CGA **GGA TCC** GTT GAT CAT CGC GGC-3', where the bases that were non-complementary to the template are shown in bold. This changed RsaA amino acids D222/L223 to G222/S223.The *Eco*RI-*Sst*I fragments from the resulting Quikchange products were excised and ligated into the pWB9 plasmid that had been cut with *Eco*RI-*Sst*I, creating pWB9:*rsaA*ΔP:B7Hps1MCCΔ, pWB9:*rsaA*ΔP:B154Hps1MCCΔ, and pWB9:*rsaA*ΔP:B222Hps1MCCΔ.

pWB9:*rsaA*ΔP:Taq29Hps1MCCΔ, pWB9:*rsaA*ΔP:Mps4Hps1MCCΔ

The *Eco*RI-*Not*I fragments from pTZ18UB:*rsaA* (*Taq*I29*Bam*HI) (8) or the pTZ18UB:*rsaA* (*Msp*I69*Bam*HI) (7) were excised and ligated into pTZ18UB:*rsaA* (*Hin*PI277*Bam*HI) plasmid with the MCC cassette at RsaA position 277 (described above) that had been cut with *Eco*RI-*Not*I. The *rsaA Eco*RI/*Sst*I fragments were then excised from the resulting plasmids and subsequently ligated into the pWB9 plasmid that had been cut with *Eco*RI-*Sst*I, creating pWB9:*rsaA*ΔP:Taq29Hps1MCCΔ and pWB9:*rsaA*ΔP:Mps4Hps1MCCΔ.

pBBR3ΔSD:Taq169Hps1MCCΔ

The pTZ18UB:*rsaA* (*Hin*PI277*Bam*HI) plasmid with the MCC cassette at RsaA position 277 (described above) was cut with *Pst*I and then recircularized by ligation, eliminating two undesired *Cla*I sites in the released non-coding *Pst*I-*Pst*I fragment, creating plasmid pTZ18UB:*rsaA* (*Hin*PI277*Bam*HI)MCCΦ*Pst*I. The *rsaA Eco*RI/*Cla*I fragment from RB404-derived pTZ18UB:*rsaA* (*Taq*I169*Bam*HI) (8) was excised and then ligated into RB404-derived, *Eco*RI/*Cla*I cut pTZ18UB:*rsaA* (*Hin*PI277*Bam*HI)MCCΦ*Pst*I. From the resulting plasmid, the *rsaA Eco*RI/*Hind*III fragment was excised and ligated into *Eco*RI/*Hind*III cut pBBR3ΔSD, creating pBBR3ΔSD:Taq169Hps1MCCΔ.

2.6 – Construction of plasmids used for gene disruptions

pAL1

pAL1 plasmid construction was carried out by Assaf Levi. Plasmid pAL1 was constructed in order to create an in-frame deletion of the complete rsaA coding region. A

PCR product encoding a 1.0 kB region upstream of the *rsaA* gene was created using NA1000 chromosomal DNA as the template and using the primers 5'-GGA TCC GGC GTT CGA GCT GCT GCT GA-3' and 5'-GAA TTC TCA CCT GGC GGG TGA GTG AG-3', introducing *Bam*HI and *Eco*RI sites. Another PCR product was created using the primers 5'-GAA ATT CCG CTC GCC TAA GCG AAC GTC-3' and 5'-ACT AGT GGC CGA GAT CTT GCC GTC GA-3', amplifying a 1.0 kB region containing the end of *rsaA* and incorporating *Eco*RI and *Spe*I sites. Fragments were ligated into the pGEM-5ZT(+) vector at the *Eco*RV site using the pGEM-T® easy kit (Promega). The resulting fragments were digested with *Eco*RI, *Bam*HI, and *Spe*I, and ligated into *Bam*HI and *Spe*I cut pNPTS (32) plasmid. This resulted in the creation of pAL1, which was transformed by electroporation into the *E. coli* DH10B strain (Invitrogen) and selected by blue-white screening.

pK18mobsacB*manB*ΔNΔC

This plasmid was created by Dr. Peter Awram, and was used to create a strain that was null for Sap. A PCR product encoding *manB* that was deleted in the regions encoding the N- and C-termini of ManB was generated using NA1000 chromosomal DNA and the primers 5'-CCT GGG TCT GGG AAC CTA TAT CC-3' (ManB 169) and 5'-CAG TGC GGG CTC ATG GTC AG-3' (IManB 1202), and then blunt-end ligated into *Eco*RV-cut pBSKIIEEH (71). The *Eco*RI/*Hind*III fragment from the resulting plasmid (containing the desired deleted form of *manB* was then ligated into *Eco*RI/*Hind*III-cut pK18mobsacB (60), giving pK18mobsacB*manB*ΔNΔC.

pK18mobsacB:*pwb*ΔR

This plasmid, created by Theo Blake, was used to create an internal deletion in the S-layer-Associated Protease gene, *sap* (formerly known as *pwb*). A PCR product encoding *sap* was created using JS4000 chromosomal DNA as the template and the primers 5'-CCG CCC GAG CGA GCG CTG TGC GAA C-3' and 5'- ACC TTT TCG GGG GAG GGC CGC CCG C-3' and then blunt-end ligated into *Eco*RV-cut pBSKII (Stratagene). The *sap* gene was then excised from the resulting plasmid as an *Eco*RI/*Hind*III fragment, and ligated into *Eco*RI/*Hind*III-cut pBSKIIESH (71). The resulting plasmid was cut with *Pst*I, which removed 1023 bp of the *sap* sequence (approximately 1/2 of the *sap* gene), and the remaining plasmid was recircularized by ligation. The internally-deleted form of sap was then excised as an *Eco*RI/*Hind*III fragment, and ligated into *Eco*RI/*Hind*III-cut pK18mobsacB, creating pK18mobsacB:*pwb*ΔR.

pK18mobsacB:*rsaFa*ΔKP

The plasmid, created by Mike Toporowski, was used for the internal deletion of *rsaFa*, and contained some of the *rsaFa* flanking regions to encourage homologous recombination. A PCR product containing the *rsaFa* gene and flanking regions of 1008 bp 5' and 139 bp 3' was generated using NA1000 chromosomal DNA as the template, and the primers 5'-GCC ACG CCC GGC GTC CAG TCC GA-3' and 5'-GAG CTC CCT AGA GCG TTC TCC GAT CCG TGC G-3'. This fragment was blunt end ligated into the pBSKI+ plasmid (Stratagene) at the *Eco*RV site and the resulting construct was called pBSKI:*rsaFa*EX. The pBSKI:*rsaFa*EX plasmid was digested with *Kpn*I and *Pst*I and

blunt-ended using T4 DNA polymerase and ligated. The resulting plasmid has an 852 bp deletion. The internally-deleted version of *rsaFa* was excised as an *Eco*RI-*Hind*III fragment, and then ligated into *Eco*RI-*Hind*III cut pK18mobsacB, creating pK18mobsacB:*rsaFa*ΔKP.

pK18mobsacB*rsaA*353ΦB

This plasmid was created by Dr. John Nomellini. The *Bam*HI site in pTZ18UB:*rsaA* (*Aci*I353*Bam*HI) (7) was destroyed by cutting the plasmid with *Bam*HI, filling in the recessed ends with the Klenow enzyme, then ligating the Klenow-blunted ends together to recircularize the plasmid. This resulted in putting the *rsaA* sequence out of frame, introducing an early stop codon at a position corresponding to RsaA amino acid 358. The *Eco*RI/*Hind*III fragment containing the mutated *rsaA* gene was then ligated into *Eco*RI/*Hind*III cut pK18mobsacB, creating pK18mobsacB*rsaA*353ΦB.

2.7 – Construction of gene disruptions

JS1010

Knockout of *rsaFa* in NA1000 was done by Mike Toporowski using the pK18mobsacB:*rsaFa*ΔKP plasmid. Primary recombination of the plasmid was selected for using Km-resistance. Three consecutive sub-culturing events were done to encourage a second recombination event. Secondary selection on 5% sucrose PYE plates and subsequent replica plating on PYE and PYE Km plates was used to confirm a second recombination event. Colonies were then screened using the primers 5'-CGC CGG CTT CGC AGC GAT GA CCC-3' and 5'-CCC GGA GGC CTC CCA GGC GGC GTA-3' to

confirm that the appropriate gene replacement occurred. A strain confirmed to possess only the internally-deleted form of *rsaFa* was designated JS1010.

JS1011

Knockout of *rsaFa* in JS1008 (70) was done as described above for the knockout of *rsaFa* in NA1000, except that colonies determined to have had a second recombination event were first screened by colony western (7) using RsaA antiserum. Colonies that were S-layer negative according to the colony western were further screened by PCR using the same primers as described above to confirm that the appropriate gene replacement occurred. A strain confirmed to possess only the internally-deleted form of *rsaFa* was then subjected to the knocking out of *rsaA*, using the pAl1 plasmid. Primary recombination of the plasmid was selected for using Km-resistance, as well as Cm-resistance resulting from the Cm-cassette already integrated into the chromosome as a result of the *rsaFb* knockout in this strain. Three consecutive sub-culturing events in the presence of Cm (to maintain the *rsaFb* deletion) but not Km were done to encourage a second recombination event. Secondary selection on 5% sucrose PYE Cm plates and subsequent replica plating on PYE Cm and PYE Km Cm plates was used to confirm a second recombination event. Colonies were then screened by PCR using the primers 5'-GCG GCG GAG GTC TTG CAC CT-3' and 5'-CAT CTG GAT CGG GTT CTT GGT G-3'. A strain confirmed to possess only the internally-deleted form of *rsaA* was designated JS1011.

JS1012

The plasmid pK18mobsacB:*pwb*ΔR was used by Andrea Prusic to knockout *sap* in JS1001, replacing the wild type gene with and internally-deleted version of sap that was missing ~1/2 of the *sap* sequence. Primary recombination of the plasmid was selected for using Km-resistance. Three consecutive sub-culturing events were done to encourage a second recombination event. Secondary selection on 5% sucrose PYE plates and subsequent replica plating on PYE and PYE Km plates was used to confirm a second recombination event. Gene replacement was confirmed by PCR using the primers 5'-CCG CCC GAG CGA GCG CTG TGC GAA C-3' and 5'- ACC TTT TCG GGG GAG GGC CGC CCG C-3'. Next, the *rsaA* gene was knocked out using the pAL1 plasmid. Primary recombination of the plasmid was selected for using Km-resistance. Three consecutive sub-culturing events were done to encourage a second recombination event. Secondary selection on 5% sucrose PYE plates and subsequent replica plating on PYE and PYE Km plates was used to confirm a second recombination event. Colonies were then screened by PCR using the primers 5'-GCG GCG GAG GTC TTG CAC CT-3' and 5'-CAT CTG GAT CGG GTT CTT GGT G-3'. A strain confirmed to possess only the internally-deleted form of *rsaA* was designated JS1012.

JS1013

Dr. John Nomellini used pK18mobsacB*rsaA*353ΦB to knockout *rsaA* in NA1000, replacing the wild type *rsaA* gene with *rsaA* containing an early stop codon. Primary recombination of the plasmid was selected for using Km-resistance. Three consecutive sub-culturing events were done to encourage a second recombination event. Secondary

selection on 5% sucrose PYE plates and subsequent replica plating on PYE and PYE Km plates was used to confirm a second recombination event. Colonies determined to have had a second recombination event were screened by colony western (7) using RsaA antiserum. Colonies that were S-layer negative were further determined to have no surface-presented RsaA by low pH extraction (see protein techniques below).

JS1014

pK18mobsacB*manB*ΔNΔC was used to knockout *manB* in JS1013, leaving two non-functional copies of the gene in the chromosome, one missing the part of the gene encoding the ManB N-terminus and one missing the part of the gene encoding the ManB C-terminus. Recombination of the plasmid pK18mobsacB*manB*ΔNΔC was selected for using Km-resistance. A secondary recombination event was not sought after, thus stability of the primary recombination event was assured by always using Km when growing JS1014.

CB15Δ*rsaA*.

Knockout of *rsaA* in NA1000 to create CB15Δ*rsaA* was done using the plasmid pAL1 by Assaf Levi. Delivery of the pAL1 plasmid into NA1000 was done by conjugation with the *E. coli* LS980 (match maker) and MT607 helper strain (D. Alley). The helper strain utilizes vector pRK600, a derivative of pRK2013, Cm-resistance, containing a Tn9 insertion, ColE1 ori, and tra functions from pRK2013 (62). Gene replacement was confirmed by PCR analysis (not shown) and the resulting strain was named CB15Δ*rsaA*.

CB15ATn5F9/CB15ATn5F23

The CB15A Tn5 mutants F9 and F23 were made/confirmed as previously described (2).

JS4022

JS4022 was constructed by Dr. John Nomellini in the Sap-negative strain JS4015. The *repBAC* genes were introduced into the *recA* gene in the same way that resulted in the construction of JS4019, described previously (73).

JS4023

Knockout of *rsaFa* in JS4000 was done by Mike Toporowski using the pK18mobsacB:*rsaFa*ΔKP plasmid as described above for the creation of JS1010.

JS4024

Knockout of *manB* in JS4015 (72) was achieved using the pK18mobsacB*manB*ΔNΔC plasmid in the same manner utilized to create strain JS1014 (described above).

JS4025

Knockout of *rsaFb* in JS4023 was done via insertional inactivation using an N- and C-terminally deleted *rsaFb* fragment. The non-replicating plasmid pTZ18UCHE:*rsaFb*ΔNΔC (71) was electroporated into NA1000 competent cells and the Cm-resistance cassette was used for plasmid selection for insertional inactivation. Recombination of the pTZ18UCHE:*rsaFb*ΔNΔC plasmid resulted in loss of the full *rsaFb* gene, leaving independent N-and C-terminally-deleted *rsaFb* gene fragments.

Recombination of the plasmid was confirmed by PCR using the primers 5'-GAG GCC TAC TCT TCC TTT TTC AAT ATT ATT GAA-3' and 5'-GGA CGA CGC TGA CCA GCA CCC CCT GCT-3'.

2.8 – S-layer reattachment assays

Co-culturing assay

Co-culturing of S-layer donors (JS1001) with S-layer recipients (JS1003 & JS1004) was done as follows. JS 1001, JS1003 and JS1004 were grown to mid log phase. Aliquots of JS1001 and either JS1003 or JS1004 in a cellular ratio of 8 donors: 1 recipient were used to inoculate 10 mls of PYE. These co-cultures were then grown to mid-log phase, then filtered through Whatman 52 hardened filter paper (Whatman, Kent England) to remove any aggregated protein. Equivalent numbers of cells were then subjected to low pH extraction (see Protein techniques below) to assess the extent of S-layer reattachment.

RsaA reattachment assay

To produce soluble protein for reattachment purposes, cultures of smooth lipopolysaccharide (SLPS)-negative cultures (JS1001, for wild type RsaA), or SLPS-negative, S-layer-negative *C. crescentus* strains that are Sap-positive (JS1004) or Sap-negative (CB15ACa5ΔP6Δ*rsaA*) harboring the plasmids encoding RsaA or RsaA mutants were grown to OD~0.9, pelleted by centrifugation three times at 13, 000 rpm for 10 min at 4^{0}C; the pellet was discarded after each centrifugation in an effort to clear the supernatant of any cells. Supernatants were stored at 4^{0}C until needed. S-layer-negative target cells possessing various levels of SLPS (JS1003, JS1004, and JS4024) were grown

to OD_{600}~0.9, then pelleted by centrifugation at 13, 000 rpm for 5 min at 4^0C. Cell pellets were washed with 1ml of PYE then resuspended with the supernatants containing the soluble RsaA or RsaA mutant. The volume of supernatant used for resuspension was 1.2-1.5 times the volume of the target cells initially pelleted. The resulting mixtures were incubated at room temperature for 10-30 minutes with slow inversion. These cultures were then pelleted, washed twice with 10 mM HEPES pH 7 for subsequent low pH extraction, or washed twice with 10 mM Tris-HCl pH 8 for subsequent boiling of the entire sample or for subsequent whole cell protein preparations.

2.9 – Protein techniques

Low pH extraction

Surface protein from *C. crescentus* cells was extracted by low pH extraction or by EGTA-treatment as previously described (75). Briefly, cell pellets were washed twice using 10 mM HEPES pH 7.2 and then release of the S-layer was facilitated using 100 mM HEPES, pH 2.0 (low pH extraction) or 10 mM ethylene glycol bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA) in 10 mM HEPES pH 7.2. To compare the amounts of S-layer protein from *C crescentus* cells expressing RsaA to S-layer that had been reattached to S-layer-negative cells, equivalent amounts of cells (determined by OD_{600}) growing at log phase were used and equal amounts of extracted protein samples were loaded onto protein gels.

Whole cell protein preparation

Whole cell protein preparations were done with equivalent amounts of cells (determined by OD_{600}) growing at log phase. The cultures were centrifuged and the cell pellets were washed twice with 10 mM Tris-HCl pH 8. The cells were resuspended in 10 mM Tris-HCl pH8 and lysozyme (100 µg/ml) was added and the mixture incubated at 25°C for 15 minutes. RNaseA (50 µg/ml) and DNaseI (1 µg/ml) were added and the incubation continued at 37°C for 30 minutes. Equal amounts of whole cell protein preparations were loaded onto protein gels. Some samples were subjected to low pH extraction before whole cell protein preparation was done to disrupt RsaA monomer-monomer interactions, in order to assess RsaA that was directly anchored to the cell surface.

Whole cell protein preparation by boiling

As an alternative to whole cell protein preparations, equivalent numbers of cells were washed twice with 10 mM Tris-HCl pH 8 then subsequently boiled for 5 minutes.

Collagenase digests

Collagenase digests performed on soluble RsaA carrying a collagenase recognition sequence were done in PYE supplemented with collagenase buffer as follows. Supernatant from S-layer shedding *C. crescentus* strains containing the desired soluble RsaA protein were harvested as described above (see RsaA reattachment assay). The resulting culture supernatant was supplemented such that final concentrations of the following substances were achieved: 10 mM NaCl, 4 mM $CaCl_2$, 2 mM Tris-HCl pH 7, 2 mM ß-mercaptoethanol). Collagenase was added to 7 U/ml, and digestion was performed

for various lengths of time at 37^0C, and subsequently stored at 4^0C (if required) prior to use.

SDS-PAGE and Western blotting

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was done using 4% stacking, and 7.5% or 12% (as indicated) separating gels. Coomassie stained SDS-PAGE gels and Western immunoblotting were done as previously described (56). After transfer of proteins to 0.2 μm BioTrace NT nitrocellulose membrane (Life Sciences, Pensacola, FL), membranes were blocked using 3% skim milk, 0.9% NaCl, and 20 mM Tris-HCl pH8. Western blots were probed with primary rabbit polyclonal antibodies, and antibody binding was visualized by colorimetric developing methods. Colorimetric blotting was done using goat anti-rabbit Ig coupled to horseradish peroxidase and color forming reagents as previously described (66).

Antibodies

RsaA Δ188-784 antiserum (71) was used at a 1/10, 000 dilution and SLPS antiserum (74) was used at a 1/6, 000 dilution for colorimetric detection.

3. RESULTS – RsaA anchoring studies

3.1 – Assay development elucidates the importance of RsaA secondary structure for wild type RsaA anchoring

Full-length RsaA can be obtained by several different methods. For example, treating cells to low pH conditions extracts RsaA from the cell surface. EGTA-treatment of S-layer possessing cells also removes the S-layer. Cells deficient in smooth lipopolysaccharide (SLPS) shed their S-layer into the culture medium, and a fraction of this protein aggregates and precipitates out of solution. This protein can be collected by simple filtration, and solubilized by urea treatment. The remaining fraction of this shed S-layer remains soluble in the supernatant of these shedder cultures (this study). This soluble RsaA fraction can be collected by centrifugation of the culture and collection of the supernatant.

Our lab had previously found that to achieve in vitro recrystallization of RsaA onto lipid vesicles, the best method for RsaA preparation was the low pH extraction (45), but at that time it was not known that soluble RsaA is found in the supernatant of shedder cultures. I wanted to find the best method to achieve in vivo recrystallization of RsaA onto S-layer-negative cells rather than lipid vesicles, since I was trying to investigate RsaA attachment to the cell surface. To test the ability of RsaA to reattach to S-layer negative cells, I first obtained RsaA by low pH or EGTA extraction (see Materials and Methods) of wild type *C. crescentus* cells (NA1000). These RsaA preparations were then incubated with S-layer negative cells that either possessed wild type (JS1003) or deficient (JS1004) levels of SLPS (since SLPS is known to be involved in S-layer assembly on the cell surface), in water supplemented with $CaCl_2$ (since Ca^{2+} is required for normal levels

of S-layer assembly). The results indicated that RsaA obtained by EGTA extraction did not reattach to cells, while RsaA obtained by low pH extraction did reattach to cells in an SLPS-dependent manner (Fig. 2-1). Unfortunately, S-layer recrystallization did not occur to wild type levels; it appears that S-layer recrystallization occurred to about 10% of wild type S-layer levels (Fig. 2-1).

My next hypothesis was that perhaps S-layer recrystallization could be optimized by incubating S-layer protein with cells in the normal growth media PYE, rather than in calcium-supplemented water, and that dialyzing the prepared RsaA might render the protein more amenable to reattachment. To test this hypothesis, RsaA was first obtained by low pH or EGTA extraction of wild type *C. crescentus* cells (NA1000) and subsequently dialyzed (or not) against water. These RsaA preparations were then incubated with S-layer-negative cells that either possessed wild type (JS1003) or deficient (JS1004) levels of SLPS, in the normal *C. crescentus* growth media, PYE. The results show that again S-layer reattachment was achieved in an SLPS-dependent manner (except for the EGTA-extracted RsaA that was not dialyzed against water prior to addition to S-layer-negative cells), but unfortunately, not back to wild type levels (Fig. 2-2).

Co-culturing of S-layer donors (strains that lack SLPS and shed their S-layer) and S-layer recipients (strains that possess SLPS but lack S-layer) achieves S-layer recrystallization in *Aeromonas salmonicida* (25). I thought that perhaps the same experiment could be done with *C. crescentus*, and that this method might achieve better S-layer recrystallization than that achieved by the addition of low pH-extracted RsaA to S-layer-negative cells, since the acidic conditions during the low pH extraction might

	100%					10%
Amount of SLPS on cells:	wt	wt	d	wt	d	wt
Input RsaA extracted by:	N/A	EGTA		low pH		N/A

100 kDa

Fig. 2-1. S-layer reattachment in calcium-supplemented water.
Wild type RsaA was extracted from the *C. crescentus* cell surface by low pH or EGTA treatment. Resulting protein was subsequently incubated with equivalent amounts of S-layer negative cells that possess wild type (wt) or deficient (d) levels of SLPS, in calcium-supplemented water. Reattached RsaA was subsequently extracted by low pH treatment. Extractions were separated on a 7.5% SDS-PAGE gel, and RsaA was detected by western blotting using RsaA antiserum. For comparison, the lane labeled 100% is a low pH extraction of an equivalent number of cells expressing wild type levels of S-layer, and the lane labeled 10% is a low pH extraction of 10% of the number of cells used in the reattachment assay.

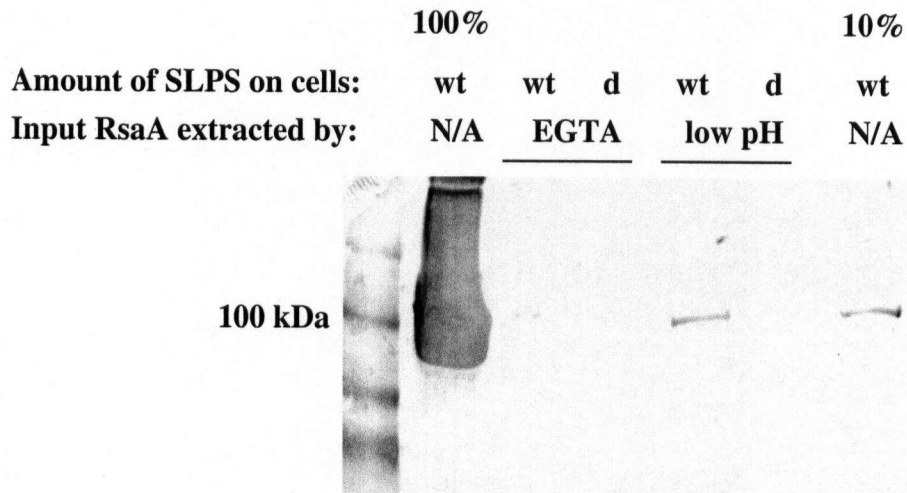| Amount of SLPS on cells:	wt	wt	d	wt	d	wt	d	wt	d
Input RsaA extracted by:	N/A	EGTA/dial		EGTA		low pH/dial		low pH	
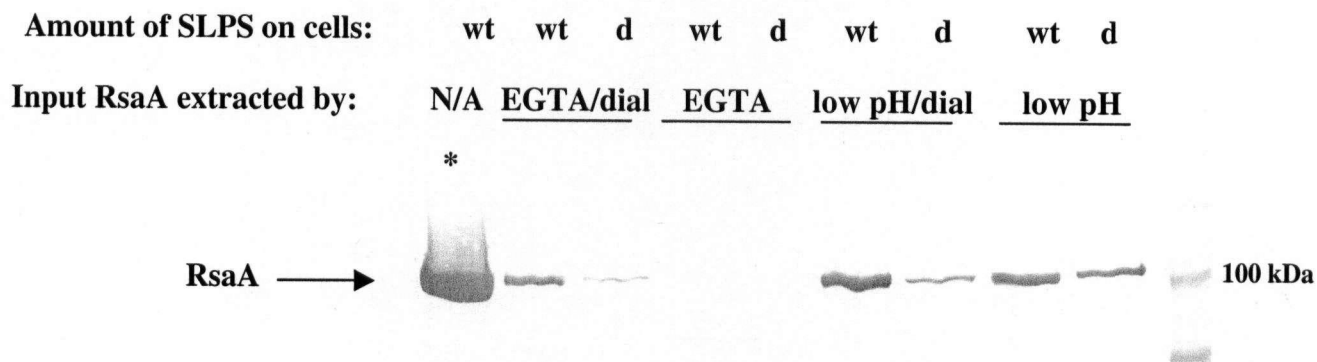
Fig. 2-2. S-layer reattachment in PYE.
Wild type RsaA was extracted from the *C. crescentus* cell surface by low pH or EGTA treatment, and the resulting protein was dialyzed (dial) or not against dH2O. These RsaA preparations were subsequently incubated with equivalent amounts of S-layer negative cells that possess wild type (wt) or deficient (d) levels of SLPS. Reattached RsaA was subsequently extracted by low pH treatment. Extractions were separated on a 7.5% SDS-PAGE gel, and RsaA was detected by western blotting using RsaA antiserum. For comparison, the lane labeled with * is a low pH extraction of an equivalent number of cells expressing wild type levels of S-layer.

cause loss of RsaA secondary structure that might be important for RsaA anchoring. To test this hypothesis, S-layer shedding cells (JS1001) were co-cultured with S-layer-negative cells that possessed wild type (JS1003) or deficient (JS1004) levels of SLPS. As predicted, more S-layer reassembled selectively to SLPS-positive cells (JS1003) in this method as compared to the methods aforementioned: SLPS-dependent S-layer-recrystallization (Fig. 2-3, lane 3) occurred to levels approaching those of wild type cells (Fig. 2-3, lane 1). These results suggested that perhaps that soluble, reattachment-competent RsaA is present in the supernatant of S-layer shedding strains, in addition to the RsaA that aggregates into macroscopic particles in these cultures. Accordingly, supernatant from an S-layer-shedding *C. crescentus* culture (JS1001) was analyzed, and soluble RsaA was indeed found in appreciable quantity (Fig. 2-4, lane 1). Protein prepared in this way was then tested for its ability to reattach to S-layer negative cells. RsaA obtained in this manner reassembled on S-layer-negative (JS1003) cells to wild type (NA1000) levels (Fig. 2-4, compare lane 6 to lane 2). These results suggest that RsaA obtained in this manner retains the proper secondary structure for optimum levels of S-layer recrystallization.

The varied ability of RsaA prepared in different ways to reattach to S-layer-negative cells suggests that obtaining RsaA by some methods causes a loss of secondary structure that is important for RsaA anchoring. These results not only address the importance of the maintenance of proper S-layer folding for normal levels of S-layer presentation on the cell surface, but also provide the basis of an assay to test S-layer-mutants for their ability to reattach, in an effort to investigate the importance of RsaA regions or residues for RsaA anchoring.

44
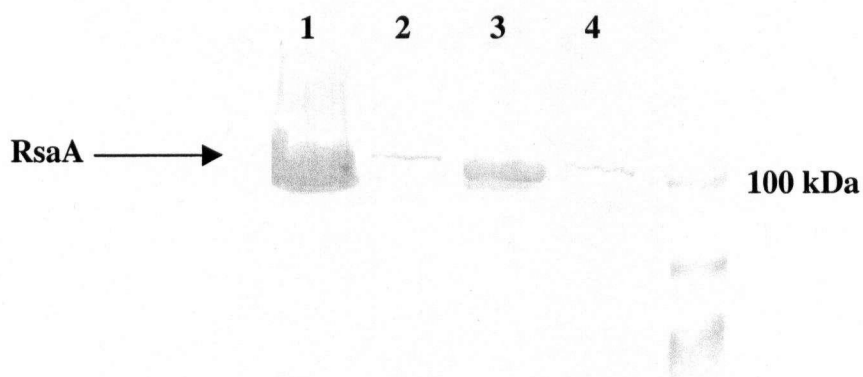
Fig. 2-3. S-layer reattachment by co-culturing S-layer donors and recipients.
S-layer shedding cells (donors) were co-cultured with S-layer-negative cells (recipients) that possess wild type or deficient levels of SLPS, in a cell ratio of 8 donors:1 recipient. Equivalent numbers wild type cells alone (lane 1), donor cells alone (lane 2), donors + wild type-SLPS recipients (lane 3), or donors + deficient-SLPS recipients (lane4) were subjected to low pH extraction. Extractions were separated on a 7.5% SDS-PAGE gel, and RsaA was detected by western blotting using RsaA antiserum. SLPS-dependent S-layer-recrystallization (lane 3) occurred to levels close to wild type levels (lane 1).

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Fig. 2-4. S-layer reattachment using soluble RsaA from the supernatant of shedder cultures.

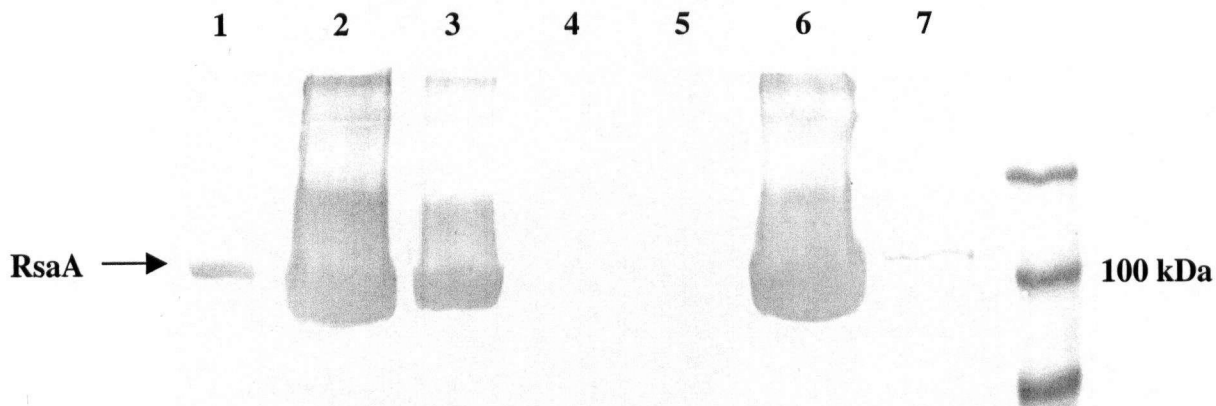Soluble RsaA harvested from the supernatant of an S-layer shedding strain was incubated with an equivalent number of S-layer negative cells that possess wild type or deficient levels of SLPS. Reattached RsaA was subsequently extracted by low pH treatment. Extractions were separated on a 7.5% SDS-PAGE gel, and RsaA was detected by western blotting using RsaA antiserum. Lanes: 1, 12 uL supernatant from shedder strain; 2 & 3, to assess the extent of S-layer reattachment, low pH extractions of 100% (lane 2) and 10% (lane 3) of the number of cells used in the reattachment assay were performed; 4, low pH extraction of S-layer-negative cells possessing wild type levels of SLPS (control); 5, low pH extraction of S-layer-negative cells possessing deficient levels of SLPS (control); 6, low pH extraction of S-layer-negative cells possessing wild type levels of SLPS after incubation with soluble RsaA; 7, low pH extraction of S-layer-negative cells possessing deficient levels of SLPS after incubation with soluble RsaA.

3.2 – SLPS is required for RsaA anchoring

A fraction of the lipopolysaccharide (LPS) on the surface of *C. crescentus* cells has an O antigen polymer attached to the core to form a "smooth" LPS (SLPS). This O antigen polymer consists at least in part of N-acetylperosamine, a 4-amino-4,6-dideoxymannose (2). *C. crescentus* strains that are deficient in SLPS shed their S-layer into the culture medium, suggesting that SLPS is involved in S-layer anchoring. However, our lab did not have a strain that was completely devoid of SLPS; rather, JS1004 was isolated as a strain grown in the absence of calcium which likely generated a "leaky" point mutation that drastically reduced SLPS production, but did not ablate it entirely (2). In reattachment assays of RsaA to JS1004, a very low level of RsaA still reattached to the cells (Fig. 2-4, lane 7). This left the possibility that either SLPS or some other molecule on the cell surface was mediating the low level of S-layer reattachment observed with JS1004. To determine if other molecules could mediate RsaA anchoring in the absence of SLPS, a strain completely devoid of SLPS and RsaA needed to be constructed.

The putative N- acetylperosamine biosynthetic pathway was previously proposed (2) and is shown in Fig. 2-5. ManB is a putative phosphomannomutase involved in this pathway, catalyzing the conversion of mannose 6-phosphate to mannose 1-phosphate. Previous transposon mutagenesis studies have shown that the disruption of *manB* results in the loss of SLPS production in vivo (2). To construct a strain completely devoid of SLPS and also RsaA, I disrupted *manB* in a strain that was already lacking RsaA (JS1013); the resulting strain was named JS1014. JS1014 was to be used as an S-layer-negative, SLPS-negative target for S-layer reattachment, to determine if any S-layer at all

Glucokinase → Glucose 6-phosphate

Pgl ↓ *Phosphoglucoisomerase*

Fructose 6-phosphate

ManC ↓ *Phosphomannoisomerase*

Mannose 6-phosphate

ManB ↓ *Phosphomannomutase*

Mannose 1-phosphate

ManC ↓ *Phosphomannoisomerase*

GDP-mannose

Gmd ↓ *GDP-mannose 4,6-dehydratase*

GDP-4-keto-6-deoxymannose

Per ↓ *Perosamine synthetase*

GDP-perosamine
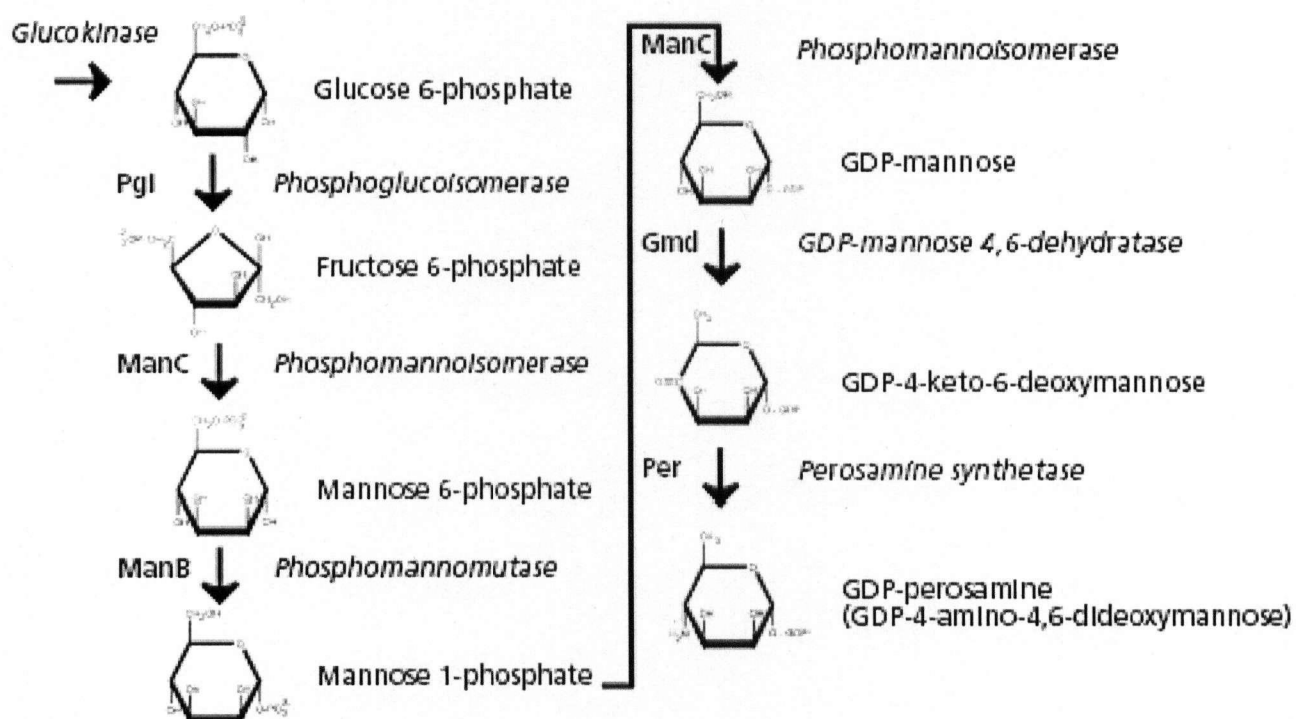(GDP-4-amino-4,6-dideoxymannose)

Fig. 2-5. Proposed N-acetylperosamine biosynthetic pathway (2).

could reattach to cells completely devoid of SLPS. The disruption of *manB* resulted in a strain completely devoid of SLPS, as expected (Fig. 2-6, lane 6).

To determine if RsaA reattachment could be mediated by another molecule in the absence of SLPS, soluble, reattachment-competent RsaA (isolated from the supernatant of JS1001) was incubated with S-layer-negative cells that had no SLPS (JS1014), low levels of SLPS (JS1004), or wild type levels of SLPS (JS1003). No S-layer reattachment was observed to cells that completely lack SLPS, a low amount of S-layer reattached to cells with low levels of SLPS, and a large amount of S-layer reattached to cells possessing wild type levels of SLPS (Fig. 2-7). These results confirm that SLPS is required for S-layer anchoring, and that no other molecule on the cell surface can mediate S-layer attachment in the absence of SLPS.

3.3 – Two anchoring regions in RsaA?

Previous work has suggested that the N-terminus of RsaA mediates cell surface anchoring, since N-terminal truncations of RsaA lead to shedding of the S-layer into the culture medium. To begin to investigate the importance of regions or residues within the RsaA anchoring region, my first goal was to find a region or regions in RsaA sufficient for RsaA anchoring. In order to generate RsaA fragments that retained proper secondary structure necessary for RsaA anchoring, I first engineered collagenase cleavage sites at various points throughout RsaA. Full-length RsaA bearing the collagenase cleavage sites at various positions were retrieved from the supernatant of *C. crescentus* shedder cultures, treated with collagenase, and the resulting RsaA fragments were tested for their ability to selectively reattach to SLPS-positive cells. For example, one collagenase
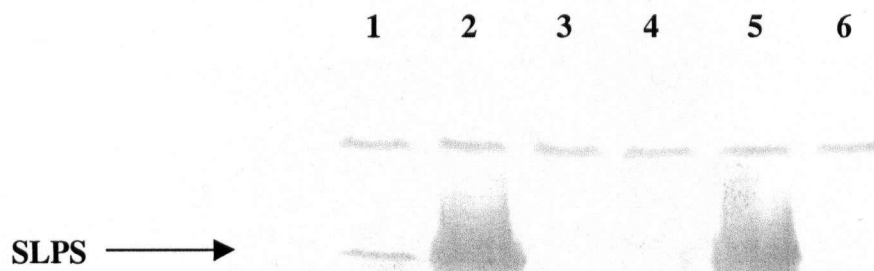
Fig. 2-6. SLPS levels in SLPS-positive, SLPS-deficient, and SLPS-negative C. crescentus strains.
Cells possessing varying amounts of SLPS were grown to mid log phase. Whole cell protein preparations from equivalent numbers of cells were performed. Samples were run on a 7.5% SDS-PAGE gel. SLPS was detected by western blotting using SLPS antiserum. Lanes: 1, Kan-cassette interruption of *rsaA* in spontaneous calcium-independent mutant (deficient levels of SLPS); 2, Kan-cassette interruption of *rsaA* (*wt* levels of SLPS); 3, Transposon insertion mutant Tn5F9 (interruption of *manB (2))*; 4, Transposon insertion mutant Tn5F23 (interruption of *manB (2))*; 5, *wt C. crescentus*; 6, *manB* knock-out in S-layer-negative strain (no SLPS).

SLPS levels
on target cells ⟶ N/A   wt   d   -

RsaA ⟶

110 kDa

90 kDa

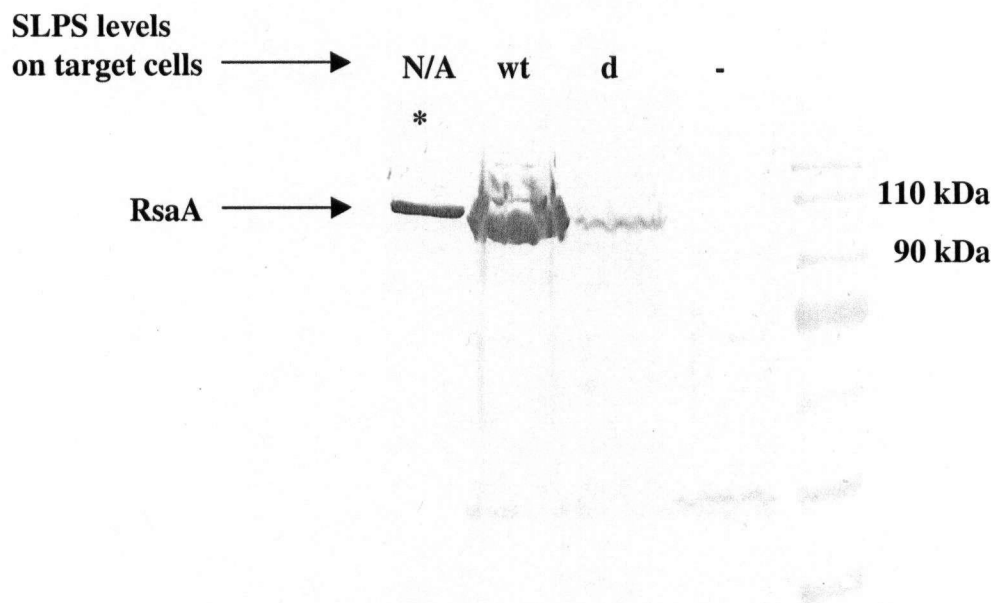*Fig. 2-7. S-layer attachment requires SLPS.*
Wild-type RsaA was incubated with cells possessing varying SLPS levels (wild type, wt, deficient, d, or none, -). Whole cell protein preparations from equivalent numbers of cells were then performed. Extracted protein was separated on a 10% SDS-PAGE gel. RsaA was detected by western blotting using RsaA antiserum. Lane marked with * is input RsaA alone.

cleavage site was engineered at a position in RsaA corresponding to amino acid 690. This protein was harvested from the supernatant of an S-layer-negative *C. crescentus* shedder culture (JS1004) harboring a plasmid-borne copy of *rsaA* with a collagenase site at the position corresponding to RsaA amino acid 690, and subsequently treated with collagenase. The resulting RsaA fragments were incubated with cells possessing varying levels of SLPS, (JS1014, JS1004, and JS1003) and then the cells were analyzed for the presence of reattached RsaA fragments. Equivalent amounts of the RsaA N- and C-terminal fragment resulted from collagenase cleavage of the RsaA mutant, as expected and as evidenced by Coomassie-staining of the digest products separated on an SDS-PAGE gel (data not shown). However, RsaA antiserum reacted poorly with the RsaA N-terminal fragment (Fig. 2-8, lane marked with *). Unexpectedly, both the N- and C-terminal RsaA fragments were detected on cells possessing wild type levels of SLPS (JS1003) (Fig. 2-8). An appreciable amount of the RsaA C-terminal fragment reattached to the cells, and given the limited reactivity of the antibody towards the RsaA N-terminal fragment, an appreciable amount of the RsaA N-terminal fragment probably reattached to those cells as well (Fig. 2-8). The phenomenon of RsaA N- and C-terminal fragment reattachment was observed for several other RsaA mutants as well (data not shown). This opened the possibility that two RsaA anchoring regions may exist in RsaA, or that some type of co-operative binding may be occurring. However, since RsaA N-terminal truncations shed into the culture medium (9), the RsaA C-terminus alone likely cannot mediate cell surface anchoring. We favored the idea that the observed RsaA C-terminal fragment reattachment was a result of its co-operative binding to reattached RsaA N-terminal fragment or to reattached full-length RsaA. The observed RsaA C-terminal

Amount of SLPS on target cells → N/A   wt   d   -

*

Full-length RsaA →

RsaA N-terminal fragment →

RsaA C-terminal fragment →

160 kDa
110 kDa

90 kDa

70 kDa

55 kDa

45 kDa
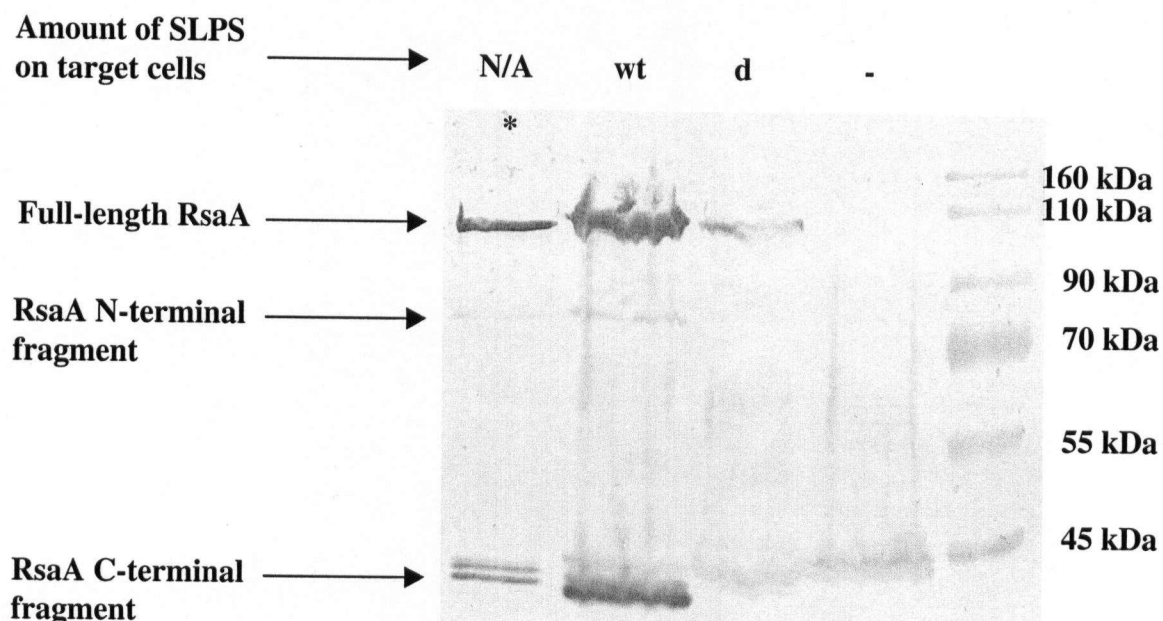
Fig 2.8. Two anchoring regions in RsaA?
RsaA fragments (resulting from collagenase treatment of mutant RsaA bearing a collagenase cleavage site at residue 690) were incubated with cells possessing varying SLPS levels (wild type, wt, deficient, d, or none, -). Whole cell protein preparations from equivalent numbers of cells were then performed. Extracted protein was separated on a 10% SDS-PAGE gel. RsaA and RsaA fragments were detected by western blotting using RsaA antiserum. Lane marked with * is input (cleaved) RsaA alone.

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fragment reattachment turned out to require full-length RsaA, as I discuss in a later part of this Results section.

3.4 – RsaA 1-277 is sufficient for RsaA anchoring

Since disruption of RsaA anchoring domain(s) and disruption of RsaA subunit-subunit interaction domains both result in the same phenotype (the S-layer is shed from the bacterium), when a particular mutation results in a shedding phenotype, it is difficult to know whether that mutation disrupted RsaA anchoring or crystallization (subunit-subunit interaction). My approach to determining the regions of the RsaA N-terminus that are important for RsaA anchoring was to first find a small RsaA N-terminal fragment that is sufficient for RsaA anchoring. Since such a protein would be lacking much of the native sequence putatively involved in RsaA subunit-subunit interactions (crystallization), I would have confidence that subsequent mutations within this RsaA N-terminal fragment that lead to loss of reattachment of this small fragment could be ascribed to disruptions in the RsaA-SLPS interaction, rather than disruptions in RsaA subunit-subunit interactions (recall that some RsaA monomers are thought to be directly anchored to SLPS on the cell surface, while others are thought to be tethered to the cell surface only by interacting with other RsaA monomers rather than by a direct interaction with SLPS).

To find a small RsaA N-terminal fragment sufficient for reattachment, I first engineered collagenase cleavage sites at various positions within full-length RsaA. These proteins were collected from *C. crescentus* shedder strains, treated with collagenase, and the resulting RsaA fragments were incubated with S-layer-negative *C. crescentus cells* to assess their ability to reattach to these cells. RsaA 1-277 was identified as a small RsaA

N-terminal fragment that was sufficient for RsaA reattachment to these cells (Fig. 2-9, lane 3), whereas RsaA 1-162 could not be detected by available RsaA antiserum (Fig 2-9, lane 2). Note that consistent with the data aforementioned, both the RsaA N-and C-terminal fragments reattached to cells in the case of RsaA fragment pair 1-723 & 724-1026, as well as full length RsaA (Fig. 2-9, lane 5), leaving the question of whether two anchoring regions exist in RsaA or whether co-operative binding is occurring unresolved. This issue is addressed in the next part of this Results section.

3.5 – The RsaA C-terminal fragment reattachment requires full-length RsaA

Results from several experiments indicated that both the RsaA N- and C-terminal fragments (resulting from collagenase treatment of RsaA bearing a collagenase site) can reattach to S-layer-negative cells. One way to resolve the issue of whether or not there are two anchoring regions within RsaA or whether co-operative binding occurs is to separate RsaA fragments resulting from collagenase digestion, then test the individual fragments for reattachment capability. I attempted to separate these RsaA fragments by several standard methods, including using Amicon Ultra Centrifugal Filter Devices™ (Bedford, Massachusetts) with the appropriate molecular weight cut-offs, size exclusion chromatography, and affinity chromatography using a Histidine-tagged version of RsaA. All attempts to separate the fragments were unsuccessful, perhaps due to the intrinsic nature of RsaA to multimerize. We have observed in the lab that S-layer fragments appear to associate with one another, rendering separation of these fragments quite difficult (Smit, unpublished). For example, a Histidine-tagged RsaA fragment will bind to a nickel column, but unfortunately the other RsaA fragment (from which separation is
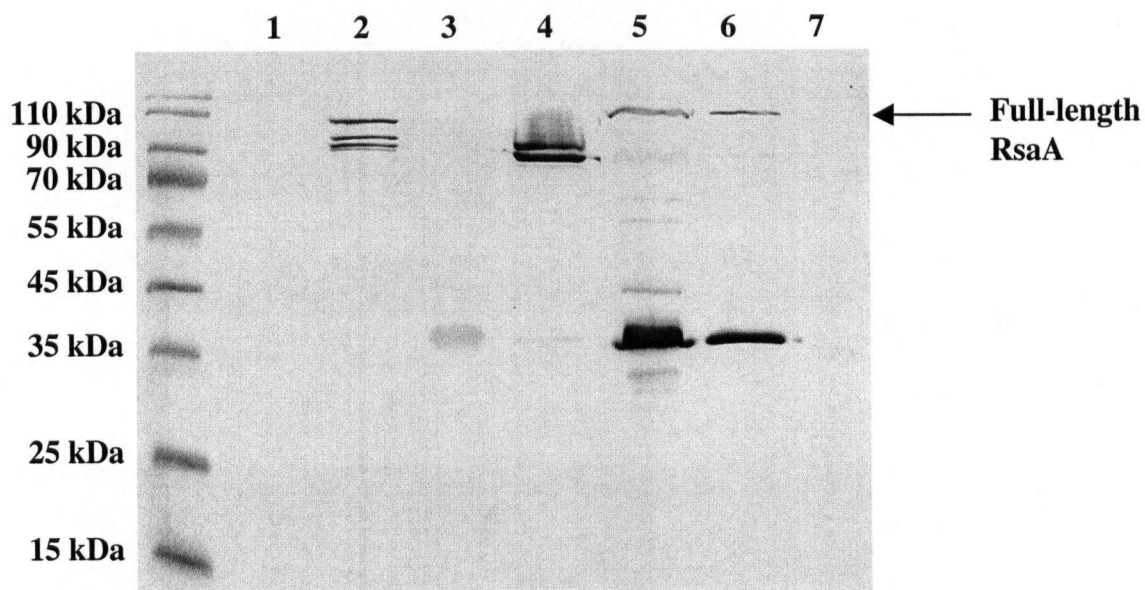
Fig. 2-9. RsaA 1-277 is sufficient for RsaA anchoring.
RsaA bearing a collagenase cleavage site at residue 162, 277 or 723 was cleaved by collagenase and the resulting RsaA fragments were incubated with Sap-negative, S-layer-negative cells. After washing, protein preparations from equivalent numbers of cells were then separated on a 12% SDS-PAGE gel. RsaA and RsaA fragments were detected by western blotting using RsaA antiserum. Lanes: 1, cleaved RsaA (cleavage site at residue 162) + cells; 2, cleaved RsaA (cleavage site at residue 162) alone; 3, cleaved RsaA (cleavage site at residue 277) + cells; 4, cleaved RsaA (cleavage site at residue 277) alone; 5, cleaved RsaA (cleavage site at residue 723) + cells; 6, cleaved RsaA (cleavage site at residue 723) alone; 7, cells alone.

desired) apparently associates with the column-bound RsaA fragment, and thus both fragments come off of the column upon elution (data not shown). Similarly, RsaA fragments tend to elute from a size exclusion column together as one peak in the void volume, even in the presence of detergents or EDTA (data not shown). This suggests that these RsaA fragments may form soluble microaggregates that cannot enter into the column matrix beads.

Upon closer inspection of the data from several experiments, it appeared that the RsaA N-terminal fragment always reattached to cells. However, the RsaA C-terminal fragment only reattached when residual full-length (uncleaved) RsaA, left over despite collagenase treatment, was available and also reattached. This suggested that the reattachment of the RsaA C-terminal fragment may be due to an association of C-terminal fragments with residual full-length S-layer, rather than direct attachment of the RsaA C-terminal fragment to the cell surface. To test this hypothesis, RsaA bearing a collagenase cleavage site at residue 277 was treated with collagenase for various lengths of time, in order to generate complete and partial digests of the protein. The resulting RsaA and RsaA fragments were then incubated with S-layer-negative cells (JS4025) to test their ability to reattach. As hypothesized, the RsaA C-terminal fragment (278-1026) only reattached when some full-length RsaA was available and also reattached (Fig. 2-10, compare lane 2 with lane 5). Since the RsaA C-terminal fragment did not reattach in the absence of full-length RsaA, these results indicate that the RsaA C-terminal fragment contained no anchoring information. In contrast, the RsaA N-terminal fragment reattaches independently of full-length RsaA (or the RsaA C-terminal fragment), indicating that the anchoring region of RsaA is located in the RsaA N-terminus.
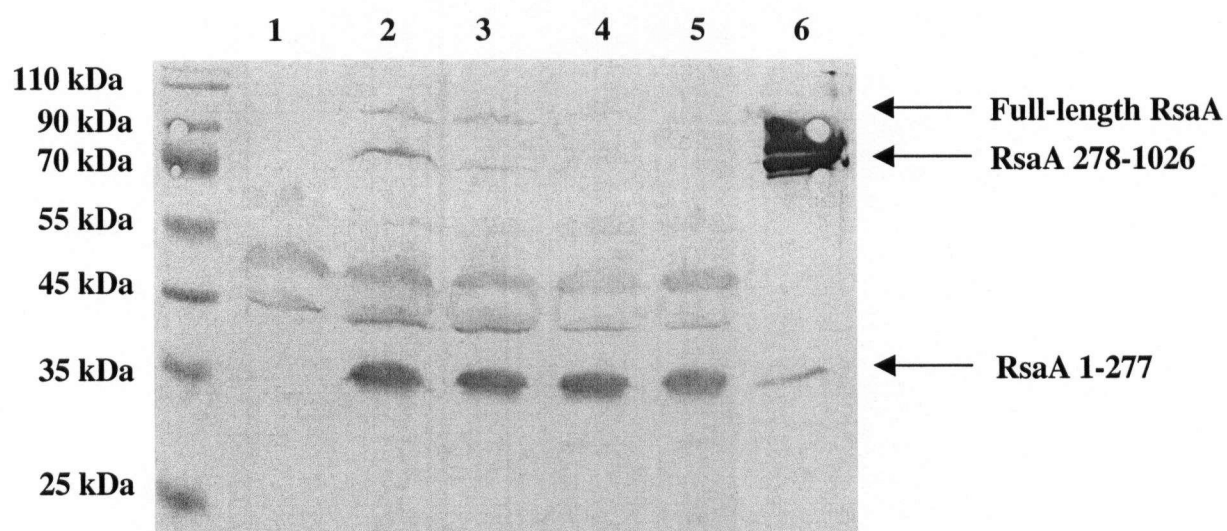
Fig. 2-10. RsaA C-terminal fragment anchoring requires full-length RsaA.
RsaA bearing a collagenase cleavage site at residue 277 was cleaved by collagenase for increasing lengths of time. The resulting RsaA fragments were incubated with SAP-negative, S-layer negative cells. After washing, protein preparations from equivalent numbers of cells were then separated on a 12% SDS-PAGE gel. RsaA and RsaA fragments were detected by western blotting using RsaA antiserum. Lanes: 1, cells (alone); 2 through 5, cells + RsaA cut by collagenase for a specific length of time: 2, 60 min; 3, 120 min; 4, 180 min; 5, overnight; 6, RsaA cut by collagenase overnight (alone).

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3.6 – Mutations in RsaA 1-277 cause the loss of RsaA anchoring

My next goal was to determine which regions within the RsaA N-terminus are important for anchoring. Accordingly, I constructed independent mutations in the N-terminus at positions in RsaA corresponding to amino acids 7, 29, 69, 154, 169, & 222 (Fig. 2-11). These mutations were chosen as an attempt to cover the entire putative RsaA anchoring region, since little information is gained from secondary structure predictions for RsaA using current algorithms. The mutations at residues 7, 154 and 222 resulted in a two amino acid exchange for Gly/Ser, while the mutations at residues 29, 69, and 169 resulted in the insertion of four amino acids (N-Asp-Gly-Ser-Val) at these positions (see Materials and Methods section). All of these mutants also possessed the collagenase cleavage site at residue 277. These full-length proteins were collected from *C. crescentus* shedder strains and subsequently treated with collagenase. The resulting fragments were then incubated with S-layer negative cells, and after washing, the cells were analyzed for the presence of RsaA and RsaA fragments. Surprisingly, all mutations in the RsaA N-terminus caused loss of anchoring (Figs. 2-12 and 2-13). These results suggest that all of the regions of RsaA that were mutated contribute in some way to the anchoring of the RsaA N-terminus to SLPS. Additionally, the digest of RsaA bearing the collagenase site at residue 277, but without further mutation in the N-terminus (the control input protein), appeared to go to completion (Fig. 2-12, lane marked with *). Although the resulting RsaA 1-277 fragment clearly reattached to SLPS-positive cells, there is no evidence of any C-terminal fragment (RsaA 278-1026) binding (Fig. 2-12, lanes marked wt). This is consistent with the aforementioned suggestion that some full-length protein is required for any C-terminal fragment to bind, and that only the N-terminus of RsaA carries the

Collagenase site at residue 277

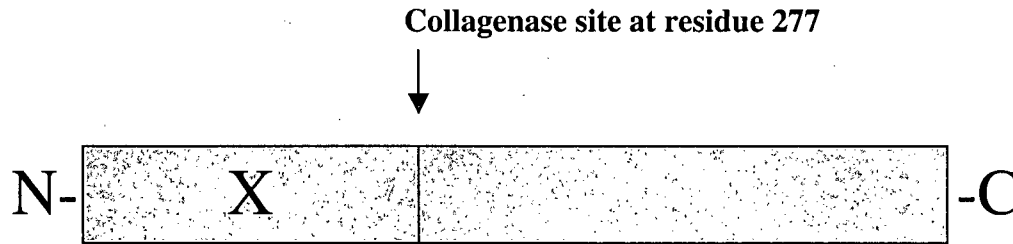*Fig. 2-11. Box model of mutant RsaA used for reattachment studies.*
X represents independent mutations at positions in the RsaA N-terminus
corresponding to amino acids 7, 29, 69, 154, 169, and 222. These full-length proteins
were isolated from *C. crescentus* shedder strains and subsequently treated with
collagenase. The resulting RsaA fragments were assessed for their ability to reattach
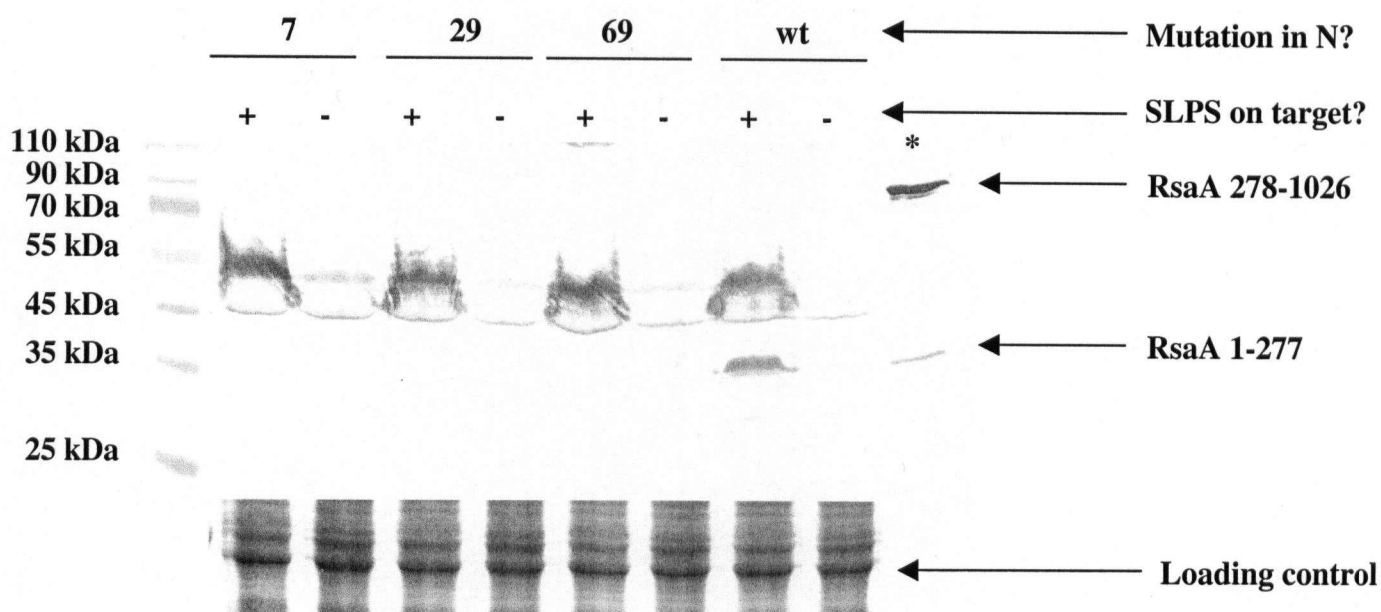to S-layer-negative cells.

Fig. 2-12. Mutations at RsaA amino acids 7, 29, and 69 disrupt RsaA anchoring.
RsaA bearing a collagenase cleavage site at residue 277 that also possessed or did not possess (wt) an additional mutation in the N-terminus were cleaved by collagenase. The resulting RsaA fragments were incubated with Sap-negative cells that either possessed (+) or did not possess (-) SLPS. After washing, whole cell protein preparations from equivalent numbers of cells were then performed. Extracted protein was separated on a 12% SDS-PAGE gel. RsaA and RsaA fragments were detected by western blotting using RsaA antiserum. The lane marked with * is a sample of the collagenase-treated input protein that did not possess an additional mutation in its N-terminus.

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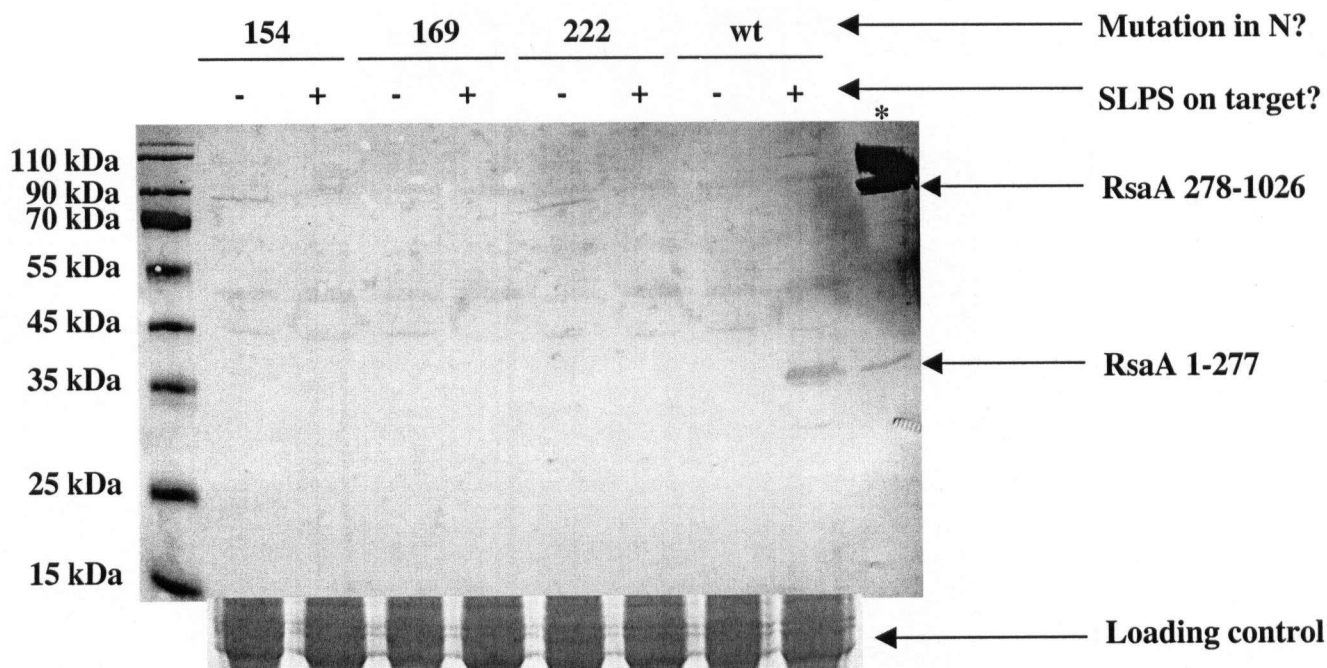
Fig. 2-13. Mutations at RsaA amino acids 154, 169, and 222 disrupt RsaA anchoring.
RsaA bearing a collagenase cleavage site at residue 277 that also possessed or did not possess an additional mutation in the N-terminus were cleaved by collagenase. The resulting RsaA fragments were incubated with SAP-negative cells that either possessed or did not possess SLPS. After washing, whole cell protein preparations from equivalent numbers of cells were then performed. Extracted protein was separated on a 12% SDS-PAGE gel. RsaA and RsaA fragments were detected by western blotting using RsaA antiserum. The lane marked with * is a sample of the collagenase-treated input protein that did not possess an additional mutation in its N-terminus.

anchoring information for the protein. In the case of the RsaA mutants, there is no evidence of any C-terminal fragment (RsaA 278-1026) binding. This is consistent with the hypothesis that only the RsaA N-terminus can mediate anchoring: if there were any residual full-length mutant protein remaining after the collagenase treatment, its mutated N-terminus would be incapable of mediating anchoring, thus preventing its - and consequently the C-terminal fragment's – reattachment to the cell surface. Taken together, these data confirm that the RsaA N-terminus mediates RsaA anchoring, and that small perturbations within the first ~225 amino acids disrupt RsaA anchoring.

4. RESULTS - Sap localization studies

4.1 – Sap cleaves RsaA 1-277

Throughout the course of my initial RsaA reattachment studies, I observed that reattachment of RsaA 1-277 to the cell surface of S-layer-deficient cells resulted in the cleavage of RsaA 1-277. Interestingly, this cleavage resulted in a product that was about 28 kDa, the same sized cleavage product previously observed for some other RsaA mutants (8). One possible candidate for this proteolytic activity was the S-layer-associated protease, Sap (72). To determine if Sap was responsible for the cleavage of RsaA 1-277, soluble RsaA bearing the collagenase site at residue 277 was isolated from cells that harbored a plasmid-borne copy of rsaA with the collagenase site engineered at residue 277; these cells were either Sap-positive (JS1004) or Sap-negative (internal deletion in *sap*, see Materials and Methods) cells (JS1012). The isolated protein was subsequently treated with collagenase, and the resulting RsaA fragments were incubated with S-layer-negative target cells that were either Sap-positive (JS1003) or Sap-deficient (JS4025). The results indicate that the source of the input protein did not matter in terms of the cleavage of RsaA 1-277, however, the use of Sap-positive target cells (JS1003) leads to complete cleavage of this protein, whereas the use of Sap-deficient target cells largely prevented this cleavage (Fig. 3-1, compare lanes 1 & 4 or lanes 2 & 5). The reason that the cleavage was not completely ablated when Sap-deficient target cells (JS4025) were used may be that in this strain, there is only a point mutation in the active site of *sap*, (72) which significantly reduces its activity, but may not abolish it completely, allowing for some residual cleavage of RsaA 1-277. These data indicate that target cell-harbored Sap is responsible for cleavage of RsaA 1-277.
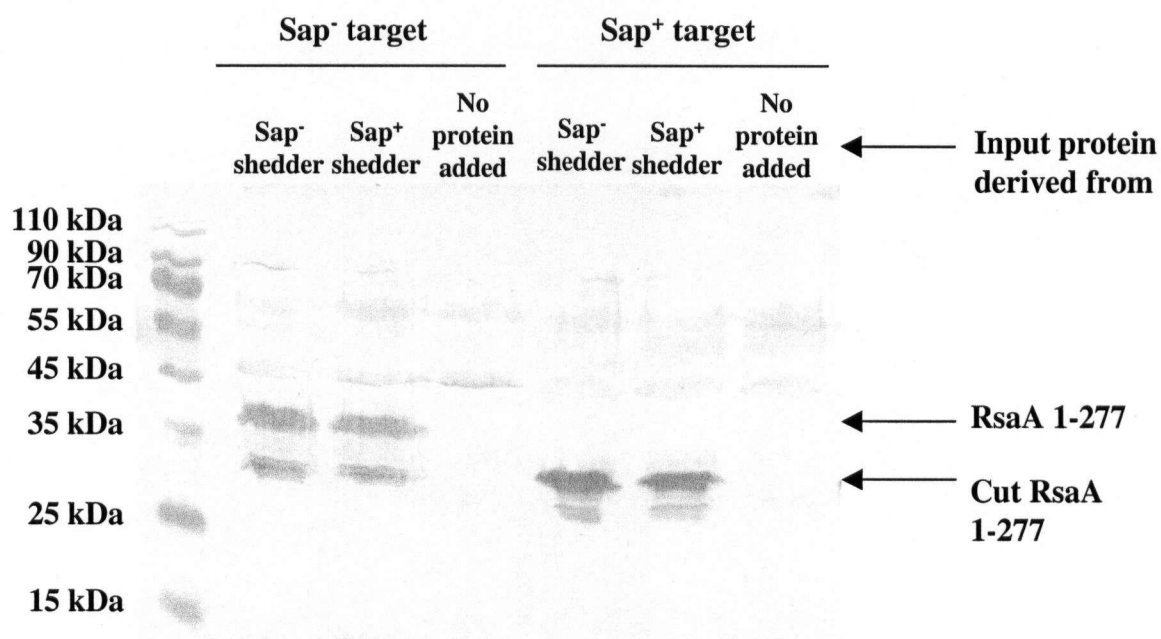
Fig. 3-1. Sap cleaves RsaA 1-277.
RsaA bearing a collagenase site at 277 was isolated from a Sap⁻ or Sap⁺ shedder strain, then treated with collagenase. The resulting RsaA fragments were then incubated with equivalent numbers of S-layer-negative cells that are Sap⁺ or Sap⁻. After washing, whole cell preparations were performed. Samples were separated on a 12% SDS-PAGE gel. RsaA fragments were detected by western blotting using RsaA antiserum.

This was a fortuitous result, since our S-layer reattachment assay involved mutating specific regions within RsaA 1-277, then testing these fragments for their ability to reattach to S-layer-negative cells. Undesired cleavage of RsaA 1-277 (that contained a particular mutation within these residues) being tested for its ability to reattach to S-layer deficient cells is prevented by using Sap-deficient cells as targets in the reattachment assay.

4.2 – Sap is an extracellular enzyme

In the S-layer reattachment assay, protein is added exogenously to intact cells. Upon analysis of reattached protein, we found that RsaA 1-277 was cleaved by Sap. Initially, to examine whether or not a particular RsaA fragment had reattached to a target cell, whole cell protein preparations were performed (using lysozyme, see Materials and Methods). This resulted in the lysis of the target cells. For Sap-positive (target) cells, it was possible that a) Sap is localized on the outer membrane or in the supernatant of the target cells and can therefore easily access and cleave RsaA 1-277 during the reattachment assay, or b) Sap is cytoplasmic, but upon cell lysis, Sap encounters and cleaves RsaA 1-277. To investigate the localization of Sap, the reattachment of RsaA 1-277 to Sap-positive target cells was performed, and then samples were either analyzed after the usual whole cell protein preparation, or by boiling samples after reattachment (see Materials and Methods). Like the whole cell protein preparations, boiling the samples would cause cell lysis. However, unlike the whole cell protein preparation, boiling would also be expected to denature the proteins in the sample, and thus Sap, if intracellular, may be released upon this boiling and thus could come into contact with RsaA 1-277, but Sap would be expected to be denatured. The denatured Sap would be

expected to be inactive and therefore unable to cleave RsaA 1-277. RsaA 1-277 was incubated with S-layer-negative, Sap-positive cells (JS1003), and then after washing, one aliquot of the resulting cells was boiled immediately, while another aliquot containing an equivalent amount of cells was subjected to whole cell protein preparation. The results indicate that in comparison to preparing the protein sample by whole cell protein preparation, boiling the samples immediately after the reattachment assay still resulted in a significant amount of cleaved RsaA 1-277 (Fig. 3-2, lane 1). This suggests that RsaA 1-277 encounters and is cleaved by Sap during the reattachment assay rather than after reattachment/cell lysis, which necessitates that Sap be localized extracellularly.

4.3 – S-layer Type I secretion OMPs Fa and/or Fb are involved in Sap secretion

Since data were accumulating that suggested that Sap is an extracellular enzyme, our focus shifted to how the protease might get secreted from the bacterium. One hypothesis is that Sap is secreted by the same Type I secretion system that RsaA utilizes. Although there is no high degree of homology between the C-terminus of RsaA and the C-terminus of Sap (i.e., no indication that Sap may contain a Type I secretion signal similar to that of RsaA), since it has been shown that another protease called AprA from *Pseudomonas aeruginosa*, whose C-terminus shares even less homology to the C-terminus of RsaA, can be secreted by the *Caulobacter crescentus* Type I secretion system (1), we thought it possible that Sap could also be secreted by this system. To test this hypothesis, a strain that I constructed (JS1011) that was devoid of the outer membrane proteins RsaFa and RsaFb involved in this system (Fig. 3-3, lane 3) as well as S-layer (data not shown) was used as a target for the reattachment of RsaA 1-277. The cleavage of RsaA 1-277 was
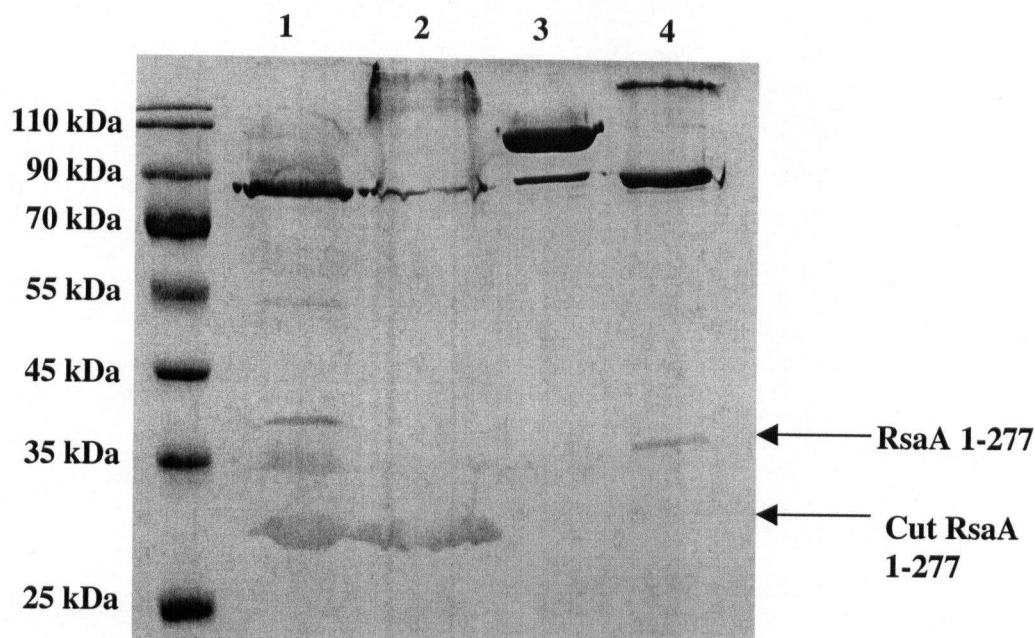
Fig. 3-2. SAP is probably an extracellular enzyme
RsaA bearing a collagenase site at residue 277 was isolated from an SAP-negative *C. crescentus* shedder strain (lane 3), then treated with collagenase (lane 4). The resulting RsaA fragments were then incubated with equivalent numbers of SAP-positive cells. After washing, cells were immediate boiled in the presence of loading buffer (lane 1), or whole cell preparations were performed (lane 2). Samples were separated on a 12% SDS-PAGE gel. RsaA fragments were detected by western blotting using RsaA antiserum.

Fig. 3.3. RsaFa and RsaFb levels in wild type and RsaF knockout strains.
Whole cell protein preparations from equivalent numbers of NA1000 (wt) or JS4000 (wt) cells, or strains derived from them that are null for RsaFa (F_a-), RsaFb (F_b-), or both Rsa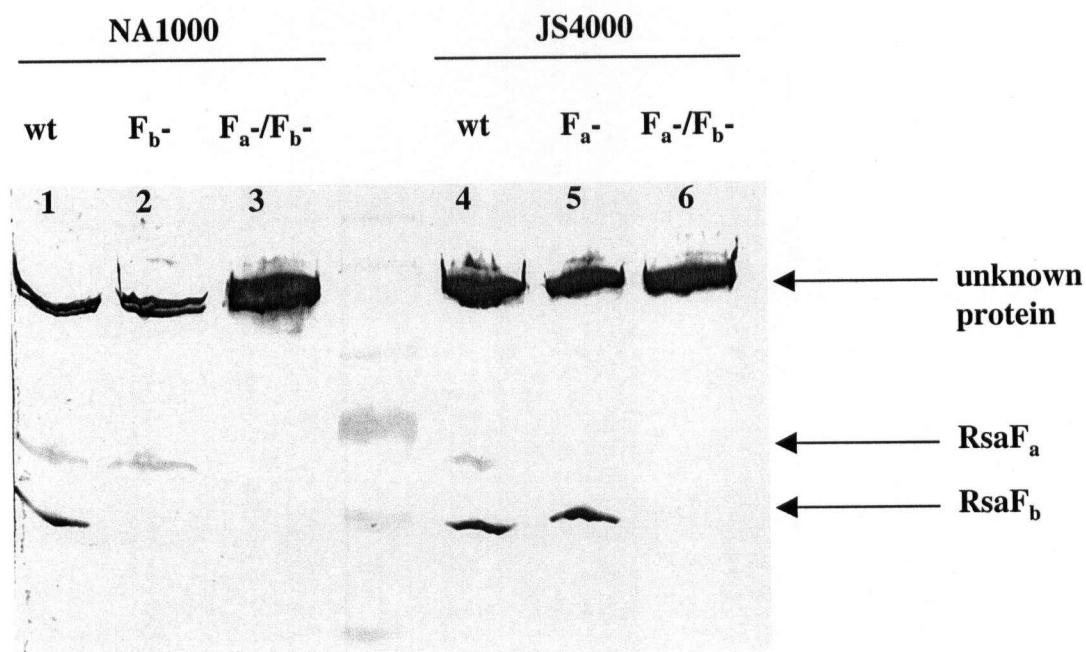Fa & RsaFb (F_a-/F_b-) were separated on a 7.5% SDS-PAGE gel. RsaFa and RsaFb were detected using RsaFa antiserum (which has cross-reactivity with RsaFb).

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significantly reduced when JS1011 was used compared to using a target (JS1015) that possesses the wild type OMPs of the S-layer Type I transporter proteins (Fig. 3-4, compare lanes 3 & 4). This suggests that RsaFa and/or RsaFb are involved in the secretion of Sap, and thus when this/these OMPs are not present, Sap does not get secreted and therefore cannot access and cleave RsaA 1-277 that has been reattached to the cell surface.

4.4 – A third F outer membrane protein may be present in strain JS4000

C. crescentus strain JS4000 is a spontaneous mutant that is deficient in S-layer production due to a frameshift mutation in the S-layer gene that results in an early stop codon a third of the way into rsaA (66). There are a few other differences between JS4000 and NA1000, including the fact that JS4000 appears to shed more heterologous protein than NA1000-derived rsaA knockouts when harboring the appropriate plasmid (Smit, unpublished). Since one focus of our laboratory has been to optimize C. crescentus protein expression/yield for various biotechnology applications, we have used JS4000 routinely for this purpose. The genome of NA1000 has been sequenced and is available (www.tigr.org). A search of the NA1000 genome suggests there are only two chromosomal OMP genes (rsaFa and rsaFb) involved in S-layer Type I secretion, and this has been confirmed experimentally (71) for this C. crescentus strain. In an unrelated study, I constructed an NA1000-derived strain that was null for rsaFa, rsaFb, and rsaA, called JS1011 (Fig. 3-3, lane 3), as well as a JS4000-derived strain that was null for rsaFa and rsaFb , called JS4023 (Fig. 3-3, lane 6). Unexpectedly, in contrast to JS1011,
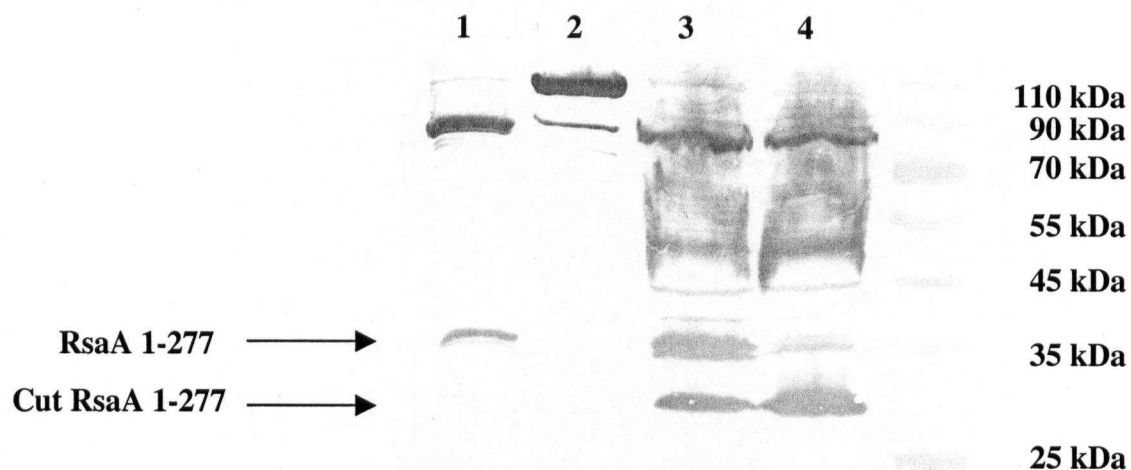
Fig. 3-4. S-layer Type I secretion OMPs Fa and/or Fb are involved in the secretion of Sap.
RsaA bearing a collagenase site at residue 277 was isolated from an Sap⁻ *C. crescentus* shedder strain (lane 2), then treated with collagenase (lane 1). The resulting RsaA fragments were then incubated with equivalent numbers of S-layer-negative *C. crescentus* cells that either possess (lane 4) or do not possess (lane 3) RsaFa & RsaFb. After washing, cells were immediate boiled in the presence of loading buffer. Equal loadings of sample were separated on a 12% SDS-PAGE, and RsaA and RsaA fragments were detected by western blotting using RsaA antiserum.

upon introduction of a plasmid-borne copy of *rsaA* into JS4023, RsaA still gets secreted to the cell surface, evidenced by a) low pH extraction of these cells yields S-layer, and b) S-layer is detected on the surface of these cells in immunofluorescence experiments (data not shown). This suggests that a third *rsaF* gene may be present on the JS4000 chromosome, allowing for RsaA secretion in this strain even when *rsaFa* and *rsaFb* have been deleted. To gather more evidence suggesting there is a third RsaF OMP in JS4000, and that Sap can utilize this putative OMP for its secretion, the cleavage of RsaA 1-277 was assessed using JS1011 or JS4023 as targets for reattachment of RsaA 1-277. As predicted, full cleavage of RsaA 1-277 was observed when JS4023 was used as a target (Fig. 3-5, lane 2) but not when JS1011 was used as a target (Fig. 3-5, lane 1). Taken together, these data suggest that a third RsaF protein may well form part of the S-layer Type I secretion system in JS4000, and that this third RsaF transporter protein is also involved in the secretion of Sap. A third OMP in JS4000-based strains may explain the improved protein expression/yield observed in our lab (Smit, unpublished) when utilizing these strains for heterologous protein production.

4.5 – Sap is unlikely to be exported into the culture media

Having implicated RsaF OMPs in the secretion of Sap, we wanted to determine whether Sap is localized to the cell surface, or secreted into the culture medium. To address this issue, an RsaA mutant that had been previously shown to get cleaved by Sap called RsaA:VP2CΔ (RsaA with the insertion of a 112 amino acid segment of a salmonid virus glycoprotein at a position corresponding to RsaA amino acid 723) (72) was first isolated from an Sap-negative shedder strain (JS1012) harboring a plasmid encoding
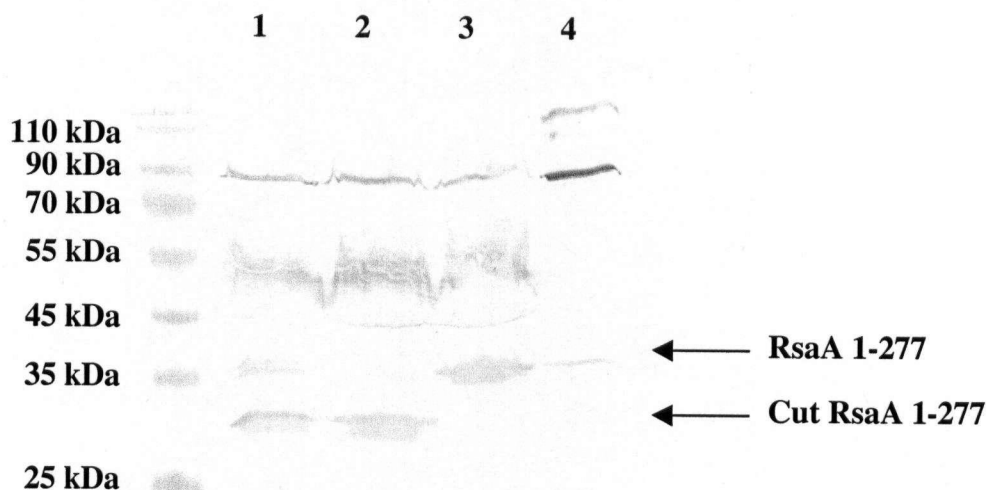
Fig. 3-5. A third F outer membrane protein may be present in strain JS4000.
RsaA bearing a collagenase site at residue 277 was isolated from an Sap⁻ *C. crescentus* shedder strain, then treated with collagenase (lane 4). The resulting RsaA fragments were then incubated with equivalent numbers of Sap-positive, S-layer-negative, RsaFa- & RsaFb-negative cells derived from NA1000 (lane 1) or JS4000 (lane 2). As a control, the RsaA fragments were also incubated with Sap-negative (JS4025) cells (lane 3). After washing, cells were immediate boiled in the presence of loading buffer. Equal loadings of sample were separated on a 12% SDS-PAGE, and RsaA and RsaA fragments were detected by western blotting using RsaA antiserum.

RsaA:VP2CΔ . This isolated protein was subsequently reattached to Sap-deficient target cells (JS4025), and then excess mutant RsaA was washed away. Next, S-layer-negative, Sap-positive cells (JS4000) or supernatant from an Sap-positive cell culture was incubated with the Sap-negative cells that had (uncleaved) RsaA:VP2CΔ reattached to them, in an effort to cleave the reattached RsaA:VP2CΔ. Cleavage of RsaA:VP2CΔ would indicate the presence of Sap on either the cell surface or in the supernatant, suggesting where Sap is localized after its secretion. Incubation of cell-bound RsaA:VP2CΔ with Sap-positive cells did not result in the cleavage of the Sap substrate (Fig. 3-6, lane 2), but this might be because Sap that is bound to the cell surface of one cell cannot effectively access or cleave its substrate when it is bound to another cell. Importantly, however, incubation of cell-bound RsaA:VP2CΔ with supernatant from the Sap-positive culture also failed to result in the cleavage of the Sap substrate, since the amount of full-length RsaA:VP2CΔ found anchored to these cells was roughly equal to that found anchored to an equivalent number of cells that were not treated with Sap-positive cells or supernatant (Fig. 3-6, compare lane 1 with lane 3). This preliminary evidence suggests that Sap is not found in the supernatant of Sap-positive cells: if Sap were secreted into the supernatant, Sap would not be hampered by being bound to a cell surface, and thus Sap would be expected to have access to its cell-bound substrate and should be able to cleave it with ease. It is therefore unlikely that Sap is exported into the culture media. Taken together with the evidence of the involvement of the OMPs Fa and/or Fb in the secretion of Sap, this suggests that Sap is an outer membrane-bound protease.

RsaA:VP2CΔ ⟶   1   2   3   4   110 kDa

Fig. 3-6. SAP is unlikely to be exported into the culture media.
RsaA with the insertion of a 112 amino acid segment of the VP2 surface glycoprotein of infectious pancreatic necrosis virus (IPNV) at residue 723 (70), hereafter called RsaA:VP2CΔ, was isolated from an Sap⁻ *C. crescentus* shedder strain. RsaA:VP2CΔ was then incubated with S-layer-negative, Sap-negative *C. crescentus* cells (lanes 1-3), or S-layer-negative, SAP-positive *C. crescentus* cells (lane 4), then excess RsaA:VP2CΔ was washed away. Aliquots of SAP-negative cells with reattached RsaA:VP2CΔ were then subsequently incubated with S-layer-negative, SAP-positive cells (lane 2) or culture supernatant (lane 1). After washing, cells were immediate boiled in the presence of loading buffer. Equal loadings of sample were separated on a 12% SDS-PAGE, and RsaA:VP2CΔ was detected by western blotting using RsaA antiserum.

5. DISCUSSION AND CONCLUSION

We have developed an assay that can be utilized systematically to investigate regions or residues of the *C. crescentus* S-layer protein, RsaA, that are important for S-layer anchoring in vivo. Through this work, we have learned that RsaA secondary structure is important for RsaA anchoring, and therefore care must be taken when preparing RsaA and RsaA mutants destined for reattachment. We demonstrated conclusively for the first time that SLPS is required for RsaA anchoring, and that in the complete absence of SLPS, RsaA anchoring does not occur. We have established that it is the N-terminus of RsaA that mediates anchoring of S-layer to the cells surface, and that the first 277 amino acids are sufficient for RsaA anchoring. Surprisingly, all of the six mutations created thus far within the first 222 amino acids have resulted in loss of RsaA anchoring, suggesting that this entire region may contribute in some way to S-layer anchoring. We have accumulated evidence suggesting that the S-layer-associated protease, Sap, is likely an OM-bound protein, rather than a cytoplasmic enzyme as previously suggested. Finally, we have implicated the S-layer Type I secretion OMPs RsaFa and/or RsaFb in the secretion of Sap to the OM.

There has been a significant amount of investigation into the S-layer anchoring regions of Gram-positive bacteria, but little work has been done to define S-layer anchoring regions of Gram-negative bacteria. In fact, to date, there has only been one other study that evaluated the ability of truncated S-layer protein to reattach to S-layer-deficient Gram-negative cells. In that study, deletion mutagenesis revealed that *C. fetus* S-layer proteins bound serospecifically to the *C. fetus* lipopolysaccharide via their conserved N-terminal regions, which include approximately 189 amino acids (16).

Those findings are comparable to the results of this study for the *C. crescentus* S-layer protein: we have found that the first ~225 amino acids of RsaA are involved in S-layer anchoring, suggesting that a large amount of residues mediate S-layer anchoring in Gram-negative bacteria. This is in accordance with anchoring domains for S-layer proteins of Gram-positive bacteria, where anchoring domains are usually 3 repeats of the ~55 amino acid S-layer homology (SLH) domain (4).

Although an S-layer anchoring motif has been defined and is well documented for Gram-positive bacteria (19), there is no such motif defined for S-layer protein for Gram-negative bacteria. In the *C. fetus* S-layer study (16), an investigation of the importance of regions or residues found within the conserved N-terminal lipopolysaccharide binding domain was not performed, thus it is not known which regions or residues within that domain are important for the anchoring of the *C. fetus* S-layer proteins. The present study is the first to elucidate the involvement of regions or residues within a defined S-layer anchoring domain in Gram-negative bacteria. Because all mutations created thus far within the putative RsaA anchoring region abolish RsaA anchoring, a complementary approach to the current mutagenesis strategy may be to complete comparative studies of S-layer sequences from other *Caulobacter* species, in an effort to search for conserved residues or motifs that may mediate S-layer anchoring. Further understanding of S-layer anchoring in *Caulobacter* may aid in the understanding of S-layer anchoring in other Gram-negative bacteria.

There have been several instances where we wondered whether or not Sap was located on the cell surface rather than in the cytoplasm. For example, when the 112-amino acid segment of the VP2 surface glycoprotein of infectious pancreatic necrosis virus (IPNV)

strain SP is inserted at a position corresponding to amino acid 723 of RsaA, Sap-positive cells subjected to low pH conditions to extract S-layer protein from the cell surface yields both the N- and C-terminal fragments resulting from Sap cleavage (72). Since the C-terminus of RsaA contains the information required for RsaA secretion (9), if Sap cleaves the heterologous S-layer protein in the cytoplasm, then only the C-terminal fragment should get secreted and thus be retrievable by low pH extraction of protein localized on the cell surface. At the time of our last publication regarding Sap we had no reason to predict that Sap could be on the cell surface (an analysis of the Sap sequence reveals no predicted N-terminal signal leader peptide, no predicted Type I secretion signal, and no predicted transmembrane domains). Additionally, no experimental data suggested that this might be the case. Therefore we previously suggested that RsaA intramolecular forces (such as hydrogen bonding) might account for both the N- and C-terminal products being secreted. Sap may cleave the particular mutant protein in the cytoplasm, but these RsaA intramolecular forces may hold the two cleavage products together and allow for their simultaneous secretion.

However we have now accumulated experimental evidence suggesting that Sap is located on the cell surface, and can access and cleave reattached RsaA 1-277. If Sap is localized on the cell surface, some Sap substrates (such as RsaA:VP2CΔ and RsaA 1-277) would likely be cleaved by Sap on the cell surface rather than in the cytoplasm, which would explain how both cleavage products are located on the cell surface. Clearly, final confirmation of the localization of Sap using Sap-specific antibody needs to be done, and efforts are underway to generate antibodies against Sap to perform these experiments.

We have observed that Sap apparently cleaves various RsaA mutants at the same position in RsaA (about a third of the way C-terminal from the N-terminus), yielding a ~28 kDa RsaA N-terminal cleavage product. This "weak Sap cleavage site" is present in wild-type RsaA, but Sap does not cleave wild-type RsaA. We hypothesize that this weak Sap recognition site is buried or inaccessible to Sap in the wild-type RsaA case due to native folding of the S-layer. In contrast, some RsaA mutants may cause altered folding of S-layer, thus exposing this weak cleavage site to Sap. It is possible that this is a Sap-mediated strategy that *C. crescentus* utilizes to rid the cell surface or RsaA mutants that disrupt normal S-layer assembly on the cell surface. Efforts are underway to determine the exact sequence and position of this weak Sap cleavage site.

Several questions arise from the Sap studies. We have implicated the S-layer Type I secretion OMPs RsaFa and/or RsaFb in the secretion of Sap. An investigation of the effect on Sap secretion in strains that are null for only RsaFa or RsaFb will suggest whether one or both of these proteins are important for Sap secretion. Furthermore, results are consistent with the presence of a third OMP in JS4000-derived JS4025 that may secrete Sap. The construction and subsequent screening of members of a JS4025 transposon library for loss of Sap secretion may aid in the identification of this putative third OMP. Identification and characterization of this third OMP might explain why protein expression/yield is improved in JS4000-based strains.

The involvement of RsaFa and/or RsaFb in Sap secretion suggests that Sap may be secreted by the S-layer Type I secretion system. Further studies such as investigations of the effects on Sap secretion in strains lacking other components of the S-layer Type I secretion system (RsaD and RsaE) will need to be done to resolve these possibilities. If

it is found that indeed the S-layer Type I ABC transporter secretes Sap as well as RsaA, this would not be the first time a Type I secretion system has been shown to secrete more than one protein. The LipBCD Type I secretion system in *Serratia marcescens* has in fact been shown to mediate the secretion of three proteins: a lipase, a metalloprotease, and the *S. marcescens* S-layer protein (33).

From primary protein sequence, RsaFa and RsaFb are predicted to be OMP beta barrel proteins. Since experimental evidence suggests that Sap is OM-bound (as is RsaA), after RsaFa and/or RsaFb-mediated translocation past the OM, Sap (and RsaA) must get transferred to molecules on the cell surface that tether these proteins to the cell surface. The question arises: if Sap is OM-bound, what OM molecule is Sap bound to? The C-terminus of Sap is homologous to the N-terminus of RsaA, and it may mediate anchoring of Sap to the cell surface. Our previous model of Sap activity in the case of heterologous S-layer was that the Sap C-terminus associated with the N-terminus of RsaA, in the cytoplasm, as it scanned for and cleaved some mutated versions of RsaA. However, in light of the data suggesting that Sap is OM-bound, it could be that the Sap C-terminal homology to the N-terminus of RsaA reflects the possibility that the C-terminus of Sap anchors to SLPS on the cell surface, in the same way that the N-terminus of RsaA anchors to SLPS. Interestingly, another *C. crescentus* OM-bound protein, HfaD, remains anchored to the cell surface in the absence of S-layer (Brun, personal communications), as does Sap. HfaD is 28% identical to the first 277 amino acids of RsaA (data not shown), suggesting that perhaps HfaD also anchors to SLPS on the cell surface. Therefore, it is possible that at least three proteins are bound to the *C. crescentus* cell surface via a conserved protein domain that interacts with SLPS. Reattachment of the

purified proteins (Sap or HfaD) to SLPS-positive but not SLPS-negative cells would confirm that these proteins anchor to the cell surface by interacting with SLPS.

The Type V Autotransporter secretion (29) and lipid-linked proteins (3) are two mechanisms by which multiple proteins are secreted and subsequently anchored to the cell envelope of Gram-negative bacteria. Both of these transport systems utilize the Sec-dependent pathway also known as the General Secretory Pathway, GSP to traverse the inner membrane, and thus generate periplasmic intermediates. These periplasmic intermediates can be subjected to proteolysis by periplasmic enzymes, and may also require chaperones for successful translocation (29), (3). In contrast to the GSP, the Type I secretion system can secrete a variety of proteins across both the inner and outer membranes, without generating periplasmic intermediates (55). If it is true that the *C. crescentus* S-layer Type I secretion system also secretes other proteins such as Sap and HfaD which subsequently anchor to the cell surface, then we may have discovered another, less cumbersome secretion pathway that *C. crescentus* utilizes to efficiently secrete and subsequently anchor proteins to the cell surface by interaction with SLPS. This concept of Type I secretion systems utilized for secretion and subsequent surface presentation of multiple proteins via the same Type I system may extend to other Gram-negative bacteria in general.

REFERENCES

1. **Awram, P., and J. Smit.** 1998. The Caulobacter crescentus paracrystalline S-layer protein is secreted by an ABC transporter (type I) secretion apparatus. J Bacteriol **180:**3062-9.

2. **Awram, P., and J. Smit.** 2001. Identification of lipopolysaccharide O antigen synthesis genes required for attachment of the S-layer of Caulobacter crescentus. Microbiology **147:**1451-60.

3. **Bernstein, H. D.** 2000. The biogenesis and assembly of bacterial membrane proteins. Curr Opin Microbiol **3:**203-9.

4. **Beveridge, T. J., and L. L. Graham.** 1991. Surface layers of bacteria. Microbiol Rev **55:**684-705.

5. **Beveridge, T. J., P. H. Pouwels, M. Sara, A. Kotiranta, K. Lounatmaa, K. Kari, E. Kerosuo, M. Haapasalo, E. M. Egelseer, I. Schocher, U. B. Sleytr, L. Morelli, M. L. Callegari, J. F. Nomellini, W. H. Bingle, J. Smit, E. Leibovitz, M. Lemaire, I. Miras, S. Salamitou, P. Beguin, H. Ohayon, P. Gounon, M. Matuschek, and S. F. Koval.** 1997. Functions of S-layers. FEMS Microbiol Rev **20:**99-149.

6. **Binet, R., S. Letoffe, J. M. Ghigo, P. Delepelaire, and C. Wandersman.** 1997. Protein secretion by Gram-negative bacterial ABC exporters--a review. Gene **192:**7-11.

7. **Bingle, W. H., J. F. Nomellini, and J. Smit.** 1997. Cell-surface display of a Pseudomonas aeruginosa strain K pilin peptide within the paracrystalline S-layer of Caulobacter crescentus. Mol Microbiol **26:**277-88.

8. **Bingle, W. H., J. F. Nomellini, and J. Smit.** 1997. Linker mutagenesis of the Caulobacter crescentus S-layer protein: toward a definition of an N-terminal anchoring region and a C-terminal secretion signal and the potential for heterologous protein secretion. J Bacteriol **179:**601-11.

9. **Bingle, W. H., J. F. Nomellini, and J. Smit.** 2000. Secretion of the Caulobacter crescentus S-layer protein: further localization of the C-terminal secretion signal and its use for secretion of recombinant proteins. J Bacteriol **182:**3298-301.

10. **Bingle, W. H., and J. Smit.** 1990. High-level expression vectors for Caulobacter crescentus incorporating the transcription/translation initiation regions of the paracrystalline surface-layer-protein gene. Plasmid **24:**143-8.

11. **Blaser, M. J., P. F. Smith, J. E. Repine, and K. A. Joiner.** 1988. Pathogenesis of Campylobacter fetus infections. Failure of encapsulated Campylobacter fetus to bind C3b explains serum and phagocytosis resistance. J Clin Invest **81:**1434-44.

12. **Boot, H. J., and P. H. Pouwels.** 1996. Expression, secretion and antigenic variation of bacterial S-layer proteins. Mol Microbiol **21:**1117-23.

13. **Brent, R., and M. Ptashne.** 1980. The lexA gene product represses its own promoter. Proc Natl Acad Sci U S A **77:**1932-6.

14. **Brun, Y. V., G. Marczynski, and L. Shapiro.** 1994. The expression of asymmetry during Caulobacter cell differentiation. Annu Rev Biochem **63:**419-50.

15. **Doig, P., W. D. McCubbin, C. M. Kay, and T. J. Trust.** 1993. Distribution of surface-exposed and non-accessible amino acid sequences among the two major structural domains of the S-layer protein of Aeromonas salmonicida. J Mol Biol **233:**753-65.

16. **Dworkin, J., M. K. Tummuru, and M. J. Blaser.** 1995. A lipopolysaccharide-binding domain of the Campylobacter fetus S-layer protein resides within the conserved N terminus of a family of silent and divergent homologs. J Bacteriol **177:**1734-41.

17. **Edwards, P., and J. Smit.** 1991. A transducing bacteriophage for Caulobacter crescentus uses the paracrystalline surface layer protein as a receptor. J Bacteriol **173:**5568-72.

18. **Egelseer, E., I. Schocher, M. Sara, and U. B. Sleytr.** 1995. The S-layer from Bacillus stearothermophilus DSM 2358 functions as an adhesion site for a high-molecular-weight amylase. J Bacteriol **177:**1444-51.

19. **Engelhardt, H., and J. Peters.** 1998. Structural research on surface layers: a focus on stability, surface layer homology domains, and surface layer-cell wall interactions. J Struct Biol **124:**276-302.

20. **Fujimoto, S., A. Takade, K. Amako, and M. J. Blaser.** 1991. Correlation between molecular size of the surface array protein and morphology and antigenicity of the Campylobacter fetus S layer. Infect Immun **59:**2017-22.

21. **Fujino, T., P. Beguin, and J. P. Aubert.** 1993. Organization of a Clostridium thermocellum gene cluster encoding the cellulosomal scaffolding protein CipA and a protein possibly involved in attachment of the cellulosome to the cell surface. J Bacteriol **175:**1891-9.

22. **Garduno, R. A., and W. W. Kay.** 1992. Interaction of the fish pathogen Aeromonas salmonicida with rainbow trout macrophages. Infect Immun **60:**4612-20.

23. **Garduno, R. A., E. J. Lee, and W. W. Kay.** 1992. S-layer-mediated association of Aeromonas salmonicida with murine macrophages. Infect Immun **60:**4373-82.

24. **Garduno, R. A., A. R. Moore, G. Olivier, A. L. Lizama, E. Garduno, and W. W. Kay.** 2000. Host cell invasion and intracellular residence by Aeromonas salmonicida: role of the S-layer. Can J Microbiol **46:**660-8.

25. **Garduno, R. A., B. M. Phipps, and W. W. Kay.** 1995. Physical and functional S-layer reconstitution in Aeromonas salmonicida. J Bacteriol **177:**2684-94.

26. **Gilchrist, A., J. A. Fisher, and J. Smit.** 1992. Nucleotide sequence analysis of the gene encoding the Caulobacter crescentus paracrystalline surface layer protein. Can J Microbiol **38:**193-202.

27. **Gilchrist, A., and J. Smit.** 1991. Transformation of freshwater and marine caulobacters by electroporation. J Bacteriol **173:**921-5.

28. **Gober, J. W., and M. V. Marques.** 1995. Regulation of cellular differentiation in Caulobacter crescentus. Microbiol Rev **59:**31-47.

29. **Henderson, I. R., F. Navarro-Garcia, M. Desvaux, R. C. Fernandez, and D. Ala'Aldeen.** 2004. Type V protein secretion pathway: the autotransporter story. Microbiol Mol Biol Rev **68**:692-744.

30. **Howard, L., and D. J. Tipper.** 1973. A polypeptide bacteriophage receptor: modified cell wall protein subunits in bacteriophage-resistant mutants of Bacillus sphaericus strain P-1. J Bacteriol **113**:1491-504.

31. **Iuga, M., P. Awram, J. F. Nomellini, and J. Smit.** 2004. Comparison of S-layer secretion genes in freshwater caulobacters. Can J Microbiol **50**:751-66.

32. **Janakiraman, R. S., and Y. V. Brun.** 1997. Transcriptional and mutational analyses of the rpoN operon in Caulobacter crescentus. J Bacteriol **179**:5138-47.

33. **Kawai, E., H. Akatsuka, A. Idei, T. Shibatani, and K. Omori.** 1998. Serratia marcescens S-layer protein is secreted extracellularly via an ATP-binding cassette exporter, the Lip system. Mol Microbiol **27**:941-52.

34. **Kilian, M.** 1981. Degradation of immunoglobulins A2, A2, and G by suspected principal periodontal pathogens. Infect Immun **34**:757-65.

35. **Kotiranta, A., M. Haapasalo, K. Kari, E. Kerosuo, I. Olsen, T. Sorsa, J. H. Meurman, and K. Lounatmaa.** 1998. Surface structure, hydrophobicity, phagocytosis, and adherence to matrix proteins of Bacillus cereus cells with and without the crystalline surface protein layer. Infect Immun **66**:4895-902.

36. **Koval, S. F., and S. H. Hynes.** 1991. Effect of paracrystalline protein surface layers on predation by Bdellovibrio bacteriovorus. J Bacteriol **173**:2244-9.

37. **Lemaire, M., H. Ohayon, P. Gounon, T. Fujino, and P. Beguin.** 1995. OlpB, a new outer layer protein of Clostridium thermocellum, and binding of its S-layer-like domains to components of the cell envelope. J Bacteriol **177**:2451-9.

38. **Lewis, L. O., A. A. Yousten, and R. G. Murray.** 1987. Characterization of the surface protein layers of the mosquito-pathogenic strains of Bacillus sphaericus. J Bacteriol **169**:72-9.

39. **Lupas, A., H. Engelhardt, J. Peters, U. Santarius, S. Volker, and W. Baumeister.** 1994. Domain structure of the Acetogenium kivui surface layer revealed by electron crystallography and sequence analysis. J Bacteriol **176**:1224-33.

40. **MacRae, J. D., and J. Smit.** 1991. Characterization of caulobacters isolated from wastewater treatment systems. Appl Environ Microbiol **57**:751-8.

41. **Matuschek, M., G. Burchhardt, K. Sahm, and H. Bahl.** 1994. Pullulanase of Thermoanaerobacterium thermosulfurigenes EM1 (Clostridium thermosulfurogenes): molecular analysis of the gene, composite structure of the enzyme, and a common model for its attachment to the cell surface. J Bacteriol **176**:3295-302.

42. **McCoy, E. C., D. Doyle, K. Burda, L. B. Corbeil, and A. J. Winter.** 1975. Superficial antigens of Campylobacter (Vibrio) fetus: characterization of antiphagocytic component. Infect Immun **11**:517-25.

43. **Mesnage, S., E. Tosi-Couture, P. Gounon, M. Mock, and A. Fouet.** 1998. The capsule and S-layer: two independent and yet compatible macromolecular structures in Bacillus anthracis. J Bacteriol **180**:52-8.

44. **Morales, V. M., A. Backman, and M. Bagdasarian.** 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene **97:**39-47.

45. **Nomellini, J. F., S. Kupcu, U. B. Sleytr, and J. Smit.** 1997. Factors controlling in vitro recrystallization of the Caulobacter crescentus paracrystalline S-layer. J Bacteriol **179:**6349-54.

46. **Nomellini, J. F., M. Toporowski, and J. Smit.** 2003. Secretion or presentation of recombinant proteins and peptides mediated by the S-layer of Caulobacter crescentus, in Expression Technologies: Current Status and Future Trends. Horizon Scientific Press, Norfolk, in press.

47. **Olabarria, G., J. L. Carrascosa, M. A. de Pedro, and J. Berenguer.** 1996. A conserved motif in S-layer proteins is involved in peptidoglycan binding in Thermus thermophilus. J Bacteriol **178:**4765-72.

48. **Pei, Z., and M. J. Blaser.** 1990. Pathogenesis of Campylobacter fetus infections. Role of surface array proteins in virulence in a mouse model. J Clin Invest **85:**1036-43.

49. **Peterson, P. K., B. J. Wilkinson, Y. Kim, D. Schmeling, and P. G. Quie.** 1978. Influence of encapsulation on staphylococcal opsonization and phagocytosis by human polymorphonuclear leukocytes. Infect Immun **19:**943-9.

50. **Poindexter, J. S.** 1964. Biological Properties and Classification of the Caulobacter Group. Bacteriol Rev **28:**231-95.

51. **Poindexter, J. S.** 1981. The caulobacters: ubiquitous unusual bacteria. Microbiol Rev **45:**123-79.

52. **Roberts, K., P. J. Shaw, and G. J. Hills.** 1981. High-resolution electron microscopy of glycoproteins: the crystalline cell wall of Lobomonas. J Cell Sci **51:**295-313.

53. **Salamitou, S., M. Lemaire, T. Fujino, H. Ohayon, P. Gounon, P. Beguin, and J. P. Aubert.** 1994. Subcellular localization of Clostridium thermocellum ORF3p, a protein carrying a receptor for the docking sequence borne by the catalytic components of the cellulosome. J Bacteriol **176:**2828-34.

54. **Salamitou, S., O. Raynaud, M. Lemaire, M. Coughlan, P. Beguin, and J. P. Aubert.** 1994. Recognition specificity of the duplicated segments present in Clostridium thermocellum endoglucanase CelD and in the cellulosome-integrating protein CipA. J Bacteriol **176:**2822-7.

55. **Salmond, G. P., and P. J. Reeves.** 1993. Membrane traffic wardens and protein secretion in gram-negative bacteria. Trends Biochem Sci **18:**7-12.

56. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

57. **Sara, M., and U. B. Sleytr.** 1996. Crystalline bacterial cell surface layers (S-layers): from cell structure to biomimetics. Prog Biophys Mol Biol **65:**83-111.

58. **Sara, M., and U. B. Sleytr.** 1987. Molecular sieving through S layers of Bacillus stearothermophilus strains. J Bacteriol **169:**4092-8.

59. **Sara, M., and U. B. Sleytr.** 2000. S-Layer proteins. J Bacteriol **182:**859-68.

60. **Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, and G. Theirbach.** 1994. Small mobilizable multi-purpose cloning vectors derived from the Escherichia

coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Cornyebacterium glutamicum. Gene **145**:69-73.

61. **Shapiro, L.** 1985. Generation of polarity during Caulobacter cell differentiation. Annu Rev Cell Biol **1**:173-207.

62. **Sharma, S. B., and E. R. Signer.** 1990. Temporal and spatial regulation of the symbiotic genes of Rhizobium meliloti in planta revealed by transposon Tn5-gusA. Genes Dev **4**:344-56.

63. **Sleytr, U. B., and T. J. Beveridge.** 1999. Bacterial S-layers. Trends Microbiol **7**:253-60.

64. **Sleytr, U. B., and P. Messner.** 1983. Crystalline surface layers on bacteria. Annu Rev Microbiol **37**:311-39.

65. **Sleytr, U. B., P. Messner, D. Pum, and M. Sara.** 1996. Crystalline Bacterial Cell Surface Proteins. Academic Press, Landes Company, Austin.

66. **Smit, J., and N. Agabian.** 1984. Cloning of the major protein of the Caulobacter crescentus periodic surface layer: detection and characterization of the cloned peptide by protein expression assays. J Bacteriol **160**:1137-45.

67. **Smit, J., H. Engelhardt, S. Volker, S. H. Smith, and W. Baumeister.** 1992. The S-layer of Caulobacter crescentus: three-dimensional image reconstruction and structure analysis by electron microscopy. J Bacteriol **174**:6527-38.

68. **Sundqvist, G., J. Carlsson, B. Herrmann, and A. Tarnvik.** 1985. Degradation of human immunoglobulins G and M and complement factors C3 and C5 by black-pigmented Bacteroides. J Med Microbiol **19**:85-94.

69. **Takeoka, A., K. Takumi, T. Koga, and T. Kawata.** 1991. Purification and characterization of S layer proteins from Clostridium difficile GAI 0714. J Gen Microbiol **137** (**Pt 2**):261-7.

70. **Thompson, S. A., O. L. Shedd, K. C. Ray, M. H. Beins, J. P. Jorgensen, and M. J. Blaser.** 1998. Campylobacter fetus surface layer proteins are transported by a type I secretion system. J Bacteriol **180**:6450-8.

71. **Toporowski, M. C., J. F. Nomellini, P. Awram, and J. Smit.** 2004. Two outer membrane proteins are required for maximal type I secretion of the Caulobacter crescentus S-layer protein. J Bacteriol **186**:8000-9.

72. **Umelo-Njaka, E., W. H. Bingle, F. Borchani, K. D. Le, P. Awram, T. Blake, J. F. Nomellini, and J. Smit.** 2002. Caulobacter crescentus synthesizes an S-layer-editing metalloprotease possessing a domain sharing sequence similarity with its paracrystalline S-layer protein. J Bacteriol **184**:2709-18.

73. **Umelo-Njaka, E., J. F. Nomellini, H. Yim, and J. Smit.** 2001. Development of small high-copy-number plasmid vectors for gene expression in Caulobacter crescentus. Plasmid **46**:37-46.

74. **Walker, S. G., D. N. Karunaratne, N. Ravenscroft, and J. Smit.** 1994. Characterization of mutants of Caulobacter crescentus defective in surface attachment of the paracrystalline surface layer. J Bacteriol **176**:6312-23.

75. **Walker, S. G., S. H. Smith, and J. Smit.** 1992. Isolation and comparison of the paracrystalline surface layer proteins of freshwater caulobacters. J Bacteriol **174**:1783-92.

76. **Welch, R. A., C. Forestier, A. Lobo, S. Pellett, W. Thomas, Jr., and G. Rowe.** 1992. The synthesis and function of the Escherichia coli hemolysin and related RTX exotoxins. FEMS Microbiol Immunol **5:**29-36.

77. **Wiklund, T., and I. Dalsgaard.** 1998. Occurrence and significance of atypical Aeromonas salmonicida in non-salmonid and salmonid fish species: a review. Dis Aquat Organ **32:**49-69.

78. **Yang, L. Y., Z. H. Pei, S. Fujimoto, and M. J. Blaser.** 1992. Reattachment of surface array proteins to Campylobacter fetus cells. J Bacteriol **174:**1258-67.