STUDIES OF THE CELL SURFACE OF *CAULOBACTER CRESCENTUS*

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Abstract

Caulobacter crescentus is a Gram-negative eubacteria which produces a surface layer (S-layer). S-layers are paracrystalline assemblies of protein that cover the outer surface of some eubacteria and archaebacteria cells. The method by which the protein subunits composing the S-layer of *C. crescentus*, RsaA, interact to form the array and attach to the cell was examined in this thesis.

The S-layer was extracted from the cell surface of *C. crescentus* NA1000 by treating cells with a pH 2 solution or a solution containing 10 mM ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA). The extracted extract was examined by sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE) and found to consist of nearly pure RsaA. The isolated S-layer was amorphous in structure but could reassemble *in vitro* into a crystalline array in the presence of calcium ions.

Two mutants of *C. crescentus* NA1000, JS1001 and JS1002, selected for the ability to grow in the absence of calcium had the additional phenotype of being unable to attach the S-layer to the cell surface although they produced a wild-type RsaA. These mutants shed the S-layer into the surrounding medium during growth. Methods were developed to identify, isolate and purify the cell surface molecules of the wild-type and S-layer attachment-defective strains. It was determined that the mutant strains did not produce a smooth lipopolysaccharide (LPS) although they produced the wild-

type rough LPS and extracellular polysaccharide. The smooth LPS (termed the S-layer associated oligosaccharide or SAO) was very homogeneous in length as determined by SDS-PAGE and silver-staining. RsaA negative strains that could form and attach an S-layer on the cell surface if RsaA was expressed on a plasmid vector were also shown to produce SAO. All S-layer attachment-defective mutants examined did not produced SAO. Two cosmids were identified that partially restored the production of SAO in the mutant strain JS1001 however restoration of S-layer attachment did not occur.

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C terminal	carboxy terminal
cm	centimeter
CFU	colony forming unit
DDW	distilled deionized water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylendiaminetetra-acetic acid
EGTA	1,2-Di(2-aminoethoxy)ethane-NNN'N'-tetra-acetic acid
EPS	extracellular polysaccharide
FWC	freshwater *Caulobacter*
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
GC	gas chromatography
g	gravity
h	hour
kDa	kilodalton
kV	kilovolts
KDO	2-keto-3-deoxyoctonate
LPS	lipopolysaccharide
L	litre
MCS	marine *Caulobacter* strain
M	molar
mg	milligram
min	minute

ml	milliliter
MS	mass spectrometry
μg	microgram
μl	microlitre
μm	micrometer
N	normal
nm	nanometer
NMR	nuclear magnetic resonance
N terminal	amino terminal
OD_{600}	optical density at 600 nm
Ω	ohms
PAGE	polyacrylamide gel electrophoresis
PCH	phenol-chloroform-hexane
PYE	peptone yeast extract
PBS	phosphate-buffered saline
RNA	ribonucleic acid
RNase	ribonuclease
SAO	S-layer associated oligosaccharide
S-layer	surface layer
SDS	sodium dodecyl sulfate
SEC	steric exclusion chromatography
TEM	transmission electron microscopy
TLC	thin-layer chromatography
Tris	Tris(hydroxymethyl)methylamine

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Dedication

This thesis is dedicated to my parents Dr. and Mrs. G. R. Walker. I would like to thank them for their love, support and guidance throughout my life. Thanks Mom and Dad.

1. Introduction

Within cells enzymes synthesize biomolecules by the formation of covalent bonds between substrates. The biomolecules that are formed, be they proteins, nucleic acids, lipids or polysaccharides, must then be organized into functional structures. The components of the functional, or supramolecular, structure interact mainly through the formation of weak (hydrogen, ionic, hydrophobic) bonds. Supramolecular structures can be composed of identical biomolecules such as the protein actin in the case of an actin filament or can be composed of a variety of different biomolecules as in a bacterial ribosome. The supramolecular structure can be constructed either by the process of "instructed morphogenesis" or "self-assembly" (Cohen 1977; Sitte 1981).

Supramolecular structures fashioned by instructed morphogenesis require the action of components that are not retained in the final structure. Thus the final components of the structure do not contain all the information required for assembly. These additional components may take the form of a template/scaffold or a proteolytic cleavage event (Kellenberger 1990). Supramolecular structures built through self-assembly do not require any additional components to achieve the final functional form. All the information required for assembly is contained in the biomolecules that make up the structure. The only requirements are a sufficient concentration of the subunits and suitable environmental

conditions (Sitte 1981). Thus the isolated components of a supramolecular structure that forms through self-assembly can, under appropriate conditions, spontaneously reassemble into the final structure *in vitro*.

Bacterial surface layers (S-layers) are examples of supramolecular structures built through the self-assembly process. This thesis reports on studies of the mechanisms by which the protein subunits that form the S-layer of *Caulobacter crescentus* interact to produce a crystalline array and the method by which the S-layer associates with the bacterial cell surface. The introduction serves as a brief review on bacterial S-layers and the S-layer of *C. crescentus*. For more in depth information on S-layers the reader is referred to published reviews by Baumeister et al. (1988), Koval (1988), Messner and Sleytr (1992), Sleytr and Messner (1983, 1988), and Smit (1987).

1.1 Bacterial surface arrays

S-layers are two dimensional crystalline arrays of proteinaceous subunits forming surface layers on prokaryotic cells (Sleytr et al. 1988). The subunits are usually composed of a single protein or glycoprotein species which self-assembles to form a characteristic lattice (Sleytr and Messner 1983). The subunits vary in molecular weight, between species and strains, from 30 - 220 kDa. S-layers are common components of prokaryotic cell design, being found on over 200 species of eubacteria and archaebacteria, however

in comparison to other wall components little is known about these structures (Messner and Sleytr 1992). This lack of knowledge is due, for the most part, to the absence of S-layers on the enteric bacteria which are the most thoroughly studied of all prokaryotic groups.

Historically, research on S-layers has focused on structural studies to determine how bacteria maintain these layers. S-layers are identified in transmission electron microscopy studies by virtue of their characteristic periodic morphology (Smit 1987). The detailed structure of the S-layer subunits are not readily discernible however due to the limited resolution in these images (Hovmöller et al. 1988a). To overcome this, computerized image processing techniques are used to obtain an unbiased averaged image in the form of a two dimensional density map (Amos et al. 1982). By combining two dimensional information from a tilt series of the same specimen, three dimensional reconstructions with 1.3 nm resolution have been produced (Baumeister and Engelhardt 1987; Chalcroft et al. 1986). Such analytical techniques have determined that S-layer proteins, within the crystal lattice, consist of a large core domain and a smaller connecting domain. This asymmetrical conformation allows these subunits to arrange themselves into a variety of patterns or morphological units the most common containing 2, 4 or 6 monomers. Crystallization of these building blocks results in hexagonal (p6), tetragonal (p4) or linear (p2) lattice types (Saxton and Baumeister 1986). The resulting patterns are very heterogeneous between species and strains of the same species with respect to lattice

symmetry and the centre-to-centre spacing of the unit cell. Three and two dimensional reconstructions indicate that the S-layers are also asymmetric with respect to the two surfaces. The surface facing the external environment is smooth in character while the surface proximal to the cell is generally rough (Sleytr and Messner 1988). It has been assumed that these layers maintain a standard pore size under all growth conditions, however the S-layer of *Aeromonas salmonicida* (Garduño and Kay 1992; Stewart et al. 1986) and some thermophilic *Bacillus Sp.* (Sleytr and Sara 1986) have been shown to undergo structural transformations which apparently alter the porosity.

Figure 1 in appendix II illustrates some of the features of S-layers using the S-layer of *C. crescentus* as an example. Two S-layer monomers are shown in Fig. 1A (appendix II) illustrating the two domains. Six S-layer monomers assemble to produce a hexagonal "unit cell" or "morphological unit" (Fig. 1B; appendix II). Unit cells then interact to form the final array structure (Fig. 1C; appendix II). Kinetic studies have determined that crystallization of S-layer monomers into the final array proceeds by a two step mechanism as illustrated in Fig. 1 (appendix II) and the first step occurs at a faster rate than the second (Sleytr and Messner 1983).

The majority of S-layer producing bacteria have a single layer, although double S-layers have been found on *Aquaspirillum metamorphum* (Beveridge and Murray 1975), *A. "Ordal"* (Beveridge and Murray 1976c), *A. serpens* MW5 (Kist and Murray 1984), *A.*

sinuosum (Smith and Murray 1990), *Lampropedia hyalina* (Austin and Murray 1990), *Nitrocystis oceanus* (Remsen et al. 1970) and *Bacillus brevis* 47 (Tsuboi et al. 1982), and three layers have been observed on Walsby's "square bacterium" (Stoeckenius 1981). The double layer of *A. "Ordal"* (Beveridge and Murray 1976c) contains an outer layer of hexagonal symmetry and an inner layer of tetragonal symmetry. The two S-layers of *A. serpens* MW5 are both hexagonal in symmetry but are antigenically unrelated (Koval et al. 1988) while the proteins of the two S-layers of *Bacillus brevis* 47 differ in molecular weight they are produced as a cotranscriptional unit. An S-layer is usually composed of a single protein. However, the very complex S-layers of *L. hyalina* (Austin and Murray 1990), *Flexibacter polymorphus* (Ridgeway and Lewin 1983) and *Chlamydia trachomatis* (Chang et al. 1982) are composed of two or more polypeptides. The pathogen *Campylobacter fetus* produces an S-layer that undergoes an antigenic shift. A single strain can produce S-layer proteins that vary by molecular weight and antigenic character (Dubreuil et al. 1990; Wang et al. 1990).

Biochemical studies of S-layers have demonstrated that general similarities exist between the protein subunits produced by diverse species. Most subunits are held together, and to the underlying cell surface, by non-covalent (hydrophobic, ionic, hydrogen or polar) bonds and are similar with respect to amino acid composition (Koval and Murray 1984a; Messner and Sleytr 1992; Sleytr and Messner 1983). They generally contain a large proportion of acidic and hydrophobic amino acids and little or no sulphur-containing amino

acids. These proteins contain a high proportion of random coil, 20 - 35 percent beta sheet and very little alpha helix (< 2 - 14 percent) (Koval 1988; Sleytr and Messner 1988). However, not all S-layer proteins conform to these generalizations. The very thermophilic and sheathed archaebacterial (Konig and Stetter 1986), *Chlamydiace Sp.* (Newhall and Jones 1983), and the inner tetragonal layer of *A. sinuosum* (Smith and Murray 1990) have subunits that are covalently bonded and are highly resistant to denaturation by physical or chemical methods. Subunits can also be modified with covalently attached carbohydrates. Glycoprotein containing subunits were first identified in *Halobacterium salinarium* (Mescher and Strominger 1976) and have since been located in a number of archaebacterial and eubacterial species. These bacterial glycoproteins contain substantial differences compared to those in eukaryotes with respect to both the glycan chains and linkages (Messner and Sleytr 1988; Messner and Sleytr 1991).

Although similarities exist between S-layer proteins produced by unrelated species, analysis of the S-layer genes has identified little to no sequence homology (Gilchrist et al. 1992; Messner and Sleytr 1992). Genetic (Gilchrist et al. 1992; Messner and Sleytr 1992), ultrastructural (Hovmöller et al. 1988b) and biochemical (Sleytr and Messner 1983) comparisons have led to a general consensus among researchers that S-layers are of a non-conserved nature and have arisen independently in species by convergent evolution. An S-layer may evolve in a given species to fulfill a

specific function(s); However, the protein subunits of all species must be capable of three major tasks: secretion, self-assembly or bonding with adjacent subunits, and attachment to the underlying cell surface (Smit 1987). It is assumed that the similarities between S-layer subunits with respect to general amino acid composition, method of subunit bonding and gross morphology are due to the proteins all having to fulfill the above tasks.

The mechanisms by which S-layers are secreted have not been studied in great detail in comparison to the mechanisms involved in assembly and attachment to the cell surface. Of the 18 sequenced S-layer genes all but 4; *Campylobacter fetus* (Blaser and Gotschlich 1990), *Caulobacter crescentus* (Gilchrist et al. 1992), *Rickettsia prowazekii* (Carl et al. 1990), and *R. rickettsii* (Gilmore et al. 1989), contain a cleaved N-terminal signal sequence. Many outer membrane proteins are believed to be transported from the periplasm to the outer membrane through adhesion zones (Bayer 1979) However, Belland and Trust (1985) have indicated that the S-layer monomer of *Aeromonas salmonicida* is transported from the cytoplasm to the distal side of the outer membrane by a mechanism which includes a step where the protein is free in the periplasm. A linkage between S-layer and lipopolysaccharide (LPS) translocation from the cytoplasm to the outer membrane has been suggested for *Acinetobacter* 199A and *Aeromonas salmonicida* (Thorne et al. 1976; Belland and Trust 1985).

The non-covalent forces responsible for subunit-subunit and

subunit-cell surface stability in S-layers are determined by identifying conditions under which the crystalline arrays will disintegrate and then reassemble into a regular array (Beveridge 1981; Koval and Murray 1984a; Smit 1987; Sleytr and Messner 1983). Within the same S-layer, the subunit-subunit and subunit-cell surface bonds may be of a different nature although it is often difficult to differentiate between the two interactions (Smit 1987). It has been noted that when metal ions are required for reassembly an S-layer Ca^{2+} is the ion of choice. Only *Bacillus brevis* (Tsuboi et al. 1982) and *Sporosarcina ureae* (Beveridge 1979) have a absolute requirement for Mg^{2+} while *Aeromonas salmonicida* appears to require both Ca^{2+} and Mg^{2+} (Garduño et al. 1992b).

The cell surface molecules with which S-layers interact to maintain cell association have been identified in only a few bacterial species. The S-layer of *Deinococcus radiodurans* is anchored to the cell via proteins (Thompson et al. 1982) whereas *Clostridium difficile* utilizes neutral cell surface polysaccharides (Masuda and Kawata 1981). Other Gram-positive species may use anionic sites on the peptidoglycan (Beveridge 1981; Hastie and Brinton 1979). The S-layers of Gram-negative eubacteria have been shown to interact with the outer membrane via protein(s) in *Acinetobacter* 199A (Thorne et al. 1975), and perhaps *Spirillum putridiconchylium* (Beveridge and Murray 1976b), and LPS in *Aeromonas salmonicida* (Belland and Trust 1985), *A. hydrophila* (Dooley and Trust 1988) and *Campylobacter fetus* (Yang et al. 1992). *Spirillum serpens*

requires both LPS and lipid (Chester and Murray 1978). For species possessing a double S-layer, the upper layer will often only reassemble in the presence of the lower layer as illustrated with *Aquaspirillum serpens* MW5 (Kist and Murray 1983).

Although much is known about the structure and biochemical composition of S-layers no one definitive function has been proposed for these layers. Because S-layer producing bacteria are founded in almost every environmental niche it is unlikely that all S-layers have an identical function. It is assumed that S-layers have evolved to serve different functional roles due to particular environmental stresses that a species encounters in its habitat. The function S-layers serve must be important to the survival of the cell when one considers the great energetic cost of the layer. S-layer monomers account for up to 10 percent of the total cellular protein and the energy expenditure is even greater when glycosylization occurs (Sleytr and Messner 1988). Considering this cost, it is not surprising that many S-layer producing strains lose them upon laboratory cultivation in the absence of environmental stress (Blaser et al. 1985; Buckmire 1971; Luckevich and Beveridge 1989; Stewart and Beveridge, 1980).

The observation of pore-like structures formed within S-layers indicates that these layers may act as a molecular sieve and restrict the diffusion of molecules larger than the exclusion limit of the pore. S-layers with exclusion limits less than that of a harmful molecule would thus protect the cell. S-layers protect some bacterial strains from lysozyme (Nermut and Murray 1967), various proteases (Sleytr

1976), predation (Buckmire 1971; Koval and Hynes 1991) and host virulence factors (Ishiguro et al. 1981; Blaser et al. 1988) presumably by exclusion.

The most obvious function for an S-layer is in the type three archaebacterial wall, consisting of a plasma membrane surrounded by an S-layer (Kandler and Konig 1985), where it determines the cell shape (Sleytr et al. 1986a). Shape determination and structural integrity has also been attributed to the S-layer sheaths of some methanogenic archaebacteria (Patel et al. 1986). A number of other functions have been suggested for S-layers. Beveridge and Murray (1976a) proposed that the electronegative character of most S-layers may serve to concentrate essential cations from a dilute environment or protect cells by immobilizing toxic ions (Beveridge 1979). Alternatively, S-layer interaction with soluble ions may also act to buffer the environment immediately surrounding the cell and inhibit large changes in pH (Stewart and Beveridge 1980). S-layers have also been implicated as a means to promote bacterial adhesion to macrophages (Garduño et al. 1992a; Trust et al. 1983), epidermal cells (Baumeister and Hegerl 1986), porphyrin and immunoglobulin (Kay et al. 1988; Phipps and Kay 1988), fibronectin and laminin (Doig et al. 1992; Kay and Trust 1991), bacteriophage (Edwards and Smit 1991; Howard and Tipper 1973) and between bacteria via autoagglutination (Evenberg and Lugtenberg 1982).

This brief review of bacterial S-layers illustrates that although these structures appear "similar" at a superficial level (two

dimensional arrays composed of acidic proteins lacking cysteine and of similar secondary structure) detailed ultrastructural, biochemical and genetic studies have revealed that these layers are very heterogeneous, sometimes even between strains of the same species, and are evolutionaraly unrelated. Therefore, when an uncharacterized S-layer is studied it is difficult to predict how the protein subunits are secreted to the outer membrane, assembled into an array and attached to the cell surface.

1.2 The S-layer of *C. crescentus*

C. crescentus is a Gram-negative eubacterium which undergoes a sequence of morphological changes at specific polar membrane sites during its life cycle (for review, see Poindexter 1964 and 1981; Shapiro 1976). Swarmer cells express a single flagellum, bacteriophage receptors, pili and an adhesive substance termed holdfast all at one cell pole. All polar features but the holdfast are lost and a stalk develops, at the same pole, as the swarmer differentiates into a stalked cell. The stalk, which is an outgrowth of the cell envelope and contains no cytoplasmic material, remains through all subsequent generations. Swarmers are produced by growth and division of the stalked cell with the swarmer cell polar surface appendages being produced at the pole distal to the stalk cell. With the exception of the pilus (Smit and Agabian 1982a) control of the production of the polar structures is linked to DNA replication. A single round of replication occurs during the life cycle and these

polar events are initiated at the midpoint by an unknown signal. The majority of research conducted with *C. crescentus* has focused on dissecting the developmental process at the genetic level (Dingwall et al. 1990; Shapiro 1993). Throughout the entire life cycle of *C. crescentus* the cell surface is completely covered with an S-layer (Smit et al. 1981).

The *C. crescentus* S-layer is of hexagonal symmetry (Smit et al. 1981). Smit et al. (1992) have produced a three-dimensional reconstruction to a resolution of 2.0 nm (see Fig. 1; appendix II). The reconstruction shows that the morphological unit is formed by six protein subunits that are arranged on a p6 lattice. The subunits forming the array contain a heavy domain, that interacts to form a central hexagonal core, and a lighter domain that connects adjacent morphological units. The centre to centre distance between the morphological units is 22 nm. The interaction of the heavy domain regions produces a central pore that has a diameter of 2.5 - 3.5 nm. A similar size of gap is found in the space between the unit cells. These breeches in the array would allow the passage of globular proteins no larger than approximately 17 kDa (Smit et al. 1992).

The gene encoding the protein that forms the S-layer (*rsaA*) has been cloned (Smit and Agabian 1984) and sequenced (Fisher et al. 1988; Gilchrist et al. 1992). The predicted molecular weight of RsaA is 98,132 and the predicted pI is 3.46. Apart from the removal of the initial methionine no N-terminal or C-terminal processing of the protein occurs during secretion (Gilchrist et al. 1992).

RsaA is a major cellular protein accounting for 5-7% of total protein synthesis (Smit et al. 1981). Indirect immunocytochemical methods have been used to examine where new S-layer is incorporated into the crystalline lattice during cell growth. Two systems of S-layer incorporation were distinguished by Smit and Agabian (1982b). During growth newly synthesized S-layer is incorporated at random locations on the cell body but only new S-layer was incorporated onto the growing stalk and the newly formed pole. Because array components are synthesized uniformly during the cell cycle (Agabian et al. 1979) and a cell possesses only one copy of *rsaA* (Smit and Agabian 1984), control of S-layer incorporation must work at the level of assembly rather than transcription. To account for the two mechanisms of S-layer assembly, it was postulated that on the cell body S-layer arrived via transient adhesion zones between the inner and outer membrane, whereas at the newly formed pole and stalk more stable sites of membrane adhesion exist. At present no information is available on how the S-layer protein is secreted or targeted to the cell surface.

Calcium has been implicated in the assembly of the *C. crescentus* S-layer and/or in the attachment of the layer to the cell surface. Gilchrist et al. (1992) identified 4 or 5 putative calcium-binding motifs in the C-terminal region of the predicted amino acid sequence of *rsaA*. Similar glycine-rich repeats have been identified in hemolysins and proteases of other species which require calcium for biological activity. Apart from the homology analysis there is no direct demonstration that the S-layer binds calcium. Poindexter

(1982) showed that wild-type *C. crescentus* has a growth requirement for calcium and that mutants could be selected which no longer require calcium. Analysis of the mutants revealed that they could no longer attach RsaA to the cell surface and that in high density liquid cultures a macroscopic debris, presumed to consist of RsaA, was formed.

Smit et al. (1992; unpublished studies; see appendix I [method A]) isolated a number of calcium-independent mutants from *C. crescentus* NA1000. It was demonstrated that these mutants produced large sheets of non-cell-associated, but assembled S-layer, when cultured on calcium-containing agar. Smit et al. (1992) showed that these sheets were composed of two S-layers that associate via the side of the array which is proximal to the cell surface in the wild-type situation. The two layers were in such precise alignment that three-dimensional image reconstruction was required to resolve that a double layer existed. When the calcium-independent mutants were grown on calcium-free plates no assembled S-layer was detected. This data indicated that the mutants were not defective in S-layer assembly and that calcium was involved in the assembly process. The S-layer gene was cloned from three calcium-independent mutants and each was individually introduced into *C. crescentus* JS1003. The chromosomal copy of *rsaA* of the parent strain, NA1000, was deleted to produce JS1003 (Smit, unpublished). Negative-stain electron microscopy and indirect immunofluorescence microscopy (Smit et al. unpublished) showed that a wild-type S-layer

was produced on the cell surface of JS1003 when any of the S-layer genes from the calcium-independent mutants were expressed on a plasmid vector. This implied that the mutation resulting in the S-layer attachment-defective phenotype was not located in *rsaA* but in some other gene whose product is involved in the attachment the array to the cell surface.

The central goal of this thesis was to identify, isolate and characterize the cell surface molecule(s) of *C. crescentus* which interact with the RsaA in order to attach the S-layer to the cell. The cell surface of calcium-independent / S-layer attachment-defective *C. crescentus* strains and the wild-type strain, NA1000, were examined in order to identify any differences between the wild-type and mutant strains. An examination of the LPS of the *Caulobacter* strains determined that the mutants did not produce a smooth LPS. The smooth LPS, termed the "S-layer associated oligosaccharide" (SAO), was produced by all wild-type / S-layer attachment-competent strains but not by any of the calcium-independent / attachment-defective mutants.

Some of the data presented in this thesis has been previously published as Ravenscroft et al. 1991, Ravenscroft et al. 1992, Walker et al. 1992 and Walker et al. 1994.

2. Materials and Methods

2.1 Chemicals

Unless otherwise stated all chemicals were purchased from Sigma (Sigma Chemical Company, St. Louis, MO) and were of analytical grade.

2.2 Bacterial strains

The *Caulobacter* strains used in this thesis are described in Table I. All *Caulobacter* strains were grown at 30°C and *Escherichia coli* B and DH5α were grown at 37°C.

2.3 Growth media

Peptone-yeast extract (PYE) medium (Poindexter 1964) was used for the growth of of all *Caulobacter* strains unless otherwise specified and contains per litre; 2 g peptone (Difco Laboratories, Detroit, MI), 1 g yeast extract (BDH Inc., Darmstadt, Ger.), 0.01% $CaCl_2$ (BDH), and 0.02% $MgSO_4$ (BDH). Cells grown in PYE medium were routinely harvested for experiments at mid-logarithmic growth phase (OD_{600} = 0.6 - 0.7). Solid media, of all types, contained 1.2% agar (Difco). For growth of freshwater *Caulobacter* isolates PYE liquid was supplemented with riboflavin at 2 μg/ml.

M_3Higg medium (Smit et al. 1981) contains 5 mM imidazole - HCl (pH 7.0), 2 mM KH_2PO_4 (pH 6.8), 0.3% glucose, 0.3% L-glutamic acid (monosodium salt; pH 7.0), 0.05% NH_4Cl, and 1% modified Hutner

TABLE I. Bacterial strains

Bacterial strain	Description or genotype	Reference of source
C. crescentus		
CB2A	S-layer minus variant of wild-type CB2, Tp^r, Rf^r, Ap^r.	Smit and Agabian 1984
CB2NY66R	Spontaneous S-layer plus mutant of CB2, Tp^r, Ap^r.	J. Poindexter*
CB2NY66Rmgl	Calcium-independent mutant of CB2NY66R, Tp^r, Ap^r.	J. Poindexter*
NA1000	Variant of wild-type strain CB15, ATCC19089, synchronous cultures readily prepared from this strain, Tp^r, Ap^r.	Smit and Agabian 1984
JS1001	Calcium-independent of NA1000.	J. Smit*
JS1002	Calcium-independent of NA1000.	J. Smit*
JS1003	NA1000 with *rsaA* interrupted with KSAC Km^r cassette.	J. Smit*
JS1004	JS1001 with *rsaA* interrupted with KSAC Km^r cassette.	J. Smit*
JS1005	JS1002 with *rsaA* interrupted with KSAC Km^r cassette.	J. Smit*
Fresh Water Caulobacter isolates		
42 FWC strains	Isolated from aquatic and wastewater sources.	MacRae and Smit, 1991

Legend: * unpublished strain, Km^r = kanamycin resistant, Tp^r = trimethoprim resistant, Ap^r = Ampicillin resistant, Rf^r = Rifampicin resistant

mineral base (Cohen-Bazire et al. 1957). Cells grown in M_3Higg or M_{10}Higg were routinely harvested for experiments at mid-logarithmic growth phase (OD_{600} = 2.0 - 3.0). M_3Higg and M_{10}Higg media were used for physiological studies to determine the cation requirements for *Caulobacter* growth and their influence on S-layer structure. The final metal ion concentration in M_3Higg medium is: 2.2 mM $MgSO_4$; 454 µM $CaCl_2$; 38 µM $ZnSO_4$; 25.1 µM $FeSO_4$; 9.1 µM $MnSO_4$; 1.6 µM $CuSO_4$; 0.9 µM $Co(NO_3)_2$; 0.5 µM $Na_2B_4O_7$; and 0.15 µM $(NH_4)_6Mo_7O_{24}$.

M_{10}Higg medium is identical to M_3Higg medium except that the Hutner mineral base was prepared without $CaCl_2$ and 18 MΩ distilled - deionized water, produced by a Barnsted "NANOpure" ultrapure water system, was used. All containers used to prepare or store M_{10}Higg medium were washed with 10 mM Na-EDTA (Fisher Scientific Co., Nepean, Ont.) (pH 8.0), to remove any trace calcium, then washed with 18 MΩ distilled - deionized water. Only new 16 x 150 mm S/P® diSPo® culture tubes (Baxter Healthcare Corporation, McGaw Park, IL) were used for growth of cells in M_{10}Higg medium. The agar used for M_{10}Higg medium plates was suspended in 50 mM EDTA (pH 8.0) and allowed to settle. The supernatant was decanted and the procedure was repeated four times. The agar was then washed in, the same manner, twice with 18 MΩ distilled - deionized water. The slurry was then suction-filtered through a hardened - ashless Whatman (Whatman International Ltd., Maidstone, England) 541 filter. The agar was then dried under negative pressure in a

dessicator oven at 85°C. Plastic petri dishes (Fisher) were used for solid M_{10}Higg medium.

L medium was used for the growth of *E. coil* (Miller, 1972) and contains per L; 10 g tryptone (Difco), 5 g yeast extract and 5 g NaCl.

When required antibiotics were added to media at the following concentrations (in μg/ml): Ap(100) [sodium salt], Km(50) [sulfate salt] (ICN Biomedicals, Inc., Cleveland, OH), Sm(50 or 10 when pSUP2021 [Simon et al. 1983] was used in NA1000) [sulfate salt], Tc(10 or 4 when pLAF5 [Keen et al. 1988] was used in JS1004) [chloride salt] (P-L Biochemicals, Inc., Milwaukee, Wis). All antibiotics were prepared and stored as recommended by Sambrook et al (1989).

2.4 Isolation of calcium independent mutants

The calcium independent mutant CB2NY66Rmgl was isolated by Dr. J. Poindexter (unpublished). The calcium independent mutant mutants JS1001 and JS1002 were isolated by Dr. John Smit (unpublished). See appendix I (method A) for the method by which these mutants were selected.

2.5 Growth studies

For studies on the ion requirements of NA1000 and JS1001 the following procedure was used. Cells were grown in M_3Higg medium to mid-log phase (OD_{600} = 2.0-3.0), harvested by centrifugation, and washed four times in M_{10}Higg by centrifugation and resuspension.

Five ml of M_{10}Higg (with or without a metal ion [chloride salt] supplement) was inoculated with 5 x 10^6 washed cells and incubated at 30°C on a tube roller (VWR Scientific Canada, Ltd., London, Ont) at 60 rpm. Plate counts determined that an OD_{600} = 1.0 of *C. crescentus* NA1000 grown in M_3Higg medium contains approximately 1 x 10^9 CFU's per ml. Growth rates were estimated between OD_{600} = 0.100 and 1.000.

2.6 Colourimetric assays

An LKB Biochrom Ultraspec II UV/VIS spectrophotometer was used for all colourimetric assays. Protein levels were determined by the method of Markwell et al. (1978) using egg white lysozyme as a standard or using the Bio-Rad™ protein assay (Bio-Rad laboratories, Missassauga, Ontario), which is based on the method of Bradford (1976), using bovine gamma globulin as a standard. 3-deoxy-2-octulosonic acid (KDO) was estimated by the method of Karkhanis et al. (1978) using authentic KDO (ammonium salt) as a standard. Inorganic phosphate was determined by the method of Ames and Dubin (1960) using K_2HPO_4 as a standard. Sugars were estimated by the method of Dubois et al. (1956) using D-glucose as a standard. Uronic acids were estimated by the method of Dische (1947) using D-glucuronic acid as a standard.

2.7 Isolation and purification of cell surface molecules

2.7.1 LPS isolation. *C. crescentus* strains CB2A and NA1000 were grown in PYE as 500 ml cultures, in 2 L Erlenmeyer flasks, on a

rotary shaker (200 rpm) and harvested during late log-phase (OD_{600} = 0.6-0.7). Cells were harvested with a Sorvall RC-5B centrifuge (Du Pont Instruments, Wilmington, Delaware) using a GSA rotor (Du Pont) (10,000 x*g* for 10 min) and washed once with 0.1 M HEPES (Research Organics, Inc., Cleveland, Ohio) buffer (pH 7.2). LPS was isolated using a modification of the method of Darveau and Hancock (1983). After nuclease digestion of the disrupted cells, the cell lysate was made to contain 0.1 M EDTA, 2% SDS, and 10 mM Tris-HCl (pH 8.0) and was then incubated at 37°C for 2 h. The extended incubation was required to completely dissociate the *Caulobacter* cell membranes. The published procedure was followed until completion of the final ultracentrifugation step. The supernatant from this ultracentrifugation step contained "crude" EPS. The LPS pellet was resuspended in 10 mM Tris-HCl (pH 8.0) and washed five times by ultracentrifugation (200,000 x*g* for 2 h at 15°C), using a Beckman (Beckman Instruments, Inc., Palo Alto, CA) L8-55 ultracentrifuge and a Type 60Ti rotor (Beckman), and resuspension. The final pellet was considered to be the "crude" LPS fraction.

The crude LPS was extracted following the Sonesson et al. (1989) modification of the Galanos et al. (1969) procedure. The freeze-dried crude LPS preparation was extracted three times with phenol:chloroform:hexane, 2:5:8, (PCH) (at 8 ml per g of original dry weight of the cells) and centrifuged at 2000 x*g*. The pooled supernatants were evaporated using a rotary evaporator and the phenol was removed by dialysis against distilled deionized water (4

x 2L). The LPS was recovered by lyophilization and washed three times with 10 ml of chloroform:methanol (2:1) and insoluble material was pelleted by centrifugation (200,000 x*g* for 2 h). The precipitate was dried under a stream of nitrogen gas, dissolved in distilled deionized water then freeze-dried to yield the "pure" LPS fraction.

2.7.2 EPS isolation. The "crude" EPS obtained during the LPS isolation procedure (see above) was freeze dried, resuspended in DDW to 1/10 the original volume, treated with bovine pancreatic RNase (25 µg/ml at 37°C for 24h), dialyzed against 4L DDW at 4°C for 24h and then ultracentrifuged at 200,000 x*g* (30 hr at 4°C) to remove any remaining LPS. The EPS was then freeze dried, resuspended in 0.1 M pyridinium acetate buffer (pH 7.0) and fractionated by steric-exclusion chromatography (SEC) on a Sephacryl S-400 (Pharmacia LKB Biotechnology, Uppsala, Sweden) column (60 x 2 cm) using 0.1 M pyridinium acetate buffer (pH 7.0) as eluent. Fractions (2.5 ml) were collected using a Pharmacia FRAC-100 fraction collector. Each fraction was analysed for carbohydrate to determine peak locations.

The nature of the cell surface EPS was investigated by assessing the degree to which the carbohydrate remained associated with the cells. The yield of EPS from unwashed cells was compared to yield from cells that were washed 5 times by centrifugation and resuspension in 0.1 M HEPES buffer (pH 7.2).

2.7.3 RsaA isolation. *C. crescentus* NA1000 was used in a series of experiments to determine an effective method for extracting the S-layer protein from the cell surface. In a typical experiment a 100 ml culture was grown in PYE to an OD_{600} = 0.6-0.7 and harvested by centrifugation (10,000 x*g* for 10 min). The cell pellet was washed twice in 1 volume of 10 mM HEPES buffer (pH 7.2) by suspension and centrifugation and then resuspended in 15 ml of the same buffer. One ml of the cell suspension was placed into 1.7 ml microfuge tubes, pelleted and the supernatant removed. Two hundred µl of one of the following agents was used to resuspend the cells: 10 mM EDTA in 10 mM HEPES buffer (pH 7.5); 10 mM EGTA in 10 mM HEPES buffer (pH 7.5); 100 mM HEPES (pH 2, 4, 6, 7.5, 8 or 10); 200 mM glycine-HCl buffer (ICN) (pH 2, 3 or 4); 100 mM TRIS buffer (ICN) (pH 7.2); 0.5% ß-mercaptoethanol (Bio-Rad) in 10 mM HEPES (pH 7.5); 1 M urea (BDH); 1 M guanidine-HCl (BRL); 10 mM NaCl (Fisher); 10 mM $CaCl_2$; and 100 mM HEPES (pH 7.5) with incubation at 65°C. The samples were incubated for 15 min at room temperature (unless otherwise stated) and then the cells were pelleted by centrifugation. Ten µl of supernatant was analyzed by SDS-PAGE and the proteins in the gel were visualized by Coomassie blue staining (See below).

Subsequently, as a standard method for isolation of S-layer protein, 5 ml cultures of *Caulobacter* strains were grown to OD_{600} = 0.6 and the cells harvested by centrifugation. The cells were washed twice by centrifugation and resuspension with 5 ml of 10 mM HEPES

(pH 7.2) and then the washed pellet was suspended in 200 µl of 100 mM HEPES (pH 2) or 200 µl of 10 mM EGTA in 10 mM HEPES (pH 7.5). The cell suspension was incubated for 10 min at 20°C, pelleted by centrifugation and the supernatant retained for examination by SDS-PAGE. The acid samples were immediately adjusted to pH 7 with 5 N NaOH (BDH).

JS1001 and JS1002 produce a macroscopic particulate "debris" in high density cultures. A debris sample (approximately 100 mg wet weight) was collected with a pasteur pipet and suspended in 10 mM HEPES buffer (pH 7.2). The material was pelleted in a microcentrifuge (5 sec, 14,000 rpm) and the supernatant discarded. The pellet was suspended in the same buffer and washed a total of three times. The washed pellet was suspended 400 ml of 8 M urea (pH 8.5) at room temperature for 8 h. Insoluble material was removed by centrifugation for 10 min in an Eppendorf centrifuge and the supernatant was dialyzed against 10 mM HEPES (pH 7.2) at 4°C.

2.7.4 SAO isolation and purification. SAO was isolated and purified from JS1003 cultured in PYE. All procedures were carried out at room temperature (20 - 22°C). Cells were washed by suspension and centrifugation (10,000 xg; 10 min) with 20 mM HEPES (pH 7.2). The 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 (Kabir 1986)], stirred for 5 min and then pelleted by centrifugation. The supernatant was saved and the pellet was extracted a second

time. The combined supernatants were ultracentrifuged at 225,000 xg. The pellet was resuspended in PBS (consisting of 1.23 g Na_2HPO_4, 0.18 g NaH_2PO_4 and 8.5 g NaCl per L) by sonication and dialyzed against PBS at 4°C. The extracts, containing approximately 5 mg protein and 450 μg KDO per ml, were mixed with an equal volume of SDS-PAGE sample buffer and heated at 100°C for 10 min then cooled to room temperature. Proteinase K 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 (12 x 14 cm separating gel cast using 1.5 mm spacers). The region of the gel containing SAO was excised, using prestained molecular weight markers (Gibco BRL Life Technologies, Inc.) as a guide to estimate its location, placed in a dialysis membrane [12 - 14 Kda cutoff. (Spectrum)] containing SDS-PAGE running buffer and electroeluted at 100 mA. The SAO was concentrated and washed extensively with water using an Centricon-30 microconcentrator (Amicon Canada Ltd., Oakville, Ont.).

2.8 Antisera production

2.8.1 Production of antiserum to low pH extracted RsaA. S-layer protein was extracted from NA1000 using 100 mM HEPES (pH 2) as described above. A New Zealand white female rabbit was immunized with this preparation, after combining with an equal quantity of Freund's incomplete adjuvant, by an initial injection containing 1 mg protein and subsequent booster injections at days

21, 28 and 35 with 0.3 mg protein. Sera with the highest titer was collected on days 55 and 62 and was processed by standard methods according to Heide and Schwick (1978). Serum activity was determined by the Ouchterlony double diffusion assay (Ouchterlony 1949) and Western immunoblot analysis. The antisera (α-RsaA) was adsorbed against whole cells of JS1003, and against Western immunoblots of JS1003 cell lysate. The pre-immune sera gave no activity against RsaA by Ouchterlony or Western immunoblot assays. Unless otherwise noted the antibody was used at a concentration of 1:50,000 for use in Western blot experiments.

2.8.2 Antisera to SAO. Smit and Merker (unpublished) produced a sera that completely labeled the cell surface of S-layer negative *C. crescentus* strains while not labeling S-layer producing strains. The epitopes on the cell surface that the antibody recognized were unknown. See appendix I (method B) for details on antigen preparation.

2.9 *In vitro* crystallization of S-layer

Low pH and EGTA extracted NA1000 S-layer samples and S-layer isolated by urea extraction of the macroscopic debris from from JS1001 cultures were dialyzed [Spectra/Por cellulose dialysis tubing with a molecular weight cutoff of 12 - 14 KDa (Spectrum Medical Industries, Inc., Los Angeles, CA)] 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 remainder was dialyzed overnight at 4°C against 10 mM HEPES (pH 7.5) containing one of: 1 mM $MgCl_2$, 1 mM $SrCl_2$, 1 mM, 5 mM or 10 mM $CaCl_2$. Each sample was then examined by negative-stain TEM.

2.10 Electrophoretic methods

2.10.1 SDS-PAGE. Samples were analysed by SDS-PAGE using the buffer system of Laemmli (1970). Polyacrylamide (Bio-Rad) - bisacrylamide (Bio-Rad) stock solutions contained 29.2 g and 0.8 g, respectively, per 100 ml. Sample buffer consisted of 40% 0.5M Tris-HCl (pH 6.8), 40% glycerol, 4% SDS, 4% β mercaptoethanol and 0.005% bromophenol blue (Bio-Rad). Samples loads were normalized by assaying for protein or KDO. When *C. crescentus* S-layer was to be analysed by SDS-PAGE, samples were not heated prior to electrophoresis because little or no S-layer protein will enter the gel if heated to 100°C in sample buffer (Smit and Agabian 1984). Protein molecular weights were estimated using Bio-Rad™ low molecular weight protein standards. If gels were to be used for Western blotting, prestained protein molecular weight standards (BRL Life Technologies, Burlington, Ontario) were employed. Gels were stained with: A) 0.1% Coomassie brilliant blue R-250 (Bio-Rad) in fixative (40% methanol [Fisher], 10% glacial acetic acid [Fisher]) then destained in fixative or B) one of the silver stains outlined below.

2.10.2 Western Blotting. Following SDS-PAGE proteins or carbohydrates were transferred to nitrocellulose membranes (Schleicher and Scheull, Inc., Keene, N.H.) by the method of Burnette (1981). After blotting, membranes were processed as described by Smit and Agabian (1984). All primary antibody was used at a dilution of 1:50,000 for α-RsaA and 1:20,000 for α-SAO. Goat α-rabbit antibody coupled to horseradish peroxidase (Antibodies Inc., Davis, CA) secondary antibody was used at a dilution of 1:2000. The blots were developed using 4-chloro-1-naphthol as described by Smit and Agabian (1982b).

2.10.3 Sample preparation. Five ml of PYE grown cells (OD_{600} = 0.6-0.7) or 1 ml of M_3Higg/M_{10}Higg grown cells (OD_{600} = 2.0-3.0) were pelleted and washed with 10 mM HEPES (pH 7.2) by suspension and centrifugation. The pellet was suspended in 250 µl of 10 mM Tris-HCl / 1 mM EDTA (pH 7.2), frozen at -20°C and then thawed at room temperature. A sample was removed to estimate protein concentration. To the remainder of the sample 1 µl of proteinase-free bovine pancreatic DNase (0.5 mg/ml), 20 µl of lysozyme (10 mg/ml) (ICN), and 3 µl of 1 M $MgCl_2$ were added and the sample lysate was incubated at room temperature for 15 min. If lysate was to be used for detection of LPS, cell lysate containing 1 µg of KDO was suspended in 20 µl of SDS-PAGE sample buffer, heated at 100°C for 10 min, cooled to room temperature, made to 0.5 mg/ml with

Proteinase K and incubated at 60°C for 1 h. This method is referred to as the modification of the sample preparation method of Hitchcock and Brown (1983) for the qualitative analysis of *Caulobacter* LPS.

Cells were prepared for analysis of LPS by the method of Hitchcock and Brown (1983) as follows. The cells from 5 ml of culture (OD_{600} = 0.6-0.7) were pelleted and washed with 10 mM HEPES (pH 7.2) by suspension and centrifugation then resuspended to a concentration of 200 Klett units (blue filter, Klett-Summerson colourimeter) in the same buffer. One and one half ml was then pelleted and the pellet was resuspended in 50 µl of a sample dissociation solution containing 2% SDS, 1 M Tris-HCl (pH 6.8), 4% β mercaptoethanol, 10% glycerol, and 0.005% bromophenol blue. The sample was heated at 100°C for 10 min, cooled to room temperature and 10 µl of the above sample dissociation solution containing 2.5 mg/ml of proteinase K was added. The sample was then incubated at 60°C for 1 h.

2.11 Silver staining

Following electrophoresis gels were stained using the Bio-Rad™ silver stain kit (Merril et al. 1981), a modification of the Bio-Rad™ silver stain kit (Cava et al. 1989), the method of Tsai and Frasch (1982) or a modification of the method of Tsai and Frasch.

The Bio-Rad™ silver stain kit was used as directed by the manufacturer. In the modification of the Bio-Rad™ silver stain kit the oxidizer solution was replaced with 0.7% sodium metaperiodate (BDH) dissolved in 0.65% isopropanol and 0.26% glacial acetic acid.

Briefly, the method of Tsai and Frasch (1982) involves an overnight fixation of the gel in 40% ethanol-5% acetic acid followed by a 5 min oxidation with 0.7% periodic acid in 40% ethanol-5% acetic acid. After washing with water the gel is stained for 10 min in an ammonium-silver reagent then washed extensively with water. The gel was developed at 25°C with a citric acid-formaldehyde solution. This development temperature inhibits the visualization of proteins (Hitchcock and Brown 1983). The Tsai and Frasch (1982) procedure was modified in that the gels were fixed for only 1 h with two changes of the fixation solution, the periodic acid oxidation step was extended from 5 to 15 min, and the staining step was extended to 20 min.

2.12 Cell preparation for thin-section electron microscopy

Treated and control cells were pelleted in a microcentrifuge tube and resuspended in Burdett's buffer (Burdett and Murray 1974) [5% acrolein, 0.25% glutaraldehyde (J. B. E. M. Services Inc., Point Claire - Durval, PQ) in 50 mM cacodylate (Electron Microscopy Sciences, Fort Washington, PA) buffer (pH 7.4)] and incubated at room temperature for 1 h then at 4°C overnight. Cells were then pelleted, washed twice with 50 mM cacodylate buffer (pH 7.4) by centrifugation and resuspension, and the washed pellet was then resuspended in 0.8% tannic acid (Mallinckrodt, Inc., Paris, KY) (in 50 mM cacodylate buffer [pH 7.4]) and incubated at room temperature for 30 min. The cells were again washed twice in 50 mM cacodylate

buffer (pH 7.4) and enrobed in 2% nobel agar (in 50 mM cacodylate buffer [pH 7.4]). Blocks were post fixed (1% OsO_4 [J. B. E. M.] and 0.5 mg/ml ruthenium red in 50 mM cacodylate buffer [pH 7.4]) for 1h at 4°C, washed three times with 50 mM cacodylate buffer (pH 7.4), twice with water and en bloc stained in saturated aqueous uranyl acetate (Fisher) for 1h. Blocks were then washed twice with water and dehydrated in an ethanol series, infiltrated with Spurr's (J. B .E. M.) resin/ethanol [(1:1) for 30 min], Spurr's resin [100% for 30 min two times] and finally embedded in Spurr's resin and heat polymerized at 65°C for 24h. Thin sections were cut then stained with uranyl acetate and Reynolds' lead citrate (Reynolds 1963). Specimens were viewed in a Siemens 101A electron microscope operating at 80 kV.

2.13 Negative stain electron microscopy

To examine colonies for the presence of S-layer, a colony was suspended in 10 µl of water containing 1 µl of bacitracin (1 mg/ml) and a carbon-stabilized, parlodion (J. B. E. M.)-coated 400 mesh copper grid was floated on top of the drop for a few min. To examine samples for *in vitro* reassembly of S-layer a grid was placed on a droplet containing RsaA. Grids were then lifted and excess liquid removed by wicking with filter paper. After drying, the sample was negatively-stained using 2% aqueous ammonium molybdate (Mallinckrodt) (pH 7.5) or 2% methylamine tungstate. Specimens were examined in a Siemens 101A transmission electron microscope

operated at 60 kV.

2.14 Transposon mutagenesis of NA1000

A Tn5 library was constructed by the electroporation of pSUP2021 (Simon et al. 1983) into electrocompetent NA1000 cells. Transposition events were selected by plating on PYE supplemented with Km and Sm. pSUP2021 was isolated from *E. coli* S17-1 by a mini-alkaline plasmid preparation procedure (Sambrook et al. 1989). Electrocompetent NA1000 were prepared as described by Gilchrist and Smit (1991). A Bio-Rad™ Gene Pulser, Pulse Controller, and cuvettes with 0.2-cm interelectrodal gaps was used as described by the manufacturer. The Gene Pulser was set at 2.5 kV and 25 μF. The Pulse Controller was set at 400 Ω. Twenty-thousand Km/Sm resistant colonies were pooled to form the library.

2.15 Complementation of JS1004 for SAO production with an NA1000 cosmid library

A NA1000 cosmid library, using pLAF5 (Keen et al. 1988) as a vector, was supplied by Dr. L. Shapiro (Stanford University) and was electroporated into electrocompetent JS1004. Nine hundred and fifty Tc^r colonies were inoculated into 96 well microtiter plates containing PYE-Tc using sterile tooth picks and grown for 40 h at 30°C. Two μl of each culture, including JS1003 as a positive control and JS1004 as a negative control, was placed on a nitrocellulose sheet and allowed to dry for 1h. Sterile DMSO was added to each microtiter well to a

final concentration of 5% and the plates were frozen at -70°C. The dry nitrocellulose was processed in the same manner as a Western blot using α-SAO sera as the primary antibody. One of the 950 electroporants reacted positively in the antibody screen. The cosmid DNA was obtained from this clone by an alkaline lysis method (Sambrook et al. 1989) and the cosmid DNA was electroporated into *E. coli* DH5α (BRL Laboratories, Gaithersburg, MD). The cosmid DNA was isolated by an alkaline lysis method, digested with BamHI and electrophoresed on a 0.7 % agarose (Bio Rad) gel using a Tris-acetate EDTA buffer system (Sambrook et al. 1989). The DNA fragments running lower than the top band (pLAF5 plus some *Caulobacter* DNA) were isolated using a GENECLEAN II® Kit (Bio 101 Inc., La Jolla, CA). The isolated DNA was ^{32}P labeled by nick translation (Rigby et al. 1977) and used to screen a cosmid library by colony-blot hybridization to identify overlapping cosmids (Maniatis et al. 1983).

2.16 Carbohydrate and lipid chemical analysis

Detailed chemical analysis on the isolated and purified carbohydrates and lipids were carried out as described by Ravenscroft et al. (1991; 1992). The rough LPS and EPS was analysed by Dr. N. Ravenscroft and the SAO was analysed by Dr. D. N. Karunaratne.

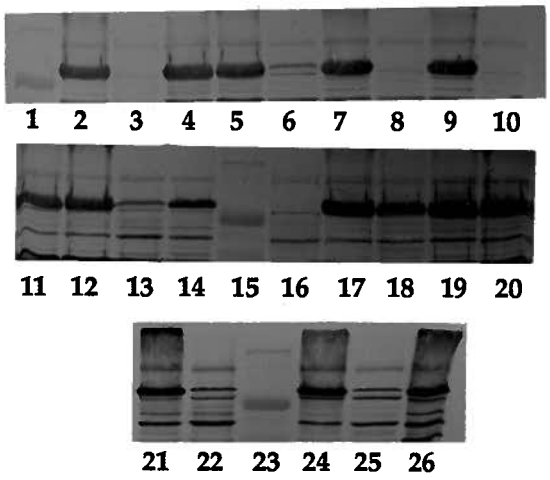
3 Results

3.1 Analysis of the S-layer attachment phenotype by Western blotting

Smit and coworkers (manuscript submitted) showed by negative-stain electron microscopy that calcium-independent mutants of *C. crescentus*, when grown on calcium-containing PYE plates, produced sheets of assembled S-layer that were not associated with the bacterial cells. The S-layer gene from the calcium-independent mutant JS1001 was cloned and expressed in the spontaneous S-layer minus *C. crescentus* CB2A and a wild-type, cell-associated, S-layer resulted. Thus although CB2A is S-layer negative it is S-layer "attachment competent". When the cloned S-layer gene from the wild-type strain NA1000 was introduced into the calcium-independent, S-layer negative, strain JS1004 non-cell associated S-layer sheets were produced. These electron microscopy studies determined that the defect in calcium-independent mutants, resulting in the inability to attach the S-layer to the cell surface, was not due to a defect in the S-layer protein but in some other cellular locus. Thus calcium-independent mutants exhibited an S-layer "attachment-defective" phenotype as well as the calcium-independent phenotype for which the mutants were selected.

Western blotting experiments were conducted to corroborate the electron microscopy observations of Smit and coworkers. S-layer attachment-defective mutants could be distinguished from wild-type

FIG. 1. Western blot analysis of whole cell lysates reacted with unadsorbed anti-RsaA antisera. "**L**" indicates cells were grown in a liquid culture and washed prior to analysis. "**P**" indicates 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**, CB2NY66Rmgl (L); **26**, CB2NY66Rmgl (P). Only the region of the gel containing S-layer is shown. The prestained molecular mass markers are 97.4 and 200 kDa. Lanes were loaded with samples containing 5 μg of protein as estimated by the method of Markwell et al. (1978). Samples were fractionated on a resolving gel containing 10% acrylamide prior to blotting onto nitrocellulose. Unless otherwise noted this and all subsequent polyacrylamide gels utilized a stacking gel containing 4 % acrylamide. The discontinuous buffer and sample dissociation method of Laemmli (1970) was used in this and all subsequent polyacrylamide gels as described in the Materials and Methods.


1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
21 22 23 24 25 26

strains on the basis of how the cells were prepared for electrophoresis. S-layer protein could be detected in colonies of calcium-independent mutants only when the mutant cells were scraped directly from plate cultures (Fig. 1; lanes 7, 9, 14, 21 and 26) whereas washed liquid-grown calcium-independent cells were almost negative for RsaA (Fig 1; lanes 6, 13, 22 and 25). 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. 1; lanes 3 and 16). Washed liquid medium-grown cells of strains that were S-layer negative but attachment-competent produced a positive S-layer blot when any cloned *rsaA* gene, including those from attachment-defective strains, was expressed in the cell on a plasmid vector (Fig. 1; lanes 4, 5, 12, 17, 18,19 and 20). When *rsaA*, cloned from an attachment-competent strain, was expressed in an attachment-defective background the attachment-defective phenotype persisted in that S-layer protein was detected only when the cells were harvested from plate cultures (Fig 1; lane 14). If the same strain is cultured in liquid medium RsaA is not detected (Fig. 1; lane 13). These results corroborated the electron microscopy observations of Smit and coworkers (manuscript submitted) which demonstrate that the defect in "calcium-independent" mutants, resulting in the inability to attach the S-layer to the cell surface, was not due to a defect in the S-layer protein but in some other cellular locus.

3.2 Isolation and purification of EPS from CB2A, NA1000 and JS1001

An EPS was identified on the surface of wild-type and calcium-independent *Caulobacter* strains. The EPS from mutant and wild-type strains was isolated and characterized to determine if an alteration in the EPS was responsible for the S-layer attachment-defective phenotype.

Fractionation, by steric-exclusion chromatography, of the crude EPS isolated from NA1000 and CB2A yielded similar profiles for both strains (Fig. 2). JS1001, a calcium-independent mutant of NA1000, produced the same fractionation profile as NA1000 (not shown). Carbohydrate analysis showed that the void volume peak (A) contained a heteropolysaccharide whereas the second peak (C) contained only ribose. Additionally, the second peak (C) contained significant amounts of phosphate and had a maximum absorbance at 260 nm so the peak was attributed to undegraded RNA. The CB2A crude EPS also contained an additional minor peak (B) between the heteropolysaccharide (A) and the RNA (C) peaks. On basis of exclusion limits reported for Sephacryl S-400 the appearance of the EPS peaks in the void volume indicates a minimum molecular mass of 1-2 million daltons.

3.2.1 Assessing the degree of EPS cell association.

Bacterial cell surface carbohydrates are qualitatively categorized as true capsules / EPS or as slime layers based on their ability to

maintain cell association. True capsules maintain cell association during growth or when the cells are subjected to mild sheer forces whereas slime layers are easily detached from the cell (Boulnois and Roberts 1990). In one trial of the EPS isolation procedure NA1000, JS1001 and CB2A cells were washed five times by centrifugation with 0.1 M HEPES buffer (pH 7.2) before extracting the EPS to determine if the EPS could be readily washed from the cells. The yield of EPS from this experiment was not significantly different than that of cells which had been washed only once or not at all (not shown). The culture supernatants from 500 ml batch cultures of CB2A, JS1001 and NA1000 were freeze-dried, dialyzed against water, freeze-dried again and analyzed for carbohydrate by gas chromatography. The analysis did not produce a sugar profile similar to that produced by purified EPS (see below). It was therefore concluded that the EPS produced by the 3 strains was significantly adherent to the cell.

3.2.2 Chemical characterization of EPS from CB2A, NA1000 and JS1001. The isolated EPS was found to be in a purified state suitable for detailed structural analysis (see appendix II, Fig. 2.). Dr. Neil Ravenscroft concluded that the EPS isolated from wild-type *C. crescentus* NA1000 and the calcium-independent mutant JS1001 were identical. The EPS produced by *C. crescentus* CB2A differed from that of NA1000.

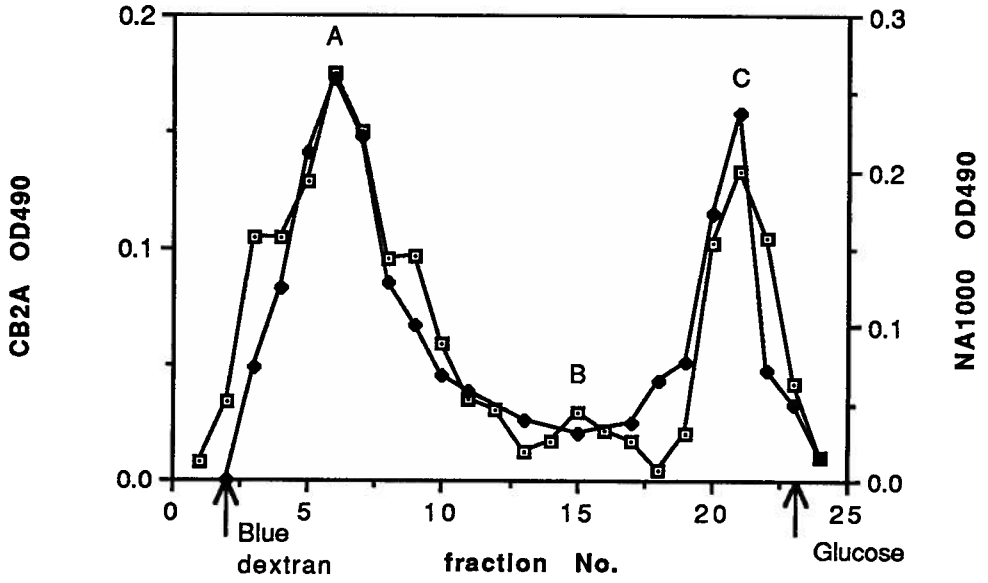


FIG. 2. Fractionation of EPS of CB2A (❑) and NA1000 (◆) on Sephacryl S-400. Carbohydrate monitored by the phenol-sulfuric acid assay as described in Materials and Methods (optical density at 490 nm). Peak A contains CB2A and NA1000 EPS fraction. Peak B contains a minor polysaccharide found only in CB2A. Peak C contains RNA. Each fraction consisted of 2.5 ml.

3.3 Isolation and purification of LPS from CB2A, NA1000 and JS1001

The LPS from mutant and wild-type *Caulobacter* strains was characterized and isolated to determine if an alteration in the LPS was responsible for the S-layer attachment-defective phenotype.

3.3.1 Electrophoretic analysis of LPS. SDS-PAGE of the purified LPS and subsequent staining, by the method of Tsai and Frasch (1982) or using the Bio-Rad silver stain kit, revealed that the *C. crescentus* strains produced a rough LPS (Fig. 3). Bands of similar mobility were detected in purified LPS preparations (Fig 3, lanes 3 - 6) No high molecular weight morphological heterogeneous forms, typical of smooth LPS, were noted. LPS profiles of these strains prepared by the method of Hitchcock and Brown (1983) also showed no high molecular weight bands (Fig. 3, lanes 1 and 2) indicating that the isolation method did not select against recovery of smooth LPS species. The heterogeneity of the LPS bands is typical of the microheterogeneity found in "rough" LPS species when examined by SDS-PAGE or TLC (Nowotny 1984). The samples prepared by the Hitchcock and Brown method (Fig. 3; lanes 1 and 2) produced bands that were broader in the horizontal plane and more condensed in the vertical plane than the bands produced by the purified samples. This is most likely a result of the Hitchcock and Brown samples containing bulk cellular components, such as undigested protein, peptidoglycan and nucleic acids which slightly alter the mobility of the LPS through the polyacrylamide gel.

The electrophoretic profile of a number of *C. crescentus*

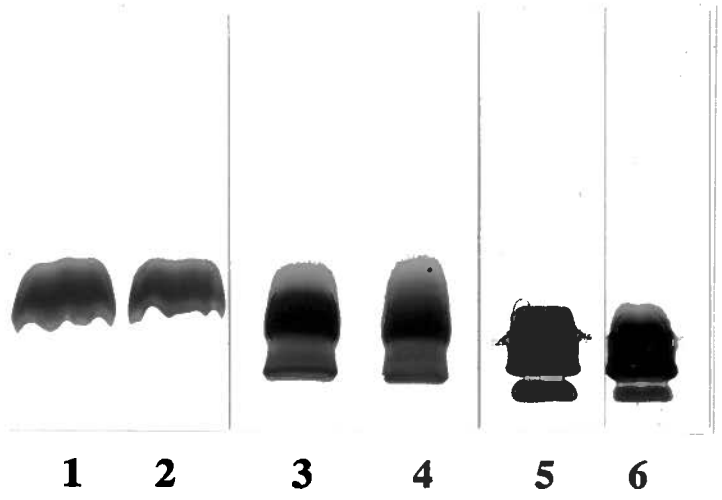


FIG. 3. SDS-PAGE of LPS prepared by the methods of Hitchcock and Brown (1983) [lanes **1** and **2**] and Darveau and Hancock (1983) [lanes **3** to **6**]. Gels were stained by the method of Tsai and Frasch (1982) [lanes **1** to **4**] or by using the Bio-Rad™ silver stain kit [lanes **5** and **6**]. Odd-numbered lanes contained LPS from CB2A; even-numbered lanes contained LPS from NA1000. All lanes were loaded with samples containing 1 μg of KDO as estimated by the method of Karknanis et al. (1978). The resolving gel contained 14% acrylamide.

strains, including the calcium-independent strains JS1001 and JS1002, were examined by the method of Hitchcock and Brown (1983) (Fig. 4). The LPS from all strains ran at the dye front of the gel. Purified LPS from JS1001 and JS1002 produced the same banding pattern (gel not shown).

3.3.2 Isolation of LPS, monitored by lipid analysis. The cold ethanol extraction procedure of Darveau and Hancock (1983) yielded the crude LPS fraction. Analysis of the fatty acids of the crude LPS showed the presence of saturated and mono-unsaturated 16- and 18-carbon fatty acids together with 3-OH-dodecanoic acid indicating contamination by phospholipids (Fig. 5A; [Lelts et al. 1982]). Extraction of the "crude" LPS by PCH and chloroform-methanol yielded a "pure" LPS product that is largely free of these C16 and C18 fatty acids (Fig. 5B). Lipid analysis of the soluble contaminates from the PCH and chloroform-methanol extraction steps revealed negligible amounts of 3-OH-dodecanoic acid indicating that little LPS was lost during these purification steps (see below). The material extracted from the final "pure" LPS was also examined by SDS-PAGE and stained with the Bio Rad™ silver stain kit and Coomassie blue. The gels showed that residual protein but not carbohydrate was extracted. Coomassie blue stained gels showed that a pronase-resistant protein of approximately 31 kDa was the major protein contaminant in the "crude" LPS (data not shown). The final freeze dried "purified" LPS was approximately 40% (by weight)

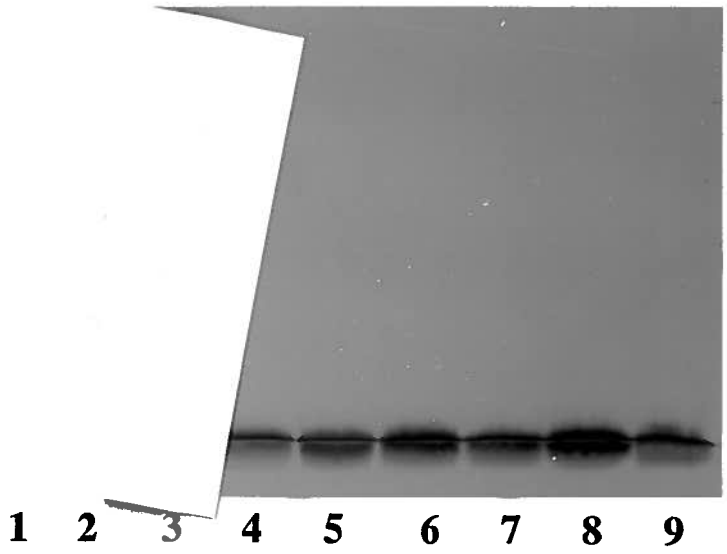


FIG. 4. SDS-PAGE of LPS prepared by the method of Hitchcock and Brown (1983) and stained by the method of Tsai and Frasch (1982). Lanes: **1**, CB2A; **2**, CB2NY66R; **3**, NA1000; **4**, JS1003; **5**, JS1002; **6**, JS1005; **7**, JS1001; **8**, JS1004; **9**, CB2NY66Rmgl. All lanes were loaded with samples containing 0.5 μg of KDO as estimated by the method of Karknanis et al. (1978). The resolving gel contained 12.5% acrylamide.

lighter that the "crude" LPS indicating that the two extraction procedures removed substantial amounts of impurities. The PCH-extraction resulted in a 10% decrease in the dry weight of the "crude" LPS whereas the chloroform-methanol wash decreased the weight by 30%.

3.3.3 Colourimetric analysis. The "pure" LPS was analyzed for protein, phosphate and KDO. No protein was detected and phosphate and KDO were found to account for 0.5% and 12% of the LPS dry weight, respectively. The molar ratio of phosphate to KDO, determined colourimetrically, was approximately 1:3. When the LPS was analysed for KDO under more severe hydrolysis conditions, than that recommended by Karkhanis et al. (1978), no increase in the amount of KDO per dry weight LPS was noted indicating that the KDO was likely not phosphorylated (Caroff et al. 1987).

The total amount of thiobarbiturate-positive material (KDO) contained in freeze dried cells of CB2A, NA1000 and *E. coli* B was also examined. For these studies identical weights of the three cell types were simultaneously analyzed along with KDO standards. The *Caulobacter* strains were found to contain less KDO per dry weight than *E. coli* B; the ratio being *E. coli* B:CB2A:NA1000 = 1:0.83:0.68.

3.3.4 Detailed chemical analysis. The purified LPS was found suitable for detailed chemical analysis. Dr. Neil Ravenscroft concluded that LPS isolated and purified from wild-type *C. crescentus* strains CB2A and NA1000 (Ravenscroft et al. 1992) and

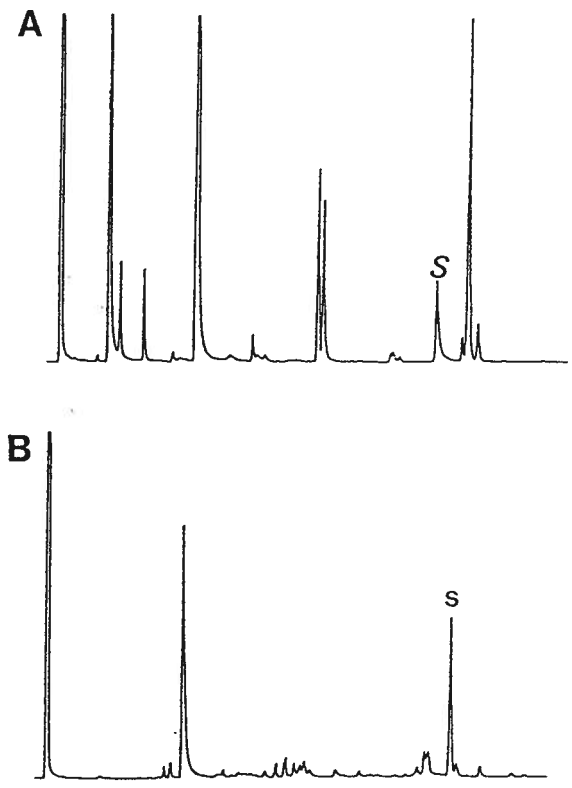


FIG. 5. A GC trace of fatty acid methyl esters from the LPS of NA1000 prepared and chromatographed as described in the Materials and Methods. (A) Crude LPS preparation prior to organic extractions. (B) Pure LPS resulting from extraction of the crude sample with phenol/chloroform/hexane followed by chloroform/methanol as described in the Materials and Methods. S = methyl ester of octadocanoic acid as an internal standard. The crude and purified LPS was prepared for GC and the major peak remaining in the purified LPS was identified as 3-OH-dodeconate (as determined by GC-MS) by Dr. Neil Ravenscroft.

the calcium-independent mutant JS1001 (Ravenscroft, unpublished) were structurally and chemically identical (see appendix II, Table I and II).

3.4 Identification of an S-layer associated oligosaccharide

During one experiment to isolate LPS from NA1000, by the Darveau and Hancock procedure (1993), an additional polysaccharide-containing molecule that migrated more slowly than LPS on SDS-PAGE was detected (Fig. 6). This molecule (which was subsequently referred to as the S-layer associated oligosaccharide or SAO) did not stain with the Tsai and Frasch silver stain procedure for LPS but was reliably stained using the more general Bio-Rad™ silver stain kit (Fig. 6; lane 1). [Note: It will be determined that the SAO is a smooth LPS species.] When this LPS preparation was examined by Western blot analysis, using anti-SAO sera (see Materials and Methods), the SAO band was specifically labeled (Fig. 6; lane 2). No reactivity was seen with the rough LPS. The various steps in the Darveau and Hancock procedure were monitored by SDS-PAGE with silver-staining and immunoblotting to determine where the SAO was lost. It was found that the SAO and significant amounts of rough LPS remained in the supernatant following the cold ethanol-Mg^{2+} precipitation procedure (data not shown).

The rapid LPS analysis method of Hitchcock and Brown did not reveal the SAO along with the rough LPS (Fig. 3 and 4). A modification of both the Hitchcock and Brown sample preparation

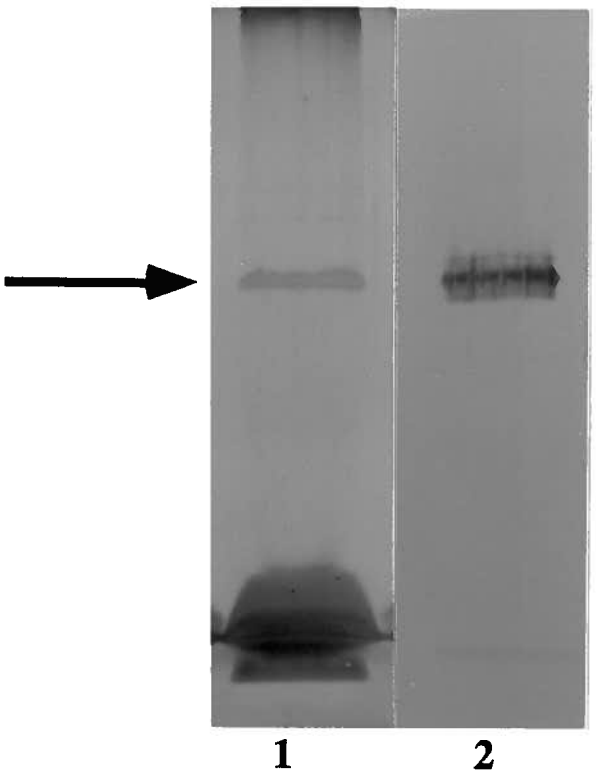
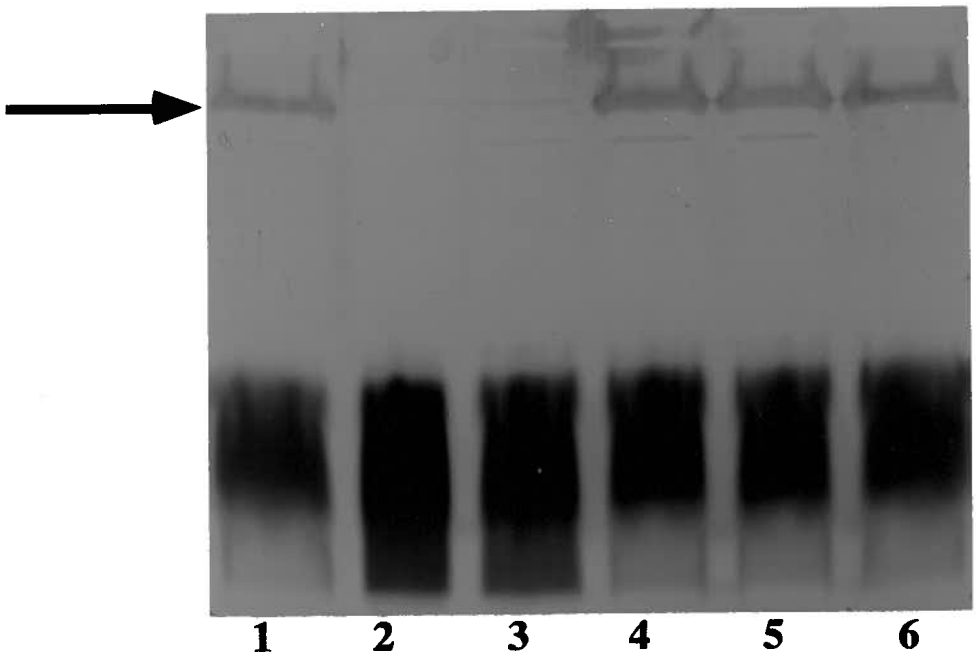


FIG. 6. Electrophoretic analysis of purified NA1000 LPS containing contaminating SAO. Lane **1**: SDS-PAGE of LPS stained using the Bio-Rad™ silver stain kit. Lane **2**: Western blot of LPS, fractionated by SDS-PAGE, reacted with α-SAO sera and visualized as described in the Materials and Methods. LPS was prepared by the method of Darveau and Hancock (1983) and fractionated using a resolving gel containing 13% acrylamide. Both lanes were loaded with samples containing 0.5 μg of KDO as estimated by the method of Karknanis et al. (1978). The arrow indicates the region of the gel containing SAO.

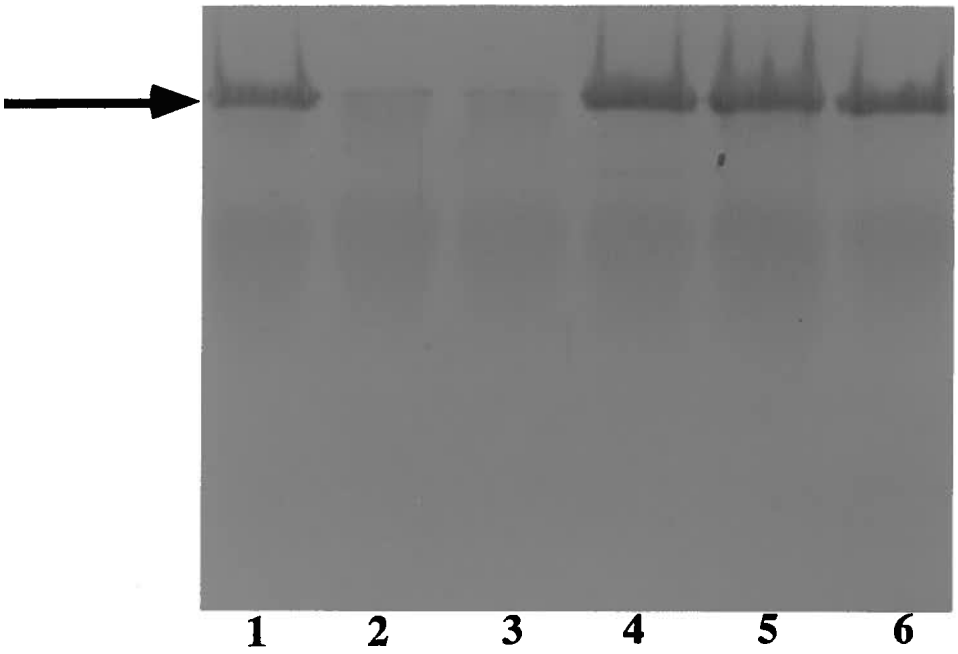
procedure and the Tsai and Frasch staining process was developed in order to visualize SAO in polyacrylamide gels. This modified procedure was used to examine a number of *Caulobacter* strains (Fig. 7A). Wild-type S-layer producing strains, NA1000 and CB2NY66R, and strains that produce a wild-type S-layer when *rsaA* is expressed on a plasmid (CB2A and JS1003), contained this additional polysaccharide (Fig. 7A; lanes 1, 4, 5, and 6). A comparable band was not detected in the S-layer attachment-defective strains JS1001 and JS1002 (Fig. 7A; lanes 2 and 3). Eight other calcium-independent strains isolated by Smit (unpublished) were also examined by these methods and none contained the SAO band (data not shown). The modified procedure of Tsai and Frasch revealed the SAO in whole cell lysates of S-layer attachment-competent *C. crescentus* strains (Fig. 7A, lanes 1, 4 - 6) although increased sample loadings were required to that which is normally used to detect LPS (note the overloading of the rough LPS in Fig. 7A). Tsai and Frasch (1982) recommends loading 1 to 5 μg of LPS per lane to obtain a satisfactory LPS profile. Western blotting of these preparations, using anti-SAO sera, showed that the SAO of all strains producing the polysaccharide cross reacted with the antibody which was raised against CB2A cell membranes (Fig. 7B). JS1001 and JS1002 showed only a faint immunoreactive band in the SAO region. These experiments were repeated using cells directly removed from plates and identical results were obtained (data not shown). This confirmed that the SAO was not sloughed off the cell surface of

FIG. 7. **A)** SDS-PAGE of whole cell lysates treated with proteinase K as described in the Materials and Methods, using a modification of the method of Hitchcock and Brown (1983), and fractionated using a resolving gel containing 13% acrylamide. The gel was stained using a modification of the method of Tsai and Frasch (1982) as described in the Materials and Methods. **B)** Western blot of samples shown in "**A**" reacted with α-SAO sera and visualized as described in Materials and Methods. Lanes in "**A**" and "**B**": **1**, NA1000; **2**, JS1001; **3**, JS1002; **4**, JS1003; **5**, CB2A; **6**, CB2NY66R. All lanes in "**A**" and "**B**" were loaded with samples containing 0.75 μg KDO as estimated by the method of Karknanis et al. (1978). The arrow in "**A**" and "**B**" indicates the region containing SAO.

A.



B.



calcium-independent mutants.

3.5 Isolation and purification of SAO

3.5.1 Extraction of cell surface molecules. *C. crescentus* is killed by the concentration of Na^+ found in phosphate-buffered saline (PBS) (Poindexter 1964). When cells incubated in PBS were examined by negative-stain electron microscopy small vesicles were observed to be extracted from the cell surface. The isolated vesicles were shown to contain SAO when examined by Western blotting using anti-SAO sera and the amount of material extracted from the cells increased if the PBS was supplemented with 10 mM EDTA (data not shown). Multiple extractions were required to completely remove all of this material from the cells and resulted in an extract that contained a low concentration of SAO. When 0.77 M NaCl / 0.12 M EDTA (pH 7.2) [25 ml/10 g (wet weight)] was used, in place of PBS / 10 mM EDTA, 95% of the cellular KDO was solubilized with two extractions. Therefore this method was used to isolate the SAO.

3.5.2 Examination of extract. The extract was subjected to ultracentrifugation and the pelleted material from NA1000 was shown to contain a protein/KDO at a ratio of 19.2 while pellets from JS1003 extract had ratio of 10.7. Proteinase K treatment of the pelleted extract followed by SDS-PAGE with silver-staining using the Bio Rad™ kit and Western blotting using anti-SAO sera revealed that the pelleted extract contained rough LPS and SAO (Fig. 8). Coomassie

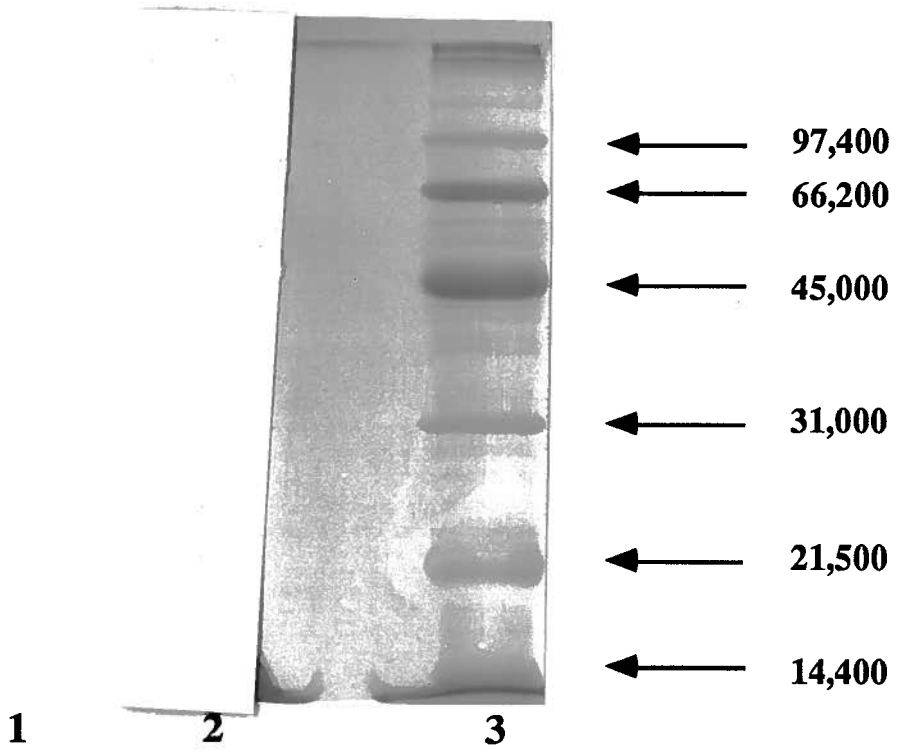


FIG. 8. Electrophoretic analysis of proteinase K-treated NaCl/EDTA extract of NA1000. Lanes: **1**, Western blot, of the polyacrylamide gel shown in lane 2, reacted with α-SAO sera and visualized as described in the Materials and Methods. **2**, SDS-PAGE stained using the Bio-Rad™ silver stain kit. **3**, Molecular mass standards in daltons. Lanes **1** and **2** were loaded with samples containing 0.5 μg KDO as estimated by the method of Karknanis et al. (1978). Samples were fractionated on a resolving gel containing 12% acrylamide. SAO is the band that runs adjacent to the 45,000 molecular mass marker. The rough LPS runs at the dye front.

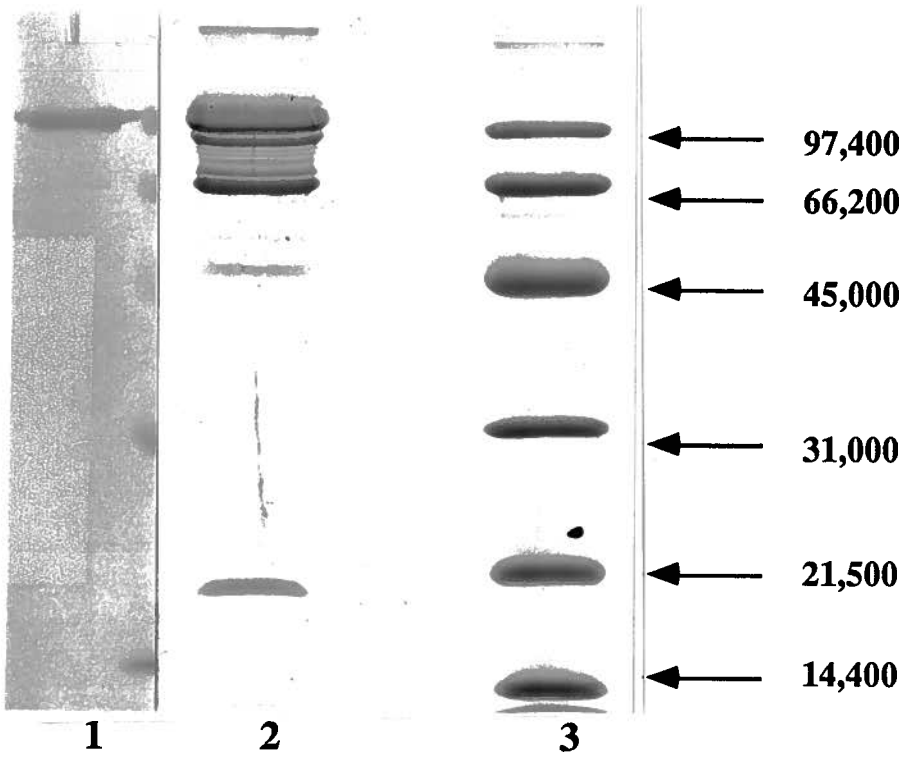


FIG. 9. Electrophoretic analysis of NaCl/EDTA extract of NA1000. Lanes: **1**, Western blot, of the polyacrylamide gel shown in lane 2, reacted with α-RsaA sera and visualized as described in the Materials and Methods. **2**, SDS-PAGE stained using Coomassie brilliant blue R-250. **3**, Molecular mass standards in daltons. Lanes 1 and 2 were loaded with samples containing 3 and 10 μg, respectively, as estimated by the method of Markwell et al. (1978). Samples were fractionated on a resolving gel containing 12% acrylamide.

blue staining of proteinase K treated extracts detected the minor band running above the SAO in lane 2 of Fig. 8 (data not shown). This band most likely represented the 66 kDa protein found in the non-proteinase K treated extracts (see below). The proteins contained in the pelleted extracts were examined by SDS-PAGE with Coomassie blue staining and Western blotting using anti-RsaA sera (Fig. 9). Many bands were detected with the major protein bands being approximately 20 kDa, 66 kDa and 105 kDa (RsaA). The 105 kDa protein reacted with α-RsaA sera confirming that it was the S-layer protein.

The supernatant following ultracentrifugation of the NA1000 extract was found to contain a high concentration of carbohydrate. Chemical analysis of this purified carbohydrate indicated that it was the EPS (Ravenscroft et al. unpublished). Figure 10 shows that the EPS was not contaminated with RNA as was the case when the EPS was isolated by the method of Darveau and Hancock (1983) before the SEC chromatographic step.

3.5.3 Examination of extracted cells. Extracted and control cells were prepared for and examined by thin-section electron microscopy (Fig. 11A and B). The extracted cells remained intact, no breeches in the peptidoglycan layer were noted, and the bilayer appearance of the outer and cytoplasmic membrane was maintained. Two major ultrastructural changes were noted. The electron-dense material between the peptidoglycan and the outer membrane was extracted from treated cells (Fig. 11A and B; arrow in inset) and

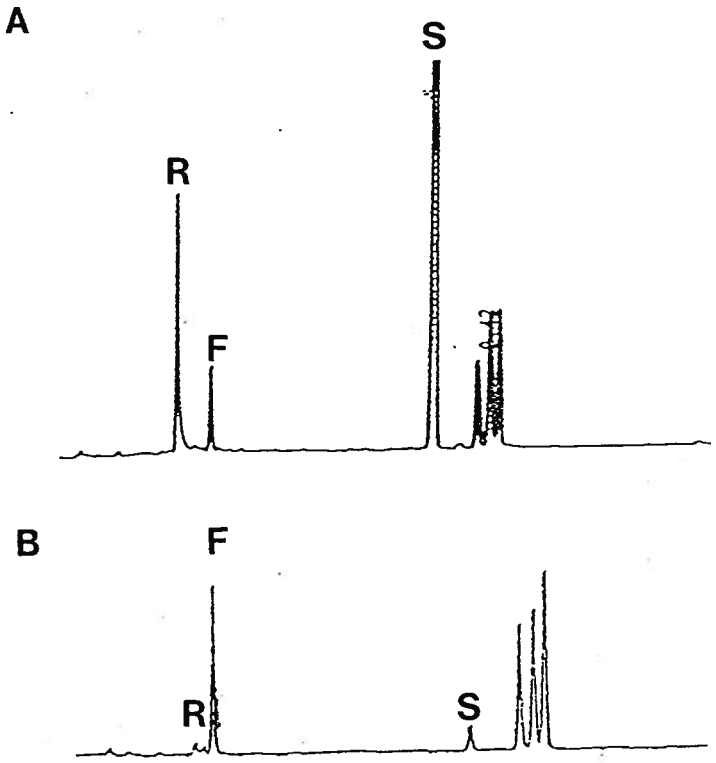
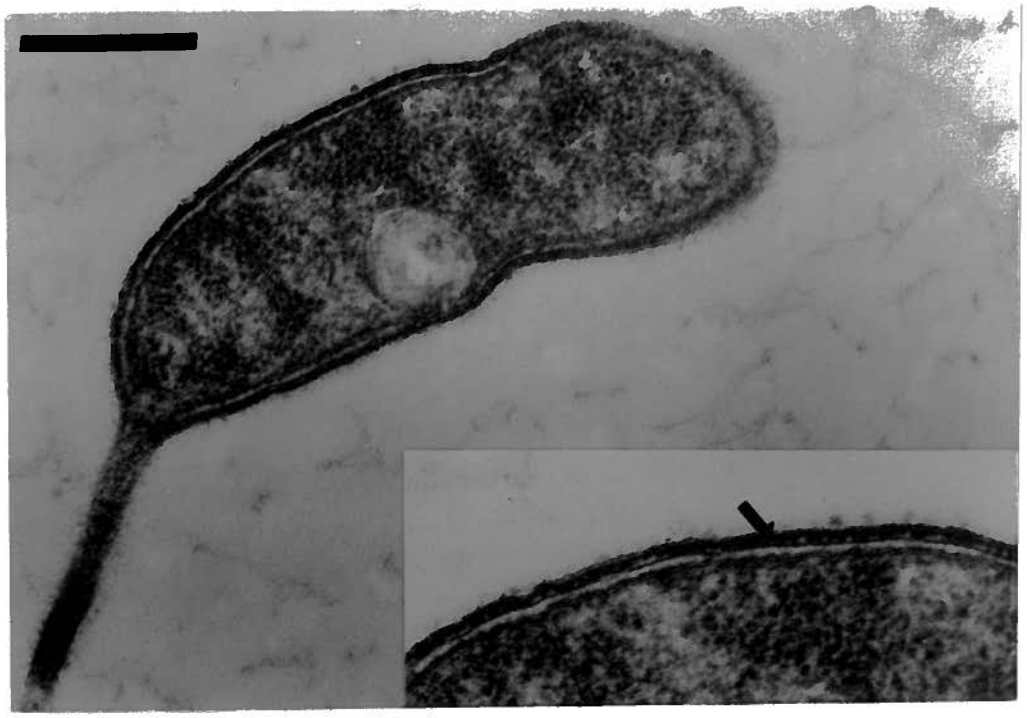


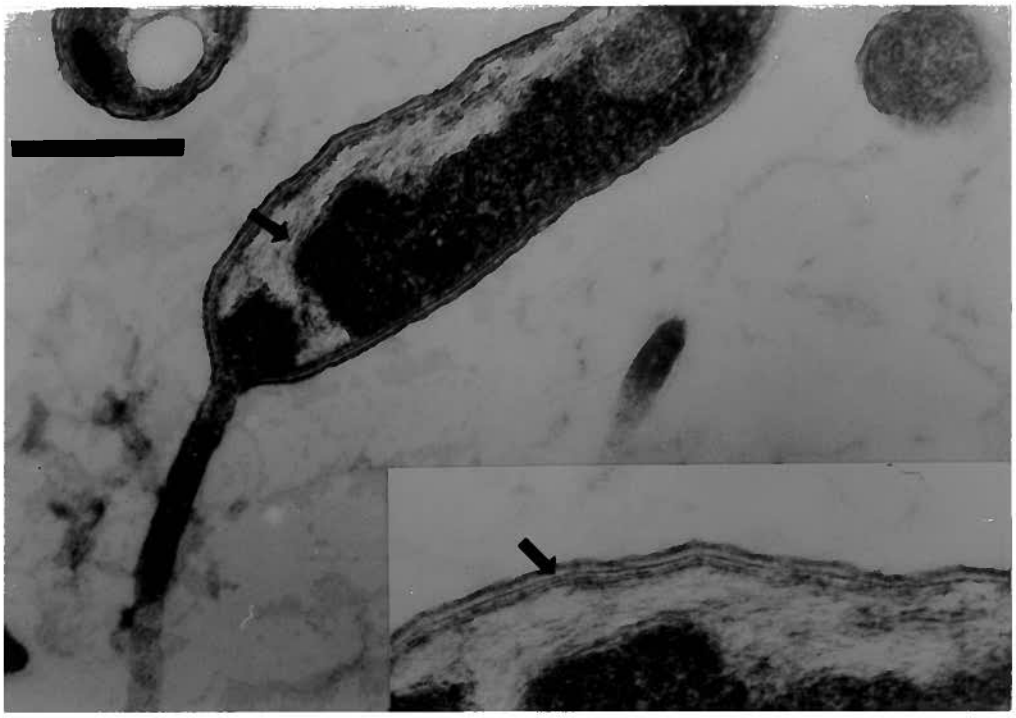
FIG. 10. GC trace of alditol acetates from EPS of NA1000 isolated using: **A**. The method of Darveau and Hancock (1983). **B**. NaCl/EDTA extraction. R = ribose; F = fucose; S = inositol standard. The same amount of inositol standard was included in both samples. The EPS was prepared for GC by Dr. Neil Ravenscroft.

FIG. 11. Thin-section TEM micrograph of: **A.** Control NA1000 cell. **B.** NaCl/EDTA extracted NA1000 cell. Note the even distribution of chromosomal material and ribosomes in control cell (**A**) and the rearrangement of cytoplasmic constituents into two distinct regions containing ribosomes or chromosomal material (**B**). Arrow in (**B**) shows ribosome-free region containing chromosomal material. Arrow in the inset figures illustrates electron-dense material between peptidoglycan layer and inner leaflet of the outer membrane (**A**) which is absent in extracted cells (**B**). All of 100 longitudinally sectioned control and extracted cells examined showed the features demonstrated in this figure. Bar = 0.5 μm. Micrograph taken by Mr. S. H. Smith.

A.



B.



there was a redistribution of the nuclear material within the cytoplasm of these cells (Fig. 11B; arrow in main figure). This redistribution has been proposed by Whitfield and Murray (1956) to be due to loss of the plasma membrane losing its selective permeability barrier to monovalent cations. A total of 100 longitudinally sectioned control and extracted cells were examined and all of the extracted cells contained the two ultrastructural changes.

3.5.4 Purification of the SAO. Proteinase K treated extracts from JS1003 were subjected to SDS-PAGE and the region of the gel containing SAO was removed. The SAO was extracted from the acrylamide by electroelution then concentrated and washed by Amicon™ filtration. A portion of the SAO was subjected to SDS-PAGE to determine its purity. Figure 12 shows that it was free of contamination by protein and rough LPS. It was noted that although both the SAO and rough LPS were stained by the Bio-Rad™ silver-stain kit this method was approximately five-fold less sensitive for the detection of rough *Caulobacter* LPS in comparison to the method of Tsai and Frasch (1982) (Gel not shown).

Colourimetric analysis indicated that the purified SAO contained, per mg dry wt; 25 μg KDO, 454 μg phenol-sulphuric positive carbohydrate, 1 μg phosphate and 4.7 μg of uronic acid. On a molar basis the KDO:Pi ratio was 3:1; the same ratio as that found in the rough LPS. Detailed chemical analysis of the SAO, preformed by Dr. D. N. Karunaratne, indicated the only major lipid present was the

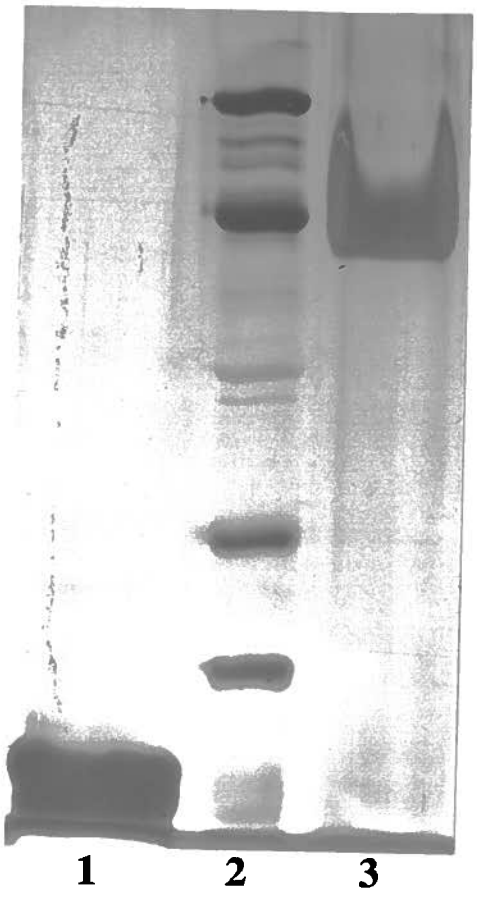


FIG. 12. SDS-PAGE of carbohydrates, purified from NaCl/EDTA extracts from JS1003, using a resolving gel containing 12% acrylamide and stained using the Bio-Rad™ silver stain kit. Lanes: **1**, purified rough LPS; **2**, protein molecular mass standard; **3**, purified SAO. Lanes **1** and **3** were loaded with samples containing 1.0 μg and 0.1 μg of KDO, respectively, as estimated by the method of Karknanis et al. (1978). Molecular mass standards are, from top, 66.2, 45, 31, 21.5, and 14.4 kDa. Note: The 31 kDa marker is poorly stained.

esterified fatty acid 3-OH-dodecanoate. Carbohydrate analysis indicated three major sugars and a number of minor sugars (see appendix II, Table III). The minor sugars were mannose, glucose, galactose and heptose which have been detected in the rough LPS. The major sugars were tentatively identified as 4,6-dideoxy-4-amino hexose, 3,6-dideoxy-3-amino hexose and glycerol.

3.6 Purification of *C. crescentus* S-layer protein

It was of interest to determine a rapid and effective method to extract and isolate a relatively pure preparation of RsaA from whole cells. With purified RsaA it will enable both the *in vitro* crystallization experiments and the production of polyclonal antisera against RsaA.

3.6.1 Extraction of the S-layer of *C. crescentus* NA1000.

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 blue staining the preparations appeared to contain nearly pure RsaA in this single step purification procedure (Fig. 13). The smearing of RsaA above the major band in the immunoblot shown in Figure 13 is commonly seen in acrylamide gels stained with Coomassie blue if sufficient RsaA protein is loaded on the lane. This is assumed to be a consequence of RsaA aggregating or polymerizing before or during electrophoresis (Smit and Agabian 1984). RsaA also stained poorly in polyacrylamide gels by Coomassie blue staining methods presumably due to the low content of basic amino acids in

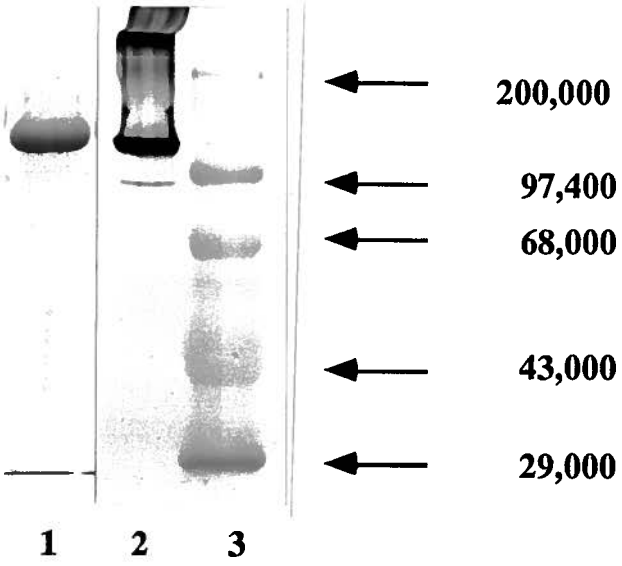


FIG. 13. Electrophoretic analysis of proteins extracted from whole cells of NA1000 using 0.1 M HEPES (pH = 2.0). Lanes: **1**, SDS-PAGE using a resolving gel containing 12% acrylamide and stained with Coomassie brilliant blue R; **2**, Western blot of gel shown in lane **1** reacted with α-RsaA sera and visualized as described in the Materials and Methods. **3**, Molecular mass markers in kDa. Lanes **1** and **2** were loaded with samples containing 3 μg of protein as estimated by the Bio-Rad™ protein assay.

the protein (Wilson 1983). Lysine, histidine and arginine account for only 2.3% of the total of amino acids in RsaA (Gilchrist et al. 1992). Occasionally, a minor amount of protein migrating with a faster electrophoretic mobility than RsaA was noted in low pH extracts. EGTA treatment also efficiently removed RsaA although the preparations were more contaminated with other protein species. Other methods were less effective: HEPES at pH 4 extracted RsaA as well as a number of other proteins while HEPES at pH 6, 7.5, 8, and 10 did not extract RsaA. Glycine-HCl at pH 2 yielded RsaA as a prominent protein but significant amounts of lower molecular weight proteins were also present. Glycine-HCl treatment at pH 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), ß-mercaptoethanol and EDTA all extracted numerous proteins without RsaA predominating. NaCl and $CaCl_2$ treatments did not yield significant amounts of protein.

The macroscopic precipitate formed in high density cultures of JS1001 was extracted with 8 M urea for 8 hrs and particulate matter was then removed by centrifugation. The urea was removed by dialysis and the solubilized protein was examined by SDS-PAGE and Coomassie blue staining (Fig. 14). Western blotting using anti-RsaA sera confirmed that the major protein species was RsaA (not shown).

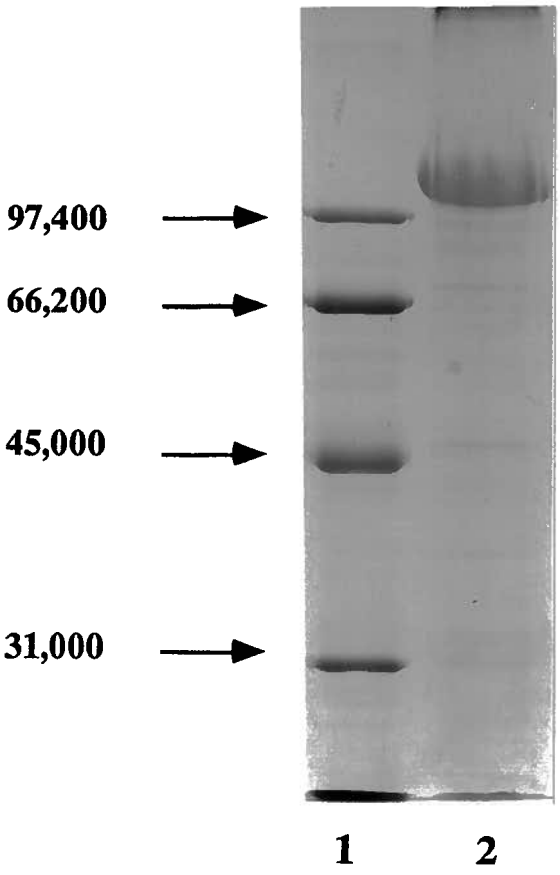


FIG. 14. SDS-PAGE using a resolving gel containing 10% acrylamide and stained with Coomassie brilliant blue R. Lanes: **1**, Molecular mass standards. **2**, Protein solubilized by urea from macroscopic precipitates produced in high density cultures of JS1001. Lane 2 was loaded with a sample containing 5 μg of protein as estimated by the Bio-Rad™ protein assay.

3.6.2 *In vitro* crystallization of the isolated NA1000 S-layer. After dialysis overnight against 100 mM HEPES buffer (pH 7.5), protein samples extracted by the EGTA or low pH methods had no visible turbidity and TEM negative-stain analysis showed only amorphous structures (not shown). Dialysis of the sample against 1 mM $MgCl_2$, or 1 mM $SrCl_2$ 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 crystallized into a regularly structured array of hexagonal symmetry (Fig. 15) with center-to-center spacing comparable to 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. RsaA solubilized by urea from the macroscopic precipitates formed in cultures of JS1001 did not recrystallize.

3.6.3 Anti-RsaA sera. The anti-RsaA sera was used at a dilution of 1:100,000 for a Western blot of whole cell lysates of NA1000 (Fig. 16). The blot indicated that the low pH extracted RsaA used as an antigen was not pure but contained a number of other proteins. Preadsorption of the antisera with cell lysates of JS1003 effectively removed antibody activity to proteins other than RsaA. The urea solubilized protein from JS1001 reacted with the anti-RsaA sera by Western blotting indicating that although it failed to recrystallize into a regular array it was in fact RsaA. The adsorbed RsaA antisera bound to NA1000 cells and did not bind to JS1003 or

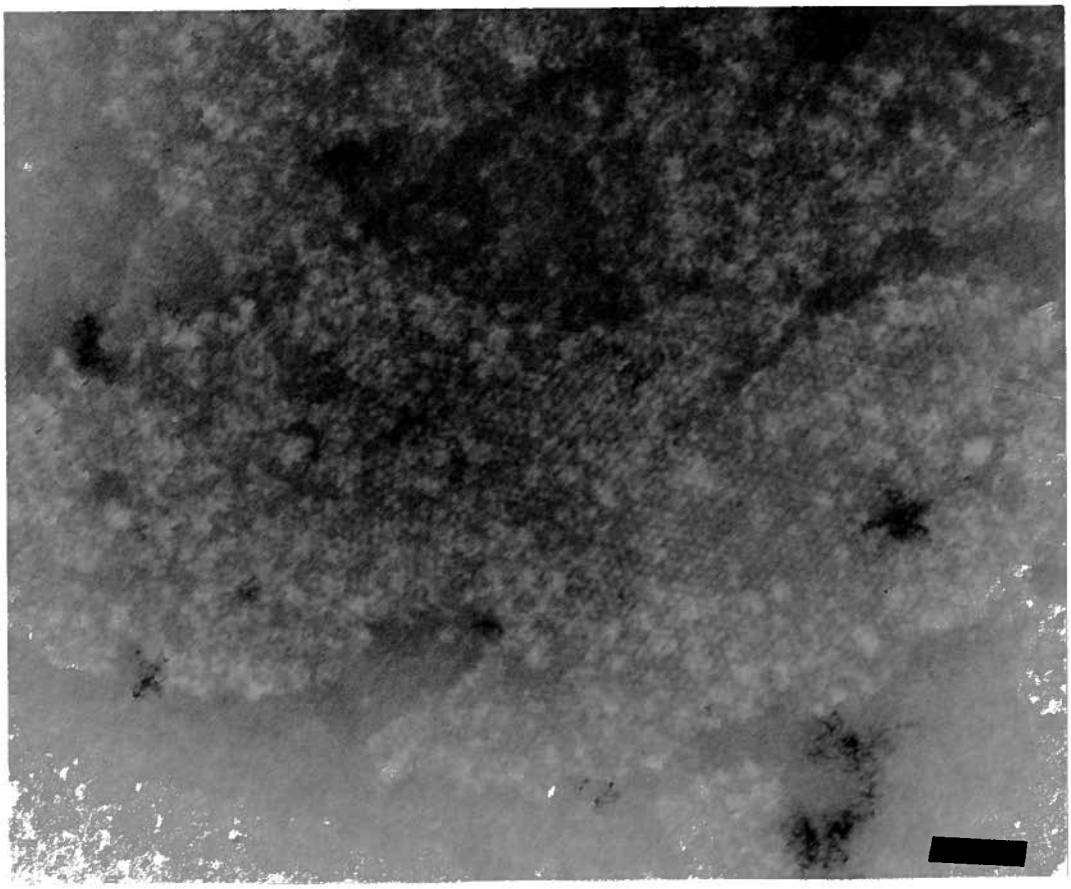


FIG. 15. Negative-stain transmission electron micrograph of the *In vitro* recrystallization of RsaA. RsaA was purified by low-pH extraction from NA1000 cells. Recrystallization was mediated by dialysis of monomeric RsaA against 10 mM HEPES buffer (pH = 7.5) containing 1 mM $CaCl_2$ at 4°C for 18 hrs. Bar = 0.1 μm. This electron micrograph was taken by Mr. S. H. Smith.

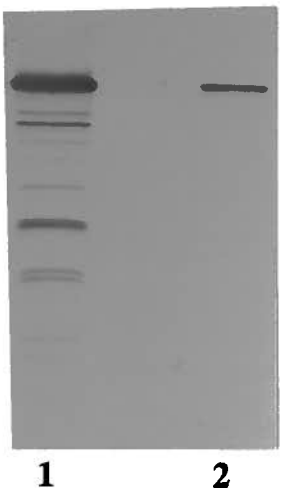


FIG. 16. Western blot reacted with unadsorbed α-RsaA sera and visualized as described in the Materials and Methods. Lanes: **1**, Whole cell lysate of NA1000; **2**, Low-pH extracted protein from NA1000. Lanes 1 and 2 were loaded with samples containing 10 and 1 μg of protein, respectively, as estimated using the Bio-Rad™ protein assay. The samples were fractionated by SDS-PAGE using a resolving gel containing 12% acrylamide.

CB2A cells in indirect immunoflurescent microscopy experiments (Smith and Smit, unpublished).

3.7 Comparison of S-layers among freshwater *Caulobacters*

It was of interest to determine if the cell surface of environmental *Caulobacter* isolates had the same general character as the laboratory strains. It will be shown that most environmental *Caulobacter* isolates produce an S-layer, rough LPS and SAO.

3.7.1 S-layer extraction. The HEPES (pH 2.0) extraction method was applied to all of the freshwater *Caulobacter* (FWC) strains and, in general, proved to be a useful technique to specifically extract the S-layer proteins. That is, only a single major high molecular weight band, characteristic of S-layer proteins from laboratory strains of *Caulobacter*, was seen by SDS-PAGE. The SDS-PAGE profiles of low pH extracts yielding an S-layer like band are shown in Figure 17A. The SDS-PAGE profiles of low pH extracts from FWC30, -38, -40, and -43 did not show a prominent S-layer like band and are shown in Figure 17A (lanes 2, 8, 30 and 31) for comparison. Strains from which an S-layer band could not be extracted using the HEPES (pH 2.0) method were extracted using HEPES buffer (pH 7.5) containing 10 mM EGTA. This treatment extracted a prominent protein in almost every case (Fig. 17B). FWC5, -14 and -21 (Fig. 17B; lanes 17, 15 and 14 respectively) did not produce an extract showing a prominent S-layer like band using HEPES buffer (pH 7.5) containing

FIG. 17A. SDS-PAGE of low pH extracted proteins from *Caulobacter* strains. Lanes: **1**, molecular mass markers; **2**, FWC38; **3**, FWC28; **4**, FWC33; **5**, FWC35; **6**, FWC31; **7**, FWC1; **8**, FWC43; **9**, NA1000; **10**, FWC15; **11**, molecular mass markers; **12**, FWC2; **13**, FWC44; **14**, FWC19; **15**, FWC17; **16**, FWC20; **17**, NA1000; **18**, FWC37; **19**, NA1000; **20**, molecular mass markers; **21**, FWC16; **22**, FWC26; **23**, FWC18; **24**, FWC22; **25**, FWC25; **26**, FWC46; **27**, FWC11; **28**, FWC23; **29**, NA1000; **30**, FWC40; **31**, FWC30; **32**, FWC39; **33**, FWC27; **34**, FWC24. The gels were stained with Coomassie Brilliant blue and the resolving gel contained 10% acrylamide. Samples contained 3 μg of protein as estimated by the Bio-Rad™ protein assay with the exception of FWC26, -30, -38, -40, -43, and -46 (see text). Molecular mass markers are: 200, 97.4, 68, 43, and 29 kDa.

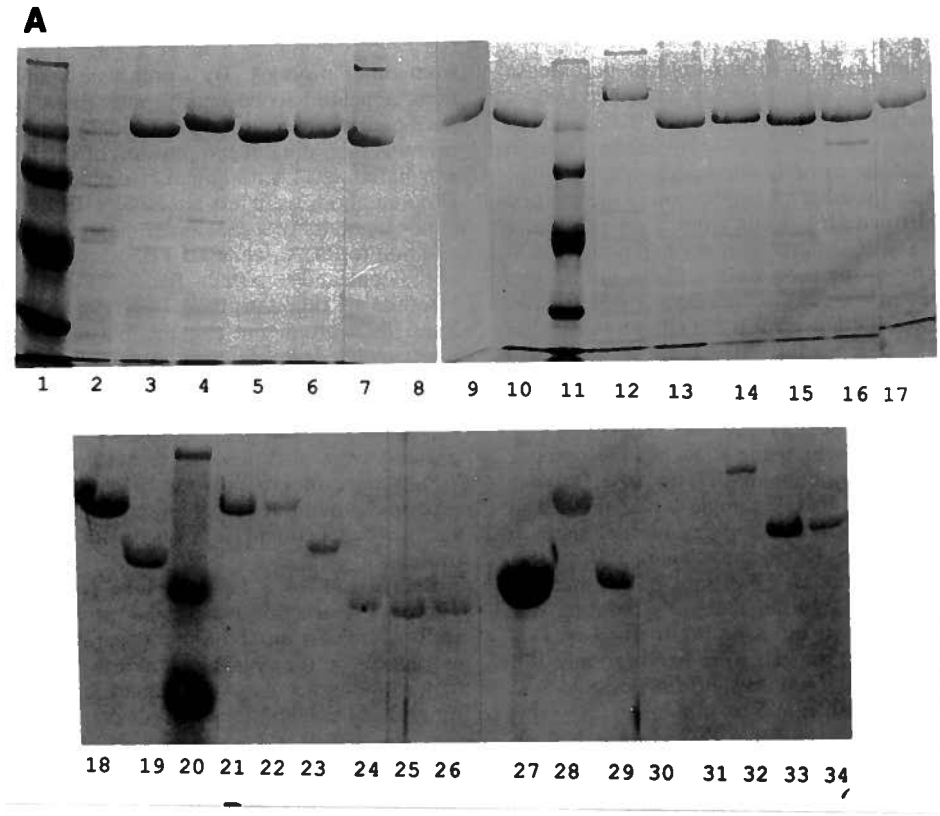
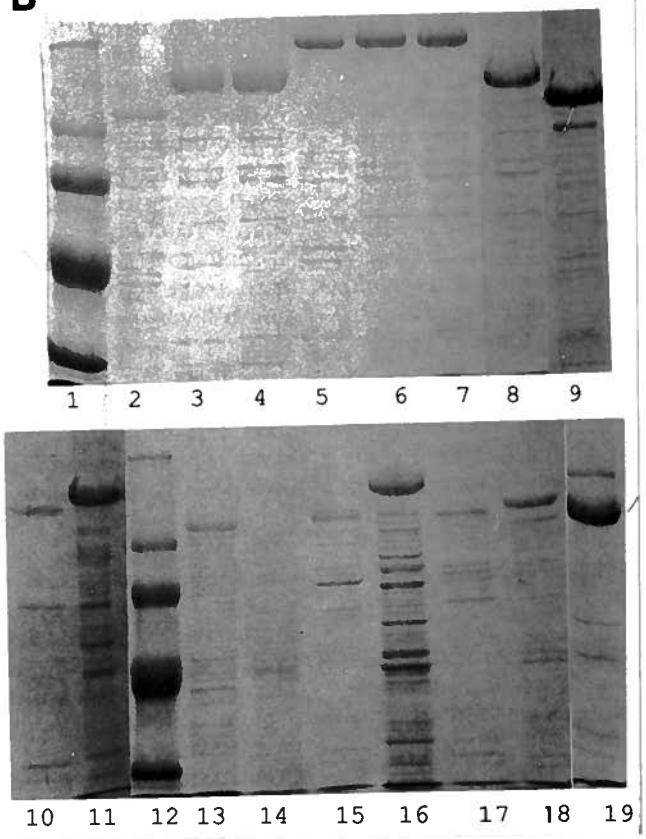

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34

FIG. 17B. SDS-PAGE of EGTA-extracted proteins from *Caulobacter* strains. Lanes: **1**, molecular mass markers; **2**, NA1000; **3**, FWC32; **4**, FWC12; **5**, FWC42; **6**, FWC7; **7**, FWC6; **8**, FWC9; **9**, FWC29; **10**, FWC41; **11**, FWC45; **12**, molecular mass markers; **13**, NA1000; **14**, FWC21; **15**, FWC14; **16**, FWC4; **17**, FWC5; **18**, FWC8; **19**, FWC34. The gels were stained with Coomassie Brilliant blue and the resolving gel contained 10% acrylamide. Samples contained 3 μg of protein as estimated by the Bio-Rad™ protein assay with the exception of FWC5, -14, -21, and -41 (see text). Molecular mass markers are: 200, 97.4, 68, 43, and 29 kDa.

B



10 mM EGTA or the low pH method. As well, FWC -30, -38, -40 and -43 did not produce an extract containing a prominent S-layer like band by using HEPES buffer (pH 7.5) containing 10 mM EGTA (not shown) or the low pH method (see above).

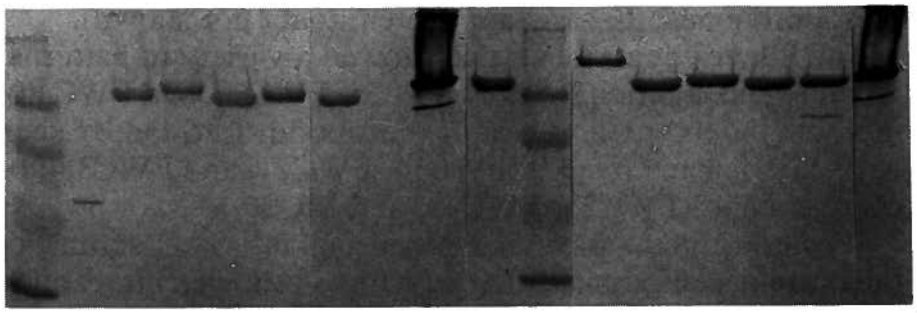
Extracts containing 3 μg of protein were examined by SDS-PAGE in Figures 17A and 17B with the exception of FWC5, -14, -21, -26, -30, -38, -40, -41, -43 and -46. Extraction of these strains by both methods resulted in extracts containing very low concentrations of protein. Therefore 3 μg of protein could not be loaded on the SDS-PAGE for these strains so 15 μl of extract from the method yielding the highest protein concentration was used.

3.7.2 Western blot analysis of extracted proteins. The protein samples used for the Coomassie blue stained gels were analyzed by Western blotting using the anti-RsaA serum (Fig. 18). All of the broad S-layer-like bands in Figure 17 gave a positive reaction with the exception of FWC23 (see Fig 17A; lane 28 and Fig.18; lane 28). The antiserum was quite specific for the suspected S-layer bands. Western blots of EGTA-extracted samples, which sometime contained non-S-layer proteins, only detected one prominent high molecular weight band.

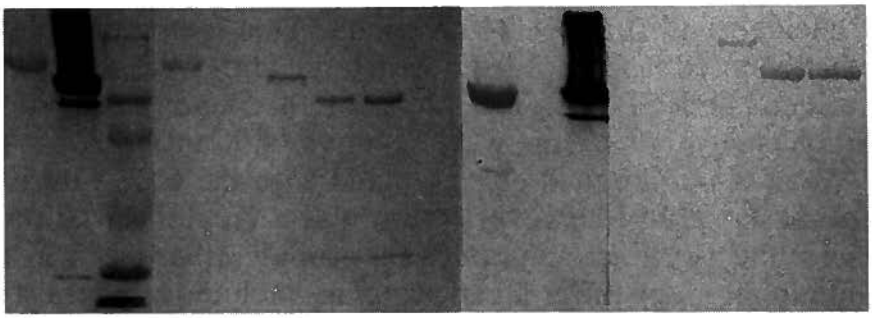
FWC5, -14, -21, -30, -38, -40, and -43 did not produce an S-layer (MacRae and Smit 1991) and did not show a high molecular weight band by Coomassie blue staining (See Fig. 17A; lanes 2, 8, 30, and 31 for FWC38, -43, -40, and -30 respectively and Fig 17B; lanes

FIG. 18. Western blot analysis of proteins extracted from *Caulobacter* strains using the low pH (**A**) or EGTA (**B**) method. Blots were reacted with α-RsaA serum. The SDS-PAGE resolving gel contained 10% acrylamide. (**A**) Lanes: **1**, molecular mass markers; **2**, FWC38; **3**, FWC28; **4**, FWC33; **5**, FWC35; **6**, FWC31; **7**, FWC1; **8**, FWC43; **9**, NA1000; **10**, FWC15; **11**, molecular mass markers; **12**, FWC2; **13**, FWC44; **14**, FWC19; **15**, FWC17; **16**, FWC20; **17**, NA1000; **18**, FWC37; **19**, NA1000; **20**, molecular mass markers; **21**, FWC16; **22**, FWC26; **23**, FWC18; **24**, FWC22; **25**, FWC25; **26**, FWC46; **27**, FWC11; **28**, FWC23; **29**, NA1000; **30**, FWC40; **31**, FWC30; **32**, FWC39; **33**, FWC27; **34**, FWC24. (**B**) 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. Samples contained 3 μg of protein as estimated by the Bio-Rad™ protein assay with the exception of FWC5, -14, -21, -26, -30, -38, -40, -41, -43, and -46 (see text). Molecular mass markers are: 200, 97.4, 68, 43, and 29 kDa. Note: The samples in this figure are not in the same order as samples in Figure 17A and 17B.

A

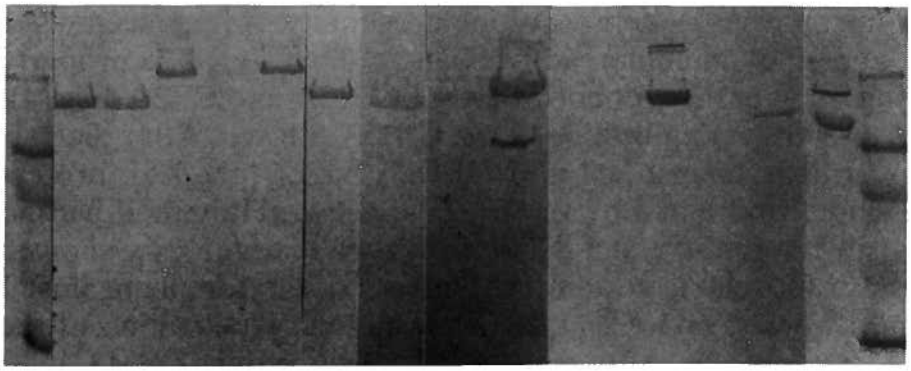


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17



18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34

B



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

14, 15 and 17 for FWC21, -14, and -5) or positive reaction by Western blotting (See Fig. 18A; lanes 2, 8, 30 and 31 for FWC28, -43, -40, -30 respectively and Fig 18B; lanes 11, 12 and 14 for FWC21, -14, and -5). The Coomassie blue stained gel of extracts from both FWC14 and 5 showed a thin high molecular weight band (Fig 17B; lanes 15 and 17) but the bands did not react to anti-RsaA sera in immunoblot experiments (Fig 18B; lanes 12 and 14). FWC23 was the only strain without an S-layer, as determined by TEM, to show a prominent band by Coomassie blue staining (Fig. 17A; lane 28) but it did not label by Western blot analysis (Fig. 18A; lane 28). FWC26, -41, and -46 have an S-layer and produced an S-layer band by the criteria of Coomassie blue staining (Fig. 17A; lanes 22 and 26 for FWC26 and -46 respectively and Fig. 17B; lane 10 for FWC41) and Western blot analysis (Fig. 18A; lanes 22 and 26 for FWC26 and -46 respectively and Fig. 18B; lane 9 for FWC41) although the bands were only weakly visible especially for the Western blots. Extraction of the S-layer protein from FWC26, -41 and -46 by both methods was considered poor in that the concentration of solubilized protein was much less that obtained from other strains (see above). The remaining 32 strains yielded significant amounts of protein with at least one of the extraction procedures and gave positive results by both Western immunoblots and Coomassie blue staining.

The molecular weights of the S-layer proteins from the FWC-strains were estimated from their mobility in SDS-PAGE relative to protein standards (Table II). The S-layer proteins were quite

TABLE II. Relevant characteristics of Caulobacter strains

Strain	S-layer protein size (kDa) [a]	Anti-RsaA response [b]	Polysaccharides detected [c]	Anti-SAO response [d]
S-layer-producing strains				
FWC1	100	+	+	1
FWC2	130	+	+	1
FWC4	130[e]	+	-	0
FWC6	180	+	+	3
FWC7	175	+	+	3
FWC8	120	+	+	1
FWC9	135	+	+	3
FWC11	110	+	+	1
FWC12	130	+	+	3
FWC15	110	+	+	0
FWC16	150	+	+	3
FWC17	105	+	+	2
FWC18	130	+	+	1
FWC19	110	+	+	2
FWC20	110	+	+	0
FWC22	105	+	+	1
FWC24	145	+	+	3
FWC25	105	+	+	1
FWC26	140	+	+	3
FWC27	145	+	+	1
FWC28	105	+	+	1
FWC29	125	+	+	0
FWC31	105	+	+	1
FWC32	135	+	+	3
FWC33	110	+	+	1
FWC34	110	+	+	2
FWC35	100	+	+	1
FWC37	150	+	+	3
FWC39	195	+	+	4
FWC41	135	+	+	3
FWC42	180	+	+	2
FWC44	105	+	+	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	155	-	+	0
FWC30	NF	-	-	0
FWC38	NF	-	Multiple bands	0
FWC40	NF	-	-	0
FWC43	NF	-	+	0

Legend for Table II.

[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-RsaA serum.

[c] Results of SDS-PAGE of proteinase K-treated samples stained for polysaccharides by a modification of the procedure of Tsai and Frasch (1982). +, single high-molecular-weight band. -, no silver-stained bands running slower than the rough 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* NA1000 SAO.

[e] S-layer presence was not confirmed by negative-stain TEM although the protein was reactive with anti-RsaA antibody.

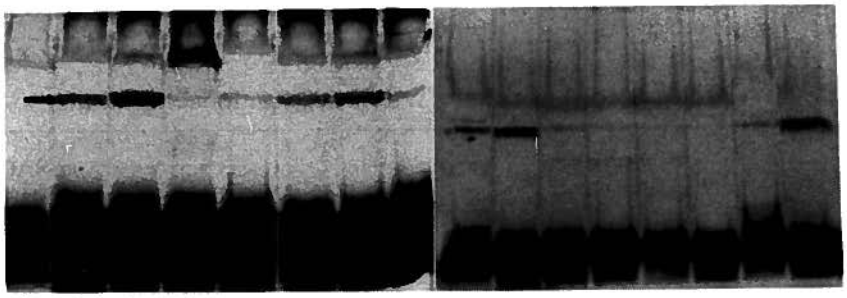
[f] NF, not found.

heterogeneous in molecular weight ranging from 100 to 190 kDa. Proteins greater than 100 kDa are difficult to size by their SDS-PAGE mobility and so the molecular weights reported are only estimates, but are useful for comparative purposes.

3.7.3 Polysaccharide analysis. The FWC strains were also examined for the electrophoretic mobility of their LPS using a rapid purification and staining procedure that was modified to visualize both SAO and rough LPS. All strains produced a low molecular weight polysaccharide-containing molecule, presumably rough LPS, which migrated at the dye front in the gel system used for these experiments (Fig. 19). All strains producing an antibody reactive S-layer protein (Fig. 18, Table II) were found to also produce a single slower-migrating carbohydrate band with the exception of FWC4 (See Fig. 19; lane 20). This SAO-like species varied in apparent molecular weight from 60 to 95 kDa (such values are only for comparative purposes reflecting the approximate molecular weight of a protein migrating at that position). Twenty two strains also showed a stained band running with the electrophoretic mobility of a 43 kDa protein (see arrow in lane 1 of Fig. 19). Lane 2 showed a moderately stained band while lane 3 contained a strongly stained band. A Coomassie blue stained gel of the proteinase K-treated samples also detected this band indicating the presence of a proteinase K-resistant protein (data not shown).

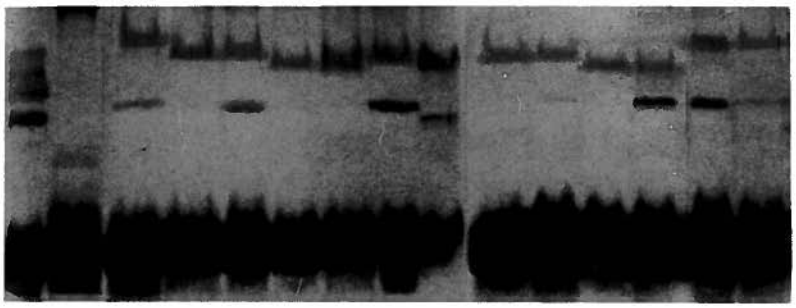
The S-layer negative strains showed a variety of high

FIG. 19. SDS-PAGE of proteinase K-treated whole cell lysates of *Caulobacter* strains stained using a modification of the method of Tsai and Frasch (1982). Lanes: **1**, NA1000; **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**, NA1000; **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**, NA1000; **39**, FWC22; **40**, FWC34; **41**, MCS6; **42**, FWC32; **43**, FWC26; **44**, NA1000; **45**, FWC31; **46**, FWC6; **47**, FWC42. Each lane was loaded with sample containing 0.75 μg of KDO as estimated by the method of Karknanis et al. (1978). The resolving gel contained 13% acrylamide. The small arrow in lane 1 indicates the running position of a proteinase-K resistant protein that was present in some samples. SAO runs as a poorly stained band above the small arrow in lane 1.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32



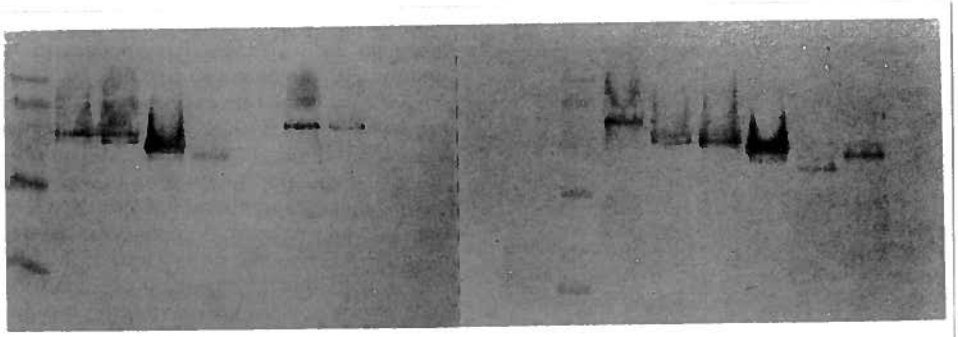
33 34 35 36 37 38 39 40 41 42 43 44 45 46 47

molecular weight carbohydrate banding patterns, including no additional bands (FWC5, -30 and -40; see Fig. 19, lanes 21, 34, and 25 respectively), multiple bands reminiscent of the smooth LPS "ladder" seen with enteric bacteria (Peterson and McGroarty 1985) (FWC14, -21, -38; see Fig 19, lanes 24, 33, and 22 respectively), or a single band (FWC23, -43 and MCS6; see Fig. 19, lanes 16, 23 and 41 respectively).

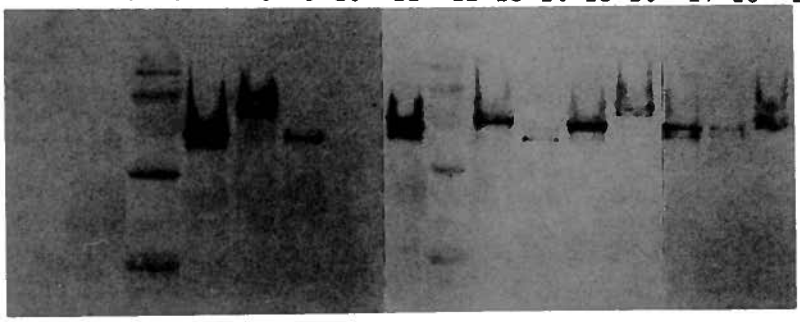
Western blot analysis, using anti-SAO serum, of the proteinase K-treated samples (Fig. 20) showed no reaction with the probable rough LPS species of any of the strains. When a reaction was seen in S-layer producing strains it coincided with the SAO-like bands. No immunoreactive bands were observed in Western blots, using anti-SAO sera, for S-layer minus strains (Table II, Fig. 20; lanes 9, 21, 11, 12, 10, 22, 6).

The majority of the "SAO-like" carbohydrates, identified by silver-staining (Fig. 19) reacted with the anti-SAO serum in Western blotting experiments (Fig. 20). However, there was a variation in the degree of immuno-reactivity between strains. A qualitative analysis of the degree to which the SAO-like molecules reacted with anti-SAO sera is presented in Table II. The laboratory strain NA1000 stained darkly and produced a major band that was smeared, reminiscent of the silver-stained image of the SAO, and a minor band running slightly faster than the major band (See Fig. 20; lanes 4, 17, 24, 28, 37, and 47). The band produced by FWC39 was as intense as that produced by NA1000 but no minor band was noted (Anti-SAO response = 4 in Table II; Fig. 20; lane 25). Eight strains produced a reaction that showed a definite smearing reaction, but was less

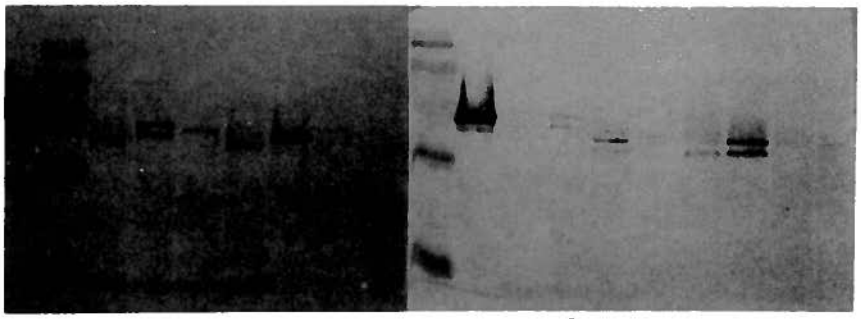
FIG. 20. Western blot analysis of proteinase-K treated whole cell lysates of *Caulobacter* strains. Blots were reacted with α-SAO sera. Lanes: **1**, molecular mass markers; **2**, FWC32; **3**, FWC26; **4**, NA1000; **5**, FWC31; **6**, FWC43; **7**, FWC6; **8**, FWC42; **9**, FWC5; **10**, FWC38; **11**, FWC21; **12**, FWC30; **13**, molecular mass markers; **14**, FWC7; **15**, FWC37; **16**, FWC12; **17**, NA1000; **18**, FWC22; **19**, FWC34; **20**, MCS6; **21**, FWC14; **22**, FWC40; **23**, molecular mass markers; **24**, NA1000; **25**, FWC39; **26**, FWC2; **27**, FWC4; **28**, NA1000; **29**, molecular mass markers; **30**, FWC9; **31**, FWC18; **32**, FWC41; **33**, FWC45; 34, FWC24; **35**, FWC27; **36**, FWC16; **37**, NA1000; **38**, molecular mass markers; **39**, FWC28; **40**, FWC33; **41**, FWC35; **42**, FWC44; **43**, FWC46; **44**, FWC8; **45**, FWC29; **46**, molecular mass markers; **47**, NA1000; **48**, FWC23; **49**, FWC1; **50**, FWC11; **51**, FWC15; **52**, FWC17; **53**, FWC19; **54**, FWC20; **55**, FWC25. Each lane was loaded with sample containing 0.75 μg of KDO as estimated by the method of Karknanis et al. (1978). Samples were fractionated by SDS-PAGE using resolving gel that contained 13% acrylamide. Note: The samples in this figure are not in the same order as samples in Figure 19.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36



37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55

intense than that seen for NA1000, and produced a minor band below the smearing band (Anti-SAO response = 3 in Table II; see Fig. 20; lane 3 [FWC26] as an example). Three strains, FWC6, 7 and 9 produced the definite smearing band but did not contain the lower minor band (Anti-SAO response = 3 in Table II; see Fig. 20; lane 7 [FWC6] as an example). Four strains showed some minor smearing above a single band (Anti-SAO response = 2 in Table II; see Fig. 20; lane 42 [FWC44] as an example) and two strains showed minor smearing above a doublet band (Anti-SAO response = 2 in Table II; see Fig. 20; lane 53 [FWC19] as an example). Eight strains produced a doublet band with no smearing above them (Anti-SAO response = 1 in Table II; see Fig. 20; lane 50 [FWC11] as an example) while four strains showed only a single immunoreactive band (Anti-SAO response = 1 in Table II; see Fig. 20; lane 26 [FWC2] as an example). Three strains producing a silver-stain positive band did not produce any immunoreactive bands (Anti-SAO response = 0 in Table II; FWC15, -20, -29). Figure 20 illustrates that the "SAO-like" carbohydrate produced by freshwater *Caulobacters* differed from strain to strain with respect to electrophoretic mobility and immunological reactivity to the anti-SAO sera.

3.8 Metal ion requirements for *C. crescentus* growth and S-layer assembly

It has been shown that wild-type *C. crescentus* NA1000 requires calcium for growth. The experiments described below

examine this calcium requirement in more detail.

3.8.1 Influence of calcium on growth of NA1000 and JS1001. Washed M_3Higg grown NA1000 and JS1001 cells were inoculated into M_{10}Higg medium supplemented with various concentrations of calcium and the optical density was measured after 48 h of incubation (Fig. 21). Calcium concentration had little influence on the growth of the calcium-independent mutant JS1001 whereas calcium concentrations of less than 75 μM resulted in decreased growth yield of NA1000. Figure 22 illustrates that calcium became growth rate limiting for NA1000 below 250 μM. When JS1003, which was NA1000 with rsaA interrupted with a Km^r cassette, was used in place of NA1000, the same growth patterns were observed. The washed NA1000 cells used to inoculate M_{10}Higg medium containing no metal ion supplement did not lyse or die. After 48 hrs of incubation, phase contrast microscopy showed the cells were elongated, tapered consisting of a swarmer and a stalked cell frozen in mid-cell division. Addition of calcium resulted in growth following a brief lag period.

3.8.2 Influence of metal ions on the growth rate of NA1000 and JS1001. M_{10}Higg medium, which contains 2.2 mM Mg^{2+}, was supplemented with various cations to a final concentration of 500 μM and the growth rate of NA1000 was determined by the method outlined in the Materials and Methods section (Fig. 23). NA1000 did not grow in M_{10}Higg medium or M_{10}Higg medium

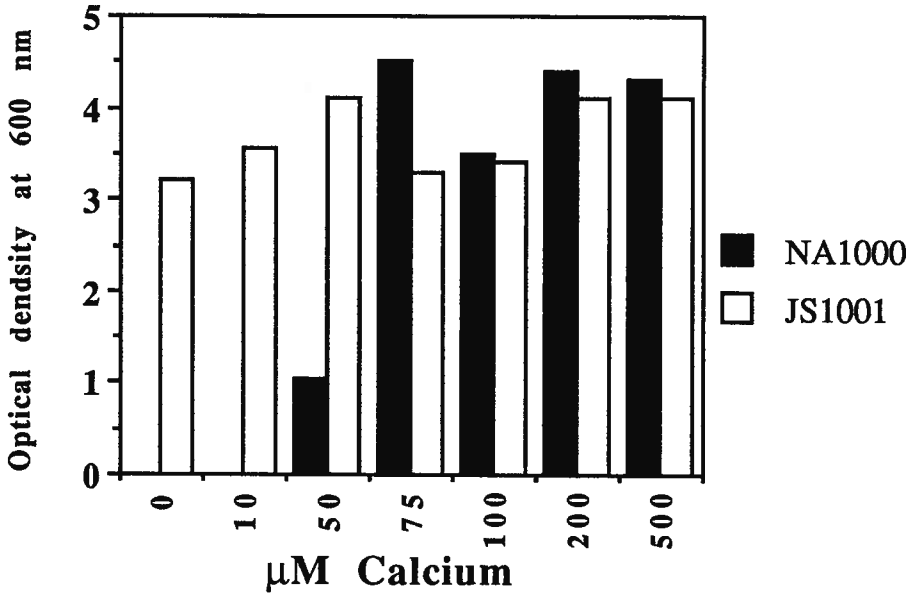


FIG. 21. The influence of calcium on growth of NA1000 and JS1001. 5 x 10^6 washed mid-logarithmic cells were inoculated into 5 mls of M_{10}Higg medium containing 0 to 500 μM calcium. Cultures were incubated at 30°C and the optical density at 600 nm was measured after 48 hrs. Duplicate tubes were used for all concentrations of calcium and the experiment was repeated 3 times. The final optical densities for each calcium concentration varied by less than 5% between experiments.

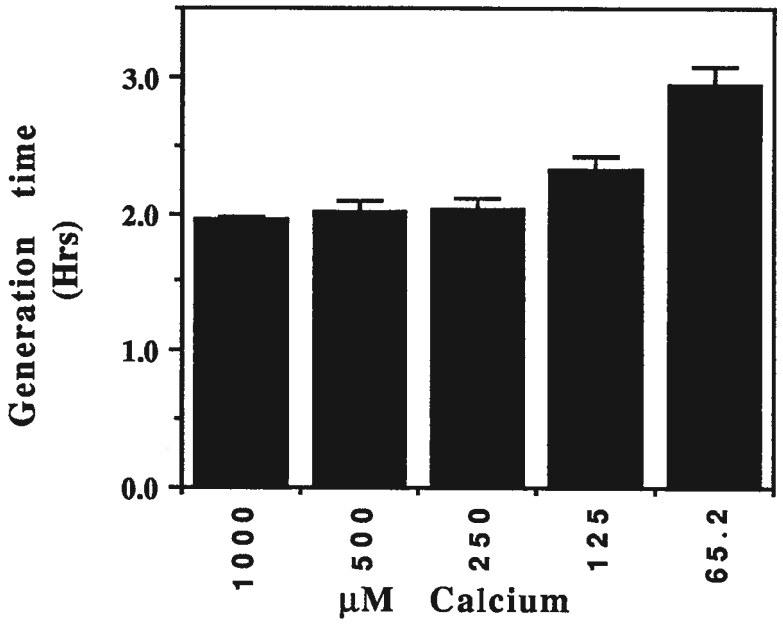


FIG. 22. Influence of calcium on the generation time of *Caulobacter crescentus* NA1000. 5 x 10^6 washed mid-logarithmic cells were inoculated into 5 mls of M_{10}Higg medium containing 65.2 to 1000 μM calcium. Cultures were incubated at 30°C and the optical density at 600 nm was followed during growth. The mean generation time was determined for cultures between OD_{600} = 0.100 to 1.000. Duplicate tubes were used for all concentrations of calcium and the experiment was repeated 3 times. The bar indicates the standard deviation.

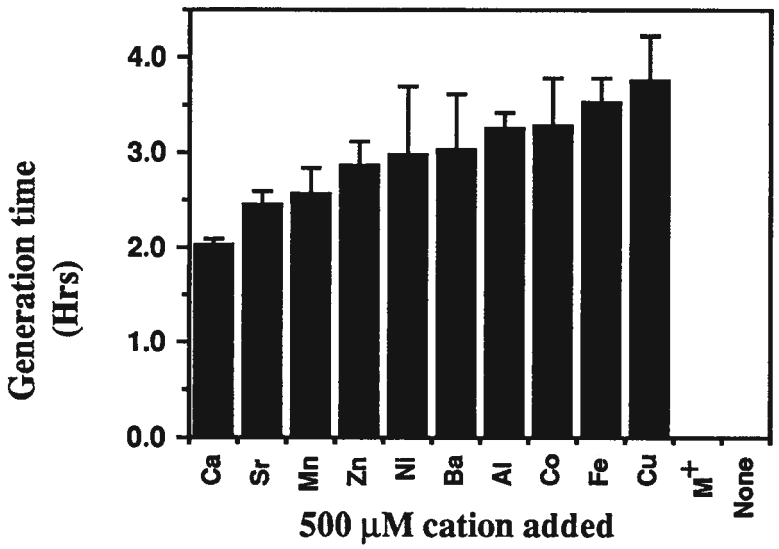


FIG. 23. Influence of the metal ion supplement on the generation time of *Caulobacter crescentus* NA1000 cultured in M_{10}Higg medium. 5 x 10^6 washed mid-logarithmic cells were inoculated into 5 mls of M_{10}Higg medium supplemented to 500 μM with a chloride metal salt. Cultures were incubated at 30°C and the optical density at 600 nm was followed during growth. The mean generation time was determined for cultures between OD_{600} = 0.100 to 1.000. Duplicate tubes were used for all metal salts and the experiment was repeated 3 times. No growth was noted in unsupplemented medium or medium supplemented with a monovalent cation. M^+ = sodium, potassium or lithium. Unsupplemented M_{10}Higg medium contained 2.2 mM magnesium chloride. The bar indicates the standard deviation.

supplemented with the mono-valent cations lithium, sodium or potassium (chloride salts). Supplementation of M_{10}Higg medium with one of 8 divalent or 2 trivalent cations allowed NA1000 to grow, although the resulting generation times were greater than those observed in medium supplemented with calcium. Figure 24 indicates that the growth of JS1001 was not greatly decreased by growth in the presence of any of the cations tested. The cation used to supplement M_{10}Higg medium also had a pronounced influence on the lag time for NA1000 (Fig. 25) that was not noted in JS1001 cultures (Fig. 26). The lag phase was defined as the number of hours for the culture to reach an OD_{600} of 0.100. Increasing the concentration of magnesium in M_{10}Higg medium to 3.0 mM did permit limited growth of NA1000, although culture lysis occurred as the OD_{600} approached 1.5 (data not shown).

3.8.3 Influence of metal ions on S-layer crystallization. TEM was used to determine if crystallized S-layer was formed on the cell surface of NA1000 or in non-cell associated sheets in cultures of JS1001. Assembled S-layer was observed in cultures of NA1000 or JS1001 only when M_{10}Higg medium was supplemented with calcium or strontium. Figure 27 illustrates the non-cell associated sheets of S-layer produced by JS1001 cultured in the presence of strontium. Identical sheets were produced when JS1001 was cultured on M_{10}Higg medium supplemented with calcium. TEM of NA1000 cells demonstrated the presence of crystallized S-layer on the cell surface when grown on M_{10}Higg medium supplemented with calcium or

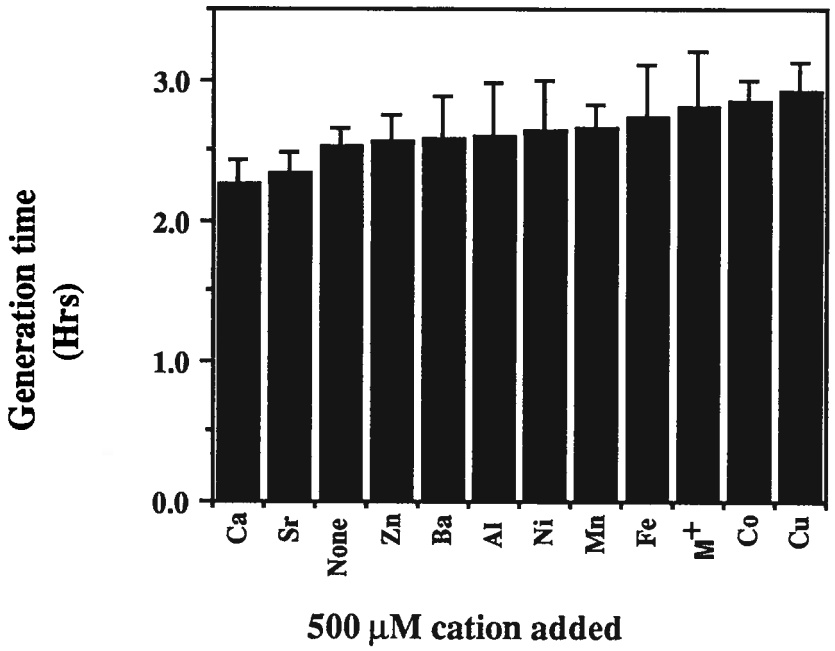


FIG. 24. Influence of the metal ion supplement on the generation time of *Caulobacter crescentus* JS1001 in M_{10}Higg medium. 5 x 10^6 washed mid-logarithmic cells were inoculated into 5 mls of M_{10}Higg medium supplemented to 500 μM with a chloride metal salt. Cultures were incubated at 30°C and the optical density at 600 nm was followed during growth. The mean generation time was determined for cultures between OD_{600} = 0.100 to 1.000. Duplicate tubes were used for all metal salts and the experiment was repeated 3 times. M^+ = sodium, potassium or lithium. Unsupplemented M_{10}Higg medium contained 2.2 mM magnesium chloride. The bar indicates the standard deviation.

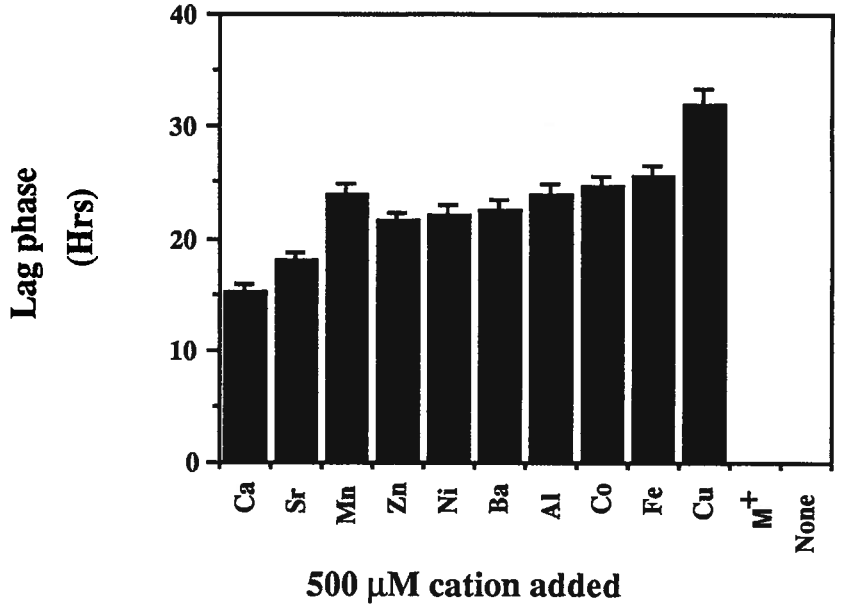


FIG. 25. Influence of the metal ion supplement on the lag phase of *Caulobacter crescentus* NA1000 in M_{10}Higg medium. 5 x 10^6 washed mid-logarithmic cells were inoculated into 5 mls of M_{10}Higg medium supplemented to 500 μM with a chloride metal salt. Cultures were incubated at 30°C and the optical density at 600 nm was followed during growth. Lag phase was defined as the number of hours required for a culture to reach an OD_{600} = 0.100. Duplicate tubes were used for all metal salts and the experiment was repeated 3 times. No growth was noted in unsupplemented medium or medium supplemented with a monovalent cation. M^+ = sodium, potassium or lithium. Unsupplemented M_{10}Higg medium contained 2.2 mM magnesium chloride. The bar indicates the standard deviation.

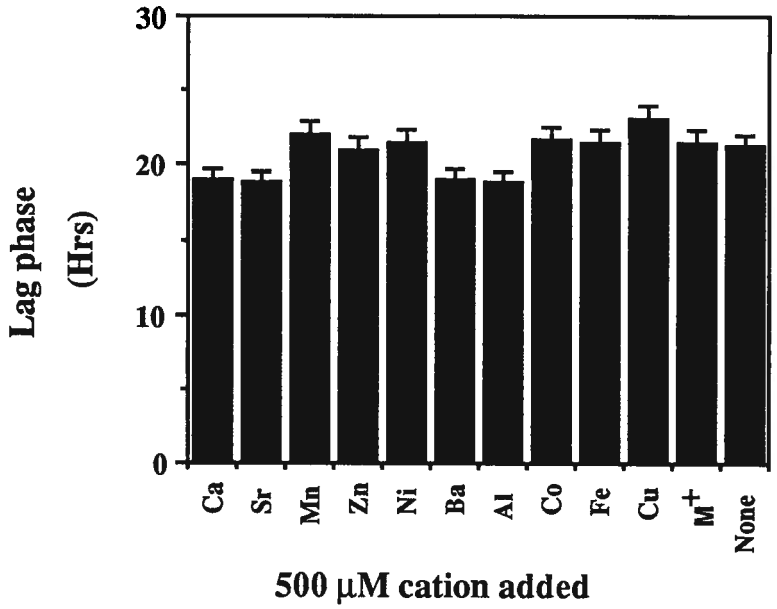


FIG. 26. Influence of the metal ion supplement on the lag phase of *Caulobacter crescentus* JS1001 in M_{10}Higg medium. 5 x 10^6 washed mid-logarithmic cells were inoculated into 5 mls of M_{10}Higg medium supplemented to 500 μM with a chloride metal salt. Cultures were incubated at 30°C and the optical density at 600 nm was followed during growth. Lag phase was defined as the number of hours required for a culture to reach an OD_{600} = 0.100. Duplicate tubes were used for all metal salts and the experiment was repeated 3 times. M^+ = sodium, potassium or lithium. Unsupplemented M_{10}Higg medium contained 2.2 mM magnesium chloride. The bar indicates the standard deviation.

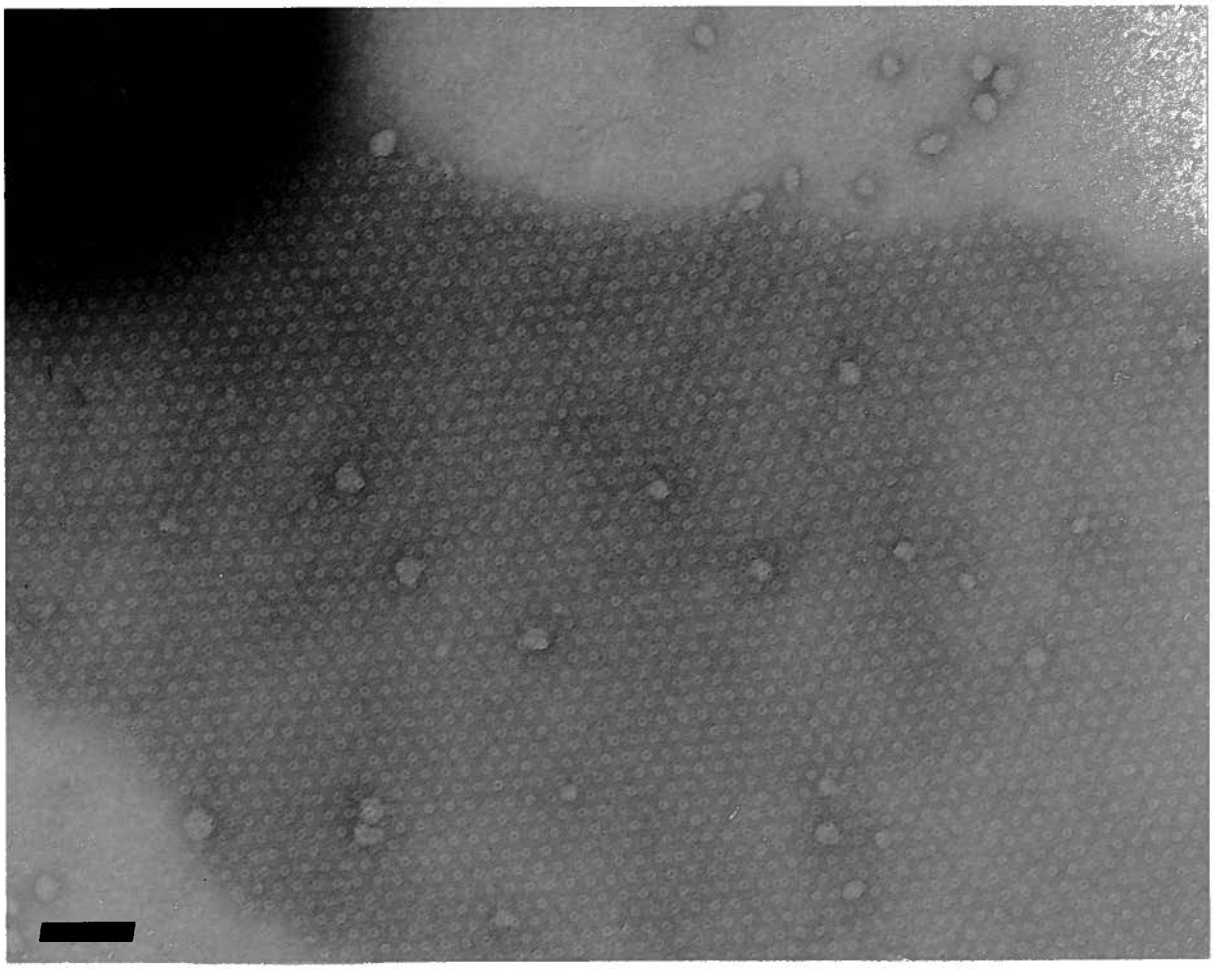


FIG. 27. Negative-stain TEM micrograph of strontium mediated crystallization of RsaA formed in a *Caulobacter crescentus* JS1001 colony. A double S-layer sheet, as determined by optical diffraction, that is not associated with the bacterial cells is formed. This electron micrograph was taken by Dr. J. Smit. Bar = 0.1 μM.

strontium (not shown). TEM of NA1000 cells grown on M_{10}Higg medium supplemented with ions other than strontium or calcium showed that large amounts of material, resembling cell membranes, sloughed off the cells and that no crystallized S-layer could be found.

3.8.4 Influence of calcium or strontium concentration on S-layer crystallization. NA1000 and JS1001 were grown on M_{10}Higg plates that were supplemented with 5, 3, 1 and 0.5 mM calcium or strontium. Colonies were examined by TEM for the presence of crystallized S-layer. Table III demonstrates that S-layer crystallization in the presence of calcium or strontium was concentration dependent. Higher concentrations of strontium or calcium were required for non-cell associated S-layer sheets to form in cultures of JS1001 than were required to allow detection of S-layer assembled on the cell surface of NA1000. It was also noted that higher concentrations of strontium were required for observation of crystallized S-layer in both NA1000 and JS1001 cultures than that required for calcium mediated crystallization of S-layer.

3.8.5 Localization of non-crystallized S-layer protein. Washed NA1000 cells were used to inoculate M_{10}Higg liquid medium supplemented to 500 μM with a cation. After growth to mid-logarithmic phase the cells were pelleted by centrifugation and washed twice with 20 mM HEPES buffer (pH 7.2). Whole cell lysates

TABLE III. The influence of template and cation concentration on the crystallization of RsaA.

Metal ion[a]	Crystallized S-layer[b]	
	NA1000	JS1001
Calcium		
0.5[c]	+[d]	-[e]
1.0	+	+
3.0	+	+
5.0	+	+
Strontium		
0.5	-	-
1.0	+	-
3.0	+	+
5.0	+	+

[a] Chloride salt.

[b] Forming an array on the cell surface of NA1000 and non-cell associated sheets in JS1001.

[c] Concentration in mM.

[d] Crystallized S-layer observed by negative-stain TEM.

[e] Crystallized S-layer not observed by negative-stain TEM.

of the washed cells were analysed by Western blotting using anti-RsaA sera. Figure 28 shows that S-layer protein was detected only in cultures grown in the presence of calcium or strontium.

Washed NA1000 or JS1001 cells were used to inoculate M_{10}Higg plates supplemented to 1 mM with a metal cation Following growth the cells were scraped from the plate and suspended in 10 mM Tris - 1 mM EDTA buffer. The cell lysates were analysed by Western blotting using anti-RsaA sera. This procedure was used to identify any RsaA within the cells, attached to the cell surface or RsaA that was translocated to the cell surface but not attached to the cell. The method used to grow and prepare cells for analysis in Fig. 28 would wash unattached S-layer from the cell surface and thus not detect the protein. S-layer was detected only when unwashed JS1001 or NA1000 cells were cultured in the presence of calcium or strontium (Fig. 29). Unadsorbed sera was used as a control to determine if any of the non-RsaA proteins recognized by the unadsorbed sera (see Fig. 16) were repressed during growth in M_{10}Higg medium supplemented with various cations. Figure 28 and 29 illustrate that only RsaA was inhibited by growth on cations other than calcium and strontium.

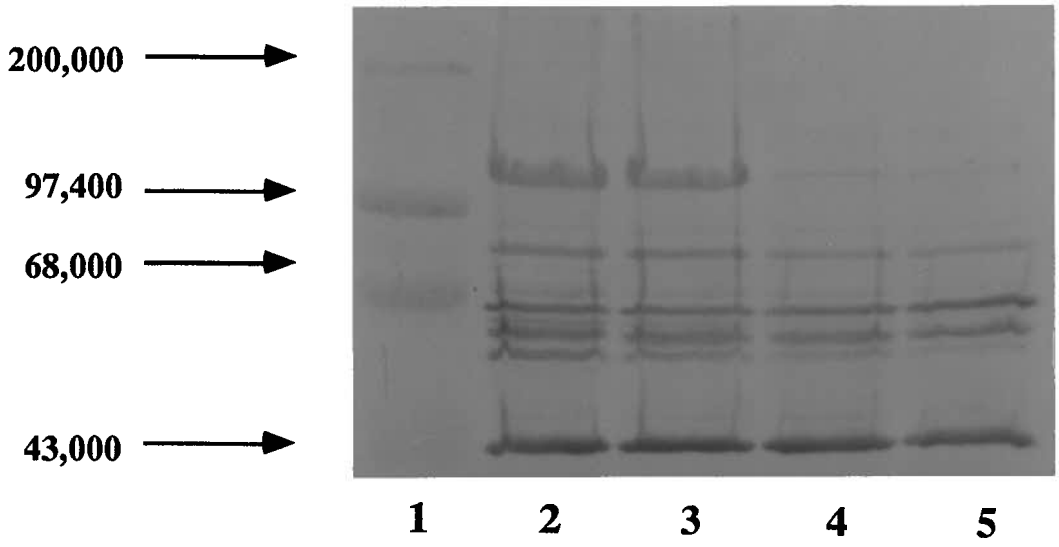


FIG. 28. Western blot reacted with unadsorbed α-RsaA sera of whole cell lysates of washed *Caulobacter crescentus* NA1000 cells grown in liquid M_{10}Higg medium supplemented to 500 μM with a chloride metal salt. Lanes: **1**, Molecular mass markers in daltons; **2**, calcium; **3**, strontium; **4**, manganese; **5**, nickel. Lanes 2 to 5 were loaded with 10 μg of protein as estimated by the method of Markwell et al. (1978). Samples were fractionated by SDS-PAGE using a resolving gel containing 10% acrylamide. Unsupplemented M_{10}Higg medium contained 2.2 mM magnesium chloride.

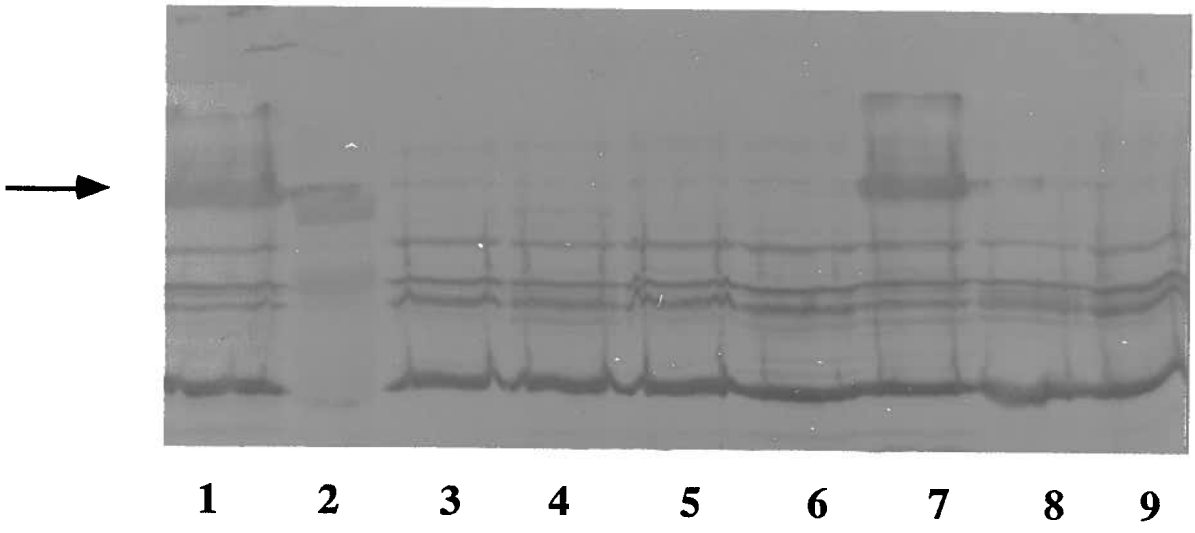


FIG. 29. Western blot of whole cell lysates from unwashed *Caulobacter crescentus* cells. Cells were grown on M_{10}Higg medium plates supplemented to 1 mM with a chloride metal salt. The blot was reacted with unadsorbed α-RsaA sera. Lanes **1**, **3**, **4**, and **5** = NA1000. Lanes **6**, **7**, **8**, and **9** = JS1001. Lanes: **1**, calcium; **2**, molecular mass markers; **3**, nickel; **4**, manganese; **5**, zinc; **6**, no ions; **7**, calcium; **8**, manganese; **9**, zinc. All lanes but 2 were loaded with 10 μg of protein as estimated by the method of Markwell et al. (1978). Samples were fractionated by SDS-PAGE using a resolving gel containing 10% acrylamide. Unsupplemented M_{10}Higg medium contained 2.2 mM magnesium chloride.

3.9 Genetic investigation of the Calcium-independence phenotype

An attempt was made to characterize the calcium-independent, S-layer attachment-defective phenotype at the genetic level. A transposon library was constructed in attempt to isolate the gene responsible for the phenotype. A cosmid library of wild-type *C. crescentus* NA1000 was used in an attempt to complement the calcium-independent phenotype in the mutant strain JS1001.

3.9.1 Production and screening of a transposon library.

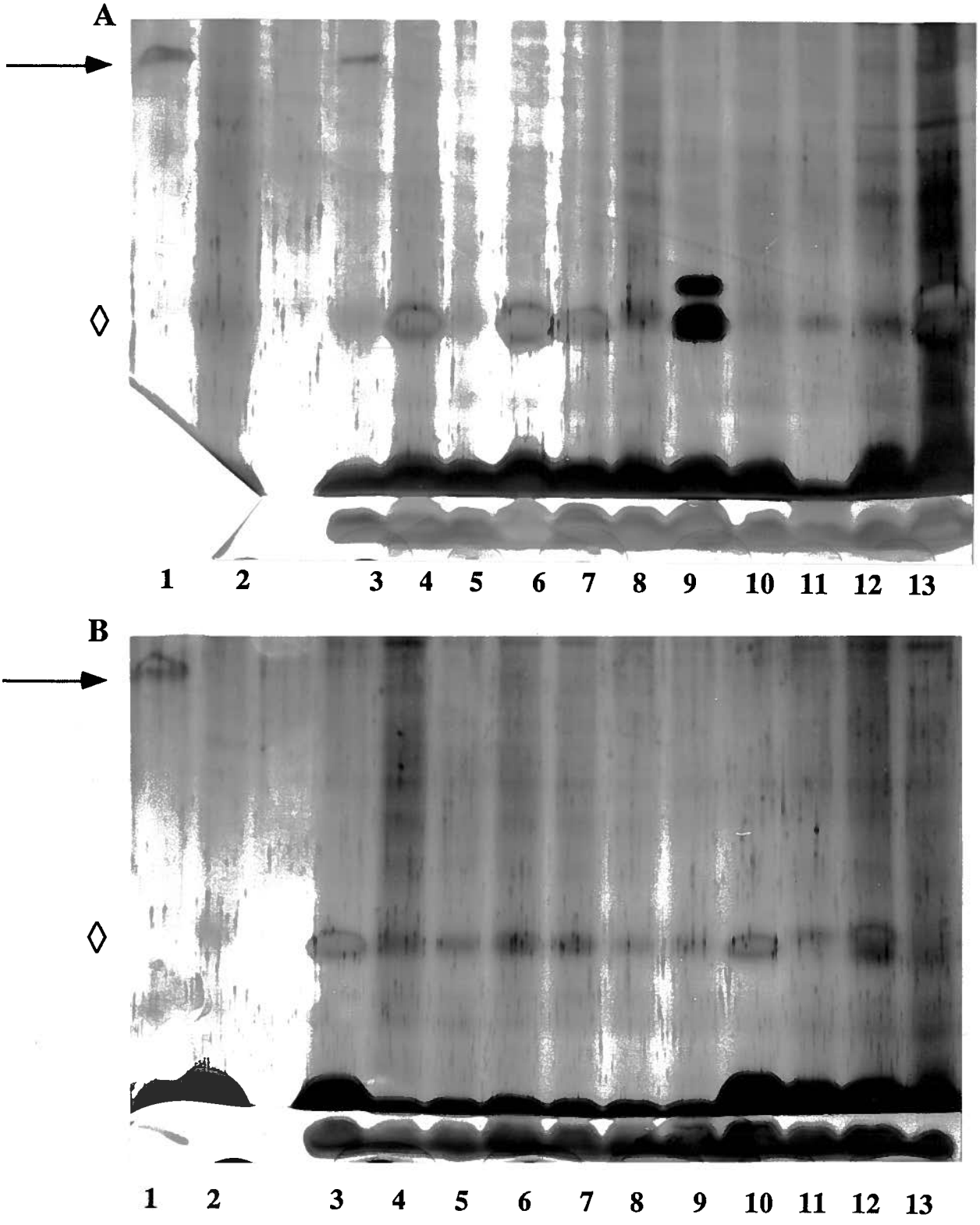
The suicide vector pSUP2021 was used to produce a transposon library of *C. crescentus* NA1000 containing 20,000 independent transposon-insertion mutants. The library was screened on calcium-free M_{10}Higg plates for the identification of calcium-independent mutants. Although the medium supported the growth of JS1001 and inhibited the growth of NA1000, no calcium-independent mutants could be isolated from the transposon library.

S-layer attachment-defective Tn5 mutants were isolated from the library by use of a colony immunoblot screen (Awram and Smit, unpublished; see appendix I [method C]). These attachment-defective mutants were analysed by SDS-PAGE and silver-staining to determine the LPS banding pattern (Fig. 30). Figure 30 demonstrates that these mutants all produce altered LPS banding patterns. However, as detailed below, none of these mutants were calcium independent.

When SAO is stained using the modification of the Bio-Rad™

silver-stain method it is visualized as a golden-yellow band while the rough LPS stains black. The arrow points to the running position of the SAO band in lane 1 of Figure 30A and 30B. The Tn5 mutants could be grouped into 6 clusters on the basis of LPS banding patterns. Cluster 1 consisted of one mutant, F1, that produced a band with the same electrophoretic mobility as SAO although the band was stained black (Fig. 30A; lane 3). The diamond (◊) beside lane 1 of Figure 30A and 30B denotes the running position of LPS species that have less electrophoretic mobility than the rough LPS in the Tn5 mutant strains. Cluster 2 consisted of 5 mutants that produced a band that was golden-yellow in colour like the SAO band although it ran with a much greater electrophoretic mobility (Fig. 30A; lanes 4, 6 and 7 for F2, 4 and 5 respectively and Fig. 30B; lanes 3 and 10 for F12 and 19 respectively). Cluster 3 consisted of 2 mutants that produced a doublet band that were golden-yellow in colour running at the same electrophoretic mobility as the cluster 2 mutants (Fig. 30A; lane 13 for F11 and Fig. 30B; lane 12 for F21). Cluster 4 consisted of one mutant that produced a doublet that stained black (Fig. 30A; lane 9 for F7). Cluster 5 consisted of 7 Tn5 mutants which produced less rough LPS and a small amount of a golden-yellow high molecular weight LPS species (Fig. 30A; lane 11 for F9 and Fig. 30B; lanes 4, 5, 6, 7, 8 and 9 for F13, 14, 15, 16, 17 and 18 respectively). Cluster 6 consisted of 5 mutants that produced a dark band at the running position of the diamond (Fig. 30A; lane 5, 8, 10 and 12 for F3, F6, F8 and F10 respectively and Fig. 30B; lane 11 and 13 for F20

FIG. 30. SDS-PAGE of proteinase K treated NaCl/EDTA extracts of *Caulobacter crescentus* Tn5 strains. The S-layer attachment defective Tn5 mutants are designated "F1 - F22". See appendix I (method C) for details on the method used to isolate the mutants. The gel was stained using the modification of the Bio-Rad™ silver-stain kit. (**A**) Lanes: **1**, NA1000; **2**, JS1001; **3**, F1; **4**, F2; **5**, F3; **6**, F4; **7**, F5; **8**, F6; **9**, F7; **10**, F8; **11**, F9; **12**, F10; **13**, F11. (**B**) Lanes: **1**, NA1000; **2**, JS1001; **3**, F12; **4**, F13; **5**, F14; **6**, F15; **7**, F16; **8**, F17; **9**, F18; **10**, F19; **11**, F20; **12**, F21; **13**, F22. The arrows designate the running position of wild-type SAO while the diamonds designate the running positions of LPS species produced by the Tn5 mutants that run with a greater electrophoretic mobility than the rough LPS. Samples containing 0.5 μg KDO, as estimated by the method of Karknanis et al. (1978), were loaded into each lane and fractionated with a resolving gel containing 13% acrylamide.


A
1 2 3 4 5 6 7 8 9 10 11 12 13
B
1 2 3 4 5 6 7 8 9 10 11 12 13

and F22 respectively). An S-layer attachment-defective Tn5 mutant from each cluster was tested for the ability to grow in the absence of calcium. None of the four mutants examined were capable of growth in M_{10}Higg liquid medium indicating that although they produced altered SAO they were not "calcium-independent" mutants.

3.9.2 Complementation of JS1004 with an NA1000 cosmid library. A cosmid library derived from NA1000 was introduced into the calcium-independent S-layer-negative strain JS1004 by electroporation. One of 680 cosmid containing clones reacted with anti-SAO sera in a dot blot immunological screen. This cosmid was designated "D". A Western blot using anti-SAO sera of JS1001 containing cosmid D indicated that although SAO was produced, it was at less than wild-type levels (Fig. 31). The *Caulobacter* DNA contained in cosmid D was isolated and used as a probe to identify 28 overlapping cosmids. Restriction digests of these cosmids indicated that 18 had unique banding patterns and these 18 were introduced into JS1001 separately by electroporation. The electroporants were screened by Western blot analysis using anti-SAO sera and two were shown to produce SAO, although at levels less than wild-type (Fig. 32). These cosmids were designated D12 and D13. Washed cultures of JS1001 containing cosmid D12 or D13 were analysed by Western blotting using anti-RsaA sera. The Western blots indicated that these cells were unable to attach most of the S-layer protein to the cell surface (not shown).

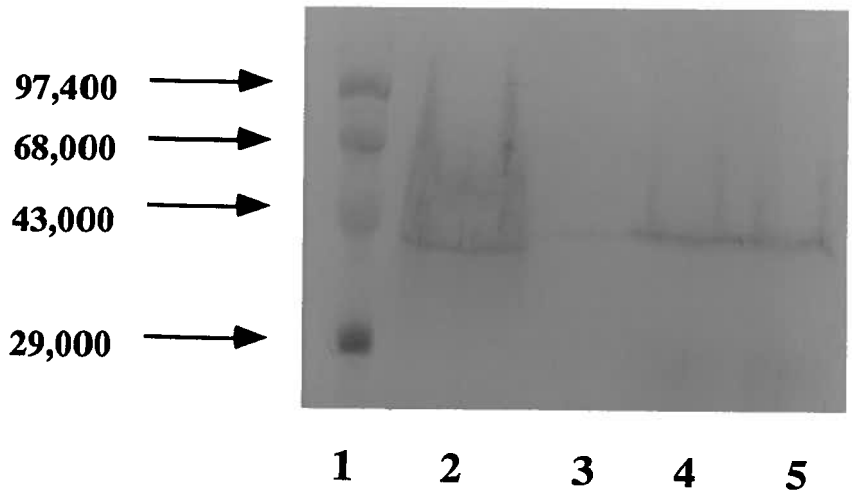


FIG. 31. Western blot reacted with α-SAO sera of proteinase K treated whole cell lysates. Cells were grown in PYE liquid media. Lanes: **1**, molecular mass markers in daltons; **2**, NA1000; **3**, JS1001; **4**, JS1004 containing cosmid D; **5**, JS1001 containing cosmid D. Samples containing 1 μg KDO as estimated by the method of Karknanis et al. (1978) were loaded into each lane and fractionated by SDS-PAGE using a resolving gel containing 13% acrylamide.

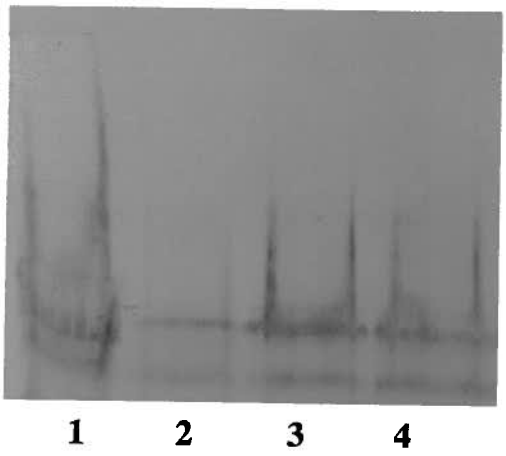


FIG. 32. Western blot reacted with α-SAO sera of proteinase K treated whole cell lysates. Cells were grown in PYE liquid media. Lanes: **1**, NA1000; **2**, JS1001 **3**, JS1001 containing cosmid D12; **4**, JS1001 containing cosmid D13. Samples containing 1 μg KDO as estimated by the method of Karknanis et al. (1978) were loaded into each lane and fractionated by SDS-PAGE using a resolving gel containing 13% acrylamide.

4 Discussion

Figure 33 is a model diagram of the *C. crescentus* cell surface based, in part, on the information contained in this thesis. The figure illustrates the presence of three major polysaccharide species one of which, the SAO or smooth LPS, plays a role in the attachment of the S-layer to the cell. Figure 33 also proposes that calcium is involved in the formation of the S-layer and may also have a role in membrane assembly. The following is a discussion of the experimental evidence which forms the basis of the model.

4.1 *C. crescentus* cell surface polysaccharides

The *C. crescentus* cell surface was examined and found to produce three types of polysaccharides: an EPS, a "rough" LPS, and a "smooth" LPS termed the SAO.

4.1.1 *C. crescentus* "rough" LPS. The presence of LPS in *C. crescentus* strains was initially revealed by SDS-PAGE analysis of washed whole cells treated with proteinase K (Fig. 3 and 4). The high electrophoretic mobility of the band sensitive to the Tsai and Frasch stain and the absence of bands of higher molecular weight indicated that the LPS species was "rough" in nature (Hitchcock et al. 1986). The LPS from all strains yielded similar electrophoretic profiles (Fig. 4).

The LPS of CB2A and NA1000 was isolated and purified using a

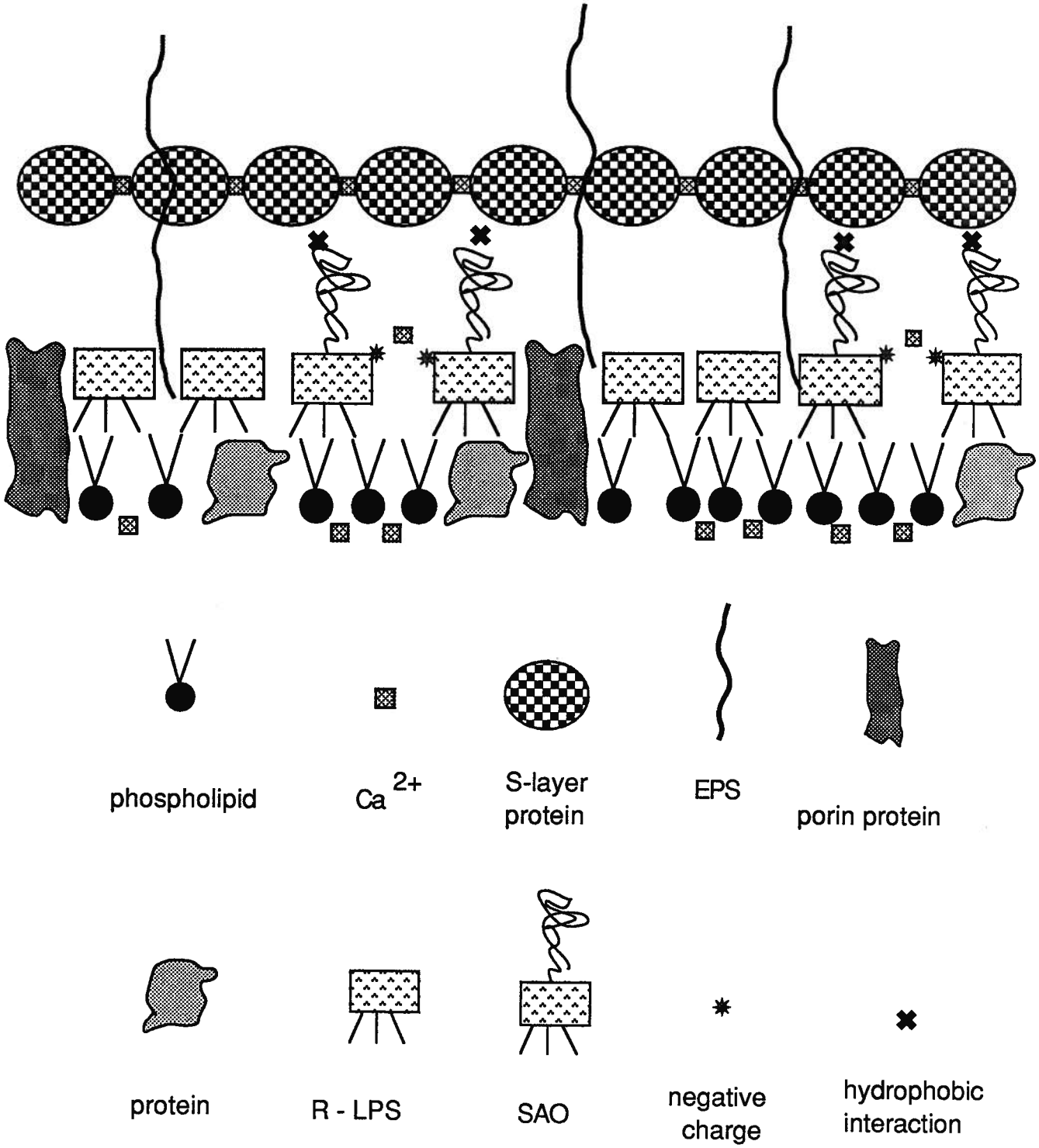


FIG. 33. Representation of the *Caulobacter crescentus* NA1000 cell surface.

modification of the method of Darveau and Hancock (1983). The purified NA1000 and CB2A LPS was analyzed by SDS-PAGE. Half of the gel was stained using the method of Tsai and Frasch (1982) while the other half was stained using the Bio-Rad silver-stain kit (Fig. 3). Both methods produced similar patterns of staining. The bands resulting from samples prepared by the method of Hitchcock and Brown (1983) (Fig. 3, lanes 1 and 2) had a slightly slower electrophoretic mobility and were wider in the horizontal plane than that of the purified LPS. This may result from these samples containing bulk cellular components, such as undigested protein, peptidoglycan and nucleic acids, which alters the mobility of the LPS though the gel. Figure 3 also demonstrates that the purified LPS did not contain contaminants. The Bio-Rad silver-stain method detects protein, carbohydrate, nucleic acid and contaminating metal salts (Bio-Rad 1987) whereas the staining procedure of Tsai and Frasch (1982) is very sensitive for detection of LPS but is insensitive for the detection of protein and nucleic acids (Hitchcock and Brown 1983). The Bio-Rad procedure allowed the visualization of contaminating protein and nucleic acid which were contained in the "crude" LPS preparation.

Detailed chemical analysis of the purified LPS from CB2A, NA1000 and JS1001 was conducted by Dr. N. Ravenscroft (Ravenscroft et al. 1992). The purified LPS was shown to consist of two definable regions: (i) an oligosaccharide region, consisting of an inner core of three residues of 2-keto-3-deoxyoctonate, two residues of α-L-glycero-D-mannoheptose, and one α-D-glycero-D-

mannoheptose and an outer core region containing one residue each of α-D-mannose, α-D-galactose, and α-D-glucose, with the glucose likely phosphorylated and (ii) a region equivalent to the lipid A of archetype LPS, consisting primarily of the esterified fatty acid 3-OH-dodecanoate (see Table I, appendix II).

The lipid A-like region was resistant to conclusive analysis. The major or only fatty acid component, 3-OH-dodecanoic acid, is less commonly found in LPS than 3-OH-tetradecanoic acid, but has been found, for example, in the Lipid A from *Pseudomonas aeruginosa* (Bhat et al. 1990). Mild acid hydrolysis readily cleaved the LPS into Lipid A and core oligosaccharide fractions. Yet despite extensive efforts, no amino or diamino sugars, typical of the "backbone" region of other Lipid A moieties (Mayer et al. 1988; Rietschel et al. 1990) were detected during multiple approaches for amino sugar analysis (Ravenscroft et al. 1992).

There is no clear explanation for the high stability of the Lipid A and thus its resistance to hydrolysis into assayable sugars. It is notable, however, that *Caulobacters* are members of the α-2 subdivision of the alpha proteobacteria, as defined by 16S rRNA sequence analysis (Stackebrandt et al. 1988; Stahl et al. 1992; Woese 1987), a group that contains members producing "unusual" Lipid A structures. The amino sugar 2,3-diamino-2,3-dideoxy-D-glucose (DAG) has been identified in the Lipid A backbone structures of some species in this phylogenetic group (Weckesser and Mayer 1988). It may be that a variation of the DAG-type Lipid A is present in *C*.

crescentus. High-voltage paper electrophoresis has proven to separate and detect such unusual Lipid A sugars in other species (Mayer et al. 1988) and may be appropriate in future studies with the *Caulobacter* Lipid A.

It has been reported that *C. crescentus* whole membranes contain from two-thirds to ten-fold less KDO than that reported for membranes of rough mutants of *Salmonella typhimurium* and wild-type *S. typhimurium*, respectively (Agabian and Unger 1978). This study showed that the total amount of KDO in whole cells of NA1000 and CB2A is less than that found in *E. coli* B, but only to the extent of 20 to 30%. This indicates that KDO could be used as an outer membrane marker during procedures to separate membrane fractions. The published methods for membrane separation and isolation in *C. crescentus* do not account for the missing KDO in the "outer membrane" fractions (Agabian and Unger 1978; Clancy and Newton 1982; Koyasu et al. 1980). Two of the protocols (Clancy and Newton 1982; Koyasu et al. 1980) used PBS in the procedure. It has been since shown that PBS extracts LPS from the envelope of *C. crescentus* (Edwards and Smit 1991; Walker and Smit, unpublished observation) and the absence of KDO in these membrane preparations might be explainable on that basis.

4.1.2 *C. crescentus* SAO. SAO was initially identified as a contaminant in a purified LPS sample (Fig. 6). This proteinase-resistant molecule was detected in whole cell lysates of wild-type S-

layer producing strains or strains that are attachment-competent using a modification of the method of Tsai and Frasch (Fig. 7). A similar band was not detected in calcium-independent strains which are unable to attach the S-layer to the cell surface indicating that it may play a role in S-layer attachment. Originally it was unclear if this band represented a species of LPS with a homogeneous length O-antigen, as seen in *Aeromonas* species (Belland and Trust 1985; Dooley and Trust 1988), or if it was a unique carbohydrate species. The SAO was not detected by the SDS-PAGE and silver-staining methods of Hitchcock and Brown (1983) or Tsai and Frasch (1982) which are used widely to identify and qualitatively characterize LPS from many bacterial species. Also, the SAO did not precipitate with the rough LPS during the cold ethanol-$MgCl_2$ step of the Darveau and Hancock (1983) procedure but remained in the supernatant along with a significant amount of rough LPS. Thus an isolation and purification procedure for SAO was determined in order to chemically characterize the molecule.

The cell surface extraction procedure using 0.77 M NaCl / 0.12 M EDTA (pH 7.2) was effective at solublizing the cell surface components without releasing large amounts of cytoplasmic constituents, although the redistribution of cytoplasmic material is indicative of the plasma membrane losing its selective permeability barrier toward ions (Whitfield and Murray 1956). This extraction method provided a convenient and rapid method to obtain rough LPS, SAO and EPS from liquid cultures ranging in volumes of 1.5 ml

to 60 liters. For detailed chemical analysis, SAO was isolated from JS1003 instead of NA1000 due to the deletion of *rsaA* in this strain which resulted in a lower protein concentration in the NaCl/EDTA extract. The SAO was then separated from the rough LPS by SDS-PAGE and isolated from the polyacrylamide gel by electroelution. Detailed chemical analysis of the purified SAO was preformed by Dr. D. N. Karunaratne (See appendix II, Table III). The SAO was shown to be composed of lipid and polysaccharide. The major fatty acid was identified as 3-OH-dodecanoate which is the same fatty acid as that found in the rough LPS. Minor amounts of the same sugars detected in the rough LPS were identified as well as large amounts of 4,6-dideoxy-4-amino hexose, 3,6-dideoxy-3-amino hexose and glycerol all in equal proportions. Proton NMR studies on the purified SAO have determined that the amino group of both dideoxyamino hexoses are acetylated (W. R. Abraham, unpublished; see Table III, appendix II). Given this data and the fact that colorimetric assays indicated that SAO and rough LPS have the same molar ratio of KDO:phosphate it is clear that SAO is a species of smooth LPS with homogeneous-length O-antigen.

Dideoxyamino sugars are regarded as "rare" and "unusual" although they have been identified as component sugars in a number of bacterial species LPS (for a list of species and references see Ashwell and Hickman 1971; Jann and Jann 1977; Kenne and Lindberg 1983; Lüderitz et al. 1968; and Wilkinson 1977). 4,6-dideoxy-4-amino hexose or 3,6-dideoxy-3-amino hexose containing O-antigens often have altered solubility characteristics than that of

most LPS. When some, but not all, species possessing these sugars are subjected to the hot phenol/water LPS isolation procedure of Westphal et al. (1952) the O-antigen is found in the phenol phase whereas the rough LPS is found in the aqueous phase. The rough and smooth LPS of most bacteria is partitioned into the aqueous phase. It has been suggested that the phenol solubility of the dideoxyamino hexose containing LPS is due to the increased number of non-polar groups (terminal methyl and N-acetyl residues) in these sugars (Hickman and Ashwell 1966). Whatever the chemical basis contributing to the phenol solubility of such an LPS it is clear that these O-antigens possesses a hydrophobic character not found in most LPS species. It is tempting to speculate that differences in hydrophobic character between the *C. crescentus* rough and smooth LPS accounted for their separation during the Darveau and Hancock (1983) procedure.

The inability to stain all components of LPS after SDS-PAGE using the standard methods of Hitchcock and Brown (1983) and Tsai and Frasch (1982) is relatively uncommon but is not unique to *C. crescentus*. *Cytophaga johnsonae* (Godchaux et al. 1990), *Campylobacter jejuni* (Preston and Penner 1987), *C. coli* (Mandatori and Penner 1989), *E. coli* O26 (Karch et al. 1984), *Coxiella burneti* (Hackstadt et al. 1985), and *Neisseria gonorrhoeae* (Mandrell et al. 1986) also have LPS species which do not stain using the standard LPS silver stain and immunological or alternative staining procedures must be used to visualize these molecules. The Bio-Rad™ silver-stain

procedure detected both the rough LPS and the SAO (Fig. 8) although its sensitivity towards the rough LPS was approximately five-fold less than that obtained using the method of Tsai and Frasch (1982). Therefore, different staining methods are required depending what species of *Caulobacter* LPS are being examined.

The mechanism by which macromolecules are stained during various silver staining protocols is unknown (Deh et al. 1985; Goldman and Merril 1982; Kropinski et al. 1986). Silver stains are based on methods using either ammoniacal silver solutions (Oakley et al. 1980) or silver nitrate (Merrill et al. 1981). The method of Tsai and Frasch (1982) uses ammoniacal silver whereas the Bio-Rad silver stain kit uses silver nitrate. A modification of the Bio-Rad silver stain (Cava et al. 1989) which substitutes sodium periodate for dichromate in the oxidation step was shown to stain SAO in polyacrylamide gels (Fig. 30). SAO was not stained when periodic acid was used to oxidize molecules prior to staining with ammoniacal silver in the method of Tsai and Frasch (1982) (Fig. 3 and 4). This indicates that the oxidized SAO does not react with ammoniacal silver. However, more detailed comparisons between the two LPS staining methods will have to be carried out in order to determine the precise reason why SAO does not stain by the method of Tsai and Frasch (1982).

When SAO is detected by the Bio-Rad™ method or the modification of the Bio-Rad™ method it is stained a yellow-orange colour which is a common staining characteristic of O-glycosidically

linked carbohydrate containing molecules (Deh et al. 1985). When photographing such gels with black and white film the resulting image of the SAO is much less intense in comparison to the rough LPS which stains dark black. Therefore, the photographs in Figure 30 are not accurate representations of the original polyacrylamide gel.

Figures 8 and 12 show that the SAO was not resolved into discrete bands as has been shown for other O-antigen of homogeneous length (Chart et al. 1984; Dooley et al. 1985). Although a variety of acrylamide concentrations and a number of gel protocols were used, heterogeneity in this region was not identified. The purified SAO was subjected to laser desorption time of flight mass spectroscopy analysis to identify any microheterogeneity in this region (A. Rüdiger, unpublished data; See Fig.3, appendix II). The analysis indicated that there is microheterogeneity present, but the average difference in mass between SAO molecules is approximately 176 daltons. Therefore SDS-PAGE, under any conditions, would be unable to detect this microheterogeneity.

The cell surface defect responsible for the S-layer attachment-defective phenotype of calcium-independent mutants of *C. crescentus* appears to be the inability to produce an O-antigen of uniform length (SAO molecule). This is reminiscent of the defect thought to be responsible for the attachment-defective phenotype in *Aeromonas salmonicida* and *A. hydrophila* (Belland and Trust 1985; Dooley and Trust 1988). In mutants of both species inability to attach the S-layer to the cell surface has been correlated with

defects in the LPS. With *A. salmonicida* strains the inability to produce a homogeneous-length smooth LPS results in an attachment-defective phenotype (Belland and Trust 1985). 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 of *A. hydrophila* producing a deep-rough LPS were, however, found to be S-layer attachment-defective (Dooley and Trust 1988). Dooley and Trust (1988) have suggested that a homogeneous-length O-antigen may be required by all Gram-negative S-layer producing species. However, the S-layer containing *Campylobacter fetus* has smooth LPS of heterogeneous-length reminiscent of the LPS of enteric bacteria (Perez-Perez et al. 1986) although Yang et al. (1992) have implicated the LPS as the cell surface molecule to which the S-layer attaches. The O-antigen of *A. salmonicida* and *A. hydrophila* extends past the S-layer into the environment (Chart et al. 1984; Dooley et al. 1988). In contrast, the *Caulobacter crescentus* (Smit, unpublished observation) and *Campylobacter fetus* (Fogg et al. 1990; McCoy et al. 1975) O-antigens do not extend past the S-layer. At present only these limited number of Gram-negative S-layer producing species have been examined by SDS-PAGE to determine the LPS profile. Therefore broad generalizations cannot be made, however, it appears that S-layers attach to the cell surface of Gram-negative bacteria via the LPS. Like S-layers themselves, the mechanism of attachment to the cell surface may prove to be a product of convergent evolution.

4.1.3 ***C. crescentus*** **EPS.** CB2A, NA1000 and JS1001 produced sufficient quantities of an EPS during growth in broth culture for it to be isolated in an aqueous phase as a by-product of the general purpose LPS isolation procedure of Darveau and Hancock (1983). The inability to wash the EPS off the surface by repeated centrifugations and suspensions and the lack of significant amounts of polysaccharide located in the culture medium following growth indicated that the polymers were not a loosely associated "slime" layer but a true capsule or EPS layer (Ørskov and Ørskov 1990). Carbohydrate analysis of the purified EPS from NA1000, JS1001 and CB2A was conducted by Dr. N. Ravenscroft (Ravenscroft et al. 1991; see Fig. 2, appendix II). These studies showed that NA1000 and CB15A produce a unique neutral EPS. The EPS of CB2A contained D-glucose, D-gulose and D-fructose in a ratio of 3:1:1 whereas the NA1000 EPS contained D-galactose, D-glucose, D-mannose and D-fucose in approximately equal amounts. NMR and methylation analysis confirmed that the polymers consist of repeating units, NA1000 consisting of a tetrasaccharide and CB2A a pentasaccharide, containing both α- and β-linked sugar residues. The repeating sugar units indicated that the isolated polymers had the general features of a bacterial EPS or capsule. The chemical differences in the EPS of CB2A and NA1000 illustrate that they have evolved independently so as to present different chemical motifs to the external environment. Like other bacterial species *C. crescentus* strains may produce many different EPS chemotypes. The EPS isolated from the

calcium-independent S-layer attachment-defective mutant JS1001 was chemically identical to that found in the parent strain NA1000.

The classification of cell surface polysaccharides as capsules (EPS), slime layers or LPS are based both on chemical analysis of the polysaccharide as well as the biophysical characteristics of the material (Costerton et al. 1981). The firm attachment of the *Caulobacter* EPS to the surface is a property shared with the LPS and it might be argued that the EPS is an LPS with a very long O-antigen. LPS is often differentiated from EPS based on the criterion that the LPS is pelleted by ultracentrifugation at 200,000 xg for 30 h in aqueous solution whereas the EPS remains in the supernatant (Whitfield and Valvano 1993). Based on this sedimentation definition the carbohydrate described above is considered to be an EPS. This term is also appropriate based on the chemical studies of the LPS and SAO that are discussed above. The "rough" LPS and SAO had a completely different chemical composition from the EPS in both CB2A and NA1000 and no KDO, a constituent of all LPS, was detected in the EPS fraction (compare Fig. 2 with Table II and Table III, all in appendix II).

It is still possible however, that the EPS fraction is technically a large species of "smooth" LPS due to the method by which it is attached to the cell surface. The long 4 or 5 sugar repeat structure oligosaccharide might be anchored to the outer membrane by attachment to a single rough LPS molecule (consisting of lipid A and core oligosaccharide moieties). Such an anchoring arrangement has

been suggested for the group I capsular polysaccharide antigens of *Escherichia coli*. (Jann and Jann 1990). Anchoring of the EPS to the surface might also be mediated by other lipids as has been shown for group II capsular polysaccharides of *E. coli* where the EPS is linked to the cell surface by phosphatidic acid (Jann and Jann 1990). At this point, the means of apparent surface adherence for the *Caulobacter* EPS is unresolved because of the difficulty in purifying sufficient quantities of the "anchor" portion of an EPS from the large excess of polymerized repeat unit after the two regions of the molecule are cleaved.

Since the EPS remained on the cell following washing of the cells by centrifugation and resuspension it might be expected that this layer would be visible by thin section TEM methods. However, no indication of an EPS layer on cells prepared for thin section TEM by standard methods have been reported even when dyes commonly used to reveal polysaccharides (eg, ruthenium red) were incorporated into the procedures (Poindexter 1964, Ravenscroft et al 1991; Smit et al. 1981). Graham et al. (1991), as part of a larger study on the use of freeze-substitution methods, did not visualize an EPS layer in strain NA1000. Yet the same cryofixation/freeze substitution technique has been used to successfully preserve and visualize the EPS layer on *Leptothrix discophora* (Beveridge 1988) and *E. coli* K30 (Whitfield et al. 1989). However, the EPS of both *Caulobacter* strains contain only neutral monosaccharides and the cationic dyes and heavy metals used to stain surface polysaccharides

may not react with the neutral polymers and thus they remain. Other methods of capsular stabilization, such as pre-treatment with antibody directed against the EPS or chemical dehydration and Lowicryl embedding (Bayer 1990), may be required to visualize the layer by transmission electron microscopy. Ravenscroft et al. (1991) visualized an EPS-like structure on *C. crescentus* CB2A using a cryofixation/freeze substitution scanning electron microscopy technique. In that procedure the sample is sputter coated with heavy metals following cryofixation/freeze substitution. Thus even neutral molecules, such as the EPS, are rendered electron dense and therefore be visible when examined by electron microscopy.

The composition of the EPS polymers differed sufficiently between CB2A and NA1000. In this context it is of interest to note that CB2A no longer produces an S-layer (Smit et al. 1981) but does produce and correctly assemble the S-layer protein from NA1000 when *rsaA* is introduced into CB2A on a plasmid (Smit et al. unpublished; See Fig. 1; lane 17). Apparently S-layer assembly is not affected by differing EPS molecules. Since the EPS produced by the S-layer attachment mutant JS1001 does not differ from its S-layer attachment competent parent strain, NA1000, it is considered unlikely that EPS plays a role in S-layer attachment to the cell surface.

4.2 RsaA extraction and *in vitro* recrystallization

RsaA could be selectively extracted from whole cells of NA1000

using low pH. Coomassie blue stained gels indicated that the extracts contained almost exclusively RsaA (Fig. 13). However, Western blots of whole cell lysates of NA1000 probed with the unadsorbed anti-RsaA sera indicated that the sample used as an antigen contained contaminating proteins (Fig. 16). The presence of contaminating non-RsaA in the low pH extracts indicated that further purification by gel exclusion chromatography or HPLC would be required if the protein was to be studied by high resolution methods. However, the purification of RsaA by the one step low pH extraction was sufficient for conducting the experiments discussed below.

Low pH extraction has also been used for selective purification of the S-layer of other bacteria including *Spirillum* "Ordal" (Beveridge and Murray 1976a), *S. putridiconchylium* (Beveridge and Murray 1976b,c), *Aeromonas hydrophila* (Dooley and Trust 1988) and *Campylobacter fetus* (McCoy et al. 1975). For the *Aeromonas* and *Campylobacter* species a low pH extraction procedure using a glycine-HCl buffer was effective. In contrast, better results in selectively removing RsaA were obtained using HEPES at low pH, recognizing that it is not a buffer in that range. Perhaps with *C. crescentus*, the protonated amino group of glycine at low pH also disrupts other membrane-associated proteins.

The reassembly studies with the purified NA1000 protein provided definitive data that only RsaA is responsible for the visible repeated structure. Previously, Smit et al. (1981) had reported that S-layer preparations from NA1000 (consisting of shed S-layer fragments isolated by differential centrifugation) contained two

other proteins, the "74K" and "20K", and membrane material. It could not be resolved whether the additional proteins were membrane-derived or were part of the visible S-layer structure. Since the reassembly experiments reported here involved preparations with very little contamination from the 74K and 20K proteins it seems clear that these additional proteins are not part of the S-layer structure. The *in vitro* reassembly experiments also reinforce that calcium is specifically required for S-layer assembly; even the divalent strontium ion, which has a hydrated molecular diameter most similar to calcium and which has substituted for calcium in the *in vitro* reassembly of other S-layers (Beveridge 1976c), was unable to replace calcium. However, as discussed below, strontium is capable of mediating *in vivo* RsaA assembly into an S-layer.

4.3 Distribution of RsaA- and SAO-like molecules in environmental *Caulobacter* isolates

NA1000, and its parent CB15 (Poindexter 1964), have been maintained in pure culture as laboratory strains for almost thirty years. Therefore it is of interest to determine if *Caulobacters* in their natural environment possess equivalent cell surfaces as that found on strain NA1000 (see Fig. 33). It is generally accepted that the cell surface of many "domesticated" bacteria bare little resemblance to that of strains growing in their natural environment (Beveridge and Graham 1991, Costerton et al. 1981; 1987). This

study was undertaken to determine if the cell surface of *Caulobacters* in nature have a similar S-layer and LPS composition as NA1000 and to determine the degree to which the S-layers produced by various environmental strains are conserved.

Table II is a summary of the results of this study. These results indicate there is a similarity between the S-layers of the FWC isolates and that most of the isolates have a cell surface which resembles that of NA1000. The similarity was demonstrated at several levels. The disruption methods used appeared in all cases to specifically disrupt and extract the S-layer and the solubilized protein was also, in all cases but one (FWC23), immunologically cross-reactive with anti-RsaA sera. It is conceivable that as in strain NA1000, calcium (or another divalent cation) is required for S-layer attachment or crystallization in the various freshwater isolates and the two extraction methods used would disrupt calcium-mediated ionic bonding (i.e., EGTA is a calcium-selective chelator and the protons of the low pH treatment would compete with calcium for anionic sites). In addition, oligosaccharide-containing molecules similar to SAO were present in all but one (FWC4) S-layer producing strain and in most cases the oligosaccharide had at least a degree of immunological reactivity with the anti-SAO sera. It can be argued then that there is not only a degree of conservation among *Caulobacter* S-layer proteins but also a conservation of an SAO-like molecule which may participate in surface attachment. Conversely, in the case of the atypical *Caulobacters*, when there is no S-layer,

there seems to be a different surface architecture as well. However, more detailed examination of the S-layer-like proteins and SAO-like polysaccharides would have to be conducted to strengthen these initial findings, which indicate that most *Caulobacters* in the environment have similar cell surface features as NA1000.

FWC23 and FWC4 were exceptional strains in this study in that they could not be grouped with the typical or atypical *Caulobacter* strains. A single prominent S-layer-like protein was extracted from FWC23 and an SAO-like carbohydrate was detected by silver-staining but neither the extracted protein or the carbohydrate reacted in Western blots (See Fig. 17A; lane 28; Fig. 18; lane 28; Fig. 19; lane 16; Fig. 20; lane 48). A regularly structured array was not identified on FWC23 by negative-stain TEM. EGTA extraction of FWC4 yielded a high molecular weight S-layer like band, as well as a large number of lower molecular weight bands, that reacted with the anti-RsaA sera by Western blotting (see Fig. 17B; lane 16 and Fig 18; lane 13). However, FWC4 lacked an SAO-like polysaccharide as determined by silver-staining and Western blotting (see Fig. 19; lane 20 and Fig. 20; lane 27). Negative-stain TEM has also failed to visualize a regular structure on the surface of this strain.

In a study by Stahl et al. (1992) involving 16s rRNA analysis of a number of these strains it was learned that 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 (Stahl et al. 1992). Nevertheless,

Caulobacters in the typical group were still measurably dissimilar. Since the group of S-layer producing *Caulobacters* are 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 might be expected that the S-layer proteins of a collection of *Caulobacter* strains would show significant differences because they are not, for example, pathogenic strains with an S-layer attuned to parasite-host interactions, as with some other S-layer producing species (Dooley and Trust 1988; Dubreuil et al. 1990; Kay et al. 1984; Murray et al. 1988). Thus, there might seem to be little reason for genetic selection to favor a specific S-layer structure, particularly at the level of immunological similarity.

The anti-RsaA sera cross-reaction was specific in the Western blotting experiments of FWC's, 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 protein is 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 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 information. Dubreuil et al. (1990) made a similar prediction of structurally nonessential regions in the S-layer of *Campylobacter fetus* strains.

The immunological findings are also reminiscent of gene hybridization studies which analogously showed that the NA1000 S-layer gene (*rsaA*) could be used to identify most *Caulobacters* isolated from the environment, since most produced S-layers, but only under reduced stringency conditions (MacRae and Smit 1991). It was hypothesized that conserved regions of the S-layer genes may be responsible for the hybridization noted. Therefore, the data presented in this thesis, that of MacRae and Smit (1991), and that of Stahl et al. (1992), indicates that the degree of S-layer structural conservation noted between *Caulobacter* isolates may be a consequence of common mechanisms of self-assembly, surface attachment, and possibly export mechanisms conserved during the evolution of the various *Caulobacter* strains.

Comparative studies between the S-layer proteins of related bacterial strains have been conducted in other species. In *Aeromonas salmonicida* there is a significant degree of structure conservation among the S-layer protein of strains isolated from diverse locals, as judged by N-terminal protein sequencing, Western immunoblot and ELISA analysis of a few strains and immunofluorescence analysis of a larger group using antibody prepared against one of the S-layer proteins (Kay et al 1984). 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 between two *A. hydrophila* strains (Dooley et al. 1988). 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 was occurring (Dubreuil et al. 1990). In *Aquaspirillum serpens*, two strains were examined by peptide mapping and immunological methods; there is apparently a degree of similarity between the S-layer proteins (Koval et al. 1988). A more general study of 39 *Bacillus stearothermophilus* strains, focussing primarily on molecular weight of the S-layer protein and appearance by electron microscopy, indicated remarkable variety not only in the presence or absence of S-layer but also the basic geometry of the paracrystalline structure and the size of the protein involved (Messner et al. 1984). A similar finding was made for several species of *Desulfatomaculum nigrificans* (Sleytr et al. 1986b). Studies with strains of *Bacillus sphaericus* also noted variation in presence or absence, molecular weight and antigenicity of the S-layer proteins (Lewis et al. 1987; Word et al. 1983). A study of *Bacillus brevis* strains, which often produce a double S-layer, showed that the middle wall protein to be immunologically conserved between strains whereas the outer wall protein was not (Gruber et al. 1988). Overall, these studies show that the degree of S-layer conservation within related strains varies from species to species.

4.4 Ionic requirements for *C. crescentus* NA1000 growth and expression / crystallization of RsaA

Wild-type *C. crescentus* NA1000 does not grow in M_{10}Higg

medium and calcium titration experiments indicated that cultures became growth rate limited at concentrations less than 250 mM calcium (Fig. 22). A number of other cations could substitute for calcium and permit NA1000 to grow in the M_{10}Higg minimal medium. All divalent cations, with the exception of magnesium, and trivalent cations tested permitted growth. Growth did not occur in minimal medium supplemented with the mono-valent cations sodium, potassium or lithium. Cell growth in the presence of ions other than calcium occurred with greater mean generation times and lag periods. Furthermore, the growth rates with these other ions varied from experiment to experiment resulting in greater standard deviations in comparison to calcium grown cells. Negative-stain electron microscopy determined that cells from non-calcium supplemented M_{10}Higg medium blebbed large quantities of membranous material with the exception of cells grown in strontium supplemented M_{10}Higg medium. This observation suggests that calcium and strontium act to maintain the integrity of the cell membranes. Metal ions are known to play an important role in maintaining the cell membranes of other bacterial species (Beveridge 1981). These growth studies indicated that ions other than calcium or strontium could permit growth in M_{10}Higg medium although such cells were less healthy.

The growth characteristics of NA1000 in M_{10}Higg medium supplemented with 0.5 mM of one metal ion indicate that calcium and strontium are the preferred ions. The ability of other ions to

substitute for the preferred ions indicates that the membrane is somewhat flexible with respect to the cations used for stabilization if membrane stabilization is the role of these cations in the physiology of NA1000. Magnesium is clearly the poorest substitute for calcium or strontium. The M_{10}Higg medium, containing 2.2 mM magnesium, required an additional 800 mM magnesium to support any growth of NA1000. Cells cultured in medium containing 3 mM magnesium had the longest lag periods and greatest mean generations times. This is in contrast to the metal ion preference shown by *Escherichia coli* and *Pseudomonas aeruginosa* where magnesium is the preferred ion for stabilization of the outer membrane (Coughlin et al. 1983; Ferris and Beveridge 1986; Nicas and Hancock 1983). Nicas and Hancock (1983) determined that growth medium containing 0.5 mM Mg^{2+} produced a wild-type outer membrane whereas medium containing 0.02 mM Mg^{2+} produced altered outer membrane.

The ability of various divalent and trivalent metal ions to substitute for calcium is somewhat surprising. However, once a metal ion is introduced into an aqueous environment such as a minimal medium, it is difficult to predict what chemical form the metal will adopt. For example, metals can be in the free ion form, adopt a form via the interaction with water molecules, or form a complex through the interactions with hydroxyl or carbonate species (Collins and Stotzky 1989). Therefore, it is difficult to predict the size and charge of the species of the metal ion that is active to permit cell growth. In order to unambiguously show that the metal ions permitting growth are acting to stabilize the cell membranes,

quantitative studies of the metal content of the inner and outer membrane need to be conducted. Unfortunately, at present there is no method available to separate the inner and outer membrane of *C. crescentus* and all cell disruption methods used to isolate the cell membrane fraction from the cytoplasm has resulted in the solublization of large amounts of LPS (Walker and Smit, unpublished data). However, quantitation of the cations bound to purified LPS and to whole cells grown in the presence of various metal ions may prove to be informative.

Negative-stain electron microscopy indicated that crystallized S-layer could be found on the cell surface of NA1000 only when grown in M_{10}Higg supplemented with calcium or strontium, but higher concentrations of strontium were required. Higher strontium concentrations were also required for the crystallization of S-layer sheets in cultures of JS1001. The native template for the S-layer, the cell surface of NA1000, could use a lower concentration of either divalent cation to mediate S-layer crystallization than the template provided by apposed S-layer subunits to produce the double sheets in JS1001 cultures. The *in vitro* crystallization experiments, discussed in section 4.2, indicated that under those conditions strontium would not substitute for calcium. The requirement for a suitable template and the effect that the template has on the cation concentration required for crystallization is illustrated by Table III. The data indicates that if very high strontium concentrations had been used *in vitro*, crystallization of RsaA may have occurred. The

effect of template quality on the concentration of cation required to mediate crystallization has been noted in other species. Koval and Murray (1984b) demonstrated that 10 mM calcium was required for S-layer to crystallize on naked envelopes of *Aquaspirillum serpens* whereas 0.5 mM calcium would mediate S-layer crystallization on the denuded cell surface.

Unlike *C. crescentus* NA1000, other Gram-negative S-layer producing species will grow under sever calcium limitation. The growth of *Aquaspirillum serpens* VHA, *Spirillum putridiconchylium*, and *Azotobacter vinelandii* in medium containing no added calcium has been studied. *Aeromonas salmonicida* has been studied in growth medium containing 0.5 μM calcium. *Aquaspirillum serpens* VHA will grow but continuous subculturing in such medium results in eventual cell lysis (Koval and Murray 1984b). *Spirillum putridiconchylium* grows, however, the cells exuded membranous material into the medium (Beveridge and Murray 1976c). For both *Azotobacter vinelandii* (Doran et al. 1987) and *Aeromonas salmonicida* (Garduño et al. 1992b), the cells grow but produce an S-layer with an altered conformation.

The substitution of calcium with a number of cations allowed the growth of *C. crescentus* in M_{10}Higg medium, but these cells did not produce an S-layer. Thus, the cell is somewhat flexible with respect to the ions that will allow growth but has a strict ionic requirement for S-layer production. Only strontium can substitute for calcium to mediate S-layer crystallization. The ability for

strontium to substitute for calcium for *in vivo* and *in vitro* crystallization of S-layer, in other Gram-negative species, has been noted for *Spirillum putridiconchylium* (Beveridge and Murray 1976c), *Azotobacter vinelandii* (Doran et al. 1978), *Aquaspirillum serpens* MW5 (Kist and Murray, 1984), the outer S-layer of *Lampropedia hyalina* (Austin and Murray 1990), and *Spirillum serpens* VHA (Buckmire and Murray 1970). The ability for strontium to substitute for calcium is not limited to the role these ions play in S-layer crystallization but has been noted in a number of physiological processes (Huh et al. 1991). For example, the attachment of *Rhizobium leguminosarum* to the roots of leguminous plants is mediated by a calcium salt bridge between a bacterial derived calcium binding protein, rhicadhesin, and the plant surface, and strontium can replace calcium in this interaction (Smit et al. 1991). Calcium and strontium are very similar with respect to many physicochemical properties, such as charge character and ionic radii, and this similarity is is often considered the reason that the two ions can substitute for one another in a number of biological processes (Fenton 1987; Huh et al. 1991; Martell 1961). However, there are only limited examples of strontium participating in the physiology of cells in their natural environments (Schultze-Lam and Beveridge 1994).

It was of interest to determine if the S-layer protein remained cell associated during growth on ions other than calcium or if it was released into the growth medium. During growth without calcium

Aquaspirillum serpens VHA secretes S-layer into the medium while the S-layer of *Azotobacter vinelandii* and *Aeromonas salmonicida* remains attached to the cell surface (Doran et al. 1987; Garduño et al. 1992b; Koval and Murray 1984b). The experiments using liquid cultures indicated that unless cells were grown in the presence of calcium or strontium, the S-layer was not cell associated, thus indicating that it was secreted into the medium (Fig. 28). However, when NA1000 cells were scraped off plates and examined for S-layer protein, it was detected only if the cells were cultured in the presence of calcium or strontium. The same observation was made for cultures of JS1001 even though the strain grows very well, although with a slightly greater generation time, in unsupplemented M_{10}Higg (Fig. 29). At present the location of the S-layer protein is unknown, but three possibilities exist. 1. The S-layer may be degraded by a protease unless it is folded into a crystallized array. There are no reports in the literature to indicate that *C. crescentus* produces a secreted protease, although it is known that unfolded or improperly folded proteins are often more susceptible to proteases. 2. The S-layer may also be folded or aggregated in a form that will not enter a gel during SDS-PAGE. It is known that RsaA, if boiled in the presence of SDS, will not enter a gel (Smit et al. 1981) and that the macroscopic precipitate, formed in calcium-containing liquid cultures of JS1001, composed of RsaA will not enter a gel unless first extracted into 8 M urea. However, during growth in calcium/strontium minus liquid medium no macroscopic precipitate is formed in cultures of JS1001. 3. In the absence of calcium or

strontium the S-layer may be blocked at the level of transcription, translation or secretion. It is known that calcium and magnesium act to inhibit transcription of one of the major S-layer gene promoters of Bacillus brevis 47 (Adachi et al. 1991). *C. crescentus* also contains a gene, flbF, which is very similar to a gene that is conserved in *Yersinia* species, *lcrD*, that has been implicated in calcium signal transduction (Plano et al. 1991; Ramakrishnan et al. 1991; Sanders et al. 1992). So there is some indication that *C. crescentus* may be able to sense environmental calcium levels. Clearly, further experimentation into the fate of the S-layer protein during growth on ions other than strontium or calcium is required.

4.5 Investigations of the genetic basis of the calcium-independent / S-layer attachment-defective phenotype

The inability to isolate calcium-independent mutants from the transposon library indicates that the locus defining the calcium-independent / S-layer attachment-defective phenotype is not a target for Tn5 integration or consists of more than one chromosomal site. If the latter explanation is true then the calcium-independent S-layer attachment-defective strains did not arise by a single step mutational event. The method by which the calcium-independent mutants were selected involved an "enrichment" step in liquid medium before plating and so it is possible that double mutants may have been selected (see appendix I; method A).

The Tn5 library was screened by Mr. P. Awram using a colony

immunoblot procedure and S-layer attachment-defective mutants were identified (see appendix I; method C). The cell surface of the mutants were extracted with NaCl/EDTA and the LPS was analyzed by SDS-PAGE and silver staining (Fig. 30). All of the Tn5 S-layer attachment-defective mutants were found to have altered LPS banding patterns. However, none of the mutants tested were capable of growth in unsupplemented M_{10}Higg medium. This indicates that a mutational event resulting in the attachment-defective phenotype does not also result in a calcium-independent phenotype. Calculation of the reversion rates from the spontaneous calcium-independent / attachment-defective phenotype to the wild-type phenotype would determine if the calcium-independent mutants resulted from a single or double mutational event.

A cosmid library of NA1000 was electroporated into the S-layer negative and calcium-independent strain JS1004 instead of the parent strain, JS1001, to allow better access of the anti-SAO sera to the cell surface during an immunoblot screen designed to detect renewed production of SAO. Smit et al. (unpublished) has shown that the S-layer blocks access of antibody to the SAO. Two cosmids, D12 and D13, were isolated that allowed production of SAO in JS1001 although at less than wild-type levels. However, JS1001 containing cosmid D12 or D13 were still unable to anchor the S-layer to the cell surface. Perhaps subcloning the *Caulobacter* DNA to another plasmid vector or deleting extraneous DNA will result in increased SAO production and S-layer attachment. It has been indicated in

Aeromonas salmonicida and *Acinetobacter* 199A that transport of S-layer and LPS are coupled (Belland and Trust 1985; Thorne et al. 1976). If this is the case in *C. crescentus* complementation of the O-antigen in trans may not result in a functional attachment between the S-layer and the SAO. However, more studies with cosmid D12 and D13 must be undertaken before any conclusions can be made.

4.6 Conclusions

4.6.1 The relationship between calcium-independence and loss of SAO. Many of the experiments presented in this thesis were designed to characterize the cell surface of the wild-type and the calcium-independent mutants of *C. crescentus* with the intention of discovering the defect in the mutants which rendered them S-layer attachment-defective. Figure 33 is a model of the wild-type cell surface based, in part, on the information obtained in this study. The wild-type cell produces three classes of polysaccharide containing molecules: the "rough" LPS, the SAO and an EPS. The SAO molecule was found to be absent in all of the calcium-independent mutants examined whereas the rough LPS and the EPS were unaltered in the mutants. Thus for *C. crescentus* growth without calcium apparently required a mutational event that consistently resulted in the loss of the SAO molecule. The attendant phenotype was that production of RsaA continued and the protein could crystallize into an S-layer, if calcium or strontium was available, but the S-layer did not attach to the cell surface. Since no other

alterations of the surface were detected and it was demonstrated in these mutants that the attachment-defective phenotype could not be ascribed to a change in RsaA it was concluded that SAO was a necessary surface component for the attachment of the S-layer.

On noting the absence of SAO in all calcium-independent mutants, it was hypothesized that SAO had a net negative charge. If this was the case, then the simplest explanation for the necessity to delete SAO in order for the cell to be viable in the absence of calcium was that calcium binds to and neutralizes the charge on the SAO molecule. Without calcium, it was envisioned that charge repulsion between adjacent SAO molecules would destabilize the outer membrane and inhibit cell growth. However, chemical analysis of purified SAO suggested that the O-antigen is composed of three neutral molecules. Therefore another hypothesis must be generated to account for the relationship between a mutation that allows grow in the absence of calcium and the SAO-negative phenotype.

The growth studies of NA1000 and JS1001 in M_{10}Higg medium clearly demonstrates that wild-type *C. crescentus* requires the presence of calcium or strontium ions to grow normally and produce an S-layer, whereas calcium-independent mutants no longer have this ionic growth requirement. Examination of the calcium-independent mutant cell surface revealed that SAO was not present and this was suggested as the structural basis for the S-layer attachment-defective phenotype. The Tn5 S-layer attachment-defective mutants were all found to have an altered smooth LPS, thus strengthening the argument that the S-layer interacts with the

wild-type smooth LPS to remain attached to the cell surface. However, unlike JS1001 the Tn5 mutants did not share the additional phenotype of being calcium-independent. The Tn5 attachment-defective mutants could not be isolated by plating the library on calcium-free medium and once isolated, by the immunoblot screen, they were unable to grow in calcium-free liquid medium. Taken as a whole, these results can best be explained by entertaining the hypothesis that the spontaneous calcium-independent mutants JS1001 and JS1002 did not arise from a single point mutational event. The inability to fully complement the calcium-independent mutant JS1004 with a cosmid also supports the notion of more than one mutational event rather than a single point mutation with pleiotropic effects.

If the spontaneous calcium-independent strains are double mutants and the loss of SAO is not responsible for the calcium-independent phenotype some other alteration in the cell must have occurred. It is possible that an alteration in the phospholipid composition of these mutants has taken place. *C. crescentus* has an unusual phospholipid composition when compared to that of other eubacteria in that it produces no phosphatidylethanolamine (De Siervo and Homola 1980; Contreras et al. 1978; Jones and Smith 1979). Approximately 85% of the phospholipids of *C. crescentus* consist of the acidic species phosphatidylglycerol and cardiolipin. Johnson and Ely (1977) noted that the addition of calcium to PYE medium increased both the growth rate and yield of *C. crescentus*.

Contreras et al. (1978) suggested that this was a consequence of calcium binding to and neutralizing the high negative charge in the membranes produced by the acidic phospholipids. It is known that divalent cations, and calcium in particular, interact with and influence the structure of acidic phospholipids (Cullis et al. 1983).

4.6.2 The role of calcium or strontium in the crystallization of the S-layer. Although a number of metal ions were able to replace calcium to allow growth of NA1000 only strontium or calcium could mediate S-layer crystallization. The location in the S-layer protein where calcium or strontium acting to mediate crystallization is unknown. Figure 1 in appendix II illustrates the possible locations where metal ion / protein interaction may occur. Three potential calcium binding sites on RsaA can be suggested: 1. Within the S-layer monomer thus altering its conformation to a form that will crystallize. 2. Between the large domains of the S-layer monomer allowing crystallization of the unit cell. 3. Between the small domains of the S-layer monomer allowing crystallization of the unit cells. Site directed mutagenesis of the predicted calcium binding motifs, identified by the *rsaA* sequence (Gilchrist et al. 1992), of RsaA may help determine the actual site(s) where calcium acts to allow crystallization.

4.6.3 The role of the SAO in S-layer attachment. Loss or alteration of the SAO has been shown to result in the S-layer

attachment-defective phenotype. Chemical studies of the purified SAO indicate that the O-antigen region if formed from sugars that may impart a hydrophobic character on the molecule. It is tempting to envision that the S-layer attaches to the cell surface by the interaction of hydrophobic regions on the S-layer protein and the SAO. If this is the case, the hydrophobic regions of the protein which interact with SAO could also interact between S-layer subunits to form the double S-layer sheets observed in mutant *C. crescentus* strains that do not produce SAO. Smit et al. (1992) demonstrated that the two S-layers forming the non-cell associated sheets, produced by calcium-independent mutants, interact via the surfaces of the S-layer that in the wild-type situation was proximal to the cell surface. Definitive proof that the SAO molecule is responsible for S-layer attachment may be obtained by studies of the S-layer attachment-defective mutants produced by Tn5 mutagenesis.

4.7 Summary

The information contained in this thesis has enhanced our understanding of the physiology and cell surface architecture of *C. crescentus*. Methods were devised to identify, isolate and purify three major cell surface molecules: the S-layer protein, LPS, and EPS of this organism. *In vivo* and *In vitro* studies have illustrated the importance of calcium ions for both the growth of *C. crescentus* and for the production and crystallization of RsaA.

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6 Appendix I

Appendix I outlines unpublished experimental methods conducted by other researchers that are pertinent to this thesis.

6.1 Isolation of Calcium-independent mutants.

The following method was used for the isolation of calcium-independent mutants (Smit, unpublished). *C. crescentus* NA1000 was grown in PYE medium and then subcultured to M_{10}Higg liquid medium. Four to six days of incubation were 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 two more days. Cells were then plated at appropriate dilutions onto M_{10}Higg plate medium. Colonies that grew were examined for their S-layer characteristics and their ability to grow in the absence or presence of normal concentrations of calcium.

6.2 Production of anti-SAO sera.

Antisera to the SAO was fortuitously raised during attempts to prepare antibody to the adhesive holdfast of strain CB2A (Merker and Smit, unpublished). Colloidal gold particles (which bind to the holdfast [Merker and Smit, 1988]) 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% CsCl, w/v). The colloidal gold particles (and associated material) sedimented to the bottom of the gradient; these were collected and used for rabbit immunization in a similar fashion to the RsaA immunization. Analysis of cells incubated with the sera by indirect immunofluorescence microscopy showed that the sera had little activity to the holdfast material. However, the cell surface of S-layer minus but S-layer attachment competent strains CB2A and JS1001 were completely labeled in immunofluorescence and immunoelectron microscopy experiments using this sera. When S-layer producing strains were examined by the same procedure no cell surface labeling was noted (Smit, unpublished).

6.3 Colony immunoblot for identifying S-layer attachment-defective and S-layer negative mutants.

Mr. Peter Awram developed a colony immunoblot screen using anti-RsaA sera that could differentiate between S-layer attachment-defective, S-layer negative and wild-type NA1000 strains (Awram and Smit, unpublished). While screening the NA1000 transposon library for mutants that no longer produced RsaA on the cell surface 22 S-layer attachment-defective Tn5 mutants were also isolated.

7 Appendix II

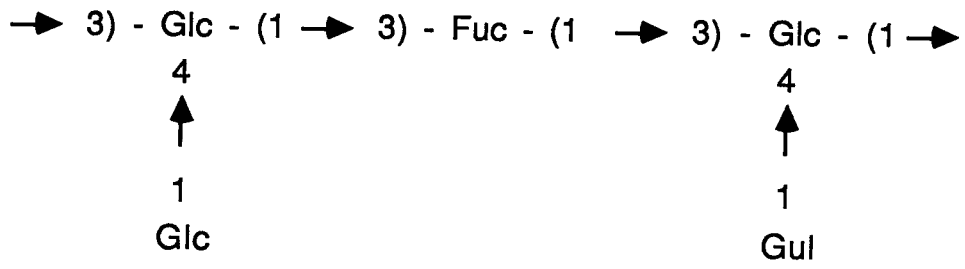
Appendix II lists the results of experiments conducted by other researchers which are pertinent to this thesis. The source of previously published data is listed in the tables or the figure legends. For unpublished data the researcher who provided the data is acknowledged.

FIG. 1. Three dimensional reconstruction of the S-layer of *Caulobacter crescentus* NA1000. **A.** S-layer monomer. **B.** The unit cell formed by crystallization of 6 S-layer monomers. **C.** The S-layer formed by crystallization of unit cells. Thin arrows in A, B and C represent possible sites of calcium interaction with the protein. This figure was adapted from Smit et al. (1992).

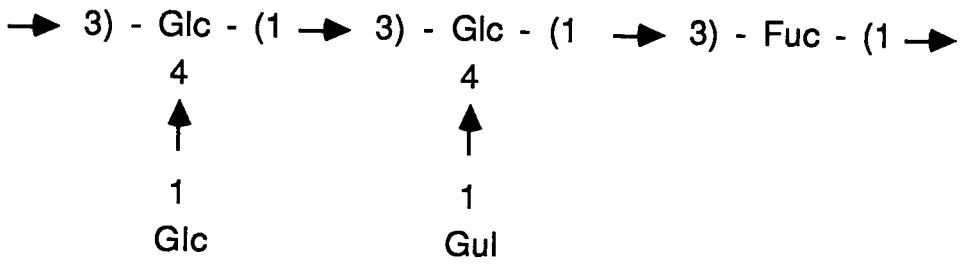
A
B
C

FIG. 2. Proposed structures for the *Caulobacter crescentus* exopolysaccharides. (A) The two possible structures for the CB2A EPS repeating unit. (B) The structure of the NA1000 EPS repeating unit. Glc = D-glucose, Fuc = D-fucose, Gul = D-gulose, Gal = D-galactose and Man = D-mannose. Data from Ravenscroft et al. 1991.

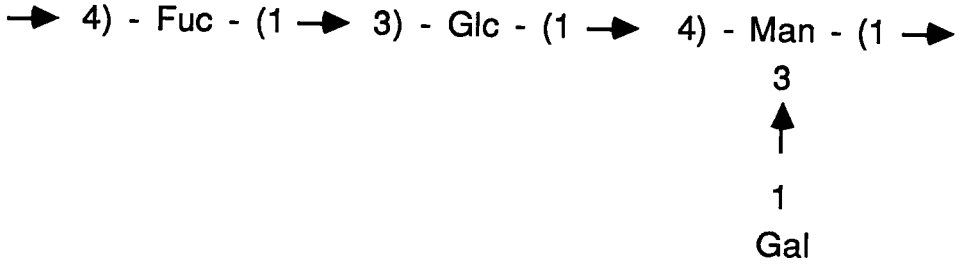
A.


→ 3) - Glc - (1 → 3) - Fuc - (1 → 3) - Glc - (1 →
4
1
Glc
4
1
Gul

or


→ 3) - Glc - (1 → 3) - Glc - (1 → 3) - Fuc - (1 →
4
1
Glc
4
1
Gul

B.


→ 4) - Fuc - (1 → 3) - Glc - (1 → 4) - Man - (1 →
3
1
Gal

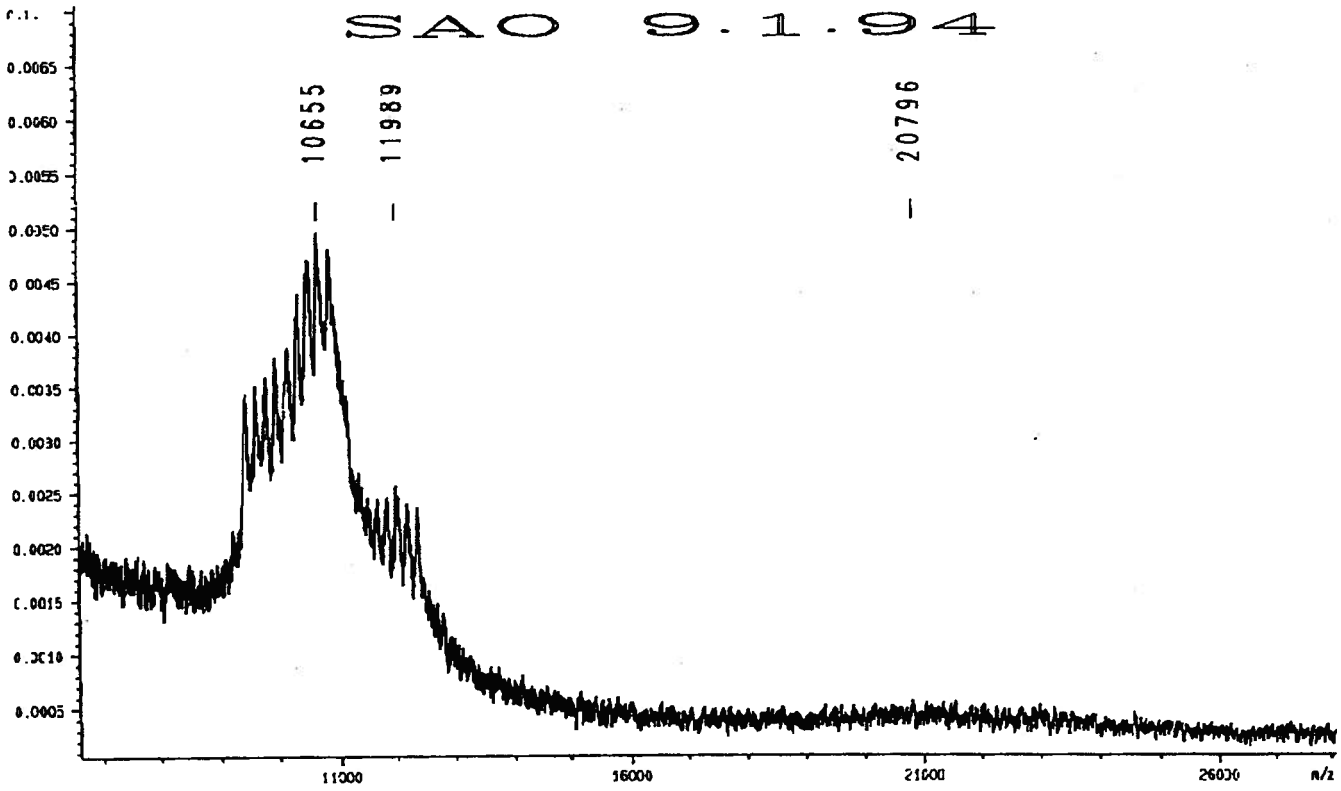


FIG. 3. Analysis of SAO by Laser desorption time of flight mass spectroscopy. The peaks are an average of 176 daltons apart. Unpublished data from A. Rüdiger (GBF-Gesellschaft für Biotechnologische Forschung).

TABLE I. Lipid analysis of *Caulobacter crescentus* rough LPS[a]

Assignment	%
3-OH-C12:0	82
2-OH-C16:1	9
C16:0	5
C18:1	4

[a] Data from Ravenscroft et al. 1992

Table II. Sugar composition of *Caulobacter crescentus* rough LPS[a]

Sugar	Residue per molecule
2-keto-3-deoxyoctonate	3
α-L-glycero-D-mannoheptose	2
α-D-glycero-D-mannoheptose	1
α-D-mannose	1
α-D-galactose	1
α-D-glucose[b]	1

[a] Data from Ravenscroft et al. 1992.

[b] phosphorylated

Table III. Sugar composition of SAO[a]

Sugar	%
Glycerol	13.6
4,6-dideoxy-4-amino hexose[b]	12.0
3,6-dideoxy-3-amino hexose[b]	15.0
Mannose	1.7
Glucose	0.2
Galactose	0.2
D-Glycero-D-manno-heptose	0.2
L-Glycero-D-manno-heptose	0.5

[a] D. N. Karunaratne, unpublished (University of British Columbia).

[b] The amino is acetylated (W. -R. Abraham, unpublished [GBF-Gesellschaft für Biotechnologische Forschung]).