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 rsaFa and rsaFb were identified by similarity to the Escherichia coli hemolysin secretion OMP TolC by using the C. crescentus genome sequence. The rsaFa gene is located several kilobases downstream of the other transporter genes, whereas rsaFb is completely unlinked. An rsaFa knockout had ~56% secretion compared to wild-type levels, while the rsaFb 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, or in the case of rsaFb, with 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 rsaFa (with normal rsaFb 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 archaeabacteria. 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 sequence 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 (26) 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,
INVOLVES TWO OMPs

TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>C. crescentus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA1000</td>
<td>Ap&lt;sup&gt;a&lt;/sup&gt;, syn-1000; variant of wild-type strain CB15 that synchronizes well</td>
<td>ATCC 19089</td>
</tr>
<tr>
<td>JS1001</td>
<td>S-LPS mutant of NA1000, sheds S-layer into medium</td>
<td>Edwards and Smit (14)</td>
</tr>
<tr>
<td>JS1003</td>
<td>NA1000 with rsa&lt;sub&gt;A&lt;/sub&gt; interrupted by KSAC Km&lt;sup&gt;a&lt;/sup&gt; cassette</td>
<td>Edwards and Smit (14)</td>
</tr>
<tr>
<td>JS1007</td>
<td>Sm&lt;sup&gt;a&lt;/sup&gt;, NA1000 rsa&lt;sub&gt;F&lt;/sub&gt;&lt;sub&gt;a&lt;/sub&gt; strain</td>
<td>This study</td>
</tr>
<tr>
<td>JS1008</td>
<td>Cm&lt;sup&gt;a&lt;/sup&gt;, NA1000 rsa&lt;sub&gt;F&lt;/sub&gt;&lt;sub&gt;b&lt;/sub&gt; strain</td>
<td>This study</td>
</tr>
<tr>
<td>JS1009</td>
<td>Cm&lt;sup&gt;a&lt;/sup&gt;, Sm&lt;sup&gt;a&lt;/sup&gt;, NA1000 rsa&lt;sub&gt;F&lt;/sub&gt;&lt;sub&gt;a&lt;/sub&gt; rsa&lt;sub&gt;F&lt;/sub&gt;&lt;sub&gt;b&lt;/sub&gt; strain</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli DH5α</strong></td>
<td>recA endA</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

| **Plasmids**                            |                          |                     |
| pHP45Ω                                  | Ap<sup>a</sup>, Sm<sup>a</sup>; source of SM cassette removed as-Smal fragment | Fellay et al. (15)   |
| pBSKIIIEH                               | Ap<sup>a</sup>; modified pBSKII cloning vector with EcoRI-, EcoRV-, HindIII-modified MCS | This study           |
| pBSKIIESH                               | Ap<sup>a</sup>; modified pBSKII cloning vector with EcoRI-, Stul-, HindIII-modified MCS | This study           |
| pTZ18UCHE                               | Cm<sup>a</sup>; cloning vector | This study           |
| pTZ18UCHE:rsa<sub>F</sub><sub>a</sub>ΔNΔC | Ap<sup>a</sup>, Sm<sup>a</sup>, rsa<sub>F</sub><sub>a</sub> gene fragment with SM cassette inserted at PstI site | This study           |
| pBSKIIIEH:rsa<sub>F</sub><sub>a</sub>ΩSm | Km<sup>a</sup>; broad-host-range plasmid derived from pBBR1 | This study           |
| pBBR4                                   | rsa<sub>F</sub><sub>a</sub> Sm<sup>a</sup>, rsa<sub>F</sub><sub>a</sub> gene inserted EcoRI/BamHI in pBBR4 | This study           |
| pBBR4:rsa<sub>F</sub><sub>a</sub>ΩP   | rsa<sub>F</sub><sub>a</sub> Km<sup>a</sup>, rsa<sub>F</sub><sub>a</sub> gene inserted EcoRI/BamHI in pBBR4 | This study           |
| pWB9/723/furin                          | Cm<sup>a</sup>, rsa<sub>F</sub><sub>a</sub> gene and rsa<sub>F</sub><sub>a</sub> promoter strain | Bingle et al. (9)    |
| pWB9/723/furin                          | Cm<sup>a</sup>, rsa<sub>F</sub><sub>a</sub> containing furin cleavage site (RKKR) in BamHI site at aa 723 | This study           |
| pGEX4T3                                  | Ap<sup>a</sup>; GST-tagged expression vector | Amersham              |
| pGEX4T3:rsa<sub>F</sub><sub>a</sub>Ω      | Ap<sup>a</sup>; GST-tagged expression vector with in-frame BamHI-EcoRI | This study           |
| pK18mobssacB                             | Kmr<sup>a</sup>, Sm<sup>a</sup>, E. coli-based suicide vector | Schafer et al. (37)  |
| pK18mobssacB:rsa<sub>F</sub><sub>a</sub>ΩSm | Kmr<sup>a</sup>, Sm<sup>a</sup>, E. coli-based suicide vector with rsa<sub>F</sub><sub>a</sub>ΩSm fragment | This study           |

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

50). Thus, it appears to interact with inner membrane translo-
cases involved in drug and cation efflux as well as protein
secretion. It has been suggested that the reason ToC is mul-
tifunctional 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 Tn<sub>5</sub> 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 (rsa<sub>F</sub><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 quantify the amount of RsaA produced by the mu-
tant strain.

In this study, we identify a second OMP gene and charac-
terize the products of rsa<sub>F</sub><sub>a</sub> and rsa<sub>F</sub><sub>b</sub>, showing that both are
the only OMP proteins involved in S-layer transport. Eliminat-
ing either protein results in decreased RsaA secretion, indicat-
ing that either can function as the OMP component but ap-
parently act cooperatively as it was not possible to restore
wild-type levels of RsaA secretion by overexpression of indi-
vidual OMPs. Interestingly, if both were present, overexpres-
sion 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, 0.02% 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 Pfu
dNA polymerase (Invitrogen) following the manufacturer’s suggested protocols.
Strain NA1000 chromosomal DNA was used as the template for all PCR prod-
ucts. Chromosomal DNA was isolated with standard phenol-chloroform extrac-
tion methods (36).

All primers used in this study are listed in Table 2. The pBSKIIIEH vector
was constructed from pBSKII (Stratagene). The BshHII fragment containing the
multiple cloning site was removed and replaced with annealed oligonucleotides EEEH-1 and EEEH-2 forming EcoRI, EcoRV, and HindIII sites. pBSKIIIESH was created similarly to pBSKIIIEEH with annealed oligonucleotides ESH-1 and ESH-2 forming EcoRI, StuI, and HindIII sites. PCR products containing the rsaF-, rsaF-2, rsaF-3, and rsaF-4 genes were generated by using primers rsaF-1 and rsaF-2 and primers rsaF-3 and rsaF-4, respectively. PCR products were ligated into the EcoRV site of the pBSKIIIEEH vector and called pBSKIIIEEHSaRF, respectively.

pBBR4 was created from plasmids pBBR1MCS and pUC4 KISS. The kanamycin resistance (Kmr) fragment from pUC4 KISS was removed with PstI and blunted with T4 polymerase. A 0.3-kbp portion of the chloramphenicol resistance (Cm') encoding gene was removed from pBBR1MCS with Dral and replaced with the blunted Kmn' fragment, producing a Kmn' broad-host-range vector that replicates in C. crescentus. pBBR3 was constructed in a similar manner to that used for pBBR4, using a blunt HindIII-cut streptomycin resistance (Sm') fragment of pHP45.

Plasmids pBBRrsaFSaRF and pBBRrsaFSaRF were made by using the BamHI-EcoRI fragment from pBSKIIIEEHSaRF and pBSKIIIESH rsaF, respectively.

pBSKIIIEEHSaRF was created by using plasmids pBSKIIIEEHSaRF and pHPI45. The streptomycin resistance insertion cassette was removed as a SmI fragment and blunt-end ligated into pBSKIIIEEHSaRF at a blunt PstI site inside rsaF. The resulting plasmid, pBSKIIIESH rsaF, was then used to make pK18mobsaRFbTaFb. The EcoRI-HindIII fragment containing the rsaFbTaFbSm fragment was cloned into EcoRI-HindIII-cut plasmid pK18mobsaRe11. A PCR product containing a truncated form of rsaFb, missing the N and C termini was generated by using primers rsaFbNC-1 and rsaFbNC-2. This PCR product was blunt-end ligated into the StuI site of pBSKIIIESH, creating pBSKIIIESH rsaFbΔSm. pTZ18U/ΔC. The double rsaFb knockout was created by homologous recombination using pK18mobsaRFbTaFbSm and JS1008 (rsaFb-competent) cells. Screening for homologous recombination of pK18mobsaRFbTaFbSm was done in the same manner as for the single rsaFbTaFbSm knockout except that chloramphenicol was used in all media to maintain the rsaFbTaFbSm. PCR confirmation of both rsaFb gene knockouts was performed with the primers and conditions described above.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>EEEH-1</td>
<td>5'-CGCGCTGATATCCGAATTCGTCAG-3'</td>
</tr>
<tr>
<td>EEEH-2</td>
<td>5'-GCGCCGCGCAGGATATCCGACAGG-3'</td>
</tr>
<tr>
<td>ESH-1</td>
<td>5'-GCGCGCTGATATCCGAATTCGTCAG-3'</td>
</tr>
<tr>
<td>ESH-2</td>
<td>5'-GCGCCGCGCAGGATATCCGACAGG-3'</td>
</tr>
<tr>
<td>rsaFb-1</td>
<td>5'-CGCGGATCTAGTTGATGCGACCGAGTCGTCAG-3'</td>
</tr>
<tr>
<td>rsaFb-2</td>
<td>5'-CGCGGATCTAGTTGATGCGACCGAGTCGTCAG-3'</td>
</tr>
<tr>
<td>rsaFb-3</td>
<td>5'-CGCGGATCTAGTTGATGCGACCGAGTCGTCAG-3'</td>
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<tr>
<td>rsaFb-4</td>
<td>5'-CGCGGATCTAGTTGATGCGACCGAGTCGTCAG-3'</td>
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<tr>
<td>rsaFbNC-1</td>
<td>5'-GAAAGGTCACTTAACCTTTCTCAGGAGCCTAGG-3'</td>
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<tr>
<td>rsaFbNC-2</td>
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<tr>
<td>che-1</td>
<td>5'-GGAAAGGATGTCGATTTTCTGCGAAGAGGG-3'</td>
</tr>
<tr>
<td>che-2</td>
<td>5'-GGAAAGGATGTCGATTTTCTGCGAAGAGGG-3'</td>
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<tr>
<td>RKKR-1</td>
<td>5'-TCGAGACCGATTGCCAGAAGAAAGGGG-3'</td>
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<td>RKKR-2</td>
<td>5'-TCGAGACCGATTGCCAGAAGAAAGGGG-3'</td>
</tr>
<tr>
<td>rsaFb-190</td>
<td>5'-GGAGACGGGCATCCGAGGTCG-3'</td>
</tr>
</tbody>
</table>

**TABLE 2. Primers used in this study**

**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 (dH2O) to remove residual C. crescentus cells. Aggregates were solubilized with 4 M urea and dialyzed against dH2O 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 RsaFb by using a glutathione S-transferase (GST)-tagged protein. The pGEX3T.rsafb 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 lysosyme (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 dH2O for 2 days to remove the urea. A rabbit was immunized as described above.

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**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-sheding JS1001 strain, culture medium was poured through a fine nylon mesh (350-μm 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-sheding 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-μm-pore-size BioTrace NT nitrocellulose membranes ( Pall Biosciences). Western blots were visualized by coloriometric 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-RsaFb 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 blot, 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 alignment (see Materials and Methods). The BLASTP sequence alignment tool (1). ClustalW alignments of the RsaF genes (accession no. NC002696, locus tag CC1015), RsaFb (accession no. NC002696, locus tag CC1318) and ToIC proteins were done with MacVector 6.0.

**Electron microscopy.** Protein A-collodial gold immunolabeling of *C. crescentus* by use of the anti-RsaFb antibody and 5-nm-diameter protein A-collodial 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 × 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 × g for 2 min, and the supernatant was discarded. Anti-RsaFb rabbit serum was added to suspend the pellet; the suspension was incubated on ice for 1 h, followed by centrifugation at 16,000 × 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* ToIC protein sequence were identified in the *C. crescentus* genome (33). These were named rsaFa and rsaFb. RsaFa has 23% identity and 45% similarity to ToIC, and RsaFb has 25% identity and 47% similarity to *E. coli* ToIC, as determined by local sequence alignment (see Materials and Methods). The rsaFa 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 rsaFb gene (1,452 bp) is located 322 kb (303 genes) away from rsaFa 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 RsaFa 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 rsaFa and rsaFb genes.** Knockouts of rsaF genes were constructed by homologous recombination of inactive rsaF genes in the wild-type *C. crescentus* strain NA1000. Disruption of rsaFa was performed by gene replacement with an rsaFb gene construct containing an antibiotic resistance cassette. A mutant resulting from a double-crossover event was confirmed by PCR and designated JS1007. The rsaFb gene was disrupted via insertional inactivation, using an N- and C-terminal-deleted rsaFb fragment (rsaFbΔNAC). The rsaFbΔNAC fragment was inserted into the chromosome via homologous recombination with a nonreplicating plasmid, resulting in two tandem, nonfunctional copies of rsaFb in the chromosome (data not shown). One confirmed mutant was selected (JS1008). To create the double knockout strain, rsaFa was disrupted and confirmed in JS1008 in the same manner used for JS1007 and was designated JS1009.

To determine that RsaFa and RsaFb were not produced in the knockout strains, Western blot analysis was performed using polyclonal anti-RsaFa antibodies. Rabbit polyclonal antibodies were generated against a GST-tagged rsaFa gene product. Cross-reactivity between the two RsaF proteins was evident, and thus anti-RsaFb antibodies were not raised since the proteins can be differentiated by size (57.5 kDa for RsaFb and 50.2 kDa for RsaFa). 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 rsaFb 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 rsaFb de-
creased RsaA secretion to 75.6% ± 1.59% (mean ± standard deviation) of wild-type levels, whereas loss of rsaFa decreased RsaA secretion to 54.5% ± 1.49% of wild-type (NA1000) levels (Table 3). RsaA in the rsaF double knockout (4.44% ± 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 bursts 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 appeared 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 rsaFa (pBBR4: rsaFa) or rsaFb (pBBR4: rsaFb) 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 RsaFa. Western blotting showed that the plasmid-derived RsaF proteins were in fact overexpressed, at levels that were 9.72 ± 0.71 (RsaFa)- and 8.01 ± 0.66 (RsaFb)-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 rsaFa and JS1009 rsaFb 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 rsaFa knockout expression of RsaA at 79.7% ± 3.36% of wild-type levels and the rsaFb knockout expressing 56.4% ± 0.47% of wild-type levels (Table 3). However, the levels of RsaA seen with the double knockout strain were higher (9.56% ± 2.16% of wild-type NA1000 levels) than those seen by low-pH extraction (4.44% ± 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

![Image](https://via.placeholder.com/150)

**FIG. 1.** (a) RsaFa and RsaFb levels in wild-type and rsaF knockout strains. Chemiluminescent anti-RsaFa Western blots of whole-culture protein preparation samples are shown. Lanes: 1, NA1000; 2, JS1008; 3, JS1007; 4, JS1009. Arrows indicate RsaFa and RsaFb. (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.

**TABLE 3.** RsaA levels compared to those of the wild type determined by whole-culture preparations or low-pH extraction

<table>
<thead>
<tr>
<th>Strain</th>
<th>% RsaA relative to wild type&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Low pH extracted</td>
<td>Whole culture</td>
<td></td>
</tr>
<tr>
<td>NA1000</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>JS1008</td>
<td>75.6 ± 1.59</td>
<td>79.7 ± 3.36</td>
<td></td>
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<tr>
<td>JS1007</td>
<td>54.4 ± 1.49</td>
<td>56.4 ± 0.47</td>
<td></td>
</tr>
<tr>
<td>JS1009</td>
<td>4.44 ± 1.74</td>
<td>9.56 ± 2.16</td>
<td></td>
</tr>
<tr>
<td>JS1003</td>
<td>0 ± 0.09</td>
<td>0 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>JS1009 rsaFa</td>
<td>78.5 ± 2.18</td>
<td>80.0 ± 1.22</td>
<td></td>
</tr>
<tr>
<td>JS1009 rsaFb</td>
<td>56.2 ± 2.08</td>
<td>57.3 ± 0.83</td>
<td></td>
</tr>
<tr>
<td>JS1001</td>
<td>NA</td>
<td>94.8 ± 1.08</td>
<td></td>
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</tbody>
</table>

<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 ± standard deviations. NA, not applicable.
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 rsaFa or rsaFb were expressed in JS1001 by addition of pBBR4:rsaFa or pBBR4:rsaFb. Both JS1001 rsaFa and JS1001 rsaFb produced levels of RsaA only slightly greater than those produced by JS1001 and the wild-type strain NA1000 (Table 4). Levels of RsaFa and RsaFb were determined by immunoblotting and densitometry analysis and were similar to those seen in the trans complementation strains described above (data not shown).

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 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 rsaFa and JS1001 rsaFb, (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 RsaFa 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. 2. (a) Complementation of JS1009 with the rsaF genes. Whole-culture preparations were compared by Western blotting and visualized by chemiluminescence using the anti-RsaFa antibodies. Lanes: 1, NA1000; 2, JS1009; 3, JS1009 rsaFa; 4, JS1009 rsaFb. (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 rsaFa; 3, JS1009 rsaFb; 4, JS1009. The arrow refers to RsaA.

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.

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.
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 rsaFa strain, and whole-culture protein levels were compared (Fig. 6). The resulting strain, JS1001 rsaFa::pWB9rsaAΔP, produced 28.3% ± 3.69% more S-layer protein than the wild-type strain JS1001 or strain JS1001::pWB9rsaAΔP. Thus, elevated secretion of RsaA is possible but requires both overexpression of RsaA and at least the rsaFa 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

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**TABLE 4. RsaA levels compared to those of JS1001 determined by whole-culture preparations**

<table>
<thead>
<tr>
<th>Strain</th>
<th>% RsaA relative to JS1001</th>
</tr>
</thead>
<tbody>
<tr>
<td>JS1001</td>
<td>100</td>
</tr>
<tr>
<td>JS1001 rsaFa</td>
<td>103.3 ± 2.95</td>
</tr>
<tr>
<td>JS1001 rsaFa</td>
<td>102.8 ± 2.38</td>
</tr>
<tr>
<td>JS1001::pWB9rsaAΔP</td>
<td>100.8 ± 1.47</td>
</tr>
<tr>
<td>JS1001 rsaFa::pWB9rsaAΔP</td>
<td>128.3 ± 3.69</td>
</tr>
</tbody>
</table>

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*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. Values are means ± standard deviations.*
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 RsaFa and RsaFb, 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 rsaFb, but does suggest that although the roles of RsaFa and RsaFb 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 RsaFa (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 C. crescentus (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 sla gene by binding to S-layer fragment to the 5′ mRNA (16). It may be that a small situation occurs in C. crescentus, and this possibility is currently being investigated.

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 sla 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 slaA gene by binding a C-terminal S-layer 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 rsaFb 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 rsaFa and rsaFb...
genes did not have RsaA levels exceeding those of the wild type. However, the strain overexpressing both RsaA and RsaFγ 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 RsaFγ. Stable expression of RsaFγ by all cells may lead to still higher levels of RsaA secretion.

These results can be compared to those on 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 pre-formed 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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