

Isolation and Comparison of the Paracrystalline Surface Layer Proteins of Freshwater Caulobacters

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Several methods for isolation of the paracrystalline surface (S) layer protein (RsaA) of *Caulobacter crescentus* CB15A were evaluated. Treatment of cells with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer at pH 2 was the most effective means of selectively removing RsaA from cells, and after neutralization, the protein was capable of reassembling into a paracrystalline structure. Ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid treatment could also be used to extract RsaA and yielded protein capable of reassembly. The success of the methods was likely related to disruption of calcium-mediated bonding; calcium was required for recrystallization, while magnesium and strontium ions were ineffective. Antibody was raised against purified RsaA and, along with the S-layer extraction techniques, was used to evaluate 42 strains of caulobacters isolated from a variety of aquatic and wastewater treatment locations. A single characteristic protein could be isolated from the 35 strains that produced an S layer; with one exception, no proteins were extracted from strains that had no S layer. The presumed S-layer proteins ranged in size from 100 to 193 kDa. All of these proteins specifically reacted with anti-RsaA serum by Western immunoblot analysis. In strain CB15A, a specific S-layer-associated oligosaccharide has been proposed to be involved in a calcium-mediated attachment of the S layer to the cell surface. This molecule was detected by Western immunoblotting with a specific antiserum and on polyacrylamide gels stained for polysaccharides. A comparable band was found in all S-layer-producing strains and for most, S-layer-associated oligosaccharide-specific antibody reacted with them in Western analysis. Overall, in freshwater caulobacters at least portions of their S-layer structures appear to be strongly conserved entities, as well as the means of attachment to the cell surface.

Caulobacters are one of many genera of bacteria that elaborate a paracrystalline surface (S) layer on their outermost surface (37). The S layer of one species, *Caulobacter crescentus*, has received the most attention, but in a survey of caulobacters isolated from wastewater treatment systems the most common type of caulobacter we encountered shares morphological similarities with *C. crescentus* strains, including a hexagonally packed S-layer structure (27).

The cloned S-layer protein gene of *C. crescentus* CB15 hybridized to specific regions of the genome for most of these S-layer-producing caulobacters, yet only under moderate-stringency conditions, and restriction length polymorphism analysis with the S-layer gene as the probe failed to reveal patterns of close relatedness between the strains (27). This was recently correlated with a 16S rRNA comparative analysis which showed that these caulobacters were a coherent group but still sufficiently different to have significant variation in their overall genomic DNA composition (41). We were interested in determining how similar their S-layer structures are from the standpoint of the protein that specifies the structure and the mechanism of interaction with the underlying cell surface.

S layers, nearly always composed of a single protein species, are common in many groups of bacteria (17, 21, 35). For most, little can be ascertained with certainty about the function of these layers; a protective barrier function is often presumed (22, 34, 40). In addition, a number of physical features are shared by many S-layer proteins, including an acidic pI, absence of cysteine residues, and a high propor-

tion of hydroxy amino acids. In several studies it has been possible to assemble the protein in the absence of the cell surface from which it was derived (24). Given such generic similarities or capabilities, it is possible that some S layers were acquired in genetic exchanges with other soil and aquatic bacteria and retained because they offered a competitive advantage, analogous to antibiotic resistance or heavy metal detoxification.

There have been only a few detailed comparative studies of S layers, within related species, to allow analysis of such a hypothesis by examining the degree of structural conservation. In *Aeromonas salmonicida*, there is a significant degree of structure conservation among the S-layer proteins of strains isolated from diverse locales, as judged by N-terminal protein sequencing, Western immunoblot, and enzyme-linked immunosorbent assay analyses of a few strains and immunofluorescence analysis of a larger group, using antibody prepared against one of the S-layer proteins (20). On the other hand, with *A. hydrophila*, there were antigenic differences among strains and no N-terminal amino acid sequence homology of the S-layer protein of one strain with that of *A. salmonicida* (8). In a similar analysis of *Campylobacter fetus*, there were significant differences in the S-layer proteins of closely related strains and the suggestion that some form of antigenic variation occurred (11). Two strains of *Aquaspirillum serpens* were examined by peptide mapping and immunological cross-reaction; there is apparently a degree of similarity between the S-layer proteins (unpublished studies cited in reference 23). A more general study of 39 *Bacillus stearothermophilus* strains, focussing primarily on the molecular weight of the S-layer protein and its appearance by electron microscopy, indicated remarkable variety, not only in the presence or absence of the S layer but also in the basic geometry of the paracrystalline struc-

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ture and the size of the protein involved (29). A similar finding was made for several species of *Desulfatamaculum nigrificans* (36). Studies with strains of *Bacillus sphaericus* also noted variation in the presence or absence, molecular weight, and antigenicity of the S layers (26, 43). A study of *Bacillus brevis* strains, which often produce a double S layer, showed that the middle wall protein is immunologically conserved, whereas the outer wall protein is not (14a). Overall, the studies show that the degree of S-layer conservation within related strains varies, with many showing a lack of similarity, but that general conclusions cannot be made.

The caulobacters in this study are an ideal group for evaluation of S-layer similarity. Isolates were taken from a variety of locales and have been characterized in a comparative sense in several ways, and since it was realized before they were collected that S layers are often rapidly lost when grown in laboratory conditions (21), the strains were stored frozen immediately after isolation. Most of the isolates have S layers that are always hexagonally packed and indistinguishable from each other in gross analysis (27).

In past studies, the S-layer protein of *C. crescentus* CB15A (39) (formerly called 130K, but now called RsaA [14]) was purified from an extracellular milieu of proteins by solubilization in sodium dodecyl sulfate (SDS) and gel filtration chromatography (38). Antibody to this preparation, however, does not label the intact S-layer structure, nor have we been able to achieve in vitro crystallization of an S layer with such preparations. We presume that complete denaturation of this large protein leads to incorrect folding when SDS is removed. A variety of S-layer protein isolation techniques have been reported for other bacteria (24); the present study focussed on methods that had the least potential for denaturation of the protein.

A specific oligosaccharide-containing surface molecule appears to mediate S-layer attachment via calcium ion bridging to the S layer (13). Loss of this molecule leads to an S-layer-shedding phenotype; sheets of crystallized protein accumulate in the area surrounding the cells, but no surface attachment occurs. The availability of a specific antiserum for this polysaccharide and a detection method also allowed comparison of this characteristic among caulobacter strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. crescentus* CB15A is the laboratory strain whose S layer has been characterized (38, 39). *C. crescentus* CB2A is a variant of CB2 whose S layer has been lost by spontaneous mutation (38). CB15AKSAC is a *C. crescentus* CB15A strain with the gene encoding RsaA, the S-layer protein, interrupted by a kanamycin resistance cartridge (12); no RsaA is produced. Most of the other caulobacters used in this study were described previously (27); most were isolated from wastewater treatment facilities. In addition, FWC2, FWC44, and FWC46 were isolated from aquatic sources by the method previously described (1, 27). The collection represents isolates from geographically separated regions throughout western North America, and all were determined to be unique isolates, minimally by comparison of protein gel band patterns. Cultures were grown at 30°C, with shaking, in peptone-yeast extract medium (PYE) (33) supplemented with CaCl₂ to 0.01% and MgSO₄ to 0.02%. The medium for the FWC strains also contained riboflavin at 2 µg/ml (27). Marine caulobacter strain MCS6 was grown as previously described (1).

Isolation of S-layer proteins. *C. crescentus* CB15A was used to determine an effective method for extracting S-layer protein. In a typical experiment, a 100-ml culture of CB15A was grown to the mid-logarithmic stage (optical density at 600 nm [OD₆₀₀] = 0.6 to 0.7) and harvested by centrifugation (10,000 × *g* for 10 min). The cell pellet was washed twice in 1 volume of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (Research Organics, Inc., Cleveland, Ohio), pH 7.2, by suspension and centrifugation and resuspended in 15 ml of buffer. One milliliter of the cell suspension was placed into microcentrifuge tubes, cells were pelleted, and the supernatant was removed. A 200-µl volume of one of the following agents was used to suspend the cells: 10 mM EDTA; 10 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA); 100 mM HEPES at pHs 2, 4, 6, 7.5, 8, and 10; 200 mM glycine-HCl buffer at pHs 2, 3, and 4; 100 mM Tris buffer (pH 7.2); 0.5% β-mercaptoethanol in 10 mM HEPES (pH 7.5); 1 M urea; 1 M guanidine-HCl; 10 mM NaCl; 10 mM CaCl₂; and 100 mM HEPES (pH 7.5) with incubation at 65°C. After incubation for 15 min, cells were pelleted by centrifugation and 10 µl of supernatant was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (25) with Coomassie blue staining.

Subsequently, as a standard method of isolation of S-layer proteins, 5-ml cultures of FWC strains were grown to an OD₆₀₀ of 0.6 and the cells were harvested by centrifugation. The cells were washed twice with 5 ml of 10 mM HEPES (pH 7.2) and then suspended in 200 µl of 100 mM HEPES (pH 2) or 200 µl of 10 mM EGTA in 10 mM HEPES (pH 7.5). Cell suspensions were incubated for 10 min at 20°C, cells were pelleted by centrifugation, and the supernatants were retained for examination by SDS-PAGE. The acidic-pH-extracted samples were adjusted to pH 7 with 5 N NaOH.

Production of antiserum to purified CB15A S-layer protein. RsaA, extracted from CB15A by using 100 mM HEPES (pH 2) as described above, was used to immunize a New Zealand White female rabbit after combination with an equal quantity of Freund's incomplete adjuvant. The initial injection contained 1 mg of protein, and booster injections at days 21, 28, and 35 contained 0.3 mg of protein. Serum with the highest titer was collected on days 55 and 62 and processed by standard methods (15). Serum activity was determined by the Ouchterlony double-diffusion assay (31) and Western immunoblot analysis (6). The antiserum (anti-RsaA) was absorbed against whole cells of CB15AKSAC and against Western immunoblots of CB15AKSAC cell lysate. The preimmune serum gave no activity by Ouchterlony or Western immunoblot assay.

Electrophoretic and Western blot analyses of S-layer proteins. The protein concentration of S-layer extracts was determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, Ontario, Canada [5]), using bovine gamma globulin as the standard. Three micrograms of protein was mixed with an equal volume of sample buffer and analyzed by SDS-PAGE using a 7.5% resolving gel. Samples were not heated prior to electrophoresis, because little or no RsaA enters the gel if it is heated to 100°C in dissociation buffer (38). Prestained protein molecular weight standards (BRL Life Technologies, Burlington, Ontario, Canada) were used to estimate the sizes of the S-layer proteins. Immunological comparison of the S layers from FWC strains with that of CB15A was done by Western immunoblot analysis using anti-RsaA serum at a dilution of 1:50,000. Antibody binding was visualized by treatment with

goat anti-rabbit serum coupled to horseradish peroxidase and color-forming reagents, as previously described (38).

Polysaccharide analysis. Lipopolysaccharide (LPS) and the S-layer-associated oligosaccharides (SAO) characterized in CB2A and CB15A and comparable oligosaccharides in other strains were examined by using a modification of the method of Hitchcock and Brown (16). Cells from 5 ml of culture ($OD_{600} = 0.6$ to 0.7) were pelleted and washed with 10 mM HEPES (pH 7.2) by suspension and centrifugation. The pellet was suspended in 250 μ l of 10 mM Tris-1 mM EDTA, frozen at -20°C , and then thawed at room temperature. One microliter of DNase (0.5 mg/ml), 20 μ l of lysozyme (10 mg/ml), and 3 μ l of 1 M MgCl_2 were added, and the samples were incubated at room temperature for 15 min. The samples were assayed for 2-keto-3-deoxyoctonate (KDO) by the method of Karkhanis et al. (18), using KDO as the standard. The amount of cell lysate containing 0.75 μ g of KDO was suspended in 20 μ l of SDS-PAGE sample buffer, heated at 100°C for 10 min, cooled to room temperature, and made to 0.5 mg/ml with proteinase K (Sigma Chemical Co., St. Louis, Mo.). After incubation at 60°C for 1 h, the material was analyzed by SDS-PAGE using a 13% separating gel. Polysaccharides were stained by using the method of Tsai and Frasch (42), except that the gels were fixed for only 1 h, with two changes of the fix solutions, and the periodic acid oxidation step was extended from 5 to 15 min.

The immunological similarity of the SAO-like bands from FWC strains to the SAO of *C. crescentus* CB2A and CB15A was assessed by Western immunoblot analysis using anti-SAO serum raised against the SAO of *C. crescentus* CB2A, the preparation of which has already been described (13).

In vitro crystallization of the CB15A S layer. A 1-liter culture of CB15A was grown to the late-log phase ($OD_{600} = 0.8$) and deflagellated in a Waring blender. Cells were pelleted by centrifugation and washed twice by suspension and centrifugation with 10 mM HEPES (pH 7.5). Half of the cell pellet was suspended in 20 ml of 10 mM EGTA-10 mM HEPES (pH 7.5); the remainder was suspended in 20 ml of 100 mM HEPES (pH 2). After incubation at room temperature for 20 min, the cells were removed by centrifugation and the supernatant was ultracentrifuged at $160,000 \times g$ for 1.5 h. The supernatants of the ultracentrifugation step were recovered, and the low-pH extract was neutralized with 5 N NaOH. The two samples were dialyzed overnight against 10 mM HEPES (pH 7.5) at 4°C . Portions of the dialyzed samples were examined by SDS-PAGE and negative-stain transmission electron microscopy (TEM). The remainders of the low-pH and EGTA extracts were each divided into dialysis bags and dialyzed overnight at 4°C against 10 mM HEPES (pH 7.5) containing 1 mM MgCl_2 ; 1 mM SrCl_2 ; and 1, 5, or 10 mM CaCl_2 . Each sample was examined by negative-stain TEM.

Electron microscopy. Parlodion-film, carbon-stabilized, 400-mesh copper grids were placed on droplets containing S-layer protein for a few seconds. The grids were lifted, and excess liquid was removed by wicking with filter paper. After drying, the sample was negatively stained by using 2% aqueous ammonium molybdate (pH 7.5) or 2% methylamine tungstate. Specimens were examined in a Siemens 101A TEM operated at 60 kV.

RESULTS

Extraction of the S layer of *C. crescentus* CB15A. Of the agents tested, 100 mM HEPES at pH 2 was the most effective at extracting RsaA with the least contamination

from other proteins. By Coomassie staining, the preparations appeared to be nearly pure RsaA in this single-step purification procedure. Occasionally, a minor amount of proteins migrating at the position of the flagellin monomers was noted. EGTA treatment also efficiently removed RsaA. Other methods were less effective. HEPES at pH 4 produced minor amounts of a few lower-molecular-weight proteins in addition to RsaA, while HEPES at pHs 6, 7.5, 8, and 10 did not significantly extract RsaA and produced increasing amounts of other proteins. Glycine-HCl at pH 2 yielded RsaA as a prominent protein, but a significant amount of lower-molecular-weight proteins was also present. Glycine-HCl treatment at pHs 3 and 4 showed further increases of other proteins. Similarly, 65°C treatment produced a prominent RsaA protein band, but many lower-molecular-weight proteins were also present. Guanidine-HCl, urea, Tris (pH 7.2), β -mercaptoethanol, and EDTA all extracted numerous proteins without RsaA predominating. As expected, NaCl and CaCl_2 treatments did not yield significant amounts of protein.

Comparison of S layers among freshwater caulobacters. The pH 2 extraction was applied to all of the FWC strains and, in general, proved to be a useful technique for specific extraction of the S-layer proteins. That is, only a single major high-molecular-weight band, characteristic of S-layer proteins from laboratory caulobacter strains, was generally seen by SDS-PAGE, and extracts from strains that were known not to have an S layer by TEM analysis (27) showed no prominent band. The S-layer-producing strains that did not show a broad high-molecular-weight band were extracted by using the EGTA method, and a characteristic band resulted in every case. Figure 1 shows the Coomassie blue-stained gels of the low-pH (Fig. 1A) or EGTA (Fig. 1B) extracts for the FWC strains and CB15A.

The protein samples used for the Coomassie blue-stained gels were analyzed by Western immunoblotting using anti-RsaA serum (Fig. 2). All of the broad S-layerlike bands in Fig. 1 reacted, except FWC23. The antiserum was quite specific for the suspected S-layer bands; note that in the EGTA-extracted samples (from which detectable amounts of non-S-layer proteins were sometimes readily extracted), only the prominent high-molecular-weight band reacted with the antiserum.

FWC5, FWC14, FWC21, FWC30, FWC38, FWC40, and FWC43, strains that do not have an S layer (27), did not show a high-molecular-weight band by Coomassie staining or a positive reaction by Western blotting. FWC23 was the only strain without an S layer to show a prominent band by Coomassie staining, but it was not labeled in Western immunoblot analysis. FWC26, FWC41, and FWC46 have an S layer and produced an S-layer band by the criteria of Coomassie staining and Western immunoblot analysis; however, extraction of the protein by both methods was poor. The remaining 32 strains yielded significant amounts of protein with at least one of the extraction procedures and gave positive results by both Western immunoblotting and Coomassie blue staining, although none of the proteins from the FWC strains gave as intense a signal by Western immunoblot analysis as did RsaA.

The molecular weights of the S-layer proteins from the FWC strains were estimated from their mobility in SDS-PAGE (Table 1); the proteins were quite heterogeneous, ranging from 100 to 193 kDa. Proteins greater than 100 kDa are difficult to size by SDS-PAGE mobility, and so the sizes reported are estimates, useful for comparative purposes. By size or presence of the S-layer protein, the FWC strains were

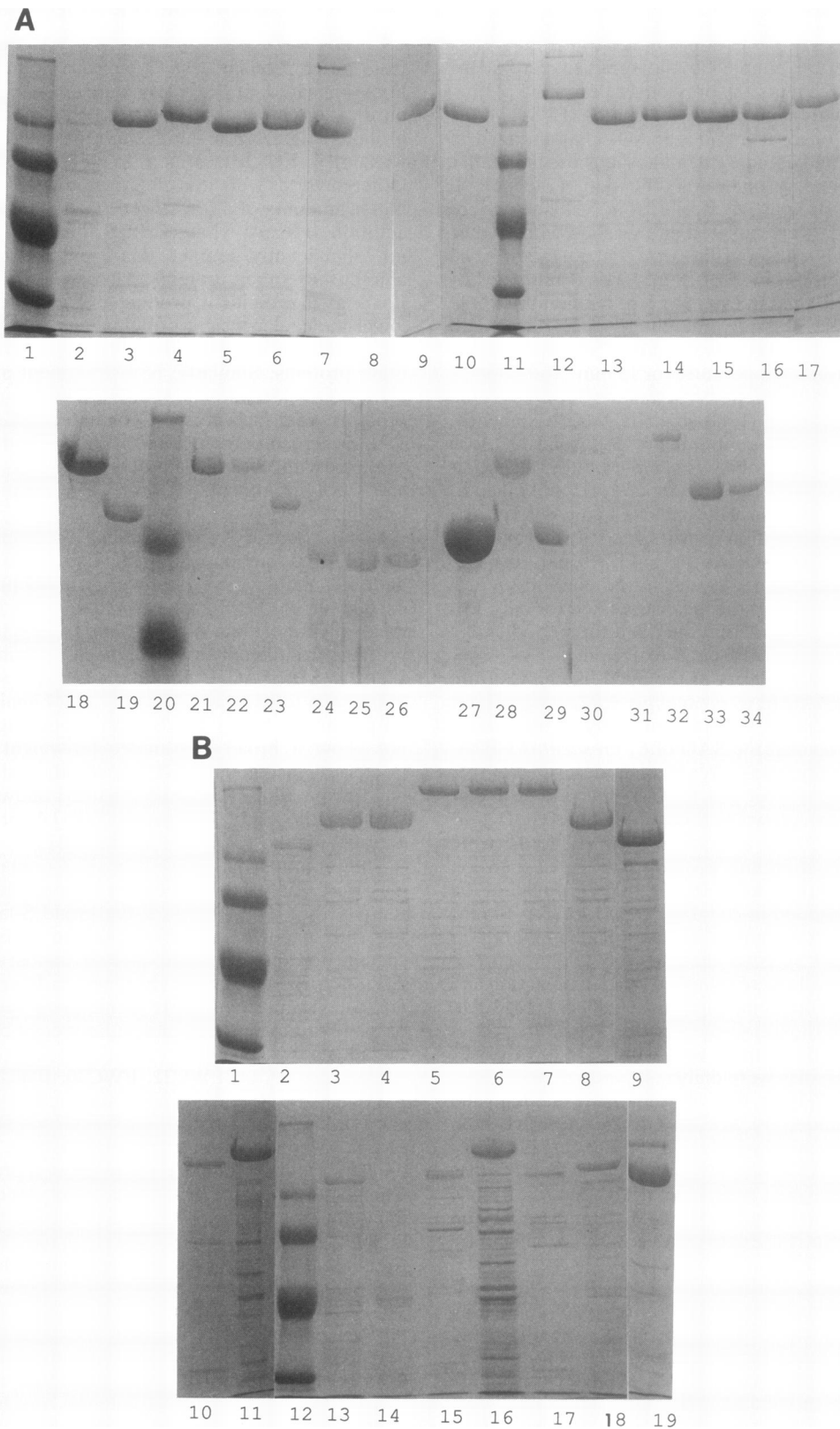


FIG. 1. SDS-PAGE of proteins extracted from various caulobacter isolates. (A) Low-pH-extracted proteins. Lanes: 1, molecular mass markers; 2, FWC38; 3, FWC28; 4, FWC33; 5, FWC35; 6, FWC31; 7, FWC1; 8, FWC43; 9, CB15A; 10, FWC15; 11, molecular mass markers; 12, FWC2; 13, FWC44; 14, FWC19; 15, FWC17; 16, FWC20; 17, CB15A; 18, FWC37; 19, CB15A; 20, molecular mass markers; 21, FWC16; 22, FWC26; 23, FWC18; 24, FWC22; 25, FWC25; 26, FWC46; 27, FWC11; 28, FWC23; 29, CB15A; 30, FWC40; 31, FWC30; 32, FWC39; 33, FWC27; 34, FWC24. (B) EGTA-extracted proteins. Lanes: 1, molecular mass markers; 2, CB15A; 3, FWC32; 4, FWC12; 5, FWC42; 6, FWC7; 7, FWC6; 8, FWC9; 9, FWC29; 10, FWC41; 11, FWC45; 12, molecular mass markers; 13, CB15A; 14, FWC21; 15, FWC14; 16, FWC4; 17, FWC5; 18, FWC8; 19, FWC34. The gels were stained with Coomassie brilliant blue. Three micrograms of protein was loaded in each lane. The molecular mass markers in this and the subsequent figures are, from the top, 200, 97.4, 68, 43, and 29 kDa.

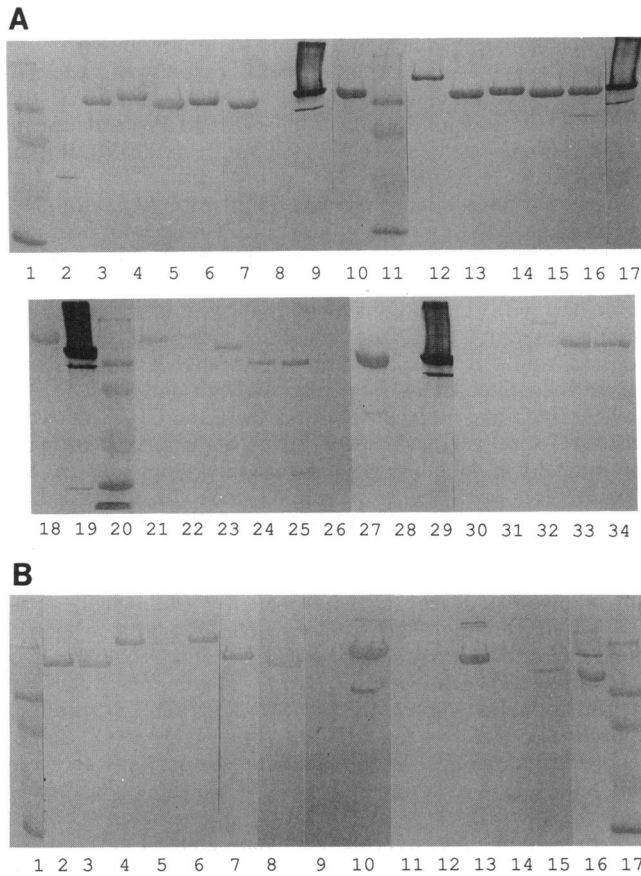


FIG. 2. Western immunoblot analysis of extracted proteins reacted with anti-RsaA serum. (A) Low-pH extracts. Lanes: 1, molecular mass markers; 2, FWC38; 3, FWC28; 4, FWC33; 5, FWC35; 6, FWC31; 7, FWC1; 8, FWC43; 9, CB15A; 10, FWC15; 11, molecular mass markers; 12, FWC2; 13, FWC44; 14, FWC19; 15, FWC17; 16, FWC20; 17, CB15A; 18, FWC37; 19, CB15A; 20, molecular mass markers; 21, FWC16; 22, FWC26; 23, FWC18; 24, FWC22; 25, FWC25; 26, FWC46; 27, FWC11; 28, FWC23; 29, CB15A; 30, FWC40; 31, FWC30; 32, FWC39; 33, FWC27; 34, FWC24. (B) EGTA extracts. Lanes: 1, molecular mass markers; 2, FWC32; 3, FWC12; 4, FWC42; 5, FWC7; 6, FWC6; 7, FWC9; 8, FWC29; 9, FWC41; 10, FWC45; 11, FWC21; 12, FWC14; 13, FWC4; 14, FWC5; 15, FWC8; 16, FWC34; 17, molecular mass markers. Three micrograms of protein was loaded in each lane.

placed into five groups. Group 1 (100 to 110 kDa) included FWC1, FWC11, FWC15, FWC17, FWC19, FWC20, FWC22, FWC25, FWC28, FWC31, FWC33, FWC34, FWC35, FWC44, FWC46, and *C. crescentus* CB15A (apparent size, 105 kDa by SDS-PAGE [38]; predicted size based on DNA sequence, 98 kDa [14]). Group 2 (122 to 153 kDa) included FWC2, FWC3, FWC4, FWC8, FWC9, FWC12, FWC16, FWC18, FWC24, FWC26, FWC27, FWC29, FWC32, FWC37, FWC41, and FWC45. Group 3 (177 to 193 kDa) included FWC6, FWC7, FWC39, and FWC42. Group 4 (no S layer and no protein detected) included FWC5, FWC14, FWC21, FWC30, FWC38, FWC40, and FWC43. Group 5 (no S layer but a prominent protein extracted) included FWC23.

Polysaccharide analysis. The FWC strains were also examined for the electrophoretic mobility of their LPS by using a rapid purification and staining procedure for LPS and similar oligosaccharide-containing molecules. Because we have

TABLE 1. Relevant characteristics of caulobacter strains

Strain	S-layer protein size (kDa) ^a	Anti-130K response ^b	Polysaccharides detected ^c	Anti-SAO response ^d
S-layer-producing strains				
FWC1	100	+	+	1
FWC2	132	+	+	1
FWC4	131 ^e	+	-	0
FWC6	181	+	+	3
FWC7	177	+	+	3
FWC8	122	+	+	1
FWC9	133	+	+	3
FWC11	108	+	+	1
FWC12	133	+	+	3
FWC15	110	+	+	0
FWC16	151	+	+	3
FWC17	106	+	+	2
FWC18	131	+	+	1
FWC19	108	+	+	2
FWC20	108	+	+	0
FWC22	107	+	+	1
FWC24	147	+	+	3
FWC25	105	+	+	1
FWC26	140	+	+	3
FWC27	145	+	+	1
FWC28	106	+	+	1
FWC29	124	+	+	0
FWC31	106	+	+	1
FWC32	133	+	+	3
FWC33	110	+	+	1
FWC34	110	+	+	2
FWC35	102	+	+	1
FWC37	149	+	+	3
FWC39	193	+	+	3
FWC41	137	+	+	3
FWC42	181	+	+	2
FWC44	106	+	+	2
FWC45	140	+	+	3
FWC46	110	+	+	2
Strains without S layers				
FWC5	NF ^f	-	-	0
FWC14	NF	-	Multiple bands	0
FWC21	NF	-	Multiple bands	0
FWC23	153	-	+	0
FWC30	NF	-	-	0
FWC38	NF	-	Multiple bands	0
FWC40	NF	-	-	0
FWC43	NF	-	+	0

^a Molecular masses were estimated on the basis of SDS-PAGE.

^b Reaction with low pH or EGTA-extracted protein by Western immunoblot analysis using anti-130K serum.

^c Results of SDS-PAGE of proteinase K-treated samples stained for polysaccharides by the silver staining procedure of Tsai and Frasch (42). +, single high-molecular-weight band. -, no silver-stained bands running slower than the lipopolysaccharide band.

^d Reaction by Western immunoblot analysis with anti-SAO serum to proteinase K-treated samples. The response is rated on a scale from 0 to 4, as follows: 0, no reaction; 1, slight reaction showing a doublet in the SAO region; 2, reaction showing the doublet and some smearing above the top doublet band; 3, definite smearing reaction reminiscent of the silver-stained image of the SAO; 4, reaction equal in intensity to that obtained with the *C. crescentus* CB15A SAO.

^e S-layer presence was not confirmed by negative-stain TEM, although the protein was reactive with anti-130K antibody.

^f NF, not found.

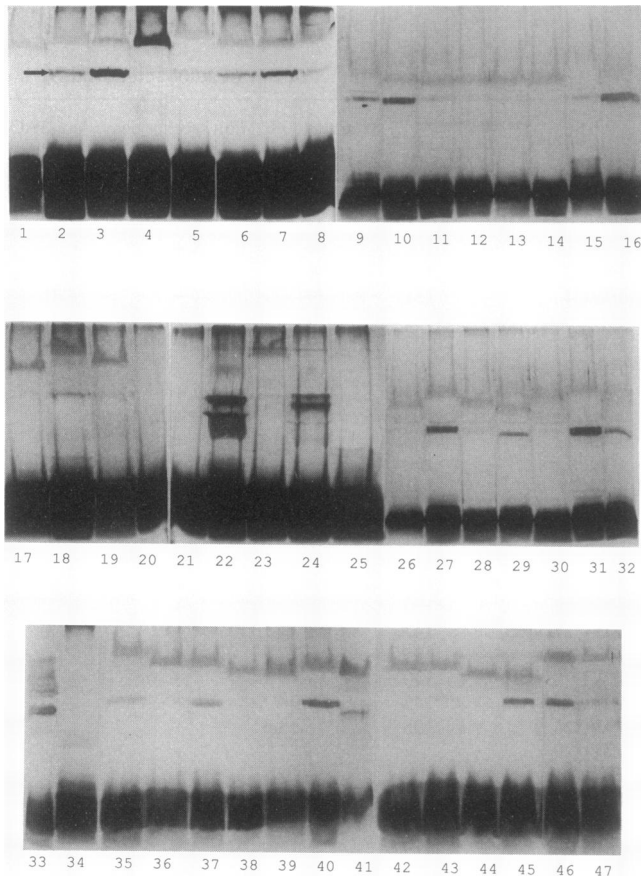


FIG. 3. SDS-PAGE of proteinase K-treated cells, stained with a method for visualization of LPS, developed by Tsai and Frasch (42). Lanes: 1, CB15A; 2, FWC9; 3, FWC18; 4, FWC41; 5, FWC45; 6, FWC24; 7, FWC27; 8, FWC16; 9, FWC25; 10, FWC20; 11, FWC19; 12, FWC17; 13, FWC15; 14, FWC11; 15, FWC1; 16, FWC23; 17, CB15A; 18, FWC39; 19, FWC2; 20, FWC4; 21, FWC5; 22, FWC38; 23, FWC43; 24, FWC14; 25, FWC40; 26, FWC29; 27, FWC8; 28, FWC46; 29, FWC44; 30, FWC35; 31, FWC33; 32, FWC28; 33, FWC21; 34, FWC30; 35, FWC7; 36, FWC37; 37, FWC12; 38, CB15A; 39, FWC22; 40, FWC34; 41, MCS6; 42, FWC32; 43, FWC26; 44, CB15A; 45, FWC31; 46, FWC6; 47, FWC42. Cell lysate containing 0.75 μ g of KDO was loaded onto each lane. The arrow in lane 1 indicates the position of proteinase K-resistant protein. SAO ran as a poorly stained band above the arrow.

identified an additional oligosaccharide in *C. crescentus* strains that is involved with calcium-mediated attachment of the S layer to the cell surface (termed SAO) (13); the presence of an SAO-like band was also monitored.

All strains produced low-molecular-weight polysaccharide-containing molecules, presumably rough LPS's, all of which migrated with the same electrophoretic mobility (Fig. 3). All strains producing an antibody-reactive S-layer protein (Fig. 2; Table 1) were found to have a single more slowly migrating band. This SAO-like species varied in apparent molecular size from 60 to 95 kDa (such values are only for comparative purposes, reflecting only the approximate size of a protein migrating at that position). Curiously, for a large proportion of the SAO-like molecules, a larger size was correlated with a larger-size S-layer protein. Many strains also showed a strongly staining doublet at approximately the position of a 43-kDa protein (Fig. 3, lane 1, arrow). A Coomassie blue-stained gel of the proteinase K-treated sam-

ples showed that the doublet did stain, indicating that this was a proteinase K-resistant protein (data not shown).

The S-layer-negative strains showed a variety of patterns, including no additional bands (FWC5, FWC30, and FWC40); multiple bands, reminiscent of the smooth LPS ladder seen with enteric bacteria (32) (FWC14, FWC21, and FWC38); or a single band (FWC23, FWC43, and MCS6).

Western immunoblot analysis, using anti-SAO serum, of the proteinase K-treated samples (Fig. 4) showed no reaction with the probable rough LPS species of any of the strains. When a reaction was seen in S-layer-producing strains, it coincided with the SAO-like bands. This is comparable to what is noted in CB2A, from which the anti-SAO serum was derived, and CB15A (13). None of the bands seen in the S-layer-negative strains were reactive with antibody.

Most SAO-like molecules reacted with the CB2A-derived anti-SAO serum; however, there was a greater variation in the intensity of labelling between samples than seen in the immunoblots of the S-layer proteins, including several that were not labeled (FWC16, FWC20, and FWC29) (Table 1). Moreover, the Western immunoblot analysis showed that most of the SAO-like bands that were labelled with antiserum showed a degree of heterogeneity, often seen as a doublet of immunoreactive bands. This too is a property shared with the authentic SAO of CB15A and CB2A (Fig. 4).

In vitro crystallization of the CB15A S layer. After dialysis overnight against 100 mM HEPES buffer (pH 7.5), protein samples extracted by the EGTA or low-pH method had no visible turbidity, and TEM negative-stain analysis showed only amorphous structures. Dialysis of the sample against 1 mM $MgCl_2$, or $SrCl_2$ did not promote crystallization of the S-layer protein. After overnight dialysis against 1 mM $CaCl_2$, the sample became turbid and TEM showed that the protein had frequently crystallized into a regularly structured array with hexagonal symmetry (Fig. 5A and B), with center-to-center spacing comparable to that of the native S layer. Higher concentrations of $CaCl_2$ also promoted turbid solutions, but ordered S-layer regions were much more difficult to detect by TEM (Fig. 5C).

DISCUSSION

The group examined here are examples of freshwater caulobacters that are common inhabitants of aquatic and soil environments. They can be roughly categorized as typical and atypical strains on the basis of our experiences in caulobacter isolation (27). That is, most caulobacters we have encountered appear similar to the laboratory *C. crescentus* strains; they have a crescent cell shape, short stalks, and a minimum of holdfast material (resulting in relatively few rosettes in culture) and elaborate a hexagonal S layer. Atypical strains have a variety of cell shapes, larger rosettes, longer stalks, and no visible S layer. In a recent study involving 16S rRNA analysis of a number of these strains, it was learned that in fact the typical strains are a relatively closely related subgroup of the freshwater caulobacters, while examples of the atypical strains were different from the typical cluster and from each other (41). Nevertheless, caulobacters in the typical group were still measurably dissimilar, exhibiting rRNA similarity values of ca. 99%. For reference, a DNA similarity of 50% generally corresponds to rRNA similarity values of 98 to 99%.

Since the group of S-layer-producing caulobacters were phylogenetically cohesive yet clearly different from one another, it was difficult to predict a priori whether the S-layer proteins would be structurally similar. Indeed, it

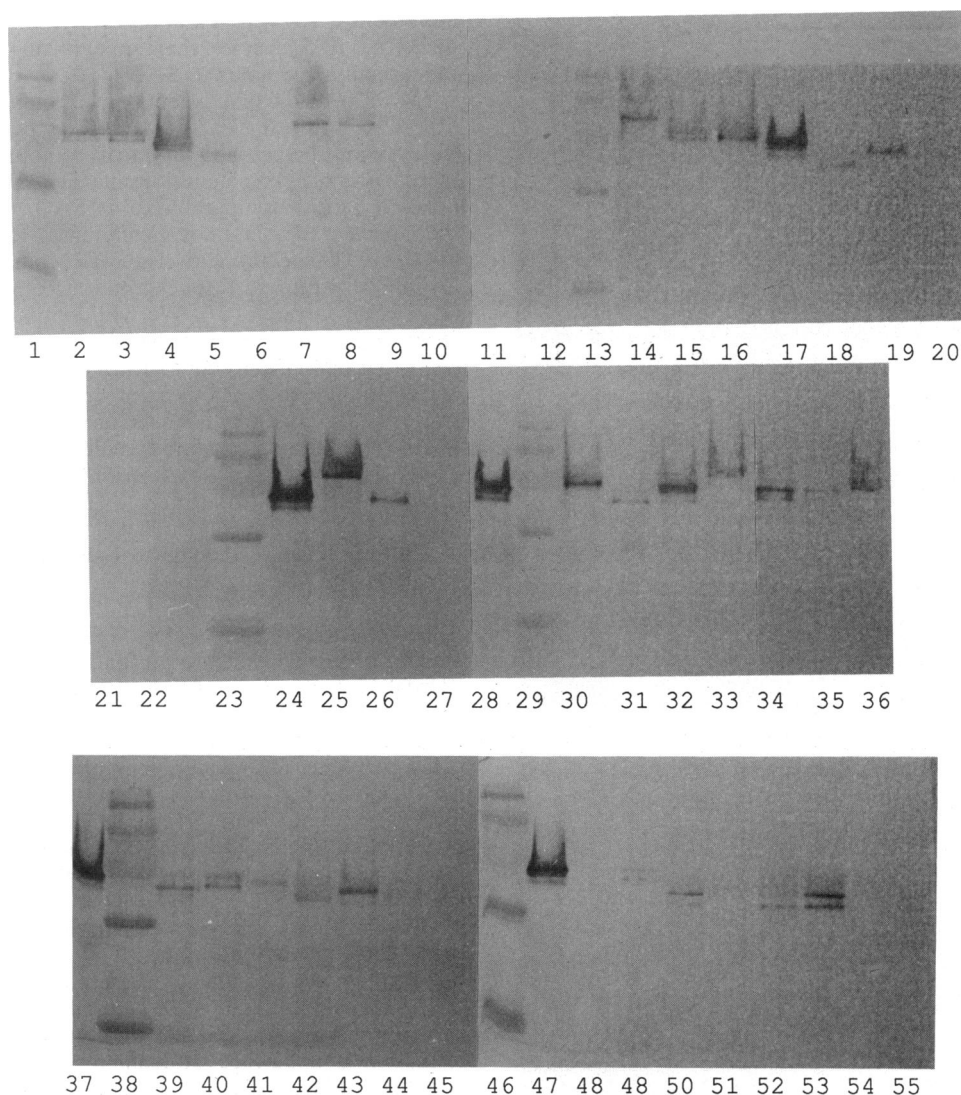


FIG. 4. Western immunoblot analysis of proteinase K-treated cells reacted with anti-SAO serum. Lanes: 1, molecular mass markers; 2, FWC32; 3, FWC26; 4, CB15A; 5, FWC31; 6, FWC43; 7, FWC6; 8, FWC42; 9, FWC5; 10, FWC38; 11, FWC21; 12, FWC30; 13, molecular mass markers; 14, FWC7; 15, FWC37; 16, FWC12; 17, CB15A; 18, FWC22; 19, FWC34; 20, MCS6; 21, FWC14; 22, FWC40; 23, molecular mass markers; 24, CB15A; 25, FWC39; 26, FWC2; 27, FWC4; 28, CB15A; 29, molecular mass markers; 30, FWC9; 31, FWC18; 32, FWC41; 33, FWC45; 34, FWC24; 35, FWC27; 36, FWC16; 37, CB15A; 38, molecular mass markers; 39, FWC28; 40, FWC33; 41, FWC35; 42, FWC44; 43, FWC46; 44, FWC8; 45, FWC29; 46, molecular mass markers; 47, CB15A; 48, FWC23; 49, FWC1; 50, FWC11; 51, FWC15; 52, FWC17; 53, FWC19; 54, FWC20; 55, FWC25. Cell lysate containing 0.75 μ g of KDO was loaded onto each lane.

might be expected that the S-layer proteins of a collection of caulobacter strains would show significant differences. They are not, for example, pathogenic strains with an S layer attuned to parasite-host interactions, as with some other S-layer-producing strains (10, 11, 19, 30). Thus, there might seem to be little reason for conservation of a specific S-layer structure, particularly at the level of immunological similarity.

However, this study indicated that there was similarity and conservation of the S layers of the caulobacters; the similarity was demonstrated at several levels. The disruption methods used appeared in all cases to disrupt and extract the S layer specifically, and the resulting released protein was also, in all cases but one, FWC 23, immunologically cross-reactive. In addition, oligosaccharide-containing molecules apparently analogous to the molecule linking the S layer to

the cell surface in *C. crescentus* strains (SAO) were also present in all S-layer-producing strains and in most cases had at least a degree of immunological similarity to the CB2A strain. It seems possible that, as with the CB15A strain, calcium (or another divalent cation) is required for attachment of the S layer to the surface (we hypothesize that this occurs via the SAO molecule) in the various caulobacter strains. That is, the most selective methods for extraction of S-layer proteins would disrupt calcium-mediated ionic bonding (i.e., treatment with EGTA, a calcium-selective chelator, and competition of calcium with protons at low pH). It can be argued then that there is not only a degree of conservation among caulobacter S-layer proteins but also conservation of a second molecule, possibly the means of surface attachment. Conversely, in the case of the atypical caulobacters, when there is no S layer there seems to be a different surface

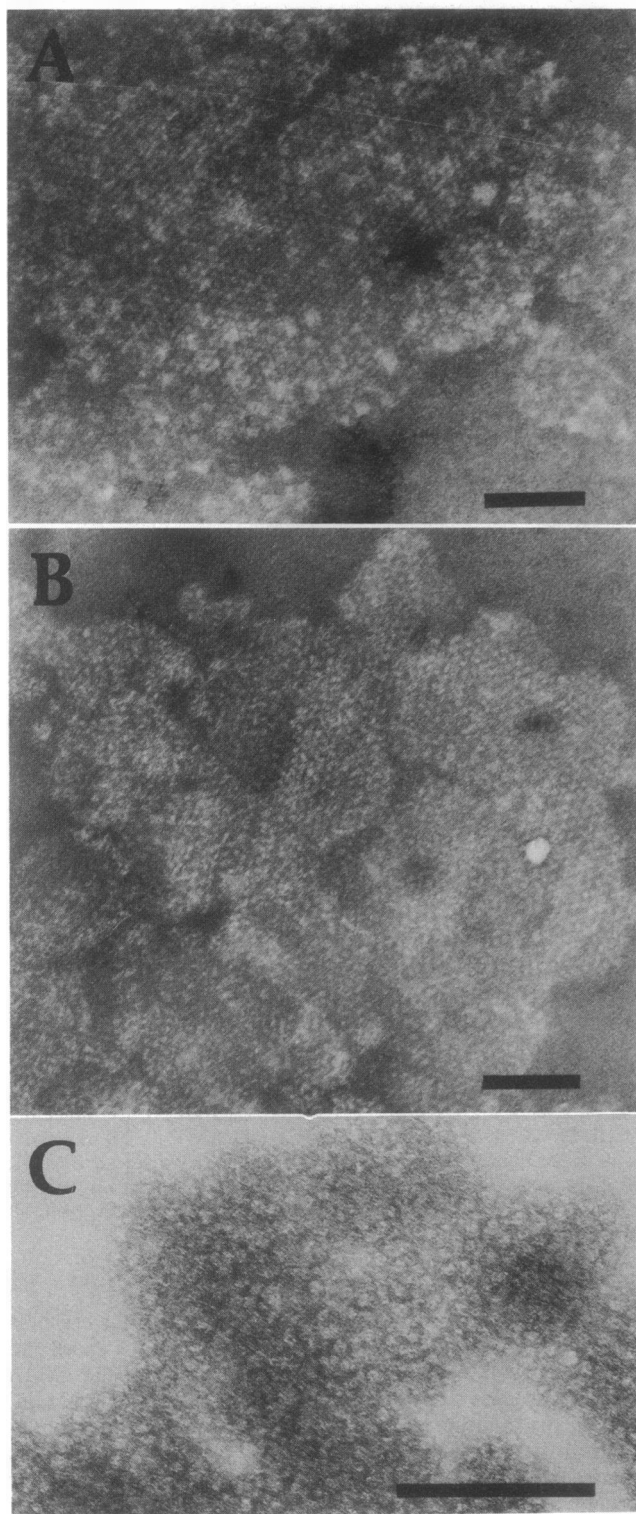


FIG. 5. In vitro recrystallization of the S-layer protein (RsaA) of *C. crescentus* CB15 into two-dimensional arrays. (A and B) Recrystallization of RsaA that was purified by the low-pH and EGTA extraction methods, respectively, followed by dialysis in 1 mM CaCl_2 . (C) Results of attempts to restore the two-dimensional array of EGTA-extracted protein by dialysis with 10 mM CaCl_2 . Negative staining with ammonium molybdate is shown. Bars, 0.25 μm .

architecture as well. Parenthetically, *A. salmonicida* produces an LPS species that appears in many ways to be functionally analogous to SAO (7, 9).

FWC23 was the one exceptional strain in the group, which otherwise showed clear demarcations between the typical (S-layer-producing) and atypical caulobacter strains. Although a single prominent protein was extracted and a single polysaccharide, comparable to an SAO, was detected, neither species was immunologically cross-reactive with S-layer- and SAO-specific antiserum. TEM analysis and hybridization with the *rsaA* gene probe also indicated that an S layer is not present (27). We will continue to examine this strain to learn whether it may produce another type of S layer that is unusually difficult to detect.

FWC4 differed from the rest of the typical caulobacter strains analyzed in that it lacked an SAO-like polysaccharide but the extraction procedures resulted in a single protein that was reactive with anti-RsaA serum. We were also unable to confirm an S-layer structure by TEM (data not shown). In the case of FWC4, it may be that a conserved S-layer protein is attached to the surface but paracrystalline order has been lost, perhaps as a consequence of the loss of an SAO-like molecule.

Low-pH treatment has also been used for selective purification of the S layer of other bacteria, including *Spirillum* "Ordal" (2), *Spirillum putridiconchylum* (3), *A. hydrophila* (10), and *Campylobacter fetus* (28). For the *Aeromonas* and *Campylobacter* species, a low-pH extraction procedure, using a glycine-HCl buffer, was effective. In contrast, we had much better results in selectively removing RsaA by using HEPES at low pH, recognizing that it is not a buffer in that range. Perhaps with caulobacters the protonated amino group of glycine at low pH also disrupts other membrane-associated proteins.

The anti-RsaA antibody cross-reaction was specific in these experiments, but the degree of labeling was relatively uniform between strains and significantly less than that obtained with RsaA. It seems possible that there are conserved regions in the S-layer proteins that are required for formation and surface attachment of the paracrystalline structure while the rest of the proteins are variable and may be dispensable. RsaA is a member of the group of smallest caulobacter S-layer proteins (ca. 100 kDa) and therefore may be one that contains the closest to the minimal amount of essential assembly-attachment information. This may mean, in some cases (e.g., FWC39), that more than half of the protein serves some purpose other than essential structure formation. Dubreuil et al. (11) made a similar prediction of structurally nonessential regions in the S layer of *C. fetus* strains.

The immunological findings are also reminiscent of gene hybridization studies, which analogously showed that the CB15A S-layer gene (*rsaA*) could be used to identify most caulobacters isolated from wastewater treatment systems (since most produced S layers), but only under reduced-stringency conditions (27). It was hypothesized that conserved regions of the S-layer genes may be responsible for the hybridization noted. Our working hypothesis, based on the data collected so far, is that the degree of S-layer structure conservation noted is likely a consequence of common mechanisms of self-assembly, surface attachment, and possibly export—mechanisms conserved during the evolution of the various caulobacter strains. Certainly, the data do not support the concept that caulobacter S layers were acquired from other bacteria.

The reassembly studies with the purified CB15A protein

provided definitive data that only RsaA is responsible for the visible repeated structure. Previously, we had reported for CB15A that S-layer preparations (consisting of shed S-layer fragments isolated by differential centrifugation) contained two other proteins, 74K and 20K, but that the preparations also contained some membrane material and it could not be resolved whether the additional proteins were membrane derived and persistently associated with S-layer preparations or were part of the visible structure (39). Since the reassembly experiments reported here involved preparations with little indication of other proteins, it seems clear that while these additional proteins may have some role in S-layer attachment or functioning, they are not part of the visible structure.

The reassembly experiments also reinforced and extended our previous findings (13) that calcium is specifically required for S-layer assembly; even the divalent strontium ion, which has a hydrated molecular diameter most similar to that of calcium and has substituted for calcium in the assembly of other S layers (4), was unable to replace calcium. We recently identified regions within RsaA that have significant homology to calcium-binding regions of other proteins that require calcium for activity (14). Analysis is under way to determine whether these regions are the reason for the high degree of specificity for calcium for S-layer surface attachment and assembly in *C. crescentus*.

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