Characterization of Mutants of *Caulobacter crescentus* Defective in Surface Attachment of the Paracrystalline Surface Layer

STEPHEN G. WALKER, D. NEDRA KARUNARATNE, NEIL RAVENSCROFT,[†] AND JOHN SMIT*

*Department of Microbiology and Immunology, The University of British Columbia,
Vancouver, British Columbia V6T 1Z3, Canada*

Received 25 May 1994/Accepted 4 August 1994

Strains of *Caulobacter crescentus* express a paracrystalline surface layer (S-layer) consisting of the protein RsaA. Mutants of *C. crescentus* NA1000 and CB2, isolated for their ability to grow in the absence of calcium ions, uniformly no longer had the S-layer attached to the cell surface. However, RsaA was still produced, and when colonies grown on calcium-sufficient medium were examined, large two-dimensional arrays of S-layer were found intermixed with the cells. Such arrays were not found in calcium-deficient medium even when high levels of magnesium ions were provided. The arrays could be disrupted with divalent ion chelators and more readily with the calcium-selective ethylene glycol-bis(β -aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA). Thus, the outer membrane surface was not needed as a template for self-assembly, but calcium likely was. The cell surface and S-layer gene of assembly-defective mutants of NA1000 were examined to determine the basis of the S-layer surface attachment defect. Mutants had no detectable alteration in the rough lipopolysaccharide (LPS) or a characterized capsular polysaccharide, but another polysaccharide molecule was greatly reduced or absent in all calcium-independent mutants. The molecule was shown to be a smooth LPS with a core sugar and fatty acid complement identical to those of the rough LPS and an O polysaccharide of homogeneous length, tentatively considered to be composed of 4,6-dideoxy-4-amino hexose, 3,6-dideoxy-3-amino hexose, and glycerol in equal proportions. This molecule (termed SLPS) was detectable by surface labeling with a specific antiserum only when the S-layer was not present. The *rsaA* genes from three calcium-independent mutants were cloned and expressed in an S-layer-negative, SLPS-positive strain. A normal S-layer was produced, ruling out defects in *rsaA* in these cases. It is proposed that SLPS is required for S-layer surface attachment, possibly via calcium bridging. The data support the possibility that calcium binding is required to prevent an otherwise lethal effect of SLPS. If true, mutations that eliminate the O polysaccharide of SLPS eliminate the lethal effects of calcium-deprived SLPS, at the expense of S-layer attachment.

Surface layers (S-layers) are a common feature on the surface of eubacteria and archaeobacteria (34). The structures are generally composed of repeated units of a single protein or glycoprotein (44). It is likely that in many bacteria the layer is the outermost layer, directly exposed to the environment (44). In such a position, S-layers may well have roles of protection for the cell or be involved with pathogenesis in the case of pathogenic bacteria (34). Many aspects of this general bacterial structure were summarized in a recent international meeting devoted to such structures (5).

Most often, S-layer proteins are assembled on (rather than within) the underlying layer, whether it is a bilayer membrane or a complex of peptidoglycan or pseudomurein (34). Thus, S-layer structural subunits are secreted proteins and must retain both their form and their function while exposed to the external environment. It follows then that to be formed into a geometric S-layer, aside from functional roles, the protein subunits must be capable of secretion, self-assembly with adjacent subunits, and attachment to the underlying cell surface.

The S-layer of *Caulobacter crescentus* NA1000 is composed of the protein RsaA (18); a similar or identical protein is found

in the related strain CB2. The *caulobacter* S-layer has been analyzed by electron microscopy and three-dimensional image reconstruction to a resolution of 2 nm (47). The repeat unit consists of a hexagonal core that is relatively widely spaced (22-nm interval), creating an open pore of 2.5 to 3.5 nm (47). The molecular details of export, self-assembly, and attachment for RsaA are being defined (7, 47, 55).

The present report describes an investigation into the means of attachment of the S-layer to the cell surface, taking the first steps to define the outer membrane components involved with the interaction. The approach is an investigation of calcium-independent *caulobacter* mutants. Species of *caulobacters* are among the few bacteria that have a requirement for calcium (53, 54a). Poindexter (38) was the first to notice that under conditions of calcium deprivation, mutants which no longer needed calcium could be selected, and in her studies a high proportion no longer assembled an S-layer, although unassembled RsaA accumulated in liquid cultures. This suggested that whatever alteration relieves the lethal effects of calcium deprivation somehow affects S-layer attachment and self-assembly.

In previous studies, some consequences of the S-layer shedding phenotype have been characterized (47). The shed RsaA protein crystallizes within colonies to produce large stacks of S-layer sheets (which often accumulate at the surface of a colony), and each sheet is composed of a mirror image double layer of the protein. We reported in a preliminary fashion (55) that an oligosaccharide was associated with S-layer attachment (and hence was termed the S-layer-associated oligosaccharide [SAO]) and that this molecule had counterparts in many other

* Corresponding author. Mailing address: Department of Microbiology and Immunology, The University of British Columbia, #300-6174 University Blvd., Vancouver, B.C. V6T 1Z3, Canada. Phone: (604) 822-4417. Fax: (604) 822-6041. Electronic mail address: jsmit@unixg.ubc.ca.

[†] Present address: Max-Planck-Institut für Medizinische Forschung, D-69120 Heidelberg, Germany.

TABLE 1. Bacteria and plasmids

Bacterial strain or plasmid	Description or genotype ^a	Reference or source
<i>C. crescentus</i>		
CB2A	S-layer-minus variant of wild-type CB2; Tp ^r Rf ^r Ap ^r	46
CB2NY66Rmgl	Calcium-independent mutant of CB2	38a
CB2NY66R	S-layer-producing CB2 strain	38a
NA1000	Variant of wild-type strain CB15, ATCC 19089; synchronous cultures readily prepared from this strain; Tp ^r Ap ^r	
JS1001	Calcium-independent mutant of NA1000 (cited in reference 16 as CB15Ca5)	This study
JS1002	Calcium-independent mutant of NA1000 (cited in reference 16 as CB15Ca10)	This study
JS1003	NA1000 with <i>rsaA</i> interrupted with KSAC Km ^r cassette (cited in reference 16 as CB15Ksac)	16
JS1004	JS1001 with <i>rsaA</i> interrupted with KSAC cassette	This study
JS1005	JS1002 with <i>rsaA</i> interrupted with KSAC cassette	This study
<i>E. coli</i>		
RB404	F ⁻ <i>dam-3 dam-6 metB1 galK2 galT22 lacY1 thi-1 tonA31 tsx-78 mtl-1 supE44</i> ; does not methylate <i>ClaI</i> sites	8
K12ΔHΔTrp	M72 Sm ^r <i>lacZ</i> (Am) Δ <i>b10</i> ⁻ <i>uvrB</i> F ⁻ Δ <i>trpE</i> A2 (λ <i>Nam7 Nam53, cI857 ΔH1</i>)	43
pKT230	Broad-host-range vector; Km ^r Sm ^r	2
pKT230-A19	pKT230 containing <i>rsaA</i> from NA1000 as a <i>HindIII</i> - <i>BamHI</i> fragment	This study
pKT230-Ca5	pKT230 containing <i>rsaA</i> from JS1001	This study
pKT230-Ca10	pKT230 containing <i>rsaA</i> from JS1002	This study
pKT230-CB2AD	pKT230 containing <i>rsaA</i> from CB2NY66Rmgl	This study
pLC28-A19	<i>rsaA</i> in <i>HindIII</i> site of pLC28, a vector containing the phage lambda heat-inducible p _L promoter; Ap ^r	46
pPR510	Derived from pUC19; Cm ^r	40
pUC13	General cloning vector; ColE1 replicon; Ap ^r	51
pUC4KSAC	Plasmid containing the KSAC Km ^r cassette	3
pUC13-A19	Wild-type <i>rsaA</i> cloned as a <i>HindIII</i> - <i>BamHI</i> fragment in pUC13	This study

^a Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Rf^r, rifampin resistant; Sm^r, streptomycin resistant; Tc^r, tetracycline resistant; Tp^r, trimethoprim resistant.

S-layer-producing caulobacter strains, on the basis of immunological similarity and staining characteristics (55).

In the present study, we investigated the calcium-independent phenotype in detail and noted that under appropriate selection conditions, all resulting calcium-independent mutants have lost the ability to attach the S-layer and that the defect lies not with the S-layer but with the SAO molecule. We then undertook an initial characterization of the composition of that molecule. Because it was found to be a smooth lipopolysaccharide (LPS) species, we have renamed it SLPS and will refer to it as such in this report.

(Preliminary accounts of some aspects of the work described here were presented at the Third International Symposium on Bacterial S-layers, held at the University of Western Ontario in September 1992 [54], and at the Canadian Society of Microbiologists/Society of Industrial Microbiology joint annual meeting, held in Toronto, Canada, August 1993 [53].)

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Strains and plasmids are described in Table 1. For normal growth and maintenance, *C. crescentus* strains were grown at 30°C in a peptone-yeast extract (PYE) medium (36) supplemented with CaCl₂ · 2H₂O to 0.01% and MgSO₄ · 7H₂O to 0.02%. For the isolation of rough LPS and extracellular polysaccharide (EPS), cultures were grown as described previously (41, 42). JS1003 was grown in a fermentor as a 60-liter batch culture for isolation of SLPS. *Escherichia coli* strains were grown at 37°C in L broth (35). Solid media contained 1.2% agar. Antibiotics were added as necessary at 50 µg/ml unless otherwise noted.

Isolation of calcium-independent mutants. *C. crescentus* NA1000 was grown in PYE medium and then subcultured to a calcium-deficient mineral salts medium, which was a modifica-

tion of HiGG medium (37). The medium (M₁₀HiGG) contained 5 mM imidazole-HCl (pH 7.0), 0.2 mM potassium phosphate, 0.05% ammonium chloride, 0.1% glucose, 0.1% sodium glutamate, and 1% Hutner's mineral salts supplement (11), except that the Hutner's supplement was prepared without calcium chloride. Four to six days of incubation at 30°C was needed to develop turbid growth (whereas with calcium-sufficient medium, growth occurred with overnight incubation). The cells were subcultured into the same medium and incubated for 2 more days. Cells were then plated at appropriate dilutions onto PYE and M₁₀HiGG solid media, solidified with nucleic acid electrophoresis-grade agarose (8 g/liter). Colonies that grew on M₁₀HiGG plates were examined for their S-layer characteristics and their ability to grow in the absence or presence of normal concentrations of calcium. From this group of mutants, JS1001 and JS1002 were selected for study.

Microscopy methods. To examine cells by negative-stain electron microscopy for the presence of S-layers, colonies were suspended in a 10-µl droplet of water, or appropriate concentrations of EDTA or EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] in some experiments, on parafilm. A carbon-stabilized, Parlodion-coated 400-mesh copper grid was floated on top of the droplet for 10 to 15 s. The grid and associated drop were then dragged into a 10-µl droplet of 2% ammonium molybdate (pH 7.5) and a 1-µl droplet of a bacitracin solution (1 mg/ml). After a mixing, excess liquid was wicked away with filter paper. Preparations were examined with a Siemens Elmiskop 101A transmission electron microscope operated at 60 kV.

The method for labeling cells with specific antibody and protein A coupled to colloidal gold in preparation for whole-mount microscopy has been described previously (45). Colloidal gold was prepared as 5-nm-diameter particles by the phosphorus reduction method (49).

FIG. 1. Electron micrograph of a large patch of S-layer found adjacent to the cells of the calcium-independent mutant JS1001 grown on calcium-sufficient PYE solid medium. Bar, 0.5 μm (inset bar, 0.1 μm).

Antibody labeling of cells for fluorescence microscopy was done by standard indirect labeling methods, using rabbit primary antisera and fluorescein-conjugated anti-rabbit serum, raised in a goat. Preparations were examined with a Zeiss epi-illuminated fluorescence microscope.

Genetic constructions. The cloning of the wild-type S-layer gene in lambda phage has been described elsewhere (46). The cloning of the analogous gene from the calcium-independent mutants JS1001, JS1002, and CB2NY66Rmgl was done similarly, except that lambda phage L47.1 (29) was used. Caulobacter chromosomal DNA was prepared by a complete *Hind*III digestion, and appropriate clones were detected by hybridization to the wild-type gene. The *Hind*III-*Bam*HI segment containing the entire *rsaA* gene (18) was ultimately placed in the corresponding sites in the broad-host-range vector pKT230.

Work with cloned DNA segments, including restriction enzyme mapping, plasmid isolation, and Southern blot hybridization, was done by standard methods (31). Plasmid constructions were introduced by transformation into *E. coli* and subsequently into caulobacters by conjugation (1) or by electroporation (19). The insertion of specific fragments into plasmids, phage, or bacterial strains was confirmed, when appropriate, by colony or plaque hybridization techniques (31). *RsaA*⁻ mutants of *C. crescentus* JS1001 and JS1002 were constructed by a gene replacement strategy using an *rsaA* gene

interrupted with a kanamycin resistance cartridge, as previously described for strain NA1000 (16).

Colorimetric assays. Quantities of protein, 3-deoxy-2-octulosonic acid (KDO), P_i , and sugars were estimated as described previously (42).

Electrophoresis and Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analysis were performed as described previously (55). Cell material was solubilized in sample buffer at room temperature instead of 100°C because little or no RsaA protein will enter the gel if it is heated to 100°C (46).

Antibody to RsaA was prepared by immunizing a rabbit with a crude inclusion body preparation from *E. coli* cells expressing RsaA. Strain K12 $\Delta\text{H}\Delta\text{Trp}$, containing pLC28-A19, was grown at 40°C to induce the expression of RsaA. Cells were harvested by centrifugation ($12,000 \times g$ for 10 min), and the cell pellet was suspended in a small amount of water. The cells were disrupted by sonication, and membranes and inclusion bodies (presumably consisting of RsaA [18]) were obtained by centrifugation for 1 h at $100,000 \times g$. After this preparation was combined with an equal quantity of Freund's incomplete adjuvant, a rabbit was immunized with an initial injection and subsequent booster injections of the preparation at days 21, 28, and 35. Serum was collected and processed as described previously (55).

When polysaccharides were examined by SDS-PAGE, sam-

ples were prepared as described previously (55) and gels were stained by a modification (55) of the method of Tsai and Frasch (50) or by using the Bio-Rad silver stain kit, which is based on the method of Merrill et al. (33).

Antiserum to the SLPS was fortuitously raised during attempts to prepare antibody to the adhesive holdfast of strain CB2A. Colloidal gold particles (which bind to the holdfast [32]) were added to cultures of CB2A cells. The cells were harvested by centrifugation, extensively treated by sonic disruption to break the cells, and treated with RNase and DNase. The preparation was then subjected to CsCl density gradient centrifugation (50% [wt/vol] CsCl). The colloidal gold particles (and associated material) sedimented to the bottom of the gradient; they were collected and used for rabbit immunization in a fashion similar to the RsaA immunization. Analysis of the serum showed little activity against the holdfast material, but in Western analysis a strong response to what is here described as SLPS, which is also selectively separated by this method and was strongly immunogenic (54b), was obtained.

Polysaccharide isolations. The method for isolation of rough LPS and extracellular polysaccharides has been described previously (41, 42). SLPS was isolated and purified from JS1003 as follows. All procedures were carried out at room temperature (20 to 22°C). Cell pellets obtained by continuous centrifugation of fermentor cultures were washed by suspension and centrifugation ($10,000 \times g$; 10 min) with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Research Organics Inc., Cleveland, Ohio), pH 7.2. Washed cell pellets were suspended in 0.77 M NaCl–0.12 M EDTA (pH 7.2) at 25 ml/10 g (wet weight) of cells (24), stirred for 5 min, and pelleted by centrifugation. The supernatant was saved, and the pellet was extracted a second time. The combined supernatants were ultracentrifuged at $225,000 \times g$. The pellet, containing rough LPS, SLPS, and three to five specific proteins (52), was resuspended in phosphate-buffered saline (PBS) by sonication and dialyzed against PBS at 4°C. The extracts, containing approximately 5 mg of protein and 450 μ g of KDO per ml, were mixed with an equal volume of SDS-PAGE dissociation buffer and heated at 100°C for 10 min. After the mixture had cooled to room temperature, proteinase K (Sigma Chemical Company, St. Louis, Mo.) was added to a final concentration of 0.5 mg/ml, and the sample was placed at 60°C overnight. The sample was then heated at 100°C for 10 min and fractionated by preparative SDS-PAGE (13% separating gel [12 by 14 cm with 1.5-mm spacers]). The region of the gel containing SLPS was excised, with prestained molecular weight markers (Gibco BRL Life Technologies, Inc.) used as a guide, placed in a dialysis membrane (12- to 14-kDa cutoff) containing SDS-PAGE running buffer, and electroeluted at 100 mA. The SLPS was concentrated and washed extensively with water by using a Centricon-30 microconcentrator (Amicon Canada Ltd.).

Carbohydrate and lipid analysis. EPS and rough LPS were analyzed as described previously (41, 42).

The following hydrolytic conditions were used for analysis of the sugars in SLPS: condition A, 2 M methanolic HCl at 100°C for 17 h and hydrolysis with 2 M trifluoroacetic acid (TFA) at 120°C for 4 h after removal of HCl; condition B, 2 M methanolic HCl at 100°C for 18 h and hydrolysis with 4 M TFA at 121°C for 1 h after removal of HCl; condition C, 4 M TFA at 120°C for 1 h; condition D, 0.5 M methanolic HCl at 85°C for 18 h, removal of HCl followed by reduction with sodium borodeuteride in anhydrous methanol, removal of borate by coevaporation with acidified methanol, and hydrolysis performed with 1 M TFA at 120°C for 1 h; and condition E, methanolysis and reduction with sodium borodeuteride per-

FIG. 2. Western blot analysis of whole-cell lysates reacted with anti-RsaA antiserum. The antiserum reliably detected RsaA but also reacted with some other caulobacter proteins, a common finding in our experience for antisera prepared from multiple antigens. Below, L indicates that cells were grown in a liquid culture and washed prior to analysis and P indicates that cells were grown on plates and were not washed prior to analysis. Lanes: 1, prestained molecular mass markers; 2, NA1000 (L); 3, JS1003 (P); 4, JS1003/pKT230-A19 (L); 5, JS1003/pKT230-Ca5 (L); 6, JS1002 (L); 7, JS1001 (P); 8, JS1004 (P); 9, JS1002 (P); 10, JS1005 (P); 11, NA1000 (L); 12, JS1003/pKT230-CB2AD (L); 13, JS1005/pKT230-A19 (L); 14, JS1005/pKT230-A19 (P); 15, prestained molecular mass markers; 16, CB2A (P); 17, CB2A/pKT230-A19 (L); 18, CB2A/pKT230-Ca10 (L); 19, CB2A/pKT230-Ca5 (L); 20, CB2A/pKT230-CB2AD (L); 21, JS1004/pKT230-Ca10 (P); 22, JS1004/pKT230-Ca10 (L); 23, prestained molecular mass markers; 24, CB2NY66R (L); 25, CB2NY66RmgI (L); 26, CB2NY66RmgI (P). Three micrograms of protein was loaded in each lane. Molecular mass markers are 200 and 97.4 kDa. Only the relevant region of the gel is shown.

formed as for condition D but hydrolysis with 1 M TFA at 120°C performed for only 0.5 h.

Gas-liquid chromatography was performed on a SE 54 capillary column with the following temperature program: 180°C for 1 min, a 5°C rise per min to 220°C, and a 10°C rise per min to 250°C. Gas chromatography-mass spectrometry (GC-MS) was performed on a DB-5 capillary column programmed at 180°C for 1 min with a 5°C rise per min to 250°C. Electron impact ionization was performed at 70 eV. Chemical ionization was performed with ammonia gas.

RESULTS

Isolation of calcium-independent mutant caulobacters. The shift of wild-type caulobacters from standard calcium-sufficient liquid medium (PYE) to calcium-deficient minimal liquid medium led to a lengthy delay (4 to 6 days) before growth was apparent, after which subsequent subcultures grew readily. When cells from several successive subcultures were plated onto M₁₀HiGG solid medium, only about 1 in 10⁶ would form colonies, relative to the CFU produced on PYE medium. Presumably, the lag in growth was an adaptation of the culture to very low calcium conditions and not the result of selection and outgrowth of calcium-independent mutants. However, in successful experiments, all colonies that grew on the solidified M₁₀HiGG continued to be calcium independent upon subculturing and displayed the S-layer shedding phenotype (see below). We hypothesize that the highly purified agarose used as a solidifying agent was free of calcium and further seques-

FIG. 3. SDS-PAGE analysis of an NA1000 rough-LPS preparation which also contained some SLPS. Lane A was stained with the Bio-Rad silver stain kit and loaded with 40 μ g of carbohydrate. Lane B shows Western blot analysis using anti-SLPS. The lane was loaded with 10 μ g of carbohydrate. The running position of SLPS (arrow) is indicated.

tered residual calcium from the medium, resulting in truly calcium-deficient conditions. In support of this, only some lots of highly purified agarose were suitable; unsuitable lots resulted in some growth of wild-type caulobacters on solid M_{10} HiGG medium. The generation time for JS1001 in M_{10} HiGG was 2.5 h, whereas in M_{10} HiGG supplemented with 1 mM $CaCl_2$ it was 2.2 h. Wild-type NA1000 had a generation time of 2.0 h in M_{10} HiGG supplemented with 1 mM $CaCl_2$.

Phenotype analysis of the calcium-independent mutants. Examination by negative-stain electron microscopy of the mutant strains that grew on M_{10} HiGG solid medium revealed a uniform phenotype; the cells had no evidence of an organized surface structure, whether or not calcium was present in the growth medium. When cells were grown in calcium-sufficient liquid medium, a macroscopic particulate debris would appear in high-density cultures. Electron microscopy examination of this debris and attempts to isolate S-layer complexes from liquid cultures by differential centrifugation (48) gave little evidence of an organized structure.

When cells in colonies were directly examined by negative-stain electron microscopy, as for the liquid-medium-grown cells, there was no visible S-layer on cell surfaces. In the calcium-sufficient PYE medium, however, large patches of S-layer were found adjacent to the cells (Fig. 1). These patches were readily found (i.e., most fields containing cells also contained an S-layer patch of some size), and typical patches showed good order (as judged by optical diffraction patterns) over large distances (47). Commonly, the patches were larger than could be accommodated on the surface of a single cell, suggesting that the patches did not slough off cells but were the result of self-assembly of RsaA away from the cell surface. Unlike S-layer preparations from wild-type cells, isolated by differential centrifugation or viewed in colonies, the array structure did not contain the amorphous structures (presumed to be outer membrane material) that usually occupy the spaces between many of the repeat units (47, 48). In a preliminary study of these S-layer patches, two-dimensional image analysis indicated that they were composed of a mirror image double layer of S-layer protein (47).

Role of calcium in S-layer assembly in the calcium-independent mutants. In comparison with the S-layer sheet formation

FIG. 4. SDS-PAGE of proteinase K-treated whole-cell lysates of *C. crescentus* strains prepared by a modification (55) of the method of Hitchcock and Brown (22). (A) Gel silver stained for polysaccharides by a modification (55) of the method of Tsai and Frasch (50). The running position of SLPS (arrow) is indicated. (B) Western blot analysis using anti-SLPS antiserum. A 0.25- μ g sample of KDO was loaded per lane. Lanes: 1, NA1000; 2, JS1001; 3, JS1002; 4, JS1003; 5, CB2A; 6, CB2NY66R.

of the calcium-independent mutants on calcium-sufficient solid medium, direct examination of colonies grown on calcium-deficient (M_{10} HiGG) solid medium showed no indication of an S-layer. This finding suggested that calcium was involved with the S-layer assembly process, in addition to whatever role it may have in surface attachment. To further examine that possibility, strains JS1001 and JS1002 were grown on PYE solid medium and colonies were suspended in increasing concentrations of EDTA (a divalent cation chelator) or EGTA (a chelator more selective for calcium) and examined by negative-stain electron microscopy. For EGTA, a 600 μ M (or lower) concentration had no visible effect on the integrity of the S-layer patches produced by JS1001 or JS1002, yet 800 μ M (and greater concentrations) completely obliterated all traces of S-layer patches. In contrast, EDTA had no effect at 1 mM and only at 10 mM was loss of the S-layer patches observed.

We also restored calcium to the M_{10} HiGG solid medium to test whether the loss of S-layer sheet formation was specifically related to the absence of calcium and not some other consequence of the shift from PYE to minimal medium; added calcium restored sheet formation for both JS1001 and JS1002. Magnesium ions did not substitute for calcium. Magnesium was present at 2.2 mM in M_{10} HiGG, and in some experiments we increased the magnesium levels to 7.2 mM; in no case were sheets found. We conclude that calcium was specifically involved with the assembly and maintenance of the S-layer patches.

Experiments with cloned S-layer genes. Negative-stain electron microscopy, immunoelectron microscopy, and immunofluorescence microscopy (using anti-RsaA serum) were used to assess the degree of S-layer expression on wild-type and mutant cells expressing cloned S-layer genes. Wild-type S-layer-producing strains NA1000 and CB2NY66R showed an S-layer on the cell surface, and S-layer sheets were not observed in colonies grown on calcium-sufficient medium. S-layer-negative mutants CB2A, JS1003, JS1004, and JS1005 showed no evidence of S-layer by microscopy. CB2A and JS1003 expressing the wild-type *rsaA* gene or any *rsaA* gene from calcium-independent mutant JS1001, 1002, or CB2NY66Rmgl cloned into pKT230 showed the wild-type S-layer phenotype. In all cases, the S-layer was surface attached and S-layer sheets were not observed in colonies. Expression of the wild-type S-layer gene in JS1004 or JS1005 resulted in the shedding phenotype. These data indicated that mutations resulting in the shedding phenotype of these calcium-indepen-

FIG. 5. Colloidal gold immunocytochemical labeling of NA1000 and JS1003 strains. (A) Wild-type S-layer-producing strain NA1000 labeled with anti-SLPS antibody. Note the absence of colloidal gold label on the cell surface. (B) *rsa4*-negative strain JS1003 labeled with the anti-SLPS antibody. Note uniform colloidal gold label on the cell surface. Bar, 0.4 μ m.

dent mutants did not occur in the S-layer gene but, rather, resulted from some alteration of the cell surface.

Western blot analysis corroborated the microscopy observations (Fig. 2). S-layer shedders were differentiated from wild-type strains in that RsaA could be detected on Western blots only when shedder cells were scraped off plate cultures (Fig. 2, lanes 7, 9, 14, 21, and 25). Western blots of washed liquid-grown shedding strains were negative for RsaA (Fig. 2, lanes 6, 13, 22, and 24). Western blots of plate-grown strains in which the chromosomal S-layer gene had been deleted or which were spontaneous S-layer-negative mutants were RsaA negative (Fig. 2, lanes 3 and 16). Washed liquid-medium-grown strains that were S-layer negative but attachment competent produced S-layer when any cloned *rsaA* gene, including those from shedder strains, was expressed in pKT230 (Fig. 2, lanes 4, 5, 12, and 17 to 20). When *rsaA* cloned from a wild-type strain was expressed in a shedder background, the shedder phenotype persisted (Fig. 2, lanes 4 and 13).

Analysis of surface polysaccharides. In an effort to identify a surface molecule(s) responsible for S-layer attachment that was altered in the calcium-independent mutants, we investigated the rough LPS and capsular polysaccharide of JS1002. The monosaccharide compositions for each class of polysaccharide in the wild type and in the mutant strain did not differ, nor was there a detectable difference in the mobility of rough LPS on SDS-PAGE gels for JS1001 or JS1002 (data not shown).

During the isolation of rough LPS by a modification (42) of the Darveau and Hancock procedure (12), we occasionally detected an additional polysaccharide-containing molecule that migrated more slowly than LPS on SDS-PAGE (Fig. 3A). This band (SLPS) did not stain with the silver stain procedure for LPS (50) but did when the more general Bio-Rad silver stain kit was used. Although the method revealed both the rough LPS and the SLPS, its sensitivity towards the rough LPS was fivefold less than that of the method of Tsai and Frasch (50) (not shown). When SLPS-contaminated rough-LPS preparations were examined by Western blot analysis using anti-SLPS antibody, the SLPS band was specifically labeled (Fig. 3B). Anti-SLPS demonstrated no reactivity with the rough LPS. In subsequent experiments which led to the SLPS isolation procedure described in Materials and Methods, we learned that SLPS represented a considerable fraction of membrane polysaccharides; upon examination of the steps in the rough-LPS isolation procedure, by immunoblotting, it was found that the SLPS remained in the supernatant following the cold ethanol-Mg²⁺ precipitation of polysaccharides by the Darveau and Hancock procedure (12) (data not shown).

The rapid LPS analysis method of Hitchcock and Brown (22) initially did not demonstrate the presence of SLPS along with rough LPS (42). A modification of the procedure along with an extended oxidation time in the Tsai and Frasch (50) staining process did reveal SLPS (55). This modified procedure was used to examine the caulobacter strains used in this study (Fig. 4A). S-layer attachment-proficient strains contained this additional polysaccharide (Fig. 4, lanes 1, 4, 5, and 6); however, a comparable band was not detected in the S-layer attachment-deficient strains JS1001 and JS1002 (Fig. 4, lanes 2 and 3). Although the modified procedure revealed the SLPS, increased sample loadings compared with what is normally used to detect LPS were required (note the overloading of the rough LPS), and the SLPS was stained less intensely than the rough LPS. Western blotting of these preparations with anti-SLPS serum showed that the SLPS of all strains producing the polysaccharide reacted with the antiserum (Fig. 4B). JS1001 and JS1002 showed only a faint immunoreactive band in the

FIG. 6. SDS-PAGE of purified polysaccharides from JS1003 stained with the Bio-Rad silver stain kit. Lanes: 1, rough LPS; 2, molecular mass markers; 3, SLPS. Lanes 1 and 3 were loaded with 1.0 and 0.25 μ g of KDO, respectively. Molecular mass markers are 66.2, 45, 31 (poorly stained), 21.5, and 14.4 kDa.

SLPS region. The process was repeated with cells removed directly from plates; identical results were obtained (data not shown), indicating that the shedding mutants did not discard SLPS along with the S-layer but, rather, did not produce SLPS. We subsequently analyzed eight other calcium-independent mutants by Western blotting and silver stain (not shown). In all strains, including CB2NY66Rmgl, which was independently isolated in another laboratory (Table 1), SLPS was absent.

Immunoelectron microscopy (Fig. 5) and immunofluorescence microscopy (not shown) with anti-SLPS serum showed that the entire cell surface of S-layer-negative but attachment-competent strains (CB2A and JS1003) was labeled, whereas S-layer-positive and attachment-competent strains (NA1000 and CB2NY66R) were unlabeled, indicating that the assembled S-layer prevents access of the antiserum to SLPS.

Purification and chemical analysis of SLPS. Purification of the SLPS was accomplished by preparative SDS-PAGE and electroelution (Fig. 6). Reelectrophoresis of the purified SLPS did not alter its electrophoretic mobility, indicating that the band did not result from aggregates of rough LPS (21). Colorimetric analysis revealed that the purified SLPS contained (per milligram [dry weight]) 25 μ g of KDO, 454 μ g of carbohydrate (detectable by the phenol-sulfuric acid assay), and 1 μ g of phosphate. On a molar basis, the KDO-phosphate ratio was 3:1, the same as that found in the rough LPS (42).

GC-MS of the purified SLPS showed that the major lipid was 3-OH-dodecanoate, the same lipid found in caulobacter rough LPS. Moreover, low levels of mannose, galactose, glucose, and heptose were noted (Table 2); these sugars are also present in the core region of caulobacter rough LPS. In addition, three carbohydrate peaks were observed at a retention time earlier than that of arabinose (used as an internal standard) (Table 2). GC-MS of the first peak unambiguously identified it as glycerol (not shown). The second and third carbohydrate peaks were observed in high percentages when methanolized, reduced with sodium borodeuteride, and thereafter hydrolyzed with TFA (hydrolytic condition E). These two sugars are referred to as peaks I and II in Table 2 and below. Peak II was absent when samples were treated under the stronger hydrolytic condition A, while peak I amounted to only 1.3%, showing that both were susceptible to strong-acid hydrolysis. With intermediate concentrations of acid (conditions B,

TABLE 2. Sugar composition of purified SAO isolated from strain JS1003

Sugar ^a	T ^b	%
Glycerol	0.46	13.6
Peak I	0.89	12.0
Peak II	0.92	15.0
Mannose	1.40	1.7
Glucose	1.42	0.2
Galactose	1.43	0.2
D-Glycero-D-mannoheptose	1.78	0.2
L-Glycero-D-mannoheptose	1.82	0.5

^a Analysis of alditol acetate derivatives by gas-liquid chromatography on an SE 54 capillary column programmed at 180°C for 1 min, 5°C increase per min to 220°C, and 10°C increase per min to 250°C.

^b T, retention time relative to that of the arabinose internal standard.

C, and D), we found a gradual increase in both peaks I and II, but the percentage of peak II was far below that of peak I under conditions B and C. The mass spectral fragmentation pattern for peak I (Fig. 7) indicated that it was either a 3-amino-5-deoxy pentitol or the methyl glycoside of 4,6-dideoxy-4-amino hexose. Peak II is thought to be a 4-amino-5-deoxy pentitol or the methyl glycoside of 3,6-dideoxy-3-amino hexose, since it has the same molecular mass as peak 1 but a different fragmentation pattern (Fig. 8). In both of these spectra, in chemical ionization mode but not in electron impact mode, an ion peak appears at an *m/e* of 272, which corresponds

to loss of a methoxy group from the molecular ion; this result favors the assignments of methyl glycosides rather than the pentitols. Since methanolysis and reduction did not show any change in the retention time or mass spectral fragmentation patterns, we concluded that there are no carboxyl groups within the sugars. Thus, we predict the presence of 6-deoxy-aminodeoxyhexose, with a likelihood of equal proportions of 3-amino and 4-amino versions. Taken as a whole, the chemical studies of purified SLPS support the contention that it is a smooth LPS species with glycerol and a relatively unusual set of hexoses attached to the core region, all in equal proportions.

This smooth species of LPS was relatively unusual in that it had a homogeneous size. SLPS could not be resolved into a cluster of bands as is often seen with other O antigens of homogeneous lengths (10, 13). Using a variety of acrylamide concentrations and a number of gel protocols, we detected no heterogeneity in this region.

DISCUSSION

For *C. crescentus*, growth without calcium apparently required a mutation event that consistently resulted in the loss of the SLPS molecule. The attendant phenotype was that production of RsaA continued and the RsaA could crystallize into an S-layer (if calcium was available) but did not attach to the cell surface. Since no other alterations of the surface were detectable and since it was demonstrated in these mutants that the shedding phenotype could not be ascribed to a change in RsaA

FIG. 7. Mass spectral fragmentation pattern of peak I as determined by electron impact (A) and chemical ionization (B).

FIG. 8. Mass spectral fragmentation pattern of peak II as determined by electron impact (A) and chemical ionization (B).

protein, we conclude that SLPS is a necessary surface component for the attachment of the S-layer.

Because growth and viability in the absence of calcium are possible only in the absence of SLPS and because the presence of SLPS has no effect on viability as long as calcium is available (whether or not RsaA is present [46]), the simplest explanation is that calcium binds to SLPS and neutralizes an inhibitory effect of SLPS. The effect may be a destabilization of the outer membrane. In addition, we have recently found that some other ions are able to substitute for calcium to allow viability of SLPS-producing strains, although growth is slower and there is evidence of membrane instability (53, 54b). Other, more circuitous explanations for the effect could be made, however, and a resolution of this issue will require direct evidence of calcium binding.

Treatments that disrupt calcium-mediated interactions (low-pH or EGTA treatments) have the effect of removing the S-layer from the surface (55). Taken together with the other findings, a reasonable hypothesis is that the S-layer is attached to the surface via binding to the SLPS molecule that is mediated (bridged) by calcium ions. In such a scenario, in the absence of SLPS the RsaA proteins might well be able to crystallize into a double layer, using the calcium-binding contact points of the crystallized RsaA and the divalent character of calcium to accomplish this. That is, another mirror image of S-layer, in perfect register, substitutes for the outer membrane as an attachment surface during S-layer assembly and the size of the two-dimensional crystals could be limited

only by RsaA availability. Image analysis has shown that such a double S-layer is formed in the shedding mutants, and thin-section analysis of colonies producing shed protein demonstrates large, extensive sheets of S-layer (47). Figure 9 restates the hypothesis in a diagrammatic manner.

The model predicts that EGTA should have a disruptive effect on shed, double-layer S-layer fragment integrity, presumably at a specific concentration (related to uniform-type and highly repetitive calcium binding site[s] on the S-layer); we noted this effect. Moreover, DNA sequence analysis of *rsaA* demonstrates regions of homology with calcium-interactive regions of other calcium-requiring bacterial proteins (18). It is possible, however, that subunit-subunit interactions are calcium mediated and that there is no involvement of calcium in surface attachment. This could occur if another type of specific bonding occurs between S-layer and SLPS, which can also occur between two copies of the RsaA surface attachment region and which can (like calcium) be disrupted by low pH or chelators.

SLPS appears to be a homogeneous species of smooth LPS, despite some ambiguous staining and immunological activity results. The anti-SLPS antiserum recognized only the O-antigen portion as determined by Western analysis, although as an immunogen, the rough LPS portion (core sugars and lipid A) must have been present. However, O antigens are an immunodominant component of gram-negative cells and often are immunodominant when whole cells are used as antigens (23). The inability to stain the smooth version of LPS (SLPS)

FIG. 9. Model for S-layer assembly and attachment based on the data presented in this report and reports of Smit et al. (47) and Gilchrist et al. (18). (A) Proposed situation in wild-type cells. The S-layer is attached to the outer membrane by divalent calcium ions, bridging RsaA with SLPS molecules. Arrows indicate presumed interaction sites on an RsaA molecule; that is, the model does not propose that the only way for RsaA molecules to organize into a geometric structure is through indirect connection to SLPS molecules or calcium ions. Question marks indicate that some interactions between RsaA subunits may be bridged by calcium ions but that the data are not conclusive. (B) In the calcium-independent mutants, the loss or severe reduction of the SLPS molecule is proposed to lead to a loss of the calcium interaction sites on the surface and consequent S-layer sheet formation as a double layer. The model predicts that in the absence of calcium ions, the outer membrane is lethally destabilized by the presence of SLPS molecules and that the reduction or elimination of SLPS in calcium-independent mutants overcomes this lethal effect.

after SDS-PAGE by the standard methods of Hitchcock and Brown (22) and Tsai and Frasch (50) is not unique to *C. crescentus*; *Cytophaga johnsonae* (20), *Campylobacter coli* (30), *Campylobacter jejuni* (39), and various enteric strains producing acidic O antigens (26), as well as *E. coli* O26, *Coxiella burnetii*, and *Neisseria gonorrhoeae* (see discussion in reference 39), also have LPS species which do not stain with the standard LPS stain.

The presence of dideoxyamino hexoses in SLPS is unusual in LPS O antigens but not unique (see reference 25 for a survey), occurring also in *Yersinia enterocolitica*, *Brucella abortus*, and *Vibrio cholerae* (9). Glycerol is an uncommon component in O antigens, especially at the levels noted here. Equal mass ratios of the two hexoses and glycerol suggest that the number of glycerol molecules is the same as the number of hexoses and allows us to entertain the possibility that the O antigen is a polymer with alternating hexose and glycerol; we are investigating that possibility.

SLPS length appeared to be precisely determined by the bacterium; it could not be resolved into even the discrete cluster of bands shown for other O antigens of homogeneous lengths (10, 13). The presence of more than one O-antigen

sugar and the fact that the dideoxyamino hexoses represented a large fraction of the total polysaccharide composition suggest a subunit structure with multiple repeats. If so, there must be a precise method of controlling O-antigen size. This could not be the result of the presence of the S-layer providing a physical limit on SLPS size; RsaA-negative mutants also demonstrated the uniform-size SLPS.

S-layer surface attachment for other gram-negative S-layer-producing bacteria has been examined in only a few cases. For *Aeromonas salmonicida* (4) and *Aeromonas hydrophila* (14), alteration in the attachment of S-layer to the cell surface is correlated with defects in the LPS. With *A. salmonicida* strains, the inability to produce a homogeneous-chain-length smooth LPS results in an attachment-defective phenotype. Wild-type *A. hydrophila* strains also produced a homogeneous-length O-polysaccharide, but mutants that generated only a core LPS could still maintain the S-layer. Mutants producing a deep rough LPS, however, were found to be S-layer attachment defective (14). In both species, the O antigen extended past the S-layer and was exposed to the environment (10, 13), in contrast to our findings with *C. crescentus*. A similar correlation of homogeneous-length O antigens and the production of an S-layer has been suggested also for strains of *Aeromonas schubertii* (27). Thus, in some ways the findings for *Aeromonas* species are comparable to those for *C. crescentus*.

Calcium limitation and the effect on S-layer have been studied in *Spirillum putridiconchylum* (6), *Aquaspirillum serpens* VHA (28), *Azotobacter vinelandii* (15), and *Aeromonas salmonicida* (17). In these studies, calcium limitation still allowed cell growth but did result in an altered outer membrane on which, with the exception of *Aeromonas salmonicida* (17), the S-layer was unable to form a crystalline array. In *Azotobacter vinelandii* (15), the S-layer protein remained attached to the cell surface under calcium limitation, whereas in *Aquaspirillum serpens* VHA (28) the S-layer protein was secreted into the growth medium. In the case of *Aeromonas salmonicida* (17), calcium limitation resulted in an alteration of the S-layer pattern. These studies indicate that in several cases calcium has a role in crystallization and/or attachment of the S-layer to the cell surface and suggest a degree of similarity or conservation of structural principles between these S-layers.

Within the S-layer-producing caulobacters, the relationship between the S-layer and a specific species of LPS suggested here may be a common theme. In a survey of caulobacters, it was noted that isolates that possessed an S-layer also produced an SLPS-like molecule with comparable staining characteristics and immunological cross-reactivity (55).

To sum up the issues surrounding S-layer attachment for *C. crescentus*, all current evidence is consistent with the model presented. The model is nevertheless speculative since several aspects require direct demonstration for the model to remain valid. Chief among these is a direct demonstration that calcium does in fact bind to SLPS and, in turn, to the assembled RsaA protein. Detailed analysis of the SLPS repeat structure is also needed; at the moment, it is not apparent to us how specific binding of calcium could be effected by a polymer composed largely of neutral sugars or how such a polymer, composed of unusual, but not unique, hexoses and glycerol could lethally destabilize the outer membrane in the absence of specific ions.

ACKNOWLEDGMENTS

We thank Jeanne Poindexter for helpful discussions regarding the correlation between calcium requirements and S-layer formation, which she was the first to notice, and for the CB2NY66R and CB2NY66Rmgl strains. We acknowledge the support of Nina Agabian, in whose laboratory some years ago the isolation by J.S. of some

of the calcium-independent mutants reported here was done, and G. G. S. Dutton and R. E. W. Hancock for providing laboratory facilities and advice regarding the LPS and EPS monosaccharide analysis. We thank Wade Bingle for assistance with some of the plasmid constructions and acknowledge the technical assistance of Mabel Wong and Patti Edwards at several stages.

This work was supported by grants from the U.S. National Institutes of Health (GM-39055), the Natural Sciences and Engineering Research Council of Canada (OGP 0036574), and the U.S. Office of Naval Research (N00014-89-J-1749) to J.S.

REFERENCES

- Anast, N., and J. Smit. 1988. Isolation and characterization of marine caulobacters and assessment of their potential for genetic experimentation. *Appl. Environ. Microbiol.* **54**:809–817.
- Bagdasarian, M., R. Lurz, B. Rückert, F. C. T. Franklin, M. M. Bagdasarian, J. Frey, and K. N. Timmis. 1981. Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene* **16**:237–247.
- Barany, F. 1985. Single-stranded hexameric linkers: a system for in-phase insertion mutagenesis and protein engineering. *Gene* **37**:111–123.
- Belland, J. J., and T. J. Trust. 1985. Synthesis, export, and assembly of *Aeromonas salmonicida* A-layer analyzed by transposon mutagenesis. *J. Bacteriol.* **163**:877–881.
- Beveridge, T. J., and S. F. Koval. 1993. Advances in paracrystalline bacterial surface layers. NATO ASI Ser. Ser. A Life Sci. **252**:v. (Preface.)
- Beveridge, T. J., and R. E. G. Murray. 1976. Dependence of the superficial layers of *Spirillum putridiconchylum* on Ca^{2+} or Sr^{2+} . *Can. J. Microbiol.* **22**:1233–1244.
- Bingle, W. H., H. D. Kurtz, and J. Smit. 1993. An all-purpose cellulase reporter for gene fusion studies and application to the paracrystalline surface (S)-layer protein of *Caulobacter crescentus*. *Can. J. Microbiol.* **39**:70–80.
- Brent, R., and M. Ptashne. 1980. The *lexA* gene product represses its own promoter. *Proc. Natl. Acad. Sci. USA* **77**:1932–1936.
- Caroff, M., D. R. Bundle, and M. Perry. 1984. Structure of the O-chain of the phenol-phase soluble cellular lipopolysaccharide of *Yersinia enterocolitica* serotype O:9. *Eur. J. Biochem.* **139**:195–200.
- Chart, H., D. H. Shaw, E. E. Ishiguro, and T. J. Trust. 1984. Structural and immunochemical homogeneity of *Aeromonas salmonicida* lipopolysaccharide. *J. Bacteriol.* **158**:16–22.
- Cohen-Bazire, G., W. R. Sistrom, and R. Y. Stanier. 1957. Kinetic studies of pigment synthesis by non-sulfur purple bacteria. *J. Cell. Comp. Physiol.* **49**:25–68.
- Darveau, R. P., and R. E. W. Hancock. 1983. Procedure for isolation of bacterial smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. *J. Bacteriol.* **155**:831–838.
- Dooley, J. S. G., R. Lallier, D. H. Shaw, and T. J. Trust. 1985. Electrophoretic and immunochemical analysis of the lipopolysaccharides from various strains of *Aeromonas hydrophila*. *J. Bacteriol.* **164**:263–269.
- Dooley, J. S. G., and T. J. Trust. 1988. Surface protein composition of *Aeromonas hydrophila* strains virulent for fish: identification of a surface array protein. *Bacteriol.* **170**:499–506.
- Doran, J. L., W. H. Bingle, and W. J. Page. 1987. Role of calcium in assembly of the *Azotobacter vinelandii* surface array. *J. Gen. Microbiol.* **133**:399–413.
- Edwards, P., and J. Smit. 1991. A transducing bacteriophage for *Caulobacter crescentus* uses the paracrystalline surface layer protein as a receptor. *J. Bacteriol.* **173**:5568–5572.
- Garduño, R. A., B. M. Phipps, W. Baumeister, and W. W. Kay. 1992. Novel structural patterns in divalent cation-depleted surface layers of *Aeromonas salmonicida*. *J. Struct. Biol.* **109**:184–195.
- Gilchrist, A., J. A. Fisher, and J. Smit. 1992. Nucleotide sequence analysis of the gene encoding the *Caulobacter crescentus* paracrystalline surface layer protein. *Can. J. Microbiol.* **38**:193–202.
- Gilchrist, A., and J. Smit. 1991. Transformation of freshwater and marine caulobacters by electroporation. *J. Bacteriol.* **173**:921–925.
- Godchaux, W., III, L. Gorski, and E. R. Leadbetter. 1990. Outer membrane polysaccharide deficiency in two nongliding mutants of *Cytophaga johnsonae*. *J. Bacteriol.* **172**:1250–1255.
- Hitchcock, P. J. 1983. Aberrant migration of lipopolysaccharide in sodium dodecyl sulfate polyacrylamide gel electrophoresis. *Eur. J. Biochem.* **133**:685–688.
- Hitchcock, P. J., and T. M. Brown. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* **154**:269–277.
- Jann, K., and B. Jann. 1977. Bacterial polysaccharide antigens, p. 247–287. In I. Sutherland (ed.), *Surface carbohydrates of the prokaryotic cell*. Academic Press, New York.
- Kabir, S. 1986. Composition and immunochemical properties of the cell surface proteins of *Vibrio cholerae*. *J. Gen. Microbiol.* **132**:2235–2242.
- Kenne, L., and B. Lindberg. 1983. Bacterial polysaccharides, p. 287–363. In G. O. Aspinall (ed.), *The polysaccharides*, vol. 2. Academic Press, New York.
- Kido, N., M. Ohta, and N. Kato. 1990. Detection of lipopolysaccharides by ethidium bromide staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Bacteriol.* **172**:1145–1147.
- Kokka, R. P., D. Lindquist, S. L. Abbott, and J. M. Janda. 1992. Structural and pathogenic properties of *Aeromonas schubertii*. *Infect. Immun.* **60**:2075–2082.
- Koval, S. F., and R. G. E. Murray. 1984. Effect of calcium on the *in vivo* assembly of the surface protein of *Aquaspirillum serpens* VHA. *Can. J. Microbiol.* **31**:261–267.
- Loenen, W. A., and W. J. Brammer. 1980. A bacteriophage lambda vector for cloning large DNA fragments made with several restriction enzymes. *Gene* **10**:249–259.
- Mandatori, R., and J. L. Penner. 1989. Structural and antigenic properties of *Campylobacter coli* lipopolysaccharides. *Infect. Immun.* **57**:3506–3511.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Merker, R. I., and J. Smit. 1988. Characterization of the adhesive holdfast of marine and freshwater caulobacters. *Appl. Environ. Microbiol.* **54**:2078–2085.
- Merrill, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211**:1437–1438.
- Messner, P., and U. B. Sleytr. 1992. Crystalline bacterial cell-surface layers. *Adv. Microb. Physiol.* **33**:213–275.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Poindexter, J. S. 1964. Biological properties and classification of the *Caulobacter* group. *Bacteriol. Rev.* **28**:231–295.
- Poindexter, J. S. 1978. Selection for nonbuoyant morphological mutants of *Caulobacter crescentus*. *J. Bacteriol.* **135**:1141–1145.
- Poindexter, J. S. 1982. S-layer assembly mutants of *Caulobacter crescentus*, abstr. CC II 1, p. 171. Abstr. 82nd Annu. Meet. Am. Soc. Microbiol. 1982.
- 38a. Poindexter, J. S. Unpublished observations.
- Preston, M. A., and J. L. Penner. 1987. Structural and antigenic properties of lipopolysaccharides from serotype reference strains of *Campylobacter jejuni*. *Infect. Immun.* **55**:1806–1812.
- Quigley, N. B., and P. R. Reeves. 1987. Chloramphenicol resistance cloning vector based on pUC9. *Plasmid* **17**:54–57.
- Ravenscroft, N., S. G. Walker, G. G. S. Dutton, and J. Smit. 1991. Identification, isolation, and structural studies of extracellular polysaccharides produced by *Caulobacter crescentus*. *J. Bacteriol.* **173**:5677–5684.
- Ravenscroft, N., S. G. Walker, G. G. S. Dutton, and J. Smit. 1992. Identification, isolation, and structural studies of the outer membrane lipopolysaccharide of *Caulobacter crescentus*. *J. Bacteriol.* **174**:7595–7605.
- Remaut, E., P. Stanssens, and W. Fiers. 1981. Plasmid vectors for high efficiency expression controlled by the p_L promoter of coliphage lambda. *Gene* **15**:81–93.
- Smit, J. 1987. Protein surface layers of bacteria, p. 343–376. In M. Inouye (ed.), *Outer membranes as model systems*. John Wiley and Sons, New York.

45. Smit, J., and N. Agabian. 1982. Cell surface patterning and morphogenesis: biogenesis of a periodic surface array during *Caulobacter* development. *J. Cell Biol.* **95**:41–49.
46. Smit, J., and N. Agabian. 1984. Cloning of the major protein of the *Caulobacter crescentus* periodic surface layer: detection and characterization of the cloned peptide by protein expression assays. *J. Bacteriol.* **160**:1137–1145.
47. Smit, J., H. Engelhardt, S. Volker, S. H. Smith, and W. Baumeister. 1992. The S-layer of *Caulobacter crescentus*: three-dimensional image reconstruction and structure analysis by electron microscopy. *J. Bacteriol.* **174**:6527–6538.
48. Smit, J., D. A. Grano, R. M. Glaeser, and N. Agabian. 1981. Periodic surface array in *Caulobacter crescentus*: fine structure and chemical analysis. *J. Bacteriol.* **146**:1135–1150.
49. Smit, J., and W. J. Todd. 1986. Colloidal gold labels for immunocytochemical analysis of microbes, p. 469–516. *In* H. Aldrich and W. J. Todd (ed.), *Ultrastructure techniques for microorganisms*. Plenum Publishing Corp., New York.
50. Tsai, C. M., and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**:115–119.
51. Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**:259–268.
52. Walker, S. G., N. Ravenscroft, and J. Smit. 1991. Isolation of the cell surface molecules of *Caulobacter crescentus*, abstr. II-41. Abstr. Annu. Meet. Can. Soc. Microbiol., Western Branch, vol. 22.
53. Walker, S. G., and J. Smit. 1993. Effect of cations on the growth of *Caulobacter crescentus* NA1000 and a calcium independent mutant, abstr. P244, p. 77. Abstr. Joint Meet. Soc. Ind. Microb. and Can. Soc. Microbiol.
54. Walker, S. G., and J. Smit. 1993. Advances in paracrystalline bacterial surface layers. Attachment of the S-layer of *Caulobacter crescentus* to the cell surface, p. 289–291. NATO ASI Ser. Ser. A Life Sci. **252**:289–291.
- 54a. Walker, S. G., and J. Smit. Unpublished data.
- 54b. Walker, S. G., and J. Smit. Unpublished observations.
55. Walker, S. G., S. H. Smith, and J. Smit. 1992. Isolation and comparison of the paracrystalline surface layer proteins of fresh-water caulobacters. *J. Bacteriol.* **174**:1783–1792.