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The *Caulobacter crescentus* Paracrystalline S-Layer Protein Is Secreted by an ABC Transporter (Type I) Secretion Apparatus

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Caulobacter crescentus **is a gram-negative bacterium that produces a two-dimensional crystalline array on its surface composed of a single 98-kDa protein, RsaA. Secretion of RsaA to the cell surface relies on an uncleaved C-terminal secretion signal. In this report, we identify two genes encoding components of the RsaA secretion apparatus. These components are part of a type I secretion system involving an ABC transporter protein. These genes, lying immediately 3′ of *rsaA*, were found by screening a Tn*5* transposon library for the loss of RsaA transport and characterizing the transposon-interrupted genes. The two proteins presumably encoded by these genes were found to have significant sequence similarity to ABC transporter and membrane fusion proteins of other type I secretion systems. The greatest sequence similarity was found to the alkaline protease (AprA) transport system of *Pseudomonas aeruginosa* and the metalloprotease (PrtB) transport system of *Erwinia chrysanthemi*. The *prtB* and *aprA* genes were introduced into *C. crescentus*, and their products were secreted by the RsaA transport system. Further, defects in the S-layer protein transport system led to the loss of this heterologous secretion. This is the first report of an S-layer protein secreted by a type I secretion apparatus. Unlike other type I secretion systems, the RsaA transport system secretes large amounts of its substrate protein (it is estimated that RsaA accounts for 10 to 12% of the total cell protein). Such levels are expected for bacterial S-layer proteins but are higher than for any other known type I secretion system.**

The gram-negative bacterium *Caulobacter crescentus* is covered with a crystalline protein surface layer (S-layer) (40) composed of the 98-kDa protein RsaA (20). Six copies of RsaA form a ringlike subunit that interconnects with other subunits to form a two-dimensional hexagonal array (39). The gene for RsaA has been cloned (38) and sequenced (20). Protein sequencing of the mature RsaA polypeptide has shown that only the initial *N*-formyl methionine is cleaved, leaving a mature polypeptide of 1,025 residues (18, 20). The S-layer is anchored to the cell surface via a noncovalent interaction between the N terminus of the protein and a specific smooth lipopolysaccharide (S-LPS) in the outer membrane (44). Ca^{2+} is required for the proper crystallization of RsaA into the S-layer, and its removal by using EGTA disrupts the S-layer structure (32, 45). It is presumed that a key function of the *C. crescentus* S-layer is to act as a physical barrier to parasites and lytic enzymes. It has been demonstrated to offer protection from a *Bdellovibrio*-like organism (26).

Because RsaA must pass through both the inner and outer membranes to form the S-layer on the outer surface of the bacterium, it is a true secreted protein. Further, an efficient secretion system is required for RsaA transport because the quantity of protein produced is significant (we have estimated that RsaA makes up 10 to 12% of the cell protein). Deletion and hybrid protein analyses have indicated that secretion of RsaA relies on an uncleaved C-terminal secretion signal located within the last 242 C-terminal amino acids of the RsaA protein (5, 7, 8). The presence of an uncleaved C-terminal secretion signal is generally diagnostic for proteins secreted by type I systems (4, 35) rather than the general secretory pathway (GSP, type II system) of the cell (33). The GSP employs a cleaved N-terminal signal sequence, and in gram-negative bacteria, secretion involves a periplasmic intermediate, unlike what is found with RsaA. The GSP is the mechanism used to secrete most S-layer proteins (9).

The best-described type I secretion systems are those required for the secretion of *Escherichia coli* hemolysin (HlyA), *Erwinia chrysanthemi* metalloproteases (PrtB), and *Pseudomonas aeruginosa* alkaline protease (AprA) (4, 35). A type I secretion apparatus consists of three components. One component, the ABC transporter, is embedded in the inner membrane and contains an ATP-binding region. It recognizes the C-terminal signal sequence of the substrate protein and hydrolyzes ATP during the transport process. Another component, a membrane fusion protein (MFP), is anchored in the inner membrane and appears to span the periplasm (13). The remaining component is an outer membrane protein (OMP) that is thought to interact with the MFP to form a channel that extends from the cytoplasm through the two membranes to the outside of the cell. Thus, in contrast to type II secretion, the type I variety does not involve the transient appearance of the secreted protein in the periplasm.

In addition to the secretion signal, the C-terminal portion of RsaA also contains five repeats of a glycine- and aspartic acid-rich region which is thought to bind calcium ions (20); such Ca^{2+}-binding motifs are found in most proteins secreted by type I systems (4). It has been suggested that these motifs are important for proper secretion signal presentation to the ABC transporter. Further, once the secreted protein has reached the external milieu, Ca^{2+} binding may trigger a conformational change in the polypeptide, helping to maintain the directional nature of the secretion process (14, 29). In the case of RsaA, then, the glycine- and aspartate-rich repeats may function (along with Ca^{2+}) both in maintaining the crystalline structure of the S-layer and in the secretion of the S-layer protein itself. The foregoing presumes that, in fact, RsaA was secreted by a type I system, but proof of this hypothesis requires identifica-

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TABLE 1. Strains and plasmids used in this study

Strain, plasmid, or cosmids	Relevant characteristics	Reference or source
E. coli DH5α	*recA endA*	Life Technologies
C. crescentus		
JS1001	S-LPS mutant of NA1000, sheds S-layer into medium	16
NA1000	Ap^r *syn-1000*; variant of wild-type strain CB15 that synchronizes well	ATCC 19089
JS1003	NA1000 with *rsaA* interrupted by KSAC Km^r cassette	16
Plasmids		
pBBR5	Tc^r; broad host range	This study
pBSKS+	ColE1 cloning vector; *lacZ* Ap^r	Stratagene
pJUEK72	$aprD^+$ $aprE^+$ $aprF^+$ $aprA^+$ $aprI^+$	22
pRAT1	$rsaA^+$ $rsaD^+$ $rsaE^+$ Ap^r	This study
pRAT4ΔH	$rsaA^+$ $rsaD^+$ $rsaE^+$ Ap^r; *rsaA* is under control of *lacZ* promoter	This study
pRAT4ΔH:pBBR5	$rsaA^+$ $rsaD^+$ $rsaE^+$ Ap^r Tc^r; pBBR5 was fused with pRAT4ΔH at the *Sst*I site	This study
pRAT5:pRK415	$rsaD^+$ $rsaE^+$ Ap^r Tc^r; pRK415 was fused with pRAT5 at the *Sst*I site	This study
pRK415	$lacZ^+$ Tc^r; broad host range	24
pRK415*rsaA*ΔPK	*rsaA* under control of *lacZ* promoter in pRK415	This study
pRUW500	$prtB^+$ Ap^r	12
pRUW500:pRK415	$prtB^+$ Tc^r pRK415 was fused with pRUW500 at the *Pst*I site	This study
pSUP2021	Carries Tn*5*, unable to replicate in *C. crescentus*	37
pTZ18UB:*rsaA*ΔP	The wild type promoter of *rsaA* has been replaced with a *lacZ* promoter	7
pTZ18R	Ap^r ColE1 cloning vector, a phagemid version of pUC18	30
pTZ18R*aprA*	$aprA^+$ ap^r; the *Eco*RI-*Bgl*II fragment from pJUEK72 containing *aprA* was inserted into the *Eco*RI-*Bam*HI sites of pTZ18R	This study
pUC8	ColE1 cloning vector; *lacZ* Ap^r	42
pUC9*rsaA*ΔNΔC	*rsaA* missing the extreme N terminus and C terminus	5, 8
pUC8*neoR*	*Hin*dIII-*Bam*HI fragment from Tn*5* containing neomycin resistance gene inserted into the corresponding sites in pUC8	This study
pTZ18R*aprA*:pRK415	$aprA^+$ Tc^r; pRK415 was fused with pTZ18R at the *Bam*HI site	This study
NA1000 cosmid library	1,000 cosmids containing 20–25 kb of NA1000 DNA	1

tion of the transport complex, since there is not a high degree of homology in the secretion signals of type I proteins (19). This report details the development of direct evidence for this hypothesis, identifying two of the three genes needed for the process.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. All of the strains, libraries, and plasmids used in this study are listed in Table 1. *E. coli* DH5α (Life Technologies) was used for all *E. coli* cloning manipulations. *E. coli* was grown at 37°C in Luria broth (1% tryptone, 0.5% NaCl, 0.5% yeast extract) with 1.2% agar for plates. *C. crescentus* strains were grown at 30°C in PYE medium (0.2% peptone, 0.1% yeast extract, 0.01% $CaCl_2$, 0.02% $MgSO_4$) with 1.2% agar for plates. Ampicillin was used at 100 μg/ml, streptomycin was used at 50 μg/ml, kanamycin was used at 50 μg/ml, and tetracycline was used at 0.5 μg/ml for *C. crescentus* and at 10 μg/ml for *E. coli* when appropriate.

Recombinant DNA manipulations. Standard methods of DNA manipulation and isolation were used (36). Electroporation of *C. crescentus* was performed as previously described (21). Southern blot hybridizations were done in accordance with the membrane manufacturer's manual (Amersham Hybond-N). Radiolabelled probes were made by nick translation using standard procedures (36).

A PCR product containing *rsaD* and *rsaE* was generated by using primers 5′-CGGAATCGCGCTACGCGCTGG-3′ and 5′-GGGAGCTCGAAGGGTCC TGA-3′. The product was generated by using *Taq* polymerase (Bethesda Research Laboratories) and following the manufacturer's suggested protocols. The template was pRAT1. Following a 5-min denaturation at 95°C, two cycles of 1 min at 42°C, 2 min at 65°C, and 30 s at 95°C were followed by 25 cycles of 1 min at 55°C, 2 min at 65°C, and 30 s at 95°C. The vector pBSKS+ was cut at the *Eco*RV site and T tailed (23), and the PCR product was ligated into this vector so that *rsaD* and *rsaE* would be in the same orientation as the *lacZ* promoter of pBSKS+. This construct was called pRAT5.

Plasmid pBBR5 was constructed from plasmids pBBR1MCS (25) and pHP45Ω-Tc (17). The Ω-Tc fragment from pHP45Ω-Tc was removed by using *Hin*dIII, and the ends were blunted by using T4 polymerase. A 0.3-kbp portion of the Cm^r-encoding gene was removed from pBBR1MCS by cutting with *Dra*I and replaced with the blunted Ω-Tc fragment, producing a Tc^r broad-host-range vector that replicates in *C. crescentus.*

Plasmid pRAT4ΔH was made by removing the *Cla*I-*Hin*dIII fragment from pTZ18UB:rsaΔP and replacing it with the *Cla*I-*Hin*dIII fragment from pRAT1 containing the C terminus of *rsaA* and the complete *rsaD* and *rsaE* genes.

Tn*5* mutagenesis. Tn*5* mutagenesis was accomplished by using narrow-host-range (ColE1 replicon) plasmid pSUP2021 (37), which is not maintained in *C. crescentus*. The plasmid was introduced by electroporation, 20,000 colonies that were streptomycin and kanamycin resistant were pooled and frozen at −70°C, and aliquots were used for subsequent screening.

Southern blot analysis of chromosomal DNA isolated from the Tn*5* library was used to assess the randomness of insertions. Hybridization with a Tn*5* probe indicated that while there were some hot spots of Tn*5* integration, Tn*5* distribution was reasonably random throughout the chromosome (data not shown).

SDS-PAGE and Western blot analysis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western immunoblot analysis were done as previously described (45). After transfer of proteins to nitrocellulose, the blots were probed with a polyclonal antibody and antibody binding was visualized by using goat anti-rabbit serum coupled to horseradish peroxidase and color-forming reagents (38).

To detect those *C. crescentus* whole cells synthesizing an S-layer, a Western colony blot assay was used (6). Briefly, cell material was transferred to nitrocellulose by pressing the membrane onto the surface of an agar plate. The membrane was air dried for 10 to 15 min, washed in a blocking solution (38) with vigorous agitation on a rotary shaker, and then processed in the standard fashion (38).

Surface protein from *C. crescentus* cells was extracted by using pH 2.0 HEPES buffer as described by Walker et al. (45). To compare the amounts of surface protein extracted from different mutants, equal amounts of cells growing at log phase were harvested and equal amounts of the protein extract were loaded onto the protein gel.

Isolation of DNA 3′ of *rsaA*. The NA1000 cosmid library was probed with radiolabelled *rsaA*; 11 cosmid clones hybridizing to the probe were isolated. Southern blot analysis was used to determine which cosmids contained DNA 3′ of *rsaA*. An 11.7-kb *Sst*I-*Eco*RI fragment containing *rsaA* plus 7.3 kb of 3′ DNA was isolated from one of the cosmids and cloned into the *Sst*I-*Eco*RI site of pBSKS+; the resulting plasmid was named pRAT1. The 3′ end of the cloned fragment consisted of 15 bp of pLAFR5 DNA containing *Sau3*AI, *Sma*I; and *Eco*RI sites.

Nucleotide sequencing and sequence analysis. *Bam*HI fragments from pRAT1 were subcloned into the *Bam*HI site of vector pTZ18R for sequencing. The 3′-end fragment was subcloned into pTZ18R by using *Bam*HI and *Eco*RI. The 5′-end fragment was subcloned into pTZ18R by using *Sst*I and *Hin*dIII. Sequenc-

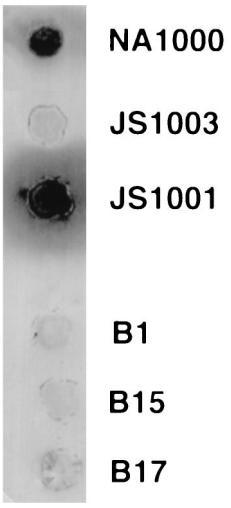

FIG. 1. Sample colony immunoblot using anti-S antibody. NA1000 has an S-layer, JS1003 does not, and JS1001 sheds the S-layer, forming a halo around the colony. B1, B2, and B5 represent samples of the S-layer-negative Tn*5* mutants that were found.

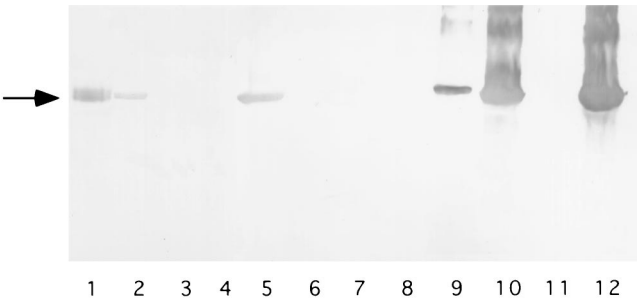

FIG. 3. Complementation of Tn*5* mutants with *rsaA*. Protein was extracted from the surfaces of the Tn*5* mutants and JS1003 carrying plasmid pRK415 *rsaA*ΔPK, which expresses *rsaA* under the control of the *lac* promoter. Protein was also extracted from the surfaces of the wild type and *rsaA* knockout mutants that did not contain any plasmid to demonstrate differences in expression. Equal amounts of surface extracts were loaded onto the gel, and a Western blot was performed by using a polyclonal antibody against RsaA. Lanes: 2 through 10, surface extracts from cells containing plasmid pRK415*rsaA*ΔPK (PK); 1, purified RsaA; 2, JS1003 (PK); 3, B9 (PK); 4, B13 (PK); 5, B1 (PK) (a Tn*5* insertion in *rsaA*); 6, B5 (PK); 7, B15 (PK); 8, B17 (PK); 9, B2 (PK); 10, B12 (PK) (a random Tn*5* insertion); 11, JS1003 (*rsaA*); 12, NA1000 (wild type). The arrow indicates full-length RsaA.

ing was performed on a DNA sequencer (Applied Biosystems model 373). After use of universal primers, additional sequence was obtained by "walking along" the DNA using 15-bp primers based on the acquired sequence. Nucleotide and amino acid sequence data were analyzed by using Geneworks and MacVector software (Oxford Molecular Group) and the National Center for Biotechnology Information BLAST e-mail server using the BLAST algorithm (2). Protein alignments were generated by using the ClustalW algorithm as implemented by the MacVector software and using the default settings.

Nucleotide sequence accession number. The sequences for *rsaD* and *rsaE* have been submitted to GenBank and assigned accession no. AF062345.

RESULTS

Identification of Tn*5* mutants lacking an S-layer. A pooled Tn*5* library was screened for S-layer-negative mutants by using a Western colony immunoblot assay (see Materials and Methods). The polyclonal primary antibodies used were α-RsaA (45) and α-S-LPS (44). α-RsaA reacts to RsaA, and α-S-LPS reacts to the S-LPS required for anchoring of the S-layer to the surface of the bacterium (44). When α-RsaA was used, colonies with an S-layer reacted with the antibody and appeared as a spot on the blot (Fig. 1). It was also found that a halo could be detected around colonies when the S-layer could not anchor to the cells (e.g., cells with a defective S-LPS). It appears that the shed S-layer diffused away from the colony and was detected in the Western colony blot as a ring around the colony (Fig. 1). These are not further described in this report. When α-S-LPS was used, the antibody only reacted to exposed S-LPS when the cells of a colony lacked an S-layer; the S-layer blocks the binding of α-S-LPS. RsaA appears to be completely degraded when it is not secreted (5, 8); therefore, cell lysis during this procedure and release of unsecreted RsaA were not a concern. In sum, by using this method, it was possible to differentiate among cells secreting RsaA, cells secreting and shedding an S-layer, and cells without an S-layer.

In total, 9,000 colonies from the pooled Tn*5* mutant library were screened by using the α-S-LPS antibody and 22,000 colonies were screened by using α-RsaA. Seventeen Tn*5* S-layer-negative mutants were found. SDS-PAGE and Western blot analysis of whole-cell lysates and culture supernatants confirmed that no S-layer was found in or on the cells or in the culture supernatant of these mutants (data not shown).

Identification of Tn*5* mutants defective in RsaA secretion. Several possible Tn*5* insertion events in addition to those in the secretion genes could result in an S-layer-negative phenotype. To screen out Tn*5* insertions in the *rsaA* gene, Southern blot analysis was performed on the S-layer-negative mutants. Eleven of the mutants were caused by insertions in *rsaA* and were not further characterized. One mutant, B12, was originally picked as having no S-layer but, on further examination, was found to have an S-layer and was kept for use as a random Tn*5* mutant. Five mutants, B5, B9, B13, B15, and B17, were caused by insertions in the DNA immediately 3′ of *rsaA*, and one mutant, B2, was located elsewhere on the chromosome (Fig. 2). These

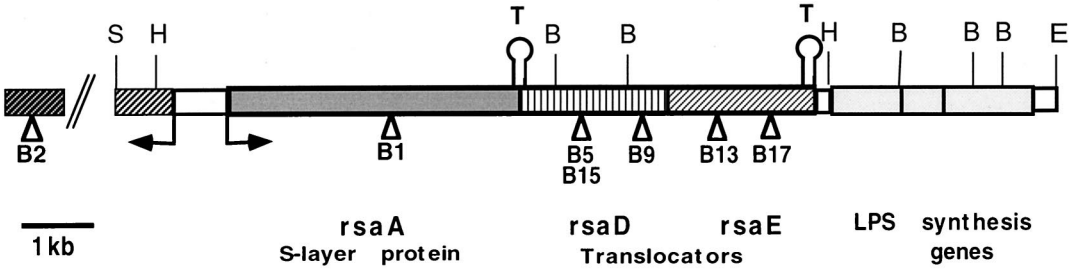

FIG. 2. Graphic representation of the DNA examined in this study. A triangle represents the approximate location of a Tn*5* insertion, a T indicates a terminator, and an arrow indicates a promoter. Restriction enzyme sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sst*I. Boxes containing patterns are ORFs or genes, as indicated below the diagram.

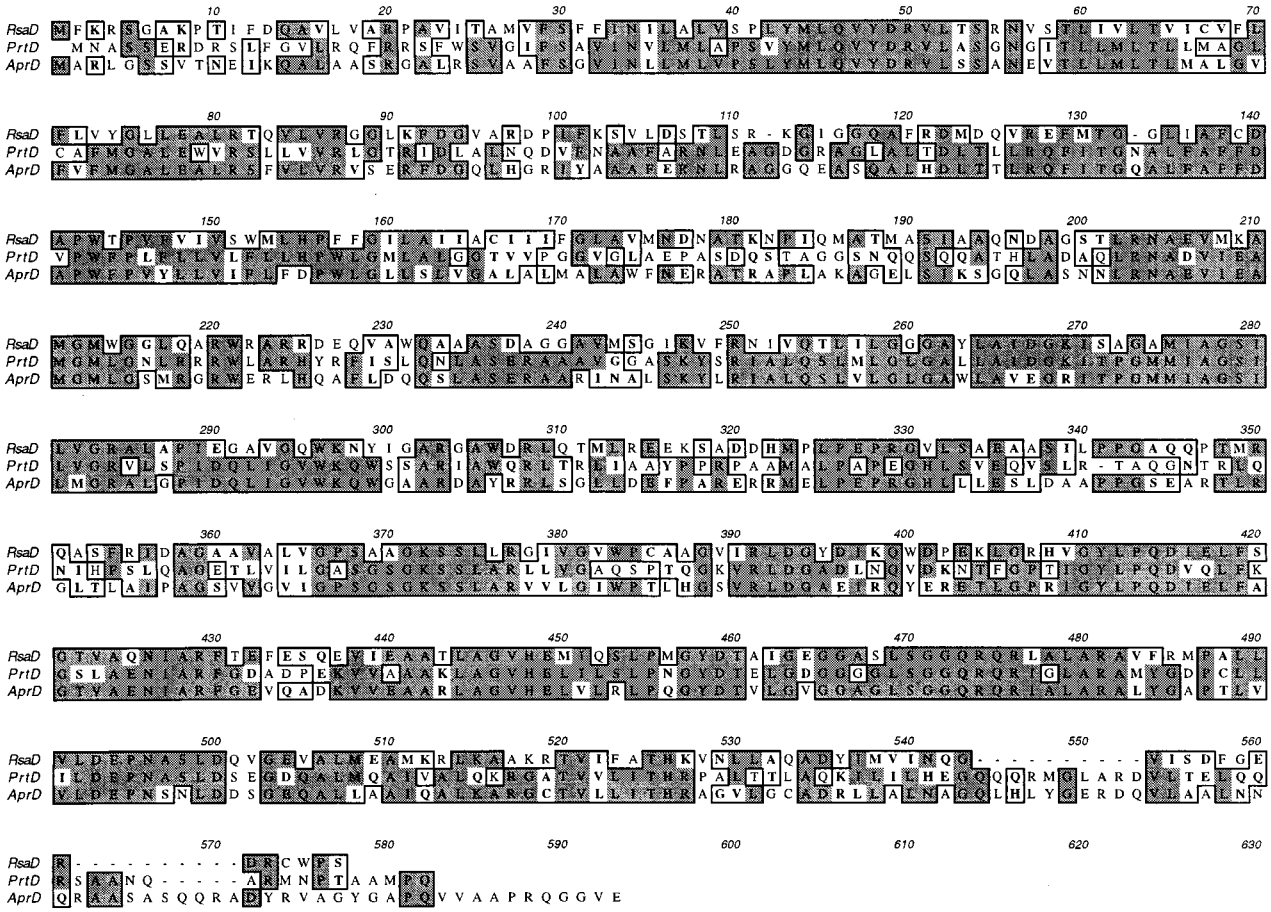

FIG. 4. Alignment of RsaD with AprD and PrtD using the ClustalW algorithm implemented by the program MacVector 6.0. Identical and similar amino acids are boxed. Identical amino acids are shaded. Similar amino acids are in boldface. The ABC transporter protein is represented by PrtD for metalloprotease transport in *E. chrysanthemi* and AprD for alkaline protease transport in *P. aeruginosa.*

last six mutants were possible RsaA translocator mutants.

To determine that loss of the S-layer was not caused by a mutation affecting the regulation of RsaA, such as a transcription regulator, *rsaA*, was expressed in the mutants under the control of a *lacZ* promoter by using plasmid pRK415*rsa*AΔPK. This construct restored RsaA production in JS1003, a mutant with an interrupted *rsaA* gene, although wild-type RsaA expression levels were not reached. An S-layer was produced in mutants that had a Tn*5* insertion in *rsaA* and none of the five mutants with a Tn*5* insertion in the DNA immediately 3′ of *rsaA* secreted RsaA when *rsaA* was present in *trans* in this manner (Fig. 3). Secretion was not restored to full wild-type levels in either JS1003 or B1 with plasmid pRK415*rsaA*ΔPK.

In addition, the one mutant, B2, in which the Tn*5* insertion was not adjacent to the *rsaA* gene also produced an S-layer. This indicates that the B2 insertion was not in a gene involved in RsaA secretion. B2 may have an interruption in a gene responsible for the regulation of RsaA production or, more likely, the Tn*5* insertion mutation is irrelevant and there was a secondary point mutation in *rsaA.*

Isolation and sequencing of DNA near *rsaA*. By using the locations of Tn*5* insertions disrupting RsaA secretion and a previously constructed cosmid library (see Materials and Methods), a DNA fragment containing *rsaA* plus 7.3 kb of 3′ DNA was isolated; cloned into pBSKS+, forming pRAT1; and sequenced to search for translocator genes. An open reading frame (ORF) was found 5′ of *rsaA*, confirming earlier results (18), and five ORFs were found 3′ of *rsaA* (Fig. 2).

A search of gene and protein databases showed that there were two ORFs immediately 3′ of *rsaA* that encoded proteins with significant similarity to the ABC transporter and MFPs of two type I secretion systems: the alkaline protease transport system of *P. aeruginosa* (22) and the metalloprotease transport system of *E. chrysanthemi* (28) (Fig. 4 and 5).

The first ORF was 1,656 bp long and started 242 bp after the termination codon of *rsaA*. This ORF was predicted to code for a 555-amino-acid protein with a predicted molecular mass of 59.8 kDa. The predicted protein is 46% identical and 69% similar to AprD from *P. aeruginosa* and 33% identical and 62% similar to PrtD from *E. chrysanthemi*. The gene was designated *rsaD* because of its similarity to these genes (Fig. 4). RsaD does not appear to have an N-terminal signal sequence but does exhibit several N-terminal hydrophobic domains that may be transmembrane regions and a possible ATP binding site in the C-terminal half of the protein.

The second ORF started 146 bp after *rsaD*, contained 1,305 bp, and encoded a protein of 435 residues with a predicted molecular mass of 48 kDa. The predicted protein is 28% identical and 50% similar to AprE from *P. aeruginosa* and 29% identical and 52% similar to PrtE from *E. chrysanthemi*. The gene was designated *rsaE* because of its similarity to these

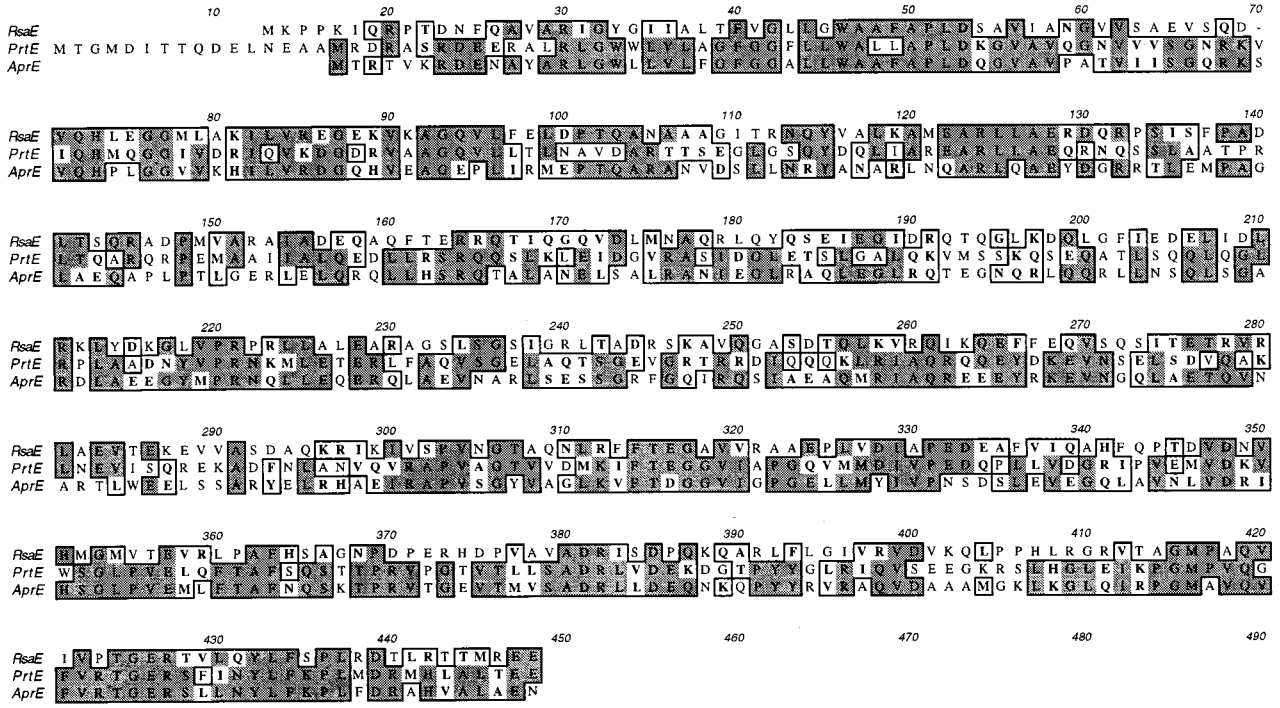

FIG. 5. Alignment of RsaE with AprE and PrtE by using the ClustalW algorithm implemented by the program MacVector 6.0. Identical and similar amino acids are boxed. Identical amino acids are shaded. Similar amino acids are in boldface. The MFP is represented by PrtE in *E. chrysanthemi* and by AprE in *P. aeruginosa*.

genes (Fig. 5). In contrast to RsaD, RsaE was predicted to have a typical N-terminal signal sequence.

With respect to transcription-translation initiation-termination signals, (i) there was no indication of a promoter immediately 5′ of either *rsaD* or *rsaE*, suggesting that they may be part of a polycistron which includes *rsaA*; (ii) there were possible ribosome binding sites 7 bp upstream of the *rsaD* ATG initiation codon and 8 bp upstream of the *rsaE* ATG initiation codon; and (iii) there was a putative rho-independent terminator immediately after the stop codon of *rsaE*.

Three more ORFs were found 3′ of *rsaE*. None of these ORFs encoded proteins similar to the third component of type I secretion systems. Instead, these ORFs encoded proteins similar to those involved in the synthesis of perosamine, a dideoxyaminohexose. The first ORF encoded a protein with similarity to GDP–D-mannose dehydratase (11, 41), the second ORF encoded a protein with similarity to UDP–*N*-acetylglucosamine acyltransferase (10, 43), and the third protein had similarity to perosamine synthetase (3, 41).

Complementation of secretion-defective Tn*5* mutants. To demonstrate that the Tn*5* insertions were definitively responsible for the secretion defect, we attempted to complement the Tn*5* mutants in *trans*. We first used cosmid 17A7, which contained the entire RSA operon, to attempt to complement the mutants. All attempts at complementation using this cosmid were unsuccessful, including an attempt to restore RsaA production in JS1003 (which contains an inactivated *rsaA* gene). Since RsaA production in JS1003 can be restored with other plasmids containing *rsaA*, we believe that something pertaining to the nature of the cosmid prevented complementation (see Discussion).

A PCR product containing genes *rsaD* and *rsaE* was generated and cloned into a suitable expression vector; the result was named pRAT5:PRK415 (see Materials and Methods). This plasmid was introduced into Tn*5* mutants B15 and B17.

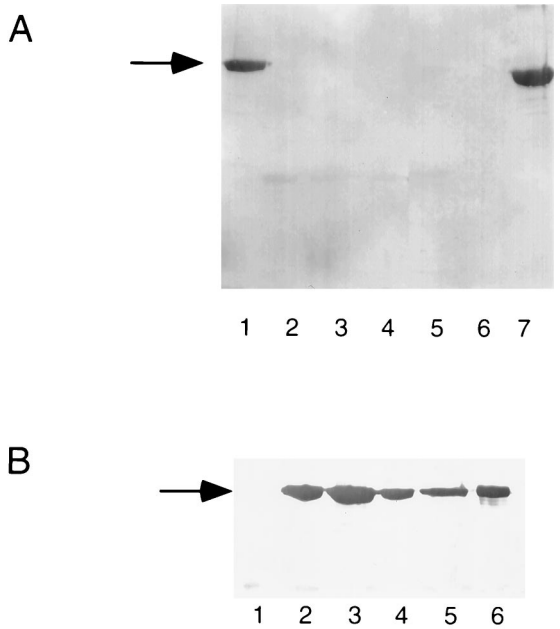

FIG. 6. Complementation of transport-deficient mutants using *rsaD* and *rsaE*. Western blots of surface-extracted protein using anti-S antibody. (A) Lanes: 1, B17 (DE); 2, B15 (DE); 3, B1 (DE); 4, B17 (17A7); 5, B15 (17A7); 6, JS1003; 7, NA1000. DE indicates that the cells carried plasmid pRAT5:pRK415 containing the genes *rsaD* and *rsaE*. 17A7 indicates that the cells carry cosmid 17A7 containing the entire RSA operon. Equal amounts of surface extract were loaded in all lanes. (B) Lanes: 1, B1 (DE); 2, B5 (DE); 3, B9 (DE); 4, B15 (DE); 5, B17 (DE); 6; NA1000. DE indicates that the cells carry plasmid pRAT5: pBBR5 expressing the genes *rsaD* and *rsaE*. Equal amounts of surface extract were loaded in all lanes except 6, where there was only one-quarter of the amount loaded in the other lanes. The arrow indicates full-length RsaA.

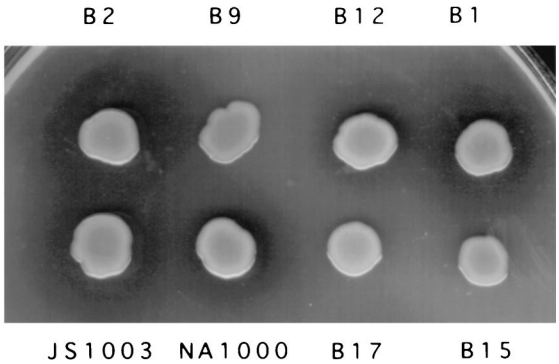

FIG. 7. Expression of *prtB* in *C. crescentus*. PrtB was expressed in all of the colonies shown by using plasmid pRK415:pRUW500. The cells were spotted onto PYE plates containing 1% skim milk. Halos around colonies indicate that active PrtB is being secreted. Note that NA1000 and B12 cells are producing RsaA, as well as PrtB, and that the halos surrounding these colonies are smaller.

With this plasmid, mutant B17 produced RsaA while, inexplicably, the B15 mutant did not (Fig. 6A).

To address the problems with B15 complementation, a new Tc^r broad-host-range vector, pBBR5, was constructed. It was hoped that this vector would have a different copy number and expression level that would alleviate the problems encountered when using pRK415 or pLAFR5. Two plasmids were constructed by using this vector (see Materials and Methods). pRAT5:PBBR5 transcribed the *rsaD* and *rsaE* genes by using the *lacZ* promoter. pRAT4ΔH:PBBR5 transcribed the *rsaA*, *rsaD*, and *rsaE* genes by using the *lacZ* promoter. When pRAT5:PBBR5 was introduced into mutants B1, B5, B9, B15, and B17, Western blot analysis showed that the mutants with defective *rsaD* and *rsaE* genes expressed RsaA on the surface while *rsaA* mutant B1 did not (Fig. 6B). When pRAT4ΔH:PBBR5 was expressed in the same mutants, RsaA was only found on the surfaces of mutants B1 and B17 (data not shown).

Secretion of the *P. aeruginosa* and *E. chrysanthemi* proteases by the RsaA secretion machinery. Due to the close similarity of the RsaD and RsaE proteins to those of cognate type I secretion proteins of *P. aeruginosa* and *E. chrysanthemi*, it seemed possible that the proteases transported by these systems would be transported by the *C. crescentus* secretion machinery. To test this idea, metalloprotease gene *prtB* and alkaline protease gene *aprA* were expressed in *C. crescentus* by introducing these genes on plasmids pRUW500:pRK415 and pTZ18R*aprA*:pRK415 into strains NA1000 and JS1003, as well as the NA1000 Tn*5* mutants. The secreted proteases formed a halo around a colony on a skim milk plate. Normally, *C. crescentus* does not form halos, even when allowed to grow for over a week, a time when significant cell lysis is expected to occur (data not shown). The proteases were not secreted in the mutants in which the *rsaD* and *rsaE* sequences were interrupted with Tn*5* but were secreted in active form when the Tn*5* interruption was in the *rsaA* gene (Fig. 7). The proteases were secreted at lower levels in wild-type strain NA1000 and S-layer-producing mutant B12, compared to mutant JS1003 or B1, in which the *rsaA* gene has been interrupted, suggesting that there was competition between RsaA and PrtB for the secretion machinery, further supporting the supposition that RsaD and RsaE are parts of a type I secretion mechanism. Identical results were found when *aprA* was expressed in the Tn*5* mutants (data not shown).

DISCUSSION

Analysis of the region 3′ of *rsaA* revealed the presence of two genes (*rsaD* and *rsaE*) encoding proteins with significant similarity to components of the type I secretion systems used by *P. aeruginosa* and *E. chrysanthemi* to secrete two different extracellular proteases (22, 28). Because interruption of *rsaD* and *rsaE* eliminated secretion of RsaA and the defects could be restored by complementation, it was apparent that their gene products make up part of the RsaA translocator machinery.

This appears to be the first reported example of an S-layer that is secreted by a type I secretion system, although we recently learned that the S-layer of *Campylobacter fetus* may also be transported in this manner (8a). If this is so, it is notable that the *C. fetus* S-layer protein has several features in common with that of *C. crescentus*. It is produced by a free-living, gram-negative bacterium, is hexagonally packed, anchors to the cell surface via its N terminus to a particular species of LPS (7, 15, 44), and so far has the greatest similarity of any S-layer protein to RsaA (20).

The genes for the ABC transporter and the MFP components of type I secretion systems are generally found in an operon that includes the transported protein (4, 35); in this respect, then, the organization of the *rsaA*, *rsaD*, and *rsaE* genes was not surprising. In contrast, the gene encoding the OMP component of type I secretion systems may or may not be closely linked to the other secretion genes. Since sequencing immediately 3′ of *rsaD* and *rsaE* has revealed ORFs that are most likely involved in S-LPS O-antigen biosynthesis and are not candidates for the third component, it is apparent that the third component is located at least many kilobases away from the region described here. We are currently exploring methods of identifying the OMP component and heterologous complementation with other type I secretion components. RsaA, RsaD, and RsaE have been expressed in *E. coli* strains carrying TolC, the *E. coli* OMP involved in hemolysin secretion, without successful secretion of RsaA (unpublished data).

The ORFs identified 3′ of *rsaE* all have significant identity to genes in *Vibrio cholerae* that encode proteins of the perosamine biosynthetic pathway (41). The structure of *C. crescentus* S-LPS has been studied in this laboratory and found to contain a dideoxyamino hexose (34). These sequencing results and the proximity to the RSA operon suggest that this sugar may be perosamine, which is a dideoxyamino hexose.

While it was possible to complement a secretion defect by using genes *rsaD* and *rsaE*, complementation did not restore secretion to wild-type levels and it was not possible to restore secretion in some instances. One reason may be due to the necessity of using tetracycline to maintain the cosmid. Tn*5* confers resistance to kanamycin and streptomycin, so it was necessary to use another antibiotic; tetracycline was the only practical alternative. *C. crescentus* cells carrying a Tc^r-encoding gene appear to be anomalous by microscopy. The cells are often severely elongated, and there are few motile cells. It was difficult to grow cultures carrying Tc^r plasmids with the RSA genes to densities high enough for extraction of sufficient protein to be seen on the Western blot. Furthermore, the fact that cosmid 17A7, carrying the entire RSA operon, could not complement the JS1003 mutant, even though other constructs can be used to complement the *rsaA* defect, suggested that some factor related to the cosmid is responsible for the inability to complement.

It was puzzling that the B15 mutant was not complemented by pRAT5:pRK415, while B17 was complemented. One possibility might be that the B15 mutant makes a partial product

that interacts with the other transporter components but cannot make a fully functional transporter complex and not enough protein is expressed from the plasmid to dilute out this effect (i.e., that a dominant effect is caused by a truncated gene product). Transcription of *rsaD* and *rsaE* was driven by the *lac* promoter on plasmid pRAT5:PRK415 and by the native promoter on the cosmid. This may have resulted in a larger amount of protein being produced from the plasmid than from the cosmid. Plasmid pRAT5:pBBR5 complemented B15 and a number of other mutants. Vector pBBR5 is considerably smaller than pRK415 and, in preliminary experiments, appeared to have a higher copy number (data not shown), which may result in larger amounts of RsaD and RsaE in the cell, diluting out the effects of truncated proteins more effectively than pRAT5:pRK415. Further, plasmid pRAT4ΔH:pBBR5 did not complement any of the *rsaD* and *rsaE* mutants except B17. In this plasmid, *rsaD* and *rsaE* either are transcribed by their wild-type promoter or are likely transcribed as part of the *rsaA* transcript as described below. In either case, a lesser transcript amount would be produced than from the *lacZ* promoter of pRAT5:pBBR5, making the construct less effective in diluting the effect of a truncated gene product.

rsaA contains a potential rho-independent terminator sequence at the end of the coding region (21). This predicted terminator results in a predicted transcript that matched closely the size of a transcript found by Northern blot analysis (18). In this study, we found no obvious indications of a promoter immediately 5′ of either the *rsaD* or *rsaE* gene. It may be that transcription of *rsaD* and *rsaE* is similar to transcription of the *hlyA*, *hlyB*, and *hlyD* genes, where a similar rho-independent terminator is found after the *hlyA* gene, and most transcripts terminate at this point. An antiterminator, RfaH, prevents termination, and when it does, a larger transcript including the *hlyB* and *hlyD* genes is made (27). Transcription of the *E. chrysanthemi* protease secretion genes appears to be accomplished by a similar method (28), and we postulate that the same is true for the RSA operon. A transcription pattern like this may account for the reduced expression found in the JS1003 and B1 mutants when they are complemented with *rsaA*. The kanamycin fragment interrupting *rsaA* in JS1003 does not have a transcription terminator and may transcribe through to the end of *rsaE*, resulting in a transcript 1.5 kb longer than that of the wild type, which would likely be more unstable and result in fewer transport complexes. In B1, it is likely that *rsaD* and *rsaE* are transcribed off one of the Tn*5* promoters, resulting in decreased transcript amounts and, in turn, transport complexes.

Type I secretion systems can be grouped into families. The RTX toxins, such as α-hemolysin (*E. coli*) and leukotoxin (*Pasteurella haemolytica*), make up one family, while extracellular proteases (e.g., AprA and PrtB), lipases, and some other proteins constitute another (4). Within the families, there is high sequence similarity and the components can be interchanged without a dramatic drop in protein transport, but when they are interchanged between families, protein transport drops significantly. Because we have demonstrated that proteins AprA and PrtB can be secreted from *C. crescentus* in active form, there is competition for the secretion apparatus between the proteases and RsaA and there is higher sequence similarity between these proteins than with RTX toxins, presumably, RsaA can likely be grouped with the protease family. It is of continuing interest to us to determine the features common to these metalloproteases and RsaA that allow transport to occur.

RsaA accounts for a large portion (10 to 12%) of the cellular protein. As far as we can determine, the RsaA secretion machinery secretes a larger fraction of the total cell protein than any other known type I secretion mechanism. This high level of protein production is apparently necessary to keep the cell completely covered with S-layer at all times and is similar to the levels noted for other bacterial S-layer proteins (31). This means that the RsaA secretion machinery is either more efficient than that of other type I secretion systems or a larger number of transport complexes exist in the membranes or a combination of both factors. This question is an important one to answer because we are currently engaged in evaluating the potential of the S-layer protein secretion system for the secretion of heterologous proteins and peptides in a biotechnological context (6, 7).

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