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BIOCHEMISTRY AND ASSEMBLY OF THE REGULAR SURFACE ARRAY
OF AQUASPIRILLUM SERPENS MW5

by

Marion Leah Kist

Department of Microbiology and Immunology

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario

London, Ontario

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ABSTRACT

Regularly-structured (RS) arrays of protein or glycoprotein subunits have been detected on the surface of a wide variety of eubacteria and archaebacteria. These paracrystalline arrays represent the outermost layers of the bacterial cell envelope and form a highly-organized barrier at the external interface with the environment. They have provided excellent models for the study of macromolecular arrangement and assembly.

The regularly-structured surface array of the Gram-negative bacterium, Aquaspirillum serbens MW5, was examined in this study. The surface array of this organism consists of two layers of hexagonally-arranged subunits which, together, produce a complex and linear surface pattern. The components of the structure were isolated and identified as proteins and the biochemical and in vitro assembly properties of these proteins were examined.

The array was removed from cell envelopes and dissociated by treatment with 6 M urea, 1.5 M guanidine hydrochloride, or 20 mM lithium 3,5-diiodosalicylate. The surface components reassembled (provided that Ca^{2+} or Sr^{2+} was present) onto the outer membrane surface from which they were removed and onto the outer membranes of a mutant of strain MW5 which had lost its surface array. The

components also reassembled onto the outer membranes of other strains of *A. serpens* (VHL and 12638, the type strain) but not onto the outer membranes of *Escherichia coli* or *Pseudomonas aeruginosa*. The isolated surface components self-assembled in vitro (again, requiring Ca^{2+} or Sr^{2+}) into planar sheets which possessed the linear pattern characteristic of the intact cell surface. Image analysis of these sheets showed that they were highly similar to the in vivo structure.

The two layers were removed sequentially from cell envelopes by a two step extraction procedure involving initial treatment with pH 10.3 buffer to remove the outermost surface layer and subsequent treatment with 6 M urea to remove the innermost layer. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the outer and inner layers of the array were composed of two proteins with molecular weights of 125,000 (125K) and 150,000 (150K), respectively.

The two layers assembled sequentially; the 150K protein formed an array, either alone by self-assembly, or on an outer membrane surface, and the 125K protein required that array as a template for its in vitro assembly.

Negative staining-electron microscopy of the 150K protein extract showed that it contained circular, doughnut-shaped units (11.5-12.0 nm in diameter) which were

similar in size and morphology to the units which comprise the inner, hexagonally-arranged surface layer. The isolated units were dissociated by heating in 1% SDS at 100C. SDS-PAGE of the intact units showed them to have an apparent molecular weight of 420,000. This suggested that each unit was a trimer, consisting of three 150K monomers. The 150K protein, therefore, can be isolated both as an oligomer and as an intact unit. In contrast, the 125K protein was always isolated in its monomeric form. The assembly of these layers, therefore, involves two steps: The assembly of monomers to form intact units (oligomers) and the assembly of the units into a hexagonal array. The latter was identified as the calcium-dependent step. The conditions required for the in vitro assembly of monomers to oligomers were not determined.

The amino acid compositions of the two surface array proteins showed them to be similar to each other and to that of the RS protein from a related strain, A. serpens VHA. They also demonstrated a number of properties in common with other bacterial RS proteins including a high proportion of acidic and hydrophobic residues. The acidic nature of the proteins was confirmed by their low isoelectric point (4.7). Carbohydrate was not detected.

The interaction of the RS proteins with calcium was investigated. Calcium was required for the in vitro

assembly of the proteins and for the retention of the array on the cell surface during growth. The 150K and 125K proteins behaved as calcium-binding proteins in a spectrophotometric assay for calcium using the chromogenic agent, cresolphthalein complexone. The electrophoretic mobility of the two proteins in SDS-polyacrylamide gels increased in the presence of calcium.

Degradation of the two proteins was observed during their isolation from cell envelopes. This degradation interfered with the attempted purification of the proteins by gel chromatography and protein precipitation methods. The proteolytic degradation of the 125K protein was minimized by including certain anti-proteolytic agents (dithiothreitol, L-cysteine hydrochloride, N-tosyl-L-lysine chloromethyl ketone [TLCK], N-tosylphenylalanine chloromethyl ketone [TPCK], p-chloromercuriphenylsulfonic acid [PCMBBS], or aprotinin) during the isolation procedure. Degradation of both RS proteins increased in the presence of EGTA but could be partially inhibited by TPCK.

The relatedness of the two RS proteins of strain MW5 was examined as well as their relatedness to the RS protein of a closely-related strain, *A. serpens* VHA. Antigenic cross-reactivity between the surface array proteins was detected using the Western blotting method. IgG specific

for the 150K RS protein of strain MW5, however, did not bind to the 125K protein and reacted only slightly with the 140K VHA RS protein. Crude antisera specific for the 150K protein cross-reacted slightly, however, with the 125K RS protein. Peptide mapping of the three proteins showed that, while all shared a certain core of sequences, differences were also apparent. The 150K and 125K proteins of strain MW5 were more similar to each other than were either to the RS protein of strain VHA. The sequence differences detected support the observed biochemical and assembly differences between the RS proteins of strain MW5.

The regularly-structured surface array of A. serpens MW5 provides an excellent model for the assembly of a complex macromolecular structure. The biochemical and in vitro assembly properties of the components of this array, examined in this study, provides a basis for the study of the in vivo assembly of the structure.

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"I'll get by with a little help from my friends..."

Lennon and McCartney.

This thesis is dedicated to my mother
and to the memory of my late father.

"And what is as important as knowledge?" asked the mind.

"Caring and seeing with the heart," answered the soul.

Anonymous

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LIST OF ABBREVIATIONS

RS	regularly-structured
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
EDTA	ethylenediaminetetra-acetate
EGTA	ethyleneglycol-bis-(β -aminoethyl ether)N,N'-tetraacetate
LIS	lithium 3,5-diiodosalicylate
BSA	bovine serum albumin
Con A	concanavalin A
CC	o-cresolphthalein complexone
IgG	immunoglobulin G
DEAE-	diethylaminoethyl-
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
TLCK	N-tosyl-L-lysine chloromethyl ketone
TPCK	N-tosylphenylalanine chloromethyl ketone
PCMBs	p-chloromercuriphenylsulfonic acid

CHAPTER 1

INTRODUCTION

In 1953, Houwink described a "macromolecular monolayer" on the surface of shadowed cells of a Spirillum species. The monolayer had a definite periodicity with its units arranged in a hexagonal pattern over the cell surface. Salton and Williams (1954) then detected a similar hexagonal pattern on the surface of Rhodospirillum rubrum while Labaw and Mosley (1954) described a tetragonal arrangement of units on the surface of an unidentified Gram-positive bacterium. In the thirty-three years which have passed since these initial reports, regularly-structured (RS) layers of protein have been detected on the surfaces of members of over fifty-five bacterial genera including representatives of every morphology, physiology and habitat (reviewed in Sleytr and Messner, 1983).

RS layers (or arrays) are thermodynamic assemblies of identical protein or glycoprotein subunits which interact non-covalently with one another and with underlying cell wall layers. They are self-assembly systems in which all of the information required for their polymerization into regular arrays lies within each subunit through their inherent bonding capacities. This, combined with their paracrystalline regularity of fine structure, makes them

excellent models for the study of macromolecular assembly and arrangement. Within a single genus (or even species) great differences in the morphology and biochemistry of the arrays can be demonstrated (Buckmire, 1970; Hollaus and Sleytr, 1972; Lapchine, 1979; Messner et al., 1984; Word et al., 1983). The RS layers of several members of the genus Aquaspirillum have been examined and provide examples of protein arrays of varying complexities (Murray, 1965; Buckmire, 1970; Buckmire and Murray, 1970, 1973, 1976; Beveridge and Murray, 1974, 1975, 1976a,b,c; Koval and Murray, 1981, 1983, 1984, 1985). Recently, the structure of the double-layered surface array of A. serpens MW5 has been examined (Stewart and Murray, 1982) and has provided an opportunity to study the assembly and biochemistry of a complex surface structure.

This introduction will be pursued in four sections. The first reviews the architecture and biochemistry of the bacterial cell envelope with emphasis on the Gram-negative cell envelope and the biochemistry of the Aquaspirillum serpens cell wall. The structures that are discussed in this thesis are intimately associated with the cell wall. Consequently, much of the work involved in isolating RS layer proteins and in determining the nature of their assembly and association with components of underlying wall layers has required an understanding of the structure and properties of the latter. There is extensive literature on this subject and it is necessary to review current

understanding to put this study into perspective. The second section of this introduction describes the properties of surface arrays in general while the third outlines current knowledge of the RS layer of Aquaspirillum serpens MW5. Finally, the goals of this study are outlined.

1.1. Architecture of the Bacterial Cell Envelope

The division of bacteria into two major groups on the basis of how they stain with the Gram reaction (Gram, 1884) has remained the primary step in the taxonomic classification of bacteria. Early electron microscopic studies demonstrated clear structural differences between Gram-positive and Gram-negative bacteria.

Thin sections of fixed and embedded Gram-positive eubacteria revealed cell wall profiles of relatively simple organization. The cell wall of Bacillus cereus appeared to consist only of a thick, densely-staining, mucopeptide layer (Chapman and Hillier, 1953). A distinct plasma membrane could not be detected in these early electron micrographs (Chapman and Hillier, 1953). The introduction of improved methods for the fixation, staining, and embedding of specimens led, however, to the clear demonstration of a 7.5-8.0 nm double-track cytoplasmic membrane in a variety of bacteria (Murray, 1957; Kellenberger and Ryter, 1958; Van Iterson, 1961).

Even the earliest of studies demonstrated the cell walls of Gram-negative bacteria to be multi-layered

complexes in contrast to the relative simplicity of structure in Gram-positive bacteria. Initial examination of thin sections of embedded Escherichia coli cells revealed a 7.0-8.0 nm double-track plasma membrane and a triple-layered wall (Kellenberger and Ryter, 1958). One of the components of this triple-layered wall was a second double-track membrane which was termed the outer membrane since it comprised the outermost layer of the complex envelope (Birch-Andersen et al., 1953). With advances in fixation (Kellenberger and Ryter, 1958) and staining techniques (Zobel and Beer, 1965), came the demonstration of a 2.0-3.0 nm murein, or peptidoglycan, layer aligned between the two membranes (De Petris, 1965, 1967; Murray et al., 1965); an organization which has been termed "trilaminar". Similar cell envelope profiles have since been demonstrated in a number of Gram-negative bacteria (Glauert and Thornley, 1969).

Many bacteria have been shown to possess cell envelopes of even greater complexity with additional layers found external to the peptidoglycan layer of Gram-positive and the outer membrane of Gram-negative eubacteria. Today it is known that an enormous variety of bacteria carry capsules, slime layers, and regularly-structured arrays of protein on their outermost surfaces (Beveridge, 1981).

The architecture of the bacterial cell envelope and the spatial organization of its components has been revealed largely through: (i) developments in electron microscopic

techniques (ie. improvements in specimen preparation and staining); (ii) the introduction of isolation methods for the various layers and their individual components; and (iii) the development of various electron microscopic-aided localization techniques.

All of these methods have contributed to our present view of the architecture of the cell envelope of Gram-positive and Gram-negative eubacteria shown in Figures 1A and B. The diagrams are not, however, representative for all bacteria. Notable exceptions are found amongst the oldest prokaryotic lineages, the archaeobacteria, which possess cell boundaries of very simple organization. The cell envelopes of some of these organisms (eg. Sulfolobus acidocaldarius, Halobacterium cutirubrum) consist only of a cytoplasmic membrane and, lying external to this, a regularly-structured array of glycoprotein subunits (Stoeckenius and Rowen, 1967; Weiss, 1974) (Figure 1C).

1.2. Components of the Bacterial Cell Envelope

1.2.1 Plasma Membrane

The plasma membranes of Gram-positive and Gram-negative bacteria are similar in structure and composition except for the presence of lipoteichoic acid in the membranes of Gram-positive bacteria (Ward, 1981). The membrane is 7.0-8.0 nm thick and appears, in stained thin sections, as a double-track (ie. electron-dense-light-dense) (De Petris, 1965, 1967), which surrounds the cytosol. The plasma

membrane is multifunctional (Salton, 1971, 1974). It possesses enzymes and other components involved in respiration and energy production, oxidative phosphorylation, the biosynthesis of wall materials, and the active transport of metabolites. It also possesses components involved in motility, (flagellar apparatus), chemotaxis, photosynthesis, and nitrogen fixation.

Chemical analyses of plasma membrane isolated from both Gram-positive and Gram-negative bacteria show them to consist of lipids (mainly phospholipid) and protein. The most common phospholipids are phosphatidylglycerol, diphosphatidylglycerol (cardiolipin) and phosphatidylethanolamine (Lechevalier, 1977). These phospholipids, as well as lyso-phosphatidylethanolamine were found to be present in cell envelopes of Aquaspirillum serpens VHA (Chester and Murray, 1975). The fatty acids of bacterial lipids are generally of the saturated, monounsaturated, or cyclopropane variety (Kaneda, 1977). The types and proportions of these vary with the conditions used for the growth of the organism (Abel et al., 1963; Marr and Ingraham, 1962).

SDS-PAGE of isolated plasma membranes indicates a wide variety of proteins present in minor quantities. Approximately 30-40 polypeptide bands are usually resolved in unidirectional SDS-polyacrylamide gels (Rogers et al., 1980). The number and variety of proteins detected reflect the multifunctional nature of the membrane (Salton, 1974).

1.2.2 Periplasmic Space

The periplasmic space is defined as the region between the plasma and outer membranes of the Gram-negative cell envelope. The periplasmic space may not be continuous since localized adhesion sites between the plasma and outer membrane have been reported (Bayer, 1968) which may represent sites of export of newly-synthesized wall and capsular material (Bayer, 1979). The peptidoglycan layer of Gram-negative bacteria is found within the periplasmic space as are a number of peripherally-located proteins of the plasma membrane and a wealth of free proteins. Proteins which have been identified in this region include solute binding-proteins (Kellermann and Szmelcman, 1980; Lo, 1979) and a number of hydrolytic enzymes (Wetzel et al., 1970; Curtis et al., 1972; Anathaswany, 1977). The periplasmic space, then, forms a microenvironment in the Gram-negative cell. The region between the plasma membrane and peptidoglycan layer of Gram-positive bacteria can also be thought of as a periplasmic space but it is generally regarded as less active since these bacteria tend to secrete more enzymes (Glenn, 1976).

1.2.3 Peptidoglycan

The peptidoglycan layer of bacterial cell envelopes is responsible for maintaining the shape and rigidity of the cell (Weidel and Pelzer, 1964). It is lysozyme-sensitive; treatment with this enzyme causes rod-shaped cells to round.

up and form spherical bodies (Weibull, 1953). The peptidoglycan, or murein, layer resides within the periplasmic space in Gram-negative bacteria and appears, in stained thin sections of some organisms, as a 2-3 nm electron-dense line. In Gram-positive bacteria the peptidoglycan layer is much thicker (15-50 nm) and contains a number of polymers including neutral polysaccharides, teichoic and teichuronic acids, and covalently-associated proteins (Rogers et al., 1980).

Peptidoglycan is a heteropolymer consisting of glycan chains which are interlinked into a meshwork surrounding the cell by cross-links formed between repeating peptide subunits. The glycan chains are the structural backbones of the polymer and consist of alternating B-1,4 linked N-acetylglucosamine and N-acetylmuramic acid residues (Jeanloz et al., 1963; Ghuysen, 1968). The tetra or pentapeptides which cross-link these chains are covalently-linked to the carboxyl group of the N-acetylmuramic acid (Schleifer and Kandler, 1972). The nature and extent of cross-linking varies with different bacteria (Schleifer and Kandler, 1972). Interchain bonds may be formed by direct linkage between the pentapeptide substituents or through short peptide bridges between them.

The peptidoglycan of Aquaspirillum serpens has been examined and found to closely resemble that of most Gram-negative bacteria in terms of its amino acid and amino sugar composition (Kolenbrander and Ensign, 1968; Martin et

al., 1972). One difference detected, however, was that the organism's peptidoglycan appeared to have a higher percentage of cross-linking (54% according to Kolenbrander and Ensign, 1968) than most other Gram-negative bacteria such as E. coli (30%, Takebe, 1965).

1.2.4 Outer Membrane

The outer membrane appears, in stained thin sections of whole cells, as a 7-8 nm bilayer external to the peptidoglycan layer of Gram-negative bacteria. Analyses of isolated plasma and outer membrane have shown that the two are structurally and functionally different. Outer membrane is composed of 20-25% phospholipid and 45-50% protein (Di Rienzo et al., 1978). However, the outer membrane also contains an unusual component, lipopolysaccharide (LPS), which is considered essential to the integrity of the membrane (Osborn, 1968; Costerton et al., 1974). The phospholipid composition of the outer membrane closely resembles that of the plasma membrane except that the former contains a much greater proportion of phosphatidylethanolamine than does the latter (Rottem et al., 1975). The phospholipid of the outer membrane is found primarily within the inner leaflet of the bilayer; the lipid A component of LPS constitutes a major proportion of the lipid of the outer leaflet (Smit et al., 1975; Funahara and Nikaido, 1980).

All Gram-negative eubacteria examined to date contain

lipopolysaccharide (Osborn, 1968; Costerton et al., 1974). LPS is an amphipathic molecule consisting of two main components: lipid A (which is embedded in the outer leaflet of the outer membrane) and a polysaccharide chain extending outwards from the surface of the cell (Luderitz et al., 1971). The polysaccharide chain itself consists of two parts: an oligosaccharide core (usually containing heptoses and ketodeoxyoctanate) directly linked to the N-acetylglucosamine of lipid A and a repeating polysaccharide side chain. The monosaccharide composition and linkages of these side chains are specific for certain bacterial strains and represent the 'O' antigen of Gram-negative bacteria (Ashwell and Hickman, 1971). The O-side chains of LPS often act as phage receptors and provide a basis for the serological typing of Gram-negative organisms (Luderitz et al., 1971).

The LPS of Aquaspirillum serpens VHA has been examined and found to contain L-glycerol-D-mannoheptose, rhamnose, glucosamine, ethanolamine, and phosphate in common with many of the lipopolysaccharides isolated from the Enterobacteriaceae although 2-keto-3-deoxyoctonate could not be detected (Chester and Murray, 1975). The LPS of strain VHA has recently been analyzed by SDS-PAGE and has been shown to be smooth with limited heterogeneity of the O-side chains (D. Wiseman and S.F. Koval, personal communication).

The protein composition of the outer membrane of Gram-negative bacteria is quite distinct from that of the

plasma membrane. It should be emphasized that the general features of outer membrane proteins which are discussed here refer, primarily, to the enteric bacteria (notably E. coli) and do not necessarily apply to all Gram-negative bacteria.

SDS-PAGE of isolated outer membrane reveals 10-20 minor proteins and 3-5 major proteins (Inouye, 1979). The major proteins consist of a peripherally-located polypeptide (Tol G or Omp A) and two types of integral polypeptides (matrix and lipoprotein).

Omp A is a surface-located major outer membrane protein as is suggested by its susceptibility to tryptic cleavage (Inouye and Yee, 1972) and by its ability to act as a receptor for bacteriophage (Datta et al., 1977; Manning et al., 1976). It is thought to function in F-pilus-mediated conjugation (Manning et al., 1976; Manning and Reeves, 1976).

The outer membrane also contains 2-3 matrix, or peptidoglycan-associated, proteins. Their molecular weights vary from between approximately 30,000-40,000 depending on the organism studied and SDS-PAGE system used (Osborn and Wu, 1980). These proteins are strongly associated with the peptidoglycan component of the envelope via ionic linkages. Cell envelopes must be heated in 2% sodium dodecyl sulphate at temperatures exceeding 60C in order to solubilize the proteins (Rosenbusch, 1974). In some organisms (eg. Salmonella, E. coli) the matrix proteins are seen as hexagonally-packed particles on the peptidoglycan following

solubilization of the outer membrane by detergent treatment (Steven, et al., 1977). Most data suggest that each particle probably represents a trimer which forms a hollow channel (Steven et al., 1977; Palva and Randall, 1978; Tokunaga et al., 1979). These hydrophilic transmembrane channels function in the passive diffusion of nutrients and waste products across the outer membrane and, for this reason, have also been termed "porin" proteins (Nakae, 1976a,b; Luktenhaus, 1977; Nakae and Ishii, 1978).

The lipoprotein (molecular weight, 7000) was first described in E. coli by Braun and Rehn (1969). One third of the lipoprotein found in the outer membrane is covalently linked to meso-diaminopimelic acid residues of the peptidoglycan (Braun and Sieglin, 1970; Braun and Wolff, 1970). Both bound and free forms of lipoprotein are strongly associated with the matrix protein trimer and contribute to the integrity of the outer membrane (Di Rienzo et al., 1978).

The outer membrane of Gram-negative bacteria also contains approximately 10-20 minor proteins (Inouye, 1979). These proteins function in the transport of essential nutrients, vitamins and metals but may also act as receptors for phage and bacteriocins (Di Rienzo et al., 1978).

Few enzymes have been identified in the outer membrane. MacGregor et al., (1979) have identified a protease in the outer membrane of E. coli and phospholipase activity has

also been reported (Bell et al., 1971).

1.3. Surface Arrays

1.3.1 Ultrastructure

The contribution of electron microscopy to our current knowledge of bacterial cell wall structure cannot be overemphasized. Without the resolving power of the electron microscope we would have no idea that structures such as RS layers even existed. The initial report of a bacterial surface array was made in 1953 when Houwink described a macromolecular monolayer on the surface of shadowed cells of a Spirillum species (Houwink, 1953). The monolayer had a definite periodicity with units arranged in a hexagonal pattern over the cell surface. Salton and Williams (1954) then detected a similar hexagonal pattern on the surface of Rhodospirillum rubrum while Labaw and Mosley (1954) described a tetragonal arrangement of units on the surface of an unidentified Gram-positive bacterium. Metal shadowing, with or without prior freeze-etching, was also used to demonstrate periodic arrays for several Halobacterium species (Houwink, 1956; Mohr and Larsen, 1953; Kushner and Bayley, 1963). In 1963, Murray demonstrated a hexagonally-packed array on the surface of negatively stained cells and cell wall preparations of Aquaspirillum serpens VHA. Regularly-structured (RS) layers of subunits were subsequently detected on the surfaces of various Bacillus species (Nermut and Murray, 1967; Ellar and

Lundgren, 1967; Holt and Leadbetter, 1969), Clostridium species (Takagi et al., 1965; Hollaus and Sleytr, 1972), Acinetobacter sp. (Thornley and Glauert, 1968), Ectothiorhodospira mobilis (Remsen et al., 1968), Lactobacillus species (Barker and Thorne, 1970; Kawata et al., 1974), Chromatium (Remsen et al., 1970), Flexibacter polymorphus (Ridgway and Lewin, 1973), Aquaspirillum sp. (Beveridge and Murray, 1974, 1975, 1976a), Campylobacter fetus (Winter et al., 1978), and Pseudomonas sp. (Lapchine, 1979). In the thirty-three years which have passed since Houwink's initial discovery, patterned surface layers have been reported for members of over fifty-five bacterial genera (Sleytr and Messner, 1983). In recent years, the ultrastructure of the RS layers of Aeromonas salmonicida (Kay et al., 1981), Caulobacter crescentus (Smit et al., 1981), Bacillus brevis 47 (Tsuboi et al., 1982), Aquaspirillum serpens MW5 (Stewart and Murray, 1982), Azotobacter vinelandii (Bingle et al., 1984), Clostridium difficile (Kawata et al., 1984), and Bacteroides sp. (Sjögren et al., 1985) have been described.

Most of the arrays consist of a single layer of subunits. The first evidence for multiple-layered arrays was provided by Chapman et al. (1963). They examined the cell envelope of Lamproedia hyalina and detected a surface array which consisted of two patterned layers. The inner (perforate) layer was a honeycomb network of hexagonally-arranged units with a center-to-center spacing

of 14.5 nm. The outer (punctate) layer was composed of outward-projecting spines, also hexagonally-arranged, but with a center-to-center spacing of 26 nm. Watson and Remsen (1970) showed that the individual layers of a multi-layered array are not necessarily of the same lattice type. They examined the two-layered surface array of Nitrocystis oceanus and found a tetragonal arrangement of subunits within the innermost layer and a hexagonal arrangement of subunits within the outermost layer. Beveridge and Murray (1974, 1975, 1976a) examined the RS layers of several Aquaspirillum species and described multi-layered arrays of varying complexities. More recently, the surface arrays of Aquaspirillum serpens MW5 (Stewart and Murray, 1982) and Bacillus brevis 47 (Tsuboi et al., 1982) have been shown to be double-layered structures.

It is apparent that the ultrastructure of bacterial surface arrays is best studied using a combination of electron microscopic methods since any one technique may prove ineffective. Several workers, for example, have reported difficulty in obtaining useful structural information from negatively stained preparations of whole cells or cell envelopes, possibly due to masking effects by surface polysaccharides (Bingle et al., 1984; Messner et al., 1984). As well, RS layers are not always easy to demonstrate by freeze-etch analysis. Smit et al (1981) found only weak indications of a regularly-structured array on the surface of Caulobacter crescentus using this technique

although the array was easily detected by negative staining. Similarly, RS layers are not always evident in thin sections of embedded cells (Thornley and Glauert, 1968).

The arrays studied to date consist of hexagonal, tetragonal, or linear arrangements of protein subunits on the cell surface. The pattern of the array, as well as the size, morphology, and center-to-center spacing of these units, vary greatly between even closely-related strains within a species such as Aquaspirillum serpens (Buckmire, 1970) and Bacillus stearothermophilus (Messner et al., 1984). The array covers the entire bacterial cell and appears uniform in lattice over the entire surface. Faults in the lattice may be found at cell poles and septation sites where the packing order of the lattice may not be able to accommodate changes in the curvature of the surface (Murray, 1963; Watson and Remson, 1969; Sleytr and Glauert, 1975). Murray (1963) showed that, in RS layers with hexagonal symmetry, compensation can be made through the insertion of pentamers at these sites. The RS layers of certain bacteria (eg. Lamproedia hyalina, Methanospirillum hungatei) encompass groups, or chains, of cells (Pangborn and Starr, 1966; Beveridge et al., 1985). For this reason, the patterned surface layer of Methanospirillum hungatei has been termed a protein "sheath" (Beveridge et al., 1985).

High resolution studies of various surface arrays have been performed on negatively stained or shadowed preparations of isolated RS layer fragments or on RS layers

which have self-assembled in vitro. Finch et al. (1967) were the first to apply image processing techniques to the analysis of a bacterial surface array. They used optical diffraction and filtering methods to examine the fine structure of the RS layer of Bacillus polymyxa studied by Nermut and Murray (1967). Aebi et al. (1973) later applied computer-assisted digital filtering to the analysis of this protein array. Since this time, the fine structure of numerous RS layers have been examined using these techniques (Crowther and Sleytr, 1977; Stewart and Beveridge, 1980; Stewart et al., 1980; Stewart and Murray, 1982; Burley and Murray, 1983; Stewart et al., 1985). To date, RS layers with hexagonal (p3, p6), square (p4), and oblique (p2) lattice symmetries have been observed. Center-to-center spacing of individual units ranges from approximately 2.8-20 nm (Sleytr and Messner, 1983; Stewart et al., 1985) and pore sizes are usually in the 2-3 nm range (Aebi et al., 1973; Crowther and Sleytr, 1977; Stewart and Beveridge, 1980; Stewart et al., 1980).

1.3.2 Isolation Procedures

Ideally, a surface layer could be removed with ease and purified without excessive complications due to extraction of other cell components. Only a few bacteria, however, such as Campylobacter fetus (Winter et al., 1978) and Caulobacter crescentus (Smit et al., 1981), excrete soluble RS protein or shed excess surface array into the growth

medium, allowing the RS proteins to be recovered from the culture supernatant.

The majority of arrays are not so easily removed because the RS layers are more firmly attached to underlying structures. Extraction of whole cells produces, then, an unmanageable mix of cell proteins. The development of effective isolation procedures for bacterial surface arrays has benefitted greatly from the introduction of cell breakage techniques which allow the preparation of cell envelopes from whole cells. Breakage of microorganisms by ultrasonic disintegration was first introduced by Harvey and Loomis (1929). With the introduction of the electron microscope, Mudd et al. (1941) was able to demonstrate that cell wall fragments of various Bacillus species could be prepared by sonicating whole cells. Salton and Horne (1951) described the ballistic disintegration of whole cells of E. coli, Salmonella pullorum, and Streptococcus faecalis using glass beads in a Mickle shaker with subsequent recovery of cell envelope fragments. Cell breakage due to shear forces was introduced with the development of the French pressure cell (Milner et al., 1950) and the Hughes press (Hughes, 1951). Schnaitman (1971) and later Filip et al. (1973) showed that treatment of bacterial cell envelopes with certain detergents (Triton X-100, Sarkosyl) effectively removed adhering plasma membrane while other wall layers remained intact.

Goundry et al. (1967) were the first to describe

the isolation of a bacterial surface array. They incubated cell wall preparations of Bacillus polymyxa in 1% SDS for 1 hour at room temperature and observed that the regularly-structured array was completely removed by this treatment. Treatment with 6 M urea only partially removed the array. Nermut and Murray (1967) examined the effects of a wide range of detergents, chaotropes, and enzymes on the surface array of Bacillus polymyxa. Their experiments showed that the concentration of the extracting agent and the length of time used for the treatment were critical. They found that the array was removed as intact sheets by treatment with low concentrations of guanidine hydrochloride while higher concentrations of the agent resulted in the dissociation of the isolated sheets into individual units. Sleytr and Plohberger (1980) later showed that the RS layer of numerous thermophilic clostridia and bacilli could also be removed intact from cell wall preparations by treatment with low concentrations of urea or guanidine hydrochloride. Intact RS layers have been isolated from several clostridia and bacilli species as well as from Sporosarcina ureae by treating cell wall preparations with lysozyme which digests the underlying peptidoglycan layer (Sleytr, 1976; Beveridge, 1979; Sleytr and Plohberger, 1980). The solubilization or extraction of the individual units of the surface arrays from cell wall preparations of Gram-positive bacteria has been achieved by treatment with high concentrations of chaotropic or chelating agents or detergents (Howard and

Tipper, 1973; Sleytr, 1976; Hastie and Brinton, 1979a; Masuda and Kawata, 1979; Sleytr and Plohberger, 1980; Tsuboi et al., 1982).

The isolation of surface arrays from Gram-negative bacteria has always proven more difficult since the arrays of these organisms are closely-associated with an underlying outer membrane which is filled with proteins also susceptible to many of the agents used for the extraction of RS components. The RS layers of Gram-negative bacteria have been isolated by treating cell envelope preparations with urea or guanidine hydrochloride (Buckmire and Murray, 1970; Thornley et al., 1974; Kay et al., 1981; Phipps et al., 1983), lithium 3,5-diiodosalicylate (Koval and Murray, 1983), detergents (Koval and Murray, 1983; Winter et al., 1978), low pH (Beveridge and Murray 1976a,c), cationic substitution (Beveridge and Murray, 1976a,c), low ionic strength (Bingle et al., 1984) and metal chelating agents (Watson and Remsen, 1969; Thornley et al., 1974; Beveridge and Murray, 1976b) with or without further purification by gel chromatography (Thornley et al., 1974; Phipps et al., 1983).

These treatments usually result in the disintegration of the RS layer into individual subunits; ie. they are not usually removed intact. One exception is the RS layer of Deinococcus radiodurans which can be removed intact from isolated outer membrane vesicles by dissolving the membranes with 2% SDS (Thompson et al., 1982). Recently, the

regularly-structured protein sheath of an archaebacterium, Methanospirillum hungatei, has been shown to be particularly resistant to chemical and enzymatic degradation. The surface structure was isolated intact by treating cells with dithiothreitol at an alkaline pH and remained fully intact even after treatment with a wide variety of detergents, chaotropes and proteolytic enzymes (Beveridge et al., 1985). Isolation procedures have also been developed for the RS proteins of those archaebacteria whose surface arrays are closely-associated with a plasma membrane. These include treatments with detergents (Weiss, 1974), organic solvents (Mescher et al., 1974), and extremes of pH (Michel et al., 1980).

The isolation procedures which have been effective suggest that the forces involved in the attachment of these protein arrays to supporting layers and the bonds between the individual subunits are non-covalent in nature and probably involve hydrogen and ionic bonding (Nermut and Murray, 1967; Buckmire and Murray, 1970; Beveridge and Murray 1976a,b,c; Howard and Tipper, 1973; Thornley et al., 1974; Sleytr, 1976; Hastie and Brinton, 1979a; Masuda and Kawata, 1980; Michel et al., 1980; Sleytr and Plohberger, 1980; Kay et al., 1981; Tsuboi et al., 1982; Bingle et al., 1984) and, in some cases, hydrophobic interactions (Thompson et al., 1982).

1.3.3 Chemical Characterization

Goundry et al. (1967) were the first to show that surface arrays were composed of protein. Amino acid analysis of the detergent-extracted RS layer of Bacillus polymyxa showed it to be rich in aspartic acid. Since this time, biochemical analyses have been done on the isolated RS layers of numerous bacteria and have shown them to consist, usually, of a single, homogeneous polypeptide species which may contain a minor (less than 12%) carbohydrate component (Mescher et al., 1974; McCoy et al., 1975; Mescher and Strominger, 1976; Sleytr and Thorne, 1976; Winter et al., 1978; Hastie and Brinton, 1979a; Michel et al., 1980; Sleytr and Plohberger, 1980; Wieland et al., 1980; Sleytr and Messner, 1983; Word et al., 1983; Kúpca et al., 1984; Messner et al., 1984).

The molecular weights of the RS proteins studied to date range from 40,000 to 200,000 (Table 2, Sleytr and Messner, 1983) and the amino acid composition of many isolated RS proteins (Table 2, Sleytr and Messner, 1983) has shown them to share many common features: (i) RS proteins contain a high proportion of acidic amino acids. This has been supported, in certain cases, by isoelectric focusing (Thornley et al., 1974; Sleytr and Thorne, 1976; Hastie and Brinton, 1979a; Smit et al., 1981; Kay et al., 1984; Koval and Murray, personal communication). (ii) Sulphur-containing amino acids are either absent or present in very minor amounts. (iii) RS proteins contain a high proportion of

hydrophobic residues. (iv) The calculated secondary structure of all RS proteins show them to consist, on the average, of 40% alpha helix and 20% beta-sheet domains (Sleytr and Messner, 1983).

1.3.4 Relatedness

Within a single bacterial species many strains can often be found which possess RS layers. Several workers have examined the surface arrays of closely-related bacteria for possible taxonomic implications.

Buckmire (1970) examined the RS layers of various Aquaspirillum serpens strains and found that, although all consisted of hexagonally-packed subunits, the center-to-center spacing of the subunits and the type of linkers connecting the subunits differed greatly between the strains. Comparative studies of the RS layers of various species and strains of Clostridium and Pseudomonas have also indicated remarkable morphological heterogeneity (Hollaus and Sleytr, 1972; Sleytr and Glauert, 1975; Lapchine, 1979). Masuda and Kawata (1983) examined 22 strains within six species of Lactobacillus and found that the molecular weights of the RS proteins of those organisms which possessed a surface array varied from 41,000 to 55,000. Antisera specific for the RS protein of L. buchneri did not precipitate the RS proteins of five other Lactobacillus species in an immunodiffusion assay. Word et al. (1983) surveyed 61 strains of Bacillus sphaericus for

the presence of an RS layer. They found that, even within a DNA homology group, surface arrays were not uniformly present or absent. Antisera specific for the RS protein of B. sphaericus P-1 precipitated the RS proteins of only 7 of 17 strains within its own homology group and only partial identity could be demonstrated in the immunodiffusion assay. Messner et al. (1984) examined the RS layers of 39 strains of Bacillus stearothermophilus and, on the basis of the great differences observed in lattice constants, subunit molecular weights, and qualitative and quantitative carbohydrate compositions, concluded that these structures could not be considered useful taxonomic markers.

Recently, however, Kay et al. (1984) examined the biochemistry of the RS proteins of three Aeromonas salmonicida strains isolated from different sources. They found that the molecular weights and amino acid compositions of the strains were quite similar and were within twenty residues of each other in terms of size. The N-terminal sequences of the proteins were compared and found to be highly conserved. Certain differences were detected in the isoelectric points and tryptic digestion patterns of the proteins; overall, however, they were quite similar. The proteins proved immunologically cross-reactive. In addition, twenty-five strains of Aeromonas salmonicida reacted positively with fluorescent antibody directed against the RS protein of A. salmonicida A450 indicating common surface-exposed peptides. Since the virulence of

this organism is dependent upon the presence of an RS layer, the surface protein may have selective value.

Further information as to the relatedness of bacterial RS proteins should become available with the application of genetic analysis. Smit and Agabian (1984) have cloned the gene for the RS protein of Caulobacter crescentus and are currently using probes to detect similarities or differences in other strains and species of Caulobacter as well as in other organisms. Comparison of two Caulobacter crescentus strains (CB15 and CB2) by the Southern blot method demonstrated homology between the surface array genes using a probe prepared from strain CB15. Significant hybridization was also obtained with approximately twenty isolates of marine Caulobacter strains (J. Smit, personal communication). No significant homology, however, could be found when the probe was tested with various Aquaspirillum species whose RS layers appear to share common properties with those of Caulobacter species (J. Smit, personal communication).

1.3.5 Assembly

The isolated surface array subunits of many bacteria are capable of reassembling back onto the cell envelope layers from which they were isolated (reattachment) or assembling into an array in the absence of a supporting layer or template (self-assembly). The reassembled products usually possess the regular pattern characteristic of the

intact cell surface. The stimulus for these in vitro assemblies may simply be the removal of the agent used for the isolation of the subunits (Sleytr, 1975, 1976; Hastie and Brinton, 1979a,b; Masuda and Kawata, 1980; Sleytr and Plohberger, 1980; Word et al., 1983). Frequently, however, the proper assembly conditions must be determined and may involve manipulation of temperature, ionic strength or composition, or pH (Buckmire and Murray, 1973, 1976; Thorne et al., 1975; Beveridge and Murray, 1976a,c; Sleytr and Plohberger, 1980; Tsuboi et al., 1982; Bingle et al., 1984).

1.3.5.1 Reattachment

Buckmire and Murray (1973, 1976) examined the in vitro assembly of the RS protein of Aquaspirillum serpens VHA. They showed that isolated RS protein reassembled (in the presence of calcium or strontium) into hexagonal arrays on the cell envelope fragments from which they were removed. Magnesium or monovalent ions failed to support reattachment of the surface array protein. The authors suggested that divalent cations may form salt linkages between the negatively-charged RS protein and an outer membrane component.

Beveridge and Murray (1976a,c) examined the assembly properties of the RS components of two Aquaspirillum species which possess complex, multi-layered surface arrays. The in vitro reassemblies of the RS proteins of A. "ordal" and A. putridiconchylium were also shown to be dependent on calcium

or strontium. A divalent cation requirement for assembly has also been demonstrated for the RS proteins of Acinetobacter sp. (Thorne et al., 1975) and Azotobacter vinelandii (Bingle et al., 1984).

Several workers have attempted to identify specific components of the cell wall which are responsible for the attachment of RS proteins. Thorne et al. (1975) chemically modified the RS protein of Acinetobacter sp. 199A and outer membrane components within the cell envelope. They found that modifying the carboxyl groups within the isolated surface protein prevented its reattachment to an outer membrane surface. The authors suggested that divalent cations may form salt linkages between carboxyl groups within the RS protein and a negatively-charged (possibly phosphate) group of a membrane protein. Masuda and Kawata (1980) showed that the RS protein of Lactobacillus brevis would not reassemble onto formamide-treated cell wall fragments which lacked neutral polysaccharide. Removal of teichoic acids from the native walls did not prevent reassembly and the authors suggested that the neutral polysaccharide component of the wall played a role in the attachment of the RS protein.

It is generally believed, however, that the attachment of RS proteins to the underlying cell wall does not involve a specific wall component, but, rather, that only a suitable surface charge and density is required. This is supported by the reassembly of isolated RS proteins onto heterologous

templates. Sleytr (1975, 1976) showed that the RS proteins of Clostridium thermohydrosaccharolyticum and Clostridium thermohydrosulfuricum (which form tetragonal and hexagonal arrays, respectively, on the surfaces of these organisms) reassembled back into their characteristic lattices on cell wall fragments prepared from the other species. Similarly, the RS proteins of Aquaspirillum serpens VHA and Lactobacillus brevis reassembled onto heterologous templates in vitro (Chester and Murray, 1978; Masuda and Kawata, 1980).

While a specific wall component may not play a role in attachment, a specific region of the RS protein may be involved. Hastie and Brinton (1979a,b) examined the reattachment of the RS protein of Bacillus sphaericus 9602 to sacculi prepared from this organism. They treated the 142,000 RS protein with pronase which cleaved the native polypeptide to a lower molecular weight form (124,000). The modified protein failed to reassemble onto cell wall fragments and the authors concluded that the missing 18,000 fragment of the protein was required for attachment to the cell wall. Similarly, Koval and Murray (1984) found that partially-degraded RS protein of Aquaspirillum serpens VHA would not reassemble onto cell envelopes.

1.3.5.2 Self-Assembly

Many isolated surface array subunits have demonstrated the ability to self-assemble in vitro into lattices similar,

or identical, to those seen on intact cells (Brinton et al., 1969; Glauert and Thornley, 1973; Sleytr, 1976; Masuda and Kawata, 1980; Michel et al., 1980; Sleytr and Plohberger, 1980; Tsuboi et al., 1982; Word et al., 1983). This is not an unusual phenomenon; the components of a great many biological structures (flagella, pili, spinae, virus capsids, enzymes, microtubules) are also capable of self-assembly (Kushner, 1969). It is a common feature of biological organization that structures of great dimension are comprised of smaller units. The units are synthesized separately and then interact with one another to form the completed structure. In self-assembly systems the individual subunits possess the inherent capacity to direct their own assembly. The final polymerized structure arises spontaneously and achieves a thermodynamic equilibrium which is determined by such conditions as temperature, pressure, pH, and ionic concentrations. Stability of the self-assembled structure is attributed to correct bonding which provides a state of the lowest free energy for the system. The bonding is not of a covalent nature but, rather, is characterized by the weaker interactions such as hydrogen and ionic bonding and hydrophobic interactions (Caspar and Klug, 1962).

Brinton et al. (1969) were the first to demonstrate the in vitro self-assembly of a bacterial surface array. They found that the RS layer of Bacillus sphaericus P-1 could be removed intact from the underlying peptidoglycan layer by

treating whole cells with a low concentration of guanidine hydrochloride. The isolated array could then be dissociated into subunits by acid treatment. Subsequent neutralization of the subunit suspension resulted in the formation of sheets which possessed the tetragonal pattern characteristic of the intact cell surface. Modification of the RS protein, however, resulted in an alteration in the type of assembly product obtained. Treatment of self-assembled sheets with pronase resulted in their conversion to cylinders which retained the tetragonal pattern.

Glauert and Thornley (1972) described the self-assembly of the RS layer of Acinetobacter sp. 199A. Surface array subunits, which had been removed from outer membrane preparations by sonication, self-assembled to form sheets which possessed the tetragonal pattern of the intact cell surface. Thorne et al. (1975) later showed that the self-assembly of the subunits was dependent on the presence of chloride ions.

Sleytr (1976) examined the self-assembly of the RS layers of two Clostridium species and described a variety of assembly products including flat sheets, open-ended cylinders, and closed spheres. Assembly was not ion dependent and simply required the removal of the chaotropic agents (urea, guanidine hydrochloride) used for the isolation of the subunits. Sleytr and Phlohberger (1980) later expanded this study to include 65 thermophilic strains of bacilli and clostridia. They showed that the type of

assembly product obtained could be selected for by the correct manipulation of assembly conditions (temperature, cation composition).

1.3.6 Synthesis and Export

While the morphology, biochemistry, and assembly of the RS layers of numerous bacteria have been studied in detail, relatively little is known about the regulation of the synthesis of RS proteins or of their export from the bacterial cytoplasm to the cell surface.

In some organisms, the control over RS protein synthesis appears to be strict; little or no RS protein has been detected in the growth media of Bacillus sphaericus (Howard and Tipper, 1973) or Aquaspirillum serpens VHA (Koval and Murray, 1985). With others, such as Acinetobacter sp. (Thorne et al., 1976), Campylobacter fetus (Winter et al., 1978), Bacillus brevis 47 (Yamada et al., 1981) and Azotobacter vinelandii (Bingle et al., 1984), this control is more relaxed since excess RS protein can be detected in the growth medium in fairly large quantities (Thorne et al., 1976; Yamada et al., 1981; Bingle et al., 1984). A better understanding of RS gene regulation should emerge with recent advances made in the cloning of RS protein genes. Smit and Agabian (1984) have cloned the gene for the RS layer of Caulobacter crescentus into a lambda vector. The 130,000 Mr protein which comprises this structure was produced as a discrete product as a result of

transcription from a lambda promoter in infected E. coli strains.

Belland and Trust (1985) have recently used transpositional mutagenesis to obtain mutants blocked at various stages in the synthesis, export, and assembly of the RS protein of Aeromonas salmonicida. They found that mutants which were unable to produce lipopolysaccharide (LPS) with O polysaccharide chains were also unable to assemble the RS protein on their outer membrane surfaces. With another class of mutation, a single Tn5 insertion resulted in the loss of both O polysaccharide and RS protein synthesis, suggesting that both may be controlled by the same chromosomal gene.

Turnover studies have examined the incorporation of newly-synthesized RS protein into the array. Results obtained with both Bacillus sphaericus (Howard and Tipper, 1973) and Acinetobacter sp. (Thorne et al., 1976) indicated that, once incorporated into the structure, the RS protein undergoes no appreciable turnover.

Labelling studies have attempted to localize the site of insertion of newly-synthesized RS protein into the array during cell division and growth. With Bacillus sphaericus (Howard and Tipper, 1973) this was found to be at the site of cell division. Bands of new RS layer subunits were also detected along the cylindrical part of the cell, probably to accommodate cell elongation. Similarly, Smit and Agabian (1982) found that newly-synthesized RS proteins of

Caulobacter crescentus also inserted at the site of an incipient cell division. Incorporation, however, into the cylindrical part of the cell appeared to be at random sites and not in discrete bands. Electron microscopic studies of two Clostridium species showed that an excess of RS layer subunits were located at the site of cell division, most likely to ensure that the newly-divided cells were fully covered (Sleytr and Glauert, 1976).

1.3.7 Function

One of the first functions proposed for surface arrays was that they contributed to the determination and retention of cell shape (Brinton et al., 1969). Today it is generally believed that the peptidoglycan component of the eubacterial cell envelope is responsible for the greater part of this function. It has been argued, however, that for those archaeobacteria whose cell envelopes consist only of a plasma membrane and a surrounding RS layer (eg. Sulfolobus, Halobacterium), the surface structure may, indeed, play a role in the determination of cell shape (Stoeckenius and Rowen, 1967; Weiss, 1974). This is supported by the observed conversion of rod-shaped halobacterial cells to spheres when the synthesis of the sugar component of the organism's RS protein is inhibited (Mescher and Strominger, 1975).

Buckmire (1970) demonstrated that RS layers may provide protection from environmental hazards. He found that those

aquaspirilla which possessed RS layers were resistant to invasion and digestion by the bacterial predator, Bdellovibrio bacteriovorus, while naked strains were susceptible. RS layers may, perhaps, prove a less 'palatable' coating to predators in the environment. Surface arrays were also shown to protect certain Aeromonas salmonicida strains from bacteriophage; for other bacteria, however, phage appear to use the protein array as receptors for attachment (Howard and Tipper, 1973; Ishiguro et al., 1984).

Ultrastructural studies have shown that RS layers completely surround the cell, forming a protein meshwork with determined pore sizes of approximately 2-3 nm. It has been proposed that the arrays may act as molecular sieves selectively retaining desirable substances within the cell and preventing less desirable ones from entering (Crowther and Sleytr, 1977; Sleytr, 1978; Stewart and Beveridge, 1980; Stewart et al., 1980; Stewart and Murray, 1982). Early studies appear to support this; the RS layers of certain Gram-positive bacteria were shown to protect the underlying peptidoglycan layer from muramidases (Nermut and Murray, 1967; Takumi and Kawata, 1974). The presence of an RS layer, however, does not guarantee such protection since Kawata et al. (1974) and, more recently, Messner et al. (1984) have reported that the peptidoglycan of many bacteria which possess surface arrays is easily digested by enzymes such as lysozyme. The intact RS layers of certain organisms

(eg. Methanospirillum hungatei) have been shown to be resistant to a wide variety of proteases suggesting that the arrays may shield the bacterial cell from various proteolytic enzymes secreted by other organisms (Beveridge et al., 1985).

The presence of a surface array on the human and animal pathogen, Campylobacter fetus (McCoy et al., 1975; Winter et al., 1978) and the fish pathogen, Aeromonas salmonicida (Ishiguro et al., 1981; Kay et al., 1981., Evenberg et al., 1982; Munn et al., 1982; Trust et al., 1983) was found to be associated with the virulence of these organisms. The RS layer appeared to contribute to antiphagocytic effects and provided protection from the host immune response. The RS layer of Aeromonas salmonicida was also shown to function in autoagglutination and adhesion to epidermal surfaces (Evenberg et al., 1982).

As additional information has become available as to the biochemical nature of RS proteins, other functions have been suggested. Beveridge (1979) has proposed that a highly-charged protein array may serve to immobilize toxic metals or offer an increased buffering capacity against Lewis acids and hydronium ions in the environment. The RS layer of Azotobacter vinelandii was found to complex with excess iron in its growth medium, perhaps to store the metal for future use by the bacterium (Page and Huyer, 1984). RS layers may act, then, by trapping limited nutrients in the environment.

The variety of functions proposed and/or demonstrated for bacterial surface arrays argues that these layers arose as a result of selective environmental pressures. This is supported by the observed loss of RS layers when these pressures are removed (ie. upon continued lab culture) (R.G.E. Murray, S.F. Koval, personal communication; M. Kist, unpublished results).

1.4. Aquaspirillum serpens MW5: Description of the organism

Aquaspirillum serpens MW5 is a Gram-negative, helical, freshwater bacterium. Phenotypically, the organism fulfills the requirements for inclusion in the species A. serpens (Hylemon et al., 1973; Boivin et al., 1985). Boivin et al. (1985) demonstrated, through DNA hybridization studies, that Aquaspirillum serpens MW5 is genotypically similar both to the type strain of the species (ATCC 12638) and to strain VHA.

1.4.1 Specific Features of the Surface Array of Aquaspirillum serpens MW5

Prior to this study work done on the RS layer of Aquaspirillum serpens MW5 had focused on the determination of the fine structure of the array. Stewart and Murray (1982) examined this surface structure using image analysis techniques (computer image processing and least squares analysis). The surface of negatively stained whole cells

appeared "ribbed" with the distance between the ribs being 16 nm. The ribs were aligned at a 90 degree angle to the longitudinal axis of the cell. This ribbed or linear pattern was also detected in negatively stained preparations of isolated RS layer fragments. In a few areas of these fragments the subunits appeared to be hexagonally-packed with a center-to-center spacing of 18.8 nm and connected by delta, or "direct", linkers. Thin sections of whole cells had revealed that the surface array was a two-layered structure--ie., two layers of subunits were found to lie external to the outer membrane of the cell envelope. The hexagonal pattern detected in negatively-stained fragments of the array likely represented a single layer of subunits and had been found in areas of the fragment where one of the two layers had been damaged or removed. The linear, or ribbed, pattern, therefore, represented a moiré involving the contributions of both layers of the array. Computer image processing was employed to further analyze the layers of the array. The linear pattern was indexed on a rectangular lattice with unit vectors corresponding to 32 and 18.5 nm. Analysis of the structure factor amplitudes indicated that the array had cmm symmetry. The hexagonally-packed layer was found to have sixfold, or p6mm symmetry. Stewart and Murray (1982) determined that the linear moiré pattern seen in negatively-stained cell envelope preparations could be produced by the superimposition of two hexagonally-packed layers of

subunits, each having $p6mm$ symmetry. One sheet was found to be staggered by one-half repeat along a hexagonal (1,0) lattice line relative to the first. The authors were unable to detect any consistent differences between the upper and lower layers although, they pointed out, the study was limited to 2.5 nm resolution and so would not be able to pick up subtle structural differences.

1.4.2 Goals

The objective of this study was to examine the biochemical and in vitro assembly properties of the surface array components of Aquaspirillum serpens MW5 in order to: (i) complement previous structural work (Stewart and Murray, 1982); (ii) provide biochemical and assembly data for a complex surface array since most studies of multi-layered arrays have concentrated primarily on their ultrastructure (Chapman et al., 1963; Watson and Remsen, 1970; Beveridge and Murray, 1974, 1975) and (iii) compare these properties with those previously described for a closely-related strain, Aquaspirillum serpens VHA (Buckmire and Murray, 1970, 1973, 1976; Chester and Murray, 1978; Koval and Murray, 1981, 1983, 1984, 1985).

CHAPTER 2

MATERIALS AND METHODS

2.1. Organisms

The surface array of Aquaspirillum serpens MW5 (University of Western Ontario Culture Collection no. 377) was examined in this study. Aquaspirillum serpens VHA (U.W.O. no. 369) was used throughout the relatedness studies for comparative purposes and the following organisms were used for the in vitro reassembly experiments: (i) a spontaneous mutant (RS⁻) of MW5 (University of Western Ontario Culture Collection no. 1191) which had lost its regular surface array; (ii) Aquaspirillum serpens strains VHL (U.W.O. no. 368), and (iii) the type strain (ATCC no. 12638); (iv) Escherichia coli (U.W.O. no. 1025); and (v) Pseudomonas aeruginosa (U.W.O. no. 912).

2.1.1 Cultivation and Harvesting

All Aquaspirillum serpens strains were grown in peptone-succinate-salts (PSS) broth (Hylemon et al., 1973), pH 7.2, containing, as a final concentration (wt/vol): 1.0% peptone (Difco Laboratories, Detroit, Mich.), 0.1% succinate, 0.1% magnesium sulphate, 0.1% ammonium sulphate, 0.0002% ferric chloride, and 0.0002% manganese chloride. The succinate and salts were all obtained from Fisher

Scientific Co., Fair Lawn, New Jersey. After autoclaving, sterile calcium chloride was added to a final concentration of 0.025% (wt/vol). The cells were grown at 30C with shaking (150 rpm; Psychrotherm incubator-shaker; New Brunswick Scientific Co., New Brunswick, N.J.). Cells were harvested in the mid-logarithmic phase of growth (approximately 12 hours post-inoculation) by centrifugation at 3,500 x g for 15 min at 4C and washed in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing 1mM calcium chloride (HEPES-Ca²⁺ buffer).

Stocks of Aquaspirillum serpens strains were routinely maintained on PSS slants (PSS broth containing 1.5% [wt/vol] agar) at 30C.

For some experiments, A. serpens MW5 was grown in a defined medium for aquaspirilla containing 0.4% glutamic acid (carbon and nitrogen source), 0.01 M NaH₂PO₄·H₂O, 0.01M KCl, 0.002 M Na₂SO₄·10H₂O, 0.002 M citric acid, 600 uM HCL (trace element solvent), 25 uM ZnO, 100 uM FeCl₃·6H₂O, 50 uM MnCl₂·4H₂O, 5 uM CuCl₂·2H₂O, 10 uM CoCl₂·6H₂O, 5 uM H₃BO₃, and 0.01 uM Na₂MoO₄ (Whitby and Murray, 1980). The pH was adjusted to 7.0 with 10 N NaOH and sterile MgCl₂ was added after autoclaving to a final concentration of 0.000125 M. An 8% (v/v) initial inoculum from a culture grown overnight (12 h) in PSS + Ca²⁺ was required for successful growth in this media.

Escherichia coli and Pseudomonas aeruginosa cells were

grown in nutrient broth (Difco Laboratories, Detroit, Mich.), pH 7.0, for 12 h at 30C with shaking. Cells were harvested and washed as described above.

2.1.2 Preparation of Cell Envelopes

Envelopes were prepared by the method of Koval and Murray (1981) as follows. Washed cells were homogenized with a Potter-Elvehjem tissue homogenizer using a Teflon pestle in order to remove their flagella. The homogenized cells were centrifuged at 3,500 x g and the supernatant fraction (containing the sheared flagella) discarded. The cells were resuspended in ice-cold HEPES-Ca²⁺ buffer and passed through a French pressure cell (Aminco) at 16,000 lb/in². The product was centrifuged at 3,500 x g for 15 min at 4C to remove any intact cells. The supernatant was then centrifuged at 48,000 x g for 30 min to pellet the cell wall-enriched fraction (envelopes): The envelopes were washed in HEPES-Ca²⁺ buffer and incubated for 1h at room temperature in the same buffer containing 0.025% magnesium sulphate, 10 ug/ml DNase and 20 ug/ml RNase. Both enzymes were obtained from Sigma Chemical Co. (St. Louis, Mo.). The envelopes were then washed four times with HEPES-Ca²⁺ buffer. The supernatants were analyzed for nucleic acids by reading the absorbance at 260 nm. The envelopes were routinely extracted for 30 min with 2% Triton X-100 in HEPES-Ca²⁺ buffer to remove contaminating plasma membrane (Schnaitman, 1971), washed repeatedly with buffer, and

stored as a pellet at -10C.

2.1.3 Identification of Peptidoglycan-Associated Proteins

The peptidoglycan-associated protein within the outer membrane of *A. serpens* MW5 was tentatively identified according to the solubility criterion established by Rosenbusch (1974). Cell envelopes were resuspended (at a protein concentration of 1 mg/ml) in 2% SDS in HEPES-Ca²⁺ buffer and incubated at either 50C, 60C, or 100C for 30 min. Aliquots of each were then removed and immediately analyzed by SDS-PAGE.

2.2. Electron Microscopy

2.2.1 Negative staining. Whole cells were negatively stained as follows. A Formvar-coated 200-mesh copper grid was inverted over a drop of the cell suspension for 1 min, washed with distilled water, and subsequently stained on a drop of 1.0% ammonium molybdate (British Drug Houses Ltd., Toronto, Canada), pH 7.0, in distilled water. The grid was dried after removing excess fluid by touching its edge to a piece of filter paper. Cell envelopes were negatively stained as described above except that 2.0% ammonium molybdate was used. Negatively stained self-assembly products were prepared by mixing samples (1:1) with 2.0% ammonium molybdate, pH 7.0. A thin film of this suspension was broken over the surface of the copper grid using a 4.0 mm wire loop.

2.2.2 Fixation, embedding, and staining. All procedures were carried out at room temperature. The prefix, fixative, and postfix solutions were all prepared in 0.05 M sodium cacodylate buffer, pH 7.2. Samples were prefixed in 0.5% tannic acid (British Drug Houses Ltd., Toronto, Canada) for 30 min and then fixed in 5.0% acrolein-0.25% glutaraldehyde (both from Polysciences Inc., Warrington, Pa.) overnight (Burdett and Murray, 1974). The samples were washed three times with buffer, enrobed in 2.0% Noble agar (Difco Laboratories, Detroit, Mich.), and postfix in 1.0% uranyl acetate (British Drug Houses) in distilled water for 2 h. The samples were then dehydrated in a 30 to 100% ethanol series and embedded in Spurr embedding medium. Sections were cut on a Sorvall Porter-Blum Ultra Microtome MT-1 (Ivan Sorvall, Inc., Norwalk, Conn.) with glass knives and stained with 1.0% uranyl acetate-lead citrate (Reynolds, 1963). Samples were examined in a Philips EM-200 or EM-300 electron microscope operating at 60kV. Micrographs were taken on Kodak fine-grain positive film.

2.3. Effects of Chaotropic Agents on the Surface Array.

Cell envelopes (1.0 mg protein) were resuspended in 1.0 ml of each of the following solutions. All solutions were prepared in distilled water unless otherwise stated.

(A) Hydrogen bond-breaking agents:

(i) 1.5 M guanidine hydrochloride (ii) 1.5 M, 3.0 M, and

6 M urea

(B) Salts:

(i) 1 M LiCl (ii) 25 ug/ml protamine chloride (iii) 0.05 M
TbCl₃ (iv) 0.5 M LaNO₃

(C) Chelating Agents:

(i) 0.2 M EDTA, pH 8.0 (ii) 0.2 M EGTA, pH 8.0 (iii) 1.0
M sodium citrate, pH 7.0 (iv) 1.0 M sodium oxalate, pH 7.0
(v) 0.05 M 8-hydroxyquinoline (in 15% ethanol, pH 9.0)
(Sigma).

(D) Detergents:

(i) 2.0% (v/v) Triton X-100 (ii) 2.0% (w/v) SDS (iii) 2.0%
(w/v) n-octylglucoside (iv) 2.0% (w/v)
cetyl-trimethylammonium bromide (CTAB) (v) 2.0% (w/v)
Brij 58

(E) Disulfide Bond-breaking Agents:

(i) 10% (v/v) B-mercaptoethanol (ii) 10% (w/v)
dithiothreitol

(F) pH:

(i) distilled water, pH adjusted with NaOH or HCl to 3.0,
3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 9.0, 9.5, 10.0, 10.5,
11.0, 11.5, or 12.0.

(ii) 0.1 M citric acid-sodium phosphate buffer, pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, or 7.0.

(iii) 0.05 M sodium borate buffer, pH 9.0, 9.5, 10.0, or 10.5.

(iv) 0.2 M sodium phosphate buffer, pH 11.0, or 11.5.

(v) 0.2 M NaOH-KCl buffer, pH 12.0.

(G) Miscellaneous Chaotropic Agents:

(i) 20 mM lithium 3,5-diiodosalicylate (LIS) (Sigma)

The cell envelopes were incubated for 1 h at room temperature with occasional mixing and then collected by centrifugation for 3 min in an Eppendorf centrifuge (Model 5412). The supernatants were removed and analyzed by SDS-PAGE. The pellets were washed twice with distilled water and examined by negative staining-electron microscopy and SDS-PAGE.

2.4. Sequential Protein Extraction Procedure

(i) High-pH buffer extraction. Cell envelopes were resuspended at a protein concentration of 1.0 mg/ml in 0.05 M sodium borate buffer, pH 10.3, and incubated for 1.5 h at room temperature with occasional vortexing. After incubation, the envelopes were collected by centrifugation

for 3 min in an Eppendorf centrifuge (model 5412) at room temperature. The supernatant was removed for SDS-PAGE analysis and examination by negative staining-electron microscopy. The pellet (pH 10.3 pellet) was washed once with distilled water and three times with HEPES-Ca²⁺ buffer, pH 7.4. A sample was removed and embedded for analysis by thin sectioning. Samples of the pH 10.3 pellet were also reserved for examination by negative staining and SDS-PAGE.

(ii) 6 M urea extraction. The pH 10.3 pellet was resuspended in unbuffered 6 M urea at a protein concentration of 1.0 mg/ml and incubated at room temperature for 2 h with occasional vortexing. After incubation, the envelopes were collected by centrifugation in an Eppendorf centrifuge. The supernatant and pellet fractions were treated and analyzed as outlined above.

2.4.1 Sequential Protein Extraction: Large Scale Extraction

For some experiments larger quantities of the surface array proteins were required. Cell envelopes prepared from 3 L of cells (approximately 250 mg protein) were extracted sequentially as described above (section 2.4.) except that the envelopes after each of the two extractions were collected by centrifugation at 48,000 x g for 30 min at 4C. The supernatants containing the solubilized proteins were then ultracentrifuged at 150,000 x g for 1 h at 4C to remove any contaminating cell envelopes or membrane vesicles. The

supernatants were routinely dialyzed overnight (18 h) against distilled water at 4C and then concentrated by membrane filtration (YM30 membrane, Amicon Corp.) and stored at either -10C or -20C.

2.4.2 Inhibition of Proteolysis During Isolation

(i) Use of Protease Inhibitors During Cell Breakage and Envelope Preparation

Cell envelopes were prepared as described (section 2.1.2) except that 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM N-tosylphenylalanine chloromethyl ketone (TPCK), or 1.0 mM dithiothreitol was included at all stages following the deflagellation of the cells. The cell envelopes prepared in the presence of these inhibitors were examined by negative staining-electron microscopy and SDS-PAGE.

(ii) Use of Protease Inhibitors During Protein Extraction

The RS proteins were extracted sequentially from cell envelopes of strain MW5 as described (section 2.4.) except that the extracting buffers included one of the following agents designed to inhibit proteolysis: 10 mM dithiothreitol (DTT), 10 mM L-cysteine hydrochloride, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM N-tosyl-L-lysine chloromethyl ketone (TLCK), 1 mM N-tosylphenylalanine chloromethyl ketone (TPCK), 100 ug/ml soybean trypsin inhibitor (type 1-S) (STI), 100 ug/ml aprotinin, 100 ug/ml p-chloromercuriphenylsulfonic acid (PCMBs), 100 ug/ml

pepstatin, or 10 mM iodoacetate. DTT, L-cysteine hydrochloride, and iodoacetate were obtained from Fisher Scientific Co. PMSF, TLCK, TPCK, STI, aprotinin, and PCMBs were all obtained from Sigma while the pepstatin was purchased from Boehringer Mannheim. Stocks of PMSF, TLCK, TPCK, and pepstatin were initially prepared in 95% ethanol and the subsequent extracting and dialysis solutions for these inhibitors contained 5% ethanol. Following the incubation period, the cell envelopes were collected by centrifugation as described and the supernatant fractions dialyzed overnight (18 h) against deionized water (or 5% ethanol) containing the same inhibitors but at one-tenth the concentration used during extraction. The dialysates were then removed from the tubing and analyzed by SDS-PAGE.

(iii) Isolation at Low Temperatures

The RS proteins were extracted sequentially from cell envelopes as described (section 2.4.) except that all operations were conducted at 4C.

2.5. Assembly

2.5.1 Urea Extraction of Surface Protein for Assembly

Experiments

Cell envelopes prepared from 3 litres of cells (approximately 250 mg of protein) were resuspended in 200 ml of unbuffered 6 M urea (Fisher Scientific Co., Fairlawn, N.J.) and incubated with constant stirring at room temperature for 2 h. After incubation, the cell envelopes

were collected by centrifugation at 48,000 x g for 30 min at 4C. The supernatant was then ultracentrifuged at 150,000 x g for 1 h at 4C to remove any contaminating membrane vesicles. A portion was removed and dialyzed overnight against distilled water at 4C for protein estimation and SDS-PAGE. The remainder of the supernatant contained urea and was the crude protein extract used for subsequent in vitro reconstitution and self-assembly studies. The extracted envelopes were washed three times with distilled water and analyzed by negative staining and SDS-PAGE.

2.5.2. In Vitro Reconstitution (Reattachment)

2.5.2.1 Reassembly onto Outer Membrane

Cell envelopes prepared from the RS⁻ strain of A. serpens MW5 or 6 M urea-extracted cell envelopes of strain MW5 were mixed with 6 M urea-extracted RS protein to give final protein concentrations of 500 and 125 ug/ml, respectively. The total volume of each mixture was 1.0 ml. The suspensions were dialyzed overnight at 4C with constant stirring against distilled water or distilled water containing 10mM NaCl, KCl, LiCl, MgCl₂, CaCl₂, or SrCl₂ (salts all obtained from Fisher Scientific Co.). The dialysates were then removed from the tubing and the envelopes were collected by centrifugation for 3 min in an Eppendorf centrifuge at room temperature. The pellets were washed twice with distilled water and examined by negative staining and SDS-PAGE.

2.5.2.2 Reassembly onto Heterologous Templates

Cell envelopes prepared from A. serpens VHL, A. serpens 12638 (type strain), and wild-type strains of Escherichia coli and Pseudomonas aeruginosa, were mixed with 6 M urea-extracted RS protein of A. serpens MW5 as described above and dialyzed overnight at 4C with constant stirring against 10 mM calcium. The dialysates were then removed from the tubing and the envelopes collected by centrifugation for 3 min in an Eppendorf centrifuge at room temperature. The pellets were washed twice with distilled water and examined by electron microscopy.

2.5.3 Sequential Reassembly onto Outer Membrane

The ability of the components of each of the two layers to reassemble in tandem onto an outer membrane surface was examined. RS⁻ cell envelopes were mixed together with either: (i) the 125K protein extract (obtained from the initial step of the sequential extraction procedure) or (ii) the 150K protein extract (obtained from the final step of the sequential extraction procedure) and dialyzed overnight at 4C against 10 mM CaCl₂ in distilled water. The pellets were collected by centrifugation and washed as outlined above for the in vitro reconstitution studies. A portion of each was retained for negative staining and SDS-PAGE. In the second step of the sequential reconstitution, the pellets from (i) and (ii) were mixed with the 150K and 125K protein extracts, respectively. These reassembly mixtures

were dialyzed and subsequently analyzed as described above.

The ability of the 125K RS protein of strain MW5 to reassemble onto the hexagonally-arranged RS layer of A. serpens VHA was also examined. Cell envelopes prepared from strain VHA (500 ug protein) were mixed with the 125K protein extract (125 ug protein) and dialyzed overnight at 4C against 10 mM CaCl₂. The dialysate was removed from the tubing and examined by negative staining-electron microscopy and SDS-PAGE.

2.5.4 Self-Assembly

2.5.4.1 Self-Assembly of the RS proteins (150K + 125K):

Parameters of Assembly

(i) Cation requirements. 6 M urea-extracted RS proteins (500 ug protein/ml) (section 2.5.1) were dialyzed overnight at 4C against distilled water or distilled water containing 10 mM NaCl, KCl, LiCl, MgCl₂, CaCl₂, or SrCl₂. An identical set of assembly mixtures were dialyzed against the same series of cations but at room temperature (22C). The dialysates were then removed from the tubing and examined by negative staining-electron microscopy. Self-assembled products were collected by centrifugation for 3 min in an Eppendorf centrifuge, washed twice with the appropriate cation (1 mM), and analyzed by SDS-PAGE.

(ii) Ionic Strength/pH. 6 M urea-extracted RS proteins (500 ug protein/ml) (section 2.5.1) were dialyzed overnight at 4C against distilled water or 10 mM HEPES buffer, pH 7.0,

containing 0.5 mM, 1 mM, or 10 mM CaCl_2 . The dialysates were removed from the tubing and examined by negative staining-electron microscopy.

(iii) Protein Concentration. Aliquots of 6 M urea-extracted RS proteins (section 2.5.1) were adjusted to protein concentrations of either 50, 100, 250, or 500 $\mu\text{g/ml}$ and dialyzed overnight at 4C against 10 mM CaCl_2 . The dialysates were removed from the tubing and examined by negative staining-electron microscopy.

2.5.4.2 Self-Assembly of the Individual RS Proteins

150K and 125K protein extracts (section 2.4) were dialyzed separately overnight against 10 mM CaCl_2 or 10 mM SrCl_2 at 4C. The protein concentration of each sample was 500 $\mu\text{g/ml}$. The dialysates were then removed from the tubing and examined by negative staining-electron microscopy. Self-assembled products were collected by centrifugation for 3 min in an Eppendorf centrifuge, washed twice with 1.0 mM CaCl_2 or 1.0 mM SrCl_2 , and analyzed by SDS-PAGE.

2.5.4.3 Image Analysis of Self-Assembly Products

Image processing of the self-assembly products was performed essentially according to the method described by Burley *et al.* (1983). Areas of micrographs free from obvious faults were selected visually. Fourier transforms were generated from these areas using an optical diffractometer (Dept. of Physics, University of Western

Ontario) with a helium-neon laser as the light source. Selected diffraction points were enhanced by filtering out, or masking, the background noise. The images were digitized using a digital image transducer and a PDP11-44 computer and the inverse Fourier transforms generated to produce the optically-reconstructed images.

2.6. Electrophoretic Methods: Ion-Exchange Chromatography, PEG Precipitation

2.6.1 SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous slab gels were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoresis was conducted according to the method of Laemmli (1970) as described by Koval and Murray (1981). Except where noted, the stacking and resolving gels contained acrylamide concentrations of 4% and 9%, respectively. The acrylamide stock contained 39% acrylamide and 1% bis-acrylamide. Samples were routinely heated in the sample buffer described by Laemmli (1970) at 100C for 3 min. The gels were stained and fixed in 0.1% Coomassie brilliant blue R250-25% methanol-7% acetic acid at room temperature overnight. They were destained by diffusion in several changes of 25% methanol-10% acetic acid. All chemicals for electrophoresis were obtained from Bio-Rad Laboratories (Richmond, Ca.).

2.6.2 Gel Densitometry

Densitometric scans of Coomassie blue stained-SDS-polyacrylamide gels were done using a Beckman spectrophotometer (Model Du-8), equipped with a linear transport accessory. Gels were scanned through a 0.5 mm slit at 560 nm with a scan rate of 2 cm/min.

2.6.3 Determination of Molecular Weight

Molecular weights of the major cell envelope proteins were determined by comparing their migration in SDS-polyacrylamide gels to those of the following calibration proteins: RNA polymerase (165,000, 155,000, and 39,000), bovine serum albumin (68,000), and soybean trypsin inhibitor (21,500) (Boehringer Mannheim, Dorval, Quebec, Canada). Myosin (205,000), β -galactosidase (116,000), and phosphorylase B (97,000) (Sigma) were used as calibration proteins for the molecular weight determination of oligomeric forms of the surface proteins.

2.6.4 Periodic Acid-Schiff Stain for Carbohydrate

Cell envelope proteins, 6 M urea-extracted RS proteins (section 2.5.1), and the protein extracts obtained from the sequential extraction procedure (section 2.4.1) were separated by SDS-PAGE and stained for glycoprotein with periodic acid-Schiff's reagent according to the method of Glossmann and Neville (1971). Fetuin and BSA were used as positive and negative controls, respectively.

2.6.5 Non-denaturing PAGE

Discontinuous native slab gels and protein samples were prepared as described above (2.6.1) except that SDS was omitted from the gel solutions, electrophoresis running buffer, and sample buffer. The stacking and resolving gels contained acrylamide concentrations of 3 and 5%, respectively. The acrylamide stock contained 30% acrylamide and 0.8% bis-acrylamide.

2.6.6 Isoelectric Focusing

40 ug of 6 M urea-extracted and 20 mM LIS-extracted and dialyzed RS proteins were loaded onto separate tube gels (11 cm in length and 3 mm in diameter) and focused in ampholine gradients (pH 4-6) according to the method of O'Farrell (1975). The focused proteins were separated in the second dimension by SDS-PAGE and the slab gel stained with Coomassie brilliant blue. The ampholines used in the focusing gels were obtained from Bio Rad.

2.6.7 Ion-Exchange Chromatography

The 150K and 125K proteins were extracted from cell envelopes as described (section 2.5.1) and simultaneously concentrated by membrane filtration (YM30 membrane, Amicon Corp.) and dialyzed against 0.01 M imidazole buffer, pH 6.5. 0.5 ml of this sample (2.0 mg protein) was applied to a DEAE-Sephrose CL-6B column (15 cm x 0.7 cm), equilibrated with the same buffer. The column was washed with 5 volumes

of buffer and the proteins eluted on a linear gradient of 60 ml of 0-1.0 M NaCl in 0.01 M imidazole buffer, pH 6.5. 1.0 ml fractions were collected and eluted protein detected by reading the absorbance of the fractions at 280 nm. Fractions corresponding to the major peaks were pooled and lyophilized. The samples were resuspended in distilled water and analyzed by SDS-PAGE.

For some experiments, the RS proteins were allowed to adsorb to the ion-exchange resin in batch. 1.0 ml of the 125K protein extract (1.0 mg protein) (section 2.4.1) was added to 2.0 ml of DEAE-Sepharose CL-6B (washed and equilibrated with 0.01 M imidazole buffer, pH 6.5) in a 30 ml Corex tube, and mixed well. The beads were allowed to settle. The supernatant was removed and analyzed by SDS-PAGE. The resin was washed with 10 volumes of buffer and a stepwise elution series of 0.25 M, 0.5 M, 1.0 M, and 1.5 M NaCl (in buffer) initiated. The beads containing the bound protein were mixed with 1.0 ml volumes of the eluents and then allowed to settle. The supernatant after each incubation was removed and checked by SDS-PAGE for eluted protein.

2.6.8 Polyethylene Glycol (PEG) Precipitation of RS protein

(i) PEG stock solutions (PEG series: 400, 1000, 1500, 8,000, and 20,000; 20% w/w in 20 mM HEPES, pH 7.0) were mixed 1:1 with separate aliquots of the 125K protein extract (500 ug protein) (section 2.4.1) to final PEG concentrations

of 10%. The solutions (total volume, 1.0 ml) were mixed and incubated at room temperature for 4 h. The precipitates were collected by centrifugation for 3 min in an Eppendorf centrifuge, resuspended in a small volume of distilled water, and analyzed by SDS-PAGE. The polyethylene glycol was obtained from Fisher Scientific Co. (PEG 400, 8,000, 20,000) and BDH (PEG 1000, 1500).

(ii) 6%, 20%, 30%, and 50% PEG 1500 stock solutions (w/w in 20 mM HEPES, pH 7.0) were mixed 1:1 with the 125K protein extract (500 ug protein) (section 2.4.1) to give final PEG concentrations of 3%, 10%, 15%, and 25%. The solutions (total volume, 1.0 ml) were mixed and incubated at room temperature for 4 h. The precipitates were collected by centrifugation as described above and analyzed by SDS-PAGE.

2.7 Biochemical Assays

2.7.1 Protein Estimation

Protein content was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.7.2 Determination of Carbohydrate Content

RS protein extracts (50-1000 ug protein) were assayed for reducing sugar content by the method of Dubois et al. (1956) using glucose as a standard.

2.7.3 Amino Acid Analysis

Cell envelope proteins of *A. serpens* MW5 were separated by SDS-PAGE. The bands were visualized by immersing the unstained gel in 4 M sodium acetate according to the method of Higgins and Dahmus, (1979). The high concentration of salt precipitates SDS not bound to protein; polypeptides, therefore, appear transparent against a white background. Transparent bands corresponding to the 150K and 125K proteins were excised using a razor blade. The bands were homogenized in 0.05 M ammonium carbonate buffer, pH 7.5, containing 0.05% SDS, using a glass homogenizer and pestle. The proteins were eluted overnight by incubating the homogenized gel slices in the same buffer with constant agitation. The eluted proteins were dialyzed against deionized water (to remove any glycine eluted from the slab gel), lyophilized, and aliquots of each reserved for SDS-PAGE. The remainder was hydrolyzed in 6 N HCl for 18 h at 115°C and the hydrolyzates analyzed in a Beckman 119CL single column analyzer.

2.8 Interaction of the RS proteins with Calcium

2.8.1 Growth in Calcium-free Media

To determine if calcium was required for maintaining the integrity of the RS layer of *A. serpens* MW5 during broth culture, the organism was repeatedly transferred in calcium-free broth. A defined medium for aquaspirilla (Whitby and Murray, 1980) was used for these experiments.

(section 2.1.1). The broth was inoculated [8% (v/v)] with a 12 h culture of MW5 (grown in PSS+Ca²⁺ broth) and incubated O/N with shaking at 30C. 4% (v/v) of this overnight culture was then transferred to fresh calcium-free media and incubated for 24 h as described. This was repeated six times. Cells were then harvested and cell envelopes prepared and examined by negative staining-electron microscopy and SDS-PAGE. Control flasks contained the same media supplemented with calcium (1.0 mM) and were treated identically.

2.8.2 Effect of Calcium on the Extraction of the RS Proteins from Cell Envelopes

Cell envelopes prepared from *A. serpens* MW5 were resuspended (at a protein concentration of 1.0 mg/ml) in 6 M urea containing 10 mM CaCl₂. The mixtures were incubated at room temperature for 1 h with occasional mixing. The envelopes were then collected by centrifugation for 3 min in an Eppendorf centrifuge and the supernatant dialyzed overnight at 4C against 10 mM CaCl₂. The dialysates were removed from the tubing and analyzed by SDS-PAGE and electron microscopy.

2.8.3 Calcium-binding Assay

The RS proteins of *A. serpens* MW5 were analyzed for bound calcium using the calcium detection procedure outlined in Technical Bulletin No. 586 (Sigma Chem. Co.). The

procedure is based on the spectrophotometric method first described by Pollard and Martin (1956) and later modified by Sarkar and Chauham (1967). *6 M urea-extracted and dialyzed surface proteins (section 2.5.1) and the 150K and 125K protein extracts (section 2.4.1) were adjusted to protein concentrations of 0.5 mg/ml in deionized water and dialyzed for 48 h at 4C against deionized water to remove any free calcium. 200 ul (100 ug protein) of each extract was added to 2.0 ml of calcium assay solution containing 225 mM 2-amino-2-methyl-1-propanol, 0.003% 8-hydroxyquinoline, DMSO, and the chromogenic agent, o-cresolphthalein complexone (CC) (Sigma diagnostic kit 586, Sigma Chemical Co.). CC binds calcium and, at alkaline pH, produces a purple colour which absorbs at 575 nm (Pollard and Martin, 1956). The presence of calcium in the samples was indicated by elevated absorbance at 575 nm as compared to deionized water.

Aliquots of each of the above dialyzed samples were then dialyzed for 24 hours against 200 mM EGTA (Sigma) in deionized water, pH 8.0, to remove bound calcium, and then dialyzed for a further 48 h against deionized water to remove the calcium-chelating agent. The samples were assayed for calcium as described above. Concanavalin A (Sigma) and BSA (Sigma) were used as the positive and negative controls, respectively. They were treated as outlined above except that they were initially incubated for 2 h in 200 mM CaCl_2 prior to the dialysis steps.

2.8.4 Effect of EGTA

MW5 cell envelopes (20 mg protein) were resuspended in 10 ml of 6 M urea (in 10 mM HEPES, pH 8.0) and incubated for 1.5 h at room temperature with constant stirring. The envelopes were collected by centrifugation at 48,000 x g for 30 min at 4C. The pellet was discarded. The supernatant was centrifuged at 150,000 x g for 2 h and then one-half was dialyzed against HEPES buffer while the other half was dialyzed against the same buffer containing 20 mM EGTA (Sigma) at 4C overnight (18 h). The dialysates were then removed from the tubing and analyzed by SDS-PAGE.

2.8.5 Inhibition of Proteolysis in the presence of EGTA

MW5 cell envelopes (1.0 mg each) were resuspended in 1.0 ml of 6 M urea (in 10 mM HEPES, pH 7.5) containing: (i) 1 mM TPCK, (ii) 5 mM EGTA, or (iii) 1 mM TPCK and 5 mM EGTA and incubated for 1.0 h with occasional mixing. The envelopes were collected by centrifugation for 3 min in an Eppendorf centrifuge and the pellets discarded. Aliquots of all three supernatants were reserved for analysis by SDS-PAGE. The remainder of supernatants (i) and (ii) were dialyzed against 0.1 mM TPCK and 5 mM EGTA respectively. An aliquot of supernatant (iii) was dialyzed against 0.1 mM TPCK while a second aliquot was dialyzed against 1.0 mM TPCK. Dialysis was performed at 4C overnight (18 h) after which the dialysates were removed from the tubing and analyzed by SDS-PAGE.

2.9. Relatedness

2.9.1 Peptide Mapping

2.9.1.1 One-Dimensional Peptide Mapping: Limited

Proteolysis in SDS-polyacrylamide Gels

The RS proteins of A. serpens MW5 were subjected to limited proteolysis in the presence of SDS and the peptides separated by gel electrophoresis according to the method of Cleveland et al. (1977). 50 ug of the 150K and 125K protein extracts (section 2.4.1) were loaded into separate wells of an SDS-polyacrylamide gel and electrophoresis conducted as described. The gels were fixed and stained with Coomassie brilliant blue and the bands corresponding to the 150K and 125K proteins were excised with a razor blade. The excised gel slices were stored in 0.125 M Tris buffer, pH 6.8 at -70C. The gel slices were pushed to the bottom of the sample wells (one slice per well) of a second SDS-polyacrylamide gel. The acrylamide concentrations of the stacking and resolving gels were 4% and 12%, respectively. Spaces around the slices were filled with an overlay buffer containing 0.125 M Tris-hydrochloride, pH 6.8, 1 mM EDTA, 20% glycerol, and 0.1% SDS (TEGS buffer). Staphylococcal V8 protease (500 U/mg of protein; Miles Laboratories) was added to the wells where desired. Electrophoresis was initiated at 4C. When the dye marker reached the resolving gel, electrophoresis was stopped. The entire slab unit was incubated at 40C for 30 min to allow proteolytic digestion of the RS proteins. The peptides

generated were then separated by electrophoresis at 4C until the dye marker reached the bottom of the resolving gel. The gel was stained with Coomassie blue and destained as described above.

2.9.1.2 Two-Dimensional Peptide Mapping

The RS proteins of A. serpens MW5 and VHA were labelled with Iodine-125, digested with either trypsin or Staphylococcal V8 protease, and separated in two dimensions on cellulose-coated thin layer chromatography (TLC) plates according to the method of Elder et al. (1977). 50 ug of each of the RS proteins were loaded into separate sample wells of an SDS-polyacrylamide gel and electrophoresis conducted as described. The gel was simultaneously fixed and stained overnight with Coomassie blue in 25% methanol-10% acetic acid and the bands corresponding to the 150K and 125K proteins excised. The gel slices were soaked in 10% methanol for 4 h with 3 changes to remove SDS and other contaminants. The gel slices were placed in separate siliconized tubes and then dried by lyophilization. The proteins were then radioiodinated with ^{125}I (Amersham; 100 mCi/ml) in the gel slice by a modification of the chloramine T method (Greenwood et al., 1963) as described by Elder et al. (1977). 22 ul of 0.5 M sodium phosphate buffer, pH 7.5, 3 ul ^{125}I (300 uCi), and 5 ul chloramine T (1mg/ml) (Fisher Scientific Co., Fairlawn, N.J.) were added sequentially to the dried gel slices and incubated for 30

min after which time the reaction was stopped by the addition of 1.0 ml of sodium bisulfite (1mg/ml). After 15 min the sodium bisulfite solution was removed and the gel slices washed with 10% methanol for 72 h with 3 changes. The gel slices were then dried by lyophilization and 50 or 100 ug of trypsin (Sigma, type XIII) or Staphylococcus V8 protease (Miles Laboratories) was added to each tube. The enzymes were prepared as 100 ug/ml stock solutions in 0.05 M ammonium carbonate buffer, pH 8.0. The labelled proteins were digested for 24 h at 37C. Alternatively, after 22 h, fresh enzyme was added at the same concentration and digestion allowed to proceed for a further 4 h. The peptide solutions were then transferred to new siliconized tubes and lyophilized overnight. The peptides were reconstituted in 20 ul of electrophoresis buffer (acetic acid:formic acid:distilled water; 15:5:80) and 1-3 ul (approximately 150,000 cpm) was spotted onto the origin of cellulose-coated TLC plates (10x10 cm) (EM Laboratories, Elmsford, N.Y.). Electrophoresis was conducted at room temperature on a high voltage electrophoresis apparatus (Camag) at 1000V for approximately 20 min. The plates were dried and chromatographed in the second dimension in buffer (butanol:pyridine:acetic acid:water, 32.5:25:5:20) containing 7% 2,5-diphenyloxazole (PPO) (w/v). The plates were dried and then analyzed by autoradiography using Kodak XAR-5 x-ray film. The autoradiographs were exposed at -70C for 16 h.

2.9.2. Antigenic Relatedness

2.9.2.1 Preparation of Antisera and Isolation of Gamma Globulin (IgG) Fractions

Antisera, specific for the 150K and 125K proteins, was raised in 2.5 kg, New Zealand white, female rabbits. A preparative SDS-polyacrylamide gel was considerably overloaded (approximately 1.8 mg of cell envelope protein) and electrophoresis conducted as usual. The gel was briefly fixed, stained, and destained and the 150K and 125K protein bands excised. The protein-containing gel slices were washed in saline and then completely homogenized by, first, grinding with a mortar and pestle and then by passage through a syringe fitted with a 26 gauge needle. Aliquots were injected into rabbits (intramuscularly) at weekly intervals for a total of 7 injections. Blood was obtained by cardiac puncture, allowed to clot at 4C, and the antisera stored at -70C. Antiserum specific for the RS protein of A. serpens VHA was prepared in a similar manner and was a gift from Dr. S. Koval, University of Western Ontario, London, Canada.

IgG fractions were isolated by passing the crude antisera through a Protein A-Sepharose column (Bio-Rad). The bound IgG was eluted with 0.1 M glycine-HCl, pH 3.0 and the absorbance of the eluted fractions measured at 280-nm. Fractions corresponding to the peaks were pooled and simultaneously concentrated and dialyzed against 10 mM phosphate-buffered saline (PBS), pH 7.4, by ultrafiltration.

IgG samples were stored at -70C.

2.9.2.2 Immunoblotting

Cell envelope proteins or RS protein extracts were separated by SDS-PAGE and then electrophoretically transferred to nitrocellulose paper (40 V, 3 hours) by the method of Towbin et al. (1979). The paper was briefly stained (3 min) with 1% Amido black in 25% methanol, 10% acetic acid and then destained in 10% methanol, 10% acetic acid in order to visualize the transferred protein. Individual lanes were excised, and washed, first with distilled water, and then with PBS. The strips were stored in buffer overnight at 4C. Available binding sites were then blocked by incubation in 1% BSA in PBS for 2 h with gentle agitation and the strips were then washed for 30 min with PBS (6 changes). The strips were then incubated for 2 h with the appropriate IgG sample (10 ug protein/lane), washed with PBS, and incubated for 2 h with goat anti-rabbit IgG-horseradish peroxidase conjugate (Bio Rad) (8 ul/lane). The strips were washed overnight (18 h) with PBS and the bound IgG visualized with 4 chloro-1-naphthol. The strips were rinsed with distilled water; allowed to air dry, and photographed using Panatomic X film (Eastman Kodak Co.).

Alternatively, for some experiments, the strips containing the transferred proteins were incubated (after the BSA blocking stage) for 2 h with the appropriate crude antiserum (10 ul/lane), washed with PBS, and incubated with

^{125}I -Protein A (approximately 10,000 cpm, New England Nuclear) for 2 h. The strips were then washed for 2 h with PBS (6 changes), allowed to air dry, and were autoradiographed using Kodak XAR-5 x-ray film. The autoradiographs were exposed at -70C for 18 h.

CHAPTER 3

RESULTS

3.1. Observations of Whole Cells

Negatively stained whole cells (Fig. 2a) displayed the characteristic ribbed, or linear, surface pattern described by Stewart and Murray (1982) and showed a moiré effect resulting from the superimposition of two layers of hexagonally-arranged subunits. The ribs were aligned at 90 degree angles to the longitudinal axis of the cell. The center-to-center spacing of the ribs was 16 nm and matched that described by Stewart and Murray (1982).

Thin sections of embedded cells demonstrated the presence of two additional layers lying external to the outer membrane layer of the cell envelope (Fig. 2b) and confirmed that the surface array of Aquaspirillum serpens MW5 was a double-layered structure.

3.2. Observations of Cell Envelopes

3.2.1 Electron Microscopy. Negatively stained cell envelopes (Fig. 3) also displayed the moiré surface pattern described by Stewart and Murray (1982) except that linearity of the array was not as well defined as with whole cells. This was probably due to forced curvature of wall layers as a result of envelope preparation techniques.

Thin sections of these envelopes showed that both layers of the array were present and had not been removed during preparation of the envelope fractions.

It should be noted that, in some cases, the moiré surface pattern was not continuous over the entire cell envelope surface; a few small patches of hexagonally-arranged subunits could be detected on the surface of some cell envelopes by negative staining-electron microscopy. This was particularly true of envelope preparations which had been stored for long periods of time (or which had been frozen and thawed several times) and suggested that the outer layer of the array had been partially removed or damaged.

3.2.2 SDS-PAGE. SDS-PAGE of the cell envelope preparation revealed three major polypeptides having apparent molecular weights of 150,000 (150K), 125,000 (125K), and 32,000 (32K) along with several (approximately 15-20) other minor proteins (Figs. 3,4). The polypeptides were component proteins of the outer membrane and surface array because Triton X-100 treatment of cell envelopes (to solubilize plasma membrane proteins) did not significantly alter the SDS-PAGE polypeptide pattern (data not shown).

The relative amounts of the Coomassie blue-stained polypeptides were detected by a densitometric scan of the electrophoretically-separated envelope proteins (Fig. 5). The 32K polypeptide was the most abundant protein within

the cell envelope. The ratio of the 150K:125K proteins was 1.35:1.

3.2.3 Identification of Peptidoglycan-Associated Protein

All cell envelope proteins were completely solubilized by heating at either 60C or 100C in 2% SDS for 30 min (Fig. 6). Only a very minor amount of the 32K protein was solubilized when envelopes were heated at 50C in 2% SDS for the same length of time. A slight reduction in the amount of solubilized 150K polypeptide was also observed. Coomassie blue-stained material was detected both in the sample wells of the stacking gel and at the interface between the stacking and resolving gels. The insolubility of the 32K polypeptide in SDS at temperatures below 60C, together with its molecular weight, suggests its classification as a peptidoglycan-associated protein (Rosenbusch, 1974).

3.3. Effects of Chaotropic Agents

Stewart and Murray (1982) had previously determined that the surface array of *A. serpens* MW5 consisted of two layers of hexagonally-arranged subunits which overlapped to produce a moiré surface pattern. This structural information aided in assessing the effects of various chaotropic agents on the array. Cell envelopes treated with the agents were examined by negative staining-electron microscopy. The results are summarized in Table 1 and

involved no effect (moiré surface pattern), removal of one layer (individual or hexagonally-arranged units), removal of the entire array (outer membrane), or removal and damage to the outer membrane (disrupted outer membrane).

3.3.1 Electron microscopy. The surface array of A. serpens MW5 was completely removed from cell envelopes by treatment with 6 M urea, 1.5 M guanidine hydrochloride, or 20 mM LIS. Cell envelopes treated with these agents displayed a smooth outer membrane surface when examined by negative staining-electron microscopy (Fig. 7). Treatment with 2% SDS or strong base (pH 12) also appeared to remove the surface array but severely disrupted the integrity of the outer membrane (Figs. 7d, 11d).

Treatment of cell envelopes with 3 M urea, metal chelating agents (EDTA, EGTA, sodium oxalate, sodium citrate), salts (lanthanum nitrate, protamine chloride), or alkali (pH 9.5-11) appeared to partially solubilize the array since either individual or hexagonally-arranged subunits could be detected on the outer membrane surfaces of negatively stained samples (Figs. 9, 11). Treatment of cell envelopes with either EDTA or EGTA disrupted the integrity of the outer membrane and significant blebbing of the membrane could be detected (Fig. 9b,c).

Non-ionic and cationic detergents, disulfide bond-breaking agents, and low concentrations of chaotropes (eg. 1.5 M urea) had no visible effect on the surface

structure.

3.3.2 SDS-PAGE. SDS-PAGE of envelopes treated with 6 M urea, 20 mM LIS, or 1.5 M guanidine hydrochloride showed that both the 150K and 125K polypeptides as well as a number of minor proteins were solubilized by these treatments (Fig. 8). Considerable degradation of the two major polypeptides was observed when guanidine hydrochloride was used as the extracting agent (Fig. 8, lane C). The major 32K polypeptide was not solubilized by these treatments and remained with the cell envelope pellets.

SDS-PAGE of envelopes treated with chelating agents showed that for those samples for which a hexagonal surface pattern or individual units were detected, the 125K protein was almost completely extracted from the cell envelopes (Fig. 10). Similarly, treatment with lanthanum nitrate, protamine chloride, or 3 M urea resulted in the solubilization of the 125K polypeptide (data not shown).

SDS-PAGE of envelopes treated with a wide pH range (Figs. 12, 13) indicated that between pH 9.5 and 11.0, the 125K protein was preferentially solubilized (Fig. 13). Treatment with strong base (pH 12.0) solubilized most envelope proteins (Fig. 13).

3.4. Sequential Extraction of the Surface Array Components

The results described in section 3.3. suggested the

Table 1. Effect of Chaotropic Agents on the Surface Array

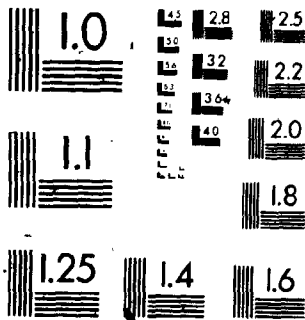
<u>Agent</u>	<u>Effect on Array^a</u>	<u>Appearance by Negative Staining- Electron Microscopy.</u>
1.5 M urea	-	moiré
3.0 M urea	+/-	hexagonal
6.0 M urea	+	outer membrane
1.5 M GuHCl	+	outer membrane
0.02 M LIS	+	outer membrane
1.0 M LiCl	-	moiré
25 ug/ml protamine chloride	+/-	hexagonal
0.05 M TbCl ₃	-	moiré
0.5 M LaNO ₃	+/-	hexagonal
0.2 M EDTA	+/-	units/disrupted OM
0.2 M EGTA	+/-	units/disrupted OM
1.0 M sodium oxalate	+/-	hexagonal
1.0 M sodium citrate	+/-	hexagonal
0.05 M 8OH-quinoline	-	moiré
pH 3.0-4.0	+/-	disorganized array/ disrupted OM
pH 4.5-9.0	-	moiré
pH 9.5-11.0	+/-	hexagonal
pH 11.5-12.0	+	disrupted OM
2.0% SDS	+	disrupted OM
2.0% Brij 58	-	moiré
2.0% n-octylglucoside	-	moiré
2.0% CTAB	-	moiré

a: + RS array entirely removed from cell envelopes
 - no effect
 +/- partial solubilization of the array

Abbreviations: GuHCl, guanidine hydrochloride; LIS, lithium diiodosalicylate; EDTA, ethylenediaminetetraacetate; EGTA, ethyleneglycol-bis-(B-aminoethyl ether)-N,N'-tetraacetate; SDS, sodium dodecyl sulphate; CTAB, cetyltrimethylammonium bromide; OM, outer membrane.

2

MICROCOPY RESOLUTION TEST CHART
NBS 1010a
(ANSI and ISO TEST CHART No. 2)



use of a sequential extraction procedure for the isolation of the components of the array: alkali extraction of the outer layer followed by 6 M urea extraction of the inner layer. The procedure was monitored by both SDS-PAGE (Fig. 14) and electron microscopy (Fig. 15).

3.4.1 SDS-PAGE. Treatment of cell envelopes with a high-pH buffer followed by 6 M urea resulted in the sequential extraction of the 125K and 150K proteins, respectively. The initial high-pH treatment solubilized most of the 125K protein (Fig. 14, lane b), and only small amounts of other polypeptides. The pH 10.3-extracted pellet retained only a small amount of the 125K protein (lane c). Subsequent treatment of these envelopes with 6 M urea resulted in the extraction of the 150K protein (lane d). This extract also contained other minor protein bands. The final pellet from this two-stage extraction (lane e) did not contain either of the major high-molecular-weight polypeptides.

3.4.2 Electron microscopy. Negatively stained control envelopes (Fig. 15a) possessed the characteristic moiré surface pattern of intact cells. Thin sections of these envelopes confirmed the presence of both surface layers. The surface pattern changed on the envelopes extracted at pH 10.3 (panel c) and showed individual subunits arranged in a hexagonal pattern. The center-to-center spacing

between the individual subunits was about 17 nm. This suggested that the outermost layer of the array had been removed by the high pH treatment. Thin sectioning of these envelopes confirmed the presence of only one superficial layer. A regular surface pattern could not be detected on negatively stained envelopes which had been extracted initially by the high-pH buffer, and, subsequently, with 6 M urea (panel e); thin sections of these envelopes confirmed that both layers had been removed.

3.5. Assembly In Vitro

3.5.1. Reassembly onto Outer Membrane--Homologous Reattachment

The ability of the 150K and 125K proteins to reassemble onto the outer membrane surface of RS⁻ cell envelopes was tested under a variety of ionic conditions. 6 M urea-extracted surface array proteins were used for the in vitro studies described despite the presence of several other minor proteins in addition to the 150K and 125K polypeptides (Fig. 17, lane a). Cell envelopes prepared from the RS⁻ variant of strain MW5 (Fig. 16a) did not possess a surface array and did not contain the 150K and 125K proteins. These envelopes were used as the template for the in vitro reconstitutions because they would serve as an effective indicator of reassembled surface protein.

3.5.1.1 Electron microscopy. The RS proteins of A. serpens MW5 reassembled onto the outer membrane surface of RS⁻ cell envelopes in the presence of 10 mM Ca²⁺ or Sr²⁺ (Fig. 16c). The proteins reassembled to form the ribbed, or linear, pattern characteristic of the intact cell surface. The center-to-center spacing of the reassembled linear elements averaged 16 nm and matched that reported for whole cells (Fig. 2a). The continuity of the in vivo subunit pattern, however, was not duplicated; instead, a mosaic of regular "patches" could be detected. This 'crazy paving' effect (Sleytr, 1976) suggested that the reassembly was initiated at, and extended from, discrete sites which were variable distances apart. Dialysis against distilled water or other cations failed to induce the reassembly. Small, random patches of hexagonally-arranged subunits could be detected, however, on a few of these envelopes (Fig. 16b). Identical results were obtained when cell envelopes of strain MW5 (which had been stripped of the surface array with 6 M urea) were used as the template for the in vitro reconstitutions or when higher (20 mM) concentrations of the monovalent cations and MgCl₂ were used (results not shown).

3.5.1.2 SDS-PAGE. The 150K and 125K proteins were only found associated with the cell envelopes of those reassembly mixtures which had been dialyzed against Ca²⁺ or Sr²⁺ (Fig. 17). The cell envelopes of the other

preparations contained only a small amount of the 150K protein.

3.5.2 Reassembly onto Heterologous Templates

The 6 M urea-extracted surface proteins of A. serpens MW5 reassembled onto the outer membrane surfaces of cell envelopes prepared from A. serpens VHL and, less effectively, onto cell envelopes prepared from the type strain, A. serpens 12638 (Fig. 18). The reassembled surfaces appeared similar in pattern to the reconstituted envelopes of A. serpens MW5 (Fig. 16c). The RS proteins did not reassemble onto cell envelopes of E. coli or Pseudomonas aeruginosa using identical experimental conditions (Fig. 18).

3.5.3. Sequential Reassembly onto Outer Membrane

The ability of the components of each of the two layers to reassemble in tandem onto an outer membrane surface was examined. The two surface layers could be reassembled in sequence onto the RS⁻ cell envelopes, but the order was critical.

3.5.3.1 Electron microscopy. The 150K protein reassembled, in the presence of Ca²⁺, into a hexagonal array on the outer membrane surface (Fig 19a). The center-to-center spacing between the subunits averaged 17 nm and matched that reported for the pH 10.3-extracted

envelopes (Fig. 15c). The 125K protein failed to reassemble onto a similar template under identical conditions. The 125K protein did reassemble onto cell envelopes on which the 150K protein had previously ordered itself into a hexagonal array. Negatively stained cell envelopes revealed random patches of linear array in various orientations (Fig. 19b), indicating that the second, or outer, surface layer had been added to the inner protein layer. The center-to-center spacing of the ribs averaged 16 nm. The 125K protein did not reassemble onto the hexagonally-packed RS layer of A. serpens VHA.

3.5.3.2 SDS-PAGE. RS⁻ cell envelopes lacked the 150K and 125K proteins (Fig. 20, lane b). The 150K protein reassembled onto these envelopes (lane d) and provided a template for the reassembly of the 125K protein (lane f). The protein composition of the fully reconstituted envelopes matched that of the control envelopes (lane g).

3.5.4. Self-Assembly

The 150K and 125K proteins, extracted from cell envelopes with 6 M urea, self-assembled in vitro to form planar sheets when the urea was removed by dialysis against 10 mM CaCl₂ or SrCl₂ (Fig. 21). The self-assembled products possessed the ribbed pattern characteristic of the intact cell surface, indicating that both layers of the array had reassembled. The center-to-center spacing

between the ribs was 16 nm. Similar products were obtained when the assemblies were conducted at room temperature, when 10 mM HEPES buffer (pH 7.0) (containing 10 mM Ca^+ or Sr^{2+}) was used instead of distilled water, or when lower concentrations (0.5 mM, 1.0 mM) of CaCl_2 were used to induce the self-assembly (Fig. 21). Cation composition and protein concentration proved to be the most important parameters of assembly; dialysis against distilled water alone or 10 mM NaCl , KCl , LiCl , or MgCl_2 , failed to induce self-assembly as did samples in which the protein concentration was less than 100 $\mu\text{g}/\text{ml}$.

SDS-PAGE of these products confirmed that both the 150K and 125K proteins were participating in the self-assembly (Fig. 22).

The 150K protein self-assembled to form patches of hexagonally-arranged subunits when dialyzed alone against 10 mM CaCl_2 or SrCl_2 (Fig. 23). SDS-PAGE of these assembly products showed that they were composed of the major 150K polypeptide although a number of minor polypeptides were also present in these preparations (Fig. 23). The 125K protein failed to self-assemble to an ordered array using identical conditions. Subsequent dialysis of the 125K protein against higher concentrations (20 mM, 50 mM) of CaCl_2 or SrCl_2 in distilled water or buffer (10, 20, or 50 mM HEPES, pH 7.0), also failed to induce self-assembly even when the protein concentration of the sample was increased to 2 mg/ml (results not shown).

3.5.4.1 Image Analysis of Reassembly Products

Appropriate regions of the negatively stained self-assembly products were selected for further examination by standard image analysis methods. Visual inspection of the hexagonally-arranged layer (assembled 150K protein) (Fig. 24a) suggested that the array had sixfold symmetry and this was clearly demonstrated by its diffraction pattern (Fig. 24b). Negatively stained double-layered sheets (assembled 150K + 125K protein) (Fig. 25a) demonstrated the ribbed, or moiré, pattern characteristic of the intact cell surface. The overlapping of the two layers resulted in a somewhat 'noisy' diffraction pattern but one which still illustrated the linear banding observed with the negatively stained image (Fig. 25b). The reconstructed images of the self-assembly products (Fig. 26) were highly similar (but not identical) to those previously obtained for the *in vivo* array (Stewart and Murray, 1982). The single, hexagonally-arranged layer (assembled 150K protein) was composed of doughnut-shaped units connected by 'delta' or direct linkers (Fig. 26a). A moiré, or overlapping, pattern was obtained for the double-layered sheet (assembled 150K and 125K protein) (Fig. 26b). The reconstructed image confirmed that it is the additive effect of two superimposed protein layers which produces the characteristic linear pattern of the assembled product.

3.6. Biochemical Properties of the RS Proteins

3.6.1 Effect of Heating on the Mobility of the RS proteins in SDS-polyacrylamide Gels

All protein extracts were routinely heated at 100C in the sample buffer of Laemmli et al. (1970) for 3 min prior to electrophoresis. To test the effect of heating on the mobility of the 150K and 125K proteins, aliquots of 6 M urea-extracted and dialyzed surface protein (at 2 mg protein/ml) were mixed 1:1 with sample buffer and either heated as described or not heated prior to electrophoresis. The RS proteins in the heated samples migrated to their usual 150K and 125K positions in an SDS-polyacrylamide gel (Fig. 27). The 125K protein in the unheated sample migrated to the same position as in the heated sample. The mobility of the 150K protein, however, was greatly reduced when the sample was not heated. The 150K protein migrated to a position corresponding to an apparent molecular weight of 420,000 (Fig. 28). The same heating effect was observed with the 150K protein isolated by the sequential protein extraction procedure (section 2.4) (Fig. 29). Heated and unheated aliquots were examined by negative staining-electron microscopy (Fig. 29). Circular, doughnut-shaped structures (11.5-12.0 nm in diameter) could be detected in the 150K protein extract which was not heated. These units were similar in size and morphology to the units which comprise the inner, hexagonally-arranged, surface layer. The doughnut-shaped structures were no

longer visible upon heating of the sample. The 150K protein, therefore, can be isolated both as an intact unit of the array and as an oligomeric protein. The approximate molecular weight of the oligomer (420,000) (Fig. 28) suggested that it was composed of three 150K subunits. Similar doughnut-shaped units could not be detected in negatively stained samples of the 125K protein extract.

SDS-PAGE analysis of unheated 150K protein extracts (which had been stored at -10C or -20C for long periods of time) indicated that some of the 420K oligomers had dissociated into their component 150K monomers (results not shown). Intermediates (eg. dimers) were never detected.

3.6.2 Non-denaturing PAGE

The 150K and 125K proteins were not easily separated by electrophoresis in a native 5% polyacrylamide gel (Fig. 30). The 125K polypeptide showed slightly greater mobility than the 150K protein. 6 M urea-extracted and dialyzed surface proteins were resolved into separate bands only when the gel sample was underloaded.

3.6.3 Determination of Isoelectric Point

The 150K and 125K proteins were not separated by isoelectric focusing in an ampholine gradient of pH 4-6 (Fig. 31). Both were shown to have isoelectric points of 4.7.

3.6.4 Determination of Carbohydrate

Carbohydrate was not detected in RS protein extracts using the phenol-sulfuric method of Dubois et al. (1956). The 150K and 125K polypeptides, separated by electrophoresis in an SDS-polyacrylamide gel, did not react positively for carbohydrate using the periodic acid-Schiff method.

3.6.5 Amino Acid Analysis

The amino acid profiles of the 150K and 125K proteins (Table 2) were similar to each other and to that of the RS protein of A. serpens VHA (included in Table 2 for comparison). The amino acids of the 150K and 125K proteins included a high proportion of acidic (asp, glu: 23.3% and 21.6%, respectively) and non-polar types (val, leu, ile, phe, ala, pro: 42.1% and 40.4%, respectively). The 125K protein contained a slightly higher proportion of polar residues (thre, ser, tyr: 20.5%) than did the 150K protein (15.5%).

3.7. Attempted Purification of the RS Proteins; Stability

3.7.1 Ion-Exchange Chromatography

The 150K protein was eluted from a DEAE-Sepharose CL-6B column over a range of 0.05-0.1 M NaCl (Fig. 32A). The sample, however, contained several minor polypeptides in addition to the major 150K protein. The use of a steeper elution gradient (0-0.25 M NaCl), the collection of

Table 2. Amino acid compositions of the RS proteins of Aquaspirillum serpens strains MW5 and VHA

Amino acid ^a	Proportion ^b		
	MW5-150K	MW5-125K	VHA-140K ^c
asp	17.0	16.3	16.8
thr	9.4	13.2	14.4
ser	4.6	6.6	5.6
glu	6.3	5.3	4.3
pro	1.5	0.4	1.5
gly	11.0	12.2	14.0
ala	14.9	15.5	15.0
val	9.6	7.6	7.5
ile	4.3	5.5	4.8
leu	8.4	8.4	8.0
tyr	1.5	0.7	1.2
phe	3.4	3.0	2.0
his	0.7	0.4	0.3
lys	4.8	4.3	3.3
arg	2.0	0.6	1.3

a: tryptophan is destroyed in the acid hydrolysis of the protein; cysteine, methionine not determined.

b: expressed as % (mole/mole).

c: taken from Buckmire and Murray (1973)

smaller fraction volumes (and analysis of individual fractions) or attempted separation by Sephadex G-200 chromatography failed to eliminate the minor polypeptides (data not shown). The 125K protein bound to the column but was not eluted by the NaCl gradient. Similar gradients of other salts (KCl) also failed to elute the protein as did a decreasing pH gradient (0.01 M imidazole buffer, pH 6.5-4.0). Similarly, the 125K protein adhered to a Sephadex G-200 column but was never eluted (data not shown). The 125K protein (and its major degradative form) bound to the ion-exchange resin in batch tests and was only eluted at NaCl concentrations of 1.0 M or 1.5 M. SDS-PAGE showed, however, that the protein eluted at these concentrations consisted of a number of minor polypeptides of molecular weights less than 125K which were not present in the original sample (Fig. 32B). These were believed to be degradation products of the 125K polypeptide.

3.7.2 Polyethylene Glycol Precipitation of RS Protein

The 125K RS protein does not self-assemble in vitro. Other protein precipitation methods, therefore, were tested. Attempts were made to eliminate some of the minor, lower-molecular-weight polypeptides detected by SDS-PAGE of the 125K protein extract by selective PEG precipitation of either these minor bands or the major RS protein. Initial testing showed that the 125K protein was precipitated by 10% concentrations of either PEG 400, 1000, 1500, 8000, or

20,000 although the latter three proved the most efficient (ie. precipitated the most protein). SDS-PAGE of these precipitates, however, showed that most of the minor polypeptides present in the original sample co-precipitated with the major polypeptide (results not shown). A variety of PEG 1500 concentrations were then tested. Subsequent mixing of the 125K protein extract with PEG 1500 (to give final PEG concentrations of 3%, 15%, and 25%) did not, however, separate the major 125K protein from the lower-molecular-weight polypeptides (Fig. 33).

3.7.3 Stability of the RS Proteins: Inhibition of Proteolysis

At least some of the minor, lower-molecular-weight, polypeptides detected by SDS-PAGE of the sequentially-extracted RS proteins were believed to be degradation products of the major 150K and 125K proteins because antisera specific for each of the two proteins cross-reacted with most of these minor proteins (Fig. 34). The number and intensity of the minor bands increased following overnight dialysis at 4C to remove the extracting agents and, again, upon subsequent storage at -10C (Fig. 35). The 150K protein appeared more stable than the 125K protein since more extensive degradation was observed with the latter over time.

The inclusion of certain protease inhibitors (PMSF, TPCK, dithiothreitol) during cell breakage and envelope

preparation did not alter the SDS-PAGE polypeptide pattern of the envelopes nor did carrying out all steps of the sequential protein extraction procedure at 4C lessen the extent of RS protein degradation (data not shown). The inclusion of anti-proteolytic agents during the extraction and dialysis stages, however, minimized the proteolytic degradation of the 125K protein (Fig. 36). The 125K polypeptide partially degrades to a major lower-molecular-weight form following overnight dialysis against deionized water at 4C. This degradation was inhibited to varying extents by dithiothreitol, L-cysteine hydrochloride, TLCK, TPCK, aprotinin, and PCMBs but several minor polypeptide bands could still be detected in these preparations. Anti-proteolytic agents added during the extraction and dialysis stages did not significantly alter the degradation of the 150K protein (results not shown).

3.8. Role of Calcium :

3.8.1 Growth in Calcium-free Media

Cell envelopes prepared from cells grown in either FSS broth or a defined medium for aquaspirilla (Whitby and Murray, 1980) containing 1.0 mM CaCl_2 possessed a surface array as detected by negative staining-electron microscopy (Fig. 37a). Cells grown in defined media containing CaCl_2 and then repeatedly transferred in calcium-free defined media, lost the regularly-structured surface layer. Small patches of hexagonally-arranged units were detected on only

a few of these envelopes (Fig. 37b). SDS-PAGE of the envelope preparations (Fig. 38) showed that those envelopes which had lost their RS layer were also deficient in, or lacked altogether, the 150K and 125K polypeptides.

3.8.2 Effect of Calcium on the Electrophoretic Mobility of the RS Proteins

The 150K and 125K proteins, extracted from cell envelopes with 6 M urea, showed identical mobilities by SDS-PAGE whether the urea was removed by dialysis against deionized water or 10 mM CaCl_2 (Fig. 39). However, when the RS proteins were extracted from cell envelopes with 6 M urea containing 10 mM Ca^{2+} and then dialyzed against 10 mM Ca^{2+} to remove the urea, the electrophoretic mobility of the two major polypeptides increased (Fig. 39).

3.8.3 Calcium-binding Assay.

The 150K and 125K proteins behaved as calcium-binding proteins using a spectrophotometric method for the detection of calcium (Table 3). The chromogenic agent, o-cresolphthalein complexone (CC), was added to separate solutions of bovine serum albumin (BSA), concanavalin A (Con A), and the two RS proteins of MW5. Cresolphthalein complexone reacts with calcium to form a purple colour which absorbs at 575 nm (Pollard and Martin, 1956). The optical density, measured at this wavelength, is directly proportional to the amount of calcium present (Pollard and

Martin, 1956). BSA and Con A were both initially incubated with calcium. The protein solutions were then dialyzed against deionized water and CC added. Extensive dialysis of all samples against deionized water ensured that any calcium detected would reflect that bound to the proteins and not free calcium. BSA (a non-calcium-binding protein) failed to bind any of the provided calcium (Table 3). Con A (a calcium-binding protein) did bind calcium as was reflected by the increased absorption at 575 nm (Table 3).

This bound calcium was removed after treatment with EGTA, a calcium-chelating agent. The RS proteins of *A. serpens* MW5 were treated as outlined above except that they were not initially incubated with calcium. Any bound calcium, therefore, would be that scavenged from the growth medium or from buffers used during the preparation of cell envelopes prior to the extraction procedure. Both proteins were found to contain bound calcium (Table 3), and the 125K protein appeared to bind calcium to a greater extent than did the 150K protein. As with Con A, the calcium could be effectively removed by subsequent treatment with EGTA.

3.8.4 Effect of EGTA

Dialysis of 6 M urea-extracted surface proteins against 20 mM EGTA (a calcium-chelating agent) or extraction of the surface proteins using 6 M urea which contained EGTA resulted in extensive degradation of the 150K and 125K polypeptides (Fig. 40, 41). Identical

Table 3. Spectrophotometric Determination of Calcium

<u>Sample</u>	<u>Optical Density (575 nm)</u>	
	<u>After Dialysis vs DW</u>	<u>After Dialysis vs EGTA</u>
BSA + Ca ²⁺	0.001	0.000
Con A + Ca ²⁺	0.115	0.001
MW5 RS proteins (150K + 125K)	0.090	0.000
150K protein	0.040	0.002
125K protein	0.125	0.001

Abbreviations: BSA, bovine serum albumin;
 Con A, Concanavalin A;
 EGTA, ethyleneglycol-bis-(β -aminoethyl-
 ether)N,N'-tetraacetate;
 DW, deionized water.

results were obtained when EDTA was substituted for EGTA (data not shown). This degradation was partially inhibited by including 1 mM TPCK in the extraction buffer (Fig. 41).

The inhibitory effect was lost upon dialysis against a reduced concentration of inhibitor (0.1 mM TPCK) but could be partially maintained if the inhibitor concentration remained at 1.0 mM throughout dialysis.

3.9. Relatedness

3.9.1. Peptide Mapping

3.9.1.1 One-dimensional Peptide Mapping: Limited

Proteolysis in SDS-polyacrylamide gels

Limited proteolysis of the 150K and 125K polypeptides using Staphylococcus aureus V-8 enzyme produced different peptide profiles (Fig. 42). Twelve bands could be resolved in the 150K protein sample (lane C) while 15 bands were detected for the 125K protein (lane D). Of these, only two were found to be common to both proteins (indicated by arrows) and these were present in different proportions.

3.9.1.2 Two-dimensional Peptide Mapping

The 150K and 125K proteins of A. serpens MW5 and the 140K RS protein of A. serpens VHA, proteolytically-digested with either trypsin or Staphylococcus aureus V8 enzyme, gave reproducible peptide patterns (Figs. 43, 44). The use of higher concentrations of enzyme, longer digestion times, or the addition of fresh enzyme after an initial incubation

period, did not alter the profiles. The 150K and 125K proteins shared approximately 12 peptides (out of a total of about 28) when digested with trypsin and 8 peptides when digested with the V8 enzyme. The 150K and 125K proteins each had approximately 4-5 peptides in common with the VHA RS protein using either enzyme. Overall, the 150K and 125K proteins appeared more similar to each other than did either to the 140K protein of strain VHA

3.9.2 Antigenic Relatedness (Immunoblotting)

Crude antisera raised against each of the two RS proteins of strain MW5 did not react with either of the 150K or 125K proteins in Ouchterlony double diffusion assays or by crossed immunoelectrophoresis (data not shown). This may have been due to the fact that the antibody was raised in response to challenge by SDS-denatured protein and may not, therefore, recognize native protein. Alternatively, a weak immune response may have been elicited. To increase the sensitivity of the reactions, IgG was isolated from the crude antiserum preparations and the Western blotting method used to test for cross-reactivity (Fig. 45). IgG isolated from antisera raised against the 150K protein (anti-150K IgG) did not bind to the 125K protein of strain MW5 and reacted only very slightly with the 140K RS protein of strain VHA although anti-150K IgG did react with the 150K polypeptide (Fig. 45). Crude antisera specific for the 150K protein

did cross-react, however, with the 125K protein using ^{125}I -labelled Protein A to detect bound antibody (Fig. 34). Anti-125K IgG reacted with both the 150K protein and the VHA RS protein (Fig. 45). Anti-VHA IgG bound to both of the RS proteins of strain MW5 although greater reactivity was shown with the 125K protein. A few of the minor cell envelope proteins also reacted slightly with each of the IgG samples as did the peptidoglycan-associated proteins of the cell envelopes of both MW5 and VHA.

CHAPTER 4

DISCUSSION

Bacterial surface arrays possess a number of features which make them attractive for study. Their paracrystalline regularity of fine structure make them excellent subjects for analysis by image processing techniques and, therefore, for the study of macromolecular arrangement. They are also, however, self-assembly systems in which all of the information necessary for their polymerization into regular arrays resides within each protein or glycoprotein subunit through their inherent bonding capacities. They have, therefore, in more recent years, been recognized as excellent models for the study of macromolecular assembly.

This study has focused on the assembly and biochemistry of the surface array of A. serpens MW5 whose two-layered array provides a more complex model for the assembly of a biological structure. Previous studies in this laboratory have examined the RS layers of a number of Aquaspirillum sp. (Murray, 1963; Buckmire, 1970; Buckmire and Murray, 1970, 1973, 1976; Beveridge and Murray, 1974, 1975, 1976, a, b, c; Koval and Murray, 1981, 1983, 1984, 1985). The present study provided an opportunity to compare the biochemical and assembly properties of the RS

proteins of A. serpens MW5 to those of other aquaspirilla and, in particular, to the RS protein of a closely-related strain, A. serpens VHA.

4.1 Identification of Surface Array Components

Analysis of the protein composition of A. serpens MW5 cell envelopes provided two possible candidates for the surface array components, the 150K and 125K polypeptides. Both were present in the large proportions required for a structure which surrounds the entire bacterial cell. The major 32K polypeptide and multiple minor protein bands were characteristic of the electrophoretic patterns commonly found for the outer membrane proteins of Gram-negative bacteria (Lugtenberg et al. 1977; Osborn and Wu, 1980) and was quite similar to that previously demonstrated for a related strain, A. serpens VHA (Koval and Murray, 1981). The 32K polypeptide was tentatively identified as a peptidoglycan-associated protein since heating the cell envelopes at temperatures above 50C in the presence of 2.0% SDS was required for its solubilization (Rosenbusch, 1974).

The subunits comprising the RS layers of a number of different bacteria have been found to interact with one another and with underlying cell wall components through non-covalent forces including hydrogen bonding, ionic bonding, and hydrophobic interactions (Nermut and Murray, 1967; Buckmire and Murray, 1970; Beveridge and Murray, 1976a,b; Howard and Tipper, 1973; Thornley et al., 1974;

Sleytr, 1976; Hastie and Brinton, 1979a; Masuda and Kawata, 1980; Michel et al., 1980; Sleytr and Plohberger, 1980; Kay et al., 1981, Tsuboi et al., 1982; Thompson et al., 1982; Bingle et al., 1984). Agents which effectively disrupt these forces have been widely used in the isolation of these structures (reviewed in Sleytr and Messner, 1983; Koval and Murray, 1984). The development of effective isolation procedures allows the identification of surface array components and provides an indication as to the nature of the attachment of the array to underlying wall layers and the nature of the subunit-subunit interactions within the protein array. 6 M urea, 1.5 M guanidine hydrochloride (both hydrogen bond-breaking agents), and 20 mM LIS (a peptide-solubilizing agent) completely removed the surface array from A. serpens MW5 cell envelopes and concomitantly extracted the 150K and 125K proteins as well as several minor polypeptides. These results strongly implicated the two major high-molecular-weight proteins as surface array components and suggested that attachment of the array to the outer membrane was mediated by hydrogen bonds.

The ability of metal-chelating agents, trivalent salts, and alkali to selectively solubilize the outermost RS layer suggested that the interaction between the two protein layers was primarily ionic and probably mediated by divalent cations. The extraction of the outer layer by treatment with EGTA suggested that calcium was involved.

Magnesium did not appear to play a role because treatment of cell envelopes with 8-hydroxyquinoline (a Mg^{2+} -specific chelating agent) did not solubilize the outer layer.

The sequential protein extraction procedure provided the means of testing independently the contributions of each of the 150K and 125K proteins to the structure of the surface array. The two step procedure, involving initial treatment of cell envelopes with a high pH buffer followed by 6 M urea treatment, effected the sequential extraction of the 125K and 150K proteins. These events, monitored by electron microscopy, demonstrated a correlation between the extraction of the 125K protein and the removal of the outermost surface layer and between the extraction of the 150K protein and the removal of the innermost layer. The two layers of A. serpens MW5 appeared to be composed, therefore, of different proteins. Although chemical analyses of the isolated surface arrays from several bacteria have found each to be composed of a single protein species (Sleytr and Messner, 1983), it should be noted that in each instance the surface structure examined consisted of only a single layer of subunits. Yamada et al. (1981) and Tsuboi et al. (1982) examined the double-layered surface array of Bacillus brevis 47 and found the two layers to be composed of different proteins.

Densitometric scans of cell envelope proteins (separated by SDS-PAGE) showed that the ratio of 150K:125K protein was 1.35:1. This was in agreement with the

observation that the moiré surface pattern was not always continuous over the cell envelope surface; small patches of hexagonally-arranged subunits were occasionally detected (by negative staining-electron microscopy) which suggested that certain areas within the outer layer of the array had been damaged or partially removed. This was particularly true of cell envelopes which had been frozen for long periods of time or frozen and thawed repeatedly.

4.2. Assembly In Vitro

4.2.1 Sequential Reassembly In Vitro

Sequential reassembly experiments provided further support for the identification of the surface array components of A. serpens MW5 because the proteins comprising the two surface layers were able to reassemble onto a cell envelope template only in their natural order. The inability of the outer layer protein (125K protein) to reassemble onto an outer membrane surface suggested that the two RS proteins of strain MW5 are structurally suited to take part in different types of molecular interactions. This difference may be responsible for restricting the structure to two layers--ie. the layers are not infinitely 'stackable'. The reassembly of the 125K protein onto a protein template appeared to involve some specificity because the 125K protein would not reassemble onto the hexagonally-packed RS layer of A. serpens VHA. The RS layer of strain VHA and the innermost hexagonally-packed

layer of strain MW5 differ structurally, however, in terms of subunit-subunit spacing and in the types of linkers which connect neighboring units. These differences appeared sufficient, in this case, to prevent an effective reassembly.

4.2.2 Reassembly onto Outer Membrane

The 150K and 125K array proteins of strain MW5 reassembled together in vitro to form a double-layered array on an outer membrane surface in the presence of either Ca^{2+} or Sr^{2+} . The reassembly of isolated RS proteins onto the walls from which they had originally been removed is a common observance (Buckmire and Murray, 1973, 1976; Thorne et al., 1975; Sleytr, 1975, 1976; Beveridge and Murray, 1976a,c; Hastie and Brinton, 1979a,b; Masuda and Kawata, 1980; Tsuboi et al., 1982, Bingle et al., 1984). The observed ionic dependence is not an unusual feature in that several other Aquaspirillum spp. have been found to require certain ions (Ca^{2+} and Sr^{2+} in particular) for both the assembly and retention of their RS layers (Buckmire and Murray, 1970; 1973; 1976; Beveridge and Murray, 1974; 1975; 1976a,b,c). The in vitro assemblies of the RS proteins of Acinetobacter sp. (Thorne et al., 1975), Bacillus brevis 47 (Tsuboi et al., 1982), and Azotobacter vinelandii (Bingle et al., 1984) have also demonstrated ionic requirements. One explanation for a cation requirement lies in the nature of the surface array

proteins which contain a high proportion of acidic amino acids. Buckmire and Murray (1976) have suggested that cations may serve to reduce the surplus of negative charges surrounding these proteins to allow the close interactions necessary for their assembly into periodic arrays or, alternatively, divalent cations may act as salt bridges linking two negatively charged subunits. Calcium or strontium was required for the reattachment of the 150K protein to an outer membrane surface which suggests that divalent cations may form salt links between the RS protein and a negatively-charged outer membrane component. As is the case with other Aquaspirillum RS proteins (Buckmire and Murray, 1976), Mg^{2+} would not support the in vitro reassemblies. Beveridge and Murray (1976b) suggested that this may be due to differences in the shell sizes of the divalent cations since the ionic radius of Mg^{2+} (0.65 \AA) is considerably smaller than that of either Ca^{2+} (0.99 \AA) or Sr^{2+} (1.13 \AA) (Weast, 1971). These results were in agreement with the observation that 8-hydroxyquinoline (a chelating agent specific for magnesium) had no disruptive effect on the structure of the array and failed to solubilize any of the surface components.

Reassembly studies which used cell envelopes prepared from closely-related and different bacteria as assembly templates attempted to determine whether or not specificity was involved in reattachment. The RS proteins of strain MW5 reassembled onto the outer membrane surfaces of cell

envelopes prepared from Aquaspirillum serpens VHL and, less efficiently, onto those prepared from Aquaspirillum serpens 12638 (type strain). The assembly of the RS proteins of other bacteria onto heterologous templates has previously been demonstrated (Sleytr, 1975, 1976; Chester and Murray, 1978; Masuda and Kawata, 1980). The inability of the RS proteins of strain MW5 to reassemble onto the outer membrane surfaces of wild-type strains of E. coli or Pseudomonas aeruginosa may be explained in one of two ways. One explanation is that these organisms may lack, or possess a modified, outer membrane component specifically required for the reattachment of the RS proteins which the related strains do possess. It is more likely, however, that the outer membranes of the three Aquaspirillum serpens strains simply possess LPS, phospholipid, and proteins which share common properties and provide suitable surfaces for the reassembly of the RS proteins of strain MW5.

4.2.3 Self-Assembly

The two RS proteins of strain MW5 reassembled in vitro to form a double layer in the absence of a cell envelope template. Self-assembly is a phenomenon common to many structures composed of identical subunits (Caspar and Klug, 1962; Kushner, 1972). Although the RS proteins isolated from a number of different bacteria have demonstrated the ability to self-assemble in vitro (Brinton et al., 1969; Glauert and Thornley, 1973; Sleytr, 1976; Masuda and

Kawata, 1980; Michel et al., 1980; Bleytr and Plohberger, 1980; Tsuboi et al., 1982; Word et al., 1983), the phenomenon has not been reported for Aquaspirillum spp. The in vitro assemblies of the Aquaspirillum RS proteins have all previously been characterized as template-dependent (Buckmire and Murray, 1973; 1976; Beveridge and Murray, 1976a,c). The surface array components of A. serpens MW5 self-assembled to form planar sheets which possessed the ribbed or linear pattern characteristic of the intact cell surface. Image analysis of the self-assembled sheets showed them to be highly similar and quite comparable to the in vivo surface array material previously examined by Stewart and Murray (1982). The method used for the extraction of the RS proteins from cell envelopes did not exclude or alter, therefore, any of the components of the array. SDS-PAGE of the self-assembled sheets showed them to be composed of the 150K and 125K proteins and several minor polypeptides. The latter were suspected to be degradation products of the major RS proteins, although it is possible that some of these additional polypeptides represent minor components of the array (eg. linkers).

The two RS proteins demonstrated different individual assembly properties. The 150K protein self-assembled in vitro to form patches of hexagonally-arranged subunits connected by direct or 'delta' linkers. Image analysis of these structures showed them to be similar to the in vivo single, hexagonally-packed layer of strain MW5 previously

examined by Stewart and Murray (1982). The 125K protein failed to self-assemble despite attempts at numerous variations of assembly conditions. The method of isolating the 125K protein from cell envelopes was also varied in case the pH of the extracting buffer (10.3) was causing the irreversible denaturation of the protein and preventing self-assembly. The 125K protein, solubilized from cell envelopes by treatment with protamine chloride, lanthanum nitrate, or a variety of metal chelators (EDTA, EGTA, sodium oxalate, sodium citrate), also failed to self-assemble into a hexagonally-packed array in vitro (M. Kist, unpublished data).

One possible explanation for the inability of the 125K protein to self-assemble was provided by the examination of each of the 150K and 125K protein extracts by negative staining-electron microscopy. Negative staining of the 150K protein extract found it to consist of circular, doughnut-shaped units; similar in size and morphology to those which comprise the inner, hexagonally-packed, layer of the array. The 150K protein, therefore, is isolated as an oligomer or an intact unit. In contrast, the 125K protein is always isolated in its monomeric form.

Structural units could never be detected in 125K protein extracts despite the use of different extracting agents and conditions. The inability of the 125K protein to self-assemble in vitro into a hexagonally-packed array can be explained in light of these findings. The assembly of

the protein arrays is actually a two step process (Fig. 46) in which monomers first assemble into oligomers (or intact units) and then units assemble to form hexagonal arrays. The second step appears to be the calcium-dependent step of the assembly process since addition of calcium to the 150K protein extract induces self-assembly of the units into hexagonal arrays. The conditions for the assembly of 125K monomers into intact units was not determined although several variations of pH, ionic strength, and cation composition were tested. The observation that alkali, metal chelating agents, 1.5 M urea, and various salts all dissociated the units comprising the outer layer of the array but not the units of the inner layer, indicates different types of monomer-monomer associations within the units of the two layers. The association of the monomers within the intact 420K unit probably involves hydrophobic interactions because heating the units in detergent (SDS) was the only effective means of dissociation.

It should be noted that intact units have never been detected by negative staining-electron microscopy of the isolated 140K RS protein of *A. serpens* VHA which, like the 125K protein of strain MW5, is unable to self-assemble in vitro (S.F. Koval, personal communication). Both dissociated proteins, however, are able to reassemble onto a template in vitro which suggests that assembly from the monomeric state requires a nucleation site.

SDS-PAGE analysis of the doughnut-shaped units

comprising the inner layer of the array showed that these structures had a molecular weight of approximately 420,000. Heating the units in SDS resulted in their dissociation into component monomers with an apparent molecular weight of 150K. These results suggested that the protein which comprises the inner, hexagonally-packed, layer of the array was a trimer composed of three 150K monomers. Oligomeric forms have been demonstrated for other bacterial RS proteins (Phipps *et al.*, 1983; Ridgway and Lewin, 1983; Kay *et al.*, 1984). Glaeser *et al.* (1980) and Baumeister *et al.* (1982) estimated (from electron microscopy measurements) the molecular weights of the units which comprise the hexagonally-packed RS layers of *A. serpens* VHA and *Deinococcus radiodurans* (R1), respectively. In both cases the units were found to be hexamers, each consisting of six monomers. Trimers or hexamers are both compatible with the structure of the hexagonally-arranged layer of strain MW5. If the units are hexamers then the 150K protein may represent the dimer form of a 75K monomer which fails to dissociate further by SDS-PAGE.

4.3. Biochemical Properties of the RS Proteins

4.3.1 Amino Acid Composition; Isoelectric Point;

Calcium-binding Activity

The amino acid compositions of the RS proteins of *A. serpens* MW5 were similar both to each other and to that of a related strain, *A. serpens* VHA. They shared features in

common with other bacterial RS proteins, most notably, a high proportion of acidic amino acids and hydrophobic residues.

The acidic nature of the two proteins was confirmed by their low isoelectric point (4.7) which was comparable to that of the RS protein of strain VHA (4.6) (S.F. Koval, personal communication) and within the 4.0-6.0 range reported for other bacterial RS proteins (Thornley *et al.*, 1974; Sleytr and Thorne, 1976; Hastie and Brinton, 1979a; Smit *et al.*, 1981; Kay *et al.*, 1984). Although the 150K and 125K RS proteins were shown to possess identical isoelectric points, the proteins demonstrated differences in their binding to DEAE-Sepharose (an ion-exchange resin) which suggests differences in charge distribution.

Perhaps one of the more interesting biochemical features of the RS proteins of *A. serpens* MW5 was their behaviour as calcium-binding proteins. The spectrophotometric assay used was designed for the quantitative determination of calcium in serum, plasma, or urine (Sigma Technical Bulletin, no. 586). Difficulties arise when trying to adapt the assay for the quantitative detection of bound calcium because calcium may be buried within the protein and not accessible to the chromogenic agent, cresolphthalein complexone. The assay may still be used, however, for the qualitative detection of surface-bound calcium. Concanavalin A, a known calcium-binding protein (Van Eldik *et al.*, 1982), was shown

to contain bound calcium by this method. The two RS proteins of strain MW5 also contained bound calcium which was not unexpected given the calcium requirements of the two proteins for in vitro assembly and retention on the cell surface during culture. Of greater significance would be the examination of possible conformational changes within the structure of these proteins upon calcium-binding by circular dichroism studies using purified RS protein. The calcium-binding properties of the two RS proteins should be considered when biochemical tests which depend on the charge of the proteins are performed. The behaviour of the proteins in isoelectric focusing gels or their interaction with ion-exchange resins may, for example, be altered by tightly-bound calcium.

4.3.2 Stability and Purification of the RS Proteins

Degradation of the two RS proteins was observed during their extraction from cell envelopes. This degradation increased with subsequent dialysis and concentration steps in the isolation procedure and hindered attempts at purification. The 150K RS protein of A. serpens MW5 proved more stable than the 125K RS protein. Part of the reason for this may be morphological in that a multimeric protein (oligomer) is usually more stable than its component monomers and an assembled structure (intact unit) more stable than its dissociated subunits (Friedman and Beychok, 1979). The 150K protein is isolated both as an oligomer

(420K protein) and an intact unit unlike the units comprising the outer layer of the array which are always dissociated into 125K monomers during the isolation procedure. The greater degradation observed with the 125K protein may simply be due to a greater number of sites available for proteolytic digestion. The partial inhibition of 125K protein degradation by anti-proteolytic agents indicated that a protease was present in the protein extracts which may have been co-extracted from cell envelopes during the isolation procedure. Proteolytic degradation of both the 150K and 125K RS proteins was observed when the two proteins were extracted from cell envelopes with 6 M urea containing EGTA and this degradation could be partially inhibited by TPCK. This also suggested the presence of a co-extracted protease although a putative protease could never be completely inhibited nor separated from the RS proteins despite attempts at a variety of inhibitors and gel chromatography. Although only a few enzymes have been found to reside in the outer membrane (MacGregor *et al.*, 1979; Regnier and Thang, 1979), it is reasonable to believe that enzymes have to be built into the wall to fulfill processing and degradative functions as growth and turnover demands. Several other workers have reported similar degradation during the isolation of the RS proteins from different bacteria (Mescher *et al.*, 1974; Thompson *et al.*, 1982; Koval and Murray, 1984). Baumeister *et al.* (1982),

however, argued that the multiple polypeptide bands detected by SDS-PAGE of the purified, intact RS layer of Deinococcus radiodurans were due to proteolysis of the surface protein in vivo and not during the isolation procedure. The same pattern of electrophoretically-separated polypeptides was observed despite the use of different isolation strategies and the inclusion of a variety of protease inhibitors. It is difficult to estimate at which point proteolytic degradation of the RS proteins of A. serpens MW5 begins. While it is possible that the RS proteins could be partially cleaved in vivo, the degradation of the two proteins clearly increased with subsequent steps in the isolation procedure and the inclusion of certain protease inhibitors at least partially inhibited this degradation. It is most likely that the degradation of the RS proteins begins immediately upon cell breakage and envelope preparation when many proteolytic enzymes are released from the cell cytoplasm and wall fractions.

Koval and Murray (1984) previously reported enhanced degradation of the 140K RS protein of A. serpens VHA in the presence of EGTA and suggested that calcium stabilized the conformation of the protein. As is the case with the two RS proteins of A. serpens MW5, calcium is required for the in vitro assembly of the surface array protein of strain VHA and for its retention on the cell surface during culture. The authors proposed that removal of calcium by

EGTA resulted in localized unfolding within the protein and the exposure of a greater number of sites available for proteolytic attack. This may also be true for the RS proteins of *A. serpens* MW5. The two proteins were almost completely degraded during their isolation from cell envelopes when EGTA (or EDTA) was included in the extraction buffer. The proteins would be likely to unfold in the presence of 6 M urea alone but the unfolding may be enhanced in various regions of the protein by the removal of bound calcium by EGTA. As well, the efficient refolding or renaturation of the proteins (upon removal of the urea) would be prevented in the presence of EGTA if calcium was a determining factor in their tertiary structure. Koval and Murray (1984) found that if calcium was included at all stages during the isolation of the RS protein of *A. serpens* VHA, less degradation occurred than if calcium was omitted during the procedure. Attempts to stabilize the RS proteins of strain MW5 during their isolation using a similar strategy were unsuccessful. There was no significant decrease in the number of minor, lower molecular weight, polypeptide bands detected by SDS-PAGE analysis when the proteins were extracted and dialyzed in the presence of calcium. As with the 140K RS protein of strain VHA, however, the mobility of the extracted 150K and 125K proteins did increase which may suggest a tighter folding of the proteins in the presence of calcium. Alternatively, the enhanced degradation of the RS proteins

in the presence of EGTA (or EDTA) may be due, not to the unmasking of a greater number of sites for proteolysis, but, rather, to an EGTA- or EDTA-activated protease. Certain proteases (eg. thiol proteinases) are inhibited by metals (Mihalyi, 1978) and the chelation of the latter by EGTA (or EDTA) could effectively activate such proteases. TPCK and PCMS (protease inhibitors effective against thiol proteinases) both inhibited the degradation of the 125K protein to its major, lower-molecular-weight form.

The purification of the two RS proteins of A. serpens MW5 was never achieved and was hindered, primarily, by the degradation problems discussed. There were an exceptionally high number of additional lower molecular weight polypeptides present in each of the protein extracts which cross-reacted with antisera specific for the major RS proteins. Separation of these polypeptides on the basis of charge differences and precipitation properties proved ineffective. The self-assembly of a structure is the simplest precipitation method. SDS-PAGE of the self-assembled layers, however, showed that the minor polypeptides were not eliminated by the assembly. The cycling of the RS proteins through repeated steps of assembly-dissociation-assembly (in order to dilute out the additional proteins) also failed to eliminate the minor polypeptides (M. Kist, unpublished data).

The best strategy for purification, given the problems presented, would likely involve the following features:

(i) The inclusion of a variety of protease inhibitors during cell breakage, cell envelope preparation, and subsequent extraction of the RS proteins, followed by (ii) immediate and rapid separation of polypeptides by a chromatographic method of high resolution (eg. HPLC). In the end, however, Aquaspirillum RS proteins may simply prove to be very labile proteins. Purified bacterial adenosine triphosphatases (ATPases), for example, have been shown to be cold labile (Vogel and Steinhart, 1976).

4.3.3 Relatedness

Stewart and Murray (1982) previously examined the fine structure of the surface array of A. serpens MW5 and could not detect any structural differences between the inner and outer hexagonally-arranged protein layers. They pointed out, however, that their analysis was limited to a resolution of 2.5 nm which may not have detected minor differences. Proteins which form such similar structures would be expected to be highly related. It would seem an inefficient expenditure of energy for a cell to synthesize two different types of proteins which are to comprise two structurally-similar surface layers. One might expect the synthesis of a slightly modified form of one of the two. The amino acid compositions of the two proteins were quite similar and the possibility existed that the 125K protein

represented a modified or cleaved form of the 150K protein. As well, the molecular weight differences observed between the outer (125K) and inner (150K) layer proteins by SDS-PAGE may simply reflect differences in SDS-binding. The difference in electrophoretic mobility between the two was significantly reduced when the proteins were separated in a polyacrylamide (native) gel where SDS was omitted. Peptide mapping of the two RS proteins of strain MW5, however, showed that, while the proteins shared certain sequences, there were differences between them. One protein did not appear to be a simple modification of the other. The 150K and 125K RS proteins of strain MW5 and the 140K RS protein of strain VHA did share certain sequences and it is intriguing to think of these as representing common structural and/or functional domains. Kay *et al.* (1984) examined the RS proteins of three Aeromonas salmonicida strains and found that, while the proteins shared similar molecular weights, amino acid compositions, and N-terminal sequences, tryptic peptide mapping demonstrated differences between them. The authors, however, attributed these differences to minor positional differences in certain amino acid residues within the RS proteins. The differences observed between the RS proteins of the A. serpens strains examined must be treated with caution for the same reason. The differences observed may also be exaggerated because of the molecular weight differences between the proteins.

Antigenic cross-reactivity was observed between the RS proteins of strains MW5 and VHA although anti-150K (MW5) IgG did not bind to the 125K protein of strain MW5 and reacted only very slightly with 140K RS protein of strain VHA. Nevertheless, IgG specific for these latter proteins cross-reacted with the 150K protein. This result was consistently observed and suggests that the 150K protein may possess an immunogenic region not found or recognized on the other RS proteins. Interestingly, the 150K RS protein is the only one of the three which can be isolated as an oligomeric protein. Crude antisera specific for the 150K protein did cross-react, however, with the 125K RS protein using ^{125}I -labelled Protein A to detect bound antibody. Antigenic cross-reactivity would be expected given the shared sequences demonstrated by peptide mapping and also because antibody to the proteins was, in each case, obtained by challenging rabbits with SDS-denatured RS protein. Unfolding of the proteins should offer a greater number of similar sites for cross-reactivity. Antibody raised in response to challenge by native protein would perhaps offer a different perspective. Anti-140K (VHA) IgG reacted with both of the RS proteins of strain MW5. Similar antigenic cross-reactivity has been detected between the RS proteins of related strains within other bacterial species such as Aeromonas salmonicida (Kay et al., 1984). Word et al. (1983), however, found that antibody specific for the RS protein of Bacillus sphaericus

strain P-1 precipitated the RS proteins of only 7 out of 17 strains within its own DNA homology group and only partial identity could be demonstrated in immunodiffusion assays.

Perhaps the whole question of relatedness between bacterial RS proteins would be best answered by a genetic approach to the problem. Smit and Agabian (1984) recently cloned the gene for the RS protein of Caulobacter crescentus strain CB15 and, using a probe for the gene, found significant hybridization with the surface array genes of related strains using the Southern blot method. Unless similar genetic techniques are employed, it would be difficult to consider the RS proteins of A. serpens strains as useful taxonomic markers given the observed morphological, biochemical, and antigenic differences between them. The conservation of a protein throughout evolution depends greatly on its significance to the cell and on how great the selective pressure is to retain the structure of that protein. Random changes through mutation take place over time and are acceptable as long as the modified proteins still perform the same function and are effective in the same selective system. The function of the two RS proteins of Aquaspirillum serpens MW5 is not known but is suspected to be a protective one. The RS layer of Aquaspirillum serpens VHA has been shown to protect the organism from invasion by the bacterial predator, Bdellovibrio bacteriovorus (Buckmire, 1971). Perhaps the cell tolerates minor, or even major, changes in

these proteins as long as the modified proteins continue to form paracrystalline surface layers which afford the organism protection from such environmental hazards. For other bacteria (eg. Aeromonas salmonicida) whose RS proteins fulfill other functions, severe changes may not be as acceptable and so the proteins may, indeed, be highly conserved.

If the function of the surface array of A. serpens MW5 is, in fact, a protective one then it might be an effective strategy for the cell to make the two layers of its double-layered surface array out of different components if only to ensure that, in the event of one layer being destroyed or lost, a second back-up one remains. Certainly the outer protein layer proved much more labile than the inner layer. Differences between the two could also be expected simply because the proteins take part in different types of molecular interactions. The protein comprising the inner layer (150K) must attach to an outer membrane surface while the 125K protein (outer layer) interacts with the 150K protein layer and also with the environment. The two RS proteins comprising the surface array of A. serpens MW5 demonstrated differences in their stability, solubility and assembly properties, and calcium-binding activities. The two proteins are not, however, unrelated. They shared similar isoelectric points, amino acid compositions, and certain amino acid sequences. What is difficult to estimate is the actual extent of the structural differences

between the two proteins. They may, in fact, share a certain core of sequences with the remaining sequences accounting for the observed differences in their biochemistry and assembly.

4.4. Conclusions

This study examined the regularly-structured surface array of A. serpens MW5 and showed that many rules which govern the assembly of the components of single-layered arrays are also applicable to more complex, double-layered, structures. The dependence on calcium for assembly in vitro is a recurring theme amongst Aquaspirillum RS proteins (Buckmire and Murray, 1976; Beveridge and Murray 1976a,c) and this study illustrated the calcium-binding properties of the RS proteins of strain MW5 through both assembly studies and a spectrophotometric assay. Calcium-binding has proven to be one of the more interesting biochemical features of these proteins and the mechanism of this binding and the contribution of the cation to the conformation of the proteins should be explored in future studies.

This study emphasized the need to differentiate between the morphological units of the array and their component subunits and emphasized that the assembly of these structures actually involves two assembly processes: monomers to intact units (oligomers) and units to an assembled array. The biochemical differences between the

two RS proteins of strain MW5 were reflected by differences in their assembly properties such as the inability of the 125K protein to self-assemble in vitro and the ability of the two proteins to reassemble onto an outer membrane surface only in their 'natural' order. The differences between the components of the two layers reflect an effective strategy on the part of the cell which has provided itself with a back-up layer should one be lost or damaged.

This study also examined some of the degradation problems associated with the purification of bacterial surface array proteins and these results should be considered when planning future purification strategies for Aquaspirillum RS proteins.

RS proteins represent large proportions of the total cellular protein and a large part of the cell's energy, therefore, must be directed towards their synthesis. The majority of studies of bacterial RS layers have concentrated on the ultrastructure, biochemistry, and in vitro assembly of these structures. Advances have recently been made, however, toward the study of RS gene regulation, synthesis, export, and in vivo assembly of these proteins. Initial experiments in these efforts are, quite understandably, being conducted with organisms which possess single-layered arrays such as Caulobacter crescentus (Smit and Agabian, 1984) and Aeromonas salmonicida (Belland and Trust, 1985). The surface array of strain MW5 will

provide, in the future, an excellent model for the in vivo assembly of a more complex surface structure. Of particular interest are questions regarding coordinate gene regulation, possible post-transcriptional or translational modification of one of the two proteins (given the shared amino acid sequences), and the task of assembling not one, but two protein layers on the cell surface in their correct order. It is hoped that the biochemical and in vitro assembly properties of the RS proteins of *A. serpens*, MW5 examined in this study will aid in this effort.

Figure 1. Schematic cross-sections of the cell envelopes of 'typical' (a) Gram-positive, (b) Gram-negative, and (c) archae- bacteria.

1. Cell envelopes as seen in thin sections.
2. Distribution of cell envelope components.

Abbreviations: PM, cytoplasmic membrane; PG, peptidoglycan; OM, outer membrane; RS, regularly-structured layer; LPS, lipopolysaccharide; PR, protein; PL, phospholipid ; TA, teichoic/teichuronic acid; LTA, lipoteichoic acid. Modified from Sleytr.(1981).

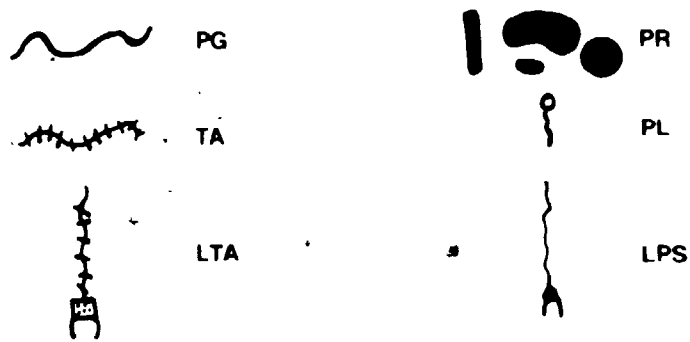
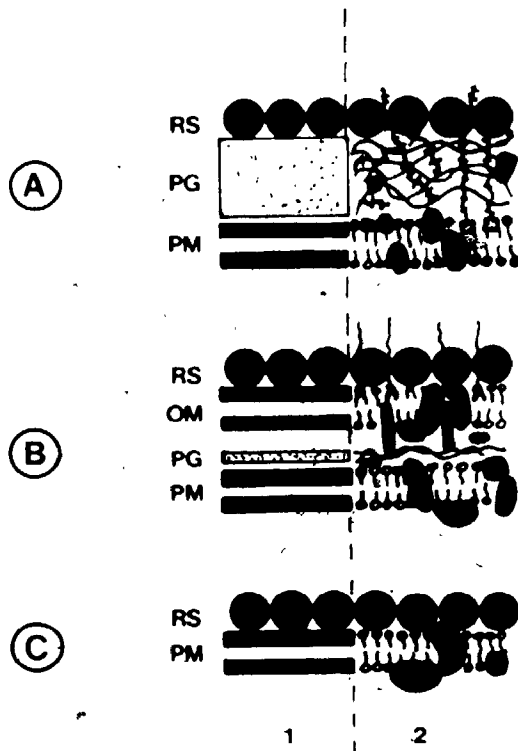


Figure 2. Demonstration of a regularly-structured array on the surface of A. serpens MW5 by electron microscopy.

Figure 2a. Whole cells of A. serpens MW5 negatively stained with 1.0% ammonium molybdate. The cell has partially plasmolyzed which makes the RS layer more visible by negative stain. The 'ribs' of the array are aligned at 90 degree angles perpendicular to the longitudinal axis of the cell. Bar = 100 nm.

Figure 2b. Thin section of A. serpens MW5 which demonstrates the presence of two additional layers lying external to the outer membrane of the cell envelope. Abbreviations: ISL, inner structured layer; OSL, outer structured layer
Bar = 100 nm.

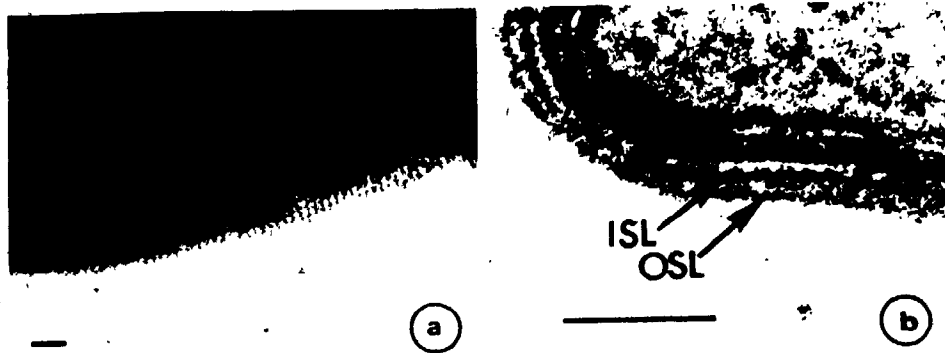
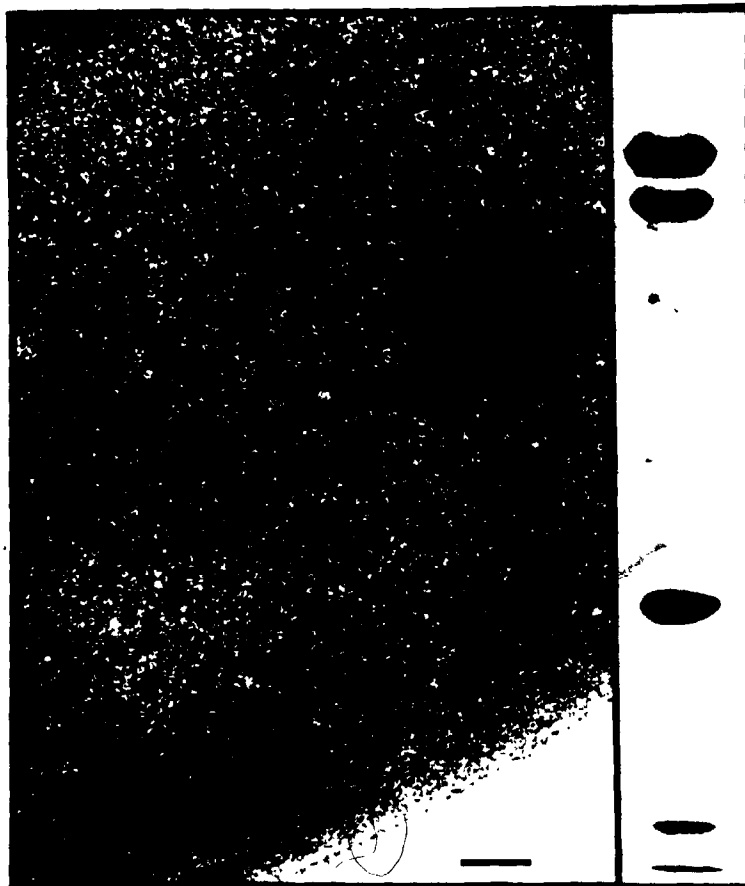


Figure 3. Negatively stained cell envelopes of A. serpens MW5 which demonstrate the moiré surface pattern. Bar = 100 nm. SDS-PAGE of these envelopes show the presence of three major polypeptides and several minor proteins.




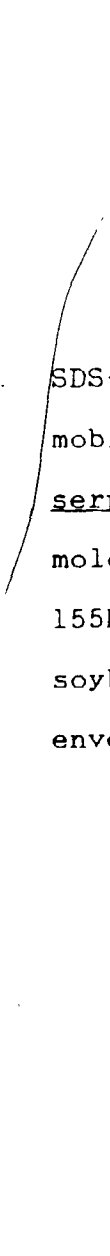



Figure 4. SDS-PAGE comparing the electrophoretic mobilities of the cell envelope proteins of A. serpens MW5 with protein standards of known molecular weight. Lane A, RNA polymerase (165K, 155K, 39K), bovine serum albumin (68K), and soybean trypsin inhibitor (21.5K); lane B, cell envelope proteins of A. serpens MW5.



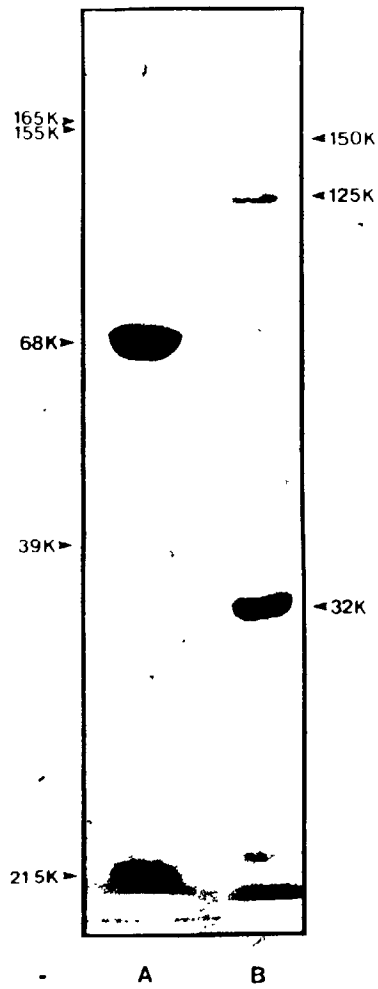


Figure 5. Densitometric scan of cell envelope proteins of A. serpens MW5 separated by SDS-PAGE and stained with Coomassie Blue.

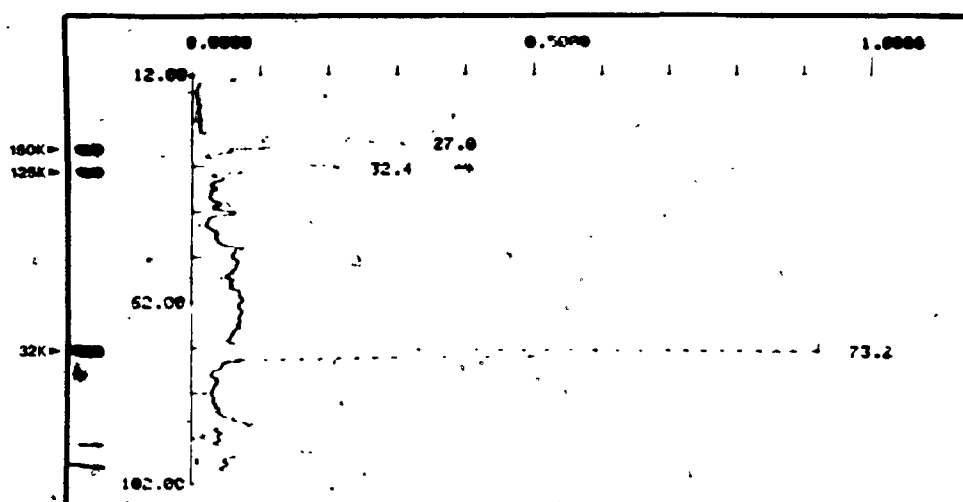


Figure 6. SDS-PAGE of A. serpens MW5 cell envelopes heated for 30 min in 2% SDS at 50C (lanes A, B), 60C (lanes C, D), and 100C (lanes E, F).

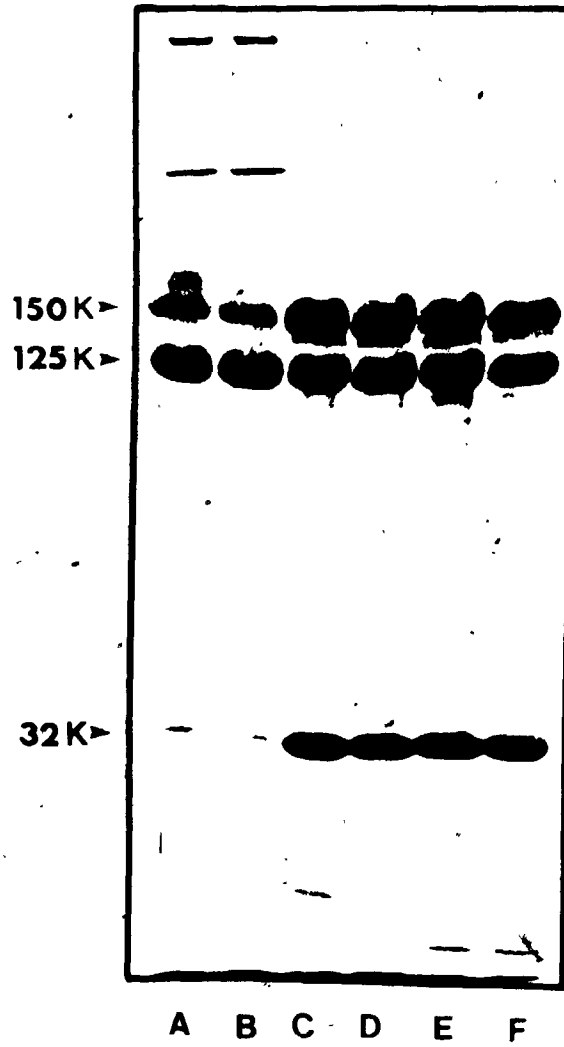


Figure 7. Negatively stained cell envelopes of A. serpens MW5 following treatment with (a) 6 M urea, (b) 1.5 M guanidine hydrochloride, (c) 20 mM LIS, and (d) 2% SDS. Abbreviations: OM, outer membrane; OMV, outer membrane vesicles. Bars = 100 nm.



(a)



(b)



(c)



(d)



Figure 8. SDS-PAGE of *A. serpens* MW5 cell envelopes following treatment with 6 M urea (lane B), 1.5 M guanidine hydrochloride (lane D), and 20 mM LIS (lane F). The cell envelope proteins solubilized by these chaotropic agents are shown in lanes A, C, and E, respectively.

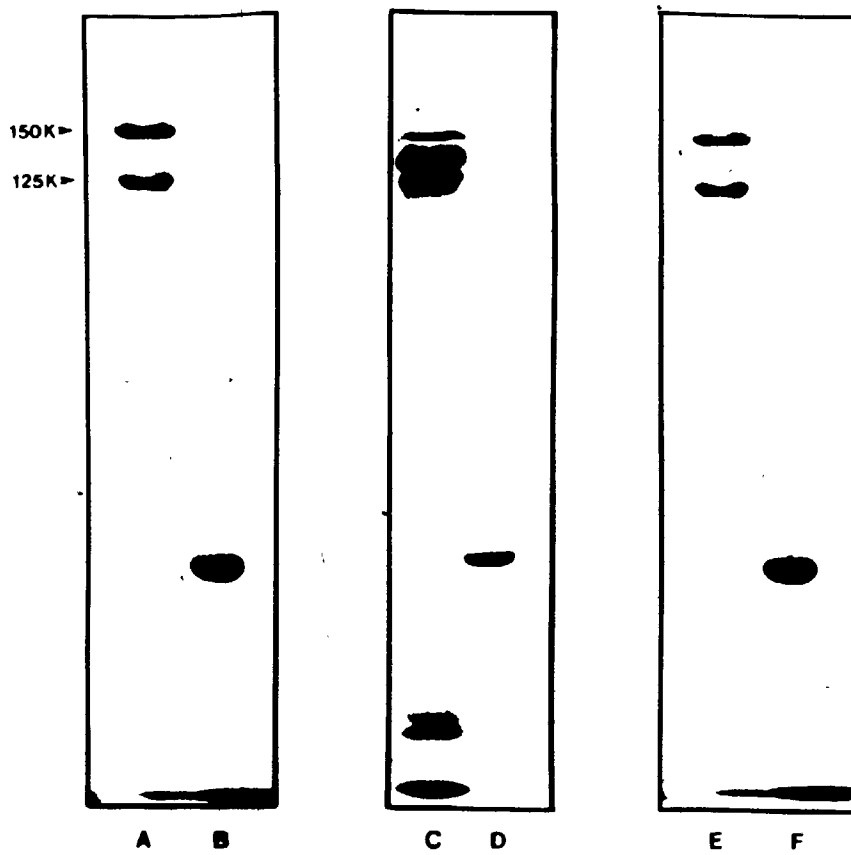


Figure 9. Negatively stained cell envelopes of *A. serpens* MW5 following treatment with (b) 0.2 M EDTA, (c) 0.2 M EGTA, (d) 1.0 M sodium citrate, (e) 1.0 M sodium oxalate, and (f) 0.05 M 8-hydroxyquinoline. Control envelopes are shown in panel a. Arrows in panels b and c indicate outer membrane blebs. Abbreviations: HEX, hexagonal arrangement of subunits. Bars = 100 nm.

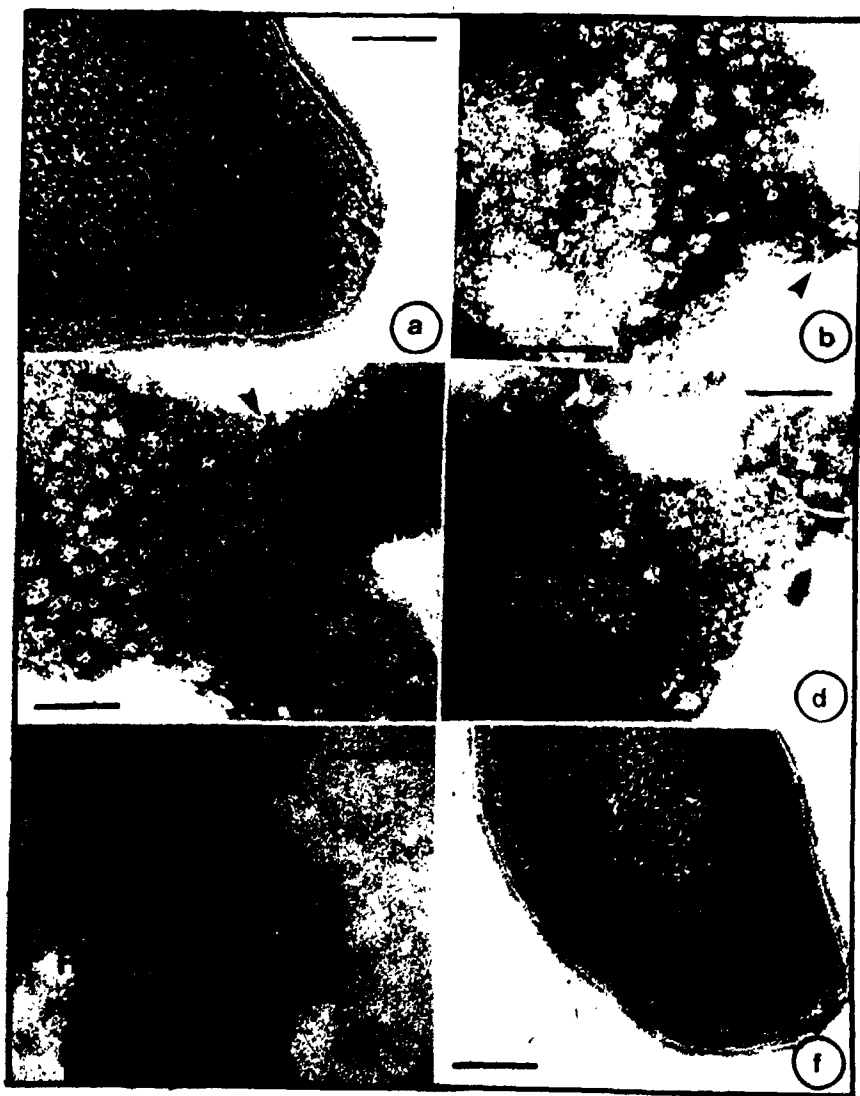


Figure 10. SDS-PAGE of *A. serpens* MW5 cell envelopes following treatment with 0.2 M EDTA (lane B), 0.2 M EGTA (lane C), 1.0 M sodium citrate (lane D), 1.0 M sodium oxalate (lane E), and 0.05 M 8-hydroxyquinoline (lane F). Control envelopes are shown in lane A.

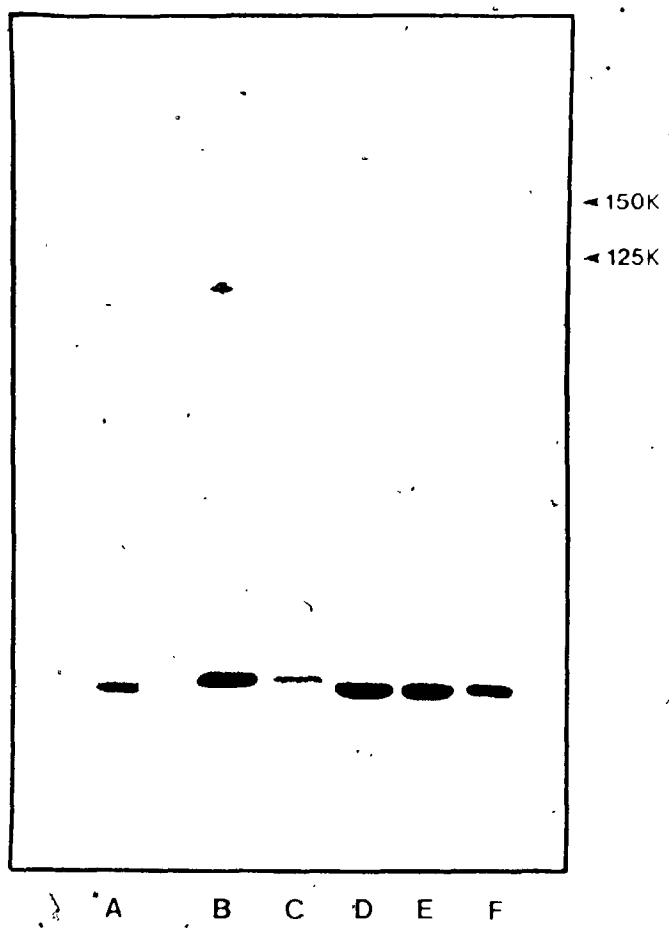


Figure 11. Negatively stained cell envelopes of A. serpens MW5 following incubation in buffers adjusted to pH (a) 3.0, (b) 7.0, (c) 10.0, and (d) 12.0. Abbreviations: om, outer membrane. Bars = 100 nm.

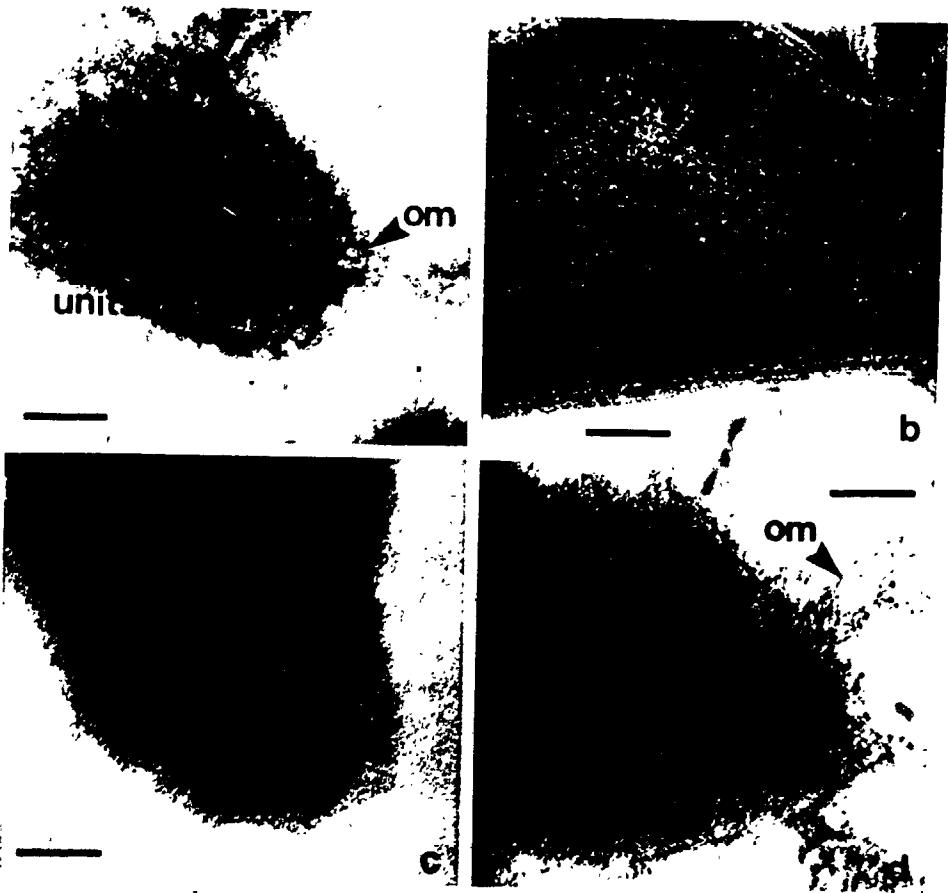


Figure 12. SDS-PAGE of *A. serpens* MW5 cell envelopes following incubation in buffers adjusted to pH 3.0 (lane B), 3.5 (lane D), 4.0 (lane F), 4.5 (lane H), 5.0 (lane J), 5.5 (lane L), 6.0 (lane N), and 7.0 (lane P). The cell envelope proteins solubilized by these treatments are shown in lanes A, C, E, G, I, K, M, and O, respectively.

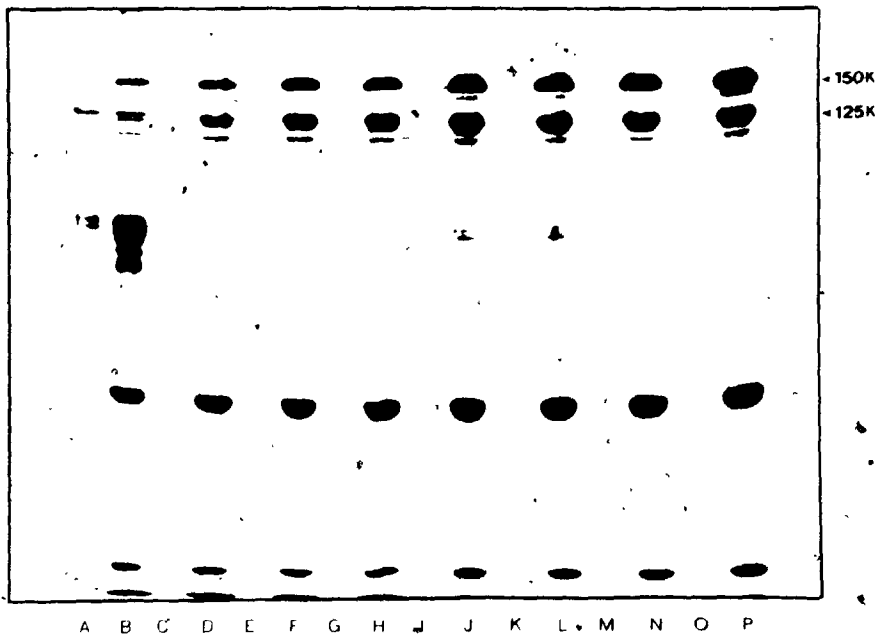


Figure 13. SDS-PAGE of *A. serpens* MW5 cell envelopes following incubation in buffers adjusted to pH 7.0 (lane B), 9.0 (lane D), 9.5 (lane F), 10.0 (lane H), 10.5 (lane J), 11.0 (lane L), 11.5 (lane N), and 12.0 (lane P). The cell envelope proteins solubilized by these treatments are shown in lanes A, C, E, G, I, K, M, and O, respectively.

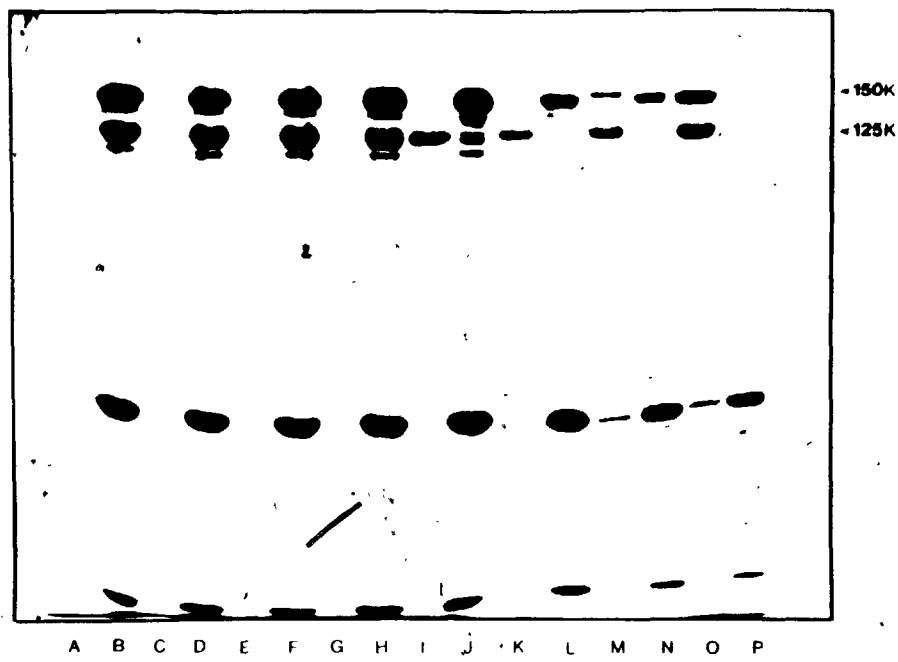


Figure 14. SDS-PAGE of the sequential protein extraction. Control envelopes (lane a) were treated initially with sodium borate buffer, pH 10.3. The solubilized proteins are shown in lane b. The extracted envelopes (lane c) were then treated with 6 M urea. Lane d, 6 M urea-extracted protein; lane e, 6 M urea-insoluble envelopes.

Figure 15. Negative stains and corresponding thin sections of the cell envelope fractions from the sequential protein extraction. Control envelopes (a and b) possessed both the inner and outer surface layers (ISL and OSL), whereas pH 10.3-extracted envelopes (c and d) possessed only a single surface layer (ISL). Envelopes extracted initially with pH 10.3 buffer and subsequently with 6 M urea (e and f) contained no additional layers external to the outer membrane. Abbreviations: ISL, inner structured layer; OSL, outer structured layer; HEX, hexagonal arrangement of subunits; OM, outer membrane. Bars = 100 nm.

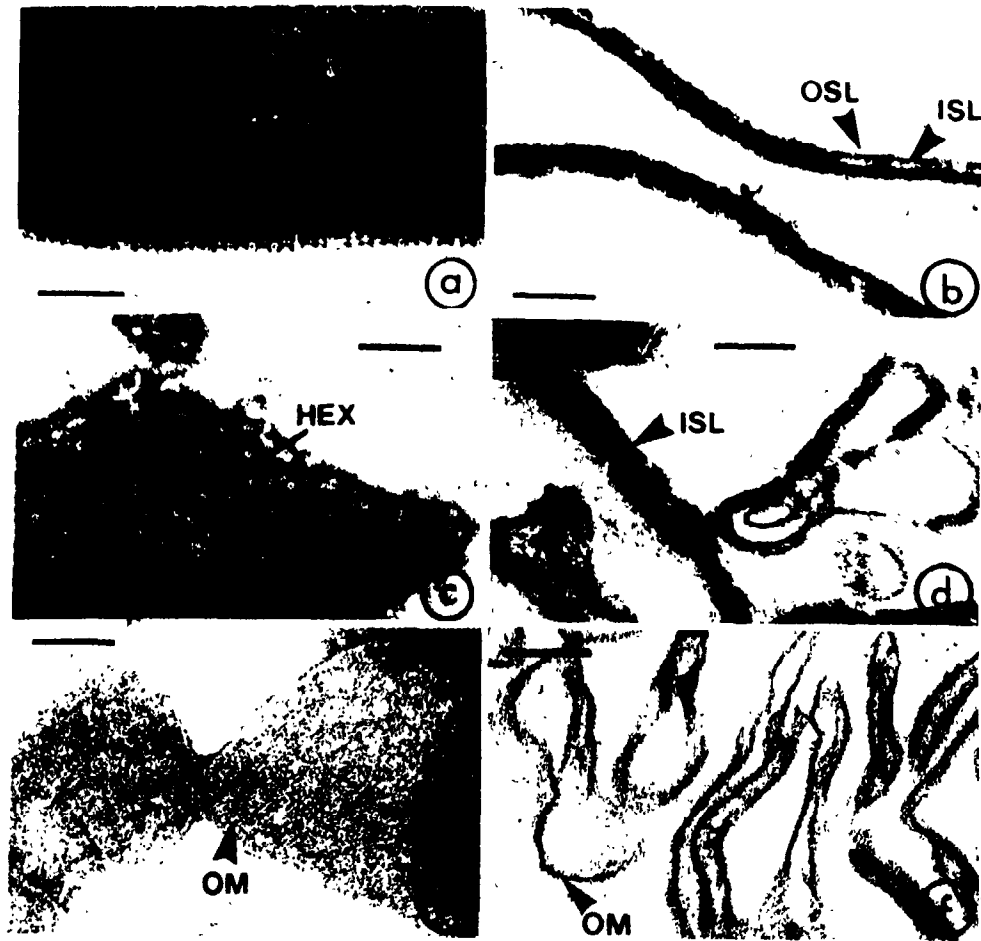


Figure 16. Reassembly of the surface array components onto RS⁻ cell envelopes (a) which do not possess an RS layer. Dialysis against distilled water, NaCl, KCl, LiCl, or MgCl₂ resulted in the reassembly of only a few patches of hexagonally-arranged subunits on a small proportion of the cell envelopes (b). Arrows indicate patches of reassembled protein. Dialysis against CaCl₂ or SrCl₂ resulted in the reassembly of both layers of the surface array as demonstrated by the presence of a linear surface pattern (c). Abbreviations: OM, outer membrane. Bars = 100 nm.

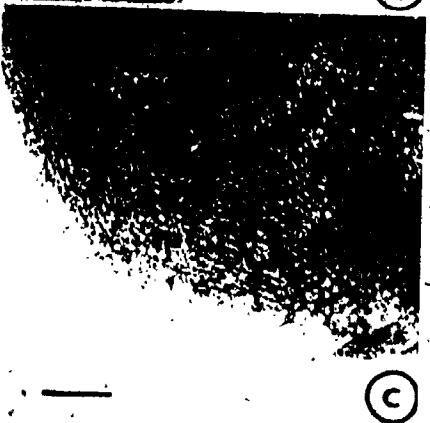
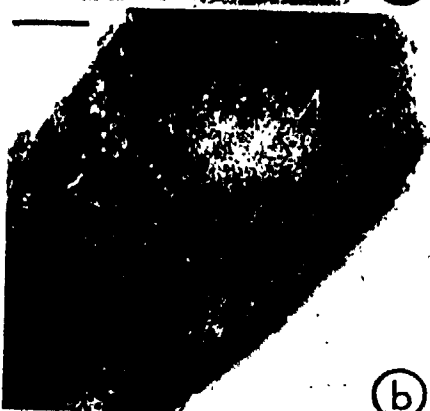
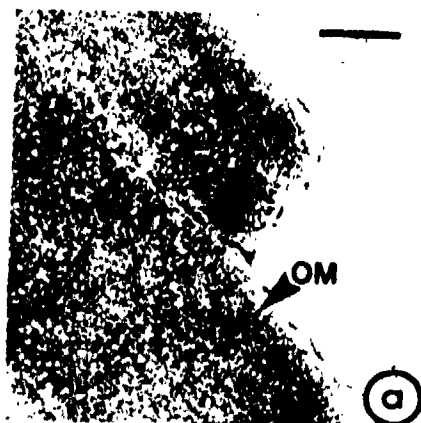


Figure 17. SDS-PAGE of reconstituted envelopes

Urea-extracted surface protein (lane a) was mixed with RS⁻ cell envelopes (lane b) and dialyzed against various salt solutions. Lanes c through i represent the envelope fractions after dialysis of the reassembly mixtures against distilled water, NaCl, KCl, LiCl, MgCl₂, CaCl₂, and SrCl₂, respectively.



a b c d e f g h i

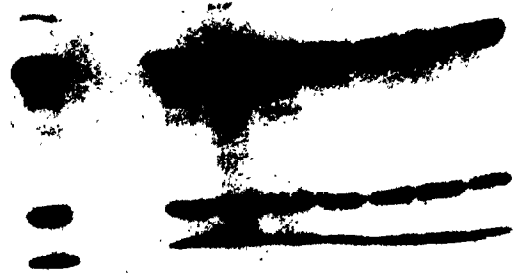


Figure 18. Heterologous reattachment. 6 M urea-extracted surface protein of *A. serpens* MW5 was mixed with cell envelopes prepared from (a) *A. serpens* VHL, (c) *A. serpens* 12638 (type strain), (e) *Escherichia coli*, and (g) *Pseudomonas aeruginosa* and dialyzed against 10 mM CaCl₂. The RS proteins of strain MW5 reassembled to form linear arrays on the outer membrane surfaces of *A. serpens* strains VHL (b) and 12638 (d) but failed to reassemble onto those of *Escherichia coli* (f) or *Pseudomonas aeruginosa* (h). Bars = 100 nm.

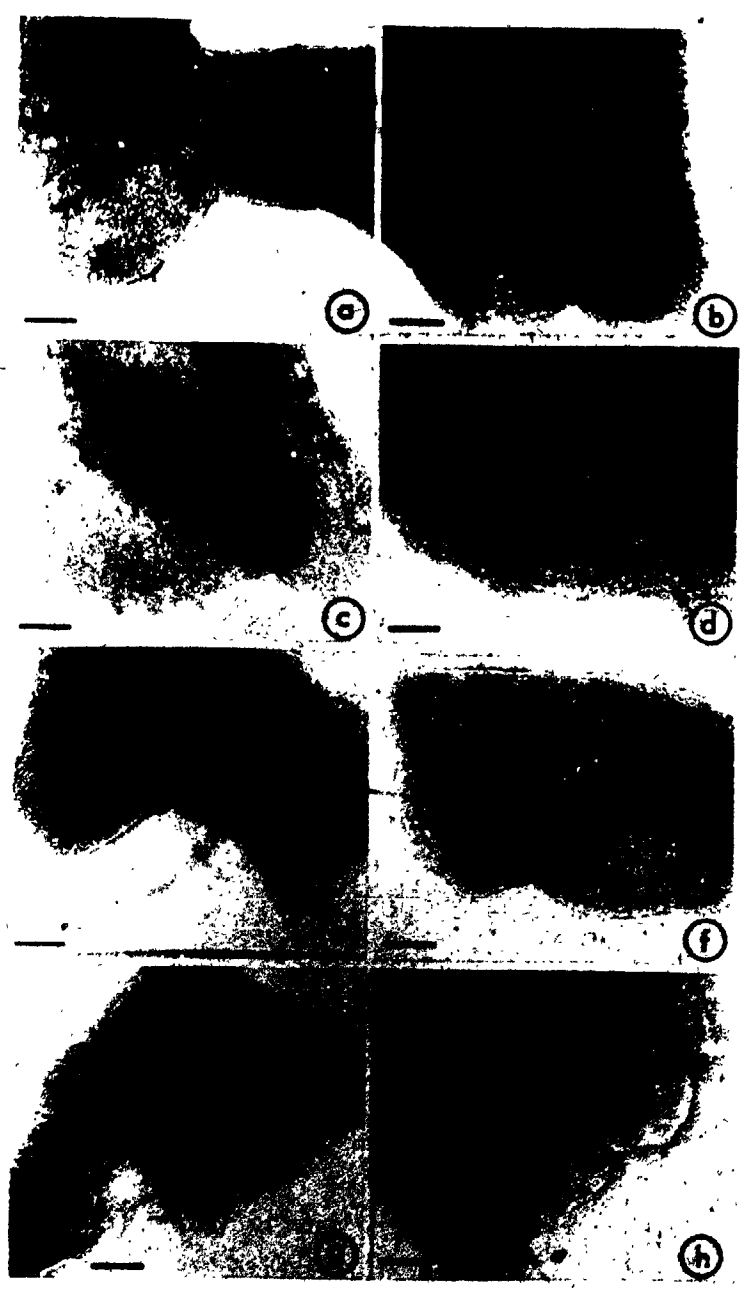


Figure 19. Negative staining of sequentially-reconstituted cell envelopes. RS⁻ cell envelopes were mixed initially with the 150K protein extract and dialyzed against Ca²⁺. The protein reassembled into a hexagonal array on the outer membrane surface (a). The addition of the 125K protein extract to these envelopes resulted in the full reconstitution of the two-layered surface array (b). Bars = 100 nm.



(b)

Figure 20... SDS-PAGE of the sequential reconstitution.

Control envelopes are shown in lanes a and g.

RS⁻ cell envelopes (lane b) were mixed with the 150K protein extract (lane c). The 150K

protein reassembled onto the RS⁻ cell envelopes

(lane d), and provided a template for the

reassembly of the 125K protein (lane e). Lane

f, fully reconstituted cell envelopes.

Figure 21. Self-assembly of the surface array components. Negative stains of the planar sheets produced when 6 M urea-extracted RS proteins were dialyzed at 4C against: (a) distilled water containing 10 mM CaCl_2 , (b) distilled water containing 10 mM SrCl_2 , or (c) 10 mM HEPES, pH 7.0, containing 10 mM CaCl_2 . Bars = 50 nm.

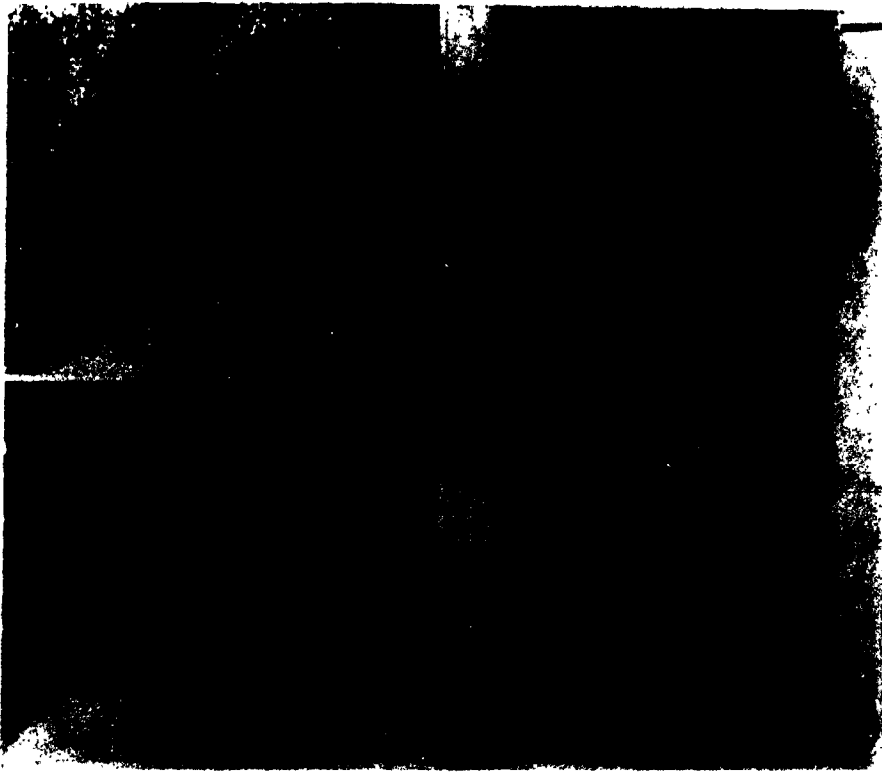


Figure 22. SDS-PAGE of the self-assembly products. Lane A, 6 M urea-extracted surface proteins; lane B, self-assembled surface array produced when the protein extract shown in lane A was dialyzed against CaCl_2 or SrCl_2 .

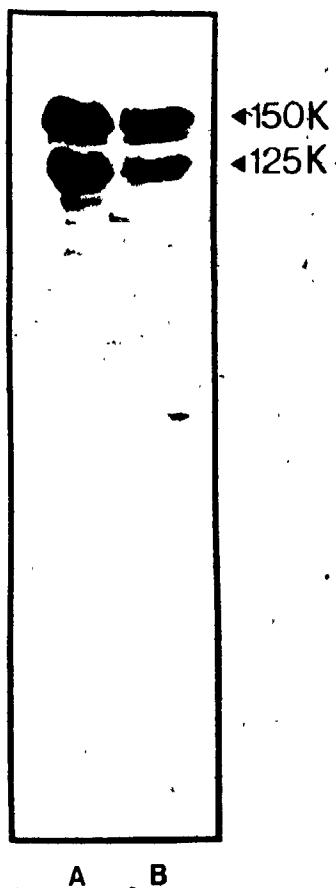


Figure 23. Self-assembly of the 150K protein. Negative stains and SDS-PAGE of the assembly products obtained when the 150K protein extract was dialyzed against 10 mM CaCl_2 . Bars = 50 nm.

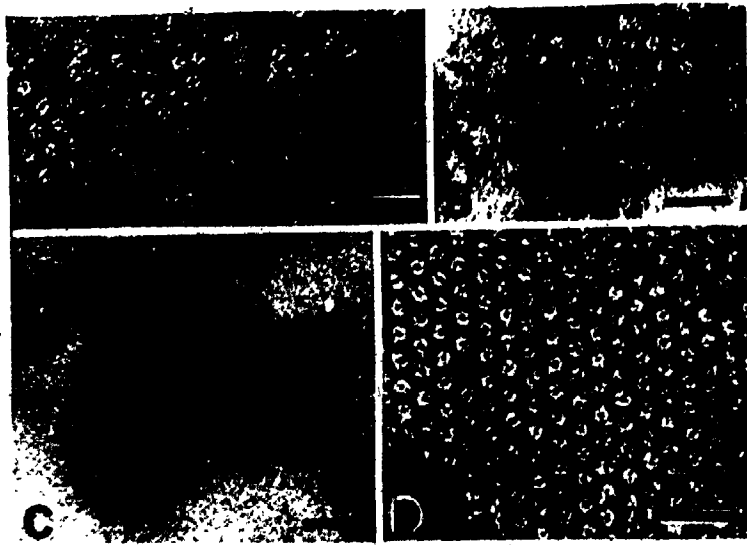
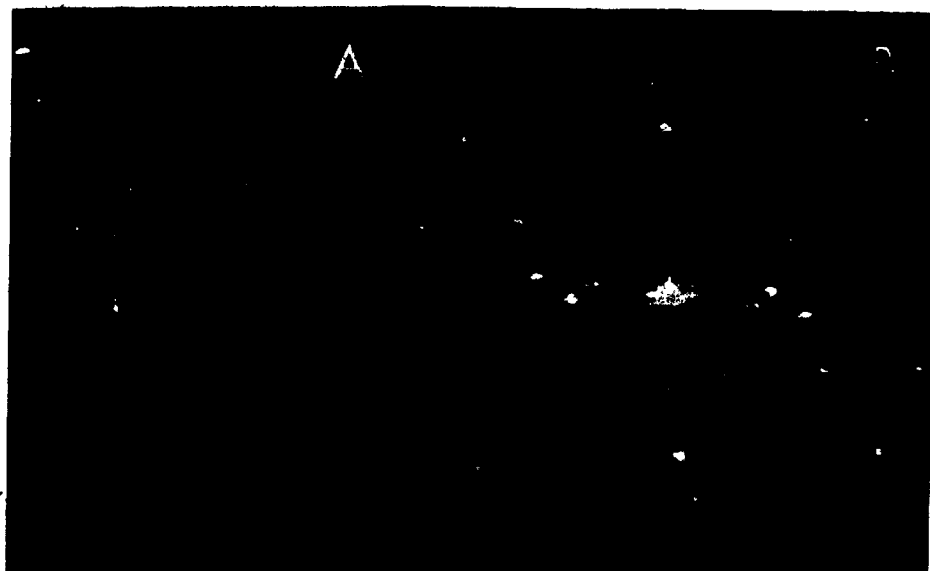


Figure 24. Image analysis of the self-assembled products. An appropriate region of a negatively stained image of the self-assembled 150K protein (a) was examined by standard image analysis techniques. Bar = 50 nm. The diffraction pattern for this array (b) confirmed a hexagonal arrangement of subunits, or sixfold symmetry.



Figure 25. Image analysis of the in vitro self-assembly of the RS proteins of A. serpens MW5 (150K + 125K). An appropriate region of the negatively stained array (A) was examined by standard optical diffraction and filtering techniques. Bar = 50 nm. The diffraction pattern for the double-layered array (B) indicates a linear arrangement of subunits.



2

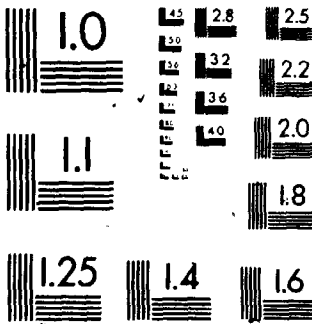
Figure 26. Reconstructed images of the self-assembled 150K protein array (a) and the self-assembled double-layered array (150K + 125K proteins) (b).

3

OF/DE

3

MICROCOPY RESOLUTION TEST CHART
NBS 1010a
ANSI and ISO TEST CHART No. 2



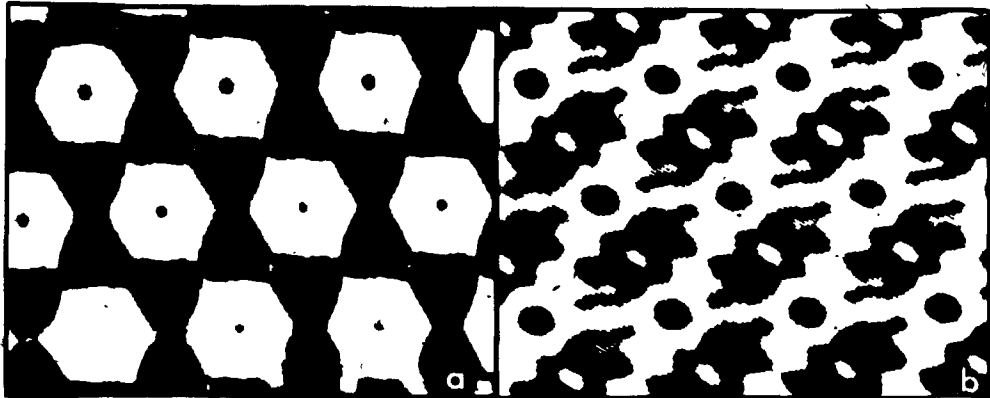


Figure 27. Effect of heating on the mobility of the RS-proteins of A. serpens MW5 in an SDS-polyacrylamide gel (5% polyacrylamide). Lane A, 6 M urea-extracted and dialyzed RS proteins heated for 3 min at 100C in sample buffer; lane B, identical RS protein extract not heated prior to electrophoresis.

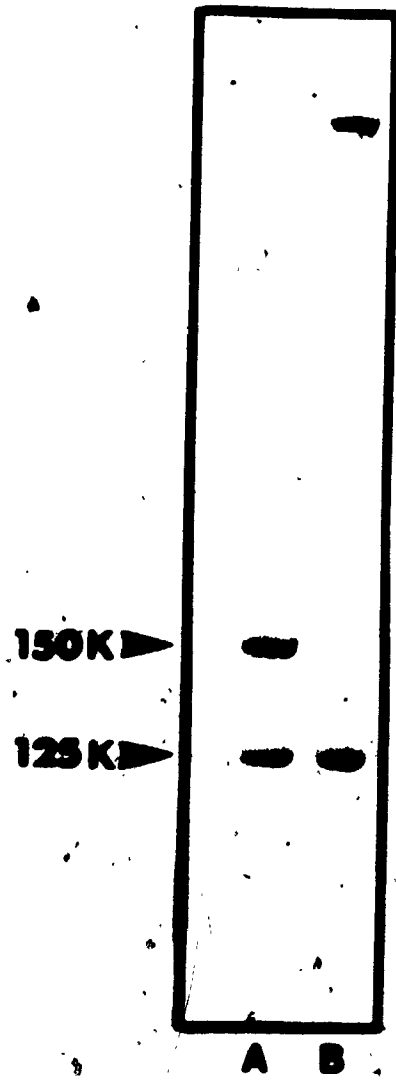


Figure 28. SDS-PAGE comparing the electrophoretic mobilities of the RS proteins of A. serpens MW5 which were heated in sample buffer at 100C for 3 min (lane B) or not heated (lane C) prior to electrophoresis, with proteins of known molecular weight (myosin, 205K; β -galactosidase, 116K; phosphorylase B, 97K) (lane A).

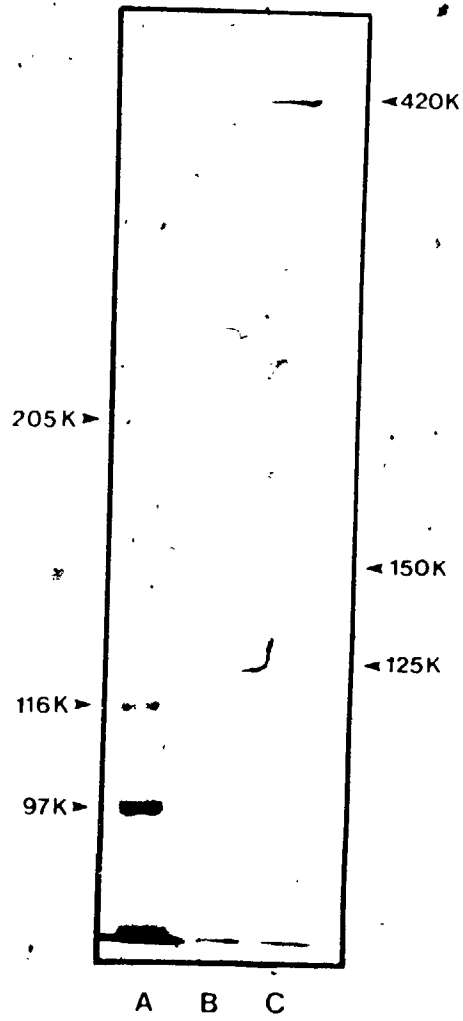
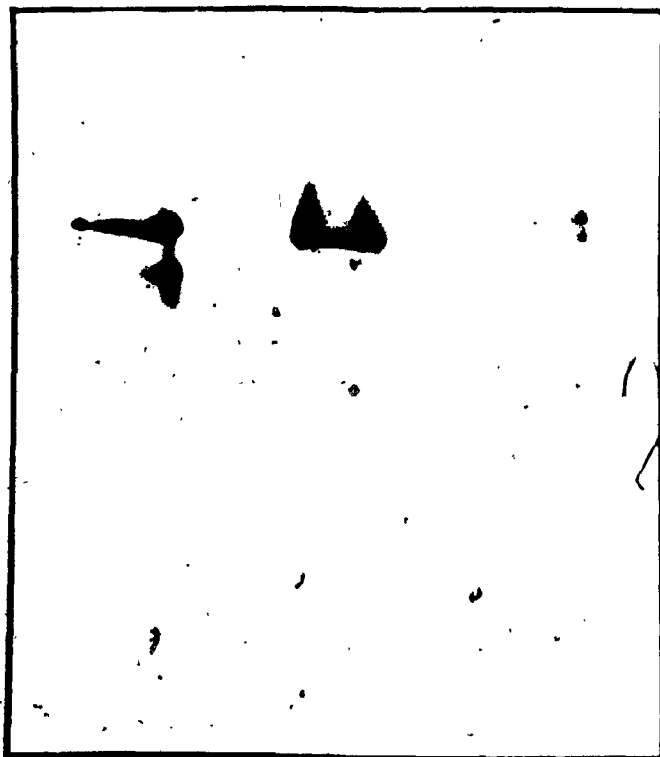


Figure 29. Negative stains of heated and unheated 150K protein extracts. Samples not heated in 1% SDS prior to electrophoresis showed decreased mobility in an SDS-polyacrylamide gel (5% polyacrylamide) (lane B) and negative stains of these extracts (panel D) demonstrated the presence of 11.5-12.0 nm doughnut-shaped units. These structures were not found in the 150K protein extracts which had been heated in the detergent at 100C (lane A, panel C).
Bars = 50 nm.



A B

Figure 30. Mobility of the RS proteins of A. serpens MW5 in a 5% polyacrylamide (native) gel. Lane A, 150K protein extract; lane B, 125K protein extract; lane C, 6 M urea-extracted and dialyzed RS proteins (150K + 125K proteins).



A

B

C

Figure 31. Determination of the isoelectric points of the RS proteins of A. serpens MW5. 6 M urea-extracted and dialyzed RS proteins (150K + 125K) were focused in the first dimension in an ampholine gradient of pH 4-6 and then separated in the second dimension by SDS-PAGE.

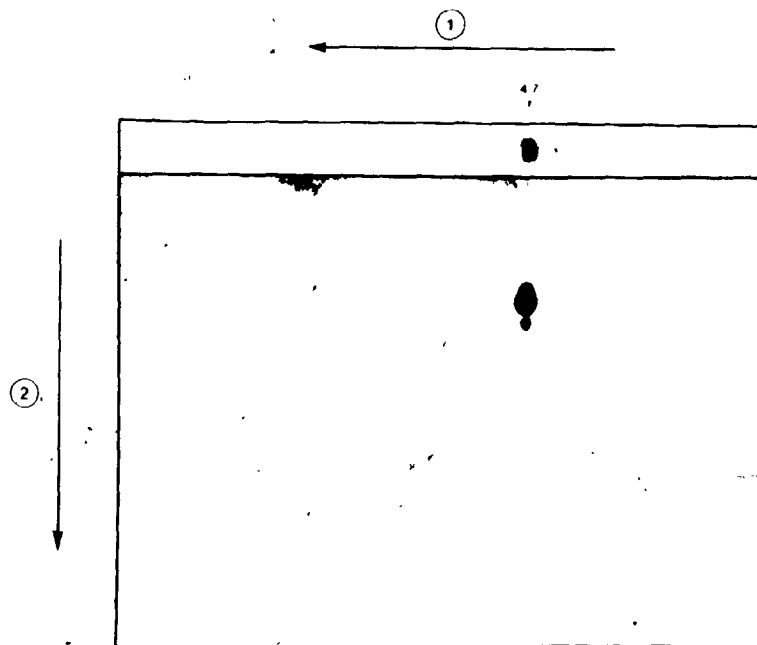
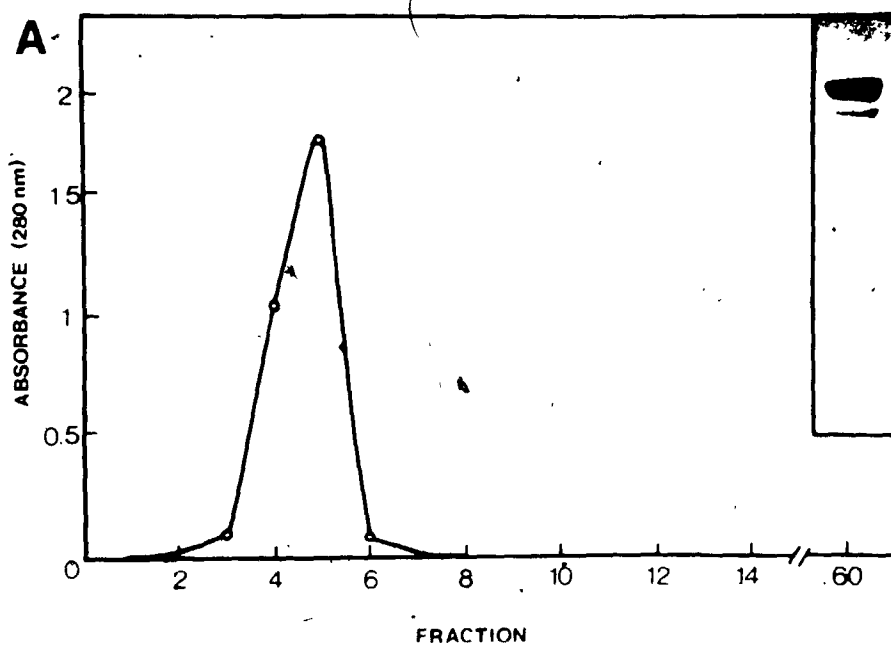


Figure 32A. Ion-exchange chromatography of the RS proteins of *A. serpens* MW5. 6 M urea-extracted RS proteins (150K + 125K) were dialyzed against 0.01 M imidazole buffer, pH 6.5, and chromatographed on a 11.0 cm x 0.7 cm DEAE-Sepharose column. The protein was eluted with a linear gradient of 0-1.0 M NaCl in 0.01 M imidazole, pH 6.5. 1.0 ml fractions were collected. A, the elution profile and SDS-PAGE analysis of the major peak which contained the 150K polypeptide and a number of minor, lower-molecular-weight proteins.

Figure 32B. Interaction of the 125K protein with DEAE-Sepharose CL-6B in batch tests. The 125K protein extract (which contained the major, lower molecular weight form of this protein and several other minor proteins) (lane A) was mixed with the resin (previously equilibrated with 0.01 M imidazole, pH 6.5) and a stepwise elution initiated. Lanes B-E, protein eluted after mixing with 0.25 M, 0.5 M, 1.0 M, and 1.5 M NaCl in 0.01 M imidazole, pH 6.5, respectively.



B

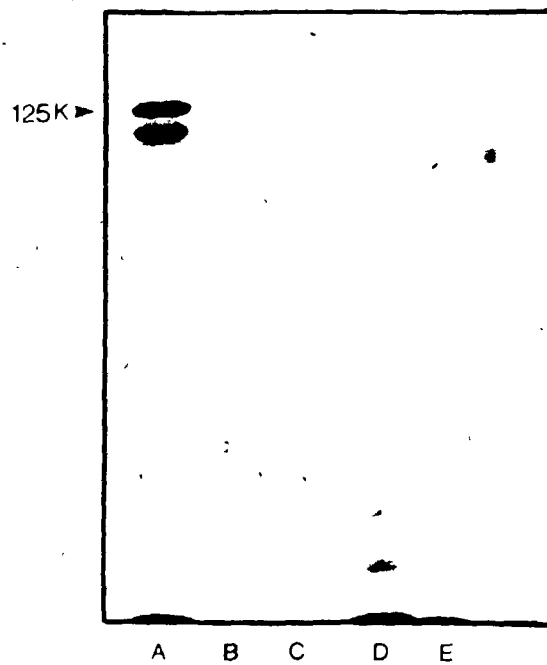


Figure 33. Polyethylene (PEG) cuts of the 125K protein extract. SDS-PAGE of the proteins precipitated when the 125K protein extract (which contained the major, lower-molecular-weight form of this protein and several other minor proteins) was mixed with 3% (lane A), 10% (lane B), 15% (lane C), and 25% (lane D) PEG 1500.

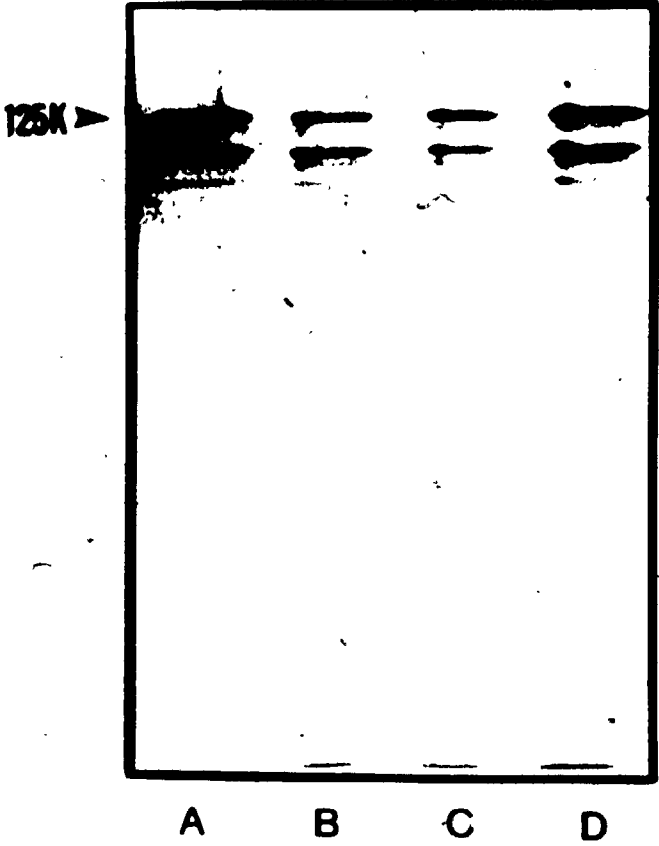
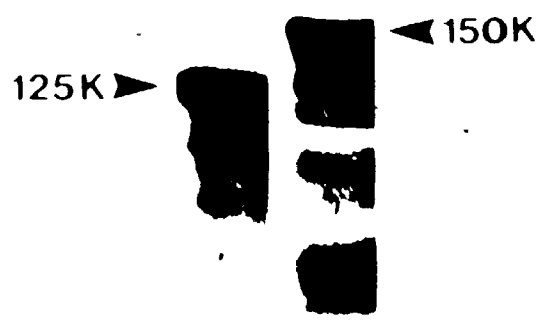
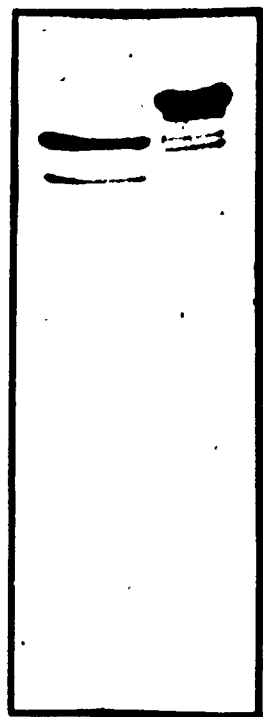


Figure 34. Immunoblotting of the RS protein extracts. The 150K and 125K proteins were extracted sequentially from cell envelopes, separated by SDS-PAGE, and electrophoretically-transferred to nitrocellulose. Lane A, the 125K protein extract; lane B, the 150K protein extract. The blotted proteins in lane A were incubated with antisera specific for the 125K RS protein and the proteins in lane B with antisera specific for the 150K RS protein. The antibody which reacted with protein was detected by incubation with ^{125}I -labelled Protein A and subsequent autoradiography for 18 h at -70C .



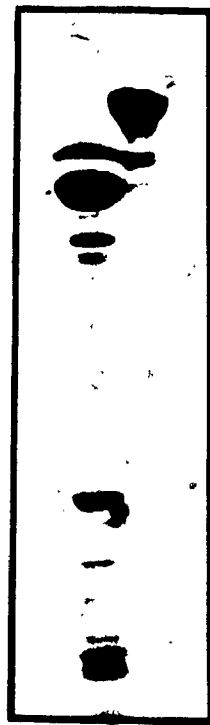
A B

Figure 35. Stability of the RS proteins of *A. serpens* MW5. The 125K and 150K proteins were extracted sequentially from cell envelopes and dialyzed, separately, against deionized water at 4C overnight. Lane A, 125K protein extract; lane B, 150K protein extract. Lanes C and D represent the same protein extracts following concentration by membrane filtration and storage for 30 days at -10C.



◀150K
◀125K

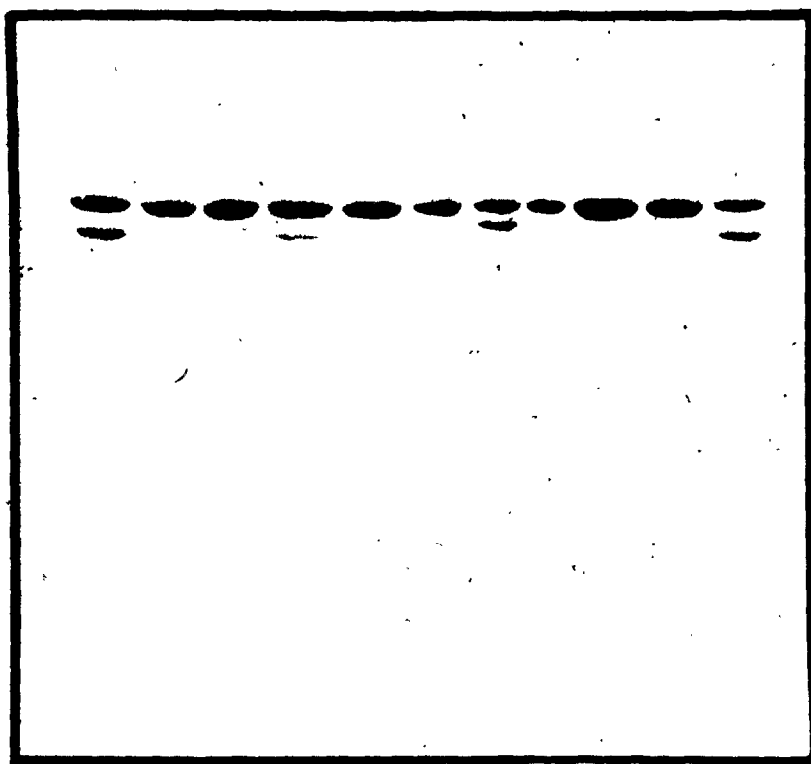
A B



◀150K
◀125K

C D

Figure 36. Extraction of the 125K RS protein from *A. serpens* MW5 envelopes in the presence of various anti-proteolytic agents. Cell envelopes were extracted with sodium borate (pH 10.3) containing either 10 mM dithiothreitol (lane B), L-cysteine hydrochloride (lane C), 1 mM phenylmethylsulfonyl fluoride (PMSF) (lane D), N-tosyl-L-lysine chloromethyl ketone (TLCK) (lane E), N-tosylphenylalanine chloromethyl ketone (TPCK) (lane F), 100 ug/ml soybean trypsin inhibitor (type I) (lane G), aprotinin (lane H), p-chloromercuriphenylsulfonic acid (PCMBS) (lane I), pepstatin (lane J), or 10 mM iodoacetate (lane K). Lanes B through K show the solubilized protein after overnight dialysis at 4C against distilled water containing the same inhibitors but at one-tenth the concentration used during the extraction. The control extract is shown in lane A.



A B C D E F G H I J K

Figure 37. Growth of *A. serpens* MW5 in calcium-free broth. Cell envelopes prepared from *A. serpens* MW5 grown in a defined medium supplemented with calcium possessed the regularly-structured array as demonstrated by negative staining-electron microscopy (a). Repeated transfer to calcium-free media resulted in the loss of the array. The majority of negatively stained envelopes prepared from these cells showed a smooth outer membrane surface although small patches of hexagonally-arranged, or individual, units could be detected on a few of these envelopes (b). Bars = 100 nm.

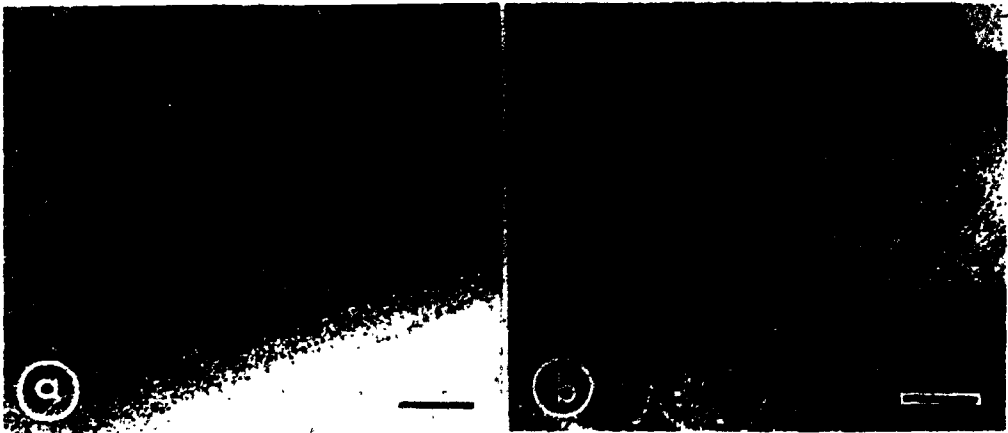


Figure 38. SDS-PAGE of *A. serpens* MW5 cell envelopes prepared from cells grown in defined medium supplemented with calcium (lane B) and upon repeated transfer in calcium-free media (lane A). Lane C, control envelopes prepared from cells grown in PSS broth supplemented with calcium.



A B C

Figure 39. Effect of calcium on the electrophoretic mobility of the RS proteins of A. serpens MW5. Lanes A and D, 6 M urea-extracted RS proteins dialyzed overnight against deionized water; lane B, the same protein extract dialyzed against 10 mM CaCl₂; lane C, RS proteins of A. serpens MW5 extracted from cell envelopes with 6 M urea containing 10 mM CaCl₂ and subsequently dialyzed against 10 mM CaCl₂.

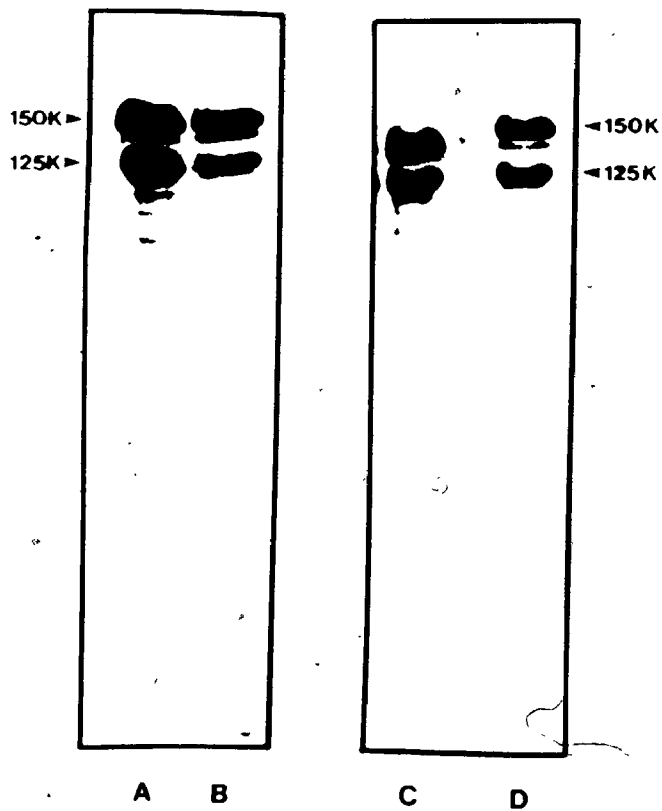


Figure 40 Effect of EGTA on the RS proteins of *A. serpens* MW5 SDS-PAGE of the RS proteins (150K + 125K) extracted from cell envelopes with 6 M urea (in 10 mM HEPES buffer, pH 8.0) and dialyzed overnight at 4C against either buffer (lane A) or buffer containing 20 mM EGTA (lane B).

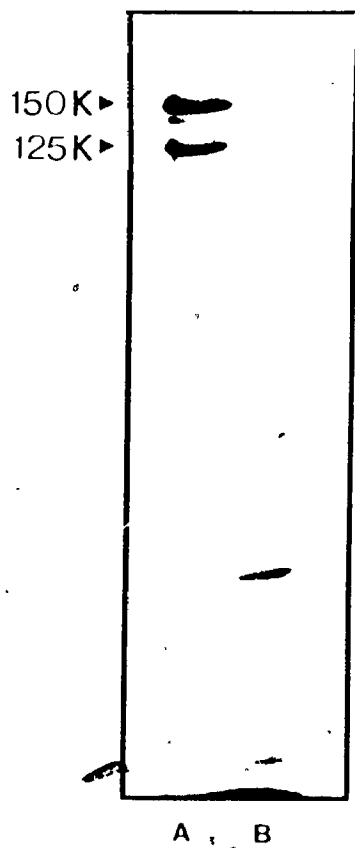
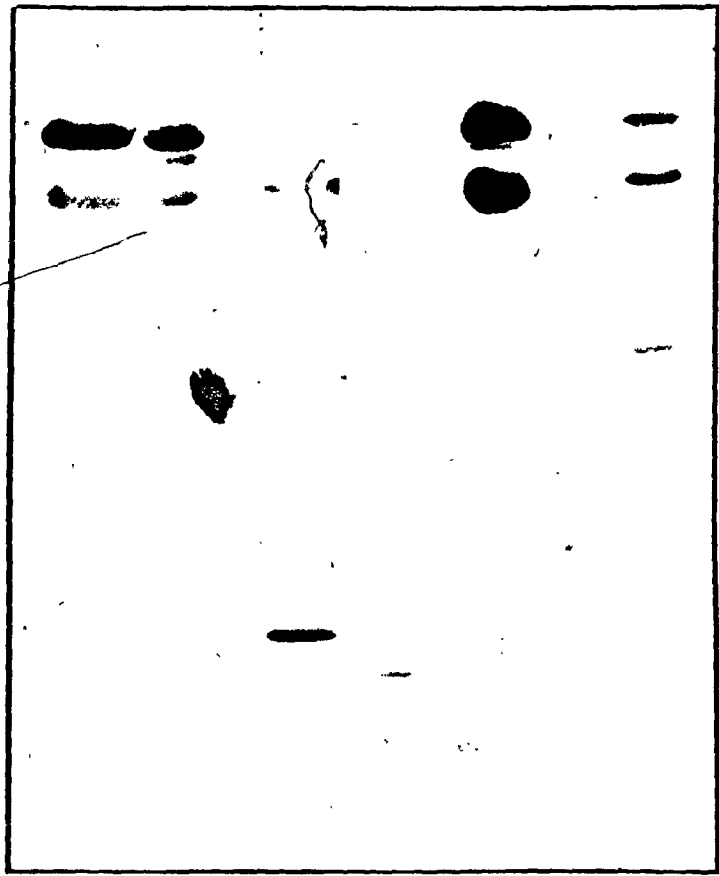


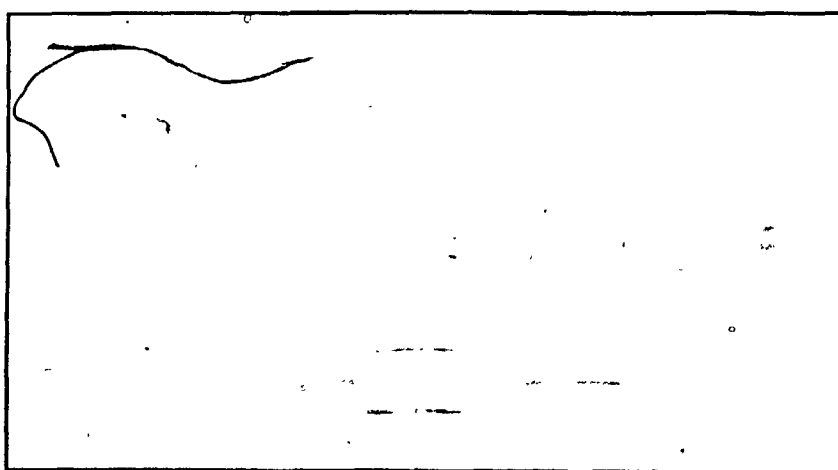
Figure 41. Inhibition of RS protein degradation in the presence of EGTA. *A. serpens* MW5 cell envelopes were treated with 10 mM HEPES buffer, pH 7.5, containing 6 M urea and 1 mM TPCK. Lane A, the solubilized RS proteins immediately after their extraction; lane B, the same protein extract following overnight dialysis against buffer containing TPCK. Extraction of the RS proteins with 6 M urea containing 10 mM EGTA resulted in the degradation of the two major polypeptides (lane C). This degradation increased upon dialysis against EGTA (lane D). The observed degradation of the RS proteins in the presence of EGTA was inhibited when TPCK was included in the extraction buffer (lane E). This inhibitory effect was lost upon dialysis against a reduced concentration of inhibitor (0.1 mM TPCK) (lane F) but could be partially maintained when the inhibitor concentration remained at 1 mM throughout dialysis (lane G).



◀150K
◀125K

A B C D E F G

Figure 42. One-dimensional peptide mapping of the RS proteins of *A. serpens* MW5. SDS-PAGE showing the peptide profiles generated when gel bands containing either the 150K (lane C) or 125K (lane D) proteins were subjected to limited proteolysis (30 min/40C) using *Staphylococcus aureus* V8 enzyme (3 ug). The enzyme migrates as a doublet in an SDS-polyacrylamide gel (lane E). Lanes A and B, control 150K and 125K proteins, respectively. Arrows indicate positions of peptide fragments common to both RS proteins.



A B C D E

Figure 43. Two-dimensional peptide mapping of the RS proteins of A. serpens MW5 and A. serpens VHA. The proteins were labelled with ^{125}I and digested with Staphylococcus aureus V8 enzyme. The peptide solutions were spotted onto cellulose-coated thin layer chromatography plates and separated in the first dimension by high-voltage electrophoresis and, subsequently, in the second dimension by chromatography in a solvent system. A, B, and C represent autoradiograms of the peptide maps generated for the 150K and 125K RS proteins of strain MW5 and the 140K RS protein of strain VHA, respectively.

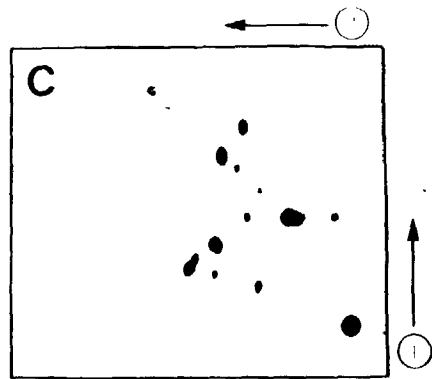
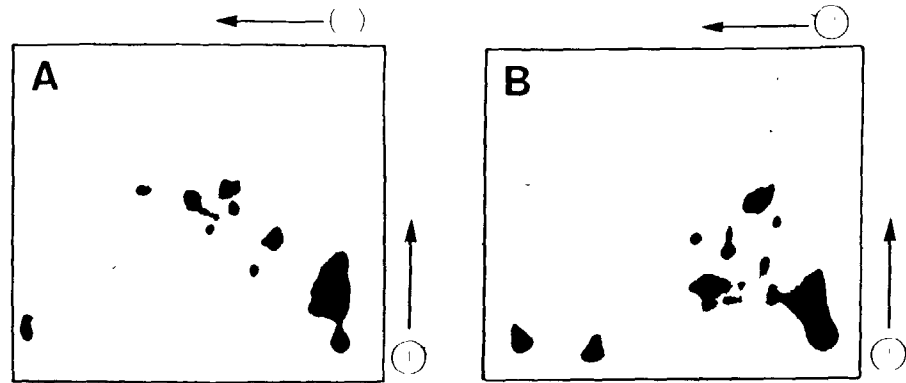


Figure 44. Autoradiograms of the two-dimensional peptide maps generated when the ^{125}I -labelled 150K (A) and 125K (B) RS proteins of *A. serpens* MW5 and the 140K RS protein of *A. serpens* VHA (C) were digested with trypsin. The peptide solutions were spotted onto the origin of cellulose-coated thin layer chromatography plates and separated in the first dimension by high-voltage electrophoresis and, subsequently, in the second dimension by chromatography.

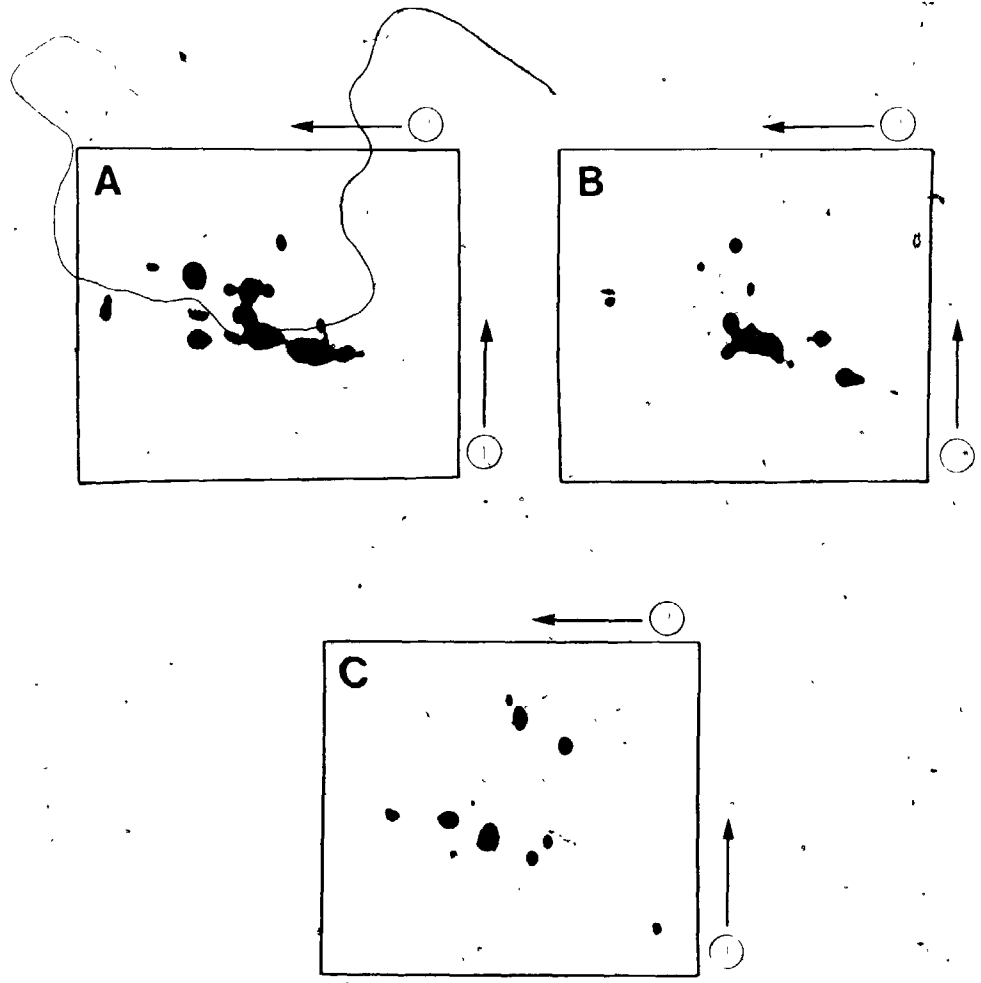
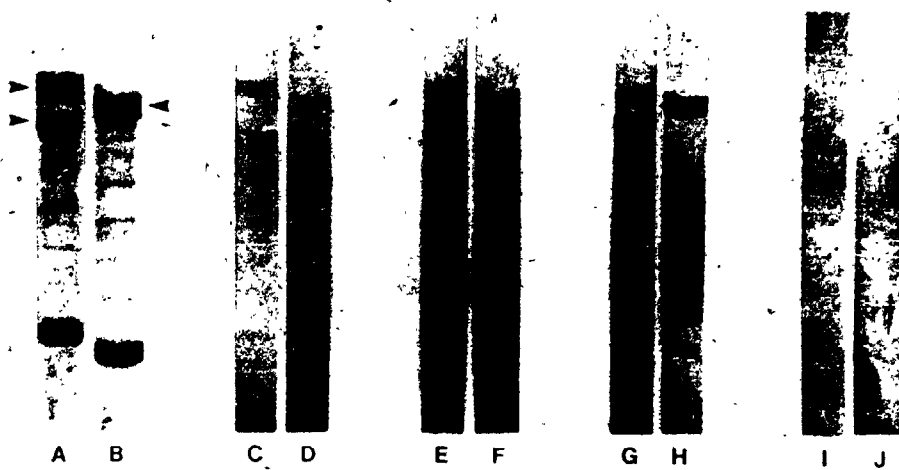
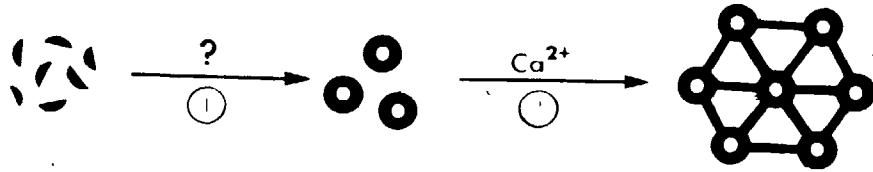


Figure 45. Antigenic relatedness of the RS proteins of A. serpens strains MW5 and VHA. Cell envelope proteins of both strains were separated by SDS-PAGE and electrophoretically-transferred to nitrocellulose sheets. Lanes A, C, E, G, I: MW5 cell envelope proteins; lanes B, D, F, H, J: VHA cell envelope proteins. The blotted proteins were incubated with : Anti-150K (MW5) IgG (lanes C, D); anti-125K (MW5) IgG (lane E, F); anti-140K (VHA) (lanes G, H); and IgG isolated from normal (pre-immune) serum (lanes I, J). Antibody which reacted with protein was detected by incubating the strips with goat anti-rabbit IgG-horseradish peroxidase conjugate followed by 4 chloro-1-naphthol. Lanes A, B: cell envelope proteins of strains MW5 and VHA stained with Amido black. Arrows indicate the positions of the RS proteins.



3

Figure 46. Model demonstrating the two-step assembly of the hexagonally-arranged RS layer. Monomers first assemble to form intact, doughnut-shaped, units (oligomers). The in vitro conditions required for this assembly were not determined. The second step of the process is calcium/strontium-dependent and involves the assembly of units into hexagonal arrays.



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