

Understanding the type I secretion of the S-layer protein RsaA in *Caulobacter crescentus*

by
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B.Sc., The University of British Columbia, 2000

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
(Department of Microbiology and Immunology)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 2004

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Title of Thesis:

Understanding the type I
Secretion of the S-layer protein RsaA
in Caulobacter crescentus

Degree:

M. Sc.

Year:

2004

Department of

Microbiology and Immunology

The University of British Columbia

Vancouver, BC Canada

ABSTRACT

The transport of RsaA, the S-layer subunit protein of *Caulobacter crescentus*, is mediated by ABC transporter (type I) secretion. The ABC transporter and membrane fusion protein (MFP) components were previously reported as downstream of *rsaA*, however the two outer membrane proteins (OMP) were not described. This study aims to elucidate the transcriptional regulation of the *rsaADE* genes as well as the role of two putative OMP genes, *rsaFa* and *rsaFb*, in the secretion of RsaA.

The *rsaADE* genes were previously thought to be transcribed as an operon in a similar fashion to that of the *Escherichia coli* alpha-hemolysin (HlyA) system. Here I show that contrary to previous hypotheses, the *rsaD* and *rsaE* genes appear to be transcribed together using a promoter found between *rsaA* and *rsaD* suggesting that they are transcribed irrespective of *rsaA*.

The outer membrane proteins of this system had been suggested, but not characterized. Two candidates for the OMP, *rsaFa* and *rsaFb*, were identified by similarity to the *E. coli* HlyA secretion OMP TolC, using the available *C. crescentus* genome sequence and were modeled using the solved TolC structure. *rsaFa* was found several Kb downstream of the other transporter genes, while *rsaFb* is in an apparently random location.

The *rsaF* genes were disrupted to determine if they were involved in RsaA secretion. Knockout of *rsaFa* reduced secretion to ~54% of wild type levels while the *rsaFb* knockout reduced secretion levels to ~76%. When expression of both proteins was eliminated there was no RsaA secretion, but a residual level of ~9% remained intact inside the cell, suggesting posttranslational down regulation. Complementation with

either of the individual *rsaF* genes using a multi-copy vector (and demonstration of overexpression) did not restore RsaA secretion to wild type levels indicating both *rsaFa* and *rsaFb* are required for normal levels of S-layer secretion. However, overexpression of *rsaFa* (with normal *rsaFb* levels) in concert with multi-copy expression of *rsaA* resulted in a 28% increase in RsaA secretion, indicating a potential for significantly increasing expression levels of an already high level type I secretion system.

This is the only known example of type I secretion requiring two outer membrane proteins to assemble a fully functional system. It appears that production of RsaA is self-regulated, such that a build up of any amount of RsaA inside the cell due to blockage of transport limits RsaA production and severely impedes cell growth showing no signs of RsaA degradation. Secretion of RsaA appears to be a function of the number and type of outer membrane proteins as well as the amount of RsaA produced.

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LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
Ap	ampicillin
BLAST	Basic Local Alignment Search Tool
bp	base pair
C-terminus	carboxy terminus
Cm	Chloramphenicol
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
g	grams
Glu	Glatamic acid/ Glutamate
hr	hour
kb	kilobases
kDa	kilodaltons
Km	kanamycin
LB	Luria-Bertani broth
LPS	lipopolysaccharide
min	minute
MFP	Membrane Fusion Protein
mg	milligram
ml	millilitre
μl	microlitre
μg	microgram
NaCl	sodium chloride
N-terminus	amino terminus
OD ₆₀₀	optical density at absorbance of 600nm
OMP	Outer Membrane Protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer salts
PCR	polymerase chain reaction
PYE	peptone yeast extract
RNAse	Ribonuclease
S-layer	surface layer
S-LPS	smooth lipopolysaccharide
SDS	sodium dodecyl sulphate
Sm	streptomycin
TBS	Tris-buffered saline (10mM tris-HCL (pH 7.5), 0.9%NaCL)
TIGR	The Institute for Genomic Research
tris	Tris (hydroxymethyl) methylamine

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. John Smit for his advice and guidance throughout the course of this project. I would also like to thank past and previous members of the Smit lab, especially Dr. John Nomellini for help and assistance as well as countless discussions about numerous aspects throughout this project. I owe him countless beers. I would like to thank Andrea Pušić and Natalie Drouillard for their technical assistance at various stages of this work. I thank Dr. Peter Awram for starting the work on the RsaA secretion apparatus. I also thank Assaf Levi for the construction of the CB15 Δ -*rsaA* clone. Last but not least, I would like to thank my parents, my brother, and my girlfriend who put up with me during my masters thesis; without their love and support I couldn't have done this.

1. INTRODUCTION

The S-layer of *Caulobacter crescentus* is secreted by a highly proficient type I secretion system. The type I secretion system was initially discovered and partially characterized in the Smit lab by Dr. Peter Awram. The ABC transporter and Membrane Fusion Protein (MFP) were characterized, but the Outer Membrane Protein (OMP) was only identified. This thesis focuses on tying up some loose ends, and in turn unraveling a few more, in the secretion of the S-layer of *C. crescentus*. Characterization of the OMP units and a better understanding of the transcriptional control of the other type I secretion sub-units has led to a greater understanding of type I secretion systems as well as proven useful for biotechnology applications of the *C. crescentus* S-layer.

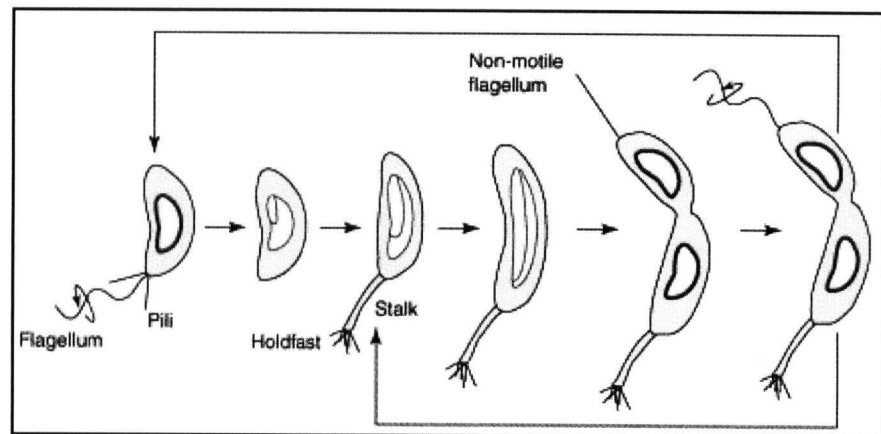
Results suggest that the type I component genes are transcribed separately from the transported RsaA. Identification of a separate promoter in between *rsaA* and *rsaD* shows that the *rsaADE* gene set is not co-transcribed like previous studies hypothesized (4, 30). Also presented is the characterization of the two OMPs (RsaFa and RsaFb) which are associated with the RsaA secretion system. The data suggests that both *rsaF* genes are required for wild type S-layer secretion levels. Regulation of RsaA production and secretion appears dependant on proper secretion, suggesting possible autoregulation. Furthermore, levels of S-layer secretion can be increased when both *rsaFa* and *rsaA* copies are increased.

1.01- *Caulobacter crescentus*

C. crescentus is a non-pathogenic Gram-negative bacterium common in fresh water and soils. Of the *Caulobacter sp.*, *C. crescentus*, which derives its name from its crescent shape, has been well studied in numerous areas of research. This bacterium has a dimorphic developmental lifestyle (18, 28, 50) switching between a motile swarmer phase and a stalked phase often being affixed to a surface (**Fig. 1-1.**). Swarmer cells have single flagellum, pili and holdfast (an adhesin) at one pole (52). Cells will then lose the flagellum and form a stalk from the cell envelope. These stalked cells will divide to produce a new swarmer cell with the flagellum being created at the pole furthest from the stalked cell. Often *C. crescentus* cells will form rosettes with multiple cells binding at one central point by the distal end of their stalks (50). Older and poorly growing cells become elongated and are often oddly shaped becoming twisted and bent. For detailed review on cell cycle see

Quardokus and Brun,
2003.

Figure 1-1. *C. crescentus* cell cycle
Cartoon representation of the dimorphic lifestyle of *C. crescentus*. (Figure from Quardokus and Brun, 2003).



1.02- Surface layer proteins

Surface layers (S-layers) are common in many genera of microorganisms, including Gram-negative bacteria, Gram-positive bacteria and Archeobacteria. S-layers are two-dimensional arrays that cover the outside of the cell. S-layers may function as protective barriers and molecular sieves, promote cell adhesion and surface recognition

and maintain cell shape and envelope rigidity (34). Thousands of copies of nearly always a single protein or glycoprotein self-assemble into a crystalline-like lattice (58). S-protein represents approximately 10-15% of the total cellular protein of the bacterial cell(16). For reviews on S-layers see Beveridge et. al., 1997; Boot and Pouwells, 1996; Sleytr and Messner, 1983.

1.03- Transport of S-layers

The majority of the S-layer transport systems that have been discovered are type II systems in which an N-terminal signal system directs export across the inner membrane using the general secretion pathway (GSP) and secretion from the bacterium then occurs via a protein specific mechanism. An example of this is the S-proteins of *Aeromonas* that are transported across the cytoplasmic membrane via the GSP, but require substrate specific terminal branches of the GSP to transport across the outer membrane. The S-layer proteins of *C. crescentus* (4) and those of *Campylobacter fetus* (68) and *Serratia marcescens*(35), however, are secreted by a type I mechanism likely allowing for high levels of S-layer secretion.

1.04- S-layer of C. crescentus

The Gram-negative bacterium *Caulobacter crescentus* is covered by an S-layer which minimally acts as a physical barrier to *Bdellovibrio*-like parasites and lytic enzymes (37). This crystalline surface layer is composed of a hexagonal array of the 98-kDa protein RsaA (63). The six RsaA subunits form a ring-like, circular structure that interconnects with other rings to form a two-dimensional hexagonal array (62) (**Fig. 1-**

2.). The S-layer is anchored to *C. crescentus* via an interaction with an outer membrane smooth lipopolysaccharide (S-LPS)(5, 71). Ca^{2+} is required for the proper crystallization of RsaA into the S-layer and its removal using EGTA disrupts S-layer structure (46, 71). Production of the *C. crescentus* S-layer has been estimated to be 10-12% of total cell protein with approximately 40,000 RsaA subunits attached to the surface of the cell(6, 9, 62). The level of secretion observed in *C. crescentus* appears to be one of the highest levels of S-layer secretion with only *C. fetus* having similar amounts. RsaA synthesis occurs without need for induction and the protein is produced continuously throughout the cell cycle (24, 60).

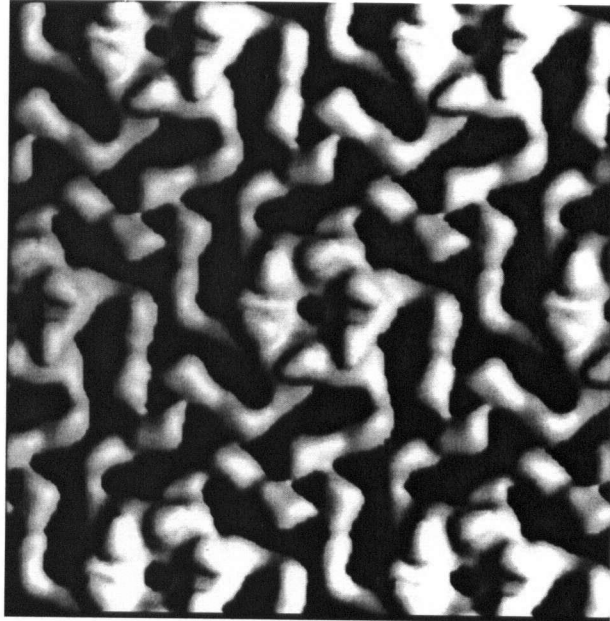


Figure 1-2. 3-D hexagonal array of the S-layer.
(figure from Smit et al, 1992)

1.05- Biotechnology applications of the C. crescentus S-layer

The S-layer of *C. crescentus* can be used for multiple biotechnology applications. Currently the S-layer protein has been used for heterologous protein production as well as protein and epitope display applications. The S-layer covers the entire surface of the bacterium. An uncleaved C-terminal secretion signal directs the secretion of RsaA (10, 12-14). When the native RsaA protein is secreted, the S-layer is attached to the outer membrane via S-LPS(5, 71). If the S-LPS is absent or disrupted, the S-layer forms aggregates which are up to 90% pure RsaA and can be easily collected. It is desirable to

produce large amounts of recombinant protein that can be easily purified. The C-terminal secretion signal and Ca^{2+} binding domain, responsible for aggregation, can be fused to a desired protein allowing recombinant proteins to be secreted by the RsaA transport system. Heterologous proteins aggregate in the culture medium and are easily purified using mesh filter. This process has been shown to be viable and recombinant proteins have been expressed and purified from *C. crescentus*(11). Up until 2003, the *C. crescentus* S-layer system was marketed as the PurePro™ expression system (Invitrogen™).

The S-layer has additional applications in the field of protein and epitope display. Initial studies used to define functional regions of the S-layer protein revealed the presence of sites that could be used for the display of small peptides. Efforts have since shown that these sites can accommodate upwards of 200 amino acid inserts (47). Since the S-layer covers the entire bacteria, this leads to thousands of copies of epitopes or proteins displayed per cell. Therefore numerous potential applications, from gene fragment display to whole cell vaccines are possible.

To increase the potential applications of this system, it is vital to understand the S-layer transport apparatus. Understanding potential zones of hindrance to RsaA transport is key to allowing secretion of all types of heterologous proteins, including those with positive charges. As well, identifying any potential bottlenecks in RsaA transport is important so that increased levels of S-layer secretion can be achieved. To address some of these questions this thesis examines the production of the RsaA secretion apparatus as well as characterization and overexpression of the OMPs.

1.06- Transport systems

There are five classes of secretion systems in Gram-negative bacteria that are well described (**Fig. 1-3.**). These systems have all been named type I through type V. The type I system requires three proteins to form a pore which spans through the inner and outer membranes allowing the protein to be secreted. This is the method by which RsaA is secreted and thus is discussed in depth below.

The type II secretion system is likely the most commonly used transport system in Gram-negative bacteria. The type II secretion system is associated with the general secretion pathway (GSP) which acts as a common protein transporter. Type II systems employ the GSP for export across the inner membrane and then use a complex of 12-16 proteins for secretion to the outside of the bacterium (67). The secreted proteins utilize a Sec-dependant N-terminal signal sequence to direct transport across the inner membrane by the Sec pathway (51). Proteins are transported across the inner membrane in an unfolded state and then fold in the periplasm. Folded proteins are often needed for the outer membrane component to recognize it for secretion.

The type III system is a Sec-independent secretion system used to translocate proteins into the extracellular environment, or directly into eukaryotic cells. These secretion systems are usually associated with bacterial pathogenesis, but are also involved in flagellum production. The systems are assembled from over 20 different structural proteins, including 10 that have counterparts in the flagellar export pathway(49). Type III systems are able to transport proteins across three membranes using a complex secretion apparatus.

Type IV secretion systems are involved in Sec dependant transport. The type IV system functions to transport DNA from bacteria to bacteria, as well as proteins from bacteria to eukaryotic cells. It has been found to facilitate the transport of multi-subunit proteins across bacterial membranes (19). It shares some similarities with the type II system utilizing the GSP system for translocation across the inner membrane. The type IV system is still not well understood, but the VirB system of *Agrobacterium tumefaciens* has been found to utilize 10 proteins for the transport mechanism.

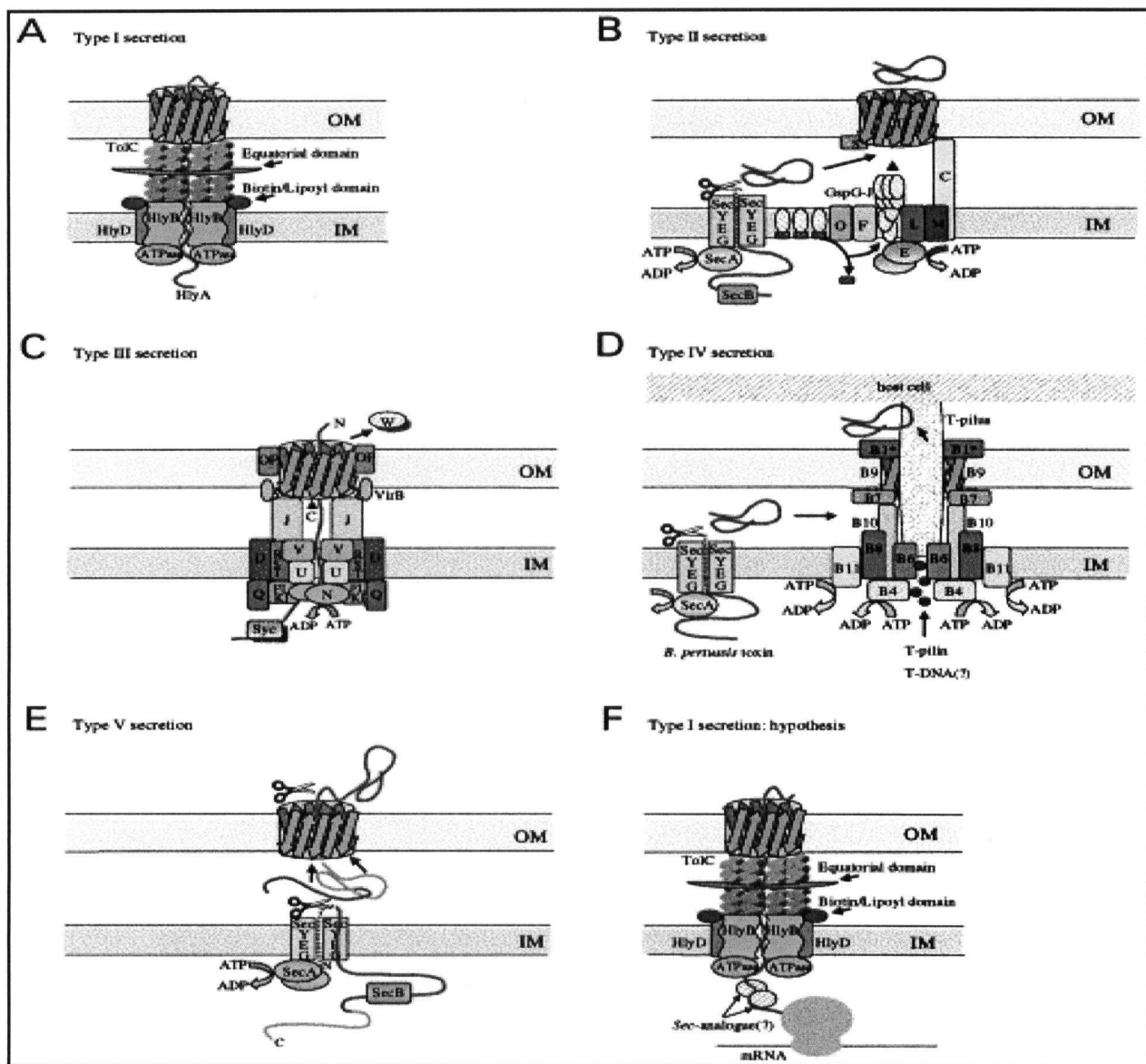


Figure 1-3. Cartoon depictions of the type I through type V transport systems.

Depicts the organization of systems in the bacterial membranes and periplasmic space. (figure from Sharff et al, 2001)

The type V system, or autotransporter system consists of both the transporter protein and the transported protein. Type V secretion is a terminal branch of the GSP that exports proteins with diverse functionalities, including proteases, toxins, adhesins, and invasins(31). A typical autotransporter contains three domains: an amino-terminal signal sequence for secretion across the inner membrane by the Sec system, an internal

passenger or functional domain, and a carboxy-terminal β -domain (31). The β -domain forms a pore in the outer membrane and then the passenger domain is transported to the outside of the cell.

1.07- Type I secretion

Type I secretion is a sec-independent pathway which secretes the protein from the cytoplasm across the outer membrane without any interaction with the periplasm. Type I proteins utilize a C-terminal secretion signal that is usually in the last 60 amino acids of the protein (7). Proteins secreted by this pathway include *Escherichia coli* α -hemolysin and other bacterial RTX toxins and proteases from *Erwinia chrysanthemi*, *S. marcescens* and *Pseudomonas aeruginosa*(54, 73). The RTX motifs contain a variable number of glycine and aspartic rich repeats in the C-terminal half of the protein. This RTX repeat is likely not the primary secretion signal, with the amino acid sequence and secondary structure of the C-terminus being more important (12, 22, 73).

The type I secretion apparatus is composed of a three-component ATP-binding cassette (ABC) based exporter. The ABC transporter, which most likely forms a homodimer in the inner membrane, engages the C-terminal sequence of the substrate protein and hydrolyzes ATP during the transport process. The membrane fusion protein (MFP) is anchored in the inner membrane by a single transmembrane domain, as well as bound to the ABC transporter, and appears to span the periplasm(67). The MFP is thought to interact with the ABC transporter protein and the last component, the outer membrane protein (OMP), forming a channel that extends from the cytoplasm through the two membranes to the outside of the cell.

1.08- The TolC protein

The *E. coli* HlyA system is the best characterized type I secretion system (15, 26, 43). In particular, the OMP TolC has been extensively characterized and is the prototype of a large family of OMPs from various Gram-negative bacteria. The structure of TolC has been solved to 2.1 Å (36) (**Fig. 1-4.**), and multiple studies have been carried out on its multifunctional nature. TolC appears to be very promiscuous, interacting with different inner membrane translocases involved in protein secretion, drug efflux and cations including the HlyA, AcrA, and CvaA systems (25, 32, 74). It has been suggested that the reason TolC is a multifunctional protein may be that its gene does not belong to any export operon (2, 3).

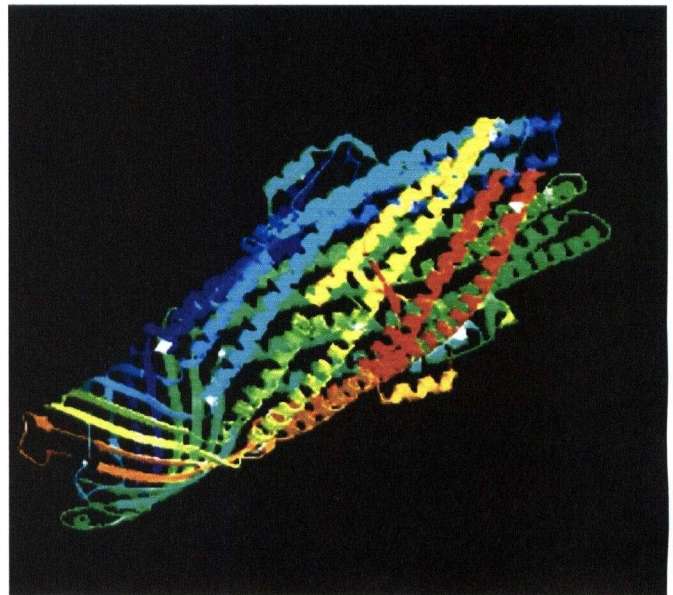


Figure 1-4. Ribbon structure of the *E. coli* TolC protein
(Figure from Koronakis et al. 2000)

1.09- Type I organization

Genome organization of most type I systems typically have the ABC transporter and MFP genes adjacent to the gene for the secreted protein on the 3' side, whereas the OMP gene location can vary. In some cases, the genes for all three transport components are immediately adjacent to the substrate gene(s) (20, 38, 68). In other type I systems, only the ABC-transporter and MFP genes are located next to the substrate gene (39, 40). The OMP commonly lies far from the other components. TolC from the *E. coli* α -

hemolysin (HlyA) system, and HasF from *S. marcescens* are examples of this and are not located near any export secretion systems (8, 74).

1.10- Transcriptional regulation

Transcriptional regulation of the type I components is not well studied. The majority of type I genes are organized together and are assumed to be transcribed together. Co-transcription of the type I transporter genes is based on previous experiments with the HlyA system of *E. coli*. Transcript analysis using rifampicin resistance suggested that *hlyABCD* were co-transcribed (75). To date, only two of the well characterized type I systems appear to have separate promoters for the transported protein as well as the transporter components. Both the *S. marcescens* (Lip) and *C. fetus* (SapA) systems have separate promoters for the S-layer gene and the transporter genes. The *sapDEF* genes are transcribed inversely from *sapA* using a separate promoter. The *lipBCD* genes are located immediately downstream of *slaA* but a separate promoter exists for *lipBCD* gene set. The type I apparatus is still not well understood and transcription of its components still appears relatively unknown.

1.11- The RsaA secretion system

The RsaA secretion system has all the usual characteristics of a type I secretion system. The secretion signal is located within the C-terminal 82 amino acids of RsaA (13). The ABC transporter (RsaD) and MFP (RsaE) have been characterized (4). The *rsaD* and *rsaE* genes are located downstream of *rsaA* and transcription of the RsaA secretion apparatus had been assumed to occur in a fashion similar to the HlyA system

with *rsaADE* being co-transcribed using the *rsaA* promoter. When initial studies were done, an OMP candidate did not immediately follow *rsaE*, indicating that that system resembles the Hly and Has systems. In that study they randomly mutagenized the genome of *C. crescentus* using the Tn5 transposon and identified several mutants that were deficient in RsaA secretion. Numerous Tn5 insertions were identified in *rsaA*, *rsaD* and *rsaE*, but none were found in an OMP component suggesting either that interfering with the OMP was lethal to the cell or that there were multiple genes involved in the formation of the outer membrane component of the secretion system. The two putative *rsaF* genes were later identified using the *C. crescentus* CB15 genome sequence, but characterization was not carried out.

1.12- Summary of the study

I was able to elucidate the transcriptional regulation of *rsaADE*. Molecular based approaches to using an *rsaA* deficient strain as well as an *rsaD* knockout strain led to the discovery that *rsaD* and *rsaE* are transcribed using a separate promoter predicted to be just downstream of the *rsaA*.

I have also identified two genes, *rsaFa* and *rsaFb* that code for OMPs involved in S-layer transport. Eliminating the function of either of these genes results in decreased RsaA secretion indicating that either protein can function as the OMP component of the secretion system. However, knocking out both genes completely eliminates RsaA secretion. Contrary to what previous studies suggested (30, 53), I demonstrate that RsaF (a and b) are involved in RsaA secretion and that both are required for maximal S-layer secretion. Data also suggests there is possible auto-regulation of RsaA expression.

Secretion is apparently a cooperative process as it was not possible to restore wild type level of RsaA secretion by overexpression of individual OMPs. Interestingly, if both OMPs were present, cooperative expression of additional RsaFa and RsaA led to levels of RsaA secretion significantly above wild type levels.

2. EXPERIMENTAL PROCEDURES

2.01- Bacterial strains, plasmids, and growth conditions

All of the strains and plasmids used in this study are listed in (Table 1.). *E. coli* DH5 α was used for all *E. coli* cloning manipulations, except for the use of Rb404 (17) for the use of non-methylated *Cla*I site digestion. As well, *E. coli* JM109 (76) was used for induced production of GST tagged RsaE. *E. coli* was grown at 37°C in Luria broth (1% tryptone, 0.5% NaCl, 0.5% yeast extract) with 1.3% agar for plates. *C. crescentus* strains were grown at 30°C in PYE medium (0.2% peptone, 0.1% yeast extract, 0.01% CaCl₂, 0.02% MgSO₄) with 1.2% agar for plates. Ampicillin was used at 50 μ g/ml, kanamycin was used at 50 μ g/ml, chloramphenicol was used at 20 μ g/ml, and streptomycin was used at 50 μ g/ml in *E.coli*. Kanamycin was used at 25 μ g/ml, chloramphenicol was used at 2 μ g/ml, and streptomycin was used at 10 μ g/ml in *C. crescentus* when needed.

JS1001 strains utilized for aggregated protein experiments were grown in 1.25 L M11 HIGG media (0.15% glucose, 0.15% glutamate, 2 mM phosphate, 0.5 mM calcium, 5 mM imidazole, 1% ammonium chloride). Cultures were grown for 4 days at 30°C at a shake speed of 60 RPM in Fernbach flasks, to maximize aeration. Aggregates were then recovered by coarse filtration using using nylon mesh as previously described (13) and cell densities (OD₆₀₀) were taken for all cultures. Aggregates were washed with dH₂O until no *C. crescentus* cells were seen attached under compound microscope. Aggregated proteins were kept at -80 °C and consequently lyophilized removing all traces of water.

Strain or plasmid	Relevant characteristics	Reference or source
Bacterial strains		
<i>C. crescentus</i>		
NA1000	Apr ^r <i>syn</i> -1000; variant of wild-type strain CB15 that synchronizes well	ATCC 19089
JS1001	S-LPS mutant of NA1000, sheds S-layer into medium	Edwards and Smit, 1991
JS1003	NA1000 with <i>rsaA</i> interrupted by KSAC Km ^r cassette	Edwards and Smit, 1991
JS1007	Sm ^r , NA1000 <i>rsaFa</i> - strain	This study
JS1008	Cm ^r , NA1000 <i>rsaFb</i> - strain	This study
JS1009	Cm ^r , Sm ^r , NA1000 <i>rsaFa</i> -/ <i>rsaFb</i> - strain	This study
B15	Km ^r , Sm ^r , NA1000 Tn5 insertion in the <i>rsaD</i> gene	Awram and Smit, 1998
Δ- <i>rsaA</i>	CB15, <i>rsaA</i> gene knocked out deleting <i>rsaA</i> promoter and portion of <i>rsaA</i> gene	This study
<i>E. coli</i>		
DH5α	<i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>hsdR17</i> , <i>SupE44</i> , <i>relA1</i> , <i>LacZYA-arfF</i>	Invitrogen
JM109	<i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>hsdR17</i> , <i>SupE44</i> , <i>relA1</i> , <i>LacIq</i> , <i>LacZM15</i>	Yanisch-Perron et al., 1985
Rb404	<i>F-dam-3</i> , <i>dam-6</i> , <i>metB1</i> , <i>galK2</i> , <i>galT22lacY1</i> , <i>thi-1</i> , <i>tonA31</i> , <i>tsx-78</i> , <i>mtl-1</i> , <i>supE44</i>	Brent and Ptashne, 1980
Plasmids		
pHP45	Apr ^r , Sm ^r , plasmid from which omega Sm cassette removed	Fellay et al., 1998
pUC4KISS	Km ^r , omega Km cassette removed for pBBR4	Taylor and Rose, 1988
pBSKI+	Apr ^r , LacZ, Cloning vector	Stratagene
pBSKII	Apr ^r , LacZ, Cloning vector	Stratagene
pBSKIIIEH	Apr ^r , modified pBSKSI cloning vector with <i>EcoRI</i> , <i>EcoRV</i> , <i>HindIII</i> modified MCS	This study
pBSKIIESH	Apr ^r , modified pBSKSI cloning vector with <i>EcoRI</i> , <i>StuI</i> , <i>HindIII</i> modified MCS	This study
pTZ18UCHE	Cm ^r , cloning vector	This study
pTZ18UCHE: <i>rsaFb</i> ΔNΔC	Cm ^r , cloning vector with <i>rsaFb</i> fragment missing N terminus and C terminus	This study
pBSKIIIEH: <i>rsaFa</i> ΔSm	Apr ^r , Sm ^r , <i>rsaFa</i> gene fragment with Sm cassette inserted at <i>PstI</i> site	This study
pBBR4	Km ^r , broad host range plasmid derived from pBBR1	This study
pBBR4: <i>rsaFa</i>	<i>rsaFa</i> +, Km ^r , <i>rsaFa</i> gene inserted <i>EcoRI</i> / <i>BamHI</i> in pBBR4	This study
pBBR4: <i>rsaFb</i>	<i>rsaFb</i> +, Km ^r , <i>rsaFb</i> gene inserted <i>EcoRI</i> / <i>BamHI</i> in pBBR4	This study
pWB9A19	Cm ^r , Sm ^r , <i>rsaA</i> gene and <i>rsaA</i> promoter strain	This study
pWB9Hps12	Cm ^r , Sm ^r , <i>rsaA</i> containing with <i>BamHI</i> site at a.a. 723	Bingle et al., 1997
pWB9:Hps12furin	Cm ^r , Sm ^r , <i>rsaA</i> containing with furin cleavage site (RKKR) in <i>BamHI</i> site at a.a. 723	This study
pGEX4T3	Apr ^r , GST tagged expression vector	Amersham
pGEX4T3: <i>rsaFa</i>	Apr ^r , GST tagged expression vector with in frame <i>BamHI</i> - <i>EcoRI</i> <i>rsaFa</i> gene	This study
pK18mobsacB	Km ^r , Suc ^s , <i>E. coli</i> based suicide vector	Schafer et al., 1994
pK18mobsacB: <i>rsaFa</i> ΔSm	Km ^r , Suc ^s , <i>E. coli</i> based suicide vector with <i>rsaFa</i> ΔSm fragment	This study
pRAT1	Apr ^r , <i>rsaA</i> +, <i>rsaD</i> +, <i>rsaE</i> +	Awram and Smit, 1998
pRAT9	Cm ^r , <i>rsaD</i> +, <i>rsaE</i> + pBBR1 based plasmid	Awram and Smit, 1998
puC19	Apr ^r , LacZ, ColE1 cloning vector	Vieria and Messing, 1982
puC19:RAT1Cm	Apr ^r , Cm ^r , containing the RAT1 gene set with a Cm cassette interrupting the <i>rsaA</i> gene	This study
pBBR4:RAT1Cm	Km ^r , Cm ^r , broad host range with RAT1 gene set, Cm cassette interrupting the <i>rsaA</i> gene	This study
pGEX4T3: <i>rsaD</i>	Apr ^r , GST tagged expression vector with inframe <i>rsaD</i> gene	This study
pGEX4T3: <i>rsaE</i>	Apr ^r , GST tagged expression vector with inframe <i>rsaE</i> gene	This study
pAL1	Sm ^r , Suc ^s , <i>E. coli</i> based pNPTS138 suicide vector with Δ- <i>rsaA</i> fragment	This study

2.02- DNA extraction, purification and separation

Chromosomal extraction was done by phenol chloroform extraction. 3 ml volumes of logarithmic phase culture were centrifuged at 16000 X g for 2 min. Cell pellets were resuspended in 0.5 ml of 10 mM tris-HCL. Lysozyme was added to final concentration of 300 µg/ml and incubated at 37°C for 10 min. SDS was added to 0.3% and incubated at 60 °C for 20 min. Proteinase K and RNaseA were added to 300µg/ml and incubated 4 hr. DNA was then purified by 2 phenol/chloroform extractions (1:1:1), one phenol extraction followed by phenol/ chloroform extraction.

Isolation of plasmid DNA was done utilizing the Qiaprep spin mini prep (Qiagen) system. Isolated plasmid DNA was eluted in TE buffer or dH₂O. Restriction enzyme digestions were done with Invitrogen or New England Biolabs Inc. enzymes and buffers as specified by manufacturers. DNA fragments were analyzed by agarose gel electrophoresis using a horizontal apparatus with a TBE buffer system. 0.9% agarose gels were used for DNA separation with 0.5 µg/ml EtBr added to the molten gel solution prior to casting. Separation of DNA fragments was conducted using a voltage range of 80-120V. DNA bands were isolated and excised from gels using a scalpel. DNA was purified using the Qiaex II gel extraction kit (Qiagen) using the manufacturers' protocols.

2.03-Plasmid and DNA manipulations (rsaADE studies)

Standard methods of DNA manipulation and isolation were used (55). Electroporation of *C. crescentus* was performed as previously described (27). All PCR products were generated using Platinum *Pfx* DNA polymerase (Invitrogen) following the

manufacturers suggested protocols, except for the pAL1 construction which utilized Taq DNA polymerase. NA1000 chromosomal DNA was used as the template for all PCR products, except the pAL1 fragments in which CB15 chromosomal DNA was used.

A fragment containing the *rsaD* gene was amplified by PCR using the primers 5'-CCGAATTCCATGTTCAAGCGCAGC-3' and 5'-GCGGCCGCTCTGGACGCGCTGCAA-3' incorporating *EcoRI* and *NotI* restriction sites. This gene fragment was inserted into the *EcoRV* site of the pBSKI⁺ plasmid. The pBSKI⁺: *rsaD* plasmid was cut with *EcoRI* and *NotI* releasing the *rsaD* fragment. This fragment was inserted into *EcoRI-NotI* cut pGEX4T3 plasmid. The pGEX4T3: *rsaD* construct is an in-frame insertion of the *rsaD* gene so that it has an attached GST tag.

Another fragment containing the *rsaE* gene was amplified by PCR using the primers 5'-CCGAATTCCATGAAGCCCCCAAG-3' and 5'-GCGGCCGCTCTCCTCGCGCATCGT-3' incorporating *EcoRI* and *NotI* restriction sites. This fragment was inserted into the pBSKI⁺ plasmid at the *EcoRV* site. The pBSKI⁺: *rsaE* plasmid was digested using *EcoRI* and *NotI* releasing the *rsaE* fragment. The fragment was then ligated into *EcoRI-NotI* cut pGEX4T3 plasmid. The pGEX4T3: *rsaE* construct is an in-frame insertion of the *rsaE* gene so that it has an attached GST tag.

Construction of the pAL1 plasmid 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 amplified using the primers 5'-GGATCCGGCGTTTCGAGCTGCTGCTGA-3' and 5'-GAATTCTCACCTGGCGGGTGAGTGAG-3' introducing *BamHI* and *EcoRI* sites.

Another PCR product was created using the primers 5'-GAAATTCCGCTCGCCTAAGCGAACGTC-3' and 5'-ACTAGTGGCCGAGATCTTGCCGTCGA-3' amplifying a 1.0 kb region containing the end of the *rsaA* gene and incorporating *EcoRI* and *SpeI* sites. Fragments were ligated into the pGEM-5ZT(+) vector at the *EcoRV* site using the pGEM-T® easy kit (Promega). The resulting fragments were digested with *EcoRI*, *BamHI* and *SpeI*, and ligated into *BamHI* and *SpeI* cut pNPTS138 plasmid(33). This resulted in creation of plasmid pAL1 which was transformed, by electroporation, into the *E. coli* DH10B strain (Invitrogen) and selected by blue–white screening.

The pUC19: RAT1 plasmid was created using the pUC19 plasmid (70) and the pRAT1 plasmid (4). The RAT1 fragment was removed from the pRAT1 plasmid as an *EcoRI*-*SstI* fragment and ligated into an *EcoRI*-*SstI* cut pUC19 plasmid. Interruption of the *rsaA* gene in the pUC19: RAT1 construct was done by antibiotic insertion. The pUC19: RAT1 Ω Cm was created using the pUC19: RAT1 plasmid and the Ω Cm cassette from the pHP45 Ω Cm plasmid (21). The Ω Cm cassette was removed from the pHP45 Ω Cm plasmid as a *BamHI* fragment and blunted using T4 polymerase. The pUC19: RAT1 plasmid was electroporated into the Rb404 *E. coli* strain to stop DAM methylation. The isolated pUC19: RAT1 construct was digested using *ClaI* and the ends were blunted using T4 DNA polymerase. The blunted Ω Cm cassette was then ligated into the blunted *ClaI* site of the pUC19: RAT1 plasmid resulting in the pUC19: RAT1 Ω Cm plasmid. The pBBR4 and pUC19: pRAT1 Ω Cm vectors were then used to create a broad host range construct containing the RAT1 Ω Cm fragment. The *EcoRI*-*SstI* cut RAT1 Ω Cm

fragment from the pUC19: RAT1 Ω Cm was ligated into the *EcoRI*–*SstI* cut pBBR4 plasmid creating the pBBR4: RAT1 Ω Cm plasmid.

2.04- Plasmid and DNA manipulations (*rsaFa* and *rsaFb* studies)

Standard methods of DNA manipulation, isolation, PCR product generation and cloning procedures were carried out as above.

Plasmids used for internal deletions were made with the *rsaF* genes (a or b) and 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 the primers 5'-GCCACGCCCCGGCGTCCAGTCCGA-3' and 5'-GAGCTCCCTAGAGCGTTCTCCGATCCGTGCG-3'. This fragment was blunt end ligated into the pBSKI⁺ plasmid at the *EcoRV* site and called pBSKI: *rsaFa EX*.

A PCR product containing *rsaFb* and flanking regions of 795 bp 5' and 858 bp 3' was generated using the primers 5'-CGCCGGCTTCGCAGCGATGAGCCC -3' and 5'-CCCGGAGGCCTCCCAGGCGGCGTA-3'. This fragment was blunt end ligated into the *StuI* site of the pBSKIIESH plasmid and called pBSKIIESH: *rsaFb EX*.

A PCR product containing *rsaFa* was generated using primers 5'-CGCGGATCCATGCGAGTGCTGTCGAAAGTTCTGTC -3' and 5'-CCGGGAATTCTAGTTGCGGGGCGCGGTCTGGAC -3'. Another PCR product containing *rsaFb* was created using primers 5'-CGCGGATCCATGTTGATGTCGAACCGTCGACGGG -3' and 5'-CCGGGAATTCTATTTGAGCCGCTCGGGGGCTT -3'. PCR products were blunt

end ligated into the *EcoRV* site of the pBSKIIEEH vector and called pBSKIIEEH: *rsaFa* and pBSKIIEEH: *rsaFb* respectively.

The pBSKIIEEH vector was constructed by Dr. John Nomellini from the plasmid pBSKII (Stratagene). The *Bss*HI fragment containing the multiple cloning site was removed and replaced with annealed oligonucleotides 5'-

CGCGCTGAATTCGGATATCTTAAGCTTGG-3' and 5'-

CGCGCCAAGCTTAAGATATCCGAATTCAG- 3' forming *EcoRI*, *EcoRV* and *HindIII* sites. Similarly, the pBSKIIESH plasmid was created from the plasmid pBSKII

(Stratagene). The *Bss*HI fragment containing the multiple cloning site was removed and replaced with annealed oligonucleotides 5'-

CGCGCTGAATTCGAGGCCTTTAAGCTTGG -3' and 5'-

CGCGCCAAGCTTAAAGGGCTCGAATTCAG -3' forming *EcoRI*, *StuI* and *HindIII* sites. These both result in smaller simpler plasmids that can be digested easily for blunt end cloning.

Dr. John Nomellini constructed the plasmid pBBR4 from plasmids pBBR1MCS and pUC4 KISS (65). The Ω -Km fragment from pUC4 KISS was removed using *PstI* and the ends were blunted using T4 polymerase. A 0.3-kbp portion of the Cm^r - encoding gene was removed from pBBR1MCS by cutting with *DraI* and replaced with the blunted Ω -Km fragment, producing a Km^r broad-host-range vector that replicates in *C. crescentus*. Plasmids pBBR4: *rsaFa* and pBBR4: *rsaFb* were made by removing the *Bam*HI- *EcoRI* fragment of the pBBR4 plasmid and replacing it with the *Bam*HI-*EcoRI* fragment from pBSKIIEEH: *rsaFa* and pBSKIIEEH: *rsaFb* plasmids respectively.

The plasmid pGEX4T3: *rsaFa* was constructed from plasmids pBSKIIEEH: *rsaFa* and pGEX4T3 (Amersham). Using incorporated restriction sites generated from the initial PCR primers, the *Bam*HI- *Eco*RI fragment containing *rsaFa* was cloned in-frame into *Bam*HI-*Eco*RI cut pGEX4T3 plasmid.

The pBSKIIEEH: *rsaFa*ΩSm plasmid was created using the plasmids pBSKIIEEH *rsaFa* and pHP45Ω(21). The ΩSm cassette was removed from the pHP45Ω plasmid as a *Sma*I fragment. This fragment was then blunt-end ligated into the pBSKIIEEH: *rsaFa* plasmid at a T4 polymerase blunted *Pst*I site inside the *rsaFa* gene. The resulting plasmid pBSKIIEEH: *rsaFa*ΩSm was then used to make the pK18mobsacB: *rsaFa*ΩSm plasmid. The *Eco*RI-*Hind*III fragment containing the *rsaFa*ΩSm fragment was cloned into *Eco*RI-*Hind*III cut pK18mobsacB plasmid.

A PCR product containing a truncated form of *rsaFb* with the N-and C-terminus missing was generated using the primers 5'-GAAGCCGACGTGCTGTCT- 3' and 5'-TGTAGGAGGTTTTTCGGGTCA-3'. This PCR product was blunt ligated into the *Stu*I site of the pBSKIIESH vector using T4 DNA ligase creating pBSKIIESH: *rsaFb* ΔNΔC plasmid. The pTZ18U CHE plasmid was constructed by Dr. Peter Awram using inverse PCR with the primers 5'-GAGGCCTAGTACTCTGTCAGACCAAGTTTACTCATA-3' and 5'-GAGGCCTACTCTTCCTTTTTCAATATTATTGAA-3' to create the backbone of the pTZ18U plasmid without the Ap^r cassette. The CHE (chloramphenicol) fragment was created as a PCR product using the pMMB206 plasmid (44) and the primers 5'-GGAAGATCTGTAACTTTTCAGGAGCTAAGGAAGCT-3' and 5'-GGAAGATCTGTAAACACAATAACTGCCTTAAAAAATTA-3'. The pTZ18U backbone product was cut with *Stu*I and blunt-ligated with *Hpa*I cut CHE fragment

creating the pTZ18UCHE plasmid. The pTZ18UCHE: *rsaFb*ΔNΔC plasmid was then made by Dr. John Nomellini using the pTZ18UCHE plasmid and the pBSKIIESH: *rsaFb*ΔNΔC plasmids. The *EcoRI*- *HindIII* fragment from the pTZ18UCHE plasmid was removed and replaced with the *EcoRI*- *HindIII* fragment containing the *rsaFb*ΔNΔC fragment from the pBSKIIESH: *rsaFb*ΔNΔC plasmid.

The pWB9: *rsaA*Δ*P* was created as previously described (12). The pWB9 Hps12furin construct was made by Dr. John Nomellini using the *Bam*HI site at a.a. 723 (Hps12) (11). Two oligonucleotides oligonucleotides 5'-TCGAGACCCGATGCGCAAGAAACGGG -3' and 5'-CCCGTTTCTTGCGCATCGGGTC -3' were annealed together and then ligated into the pUC9CXS plasmid *Xho*I - *Stu*I in a similar manner as the pillin epitope (11). The resulting plasmid was then digested using *Bam*HI releasing the RKKR furin containing fragment. This fragment was then inserted into the *Bam*HI site at a.a. 723 in *rsaA*Δ*P* and forward orientation of the fragment was confirmed by Cm resistance. The Cm^r cassette was removed by excising with *Bgl*II and then ligated back the two complementary ends. The *rsaA*Δ*P* Hps12furin fragment was then removed as an *Eco*RI-*Sst*I fragment and ligated into *Eco*RI-*Sst*I cut pWB9KSAC plasmid.

2.05- Internal deletions in the *rsaF* genes

Internal deletion of the *rsaF* genes were done using the pBSKI: *rsaFaEX* and pBSKI: *rsaFbEX* plasmids. Internal deletions were done using unique restriction enzyme sites in either *rsaF* gene. Three separate internal deletions were done in the *rsaFa* gene. The first is a deletion utilizing the *Eco*NI restriction enzyme sites. There are 3 *Eco*NI

sites in the *rsaFaEX* fragment, one 185 bp 5' of the *rsaFa* gene, one 721 bp into the gene and another 1099 bp into the gene. Digestion of the pBSKI: *rsaFaEX* at the three sites and consequent re-ligation to remove the internal fragments leads to a deletion of ~1.3 Kb and the resulting plasmid called pBSKI: *rsaFaΔN*. The second internal deletion was made in the pBSKI: *rsaFaEX* plasmid using the *EcoRV* and *PstI* restriction enzyme sites. The *EcoRV* site is 135 bp 5' of the start site and the *PstI* site is 503 bp 3' from the start of the gene. The pBSKI: *rsaFaEX* was digested with *PstI* and *EcoRV*, blunt ended using T4 DNA polymerase and then ligated together removing a fragment of 638 bp. The resulting plasmid was called pBSKI: *rsaFaΔVP*. The last internal deletion used the *PstI* and *KpnI* restriction sites. The pBSKI: *rsaFaEX* plasmid was digested with *PstI* and *KpnI* and blunt ended using T4 DNA polymerase and ligated. The resulting plasmid pBSKI: *rsaFaΔKP* has an 852 bp deletion. All *rsaFa* internal deletion fragments were removed as *EcoRI*-*HindIII* fragments and ligated into *EcoRI*-*HindIII* cut pK18mobsacB plasmid.

The one *rsaFb* gene internal deletion was made in the pBSKIIESH: *rsaFbEX* plasmid. The *EcoRV* and *NcoI* sites in the *rsaFbEX* fragment. The *EcoRV* site is 227 bp 3' of the start site and the *NcoI* site is 174 5' of the end of the gene. After digestion of the pBSKIIESH plasmid with *EcoRV* and *NcoI* and then ends were blunted using T4 DNA polymerase and ligated back together. The resulting plasmid pBSKIIESH: *rsaFbΔVN* has an internal deletion of 1051 bp. The pBSKIIESH: *rsaFbΔVN* was digested with *EcoRI* and *HindIII* releasing the *rsaFbΔVN* fragment. This fragment was ligated into *EcoRI*-*HindIII* cut pK18mobsacB plasmid.

2.06- Knockout construction

Knockout of *rsaFa* in the CB2A strain was done using the pK18mobsacB: *rsaFa* Δ KP plasmid. Primary recombination of the plasmid was selected for using Km resistance. Five concurrent sub-culturing events were used 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'-CGCCGGCTTCGCAGCGATGAGCCC -3' and 5'-CCCGGAGGCCTCCCAGGCGGCGTA-3' to confirm that appropriate gene replacement occurred. A strain confirmed to possess only the internal deletion form of *rsaFa* was designated CB2A: *rsaFa* Δ KP.

Since internal deletion knockouts were hard to obtain, alternate methods were undertaken. Knockouts of the two *rsaF* genes were done in wild type (S-layer positive) NA1000 *C. crescentus*. *rsaFa* was destroyed through gene replacement of an *rsaFa* fragment containing an internal Ω Sm cassette. The pK18mobsacB plasmid was used as a suicide vector to incorporate the antibiotic ablated *rsaFa*. The pK18mobsacB: *rsaFa* Ω Sm plasmid was electroporated into NA1000 cells. Primary selection on PYE Sm/ Km plates was used to determine if recombination had occurred. Five concurrent sub-culturing events were used to encourage a second recombination event. Secondary selection on 5% sucrose PYE plates and subsequent replica plating on PYE Sm and PYE Km plates was used to confirm a second recombination event. Colonies were then screened by PCR using primers 5'-CGCGGATCCATGCGAGTGCTGTGCGAAAGTTCTGTC -3' and 5'-CCGGGAATTCTAGTTGCGGGGCGCGGTCTGGAC -3' to determine if the

recombination event resulted in restoration of wild type *rsaFa* or incorporation of the *rsaFa*ΩSm gene fragment.

Destruction of *rsaFb* was done via insertional inactivation using an N- and C-terminally deleted *rsaFb* fragment. The non-replicatable pTZ18U CHE: *rsaFb*ΔNΔC plasmid was electroporated into NA1000 competent cells and using Cm^r cassette on the plasmid selection for insertional inactivation. Recombination of the pTZ18UCHE: *rsaFb*ΔNΔC plasmid resulted in loss of the full *rsaFb* gene leaving independent N-terminal deleted and C-terminal deleted *rsaFb* gene fragments. Colonies were screened by PCR using the primers 5'-GAGGCCTACTCTTCCTTTTCAATATTATTGAA - 3' and 5'-GGACGACGCTGACCAGCACCCCCTGCT -3'.

The double *rsaF* knockout was created by gene replacement using the pK18mobsacB: *rsaFa*ΩSm plasmid and JS1008 (*rsaFb*) competent cells. Screening for homologous recombination of the pK18mobsacB: *rsaFa*ΩSm was done in the same manner as the single *rsaFa*ΩSm knockout. The only change to the protocol was that Cm was used in all media to maintain the *rsaFb* knockout. PCR confirmations of both *rsaF* knockouts were done using the primers and conditions as stated above.

2.07- *RsaADE* gene transcription studies

The CB15 Δ-*rsaA* strain was created using the pAL1 plasmid. Delivery of the pAL1 plasmid into the CB15 strain 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^r, containing a Tn9 insertion, ColE1 ori, and tra

functions from pRK2013 (57). Gene replacement was confirmed by PCR analysis (not shown) and the resulting strain was called CB15 Δ -*rsaA*.

The CB15A Tn5 mutant B15 was made and confirmed as previously described (4). The pBBR4: RAT1 Ω Cm plasmid was electroporated into the B15 strain and cells were selected for resistance to Km, Cm, and Sm.

2.08- Antibody production

Antibodies used to detect RsaA were prepared by Dr. John Nomellini using a form of RsaA containing N and C-terminal portions of the protein, referred to as anti 188/784. This internal deletion form of RsaA was previously described in linker mutagenesis studies (12). Essentially, the N-terminal 1-188 a.a. fragment was removed as an *EcoRI*-*Bam*HI fragment and the C-terminal 784-1025 a.a. fragment was removed as a *Bam*HI-*Hind*III fragment. The two fragments were ligated together in-frame at the *Bam*HI site and then ligated into *EcoRI*-*Hind*III cut pUC8 plasmid. The *Hind*III cut pUC8: 188-784 was ligated to *Hind*III cut pKT215 vector and transformed into *C. crescentus* forming aggregated protein which was used to make antibodies against both termini. Aggregated 188/784 protein was collected and washed with dH₂O to remove any attached *C. crescentus* cells. Aggregates were solubilized with 4M Urea and dialyzed (30,000MW dialysis tubing) in dH₂O to remove all traces of urea. Samples were then injected into New Zealand white rabbits and rabbit serum was collected and processed using standard protocols (55).

Polyclonal antibodies were produced against RsaFa using a GST tagged protein. The pGEX4T3: *rsaFa* plasmid was expressed in *E. coli* (DH5 α), but unfortunately the protein was only produced in the form of inclusion bodies. Thus protein was extracted by

inclusion body preparations. Inclusion body preparations were done by growing cells at 30°C to 1.0 OD₆₀₀ and centrifuging cells and resuspending in 1XPBS buffer. Resuspended cells were incubated with lysozyme (100µg/ml) for 1hr at 25°C and then RNaseA (50µg/ml) and DNaseI (1µg/ml) were added and incubated for an additional hr at 25°C. After incubation, 10%SDS was added as well as SDS sample buffer at a 1:1:1 ratio. Samples were boiled for 5 min and then put on ice for 15 min. The inclusion body preparation was then centrifuged at 16,000g for 10 min. The pellet was then recovered and solublized using 4M urea. Protein was dialyzed (30,000MW dialysis tubing) in dH₂O for two days to remove all traces of urea. New Zealand white rabbits were injected with prepared protein samples containing a 1:1 ratio (volume) of protein to incomplete freunds adjuvant. Rabbit serum was collected and processed using standard protocols (55).

The RsaD antibodies were made in a similar fashion to the anti-RsaFa antibodies. The pGEX4T3: *rsaD* plasmid was expressed in *E. coli* DH5α and similarly, the protein was only produced in the form of inclusion bodies. Inclusion bodies were prepared same as above. Dialyzed protein was then injected into New Zealand white rabbits and the rabbit serum was collected and processed using standard protocols (55).

The RsaE antibodies were made using a GST tagged protein. The pGEX4T3: *rsaE* plasmid was expressed in the *E. coli* JM109 strain and soluble protein was produced and purified. JM109 cells with the pGEX4T3: *rsaE* plasmid were grown to OD₆₀₀ ~0.8 and the incubated with 0.1mM IPTG at 30°C for 3 hrs. Cells were then pelleted and resuspended in cold buffer (PBS/ 0.5 % Tween-20/ 1M NaCl/10mM DTT/ 1mM PMSF). Resuspended cells were sonicated and then centrifuged and supernatant saved. Supernatant was added to Glutathione Sepharose beads (Sigma) and rocked for 1 hr.

After rocking, beads were centrifuged and supernatant was aspirated. Beads were washed 3 times using 1XPBS. GST-tagged RsaE was eluted using elution buffer (50mM tris-HCl pH8/ 10 mM reduced Glutathione(Sigma)). Eluted protein fractions were then pooled and dialyzed against dH₂O. Dialyzed protein was then injected into New Zealand white rabbits and the rabbit serum was collected and processed using standard protocols (55).

2.09- Protein techniques

Surface protein from *C. crescentus* cells was extracted by low pH extraction as previously described (72). Cell pellets were washed twice using 10 mM HEPES (pH 7.2) buffer and then release of the S-layer was facilitated using 100mM HEPES at pH 2.0. To compare the amounts of surface layer protein extracted from different mutants, normalized levels of cells (determined by OD₆₀₀) growing at log phase were used and equal amounts of extracted protein samples were loaded onto protein gels.

Whole-cell-protein preparations were done with normalized levels of cells (determined by OD₆₀₀) growing at log phase. Cultures were centrifuged and cell pellets were washed twice with 10mM tris-HCl pH 8. Cells were resuspended in 10mM tris-HCl pH8 and lysozme (100µg/ml) was added and incubated at 25°C for 15 minutes, then RNaseA (50µg/ml) and DNaseI (1µg/ml) were added and incubated at 37°C for 30 minutes. Equal amounts of whole-cell-protein preparations were loaded onto protein gels. Note that whole cell preparations done to determine internal levels of S-layer were altered for certain strains. The wild type S-layer positive NA1000 has RsaA attached to its surface and thus the strain was subjected to low pH extraction before whole cell preparation was done to remove attached RsaA. As well, the S-layer shedding JS1001

strain was poured through fine mesh removing aggregates so that they did not affect RsaA levels present in the whole-cell preparation.

Whole culture preparations were done with normalized levels of cells (determined by OD₆₀₀) growing (in liquid PYE) at log phase. Whole cultures were normalized so that 1ml of 0.6 OD₆₀₀ cells were used for all cultures, thus making sure that any aggregated proteins were very small and well dispersed. Cultures were incubated with Lysozme (100 µg/ml) and incubated at 25°C for 15 minutes, and then RNaseA (50 µg/ml) and DNaseI (1 µg/ml) were added and incubated at 37°C for 30 minutes. Powdered urea was added to make the final concentration of culture equal to 2M urea. Addition of urea is needed to solubilize any micro-aggregates produced by S-layer shedding strains. Equal amounts of whole culture protein preparations were loaded onto protein gels.

Aggregated proteins were collected as previously described (13). Cultures were grown for 4 days at 30°C in 2.8L Fernbach flasks shaken at 60 rpm and then aggregated protein was collected using a fine mesh. Collected aggregates were washed with dH₂O and then centrifuged at 6000 X g for a few seconds. Washing and centrifugation was repeated until few to no *C. crescentus* cells were seen attached to the aggregates under the compound microscope. Wet weights were taken and then aggregates were kept at – 80°C. Frozen aggregates were then lyophilized until completely dry and weight taken.

2.10- 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

(55). After transfer of proteins to 0.2 μ m BioTrace NT nitrocellulose membrane (PALL), blots were blocked using Blotto (3% skim milk , 0.9% NaCl, and 20mM Tris). Western blots were probed with primary rabbit polyclonal antibodies, and antibody binding was visualized by either colorimetric or chemiluminescence developing methods.

Colorimetric blotting was done using goat anti-rabbit serum coupled to horseradish peroxidase and color forming reagents as previously described (61). Chemiluminescent blotting was done using the Amersham Biosciences ECL western blotting kit in accordance with the manufacturer's protocol.

Anti-188/784 antibodies were incubated at 1/15000 dilutions for colorimetric and 1/30000 dilutions for chemiluminescent western blotting. Incubation of primary anti-RsaFa was done at a 1/1000 dilution for colorimetric and 1/5000 dilution for chemiluminescent western blotting. Anti-RsaD antibodies were incubated at a 1/1000 dilution for colorimetric and 1/2000 dilution for chemiluminescent westerns. Anti-RsaE antibodies were used at 1/5000 dilution for colorimetric and 1/10000 dilution for chemiluminescent western blotting. Incubation of secondary anti-rabbit HRP was used at 1/5000 dilution for colorimetric and 1/15000 dilution for chemiluminescence western blotting. Kodak X-OMAT LS film was used for visualization of chemiluminescent blots while spot densitometry was done using a Bio-Rad VersaDoc 5000 system and the Quantity One (V4.3.0) program.

2.11- Electron microscopy

All electron microscopy studies were carried out by Dr. J. Smit. Protein A-colloidal gold immunolabelling of *C. crescentus* strains was performed as described

previously (60) using the anti-RsaFa antibody and 5 nm protein A-colloidal gold label prepared as previously described (59). The antibody was preabsorbed with JS1009 cells. Cells (1.0 OD_{600}) were centrifuged at $16000 \times g$ for 5 min, and then the cell pellet was resuspended in 10 mM Tris-HCl pH 8 and sonicated for 5 sec using a microprobe. Sonicated cells were then centrifuged at $16000 \times g$ for 2 min and the supernatant was removed. Anti-RsaFa rabbit serum was added to resuspend the pellet, and the suspension was then put on ice for 1 h. The serum suspension was centrifuged at $16000 \times g$ for 3 min and then the adsorbed serum was collected. Cells were imaged unstained by whole mount transmission electron microscopy.

2.12- Bioinformatic analysis and protein threading

All sequences were obtained from The Institute for Genomic Research (TIGR) *C. crescentus* genome database. The Biology Workbench website <http://workbench.sdsc.edu/> was utilized for protein sequence alignments using BLASTP sequence alignment tools (1). ClustalW alignments of RsaFa, RsaFb and TolC were done using MacVector V 6.0. Protein threading was done using the Swiss Model program(56) using the TIGR RsaFa (CC1015) and RsaFb (CC1318) protein sequences and the TolC Pdb file (IEK9). Cartoon models of the predicted threaded structures were generated using the Deep View/ Swiss-Pdb Viewer V3.7(29, 48) and the Swiss Model generated RsaFa and RsaFb Pdb files. 3D rendering was used to generate the space fill and ribbon models. Ribbon models were altered with colored sections to define α -helical and β -sheet segments. Electrostatic surfaces were predicted using electrostatic potentials of -3.00 to 3.00 .

3. RESULTS- Transcriptional regulation of the *rsaADE* genes

3.01- Identification of the *rsaADE* genes

The *rsaA* gene and its respective secretion apparatus were previously identified in our lab. The genes encoding the ABC transporter (*rsaD*) and the MFP (*rsaE*) were characterized by Dr. Peter Awram using Tn5 insertions. *rsaA* and the first two transporter components were found next to each other, but the two OMPs were located elsewhere. The promoter for *rsaA* was identified when the S-layer protein was initially characterized (61). *rsaA* contains a strong promoter and is often used in gene expression studies as a housekeeping gene(42) since *rsaA* is transcribed throughout the cell cycle with little to no change(24). A *rho*-independent terminator is found 40 bp 3' of the *rsaA* translational stop, which is located 162 bp 5' from the *rsaD* start codon. Based on studies defining the transcriptional regulation of the *E. coli* HlyA system(75), it was assumed that the promoter from *rsaA* read through the *rho*-independent terminator on occasion allowing for the transcription of *rsaD* and *rsaE*. Since no promoters were identified at the time, this hypothesis was assumed to be true.

3.02- Identification of a potential *rsaD* promoter

When I suspected that a promoter upstream of *rsaD* existed, I decided to use in-silico methods to elucidate possible promoter sites. The Softberry BPPROM program was used to predict if there were any possible promoter sequences. The nucleotide sequence from the stop codon of *rsaA* to the start codon of *rsaD* was used as a possible region of interest. When the 242 bp region was inserted into the BPPROM program it predicted a promoter ~140 bp 5' of *rsaD* (Fig. 3-1.). The putative -35 site (*rsaD*-35(1)) is located

from 147 to 141 bp upstream from the *rsaD* start codon. The putative -10 site is located 123 to 115 bp 5' of the *rsaD* start site. The ribosomal binding site was predicted to be in the region 11 to 7 bp 5' of the start codon. An alternate -35 site (*rsaD* -35(2)) was found closer to the -10 site after analysis of the intergenic space using a previously predicted consensus *C. crescentus* promoter sequence (41). The *rsaD* -35(1) site lies 19 bp away from the -10 site, whereas the *rsaD* -35(2) site is only 10 bp from the -10 site. Of the two predicted -35 sites, the latter -35 site makes the spacing of the predicted (-35, -10) binding sites similar to those of identified promoters in *C. crescentus*. As well, the *rsaD* -35(2) site has a better fit with the consensus -35 site as the TTG bases are highly conserved with the second T being conserved for all identified promoter sites (Fig. 3-2). Although the BPROM program boasts 80 % accuracy in the identification of promoters, these are based on the *E. coli* $\sigma 70$ and may not relate directly to *C. crescentus*. I therefore suggest that the architecture of the promoter region consists of the *rsaD* -35(2) site and the predicted -10 site. These results are significant as they predict a potential promoter site to support the later molecular based findings, described below.

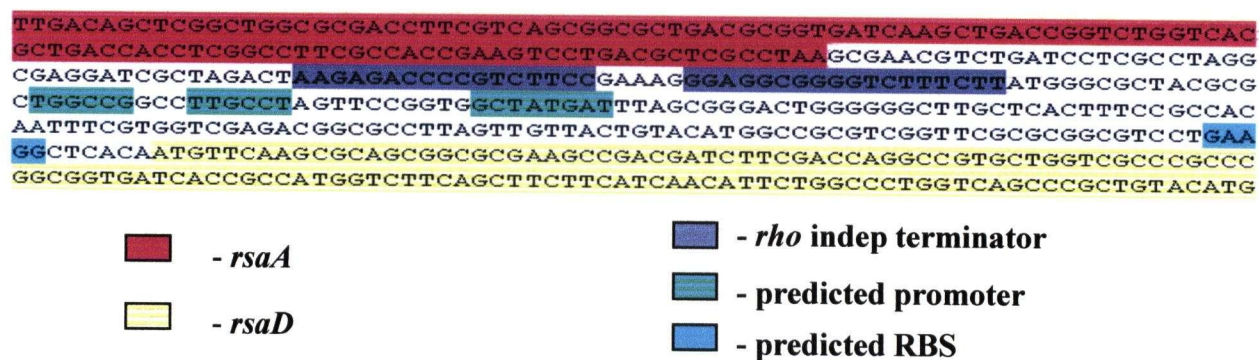


Figure 3-1. In-silico predicted *rsaD* promoter orientation.

Predicted *rsaD* promoter found using the Softberry BPROM program. Identified -10 and -35 sites are highlighted ~115 bp upstream of the *rsaD* gene. Two potential -35 sites are shown as both show similarity to consensus *C. crescentus* promoter sequences. The promoter site is located far enough away from the *rho*-independent terminator to allow for polymerase binding.

Consensus -35 – TTGACG

Consensus -10 – GCTANA_(A/T)C

rsaD -35(1) - TGGCCG

rsaD -10 - GCTATG A T

rsaD -35(2) - TTGCCT

Figure 3-2. Predicted *rsaD* promoter sites.

Predicted *rsaD* promoters as compared to predicted consensus promoter sequences. Both -35 sites appear similar to the consensus site, but the *rsaD* -35 (2) site appears a better fit due to the consensus TTG bases being identical.

In addition, the 135 bp region separating *rsaD* and *rsaE* was also analyzed to determine if there was a potential promoter. The BPROM program predicted that there were no promoters in the region, and no sites show significant similarity to predicted *C. crescentus* promoter sequences. Experiments detailed below further suggest that there is no promoter between the *rsaD* and *rsaE* genes.

3.03- Absence of *rsaE* promoter determined

As stated, *rsaE* is located directly after *rsaD* with only 135 bp separating the two genes. When studies were carried out by Dr. Peter Awram, there were no convenient tools to identify a possible promoter and thus the system was assumed to be analogous to HlyA. Creation of polyclonal antibodies to RsaD and RsaE has allowed for determination of any possible *rsaE* promoter. Using the previously created Tn5 *rsaD* knockout strain (B15) I was able to show that there was no promoter between the two genes. Whole-cell protein samples of the B15 (*rsaD*) and wild type NA1000 strains were run on SDS-PAGE gels, and western blots were probed with anti-RsaD antibodies to determine presence or absence of RsaD (**Fig. 3-3a.**). As expected, the NA1000 strain contained RsaD whereas no RsaD was present in the B15 strain. Note, that since the RsaD antibodies were

generated using protein from inclusion bodies, they resulted in high background levels. Therefore, further results show only anti-RsaE westerns, despite both westerns being carried out. Western blots using anti-RsaE antibodies were conducted, and the NA1000 strain showed the presence of RsaE while the B15 strain did not (**Fig. 3-3b.**). If there was a *rsaE* promoter located in the intergenic space between *rsaD* and *rsaE*, then presumably the promoter would still be active despite the Tn5 insertion. Thus since no *rsaE* product could be detected in the *rsaD* Tn5 mutant, the *rsaD* and *rsaE* genes must have been transcribed using the same promoter.

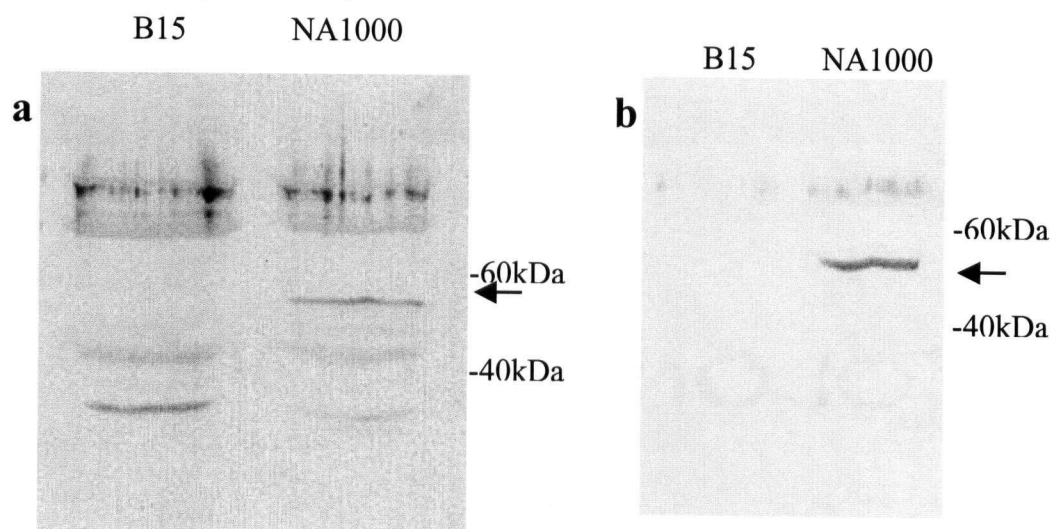


Figure 3.3 a-b. Characterization of CB15A B15.

a. Absence of RsaD in the CB15A B15 strain (*rsaD*⁻). Chemiluminescent western blot using anti-RsaD shows no RsaD (62.0 kDa) in the B15 strain but RsaD is present in NA1000.

b. Absence of RsaE in B15 strain is shown by chemiluminescent western blot. Using anti-RsaE no RsaE (48.4 kDa) is seen in the B15 strain (*rsaD*⁻) where as RsaE is present in the NA1000 strain.

3.04-Identification of the *rsaDE* promoter

To elucidate the transcriptional control of *rsaD* and *rsaE*, two separate molecular methods were utilized. The first involved the CB15 Δ -*rsaA* strain, created by Assaf Levi, which contained a deleted *rsaA* and *rsaA* promoter region. A region starting 242 bp 5' of the translational start site of *rsaA* and continuing approximately 1 kb further upstream was amplified, therefore excluding the *rsaA* promoter. Another fragment beginning a few

bp 5' of the *rsaA* translational stop codon and continuing ~1 kb 3' of *rsaA* was amplified. These two products were ligated together making a fragment that did not contain *rsaA* and its promoter (see materials and methods). The ligated product was then put into the pNPTS138 suicide vector and used for gene replacement. Loss of *rsaA* was confirmed by PCR screening for a deletion form product of *rsaA* (not shown) and the resulting strain was called CB15 Δ -*rsaA*. Western blotting using the anti 188/784 RsaA antibodies shows that no RsaA is produced by the CB15 Δ -*rsaA* strain (**Fig. 3-4 a.**).

The CB15 Δ -*rsaA* was then used to examine transcription of *rsaADE*. If transcription of *rsaD* and *rsaE* is dependent on the *rsaA* promoter, then transcription of *rsaD* and *rsaE* in the CB15 Δ -*rsaA* strain cannot occur using the *rsaA* promoter as it is deleted. On the other hand, if transcription of *rsaD* and *rsaE* is independent of the *rsaA* promoter, then another promoter must be present between *rsaA* and *rsaD*. Western blots were done to determine if RsaD and RsaE were present. Both the wild type NA1000 and the CB15 Δ -*rsaA* strain contain RsaD (not shown) as well as RsaE (**Fig. 3-4 b.**). In order to confirm that the transporter proteins are active, a plasmid borne copy of *rsaA* was complemented into the CB15 Δ -*rsaA* strain. If *rsaD* and *rsaE* are transcribed and the proteins are active, then addition of the *rsaA* gene should lead to S-layer secretion. Secretion of S-layer is evident in the complemented strain (**Fig. 3-4 c.**). Levels of S-layer secretion appear to be similar to wild-type levels, suggesting that production of RsaD and RsaE is not affected by the loss of the chromosomal copy of *rsaA* and its promoter.

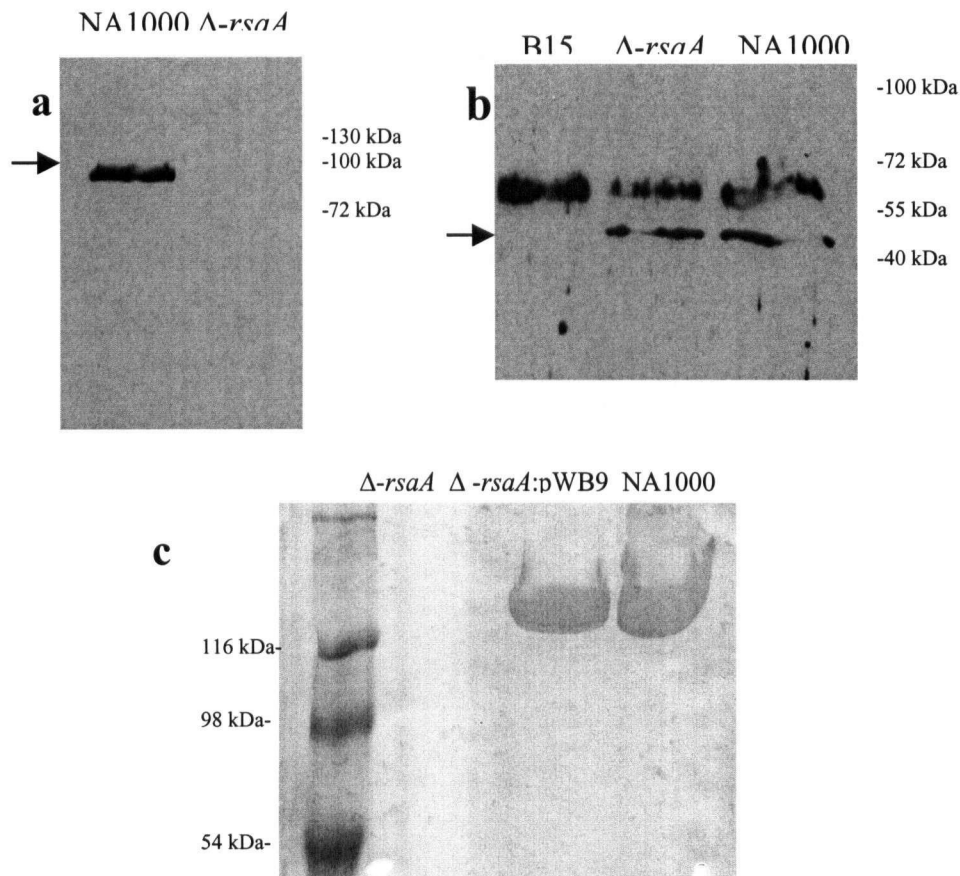


Figure 3-4 a-c. Characterization of the CB15 Δ -*rsaA* strain.

a. Anti-188/784 RsaA chemiluminescent western of whole culture preparation reveals that there is no RsaA in the CB15 Δ -*rsaA* strain (*rsaA*⁻), unlike wild type NA1000. RsaA marked by arrow.

b. Chemiluminescent western blot using Anti-RsaE shows presence of RsaE in NA1000 and CB15 Δ -*rsaA* strain (*rsaA*⁻) while the CB15A B15 strain (*rsaD*⁻) shows no RsaE. RsaE is marked with arrow.

c. Anti- 188/784 RsaA colorimetric western of complemented CB15 Δ -*rsaA* strain shows presence of RsaA. No RsaA is present in the CB15 Δ -*rsaA* strain (*rsaA*⁻) but wild type NA1000 RsaA levels are restored in the CB15 Δ -*rsaA*: pWB9: *rsaA* strain. RsaA marked by arrow.

To confirm that the putative *rsaDE* promoter was actually driving transcription of *rsaD* and *rsaE*, a second molecular method was undertaken. The B15 Tn5 clone shows no secretion of RsaA since it lacks a functional copy of *rsaD* (4). Confirmation that it lacks both RsaD and RsaE was shown above (**Fig. 3-3.**). A strategy using *trans*-complementation of a plasmid borne copy of *rsaADE* was adopted to determine transcriptional control. The RAT1 fragment(4), which includes *rsaADE* as well as flanking regions (total length ~ 11kb), was used. The RAT1 fragment was modified so that *rsaA* was knocked out, but *rsaD* and *rsaE* were not. An Ω Cm cassette was inserted into *rsaA* destroying the gene, and the possibility of read-through transcription using the *rsaA* promoter. Therefore, if complementation led to RsaA secretion then *rsaD* and *rsaE* must be transcribed using a separate promoter in the intergenic space between *rsaA* and *rsaD*. The modified RAT1 Ω Cm fragment was inserted into a broad host range plasmid and electroporated into the B15 strain. Western blots of low pH extracted protein showed that addition of the modified RAT1 Ω Cm fragment led to RsaA secretion (**Fig.3-5 a.**). This suggested that the plasmid borne *rsaD* and *rsaE* were transcribed and that the S-layer was secreted. To confirm that the plasmid borne *rsaD* and *rsaE* were transcribed, western blots were done revealing that both RsaD (not shown) and RsaE were present in the complemented strain (**Fig.3-5 b.**).

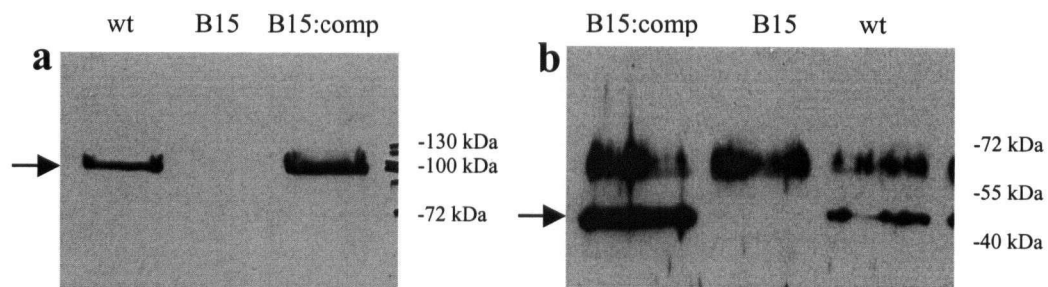


Figure 3-5 a-b. Characterization of the CB15A B15: RAT1ΩCm strain.

a. Anti-188/784 RsaA chemiluminescent western blot of the complemented B15: RAT1ΩCm (B15:comp) strain shows restoration of RsaA secretion (denoted by arrow). NA1000 (wt) shows RsaA while the CB15A B15 strain (*rsaD*⁻) does not. Complementation of the B15 strain with pBBR4: RAT1ΩCm leads to restoration of RsaA secretion suggesting *rsaD* and *rsaE* are transcribed.

b. Chemiluminescent western blot using anti- RsaE confirms presence of the *rsaE* gene product. B15: RAT1ΩCm (B15:comp) shows presence of RsaE (shown with arrow) while the B15 strain (*rsaD*⁻) does not. Levels of RsaE in the complemented strain appear higher than wild type NA1000 (wt) levels.

The combined results suggest that the *rsaA* promoter was not used for the transcription of the downstream transporter components *rsaD* and *rsaE*. Transcription must have occurred at a site just downstream of *rsaA* after the *rho*-independent terminator. Both *rsaD* and *rsaE* must be co-transcribed, as no separate promoter exists for *rsaE*.

4. RESULTS-Identification and characterization of the outer membrane proteins of the RsaA secretion system

4.01- Identification of the two *rsaF* genes

Two possible candidates for the OMP were identified in the *C. crescentus* genome (45) with similarity to the *E. coli* TolC protein sequence. These have been named *rsaFa* and *rsaFb*. RsaFa has 23% identity and 45% similarity to *E. coli* TolC, and RsaFb has 25% identity and 47% similarity to TolC as determined by local sequence alignment (see materials and methods). *rsaFa* (1581 bp) is located downstream of *rsaADE* after a gap of 5025 bp coding for five S-LPS-related genes(5). *rsaFb* (1452 bp) however, is located 322 Kb (303 genes) away from *rsaFa* and is flanked by genes of unknown function (**Fig. 4-1**). The two *rsaF* genes share 39% identity and 60% similarity which suggests that RsaFa and RsaFb may have arisen by gene duplication.

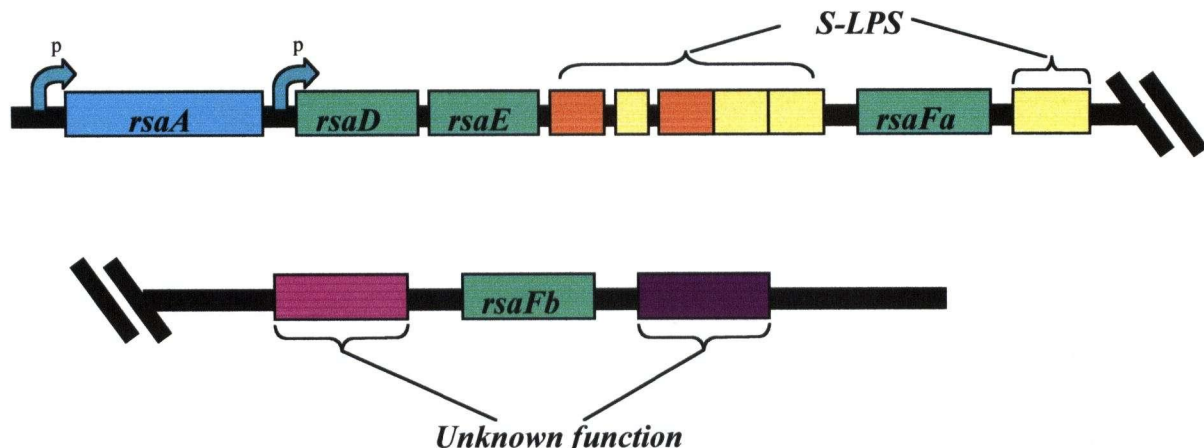


Figure 4-1. Relative location of the *rsaA* secretion apparatus.

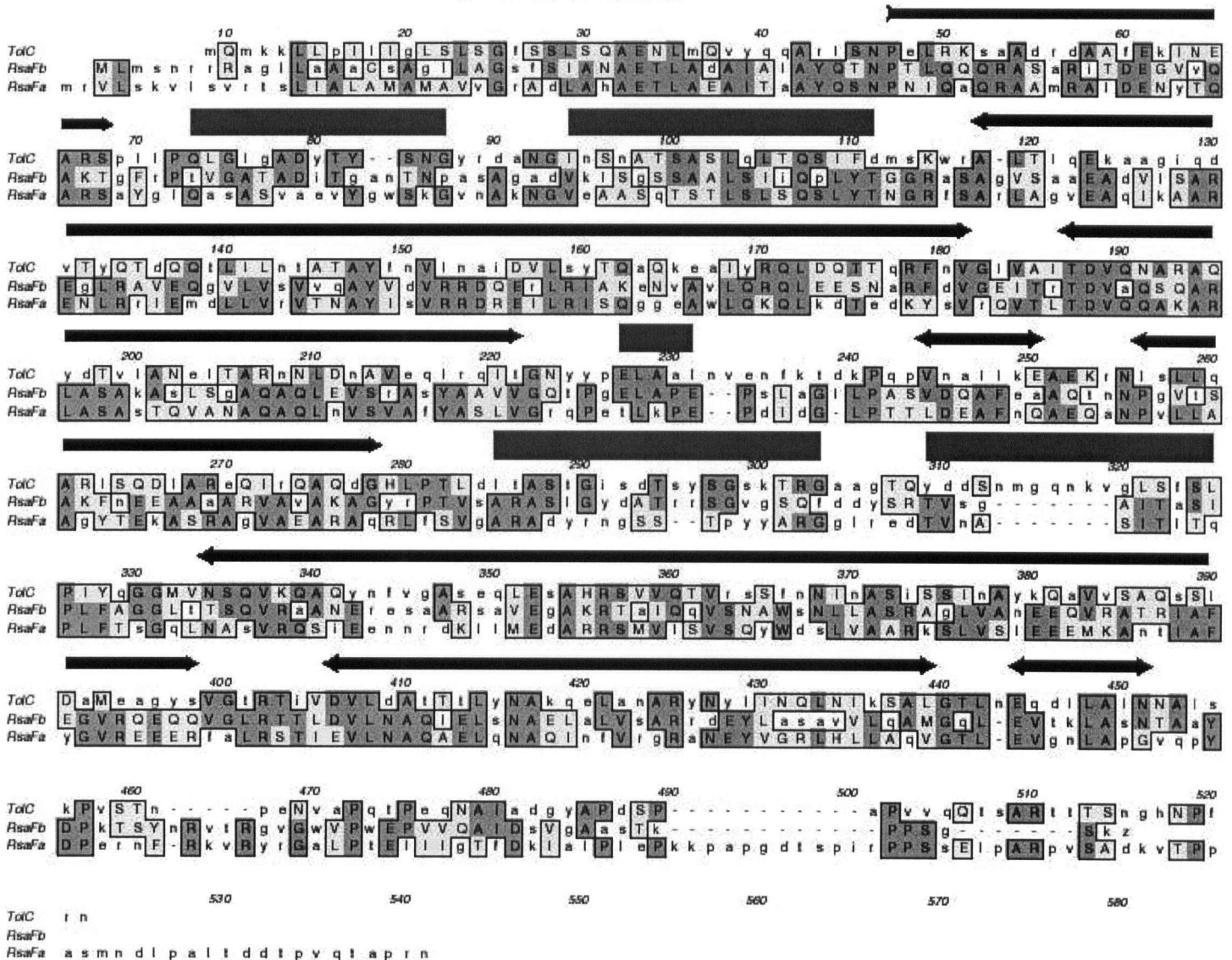
The *rsaD* and *rsaE* genes are located 3' of the *rsaA* gene. The *rsaFa* gene is downstream of 5-S-LPS genes. The *rsaFb* gene is located 322 kb away flanked by 2 genes of unknown function.

The genomic organization of these genes is different from that seen for other OMP components. In many systems all three components of the transporter system are found in sequential order and appear to be part of a single operon. Here are two separate

genes, both of which appear to be separately transcribed from the rest of the secretion components unlike that found with other OMP type I secretion components, except the *E. coli* TolC and *S. marcescens* HasF proteins (8).

The RsaF proteins were aligned with the TolC sequence using MacVector 6.0 ClustalW, and predicted α -helix and β -sheet segments were identified (**Fig. 4-2.**). The RsaFs have a similar predicted secondary structure to TolC and regions of similarity are clearly seen. To predict the tertiary protein structure, the two RsaF proteins were modeled using the solved x-ray structure of the *E. coli* TolC. Electrostatic surface models show differences between RsaFa and RsaFb as well as significant differences compared to the TolC monomer (**Fig. 4-3.**). TolC has a predicted negative charge throughout the α -helical and β -barrel segments. The RsaF models have neutral charges throughout their inner surface, with zones of positive and negative charge exposed at various regions. Both RsaFa and RsaFb exhibit a negative charge in the entrance to the α -helical periplasmic spanning portion, and a gradual progression to positive charges where the monomer shifts from α -helical to β -barrel segments. RsaFa appears to have more positively charged areas than RsaFb, but it does have zones of negative charge, facing the internal chamber of the α -helical segment, caused by clusters of Glu residues (e.g. amino acids 338-340). Both RsaF proteins have regions of positive charge at the entrance and spanning the β -barrel region of the protein.

ClustalW Formatted Alignments



α-helical regions

β-sheet regions

Figure 4-2. ClustalW alignment of the *RsaFa*, *RsaFb* and *TolC* proteins created using the MacVector 6.0 program.

ClustalW alignment shows similarity between both *RsaFa* and *RsaFb* as well as *TolC*. Inserted arrow and rectangles show regions predicted to form α-helical ribbons and β-sheets. Shown are predicted consensus regions of the three proteins and are not specific to a single protein. Areas of divergence are seen mostly in areas where loops would occur. The *RsaF* proteins likely form a similar 3D structure as *TolC* since the predicted secondary structure of the proteins appear similar. The alignment denotes identical and similar amino acids with boxes. Identical amino acids are shaded and similar amino acids are in bold type.

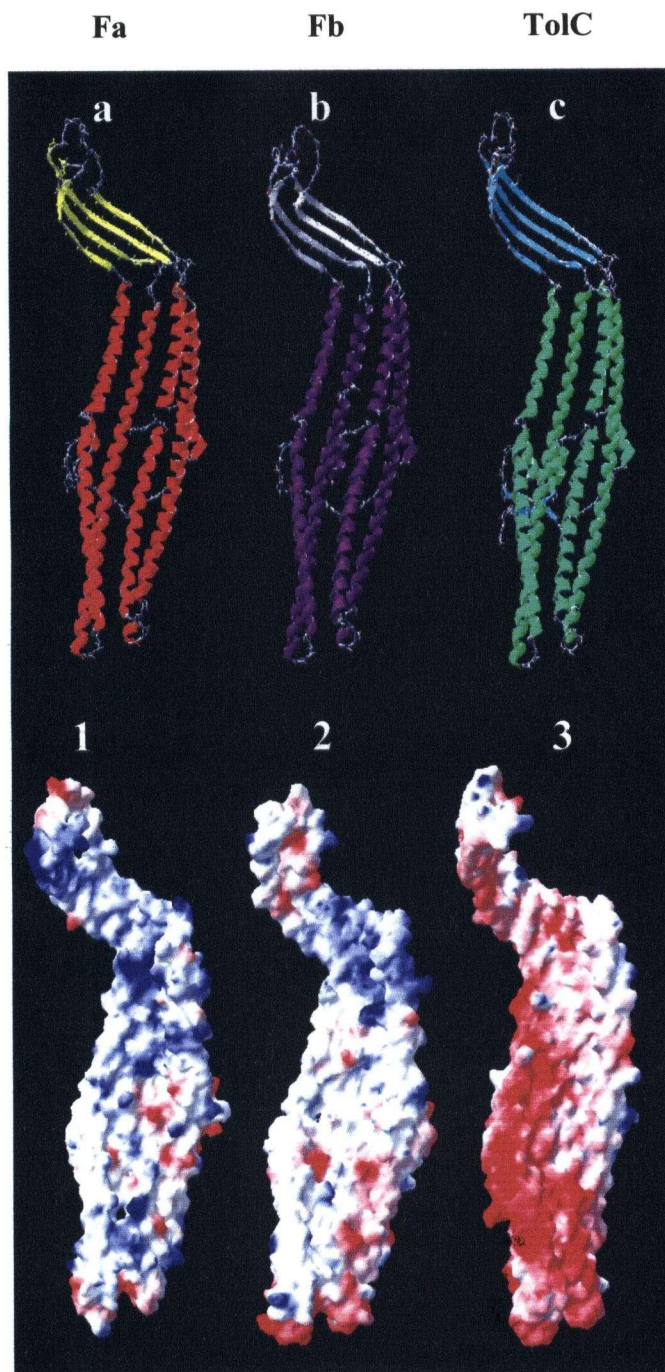


Figure 4-3. Predicted 3D-ribbon and space-fill models of RsaFa and RsaFb as well as the TolC monomer.

Threaded 3D-ribbon structures of RsaFa (a) and RsaFb (b) as well as the TolC (c) monomer as shown by Deep View/Swiss-Pdb-Viewer v3.7. Ribbon structures were predicted by the Swiss Model program and then modified with colored sections to emphasize separation of the α -helical and β -barrel regions.

Electrostatic surfaces are shown on space-fill models of the monomers. Electrostatic potentials of -3.00 to 3.00 were used to show areas of negative (red) and positive (blue) charge. RsaFa (1) and RsaFb (2) show more neutral and positively charged zones than the TolC (3) monomer. Negatively charged zones appear at the periplasmic entrance of all three proteins. Regions of positive charge appear in both RsaF proteins at the entrance to the β -barrel. As well a cluster of Glu residues (338-340) forms a zone of negative charge in the α -helical channel of RsaFa.

Identification of possible OMPs was done by searching for homologs of TolC in *C. crescentus* using the BLASTp tool on TIGR. RsaFa and RsaFb show significant similarity to the α -Hemolysin TolC protein, whereas the other protein hits appear more related to other non-type I OMPs. Initial BLAST analysis of the CB15 genome showed 4 hits. Two (RsaFa and RsaFb) had significant scores and low E values (1.1×10^{-22} (RsaFb) and 1.3×10^{-16} (RsaFa)) while the other hits (CC0806 and CC1785) had moderate scores and E values. CC0806 and CC1785 are likely not type I OMPs as a search against a non-redundant database indicated that CC0806 and CC1785 share higher percent identity with lipoproteins (approx. 40-48%) and TIGRFAMs and Pfams group them in the lipoprotein family.

ClustalW alignment of the RsaF proteins and TolC revealed significant similarity between the three proteins. The high similarity of RsaFa and RsaFb suggests that they may be orthologues formed through gene duplication. When predicted secondary regions of the RsaF proteins are compared with TolC, the predicted α -helical regions appear in stretches of similar amino acids, whereas regions where the sequences show less similarity are in predicted loop and β -sheet regions (Fig. 4-2.).

If RsaFa and RsaFb were actually crystallized, their 3-D structure may not be identical to the predicted Swiss Model files. However, the predicted structures do give greater insight into the OMPs possible form and function. Predicted charged regions are of interest as heterologous proteins expressed using the *C. crescentus* secretion system may be blocked from transport due to charge (47). It is likely that transported proteins carrying a charge would interact with either the negatively charged regions predicted to face the internal cavity of the α -helical region, or with positively charged regions in the

β -barrel region (**Fig. 4-4.**). Regions of charge may function as a ‘filtration device’ blocking the transport of improperly formed or folded RsaA monomers before they reach the bacterial surface. *C. crescentus* has been shown to possess a protease (*sapA*) (69) that scans the S-layer cleaving improperly made RsaA. Due to high levels of C-terminally secreted protein being produced the possible filtration function of RsaF may be an additional level of control in order to inhibit the passage of malformed RsaA.

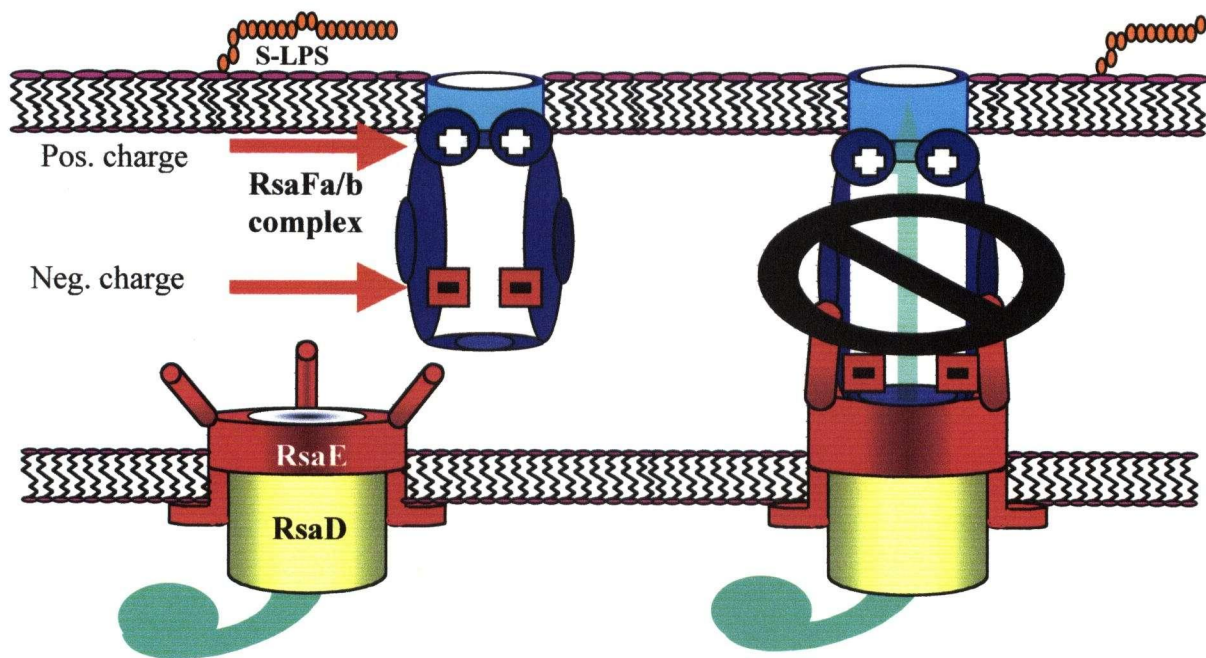


Figure 4-4. Cartoon depiction of charged regions that may block RsaA transport

Negatively charged regions in the α -helical region such as the stretches of Glu residues may block transport. Alternatively, positively charged regions located throughout the β -barrel region may also hinder RsaA secretion. Charged zones may act as a filter for improperly made or folded RsaA monomers. When improperly formed RsaA monomers interact with charged regions in the outer membrane protein, secretion is blocked and the RsaA monomer is pushed back into the cytoplasm.

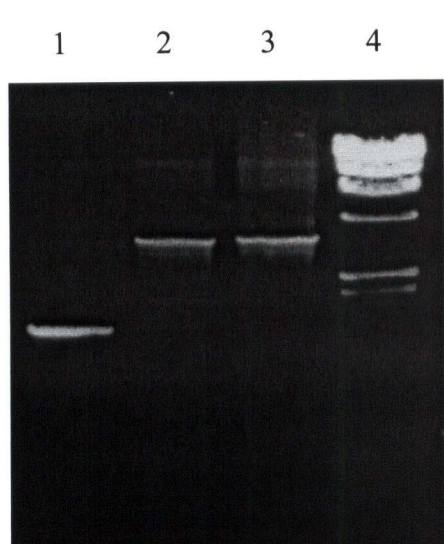
4.02- Internal deletions of the *rsaF* genes

Initially I intended to knockout *rsaFa* and *rsaFb* by gene replacement using internally deleted forms of the *rsaFs*. A 2.7 kb fragment, containing *rsaFa* and flanking regions of ~1000 bp 5' and ~200 bp 3' was amplified by PCR. Internal deletions of ~1.3 kb, 852 bp, and 638 bp were created in *rsaFa* (outlined in materials and methods). Attempts to insert an internal deletion form by gene replacement using a suicide vector (pK18mobsacB) consistently resulted in reversion to the wild type *rsaFa*. Gene replacements of altered forms of *rsaFa* were attempted in both the NA1000 (S-layer⁺) and CB2A (S-layer) strains. From the many hundreds of clones screened by PCR, only the *rsaFa* Δ KP (852 bp) internal deletion resulted in a correct gene replacement event in the CB2A strain. This gene replacement was confirmed by PCR (data not shown) and the strain was called CB2A *rsaFa* Δ KP.

rsaFb was also targeted for gene swapping using internal deletions. An internal deletion in *rsaFb* of 1051 bp was made. All attempts to get an internal deletion form to recombine properly failed. Since antibiotic insertion and N- and C-terminal methods of destroying the *rsaF* genes had worked, efforts to create internal deletions were stopped.

4.03- Disruption of the *rsaFa* and *rsaFb* genes

Since the internal deletion strategy was unsuccessful, knockouts were constructed by homologous recombination of inactivated *rsaF* genes in the wild type S-layer positive *C. crescentus* strain NA1000 using alternate methods. Disruption of the *rsaFa* gene was



performed by gene replacement of an *rsaFa* gene construct containing an antibiotic-resistance cassette. The *rsaFa*ΔSm gene fragment was inserted into the chromosome using a suicide vector and a mutant resulting from the gene replacement event was identified by PCR (Fig. 4-5.) and has been designated JS1007.

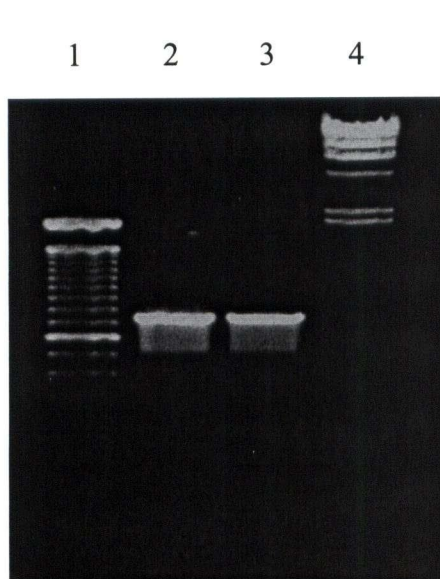
Figure 4-5. PCR confirmation of *rsaFa* knockout.

Antibiotic insertion form of *rsaFa* shown in at 3.5Kb in the JS1007 strain. Lane1- NA1000, Lane 2- JS1007, Lane 3- pBSKIIEEH: *rsaFa*ΔSm, Lane 4- λ-*Hind*III marker (sizes from top to bottom 22kb, 12kb, 6.6kb, 4.4kb, 2.4kb, 2.2kb).

The *rsaFb* gene was disrupted via insertional inactivation, using an N- and C-terminally deleted *rsaFb* gene fragment (*rsaFb*ΔNΔC). The *rsaFb*ΔNΔC gene fragment was inserted into the chromosome via homologous recombination of a non-replicating plasmid, resulting in tandem non-functional copies of the *rsaFb* gene. Confirmation by PCR showed that there were only disrupted forms of *rsaFb* in the chromosome (Fig. 4-6.). Using one primer from the plasmid and one primer from the gene I was able to confirm insertion, after which a mutant was selected and called JS1008. In order to create the double-knockout strain, the *rsaFa* gene was disrupted in the JS1008 strain in the same

manner as in the JS1007 strain. This double *rsaF* knockout strain was confirmed by PCR (not shown) for disruption of both *rsaF* genes and designated JS1009.

To determine that RsaFa and RsaFb were not produced in the knockout strains, western blot analysis was done using polyclonal anti-RsaFa antibodies. Rabbit polyclonal antibodies were generated against a GST tagged *rsaFa* gene product. The proteins can be



differentiated by size; RsaFa is 57.5 kDa and RsaFb is 50.2 kDa. Cross-reactivity between the two proteins was evident probably due to the similarity of the RsaFs. For this reason, anti-RsaFb antibodies were not produced. Curiously, the anti-RsaFa antibodies reacted better with the RsaFb protein. Whether this is due to increased binding efficiency, or relative levels of the RsaF proteins, is unknown.

Figure 4-6. PCR confirmation of *rsaFb* knockout.

N-term C-term deletion form of *rsaFb* shown in at ~600 bp in the JS1009 and JS1008 strain. Lane1- 100bp ladder, Lane 2- JS1009, Lane 3- JS1008, Lane 4- λ -HindIII marker (sizes from top to bottom 22kb, 12kb, 6.6kb, 4.4kb, 2.4kb, 2.2kb).

The cross reactivity of the polyclonal antibodies allowed us to see the loss of the RsaF proteins in the knockout strains (**Fig. 4-7.**). Progressive loss of RsaFa and RsaFb was evident in western blots of the *rsaF* knockouts. Densitometry analysis of the western blots showed that levels of the remaining RsaF in the single *rsaF* knockouts are the same as those observed in the wild-type strains. This suggests that both *rsaFa* and *rsaFb* are similar to the *E. coli tolC* and *S. marcescens hasF*, and are transcribed separately from an export/ secretion system.

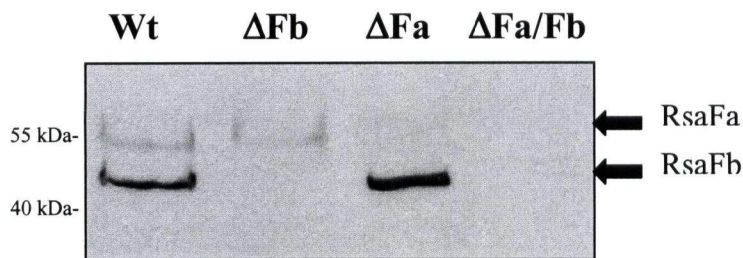


Figure 4-7. *RsaFa* and *RsaFb* levels in wild type and *rsaF* knockout strains.

Loss of RsaF proteins was determined using whole-culture-protein samples of the wild type and knockout strains. Developed film of chemiluminescent anti-

RsaFa western blot showed loss of the RsaF proteins. The amount of protein loaded per lane corresponded to 20μl of whole-culture preparation and samples were run on 12% SDS-PAGE gel and transferred to nitrocellulose membrane. Wild type NA1000, independent *rsaFa* and *rsaFb* knockouts and double *rsaF* knockouts were compared. Both RsaFa (~57kDa) and RsaFb (~50kDa) can be seen in the wild type NA1000 (wt). Loss of RsaFb was evident in the JS1008 strain (*rsaFb*⁻) (ΔFb) as only RsaFa was seen. Similarly, only RsaFb was seen in the JS1007 (*rsaFa*⁻) strain (ΔFa). The RsaF double knockout JS1009 (*rsaFa/rsaFb*⁻) (ΔFa/Fb) showed neither RsaFa nor RsaFb.

4.04- Effect of disruption of the *rsaF* genes on S-layer secretion

Independent gene knockouts were created and levels of RsaA secreted by the *rsaF* knockout strains were analyzed. Neither single knockout led to a complete loss of S-layer secretion, but levels of RsaA secretion did decrease in the two single knockouts. Because S-layer secretion was not completely abolished, a mutant was created with both *rsaFa* and *rsaFb* knocked out, resulting in what appeared to be an S-layer negative strain. When low pH extracted protein levels were compared between the knockout and wild type strains, there was a progressive decrease in levels of S-layer secretion as *rsaFa* and *rsaFb* were lost (**Fig. 4-8a and b.**). Disrupting *rsaFa* decreased S-layer secretion to a greater extent than loss of *rsaFb*, but both single *rsaF* mutants were still capable of secreting RsaA. Levels of S-layer secretion could not be easily determined through coomassie stained SDS-PAGE, as small amounts of S-layer secretion are not easily seen, and therefore western blotting was performed.

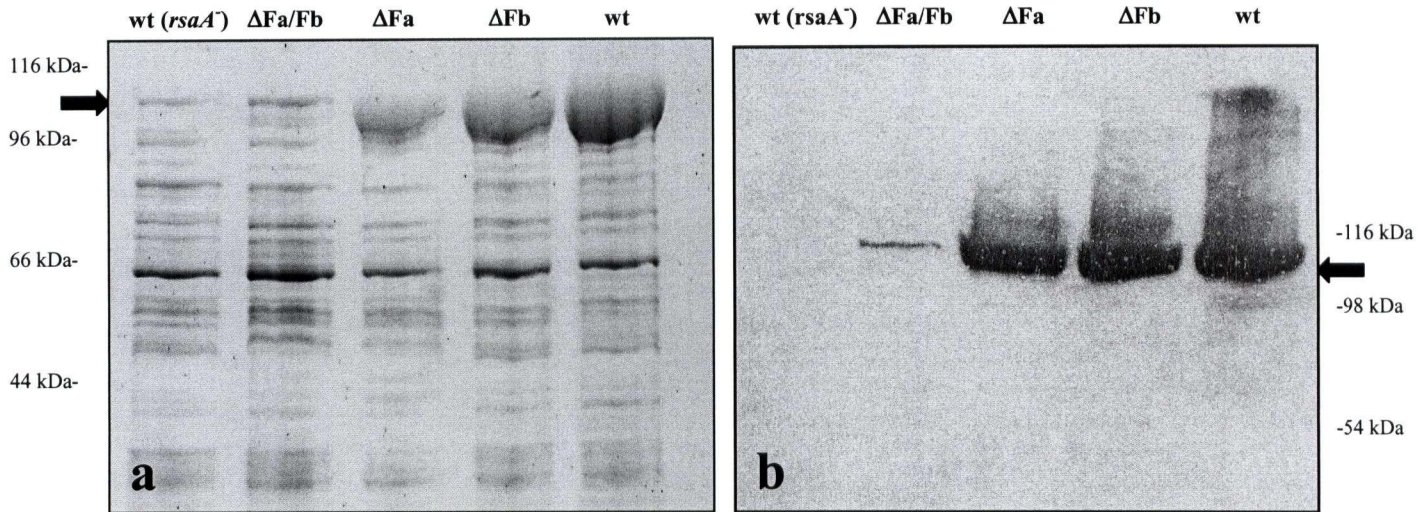


Figure 4-8. a- b. Effect of disruption of the *rsaF* genes on S-layer secretion.

Levels of RsaA as determined by low pH extraction on wild type and *rsaF* knockouts. Normalized levels of cells were used for preparations and were run on a 7.5% SDS-PAGE gel. Coomassie stained SDS PAGE (a) and chemiluminescence western blot (b) using anti-188/784 shows the effect of *rsaF* gene disruption on levels of RsaA. S-layer protein (98 kDa) can be clearly seen due to its high level of production, marked by arrows. No detectable S-layer is observed in the S-layer negative JS1003 strain (wt-*rsaA*⁻). Minor levels of S-layer can be seen in JS1009 (*rsaFa*⁻/*rsaFb*⁻) caused by burst cells during low pH extraction, whereas definite levels of S-layer can be seen in the JS1007 (*rsaFa*⁻) and JS1008 (*rsaFb*⁻) strains. Levels in both single *rsaF* knockouts do not secrete as much S-layer protein as that seen in the wild type NA1000 strain.

Quantification of S-layer secretion levels by chemiluminescence western blotting showed that disruption of *rsaFb* decreases RsaA secretion by 24% whereas loss of *rsaFa* decreases S-layer secretion by 46% from those of wild type NA1000 levels (**Table 2.**). This suggested that RsaFa is more important to S-layer secretion as loss of *rsaFa* led to a more significant decrease in RsaA secretion. Since Coomassie stained gels were not able to show small amounts of S-layer protein, western blotting was able to reveal a small amount of RsaA in the JS1009 (*rsaFa*⁻/*rsaFb*⁻) strain. S-layer protein (4% of wild type) seen in the double *rsaF* knockout strain appeared to be due to release of RsaA from cells burst during the low pH extraction and further experiments were carried out to determine if the levels of S-layer were internal RsaA.

Strain	%RsaA to wild type	
	<i>Low pH extracted</i>	<i>Whole culture</i>
NA1000	100%	100%
JS1008	76%	78%
JS1007	54%	56%
JS1009	4%	9%
JS1003	0%	0%
JS1009: <i>rsaFa</i>	78%	80%
JS1009: <i>rsaFb</i>	56%	57%
JS1001	-na-	95%

Table 2. Comparison of RsaA levels as determined by whole culture preparations or low pH extraction.

Spot densitometry of chemiluminescence western blots using polyclonal anti-188/784 were used to determine relative levels of RsaA produced by cells. Levels determined by densitometry were compared to wild type NA1000 levels. All low pH and whole culture protein preparations were normalized prior to running samples.

To confirm that levels of S-layer secretion in the double *rsaF* knockout were due to burst cells during low pH extraction, whole-cell preparations were performed to determine levels of S-layer inside the cells. To make sure that only internal RsaA was analyzed, certain strains were subjected to other protein extraction methods (outlined in materials and methods) before whole-cell-protein preparations were done. Colorimetric western blots showed levels of internal RsaA (Fig. 4-9.). Levels of RsaA produced in the double *rsaF* knockout were higher than those seen by low pH extraction, however the levels did appear very similar to levels seen in the filtered JS1001 strain. Both the JS1009 and the JS1001 internal RsaA levels were less than those seen in the NA1000 strain. The levels of RsaA in the JS1001 strain were only internal levels of RsaA, as all transported protein was shed (S-LPS⁻) and removed (filtration and washing) suggesting that RsaA seen in the double *rsaF* knockout was also held internally.

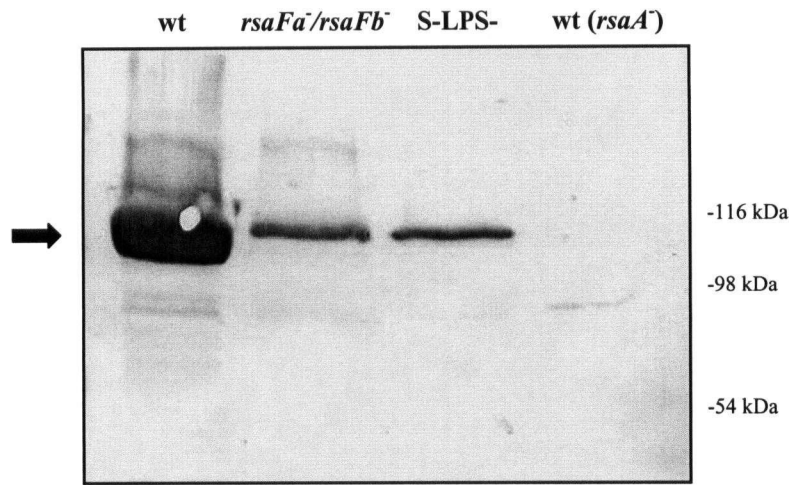


Figure 4-9. Determination of internal levels of RsaA in the *rsaF* double knockout.

Whole cell preparations of wild type, JS1001, JS1003 and JS1009 strains were run on 7.5% SDS-PAGE gel and transferred to nitrocellulose membrane for colorimetric western blot. NA1000 was subjected to low pH extraction prior to whole cell preparation, removing RsaA that was crystallized on the cell surface, to ensure that only internal RsaA levels would be observed. Similarly, JS1001 strain was poured through fine mesh filter to remove aggregated RsaA before whole cell preparation. Equal amounts of whole cell protein preparations were loaded onto the gel polyclonal anti 188/784 RsaA antibody was used for western blotting. The Arrow indicates full length RsaA. Wild type NA1000 levels (wt) appear much greater than those seen in both the knockout JS1009 (*rsaFa*⁻/*rsaFb*⁻) and JS1001 (S-LPS⁻) strains. The JS1003 strain (wt (*rsaA*⁻)) shows no visible S-layer protein. The JS1001 strain has no S-LPS, and the shed aggregates were strained off, levels of RsaA observed should be totally internal. Therefore, since the level of RsaA in the JS1009 strain is similar to the JS1001 strain it would suggest that RsaA observed is internal.

4.05- Complementation of the secretion deficient JS1009 strain

To further demonstrate that the *rsaF* knockouts were responsible for reduction or loss of the S-layer secretion, I complemented the *rsaF* knockouts in *trans* using a multiple copy broad host range plasmid. In both cases, complementation restored partial secretion (**Fig. 4-10.**). Interestingly, *trans*-complementation of the *rsaF* genes only restored S-layer secretion to levels similar to that seen in the single *rsaF* knockouts (**Table 2**). Levels of RsaA secretion in the JS1009: *rsaFa* and JS1009: *rsaFb* strains were restored to levels ~2% greater than those seen in the single *rsaF* knockouts. Due to the lack of additional antibiotic markers, complementation of both *rsaF* genes into the double knockout was not carried out.

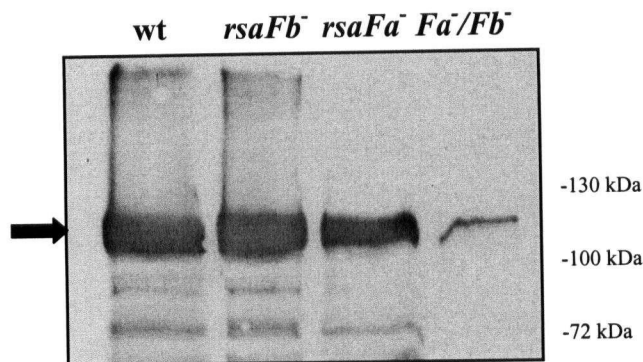


Figure 4-10. Complementation of the *rsaF* genes in trans recovers S-layer secretion.

JS1009 strain was complemented with either *rsaFa* or *rsaFb* using the medium copy pBBR4 vector. Levels of RsaA were determined by low pH extraction and run on 7.5% SDS-PAGE gel. Chemiluminescent western blotting using anti-188/784 RsaA antibodies allowed for quantification of RsaA. RsaA secretion is recovered to similar levels as those seen in the single *rsaF* knockouts with the trans-complemented JS1009 strains having fractionally higher levels of RsaA secretion. The *rsaFa* complemented strain (lane 2) has 78% of wild type levels, and the *rsaFb* complemented strain (lane 3) at 56% of wild type levels. The JS1009 strain (lane 4) was run to show effect of complementation. The trans complementation of the *rsaF* genes does suggest that the *rsaF* gene products are involved in RsaA transport.

Strains were then assessed to directly determine the extent to which the plasmid borne copies of *rsaF* were expressed. Culture preparations were done, and using polyclonal antibodies against RsaFa, expression of both plasmid borne *rsaF* genes was determined by chemiluminescence western blotting (**Fig. 4-11.**). Expression of RsaFa was 9.72 ± 0.71 times greater, and RsaFb 8.01 ± 0.66 times greater, than wild type levels. Independent knockouts suggested that there is a greater requirement for RsaFa, as loss of the protein lowers RsaA secretion by 46% whereas loss of RsaFb only leads to a 24% decrease (**Fig. 4-8.**). For this reason one would expect that if RsaFa is more capable of handling secretion of the S-layer protein, then increasing its levels in the bacterium should lead to greater RsaA secretion. However, despite significantly increasing RsaFa levels, when RsaFb is not present, recovery of wild type levels of RsaA secretion cannot be achieved. This suggested that although RsaFa appears to be more important, RsaFb is essential to achieving wild-type levels of RsaA secretion.

If the RsaF proteins formed independent homotrimeric units, then a large increase in either protein should lead to recovery of wild type S-layer secretion levels in the

JS1009 strain. Since neither complemented strains produced levels significantly above those seen in the single *rsaF* knockouts, this suggests that instead of forming independent homotrimeric units, the two OMPs might form a heterotrimeric unit that is required for maximal secretion (**Fig. 4-12.**).

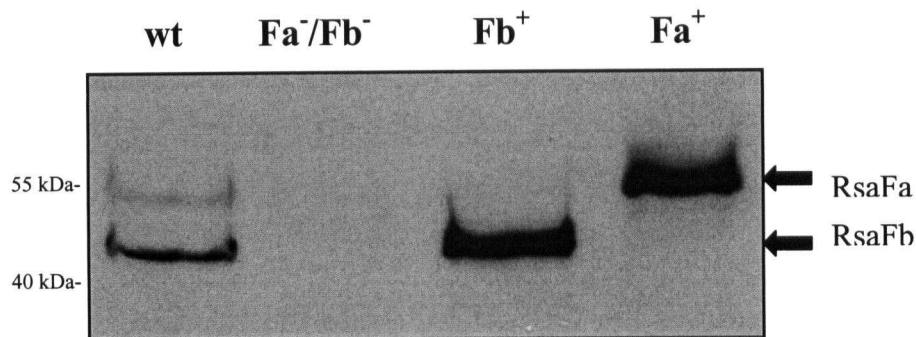


Figure 4-11. Expression of the RsaF proteins in the complemented JS1009 strain.

rsaFa and *rsaFb* were transformed into the JS1009 strain using the pBBR4 plasmid and whole-culture preparations were run on 12% SDS-PAGE gel. Chemiluminescence western blot using the anti-RsaFa antibodies showed recovery of RsaFa and RsaFb in the respective complemented JS1009 strain. The wild type NA1000 strain (wt) showed presence of both RsaFa and RsaFb. JS1009 (*rsaFa*⁻/*rsaFb*⁻) had neither RsaF protein. RsaFb was present in the JS1009: *rsaFb* complement strain (*rsaFb*⁺) and similarly RsaFa is present in the JS1009: *rsaFa* strain (*rsaFa*⁺). Western blotting revealed that not only were the plasmid borne *rsaF* genes transcribed, but that levels of RsaFa were 9.72 ± 0.72 times greater than wild type, and RsaFb was 8.01 ± 0.66 fold more than wild type levels as determined through densitometry.

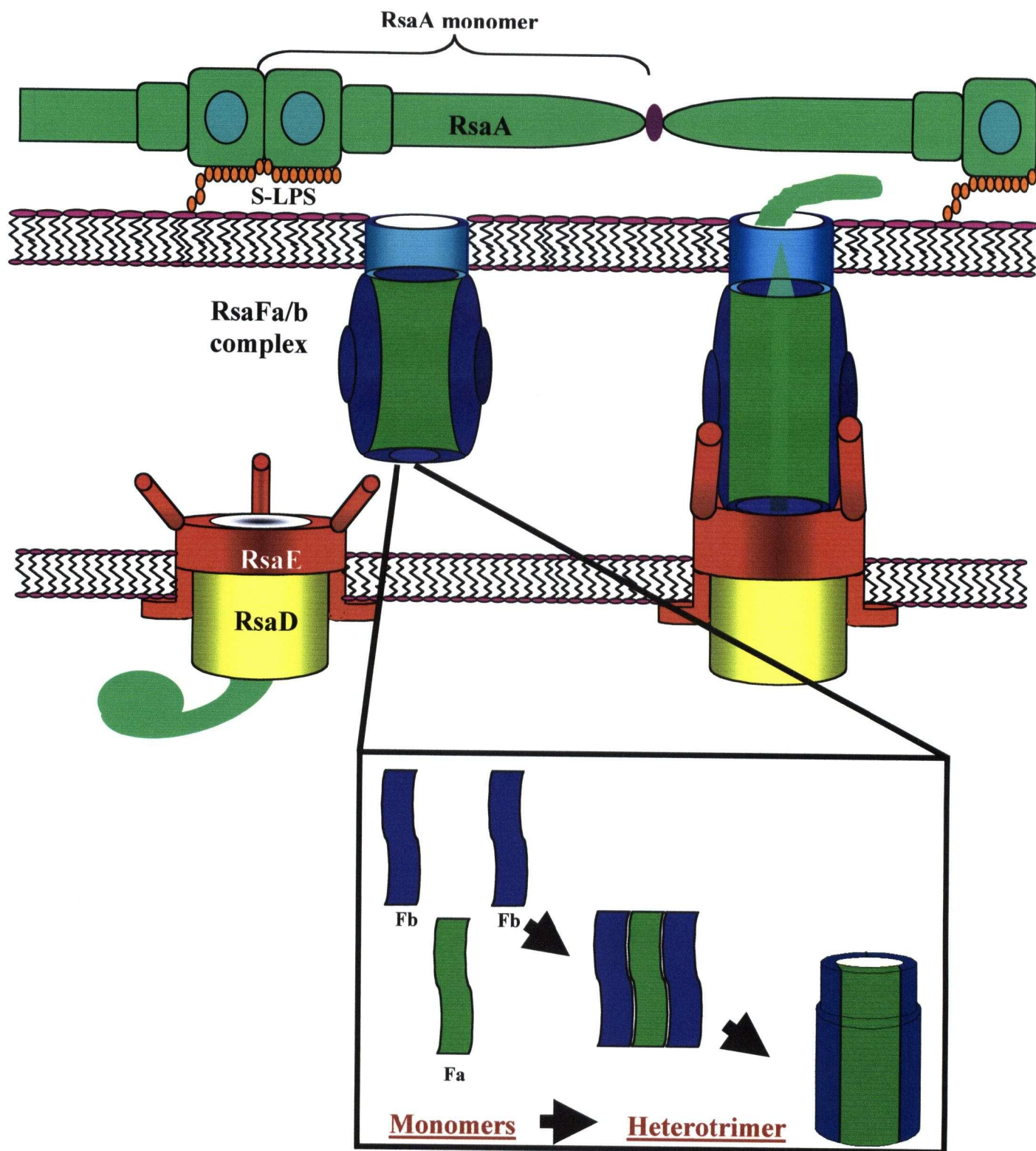


Figure 4-12. Cartoon depiction of heterotrimer formation.

A combination of three RsaF monomers would come together to form one heterotrimeric unit which would associate with the ABC transporter and MFP. The heterotrimeric unit would be some combination of the two RsaF proteins (may not be the combination depicted) with one being the more frequently utilized protein.

4.06- Production of RsaA appears to be regulated when secretion is impeded

Since the type I apparatus is efficient I sought to determine if levels of RsaA secretion and RsaA production were similar. I determined that the production of RsaA appeared to be regulated by the amount that could be transported out of the cell. Densitometry performed through chemiluminescence western blotting revealed levels of RsaA from whole-culture-protein preparations were almost identical to those observed through low pH extraction (**Fig. 4-13.**). Western blots showed the effect of knocking out *rsaFb* reduces S-layer production to 78% of wild type levels, and destruction of *rsaFa* reduces RsaA production to 56% of wild type levels (**Table 2**). Levels of RsaA seen with the double knockout strain were somewhat higher than those seen by low pH extraction at 9% of wild type NA1000 levels. The levels of RsaA found by whole-culture preparation were much more representative of total RsaA produced in the cell than those obtained through low pH extraction.

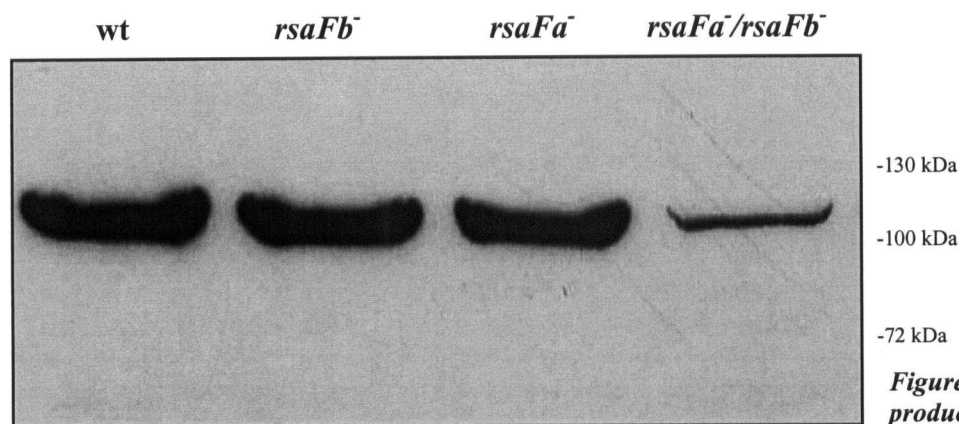


Figure 4-13. RsaA production and RsaA

secretion levels are comparable suggesting that little residual S-layer is left inside of the cell.

Chemiluminescent western blot analysis of whole-culture preparations using anti 188/784 RsaA antibodies allowed comparison of RsaA levels in the *rsaF* knockouts. Progressive decrease in RsaA levels was seen as the *rsaF* genes were knocked out. NA1000 (wt) RsaA levels were not seen in the JS1008 (*rsaFb*⁻) or JS1007 (*rsaFa*⁻) strains. Comparable levels of S-layer were seen in whole-culture preparations, to those observed by low pH extraction. The JS1009 (*rsaFa*⁻/*rsaFb*⁻) strain shows levels of internal RsaA at 9% of NA100 levels. Whole-culture preparations may be the best estimate for RsaA production levels, since internal and external levels of RsaA can be analyzed in the same preparation.

Whole-culture and low pH extracted levels of RsaA were only fractionally different, varying approximately 2%. The ability to readily compare different methods of protein extraction is not only valuable from an experimental perspective, but it also shows there is almost no level of RsaA built up inside of the cell, and that the levels seen on the surface of the cell are representative of the level of total RsaA produced.

These results suggest that the level of RsaA produced in the cell may be regulated by the amount of protein that can be secreted, as there was a definite down-regulation of RsaA production in the RsaF mutants. I decided to pursue possible RsaA regulation by using the JS1009 strain and a strain which had an inserted charged a.a. region in RsaA. Heterologous proteins with charged regions have been found to have problems being transported through the RsaA secretion apparatus, as they likely interact with charged residues in the outer membrane component (47). A plasmid borne copy of *rsaA* was modified by insertion of nucleotides coding a 4 amino acid RKKR furin cleavage site at the 723 amino acid site and inserted into the JS1003 S-layer negative strain. Protein levels of the transporter mutant and the modified RsaA mutant were analyzed. Levels of RsaA produced by the JS1003: *Hps12furin* strain are similar to that of the RsaF double knockout strain (**Fig. 4-14.**). Internal levels of RsaA were 5% in the modified RsaA strain, whereas levels in the RsaF double knockout strain were 9% of wild type levels. If RsaA is expressed constitutively then one might expect that internal RsaA would be degraded by internal proteases. Cells which can not transport the S-layer protein, would then either degrade the accumulated RsaA or down-regulate its production. However, no breakdown products of RsaA were present in the bacteria and so I presumed the latter option is the case.

The JS1009 (*rsaFa*/*rsaFb*) and JS1003: *Hps12furin* strains grew much poorer than wild type strains suggesting that accumulation of RsaA inside the cell leads to some degree of metabolic imbalance (**Table 3.**). In addition to poor growth, there were very few motile cells and most cells were misshapen and elongated. Cultures grew at normal rates until exponential phase, and then cell growth rate slowed (**Fig. 4-15 a-b.**). When *rsaFa* is inserted into the JS1009 strain, growth rates increase towards wild type levels. These results would suggest that poor growth is due to accumulation of RsaA inside of the cell. Taken together, these results suggest that there may be a ‘feedback’ loop for RsaA production, and that buildup of RsaA inside the cell leads to stoppage of *rsaA* transcription or translation.

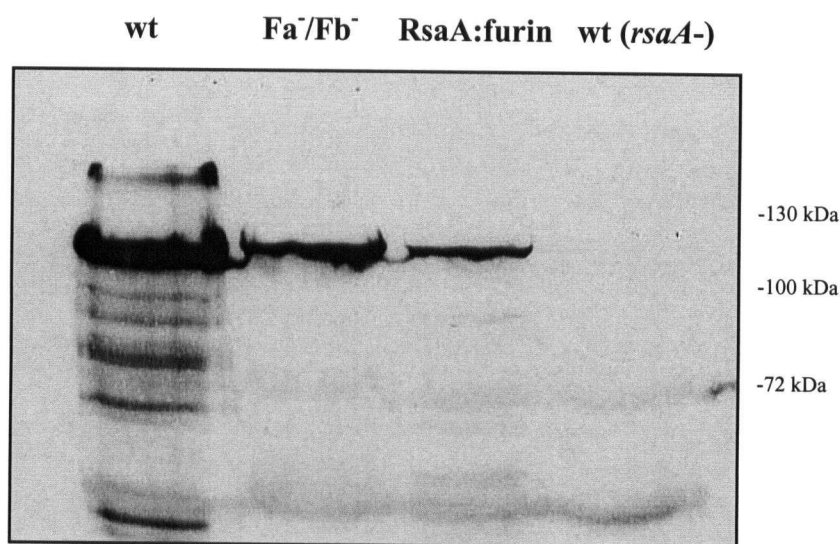


Figure 4-14. Impeded RsaA transport in *rsaF*⁻ (JS1009) mutant and RsaA (*Hps12furin*) mutant. Whole-cell preparations were loaded on 12% SDS-PAGE gels and compared by chemiluminescent western blotting using anti 188/784 RsaA antibodies. The NA1000 strain was subjected to low pH extraction before whole-cell preparations were done to minimize levels of S-layer. No RsaA breakdown products were present in the mutants. Wild type NA1000 was used as a positive S-layer control (wt). Both the JS1009 strain (*rsaFa*/*rsaFb*⁻) and the JS1003: *Hps12furin* strain (RsaA:furin) showed no evidence of breakdown products. All RsaA protein was in a non-degraded form, with levels of RsaA appearing similar for both mutant strains. The JS1003 strain showed no RsaA (wt (*rsaA*⁻)). These results suggested that when RsaA begins to accumulate inside of the cell, no breakdown occurred, and that some type of RsaA regulation was present.

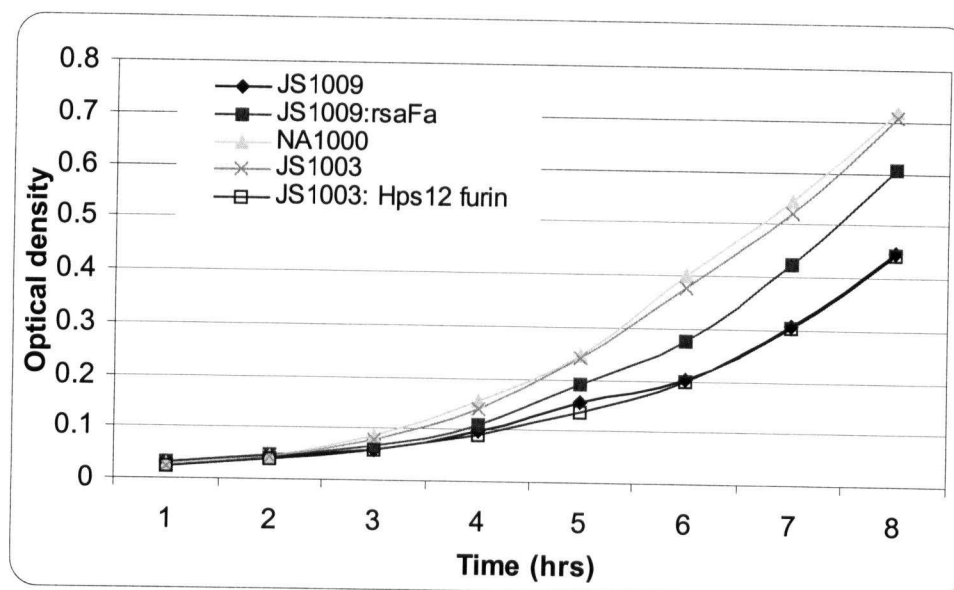


Figure 4-15 a. Exponential growth curve of knockout and modified *RsaA* strain.

The JS1009 and JS1003: Hps12furin strains grow significantly slower than wild type strains. Introduction of *rsaFa* to the JS1009 strain recovers growth rates to a degree. Plots shown are averages of three runs.

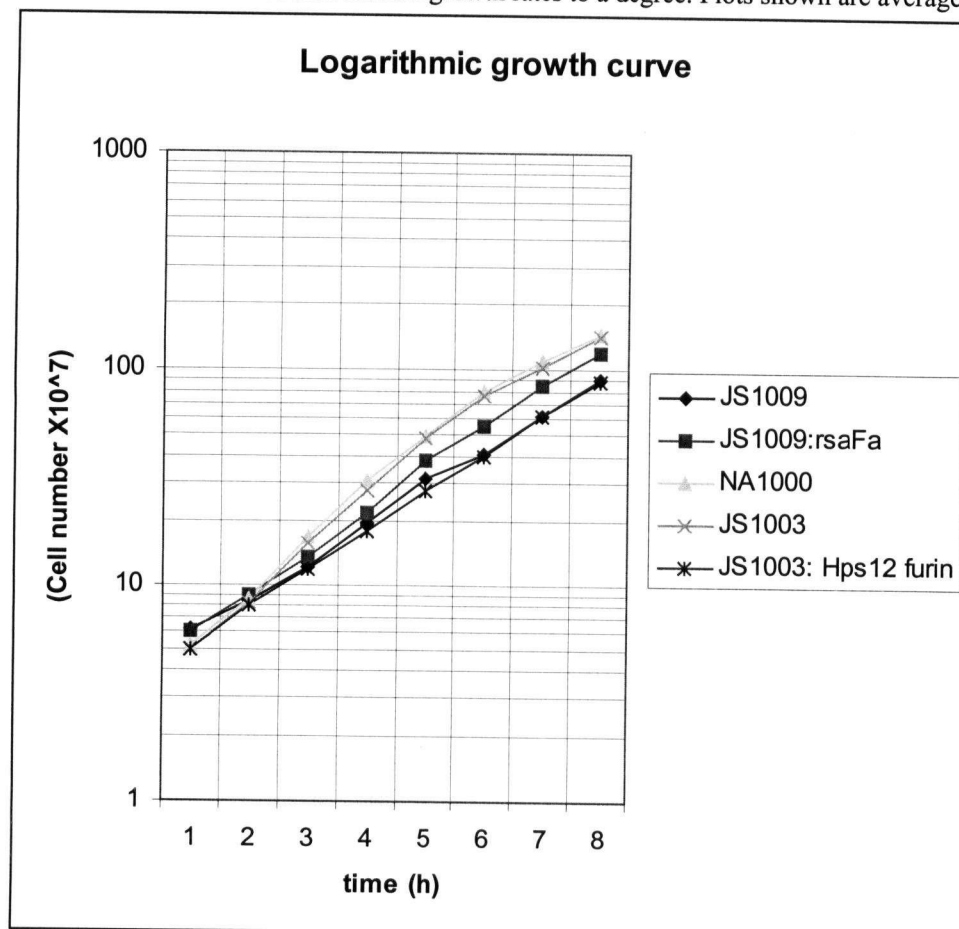


Figure 4-15 b. Logarithmic growth curve of knockout and modified *RsaA* strain.

All strains were grown at 30 °C at 200 rpm and OD600 was taken every hour. Three runs were done and average generation times were calculated for the strains.

Strain	Generation time (min)
NA1000	78
JS1009	114
JS1009: <i>rsaFa</i>	96
JS1003	79
JS1003: <i>Hps12furin</i>	108

Table 3: Generation times of the *C. crescentus* mutant strains.

rsaA is like most other S-layer genes and is transcribed continuously throughout the cell cycle(24). In *Lactobacillus brevis* the S-layer gene (*slpA*) is transcribed during stationary phase even when the S-layer protein is not produced (34). If transcription occurs continuously, then it is likely that regulation would occur at the translational level. The S-layer of *Thermus thermophilus* HB8 has been shown to autoregulate the translation of *slpA* by binding of a C-terminal SlpA fragment to the 5' end of the mRNA (23). Whether a similar situation occurs in *C. crescentus* is still unknown and further examination is needed, however it is likely that some sort of S-layer regulation exists. It is possible that built up RsaA inside of the cell binds to mRNA transcripts and halts production of RsaA (**Fig. 4-16.**).

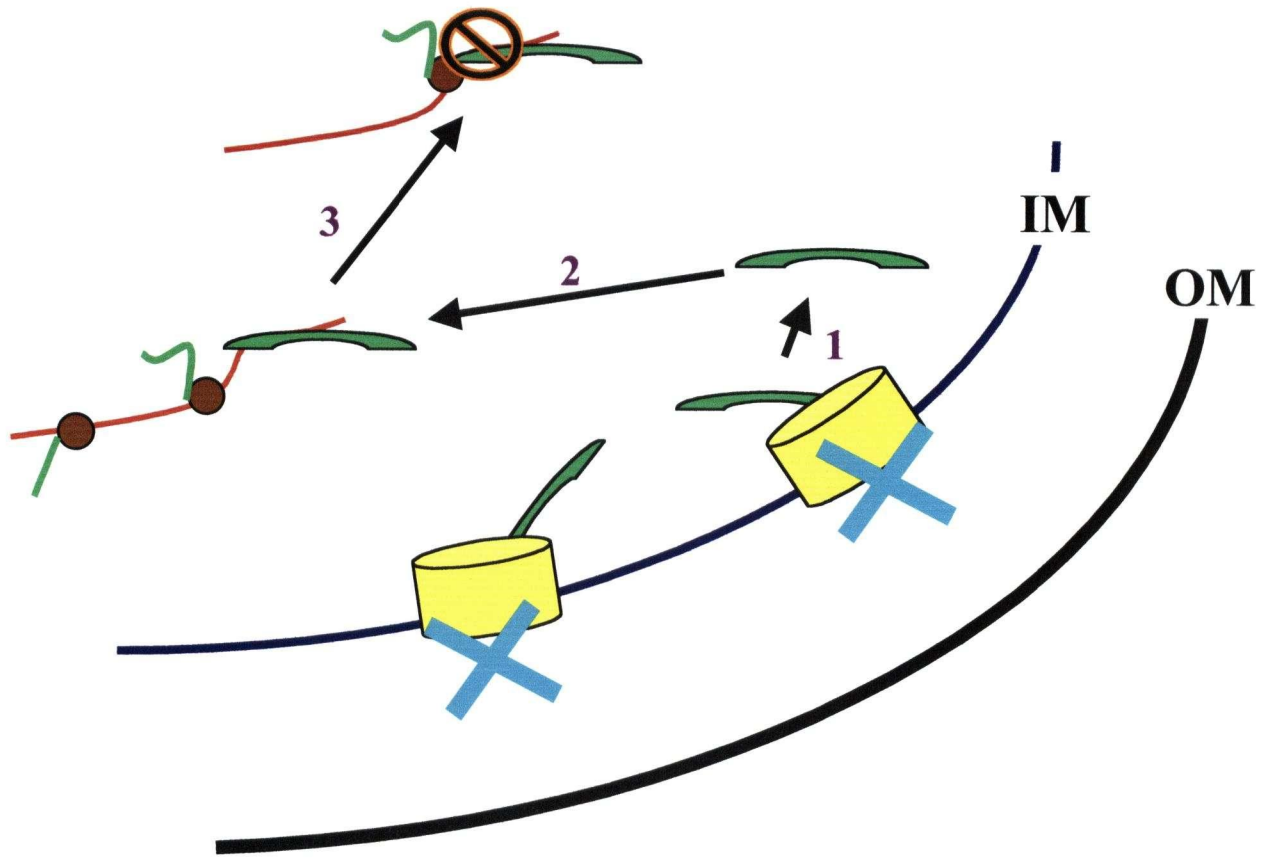


Figure 4-16. Cartoon depiction of hypothesized autoregulation of the *rsaA* gene.

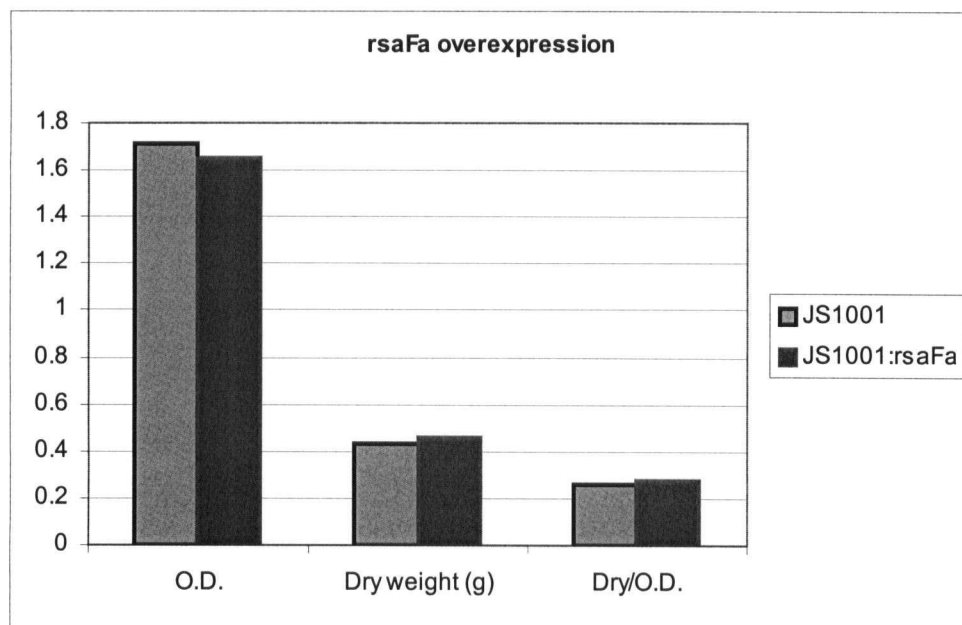
RsaA production may be affected by autoregulation, where RsaA binds to mRNA transcripts inhibiting translation. 1- Since no OMP is present (or charged proteins interfere with secretion as in *Hps12 furin* clone) RsaA is blocked from secretion and thus RsaA sits in the cytoplasm. 2- Free RsaA finds the *rsaA* mRNA transcript and binds. 3- Ribosomes moving along the mRNA transcript encounter bound RsaA and are blocked or dislodged.

4.07- Coordinate Overexpression of RsaA and RsaF

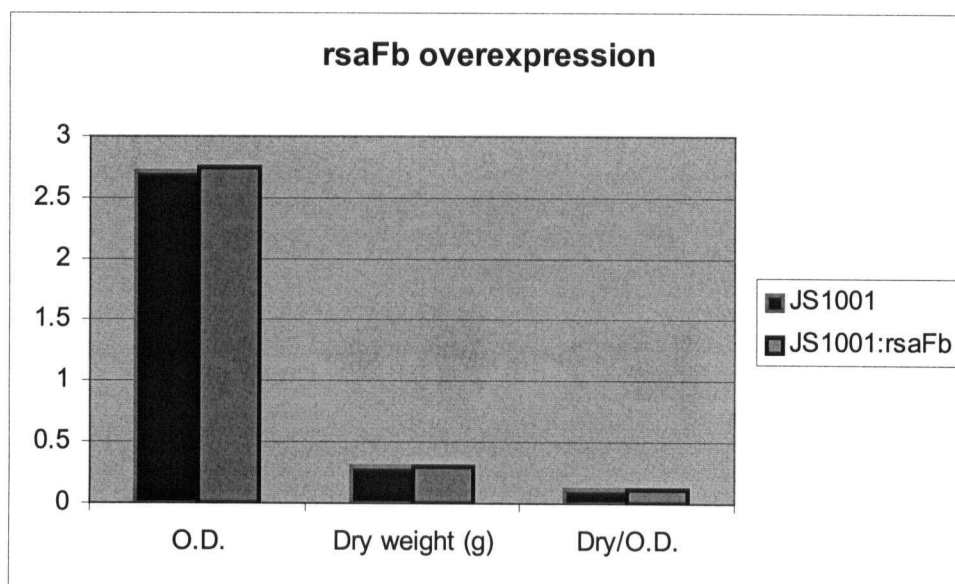
Since the RsaA secretion system has been modified for use in recombinant protein production, optimal efficiency of the system is important. Therefore, the effect of additional transporter units in wild-type (*rsaFa*⁺, *rsaFb*⁺) bacteria was examined to determine if the RsaA machinery could be induced to secrete more RsaA than normal. Vector borne copies of a single *rsaF* were inserted into S-layer positive *C. crescentus*. A S-LPS negative strain, JS1001, was used instead of the wild type NA1000 strain. This strain was used to ensure that there is no possibility of RsaA regulation by surface crystallization, or in other words, inhibition of transport when the bacterial surface is “full”. Levels of RsaA were initially determined by collection of aggregated protein at first, and later whole-culture-protein preparations were examined. The latter proved to be easier or more valuable since it ensured that any micro-aggregates were included in the sample.

Protein aggregates were collected and both wet and dry weights of the RsaA were taken. Ratios of protein to culture density (OD₆₀₀) were compared to determine if over expression was occurring. JS1001 strains containing plasmid borne copies of *rsaFa* or *rsaFb* showed little or no increase in aggregated RsaA (**Fig. 4-17 a - b.**). These results suggested that despite increased OMP gene copy RsaA secretion could not be increased. I then inserted additional copies of *rsaD* and *rsaE* into the JS1001 strain. These results showed similar variability, with the JS1001: pRAT9 clone actually showing less S-layer secretion than wild type JS1001 (**Fig. 4-17 c.**). Since levels of aggregates frequently varied despite controlled conditions, I decided to determine levels of RsaA secretion using an alternate method.

a



b



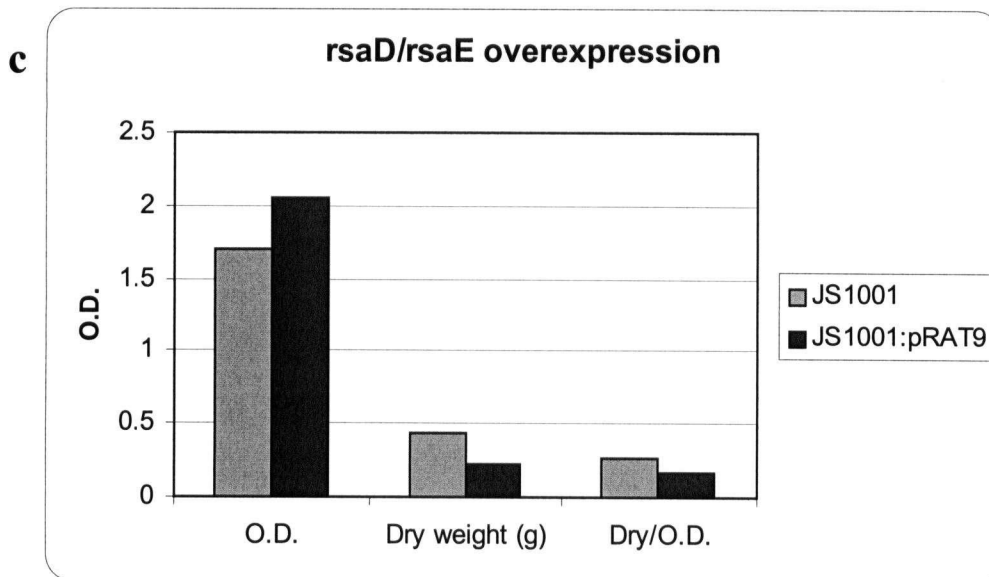


Figure 4-17. a-c. Levels of aggregate production in the JS1001 as compared to strains with additional copies of the transporter components.

a. *rsaFa* overexpression in JS1001 does not lead to significantly higher levels of RsaA. Comparison of dry weight/ OD does not show much difference as levels for JS1001 are $0.262 \text{ g/O.D}_{600} \pm 0.039$ and JS1001:*rsaFa* values are $0.279 \text{ g/O.D}_{600} \pm 0.009$.

b. *rsaFb* overexpression in JS1001 appears similar to levels in JS1001. Comparison of dry weight/ OD does not show significant difference. JS1001 levels are $0.1 \text{ g/O.D}_{600} \pm 0.08$ and JS1001:*rsaFb* values are $0.110 \text{ g/O.D}_{600} \pm 0.009$.

c. pRAT9 (*rsaD/rsaE*) overexpression in JS1001 appears deleterious to RsaA secretion and levels are lower than those seen in the *rsaFa* and *rsaFb* overexpressors. Dry weight to O.D. for JS1001 was $0.262 \text{ g/O.D}_{600} \pm 0.039$ and for JS1001:pRAT9 was $0.1627 \text{ g/O.D}_{600} \pm 0.104$. High fluctuation in the OD and dry weights of the JS1001:pRAT9 led to erratic values. These fluctuations may be due to toxicity of extra RsaD and RsaE.

Simultaneous runs were carried out for the JS1001, JS1001:*rsaFa* and JS1001:pRAT9 strains. The JS1001:*rsaFb* runs were done separately with a new set of JS1001 controls. Fluctuation of OD values and processing of aggregates for all runs led to varying values. Changes in O.D. values, etc. may be due to stability of media, starting culture, and other variable factors. For this reason, other methods were evaluated.

Whole culture protein preparations became the dominant method as it allowed for comparison of both S-LPS mutants and wild-type cells, since surface bound and/ or secreted protein was included in the preparation. In order to ensure that all secreted protein was solubilized, urea was added to 2M (final concentration).

Levels of RsaA production were not significantly increased when additional copies of *rsaFa* or *rsaFb* were expressed in JS1001 (**Fig. 4-18.**) (**Table 4**). Levels of RsaF were also determined to confirm that the *rsaF* genes were expressed, and levels of the proteins were found to be higher than in JS1001 strain (**Fig. 4-19**). The increased levels of RsaFa and RsaFb appeared similar to the complementation strains with RsaFa at levels 10.6 fold greater than wild type, and RsaFb levels 8.4 fold greater.

Figure 4-18. Effect of RsaF overexpression in the JS1001 strain.

Introduction of plasmid borne copies of either *rsaFa* or *rsaFb* in the JS1001 strain led to a slight increase in the secretion of RsaA.

Chemiluminescent western blot using anti 188-784 RsaA antibodies of normalized whole culture extracts showed similar levels of RsaA for all strains.

Spot densitometry revealed that levels were only increased by 3-4% for the JS1001: *rsaFa* (*rsaFa*⁺⁺) and JS1001: *rsaFb* (*rsaFb*⁺⁺) above wild type JS1001 (wt (S-LPS⁻)) levels.

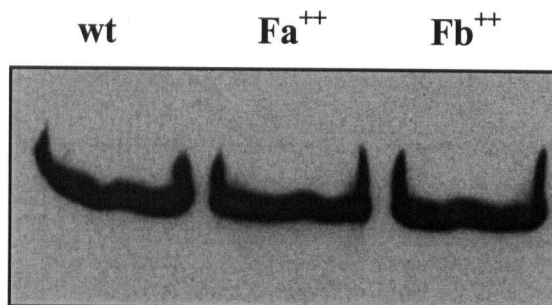
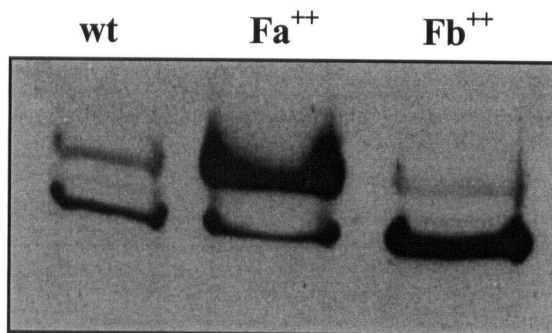


Figure 4-19. Levels of RsaFa and RsaFb in JS1001 strain.

Plasmid borne copies of *rsaFa* and *rsaFb* result in increased levels of RsaFa (10.6 X wild type) and RsaFb (8.4 X wild type). Wild type JS1001 (wt (S-LPS⁻)) has normal levels while JS1001: *rsaFa* (*rsaFa*⁺⁺) and JS1001: *rsaFb* (*rsaFb*⁺⁺) have elevated levels of their respective overexpressed RsaF.



Strain	%RsaA to JS1001
JS1001	100%
JS1001: <i>rsaFa</i>	103%
JS1001: <i>rsaFb</i>	102%
JS1001: pWB9 <i>rsaA</i> Δ P	100%
JS1001: <i>rsaFa</i> : pWB9 <i>rsaA</i> Δ P	128%

Table 4. Levels of RsaA determined by whole culture preparations.

Spot densitometry of chemiluminescence western blots using polyclonal antibodies against 188/784aa RsaA, were used to determine relative levels of RsaA produced by cells. Levels determined by densitometry were compared to JS1001 levels. All whole culture protein preparations were normalized prior to running samples.

To be assured that additional RsaFa was properly targeted to the outer membrane, protein A-colloidal gold labeling with anti-RsaF antibody was used to assess levels of RsaF detectable on the outer membrane surface (experiments carried out by Dr. J. Smit). A uniform low-level label was noted with JS1001 (**Fig. 4-20.**), indicating that some portion of the RsaF OMP was surface exposed when the oligosaccharide chains of the S-LPS fraction of total LPS were eliminated (see below). Label of JS1001: *rsaFa* and JS1001: *rsaFb* (not shown) showed two major classes of cells: those labeled at levels similar or slightly greater than that seen with JS1001 and a fraction (approximately 20% of the total) where a dense label was noted. I interpret that as an indication that plasmid copy numbers for the moderate copy number pBBR4 plasmid vary significantly from cell to cell, suggesting it is not a stably maintained plasmid. Nevertheless it appeared that some cells were expressing much higher levels of RsaFa and that it was targeted correctly to the outer membrane.

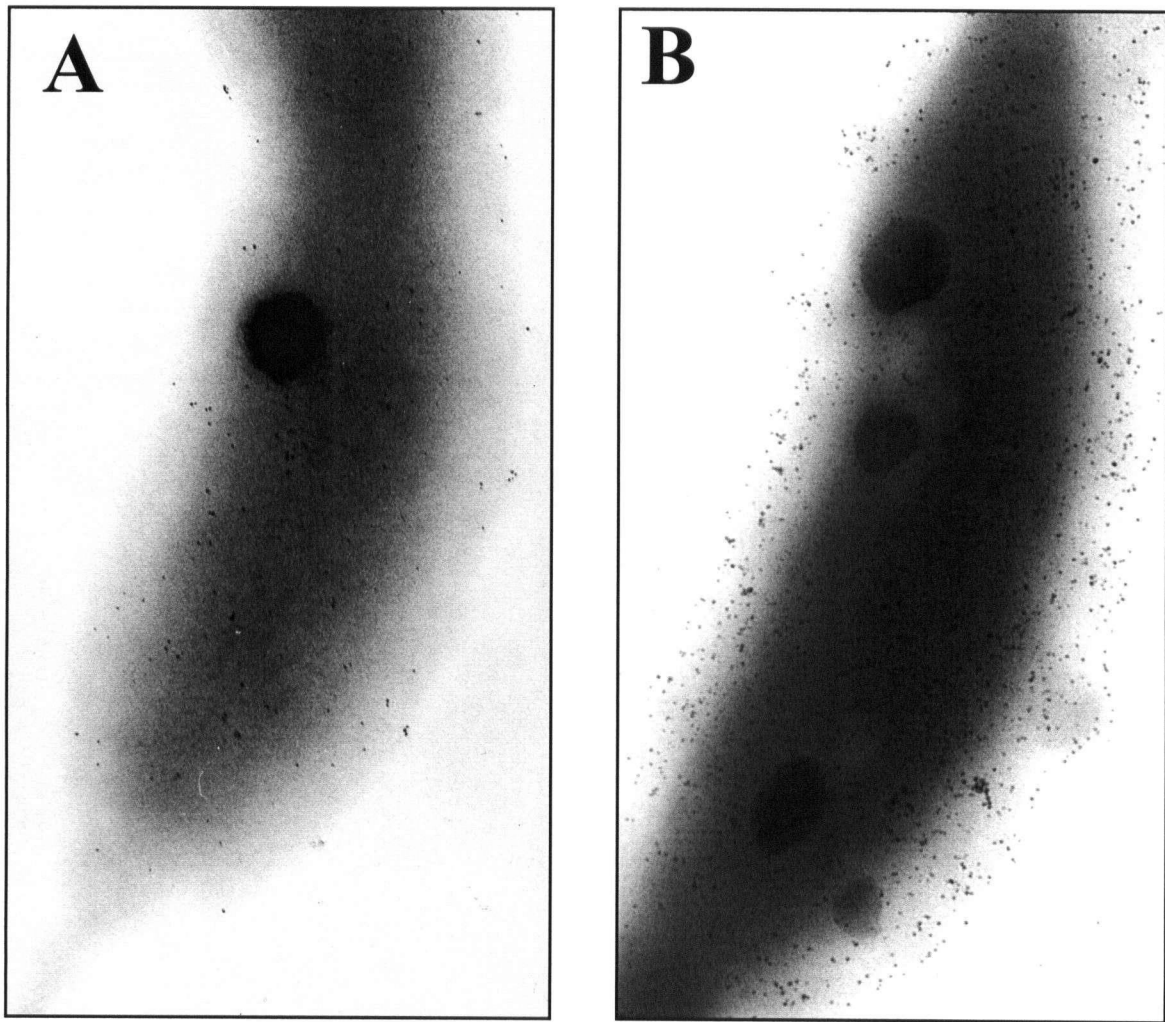


Figure 4-20. Colloidal gold labeling of surface displayed RsaF.

Surface displayed RsaFa was determined for the JS1001 strains overexpressing *rsaFa* by electron microscopy using anti-RsaFa and colloidal gold labeling (carried out by Dr. J. Smit). JS1001 (a) shows wild type levels of RsaF with moderate labeling. The JS1001: *rsaFa* strain (b) shows a significant increase in surface display of RsaF. Note that only about 20% of the JS1001: *rsaFa* cells showed significant increase in RsaF display.

Interestingly, in contrast, to JS1001 (which has no S-LPS), there was no detectable label with strain JS1003, which has no S-layer but does have a normal complement of S-LPS (not shown). Presumably this smooth form of LPS effectively blocks antibody access to the OMPs, perhaps indicating their exposure on the surface is minimal.

When both *rsaF* genes are present and additional copies of *rsaFa* are introduced, secretion of RsaA is increased only slightly. Levels of RsaA were approximately 3% greater than in wild-type JS1001 (**Fig. 4-18.**). These results are similar to experiments in which additional plasmid borne copies of *tolC* were expressed in Gram-negative bacterial strains containing the HlyA secretion apparatus (64). It was found that despite additional copies of TolC neither enhancing nor deteriorating effects occurred. The authors suggested that the HlyB and HlyD protein levels might be the limiting factor as only the outer membrane protein levels were increased. However, since aggregate experiments showed no significant change in secretion by over expressing *rsaD* and *rsaE* and likely led to deleterious effects, I thought other factors might be at play. I speculate that instead of the ABC transporter and MFP levels being the limiting factor, that the transported protein may be the limiting factor.

Since JS1001 has only the single chromosome resident copy of *rsaA* I considered whether RsaA transcription or translation (and not the OMP levels) might now determine the maximum levels of RsaA secretion. To address this possibility a multi-copy plasmid borne *rsaA* gene was introduced into the JS1001:*rsaFa* strain. The resulting strain was grown and whole-culture-protein levels were compared (**Fig. 4-21.**). The resulting strain JS1001: *rsaFa*: *rsaA* produced ~28% more S-layer protein than that seen in the JS1001 strain. Since the JS1001: *rsaA* strain did not produce more RsaA than both the wild type JS1001 and the JS1001: *rsaFa* strain, secretion must be a function of both available outer membrane proteins as well as RsaA copies. This is a remarkable increase in RsaA secretion as wild-type levels of RsaA already represent 10-12% of total cell protein and with this increase could represent upwards of 15% of the total cellular protein. I interpret

this as an indication that elevated secretion of RsaA is possible but requires both overexpression of RsaA and at least the RsaFa OMP. It is likely that RsaA secretion is dependent on a number of factors; the type and level of both RsaF proteins, and the number of *rsaA* copies (Fig. 4-22.).

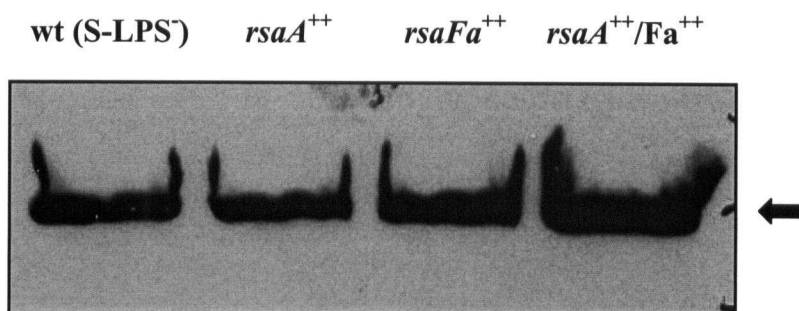


Figure 4-21. Effect of RsaFa and *rsaA* overexpression in the JS1001 strain.

Increasing RsaA secretion is a function of amount of RsaA to transport and the number of holes in the outer membrane. Chemiluminescent western blots revealed that increased copy numbers of *rsaA* and *rsaFa* in the JS1001 strain led to increased RsaA levels (marked by arrow). The JS1001 (*wt (S-LPS⁻)*), JS1001: *rsaA* (*rsaA⁺⁺*) and JS1001: *rsaFa* (*rsaFa⁺⁺*) appear to have similar levels of RsaA secretion with fractional increase of 3% seen in the JS1001: *rsaFa* strain. The JS1001: *rsaFa*: *rsaA* strain (*rsaA⁺⁺/rsaFa⁺⁺*) had a definite increase in RsaA secretion with levels 28% greater than JS1001.

The type I secretion apparatus is able to transport normal levels of RsaA produced by the cell, but cannot accommodate increased RsaA levels unless OMP levels are increased. I speculate that the ability to increase RsaA secretion may be a function of increasing the number of ‘holes’ in the bacteria. It has been shown that the HlyB and HlyD proteins exist in a pre-formed complex in the inner membrane and when HlyA is engaged, the complex recruits the TolC protein (66). The RsaA transport complex is likely analogous, and thus increasing the number of OMPs in the outer membrane may make it easier for the secretion apparatus components to find each other and transport RsaA.

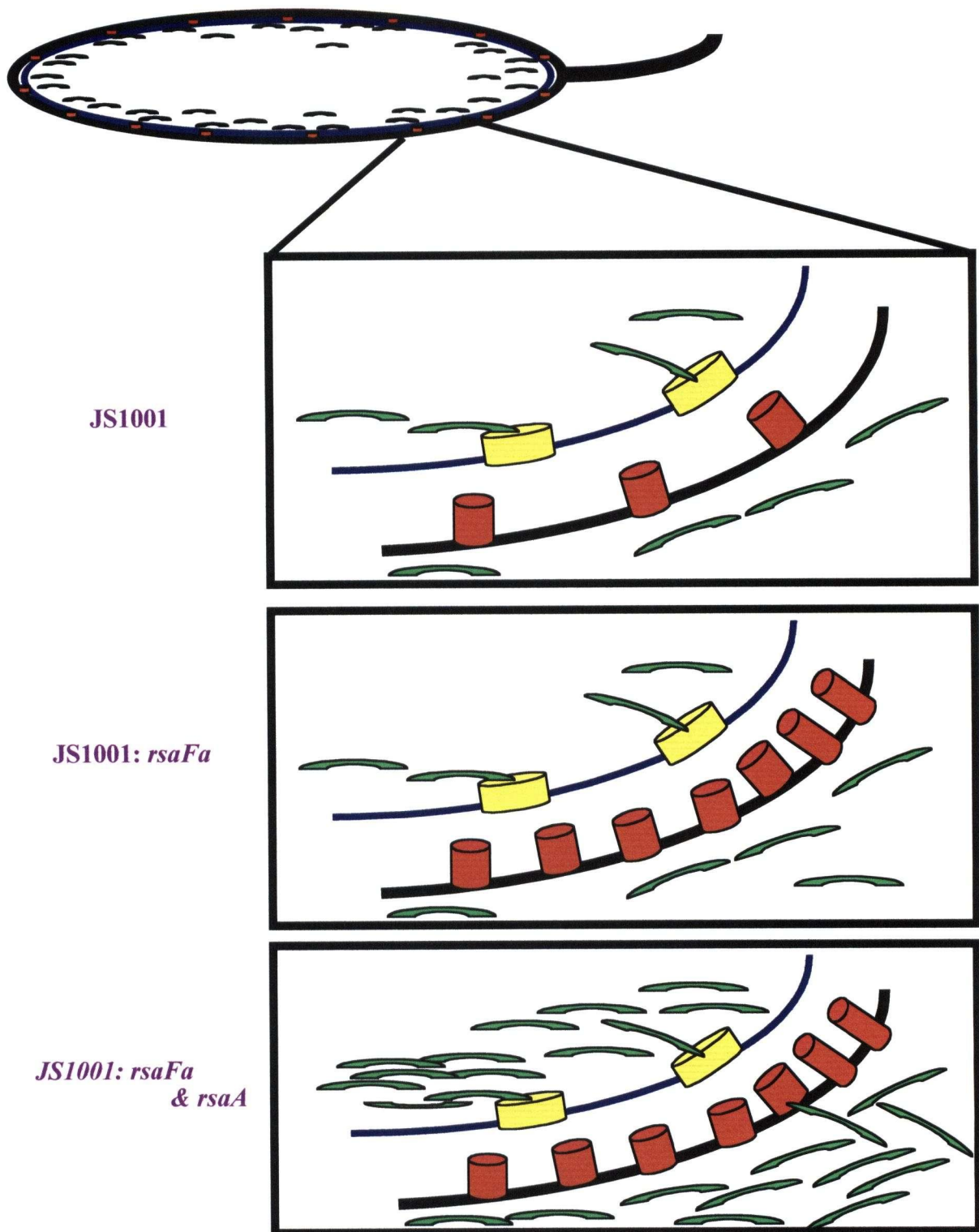


Figure 4-22. Cartoon of RsaA secretion and transport system in the overexpressing strains
 Increase in *rsaFa* gene (JS1001: *rsaFa*) copy leads to more OMP units in the outer membrane, only slightly increasing RsaA secretion. When *rsaFa* and *rsaA* are increased, more RsaA is produced and can be transported due to higher numbers of OMP's in the outer membrane.

5. DISCUSSION AND CONCLUSION

This study reports further characterization of the type I RsaA secretion apparatus, showing that two outer membrane proteins, RsaFa and RsaFb, are involved in RsaA transport. As well, it shows that unlike previously hypothesized, the type I transporter components are transcribed separately from *rsaA*. These findings may help elucidate how the RsaA secretion apparatus attains such high levels of protein secretion.

Type I organization has been based on early findings of the HlyA system. The *hlyABCD* genes are located together and were found to be co-transcribed (75). Most type I systems have been assumed to be organized and transcribed in a similar manner. To date, only the *S. marcescens* (Lip) and *C. fetus* (SapA) systems have been shown to use separate promoters for the S-layer gene and its respective transporter genes. A putative *rsaD* promoter has been predicted using in-silico methods and consensus promoter sequences. I determined that the -10 site predicted by the BPRM program was likely correct, but that an alternate -35 site was likely correct based on its location and similarity to a predicted -35 consensus sequence.

Analyzing the *rsaADE* gene set using molecular methods revealed that *rsaD* and *rsaE* were co-transcribed using a separate promoter from *rsaA*. This showed that the RsaA secretion system was not transcribed like the HlyA system, and was more like the Lip system, having a separate promoter for the ABC transporter and MFP. A mechanism where all transporter components are transcribed separately makes sense for a system secreting high levels of protein. If transcription of *rsaD* and *rsaE* relied on leakiness of the *rho*-independent terminator, it may be that not enough copies of the transport apparatus would be present to support wild-type S-layer levels. The amount of protein

secreted by the *E. coli* HlyA system is about 0.5% of the total cell protein where as the S-layer of *C. crescentus* accounts for 10-12% of the total cell protein. Separate transcription of the RsaA secretion apparatus may allow for higher levels of secretion. *C. crescentus* may have modified the type I secretion gene organization to ensure that the bacterial surface is covered at all times during the cell cycle.

In addition to gene organization, *C. crescentus* may have also adapted to accommodate high levels of protein secretion by duplicating or acquiring an additional OMP. Having two OMPs in RsaFa and RsaFb allows for increased levels of RsaA secretion. Both *rsaFa* and *rsaFb* are located away from the type I system and appear to be transcribed irrespective of the transport system. Their isolated location and expression likely allows for increased OMP units in the outer membrane, increasing the chances of complete transporters associating and in turn allowing high levels of secretion.

The two OMPs for the RsaA secretion system had been previously identified in the lab using in-silico methods and *rsaF* knockouts and complementation experiments were attempted, but did not provide any definite results. When this study started, I wanted to knockout *rsaFa* and *rsaFb* by gene replacement with internal deletion forms of the two genes. Hundreds of clones were screened by PCR, using a variety of internal deletion sizes unsuccessfully, so new methods to knockout the genes were undertaken. A double knockout was made in the NA1000 strain using antibiotic insertion, and $\Delta N\Delta C$ gene insertion of the *rsaF* genes.

Independent knockouts of *rsaFa* and *rsaFb* did not stop S-layer secretion. There was a noticeable decrease in the *rsaFa* knockout, but the *rsaFb* knockout appeared only slightly lower than wild type levels. Quantification of these levels gave us a better

understanding of the possible importance of the two OMPs. Destruction of *rsaFa* and *rsaFb* led to loss of S-layer secretion. Complementation of either gene could not restore RsaA secretion to wild type levels, despite significant increases in the respective RsaF. Indeed, when overexpressed the individual OMPs could only restore secretion to the level of their apparent contribution in the normal, single gene copy situation. This suggests that it may not be simply a matter of a limit on the number of active transporter complexes (when only one OMP is expressed) but on the transporter composition; I tentatively suggest that the trimeric OMP complex may function most effectively when assembled as a heterotrimer of RsaFa and RsaFb and that this may be the native situation. In this scenario, heterotrimers are technically not required for RsaA secretion, though survival in a natural environment may require maximal secretion of RsaA to maintain surface coverage of this protective device.

The complemented strains, and RsaA production data suggested a mechanism involving feedback down-regulation of RsaA production. I noted in such situations the cells grew much slower and there were few motile cells and most cells were misshapen and elongated. It may be that if such an autoregulation is occurring it may not be specific, impacting the synthesis of other proteins as well. If autoregulation is occurring, the levels of RsaA actually secreted and RsaA production should be quite similar. Interestingly I found that levels of RsaA production in the *rsaF* mutants were similar to the levels of RsaA secretion. Despite RsaA secretion decreasing with the loss of the *rsaF* genes, there was no accumulation of RsaA inside of the cell. Whole-culture and low pH extracted levels of RsaA protein were only fractionally different, varying by about 2%. Taken together with the effects of complete blockage of protein export, these results suggest that

the level of RsaA produced in the cell may be regulated by the amount of protein that can be secreted. This, in turn, may be set by the amount of RsaA that can remain in the cytoplasm as a result of autoregulation.

Because I had indications that RsaA autoregulated the amount of protein produced I believed it might be possible to increase expression levels by coordinate increases in RsaA translation rates mated to an increase in the number of transporters. Overexpression of an individual *rsaF* gene in the absence of the other *rsaF* gene was presumably not an appropriate course since it did not even lead to normal levels of RsaA secretion, so I instead began overexpressing individual transporter components in the presence of a normal complement of remaining transporter proteins. The effect of additional transporter units in wild-type (*rsaFa*⁺, *rsaFb*⁺) bacteria was examined to determine if the RsaA machinery could be induced to secrete more RsaA than normal. By using the S-layer shedding strain JS1001, which produces the same amount of RsaA as the parental NA1000, any RsaA feedback regulation due to the presence of a “full” S-layer on the bacterial surface would not affect the results. When both *rsaF* genes are present and additional copies of *rsaFa* were introduced, secretion of RsaA is increased only slightly. Similarly, a strain containing additional vector borne copies of the *rsaA* gene with a wild type complement of *rsaFa* and *rsaFb* genes did not exceed wild type RsaA levels. However, the JS1001: *rsaFa*: *rsaA* strain produced 28% more protein than wild type levels. Moreover, the RsaA overexpression may well be ascribed to the 20% fraction of cells that were truly overexpressing RsaFa from an unstable vector. Presumably stable expression of RsaFa by all cells may lead to still higher levels of RsaA secretion.

These experiments have led to a better understanding of the type I secretion system of *C. crescentus* and of type I systems in general. Little is known about type I secretion system organization and expression, and especially in high level secreting type I systems. I have been able to further characterize an extremely interesting transport apparatus, identifying potential bottlenecks in S-layer secretion as well as suggest possible modifications that may increase the transporter's capabilities. In doing so, I have also created new methods such as whole-culture preparations which allow for comparison of shedding and crystallizing strains, as well as internal levels of RsaA.

I propose future experiments to increase gene copies of both *rsaF* genes so that expression is stable. Finding the optimal combination of the RsaFs as well as determining if coordinate up-regulated expression of various combinations of all the transporter elements may lead to still greater levels of RsaA expression or whether other factors, such as membrane stability or available ATP to drive the transport process will set limits.

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