

## Two Outer Membrane Proteins Are Required for Maximal Type I Secretion of the *Caulobacter crescentus* S-Layer Protein

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**Transport of RsaA, the crystalline S-layer subunit protein of *Caulobacter crescentus*, is mediated by a type I secretion mechanism. Two proteins have been identified that play the role of the outer membrane protein (OMP) component in the RsaA secretion machinery. The genes *rsaF<sub>a</sub>* and *rsaF<sub>b</sub>* were identified by similarity to the *Escherichia coli* hemolysin secretion OMP TolC by using the *C. crescentus* genome sequence. The *rsaF<sub>a</sub>* gene is located several kilobases downstream of the other transporter genes, while *rsaF<sub>b</sub>* is completely unlinked. An *rsaF<sub>a</sub>* knockout had ~56% secretion compared to wild-type levels, while the *rsaF<sub>b</sub>* knockout reduced secretion levels to ~79%. When expression of both proteins was eliminated, there was no RsaA secretion, but a residual level of ~9% remained inside the cell, suggesting posttranslational autoregulation. Complementation with either of the individual *rsaF* genes by use of a multicopy vector, which resulted in 8- to 10-fold overexpression of the proteins, did not restore RsaA secretion to wild-type levels, indicating that both *rsaF* genes were required for full-level secretion. However, overexpression of *rsaF<sub>a</sub>* (with normal *rsaF<sub>b</sub>* levels) in concert with overexpression of *rsaA* resulted in a 28% increase in RsaA secretion, indicating a potential for significantly increasing expression levels of an already highly expressing type I secretion system. This is the only known example of type I secretion requiring two OMPs to assemble a fully functional system.**

Crystalline surface protein layers (S-layers) are common in many genera of microorganisms, including gram-negative bacteria, gram-positive bacteria, and archaeobacteria. S-layers may function as protective barriers and molecular sieves, promote cell adhesion and surface recognition, and maintain cell shape and envelope rigidity (23). The S-layer of the gram-negative bacterium *Caulobacter crescentus* has been shown to act as a physical barrier to a *Bdellovibrio*-like parasitic bacterium (26).

The *C. crescentus* S-layer is a two-dimensional hexagonal array composed of the 98-kDa protein RsaA (41, 42) that is anchored to the outer membrane via an interaction with smooth lipopolysaccharide (S-LPS) (4, 48). The *C. crescentus* S-layer has been estimated to be 10 to 12% of the total cell protein produced, with approximately 40,000 RsaA subunits attached to the surface of the cell (5, 8, 41). RsaA synthesis occurs without need for induction, and the protein is produced continuously throughout the cell cycle (17, 39).

While most of the characterized S-layer transport systems for gram-negative bacteria involve type II secretion (in which an N-terminal signal system directs export across the inner membrane by use of the general secretory pathway and secretion from the bacterium then occurs via a protein-specific mechanism), the S-layer protein of *C. crescentus* is secreted by a type I mechanism (3). This mechanism is also found for the S-layer of *Campylobacter fetus* (46) and the S-layer-like protein (SlaA) in *Serratia marcescens* (24). The sheer amount of protein secreted by the *rsaA* secretion apparatus suggests a well organized transport system, but we wish to determine if there

are bottlenecks in the overall secretion process that can be addressed by control of gene expression levels.

Type I secretion is a *sec*-independent pathway where protein is transported from the cytoplasm across the inner and outer membranes without interaction with the periplasm. Type I secreted proteins utilize an uncleaved C-terminal secretion signal, and, in general, the last 60 amino acids (aa) of the protein are sufficient for secretion (6). A type I secretion apparatus is composed of three components. The ATP-binding cassette (ABC) transporter, which likely forms a dimer, resides in the inner membrane, engaging the C-terminal sequence of the substrate protein and hydrolyzing ATP during transport. The membrane fusion protein (MFP) is anchored in the inner membrane by a single transmembrane domain, as well as by binding to the ABC transporter, and may span the periplasm (45). The MFP is thought to interact with the ABC transporter protein and a trimer of the outer membrane protein (OMP), to form a channel from the cytoplasm to the outside of the cell (25).

For most type I systems, the ABC transporter and MFP genes are immediately 3' of the gene for the secreted protein, 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) (13, 27, 46). In other type I systems, only the ABC transporter and MFP genes are located next to the substrate gene (28, 30) and the OMP gene lies far from the others. TolC, the OMP for the *Escherichia coli* alpha-hemolysin (HlyA) system, is an example (50).

The *E. coli* HlyA system is the best-characterized type I secretion system (12, 19, 31). The structure of the OMP (TolC) has been determined to 2.1 Å (25), and TolC has been shown to be multifunctional, interacting with numerous efflux systems, including the HlyA, AcrA, and CvaA systems (18, 22,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics <sup>a</sup>	Reference or source
<b>Bacterial strains</b>		
<i>C. crescentus</i>		
NA1000	Ap <sup>r</sup> <i>syn-1000</i> ; 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 (14)
JS1003	NA1000 with <i>rsaA</i> interrupted by KSAC Km <sup>r</sup> cassette	Edwards and Smit (14)
JS1007	Sm <sup>r</sup> ; NA1000 <i>rsaF</i> <sub>a</sub> strain	This study
JS1008	Cm <sup>r</sup> ; NA1000 <i>rsaF</i> <sub>b</sub> strain	This study
JS1009	Cm <sup>r</sup> Sm <sup>r</sup> ; NA1000 <i>rsaF</i> <sub>a</sub> <i>rsaF</i> <sub>b</sub> strain	This study
<i>E. coli</i> DH5α	<i>recA endA</i>	Invitrogen
<b>Plasmids</b>		
pHP45Ω	Ap <sup>r</sup> Sm <sup>r</sup> ; source of SM cassette removed as-SmaI fragment	Fellay et al. (15)
pBSKIIEEH	Ap <sup>r</sup> ; modified pBSKSII cloning vector with EcoRI-, EcoRV-, HindIII-modified MCS	This study
pBSKIIESH	Ap <sup>r</sup> ; modified pBSKSII cloning vector with EcoRI-, StuI-, HindIII-modified MCS	This study
pTZ18UCHE	Cm <sup>r</sup> ; cloning vector	This study
pTZ18UCHE: <i>rsaF</i> <sub>b</sub> ΔNΔC	Cm <sup>r</sup> ; cloning vector with <i>rsaF</i> <sub>b</sub> fragment missing N and C termini	This study
pBSKIIEEH: <i>rsaF</i> <sub>a</sub> ΩSm	Ap <sup>r</sup> Sm <sup>r</sup> ; <i>rsaF</i> <sub>a</sub> gene fragment with SM cassette inserted at PstI site	This study
pBBR4	Km <sup>r</sup> ; broad-host-range plasmid derived from pBBR1	This study
pBBR4: <i>rsaF</i> <sub>a</sub>	<i>rsaF</i> <sub>a</sub> <sup>+</sup> Km <sup>r</sup> ; <i>rsaF</i> <sub>a</sub> gene inserted EcoRI/BamHI in pBBR4	This study
pBBR4: <i>rsaF</i> <sub>b</sub>	<i>rsaF</i> <sub>b</sub> <sup>+</sup> Km <sup>r</sup> ; <i>rsaF</i> <sub>b</sub> gene inserted EcoRI/BamHI in pBBR4	This study
pWB9: <i>rsaA</i> ΔP	Cm <sup>r</sup> Sm <sup>r</sup> ; <i>rsaA</i> gene and <i>rsaA</i> promoter strain	Bingle et al. (9)
pWB9Hps12	Cm <sup>r</sup> Sm <sup>r</sup> ; <i>rsaA</i> containing BamHI site at aa 723	Bingle et al. (10)
pWB9:723/furin	Cm <sup>r</sup> Sm <sup>r</sup> ; <i>rsaA</i> containing furin cleavage site (RKKR) in BamHI site at aa 723	This study
pGEX4T3	Ap <sup>r</sup> ; GST-tagged expression vector	Amersham
pGEX4T3: <i>rsaF</i> <sub>a</sub>	Ap <sup>r</sup> ; GST-tagged expression vector with in-frame BamHI-EcoRI <i>rsaF</i> <sub>a</sub> gene	This study
pK18mobsacB	Km <sup>r</sup> Suc <sup>s</sup> ; <i>E. coli</i> -based suicide vector	Schafer et al. (37)
pK18mobsacB: <i>rsaF</i> <sub>a</sub> ΩSm	Km <sup>r</sup> Suc <sup>s</sup> ; <i>E. coli</i> -based suicide vector with <i>rsaF</i> <sub>a</sub> ΩSm fragment	This study

<sup>a</sup> MCS, multiple cloning site; Suc<sup>s</sup>, sucrose sensitivity; KSAC, Km<sup>r</sup> cassette derived from transposon Tn903.

50). Thus, it appears to interact with inner membrane translocases involved in drug and cation efflux as well as protein secretion. It has been suggested that the reason TolC is multifunctional may be because its gene does not belong to any export operon (2).

The RsaA secretion system has the expected characteristics of a type I secretion system. The secretion signal has been localized to the C-terminal 82 aa of RsaA (11). The ABC transporter (RsaD) and MFP (RsaE) have been characterized (3), and the *rsaD* and *rsaE* genes are found immediately downstream of *rsaA*. However, an OMP gene candidate did not immediately follow the *rsaE* gene, and despite repeated efforts, no Tn5 mutations were found in any genes encoding OMP-like products. This suggested either that the OMP was essential or that there were multiple functional OMP genes for this type I system.

Later, as part of a study characterizing the S-LPS genes that were adjacent to *rsaD* and *rsaE* (and involved with attachment of the S-layer), we identified a putative OMP gene (*rsaF*<sub>a</sub>) (4). Another study (35), however, declared that this was not an OMP gene, since disruption of the gene did not abolish RsaA secretion. Those authors apparently had not considered the possibility of multiple functional OMP homologs or made efforts to quantitate the amount of RsaA produced by the mutant strain.

In this study, we identify a second OMP gene and charac-

terize the products of *rsaF*<sub>a</sub> and *rsaF*<sub>b</sub>, showing that both are the only OMP proteins involved in S-layer transport. Eliminating either protein results in decreased RsaA secretion, indicating that either can function as the OMP component but apparently act cooperatively as it was not possible to restore wild-type levels of RsaA secretion by overexpression of individual OMPs. Interestingly, if both were present, overexpression of one led to levels of RsaA secretion significantly above wild-type levels.

## MATERIALS AND METHODS

**Strains, plasmids, and growth conditions.** The strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was grown at 37°C in Luria broth (1% tryptone, 0.5% NaCl, 0.5% yeast extract) with 1.3% agar for plates. *C. crescentus* strains were grown at 30°C in PYE medium (0.2% peptone, 0.1% yeast extract, 0.01% CaCl<sub>2</sub>, 0.02% MgSO<sub>4</sub>) with 1.2% agar for plates. Ampicillin was used at 50 μg/ml, and kanamycin was used at 50 and 25 μg/ml, chloramphenicol was used at 20 and 2 μg/ml, and streptomycin was used at 50 and 10 μg/ml in *E. coli* and *C. crescentus*, respectively.

**Plasmid and DNA manipulations.** Standard methods of DNA isolation and manipulation were used (36). Electroporation of *C. crescentus* was performed as previously described (20). All PCR products were generated using Platinum Pfx DNA polymerase (Invitrogen) following the manufacturer's suggested protocols. Strain NA1000 chromosomal DNA was used as the template for all PCR products. Chromosomal DNA was isolated with standard phenol-chloroform extraction methods (36).

All primers used in this study are listed in Table 2. The pBSKIIEEH vector was constructed from pBSKII (Stratagene). The BssHII fragment containing the

TABLE 2. Primers used in this study

Primer name	Sequence
EEH-1	5'-CGCGCTGAATTCGGATATCTTAAGCTTGG-3'
EEH-2	5'-CGCGCCAAGCTTAAGATATCCGAATTCAG-3'
ESH-1	5'-CGCGCTGAATTCGAGGCCTTTAAGCTTGG-3'
ESH-2	5'-CGCGCCAAGCTTAAGGGCTCGAATTCAG-3'
<i>rsaF<sub>a</sub></i> -1	5'-CGCGGATCCATGCGAGTGCTGTGCGAAAGTTCTGTC-3'
<i>rsaF<sub>a</sub></i> -2	5'-CCGGGAATTCAGTTGCGGGGCGCGGTCTGGAC-3'
<i>rsaF<sub>b</sub></i> -1	5'-CGCGGATCCATGTTGATGTGCGAACCGTTCGACGGG-3'
<i>rsaF<sub>b</sub></i> -2	5'-CCGGGAATTCATTTTCGAGCCGCTCGGGGGCTT-3'
<i>rsaF<sub>b</sub></i> NC-1	5'-GAAGCCGACGTGCTGTCT-3'
<i>rsaF<sub>b</sub></i> NC-2	5'-TGTAGGAGGTTTTTCGGGTCA-3'
1060	5'-GAGGCCTAGTACTCTGTCCAGCAACCAAGTTTACTCATA-3'
1920	5'-GAGGCCTACTTCTCTTTTCAATATTATTGAA-3'
CHE-1	5'-GGAAGATCTGTAACTTTTCAGGAGCTAAGGAAGCT-3'
CHE-2	5'-GGAAGATCTGTTAACACAATAACTGCCTTAAAAAATTA-3'
RKKR-1	5'-TCGAGACCCGATGCGCAAGAAACGGG-3'
RKKR-2	5'-CCCGTTTCTTTCGCGCATCGGGTC-3'
<i>rsaF<sub>b</sub></i> -190	5'-GGACGACGCTGACCAGCACCCCTGCT-3'

multiple cloning site was removed and replaced with annealed oligonucleotides EEH-1 and EEH-2 forming EcoRI, EcoRV, and HindIII sites. pBSKIIEH was created similarly to pBSKIIEEH with annealed oligonucleotides ESH-1 and ESH-2 forming EcoRI, StuI, and HindIII sites.

PCR products containing the *rsaF<sub>a</sub>* and *rsaF<sub>b</sub>* genes were generated by using primers *rsaF<sub>a</sub>*-1 and *rsaF<sub>a</sub>*-2 and primers *rsaF<sub>b</sub>*-1 and *rsaF<sub>b</sub>*-2, respectively. PCR products were ligated into the EcoRV site of the pBSKIIEEH vector and called pBSKIIEEH:*rsaF<sub>a</sub>* and pBSKIIEEH:*rsaF<sub>b</sub>*, respectively.

pBBR4 was constructed from plasmids pBBR1MCS and pUC4 KISS. The kanamycin resistance (Km<sup>r</sup>) fragment from pUC4 KISS was removed with PstI and blunted with T4 polymerase. A 0.3-kbp portion of the chloramphenicol resistance (Cm<sup>r</sup>)-encoding gene was removed from pBBR1MCS with DraI and replaced with the blunted Km<sup>r</sup> fragment, producing a Km<sup>r</sup> broad-host-range vector that replicates in *C. crescentus*. pBBR3 was constructed in a similar manner to that used for pBBR4, using a blunted HindIII-cut streptomycin resistance (Sm<sup>r</sup>) fragment of pHP45Ω.

Plasmids pBBR4:*rsaF<sub>a</sub>* and pBBR4:*rsaF<sub>b</sub>* and pGEX4T3:*rsaF<sub>a</sub>* were made using the BamHI-EcoRI fragment from pBSKIIEEH:*rsaF<sub>a</sub>* and pBSKIIEEH:*rsaF<sub>b</sub>*, respectively.

pBSKIIEEH:*rsaF<sub>a</sub>*ΩSm was created by using plasmids pBSKIIEEH:*rsaF<sub>a</sub>* and pHP45Ω. The streptomycin resistance insertion cassette was removed as a SmaI fragment and blunt-end ligated into pBSKIIEEH:*rsaF<sub>a</sub>* at a blunted PstI site inside *rsaF<sub>a</sub>*. The resulting plasmid, pBSKIIEEH:*rsaF<sub>a</sub>*ΩSm, was then used to make pK18mobsacB:*rsaF<sub>a</sub>*ΩSm. The EcoRI-HindIII fragment containing the *rsaF<sub>a</sub>*ΩSm fragment was cloned into EcoRI-HindIII-cut plasmid pK18mobsacB.

A PCR product containing a truncated form of *rsaF<sub>b</sub>*, missing the N and C termini was generated by using primers *rsaF<sub>b</sub>*NC-1 and *rsaF<sub>b</sub>*NC-2. This PCR product was blunt-end ligated into the StuI site of pBSKIIEH, creating pBSKIIEH:*rsaF<sub>b</sub>*ΔNΔC. pTZ18UCHE was constructed by using inverse PCR with primers 1060 and 1920 to create the backbone of pTZ18U without the ampicillin resistance (Ap<sup>r</sup>) cassette. The CHE (containing a chloramphenicol resistance gene) fragment was created as a PCR product by using pMMB206 (32) and primers CHE-1 and CHE-2. The pTZ18U backbone product was cut with StuI and blunt-end ligated with an HpaI-cut CHE fragment, creating pTZ18UCHE. The EcoRI-HindIII fragment from pTZ18UCHE was removed and replaced with the EcoRI-HindIII *rsaF<sub>b</sub>*ΔNΔC fragment from pBSKIIEH:*rsaF<sub>b</sub>*ΔNΔC.

pWB9:*rsaA*ΔP was described previously (10). The pWB9:723/furin construct was made using the BamHI site at amino acid (aa) 723 (Hps12). Two oligonucleotides, RKKR-1 and RKKR-2, were annealed and ligated into pUC9CXS as an XhoI-StuI fragment in a manner similar to that for the type IV pilin epitope (9). The RKKR-containing fragment was released by using BamHI and then inserted into the BamHI site at aa 723 in *rsaA*ΔP, and forward orientation of the fragment was confirmed by selection for Cm<sup>r</sup>. The Cm<sup>r</sup> cassette was excised using BglII and then ligation of the two complementary ends. The *rsaA*ΔP 723/furin fragment was then removed as an EcoRI-SstI fragment and ligated into EcoRI-SstI-cut pWB9KSAC.

**Knockout strain construction.** Knockouts of the two *rsaF* genes were constructed in the wild-type (S-layer-positive) strain *C. crescentus* NA1000. The *rsaF<sub>a</sub>* gene was destroyed by homologous recombination of an *rsaF<sub>a</sub>* gene frag-

ment containing an internal streptomycin resistance insertion cassette by using pK18mobsacB:*rsaF<sub>a</sub>*ΩSm as a suicide vector. PYE plates containing streptomycin or kanamycin were used to identify single-crossover events, and five rounds of subculturing were used to encourage a second recombination event. Bacteria were grown on 5% sucrose-PYE plates to select the second recombination event. Subsequent replicate plating on PYE-streptomycin and PYE-kanamycin plates confirmed the removal of the Km<sup>r</sup> gene. Colonies were then screened by PCR using primers *rsaF<sub>a</sub>*-1 and *rsaF<sub>a</sub>*-2 to determine if the recombination event resulted in restoration of wild-type *rsaF<sub>a</sub>* or incorporation of the *rsaF<sub>a</sub>*ΩSm gene fragment.

The *rsaF<sub>b</sub>* gene was destroyed by inactivation with an N- and C-terminal-deleted form of *rsaF<sub>b</sub>*. Selection on PYE-chloramphenicol was used to identify integration of the plasmid into the genome. Colonies were screened by PCR using primers 1060 and *rsaF<sub>b</sub>*-190.

The double *rsaF* knockout was created by homologous recombination using pK18mobsacB:*rsaF<sub>a</sub>*ΩSm and JS1008 (*rsaF<sub>b</sub>*)-competent cells. Screening for homologous recombination of pK18mobsacB:*rsaF<sub>a</sub>*ΩSm was done in the same manner as for the single *rsaF<sub>a</sub>*ΩSm knockout except that chloramphenicol was used in all media to maintain the *rsaF<sub>b</sub>* knockout. PCR confirmation of both *rsaF* gene knockouts was performed with the primers and conditions described above.

**Antibody production.** Antibodies to detect RsaA were raised against a form of RsaA containing only the N- and C-terminal portions of the protein, referred to as 188/784. This internal deletion form of the RsaA protein was previously described in reports of linker mutagenesis studies (10). Essentially, the DNA fragment coding for the N-terminal aa 1 to 188 of RsaA was removed as an EcoRI-BamHI fragment, and the portion coding for the C-terminal aa 784 to 1025 was removed as a BamHI-HindIII fragment. The two fragments were ligated together at the BamHI site and then ligated into EcoRI-HindIII-cut pUC8; this maintained the translational frame of the protein. HindIII-cut pUC8:188/784 was ligated to the HindIII-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 distilled water (dH<sub>2</sub>O) to remove residual *C. crescentus* cells. Aggregates were solubilized with 4 M urea and dialyzed against dH<sub>2</sub>O to remove urea. Samples were then injected into a New Zealand White rabbit, and serum was collected and processed by standard protocols (34).

Polyclonal antibodies were produced against RsaF<sub>a</sub> by using a glutathione S-transferase (GST)-tagged protein. The pGEX4T3:*rsaF<sub>a</sub>* plasmid was expressed in *E. coli* DH5α but produced only inclusion bodies. Inclusion body isolation was performed by growing cells to an optical density at 600 nm of 1.0 and then pelleting and resuspending them in 1× phosphate-buffered saline with lysozyme (100 μg/ml) for 1 h at 25°C; RNase A (50 μg/ml) and DNase I (1 μg/ml) were then added to the suspension, and it was incubated for an additional hour at 25°C. After incubation, 10% (starting concentration) sodium dodecyl sulfate (SDS) was added at a 1:1 ratio with SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and boiled for 5 min, put on ice for 15 min, and centrifuged at 16,000 × g for 10 min. The inclusion body pellet was then recovered and solubilized with 4 M urea. Protein was dialyzed with several changes of dH<sub>2</sub>O for 2 days to remove the urea. A rabbit was immunized as described above.



**Protein analysis methods.** Surface protein from *C. crescentus* cells was extracted by low-pH extraction as previously described (49). To compare the amounts of S-layer protein extracted from different mutants, cells were normalized and loaded onto SDS-PAGE gels.

Whole-cell protein preparations were made with normalized cultures and were pelleted by centrifugation, and the pellets were washed twice with 10 mM Tris-HCl (pH 8). The cells were resuspended in 10 mM Tris-HCl (pH 8) with lysozyme at 25°C for 15 min, and then RNase A and DNase I were added (as described above) and incubated at 37°C for 30 min. Equal amounts of whole-cell protein preparations were loaded onto protein gels. Note that whole-cell preparation methods were altered for certain strains when required to determine the internal levels of S-layer. RsaA is attached to the cell surfaces of wild-type S-layer-positive strain NA1000, which required removal by low-pH extraction and subsequent washes of the cell pellet before whole-cell preparations could be made to allow assessment of internal levels of RsaA. To remove secreted RsaA from the S-layer-shedding JS1001 strain, culture medium was poured through a fine nylon mesh (350- $\mu$ m pore size) to remove aggregates so that they did not affect the internal RsaA levels detected in the whole-cell preparation.

Whole-culture preparations were made by using normalized cells as described above. Whole-culture preparations were not centrifuged, ensuring that any aggregated RsaA was small, well dispersed, and included in the preparation. Cultures were incubated with lysozyme, RNase A, and DNase I as described above. Urea was added to the samples to give a final concentration of 2 M to solubilize any microaggregates produced by S-layer-shedding strains. Equal amounts of whole-culture protein preparations were loaded onto SDS-PAGE gels.

**SDS-PAGE and Western immunoblotting.** SDS-PAGE was performed with 7.5 or 12% (as indicated) separating gels. Coomassie blue staining of gels and Western immunoblotting were performed by use of standard protocols (36) and 0.2- $\mu$ m-pore-size BioTrace NT nitrocellulose membranes (Pall Biosciences). Western blots were visualized by colorimetric or electro-chemiluminescence (ECL) developing methods. Colorimetric detection was performed as previously described (40). Chemiluminescent blotting was done with the Amersham Biosciences ECL Western blotting kit in accordance with the manufacturer's protocol. To generate quantitative values and standard deviations, all data reported for ECL Western blots are the result of at least three and as many as seven replicate assays.

Anti-188/784 antibodies were incubated at 1/15,000 for colorimetric Western blotting and 1/30,000 for chemiluminescent Western blotting. Incubation with primary anti-RsaF<sub>a</sub> was done at a 1/1,000 dilution for colorimetric blotting and at a 1/5,000 dilution for chemiluminescent blotting. Kodak X-OMAT LS film was used for visualization of chemiluminescent blots, while spot densitometry was done with a Bio-Rad VersaDoc 5000 system and the Quantity One (version 4.3.0) program. Within the exposure limits defined by the software, quantitative densitometry using the VersaDoc 5000 allows for a linear response over several orders of magnitude.

**Bioinformatic analysis.** All sequences were obtained from the Institute for Genomic Research (TIGR) *C. crescentus* genome database. The Biology Workbench website (<http://workbench.sdsc.edu/>) was used for protein sequence alignments with the BLASTP sequence alignment tools (1). ClustalW alignments of the RsaF<sub>a</sub> (accession no. NC002696, locus tag CC1015), RsaF<sub>b</sub> (accession no. NC002696, locus tag CC1318) and TolC proteins were done with MacVector 6.0.

**Electron microscopy.** Protein A-colloidal gold immunolabeling of *C. crescentus* by use of the anti-RsaF<sub>a</sub> antibody and 5-nm-diameter protein A-colloidal gold label was performed as previously described (35). The antibody was preabsorbed with JS1009 cells. Cells (1 ml of cells with an optical density at 600 nm of 1) were centrifuged at 16,000  $\times$  g for 5 min. The cell pellet was resuspended in 10 mM Tris-HCl (pH 8) and disrupted by sonication for 5 s with a microprobe at maximum power. Sonicated cells were centrifuged at 16,000  $\times$  g for 2 min, and the supernatant was discarded. Anti-RsaF<sub>a</sub> rabbit serum was added to suspend the pellet; the suspension was incubated on ice for 1 h, followed by centrifugation at 16,000  $\times$  g for 3 min. The supernatant was used for labeling. Unstained cells were imaged by whole-mount transmission electron microscopy.

## RESULTS

**Identification of two OMP genes.** Two possible candidates for the OMP gene with similarity to the gene for the *E. coli* TolC protein sequence were identified in the *C. crescentus* genome (33). These were named *rsaF<sub>a</sub>* and *rsaF<sub>b</sub>*. RsaF<sub>a</sub> has 23% identity and 45% similarity to TolC, and RsaF<sub>b</sub> has 25% identity and 47% similarity to *E. coli* TolC, as determined by

local sequence alignment (see Materials and Methods). The *rsaF<sub>a</sub>* gene (1,581 bp) is located downstream of the *rsaADE* genes after a gap of 5,025 bp containing five S-LPS-related genes. The *rsaF<sub>b</sub>* gene (1,452 bp) is located 322 kb (303 genes) away from *rsaF<sub>a</sub>* and is flanked by genes of unknown function. The two *rsaF* genes show significant similarity with each other, sharing 39% identity and 60% similarity, and may be the result of gene duplication. A BLAST search of the *C. crescentus* genome for other candidate OMPs by using RsaF<sub>a</sub> as a query sequence identified several predicted proteins with moderate E values. However these proteins are likely outer membrane lipoproteins, as percentage identities with lipoproteins are much higher (approximately 40 to 48%), and the TIGRFAM and Pfam protein families group them in the lipoprotein family.

**Disruption of the *rsaF<sub>a</sub>* and *rsaF<sub>b</sub>* genes.** Knockouts of *rsaF* genes were constructed by homologous recombination of inactivated *rsaF* genes in the wild-type *C. crescentus* strain NA1000. Disruption of *rsaF<sub>a</sub>* was performed by gene replacement with an *rsaF<sub>a</sub>* gene construct containing an antibiotic resistance cassette. A mutant resulting from a double-cross-over event was confirmed by PCR and designated JS1007. The *rsaF<sub>b</sub>* gene was disrupted via insertional inactivation, using an N- and C-terminal-deleted *rsaF<sub>b</sub>* fragment (*rsaF<sub>b</sub>* $\Delta$ N $\Delta$ C). The *rsaF<sub>b</sub>* $\Delta$ N $\Delta$ C fragment was inserted into the chromosome via homologous recombination with a nonreplicating plasmid, resulting in two tandem, nonfunctional copies of *rsaF<sub>b</sub>*. Confirmation by PCR showed that there were only disrupted forms of *rsaF<sub>b</sub>* in the chromosome (data not shown). One confirmed mutant was selected (JS1008). To create the double knockout strain, *rsaF<sub>a</sub>* was disrupted and confirmed in JS1008 in the same manner used for JS1007 and was designated JS1009.

To determine that RsaF<sub>a</sub> and RsaF<sub>b</sub> were not produced in the knockout strains, Western blot analysis was performed using polyclonal anti-RsaF<sub>a</sub> antibodies. Rabbit polyclonal antibodies were generated against a GST-tagged *rsaF<sub>a</sub>* gene product. Cross-reactivity between the two RsaF proteins was evident, and thus anti-RsaF<sub>b</sub> antibodies were not raised since the proteins can be differentiated by size (57.5 kDa for RsaF<sub>a</sub> and 50.2 kDa for RsaF<sub>b</sub>). Western immunoblot analysis demonstrated the loss of the RsaF proteins in the *rsaF* knockout strains (Fig. 1a). Densitometry analysis of the Western blots showed that the levels of the remaining RsaF protein in the single *rsaF* knockout strains were the same as those observed in the wild-type strains (data not shown).

**Effect of disruption of the *rsaF* genes on RsaA secretion.** Levels of RsaA secreted by the *rsaF* knockout strains were analyzed. When low-pH-extracted protein levels (representing secreted protein) of knockout and wild-type strains were compared, there was a progressive decrease in amounts of RsaA secreted as the *rsaF* genes were lost (Fig. 1b). Disrupting *rsaF<sub>a</sub>* decreased S-layer secretion to a greater extent than loss of *rsaF<sub>b</sub>* did, but both single *rsaF* mutants were still capable of secreting RsaA. Elimination of both *rsaF* genes (JS1009) led to complete loss of RsaA secretion. Levels of RsaA secretion could not be easily determined by SDS-PAGE and Coomassie blue staining, as small amounts of RsaA are not readily detected; therefore, Western immunoblotting was performed.

Quantification of S-layer secretion levels by chemiluminescent Western blotting showed that disruption of *rsaF<sub>b</sub>* de-

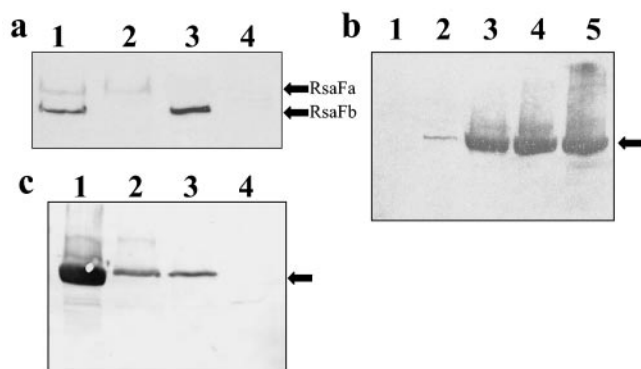


FIG. 1. (a) RsaF<sub>a</sub> and RsaF<sub>b</sub> levels in wild-type and *rsaF* knockout strains. Chemiluminescent anti-RsaF<sub>a</sub> Western blots of whole-culture protein preparation samples are shown. Lanes: 1, NA1000; 2, JS1008; 3, JS1007; 4, JS1009. Arrows indicate RsaF<sub>a</sub> and RsaF<sub>b</sub>. (b) Effect of disruption of the *rsaF* genes on S-layer secretion. Chemiluminescent Western blots of low-pH-extracted protein are shown. Lanes: 1, JS1003; 2, JS1009; 3, JS1007; 4, JS1008; 5, NA1000. RsaA is indicated by an arrow. (c) Determination of internal levels of RsaA in the *rsaF* double knockout. Whole-cell protein preparations were visualized by colorimetric Western blotting with polyclonal anti-188/784 RsaA antibody. Lanes: 1, NA1000; 2, JS1009; 3, JS1001; 4, JS1003. An arrow indicates RsaA.

creased RsaA secretion to  $75.6\% \pm 1.59\%$  (mean  $\pm$  standard deviation) of wild-type levels, whereas loss of *rsaF*<sub>a</sub> decreased RsaA secretion to  $54.5\% \pm 1.49\%$  of wild-type (NA1000) levels (Table 3). RsaA in the *rsaF* double knockout ( $4.44\% \pm 1.74\%$  of wild-type levels) was detectable only with Western blotting and was thought to be due to release of RsaA from cells that burst during the low-pH extraction.

To confirm that apparent RsaA secretion in JS1009 was due to burst cells during low-pH extraction, whole-cell preparations were analyzed to determine the RsaA levels inside the cells. To ensure that only internal RsaA was analyzed, surface protein was extracted from the cell surface (as outlined in Materials and Methods) before whole-cell protein preparations were examined. Colorimetric Western blots show levels of internal RsaA (Fig. 1c). Levels of RsaA observed with a whole-cell preparation of the double *rsaF* knockout strain ap-

TABLE 3. RsaA levels compared to those of the wild type determined by whole-culture preparations or low-pH extraction<sup>a</sup>

Strain	% RsaA relative to wild type <sup>b</sup>	
	Low pH extracted	Whole culture
NA1000	100	100
JS1008	$75.6 \pm 1.59$	$79.7 \pm 3.36$
JS1007	$54.4 \pm 1.49$	$56.4 \pm 0.47$
JS1009	$4.44 \pm 1.74$	$9.56 \pm 2.16$
JS1003	$0 \pm 0.09$	$0 \pm 0.09$
JS1009 <i>rsaF</i> <sub>a</sub>	$78.5 \pm 2.18$	$80.0 \pm 1.22$
JS1009 <i>rsaF</i> <sub>b</sub>	$56.2 \pm 2.08$	$57.3 \pm 0.83$
JS1001	NA	$94.8 \pm 1.08$

<sup>a</sup> Spot densitometry of chemiluminescent Western blots was done at least three times with polyclonal anti-188/784 antibodies and used to determine the relative levels of RsaA produced by the cells. Levels determined by densitometry were compared to wild-type NA1000 RsaA levels. All low-pH and whole-culture protein preparations were normalized prior to running samples.

<sup>b</sup> Values are means  $\pm$  standard deviations. NA, not applicable.

peared to be higher than those seen where the S-layer was removed by low-pH extraction. RsaA levels in JS1009 were similar to levels seen in the JS1001 strain, an S-layer-shedding strain, which was filtered and washed by centrifugation to remove secreted RsaA aggregates. Both the JS1009 and the JS1001 internal RsaA levels were less than those seen in the NA1000 parent strain. The levels of RsaA in the JS1001 strain were only internal levels of S-layer, as transported RsaA was shed and removed, suggesting that the RsaA detected in the double *rsaF* knockout was located internally. Furthermore, immunofluorescence studies of whole JS1009 cells with an anti-RsaA antibody did not detect the presence of any RsaA on the surface (data not shown).

#### Complementation of the secretion-deficient JS1009 strain.

To demonstrate that the *rsaF* knockouts were responsible for reduction or loss of the S-layer secretion, a multiple-copy broad-host-range plasmid containing either *rsaF*<sub>a</sub> (pBBR4:*rsaF*<sub>a</sub>) or *rsaF*<sub>b</sub> (pBBR4:*rsaF*<sub>b</sub>) was used to complement the double knockout strain. To determine if the plasmid-borne *rsaF* genes were expressed, whole-culture preparations were made using polyclonal antibodies against RsaF<sub>a</sub>. Western blotting showed that the plasmid-derived RsaF proteins were in fact overexpressed, at levels that were  $9.72 \pm 0.71$  (RsaF<sub>a</sub>)- and  $8.01 \pm 0.66$  (RsaF<sub>b</sub>)-fold higher than wild-type strain levels (Fig. 2a).

Despite overexpression mediated by multicopy vectors, in both cases complementation of the individual *rsaF* genes only restored S-layer secretion to levels comparable to that seen with corresponding single *rsaF* knockouts (Fig. 2b). The levels of RsaA secretion in the JS1009 *rsaF*<sub>a</sub> and JS1009 *rsaF*<sub>b</sub> strains were restored to levels that were only about 2% greater than those seen in the single *rsaF* knockouts (Table 3). Due to the lack of additional antibiotic selection markers, complementation of both *rsaF* genes in the double knockout was not done.

**Production of RsaA appears to be regulated when secretion is impeded.** The production of RsaA in whole-culture protein preparations was analyzed when secretion was inhibited in the RsaF mutants. Densitometry performed with Western blots showed that the levels of RsaA in the whole-culture preparations were almost identical to those observed by low-pH extraction (Fig. 3.), with the *rsaF*<sub>b</sub> knockout expression of RsaA at  $79.7\% \pm 3.36\%$  of wild-type levels and the *rsaF*<sub>a</sub> knockout expressing  $56.4\% \pm 0.47\%$  of wild-type levels (Table 3). However, the levels of RsaA seen with the double knockout strain were higher ( $9.56\% \pm 2.16\%$  of wild-type NA1000 levels) than those seen by low-pH extraction ( $4.44\% \pm 1.74\%$  of wild-type levels).

These results suggested that there was regulation of RsaA production in the cell when transport was impeded. To examine this further, the secretion of a recombinant RsaA with the charged RKKR furin cleavage sequence inserted at amino acid 723 (723/furin) was expressed in the various mutants. When this recombinant protein was expressed in JS1003, an S-layer-negative strain, no recombinant protein was secreted, apparently due to the inserted positive-charge cluster. Internal RsaA levels in the transporter mutants and JS1003::pWB9:723/furin were similar to those seen in the RsaF double knockout strain (Fig. 4): 5% of wild-type levels in the strain expressing this modified RsaA compared to 9% for the RsaF double knockout strain. There were no apparent breakdown products seen in

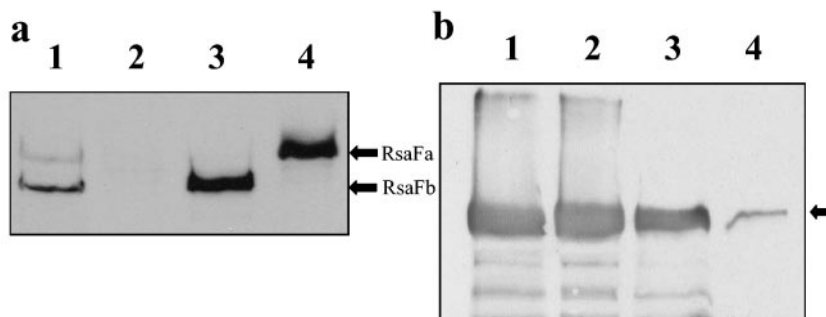


FIG. 2. (a) Complementation of JS1009 with the *rsaF* genes. Whole-culture preparations were compared by Western blotting and visualized by chemiluminescence using the anti-RsaF<sub>a</sub> antibodies. Lanes: 1, NA1000; 2, JS1009; 3, JS1009 *rsaF<sub>b</sub>*; 4, JS1009 *rsaF<sub>a</sub>*. (b) Complementation of the *rsaF* genes in *trans* restores S-layer secretion. Chemiluminescent Western blotting of low-pH-extracted protein using anti-188/784 RsaA antibodies is shown. Lanes: 1, NA1000; 2, JS1009 *rsaF<sub>a</sub>*; 3, JS1009 *rsaF<sub>b</sub>*; 4, JS1009. The arrow refers to RsaA.

any of the transporter or modified RsaA mutants. It is interesting, however, that both the JS1009 *rsaF* mutant strain and the JS1003::pWB9:723/furin strains grew much slower, with generation times of 114 and 108 min, respectively, compared to a generation time of 78 min for JS1003 and the wild-type NA1000, suggesting that retention of residual RsaA inside the cell is deleterious.

**Overexpression of RsaA and RsaF.** Because RsaF expression could be significantly increased in *trans* complementation experiments (see above), we wished to determine if the number of transporter units or the amount of RsaF was a limiting factor in the amount of RsaA that was secreted. Vector-borne copies of a single *rsaF* gene were inserted into S-layer-positive *C. crescentus* cells to determine if levels of RsaA secretion could be increased. An S-LPS-negative strain, JS1001, was used instead of the wild-type NA1000 to ensure that there was no possibility of RsaA regulation by surface crystallization, i.e., the possibility that RsaA secretion was impeded by full coverage of the bacterial surface. Levels of RsaA were determined by Western blot analysis of whole-culture preparations to ensure that any secreted RsaA was included in the sample.

Levels of RsaA production were not significantly increased when additional copies of *rsaF<sub>a</sub>* or *rsaF<sub>b</sub>* were expressed in JS1001 by addition of pBBR4:*rsaF<sub>a</sub>* or pBBR4:*rsaF<sub>b</sub>*. Both JS1001 *rsaF<sub>a</sub>* and JS1001 *rsaF<sub>b</sub>* produced levels of RsaA only slightly greater than those produced by JS1001 and the wild-type strain NA1000 (Table 4). Levels of RsaF<sub>a</sub> and RsaF<sub>b</sub> were determined by immunoblotting and densitometry analysis and were similar to those seen in the *trans* complementation strains described above (data not shown).

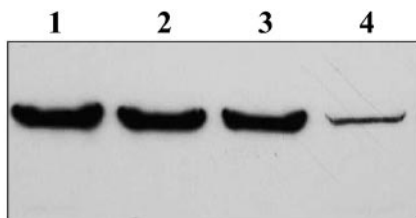


FIG. 3. RsaA production and RsaA secretion levels are comparable. Whole-culture preparations run on SDS-12% PAGE gels were transferred to a nitrocellulose membrane and analyzed with anti-188/784 RsaA antibodies. Lanes: 1, NA1000; 2, JS1008; 3, JS1007; 4, JS1009.

To be assured that additional RsaF<sub>a</sub> was properly targeted to the outer membrane, protein A-colloidal gold labeling with anti-RsaF antibody was used to assess the levels of RsaF on the outer membrane surface. A uniform low-level labeling was noted with JS1001 (Fig. 5), indicating that some portion of the RsaF OMPs were surface exposed when the oligosaccharide chains of the S-LPS fraction of total LPS were eliminated (see below). Labeling of JS1001 *rsaF<sub>a</sub>* and JS1001 *rsaF<sub>b</sub>* (data 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 dense labeling was noted (Fig. 5). We interpreted that finding as an indication that plasmid copy numbers for the moderate-copy-number plasmid pBBR4 vary significantly from cell to cell, suggesting that it is not a stably maintained plasmid. Nevertheless, it appeared that some cells were expressing much higher levels of RsaF<sub>a</sub> and that the protein was targeted correctly to the outer membrane.

Interestingly, in contrast to the findings for 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 (data not shown). Presumably, this smooth form of LPS effectively blocks antibody access to the OMPs,

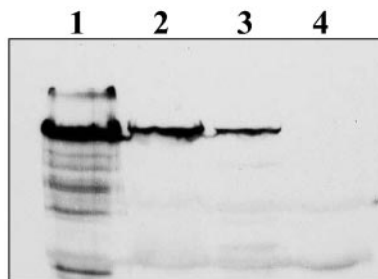


FIG. 4. Impeded RsaA transport in *rsaF* mutant (JS1009) and RsaA mutant (JS1003 Hps12furin). Whole-cell preparations were loaded on SDS-12% PAGE gels and compared by Western blotting with anti-188/784 RsaA antibodies. The NA1000 strain was subjected to low-pH extraction before whole-cell preparations were made to minimize the levels of S-layer. No RsaA breakdown products are present in the mutants. Lanes: 1, NA1000; 2, JS1009; 3, JS1003::pWB9:723/furin; 4, JS1003.



TABLE 4. RsaA levels compared to those of JS1001 determined by whole-culture preparations<sup>a</sup>

Strain	% RsaA relative to JS1001 <sup>b</sup>
JS1001 .....	100
JS1001 <i>rsaF<sub>a</sub></i> .....	103.3 ± 2.95
JS1001 <i>rsaF<sub>b</sub></i> .....	102.8 ± 2.38
JS1001::pWB9 <i>rsaA</i> ΔP .....	100.8 ± 1.47
JS1001 <i>rsaF<sub>a</sub></i> ::pWB9 <i>rsaA</i> ΔP .....	128.3 ± 3.69

<sup>a</sup> Spot densitometry of chemiluminescent Western blots was done in triplicate using polyclonal anti-188/784 antibodies and used to determine the relative levels of RsaA produced by the cells. Levels determined by densitometry were compared to JS1001 RsaA levels. All whole-culture protein preparations were normalized prior to running samples.

<sup>b</sup> Values are means ± standard deviations.

perhaps indicating that their exposure on the surface is minimal.

Since JS1001 has only the single chromosome resident copy of *rsaA*, we considered whether, in the presence of elevated OMP levels, RsaA transcription or translation might then determine the maximum levels of RsaA secretion. To address this possibility, a multicopy plasmid-borne *rsaA* gene was introduced into the JS1001 *rsaF<sub>a</sub>* strain, and whole-culture protein levels were compared (Fig. 6). The resulting strain, JS1001 *rsaF<sub>a</sub>*::pWB*rsaA*ΔP, produced 28.3% ± 3.69% more S-layer protein than the wild-type strain JS1001 or strain JS1001::

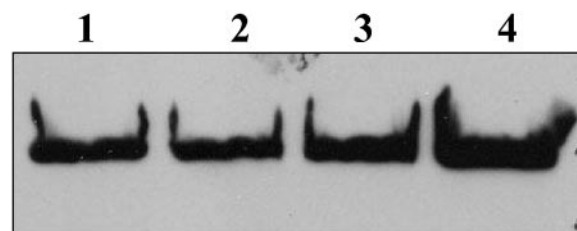


FIG. 6. Effect of RsaF overexpression in the JS1001 strain. Whole-culture preparations were run on SDS-12% PAGE gels and compared by Western blotting with anti-188/784 antibodies. Lanes: 1, JS1001; 2, JS1001 *rsaA*; 3, JS1001 *rsaF<sub>a</sub>*; 4, JS1001 *rsaF<sub>a</sub> rsaA*.

pWB*rsaA*ΔP. Thus, elevated secretion of RsaA is possible but requires both overexpression of RsaA and at least the RsaF<sub>a</sub> OMP.

## DISCUSSION

Here we have described the discovery of two OMPs that function as part of the type I secretion machinery for RsaA in *C. crescentus*. This arrangement of the OMP components, where there are two similar but independent proteins functioning as the OMP component, is different from any other known type I secretion system and is the first report of a type I

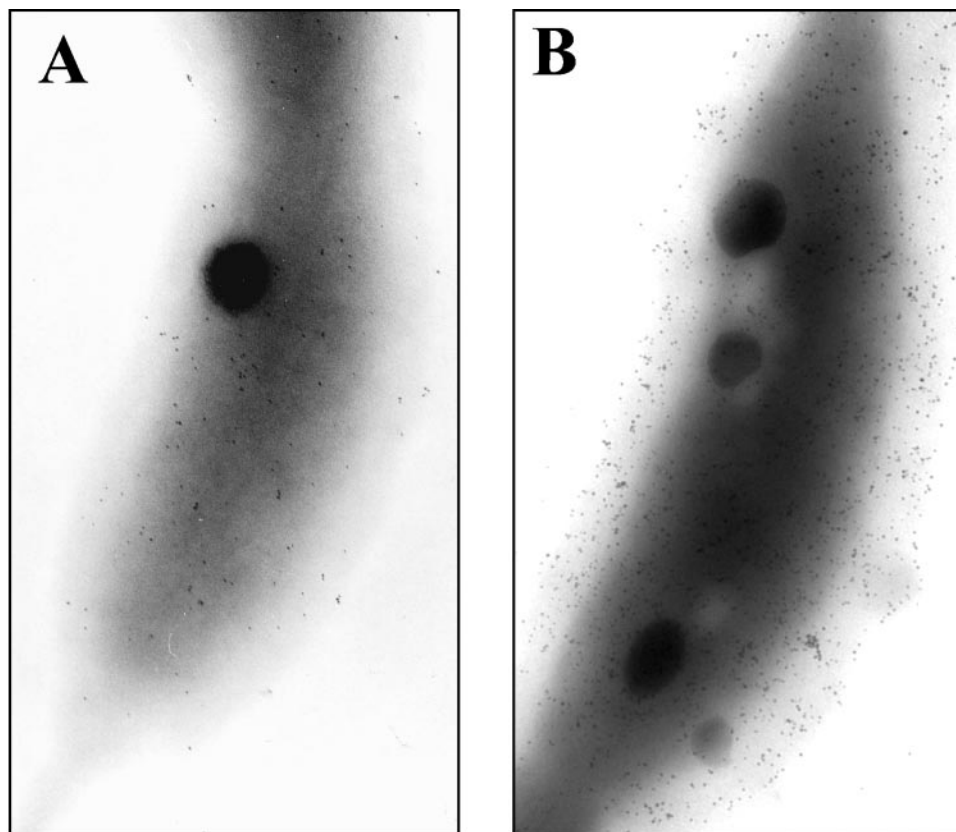


FIG. 5. Colloidal gold labeling of surface-displayed RsaF. Surface-displayed RsaF (A and B) was determined for the JS1001 strains overexpressing *rsaF<sub>a</sub>* or *rsaF<sub>b</sub>* (data not shown) by electron microscopy with anti-RsaF<sub>a</sub> and colloidal gold labeling. (A) JS1001 shows wild-type levels of RsaF with moderate labeling. (B) The JS1001 *rsaF<sub>a</sub>* strain shows a significant increase in surface display of RsaF. Note that only about 20% of the JS1001 *rsaF<sub>a</sub>* cells showed a significant increase in RsaF display.

secretion system which can utilize either of two OMPs for the secretion of the same protein. Of particular note is that while both proteins were capable of acting alone as the OMP component of the transport system, neither alone could enable wild-type secretion levels of RsaA. The amount of RsaA produced and secreted was consistently lower in the single OMP knockout strains as well as the complemented strains. Indeed, when overexpressed, the individual OMPs could restore secretion only to the level of their apparent contribution in the normal, single-gene-copy situation. This finding suggests that the level of secretion may not be limited solely by the number of active transporter complexes (when only one OMP is expressed) but also by the transporter composition. We suggest that the presumed trimeric OMP complex may function most effectively when assembled as a heterotrimer of RsaF<sub>a</sub> and RsaF<sub>b</sub>, and that this may be the native situation. This possibility is tentative, and we are presently devising approaches to study this possibility more directly. In this scenario, heterotrimers are technically not required for RsaA secretion, although survival in a natural environment may require maximal secretion of RsaA (and thereby heterotrimer formation) to maintain complete surface coverage of this protective device. Although an attractive hypothesis, heterotrimer formation is not proven, and other explanations for the inability of a single RsaF to accomplish wild-type expression levels, even when overexpressed, are possible.

A related finding is that if one *rsaF* gene is destroyed, the level of the remaining *rsaF* gene product did not increase to compensate for the loss of the other *rsaF* gene (Fig. 1a). This finding is perhaps predictable given the remote location of *rsaF<sub>b</sub>*, but does suggest that although the roles of RsaF<sub>a</sub> and RsaF<sub>b</sub> are functionally additive, their expression is not tightly coordinated. This is a significant departure from other type I systems, where (except for *E. coli* TolC and *S. marcescens* HasF) the OMP may be cotranscribed with the other transporter components (7).

We had previously assigned a tentative transporter function for RsaF<sub>a</sub> (then called RsaF) (4) and its proximity to other S-layer related genes, but had not provided experimental data to confirm a role in RsaA transport. In contrast to other opinions (21), it was our view that no predicted gene products in the *C. crescentus* genome sequence other than RsaF<sub>a</sub> and RsaF<sub>b</sub> had sufficiently high homology to TolC to be seriously considered OMP candidates. The direct genetic data presented here confirm that assessment, dismissing the notion of a third OMP (21). Although both RsaF proteins are required to secrete wild-type levels of S-layer in *C. crescentus*, RsaF<sub>a</sub> plays a more significant role as its loss reduced RsaA secretion to  $54.4\% \pm 1.49\%$  of wild-type levels whereas loss of the RsaF<sub>b</sub> protein led to only  $75.6\% \pm 1.59\%$  of wild-type secretion (Fig. 1b). For this reason, we chose RsaF<sub>a</sub> for overexpression in these initial efforts to increase RsaA secretion.

Because type I secretion relies on a C-terminal secretion signal, the target protein (here RsaA) must be completely synthesized before the export process begins. Therefore, a highly expressed type I secretion system leads to problems in biochemical logistics for the affected bacterium. As there is little primary sequence homology among various type I secretion signals yet cross-expression can often be demonstrated, it has been suggested by us and others that a degree of folding

must occur to generate a structural motif that is recognized by the secretion machinery (10, 29). Thus, if secretion is somehow impaired, rapid accumulation of large (98-kDa) monomers could be deleterious. One solution to this problem could be rapid degradation of protein that cannot be exported. We have suggested that the S-layer-associated protease (the product of *sapA*), which appears poised to recognize defects in the nascent RsaA monomer, might play such a role (47). The data here, however, gave no indication that detectable degradation occurs. Instead, we simply noted a much reduced level (9.56% of wild-type RsaA) of full-length monomer, retained inside the cells in both the double RsaF knockout and in cells attempting to export the recombinant RsaA displaying the furin cleavage site (RKKR) through a fully functional transporter complex. On this latter point, a preliminary threading of the RsaF<sub>a</sub> and RsaF<sub>b</sub> proteins onto the homologous TolC protein (for which the structure has been determined) predicted exposed charge-dense regions in the interior of the transporter channel (data not shown). We estimate that this may be the reason why the addition of the furin cleavage site (RKKR) leads to a blockage of RsaA transport, while a variety of other recombinant RsaA proteins with 100 to 200 amino acid insertions have been readily secreted (34, 47).

These data are consistent with a feedback mechanism involving down-regulation of RsaA production, though we cannot completely exclude the possibility that rapid and complete degradation of unsecreted protein occurs. In such situations, the cells grew much slower, there were few motile cells, and most cells were misshapen (data not shown). It may be that if autoregulation was triggered it may not be specific, affecting the synthesis of other proteins as well. If autoregulation of internal RsaA occurs, we suggest that the overall level of RsaA produced in the cell may in effect be determined by the amount of protein that can be secreted.

The *rsaA* gene, like other S-layer genes, is transcribed continuously throughout the cell cycle (17, 39). In *Lactobacillus brevis*, the *slpA* gene is transcribed during stationary phase even when the S-layer protein is not produced (23). Therefore, it is likely that any sort of regulation would occur at the translational level. The S-layer of *Thermus thermophilus* HB8 has been shown to autoregulate the translation of the *slpA* gene by binding a C-terminal SlpA fragment to the 5' mRNA (16). It may be that a similar situation occurs in *C. crescentus*, and this possibility is currently being investigated.

Our results suggest that RsaA expression levels may be increased only by increasing both the number of transporters and the expression of RsaA. Overexpression of an individual *rsaF* gene in the absence of the other *rsaF* gene was not an appropriate course, since it did not even lead to normal levels of RsaA secretion, so we instead began overexpressing individual transporter components in the presence of a normal complement of remaining transporter proteins. By using the S-layer-shedding strain JS1001, which produces the same amount of RsaA as the parental strain 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 were present and additional copies of *rsaF<sub>a</sub>* were introduced, the secretion of RsaA was not significantly increased (Fig. 6.). Similarly, a strain containing additional vector-borne copies of the *rsaA* gene with a wild-type complement of *rsaF<sub>a</sub>* and *rsaF<sub>b</sub>*,



genes did not have RsaA levels exceeding those of the wild type. However, the strain overexpressing both RsaA and RsaF<sub>a</sub> produced 28.3% ± 3.69% more protein than the wild type (Table 4). This is a remarkable increase in RsaA secretion as wild-type levels of RsaA already represent 10 to 12% of total cell protein. Furthermore, the vectors used for overexpression are seemingly unstable, which may mean that only 20% of the cells were truly overexpressing RsaF<sub>a</sub>. Stable expression of RsaF<sub>a</sub> by all cells may lead to still higher levels of RsaA secretion.

These results can be compared to those of experiments on hemolysin secretion where additional plasmid-borne copies of *tolC* were expressed in gram-negative bacteria containing the HlyA secretion apparatus (43). Despite the presence of additional copies of TolC, neither enhancing nor deteriorating effects were noted. The authors suggested that the HlyB and HlyD protein levels might be the limiting factor when only the OMP levels were increased. We suggest instead that translation levels of the transported protein, HlyA, may have been the limiting factor.

It is likely that RsaA secretion is determined by the type and level of both RsaF proteins and the level of *rsaA* translation. 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. The ability to increase RsaA secretion may be a function of increasing the number of transporter “holes” in the bacteria. It has been shown that the HlyB and HlyD proteins exist in a preformed complex in the inner membrane and that when HlyA is engaged, the complex recruits the TolC protein (44). Presuming that the RsaA transport complex is analogous, increasing the number of OMPs in the outer membrane may make it easier for the secretion apparatus components to engage and transport RsaA. Additional studies are under way to determine if coordinate up-regulated expression of various combinations of the transporter elements will lead to still greater levels of RsaA expression or whether other factors, such as membrane stability or available ATP pools, will set limits.

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