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John William Austin

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STRUCTURAL AND CHEMICAL CHARACTERIZATION OF THE S-LAYER OF *LAMPROPEDIA HYALINA*

by

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ABSTRACT

The complexity of the cell envelope of *Lampropedia hyalina* is reflected in both the structure of the envelope, as seen by electron microscopy, and in the number of polypeptides comprising the isolated envelopes, as seen by SDS polyacrylamide gel electrophoresis. The outer, proteinaceous S-layer, described in this thesis, is an integral part of the cell envelope but, in this case, more than usually separate in structural association and behaviour for the cell wall proper. The S-layer of *L. hyalina* is one of many similar, but usually less elaborate enclosing structural layers exhibited by large numbers of bacterial species; it consists of two separable components, an inner and an outer layer. When negatively stained and examined at low magnification by electron microscopy, the inner perforate layer appears as a sheet perforated with holes arranged in six-fold symmetry. The punctate layer is a complex structure composed of spiney units also arranged in a hexagonal pattern.

The inner perforate layer was isolated intact from cell envelopes by dissolution of all other attached structures and membranes by incubation in 2% sodium dodecyl sulphate at room temperature. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the isolated layer indicated it was composed of a single type of polypeptide with a molecular mass of 32,000. The isolated perforate layer was viewed in negative stain, and electron micrographs were digitally processed to increase the signal:noise ratio. The perforate layer, consists of block-shaped dimeric units which associate in triads joined together to form a continuous layer with p6 symmetry and a lattice constant of 14.6±0.4nm. The layer was stable in chelating and reducing agents,

and in various detergents. Dissolution of the perforate layer occurred in boiling sodium dodecyl sulphate, 6 M guanidine HCl, 8 M urea, 90% formic acid and in buffers below pH 4 and above pH 11. Amino acid analysis of the 32K polypeptide indicated a high proportion of glutamic acid and aspartic acid. This high content of acidic amino acids was confirmed by the presence of multiple fragments upon proteolysis of the soluble form of the polypeptide with endoproteinase glu-C. The assembled form of the polypeptide was not affected by endoproteinase glu-C, suggesting that the acidic amino acid residues are hidden from the protease in the assembled state. In contrast to the lack of surface located acidic amino acids, basic amino acids appear to be present on the surface of the polypeptide. This is indicated by both the sensitivity of the assembled array to hydrolysis by trypsin and the aggregation of sheets of the perforate layer at basic pH values approximating the pK_a of the amino group of lysine and the guanidinyl group of arginine.

The outer, or punctate layer, is assembled onto the outer aspect of the perforate layer. The punctate layer is composed of long, tapered cylindrical units centred on p6 symmetry axes which are connected by six fine linking arms joining at the axis of three-fold symmetry, to create a hexagonal layer with a lattice constant of 25.6±0.5nm. Extraction of cell envelopes with 100 mM Tris pH 8 containing 2% deoxycholate results in the release of the several proteins, but leaving the S-layers intact. The punctate layer was selectively extracted from this residue by incubation in 3M guanidine HCl or 3M urea. This treatment led to the release of three polypeptides with molecular weights of 60K, 66K and 240K. The

polypeptides released by guanidine extraction were reassembled using the perforate layer as a template, or self-assembled to form the native punctate layer by dialysis of the extract against 50 mM HEPES pH 7.5 containing 10 mM $CaCl_2$. Reassemblies of the punctate layer formed by dialysis against 50mM HEPES pH 7.5 containing 10mM $CaCl_2$ were composed of a 240K polypeptide and a 60K polypeptide, while calcium-dependent reassemblies onto the perforate layer contained polypeptides of 240K, 66K and 60K. Incubation of the soluble punctate layer with the perforate layer, resulted in calcium-independent binding of the 66K polypeptide to the perforate layer. Reassembly of the punctate layer required the presence of $CaCl_2$ or $SrCl_2$, and was inhibited by chelation of Ca^{2+} with EDTA or EGTA.

The polypeptides comprising the punctate layer were separated by column chromatography on hydroxyapatite, using a sodium phosphate gradient to elute the polypeptides. The 60K polypeptide appeared in negative stain as rings approximately 6 nm in diameter, while the 240K polypeptide appeared in negative stain as a long, flexible polypeptide. The 240K polypeptide assembled into arrays with six-fold symmetry with obvious Y-shaped linking elements. Negative stains of fractions containing both the 60K and the 240K polypeptides showed assembled punctate layer. A combination of antibodies to the 240K and the 60K polypeptides and protein A-colloidal gold labeling was used to confirm the location of these polypeptides in negatively stained preparations of the punctate layer, and in sections of whole cells.

When cells were fixed in the presence of Alcian blue or ruthenium red, the cationic dyes specifically bound to the punctate layer, suggesting it has a polyanionic nature. When cell envelope proteins

were separated by SDS polyacrylamide gel electrophoresis, the 240K polypeptide stained with the cationic dyes Alcian blue and Stains-all, suggesting the moiety labelled in sections by Alcian blue was the 240K polypeptide. The 240K polypeptide stained with periodic acid Schiff's stain, suggesting it is a glycoprotein.

The S-layer of *L. hyalina* offered the opportunity to study a complex assembly of protein molecules which comprise one of the most elaborate S-layers studied so far. This thesis examines the structure of this S-layer, with special attention to the determination of the number and type of component macromolecules comprising the layer and the way the components assemble to create the entire structure. Special attention was payed to the isolation of the components of the S-layer and the reassembly of these isolated proteins to determine their position and structural roles in the intact layer.

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To Joanne

"Bugs: Slurp macromolecular goo

And chemists will make a pet of you"

M. Alexander (1956) Localization of enzymes in the microbial cell. Bacteriol. Rev. 20: 67-93.

smegmatis when grown under iron limitation (Hall *et al.* 1987). Bacteria growing *in vivo* are also under iron limitation, and synthesize iron-regulated envelope proteins (Griffiths *et al.* 1983).

1.2.5.2 Lipopolysaccharide

Immunoelectron microscopy (Muhlradt and Golecki, 1975; Munford and Osborn, 1983) and enzymatic digestion of whole cells (Funahara and Nikaido, 1980) have been used to show that lipopolysaccharide is found only in the outer leaflet of the outer membrane. Indeed, the outer leaflet of the outer membrane appears to contain exclusively lipopolysaccharide, while the inner leaflet contains only exclusively phospholipid (Smit et al. 1975; Kamio and Nikaido, 1976). This assymetry of the outer membranes of enteric organisms may be required for the resistance of enterics to bile salts and endogenous phospholipases found in the gut (Nikaido and Vaara, 1985).

The chemistry and structure of LPS has been the subject of several reviews (Luderitz et al. 1982). LPS is an amphipathic molecule composed of three distinct regions. The hydrophobic lipid A portion, consisting of six or seven saturated fatty acid chains linked to glucosamine, anchors the LPS molecule into the outer membrane. The core region consists of a short carbohydrate chain containing 3-deoxy-D-*manno*-octulosonic acid (KDO) and heptose. The sugars of the R core are similar in many different species, whereas the sugars in the hydrophilic O-side chain (ie. O-antigen) differ in composition and structure, often within the same species. The LPS molecule is oriented in the outer leaflet of the outer membrane with the fatty acid side

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GLOSSARY

BSA	bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate
CTAB	cetyltrimethylammonium bromide
DOC	sodium deoxycholate
DTAB	dodecyltrimethylammonium bromide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis(oxyethylenenitrolo)tetraacetic acid
EPS	exopolysaccharide
HEPES	*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP	horse radish peroxidase
kV	kilovolts
LIS	lithium 3,5-diiodosalicylate
LPS	lipopolysaccharide
mA	milliamps
OM	outer membrane
OMP	outer membrane protein
OMV	outer membrane vesicle
PAGE	polyacrylamide gel electrophoresis
PHB	poly-β-hydroxybutyric acid
STEM	scanning transmission electron microscope
TDTAB	tetradecyltrimethylammonium bromide
Tris	tris(hydroxymethyl)amino methane

Chapter 1

LITERATURE REVIEW

1.1 *Lampropedia hyalina*

Lampropedia hyalina is an aerobic, cytochrome-containing, chemoheterotrophic gram-negative eubacterium characterized by the appearance of the colonies and by its appearance under the light microscope (Pringsheim, 1955; Murray, 1984; Puttlitz and Seeley, 1968; Starr, 1981). Individual cells are arranged in ordered square tablets one cell thick, giving rise to ruffled square colonies on solid media and a floating, hydrophobic film on the surface of static liquid media. The arrangement of cells has been described as "like a sheet of Chelsea buns in a string bag" (Murray, 1963). As the generic and specific names suggest, the tablets appear flat and individual cells contain refractile inclusions of poly-β-hydroxybutyric acid (PHB), giving rise to "glistening tablets" (Pringsheim, 1955).

Lampropedia hyalina is the sole member of the genus *Lampropedia* (Murray, 1984). Three other bacteria have been assigned to the genus in the past (DeToni and Trevisan, 1889) and were included as *species incertae sedis* in Bergey's Eighth Edition (Seeley, 1974). However *L. hyalina* is the only species for which axenic cultures exist and the other three species are no longer considered valid (Murray, 1984).

The nutritional requirements of *L. hyalina* were determined by Puttlitz and Seeley (1968). *L. hyalina* is auxotrophic for biotin and thiamine; utilizes pyruvate, lactate, butyrate, fumarate, malate, succinate (and acetate in the presence of catalytic levels of pyruvate) as sole energy sources; and utilizes alanine, arginine, tyrosine and NH_4Cl as sole nitrogen sources (Puttlitz and Seeley, 1968, Murray, 1984).

The natural habitat of *L. hyalina* remains unknown. While several observations of *L. hyalina* found in eutrophic environments are reported in the literature (Schroeter, 1886; DeToni and Trevisan, 1889; Kolkwitz, 1909), only three isolations have been reported. The first isolation of an axenic clone was from liquid manure (Pringsheim, 1955) in a dairy farmyard. This association with cattle is supported by the subsequent isolation of *L. hyalina* from rumen contents allowed to stand in an open Erlenmeyer flask for several days at room temperature (J. Kirschner unpublished; cited by Hungate, 1966), and is also supported by several sightings in rumen fluid (Clarke, 1979; Eadie, 1962; Hungate, 1966; Smiles and Dobson, 1956). The last reported isolation of *L. hyalina* was from nematodes living in the intestines of turtles (Schad *et al.* 1964). The specific habitat of *L. hyalina* remains unknown, although the organism does appear to be associated with eutrophic environments such as cow dung or rumen contents. Indeed, there may be no 'specific' habitat as tablets of *L. hyalina* (or at least "window-pane sarcina") have been observed in organic-rich cow dung and dilute (perhaps oligotrophic) water from a Winogradsky column set up from river water (Murray and Austin, unpublished).

L. hyalina has been reported to move by a form of "twitching motility", a characteristic involving quick movements of small groups of cells (Pringsheim, 1955; Pringsheim, 1966; Puttlitz and Seeley, 1968). This twitching motility, which is also observed in some cyanobacteria, prompted Pringsheim (1966) to include *L. hyalina* in the *Cyanophyceae* as an apochlorotic (colourless) species of *Merismopedia*. However, as noted by Murray (1984), several other bacteria show twitching motility, and almost always lack flagella, but possess fimbriae (Henrichsen, 1972). While *L. hyalina* has never been shown to possess flagella, the tablets within a sheet are connected by long fimbriae (Austin, unpublished results) which are perhaps involved in the twitching motility.

The sheets arise by synchronous division of the cells, with each set of divisions alternating at right angles to the previous set of divisions (Pringsheim, 1955; Murray, 1963; Kuhn and Starr, 1965). Daughter cells are separated by an 'intercalated layer' (Chapman *et al.* 1963; Murray, 1963) but remain attached by fine fibres extending between the cells (Chapman *et al.* 1963; Murray, 1963; Pangborn and Starr, 1966). These same fibres hold the complex S-layer onto the surface of the tablet. Unlike most S-layers, that of *L. hyalina* surrounds the entire tablet of cells, rather than each individual cell (Murray, 1963).

The S-layer of *L. hyalina* encloses, not individual cells, but entire tablets of cells (Chapman *et al.* 1963), cutting the sheets into tablets of 16, 32 or 64 cells (Chapman *et al.* 1963; Murray, 1963; Pangborn and Starr, 1966). The outer layer, the punctate layer, consists of spiny units connected by Y-shaped linking elements, to construct an array with hexagonal symmetry (Murray, 1963; Chapman *et al.* 1963). The

punctate layer is assembled onto an inner perforate layer, so named because it appears to be a sheet full of regularly arranged holes, which is attached to the outer membrane of the cells by the fibres of the intercalated layer (Murray, 1963; Chapman *et al.* 1963; Pangborn and Starr, 1966). Such complex S-layers have been observed on only a limited number of bacteria. This unusually complex S-layer of *L. hyalina*, the subject of this thesis, has been studied in terms of structure, composition and assembly.

1.2 The Gram-negative Cell Wall

If an obvious distinction between cytoplasmic membrane and cell wall may be made in Gram positive bacteria, the discrimination between these components in the gram-negative bacteria is more ambiguous because the cell wall includes an outer membrane. For this reason, the outer layers of gram-negative cells, ie. cytoplasmic membrane and cell wall, are better referred to collectively as the "cell envelope", as they are isolated together upon lysis of the cell. The bacterial wall has been described as the "entire fabric and associated space above the plasma membrane" (Beveridge, 1981).

Present perceptions of the gram-negative cell envelope of eubacteria suggests its basic structure consists of a cytoplasmic membrane, a peptidoglycan layer, a diffuse periplasm, an outer membrane, and, on many species, an outermost S-layer (Appendix 1). Additional layers of polysaccharide are often found on the outside of gram-negative bacteria (Costerton *et al.* 1981). Besides these layers, which completely enclose the cell, structures such as flagella, fimbriae,

pili and spinae are often found extending from the cell envelope (Holt and Beveridge, 1982). Such complexity is warranted by the many functions of the gram-negative cell envelope: provision of shape to the cell, protection from mechanical and osmotic damage, maintenance of proper ionic strength and pH, and provision of selective transport into, and out of, the bacterial cell.

1.2.1 The Cytoplasmic Membrane

The functions of the cytoplasmic membrane are mainly that of transport, bioenergetic functions (mitochondrial-type functions), biosynthetic functions (lipopolysaccharide, peptidoglycan, lipids) and anchoring of the nucleoid (Salton, 1987). The cytoplasmic membrane includes the electron transport chain (cytochromes) and the enzyme system for oxidative phosphorylation. Active transport systems are also contained within the cytoplasmic membrane. Biosynthesis of exocellular polysaccharides and wall materials also occurs at the cytoplasmic membrane.

1.2.2 The Peptidoglycan Layer

The rigid wall layer of *E. coli* B cell envelopes was first described by Weidel *et al.* (1960), and was later isolated as a bag-shaped macromolecule and characterized biochemically as murein, mucopeptide or peptidoglycan (Weidel and Pelzer, 1964). Peptidoglycan (syn. murein, mucopeptide) is the rigid wall layer of both gram positive and gram-negative organisms. Early observations of thin sections of *Escherichia coli* revealed an inner membrane, or cytoplasmic membrane, and a triple layered wall (Kellenberger and Ryter, 1958). The triple

layered wall observed by Kellenberger and Ryter (1958) was, in fact, the outer membrane and the closely applied and thin layer of peptidoglycan, subsequently resolved by Murray, Steed and Elson (1965) and dePetris (1967). Thin sections of glutaraldehyde and osmium fixed gram-negative bacteria reveal inner and outer membranes, separated by an intermediate layer, identified by Murray *et al.* (1965) to be the peptidoglycan layer. This intermediate layer, the G layer described by DePetris (1967), was thought to comprise two separate layers, a globular protein layer (g1) and a peptidoglycan layer (g2) (DePetris, 1967). The peptidoglycan layer in gram-negative bacteria is 2.0 to 3.0 nm in thickness (Murray *et al.* 1965), and can accommodate no more than 3 layers of peptidoglycan.

Recently, a new model for the structure of peptidoglycan has been proposed by Hobot *et al.* (1984). Based on sections of *E. coli* prepared by the methods of freeze-substitution and progressive lowering of temperature of glutaraldehyde fixed cells, Hobot *et al.* (1984) suggest that the peptidoglycan exists as a hydrated polymer, filling the entire 5 to 7 nm space between the cytoplasmic and outer membranes, forming a framework within which the periplasmic functions can operate. Peptidoglycan bands in Percoll density gradients at a position corresponding to a density just greater than that of water (Humphries *et al.* 1981; Hobot *et al.* 1984), supportive of the hypothesis that the peptidoglycan exists as a highly hydrated polymer in its native state.

Peptidoglycan consists of a backbone polymer of two sugar derivatives, N-acetylglucosamine and N-acetylmuramic acid, and several amino acids forming pentapeptide side chains on the muramic acid component. The glycan chains formed by the β-1,4 linked sugars are

connected by peptide cross-links formed by the amino acids, in such a way that a covalently linked structure the shape and size of the cell is formed. In gram-negative bacteria, cross-links are formed by peptide linkage of the amino group of diaminopimelic acid to the carboxyl group of the terminal D-alanine. In gram positive bacteria, the cross-links are formed by several types of peptide bridges, differing with the organism (Schleifer and Kandler, 1972).

The glycan portion of peptidoglycan is uniform throughout most of the eubacteria, the exceptions being *Planctomyces*, *Pirella*, and *Mycoplasma* which do not possess muramic acid (Liesack *et al.* 1986). N-acetylglucosamine and N-acetylmuramic acid are always linked β-1,4 to form the glycan chains. Several amino acids may make up the cross-link, including alanine, glutamic acid, diaminopimelic acid, glycine, threonine, serine and aspartic acid. Branched chain amino acids, aromatic amino acids, sulfur containing amino acids, and arginine, histidine, and proline have never been found in peptidoglycan.

1.2.3 The Periplasm

The space between the cytoplasmic and the outer membranes is referred to as the periplasm. The periplasm contains specific classes of proteins amounting to approximately 4% of the total cell protein (Nossal and Heppel, 1966), as well as several oligosaccharides (van Golde *et al.* 1973). Estimates of the volume of the periplasm have varied from 1% to 2% (Nikaido, 1979) to 7% of the total cell volume (Hobot *et al.* 1984).

While most electron micrographs show the presence of a free area between the inner and outer membranes, recent evidence suggests that the periplasm exists as a viscous gel between the outer and cytoplasmic membranes. Isolated cell envelopes fixed in glutaraldehyde and embedded in Lowicryl HM20 show only an outer membrane, cytoplasmic membrane, and a space between the two membranes (Armbruster *et al.* 1982). Hobot *et al.* (1984), using freeze-substitution embedding, have shown the periplasm to be filled with a viscous gel. To support this conclusion, Brass *et al.* (1986) have shown the lateral diffusion of maltose binding protein to be approximately 1,000 times slower in the periplasm than the rate of diffusion in aqueous solution.

1.2.4 Adhesion Zones and Periseptal Annuli

At several hundred areas of the gram-negative cell envelope, the inner and outer membranes are attached to one another (Bayer, 1968; Bayer, 1974; Bayer, 1975). These 'membrane adhesion sites' have been shown to have several functions: export of lipopolysaccharides (Bayer, 1975), capsular polysaccharide (Bayer and Thurow, 1977), and major outer membrane proteins (Bayer, 1979). Interestingly, no adhesion sites between the inner and outer membranes are observed in freeze-substituted preparations of plasmolysed *E. coli* cells (Hobot *et al.* 1984).

A new organelle, involved in cell division, has recently been described for *Salmonella typhimurium* (MacAlister *et al.* 1983) and for *E. coli* (Anba *et al.* 1984). The "periseptal annuli" flank the area of the cell involved in septum formation. The annuli are formed by association of the cytoplasmic membrane with the peptidoglycan sacculus (MacAlister *et al.* 1983). The location of periseptal annuli on

either side of the abnormal division septa in a cell division mutant of *Salmonella typhimurium* suggested that the annuli might function by segregating the site of septation from the remainder of the cell envelope (MacAlister *et al.* 1983). Envelope fractions containing adhesion sites between the cytoplasmic membrane, peptidoglycan and outer membrane have been isolated (Bayer *et al.* 1982; Ishidate *et al.* 1986), and periseptal annuli have been shown to be a part of this fraction (Chakraborti *et al.* 1986).

1.2.5 The Outer Membrane

The outer membrane acts mainly as a barrier to a wide variety of antibiotics, proteolytic and lipolytic enzymes, and bile salts of the gut (Nikaido and Nakae, 1979). The outer membrane contains selective pores, allowing certain substrates through, but impeding most others. In general, the outer membrane is impermeable to hydrophobic or high molecular weight substances.

The insolubility of the outer membrane to certain detergents (Schnaitman, 1971a, b) simplifies the isolation of this membrane. DePamphlis and Adler (1971) isolated the outer membrane by first making spheroplasts from *Escherichia coli* by the addition of lysozyme in the presence of EDTA, then by the addition of Triton X-100 were able to solubilize selectively the cytoplasmic membrane, leaving the outer membrane intact. Outer membrane isolated in this manner could be dissociated by combined treatment with Triton X-100 and EDTA, and the membrane can be reformed by dialysis against $MgCl_2$ (DePamphlis, 1971), illustrating the importance of Mg^{2+} to the integrity of the outer membrane. Although the outer membrane of *E. coli* appears to remain

intact in Triton X-100, two-thirds of the phospholipid and half of the LPS are solubilized by the detergent (Schnaitman, 1970). The cytoplasmic membranes of *E. coli* (Schnaitman, 1971) and *Fusobacterium nucleatum* (Bakken and Jensen, 1986) are selectively solubilized by Triton X-100, leaving the outer membrane intact. Sarkosyl may also be used to selectively solubilize the cytoplasmic membranes of gram-negative bacteria (Filip *et al.* 1973; Bachhawat and Ghosh, 1987; Chopra and Shales, 1980). There is some evidence that Sarkosyl extraction does not completely solubilize the inner membrane of some gram-negative bacteria, perhaps a result of extensive fusions between the inner and outer membranes (Page and Taylor, 1988).

1.2.5.1 Outer membrane proteins

Outer membrane proteins of Gram-negative bacteria generally are limited to three to five 'major' proteins as analyzed by SDS PAGE (Lugtenberg and van Alphen, 1983), although several minor bands are usually observed. The biochemistry, functions and genetics have been the subjects of several reviews (Osborn and Wu, 1980; Lugtenberg and van Alphen, 1983; Nikaido and Vaara, 1985).

The outer membrane of *E. coli* is anchored to the peptidoglycan by the low molecular weight (7.2 K) lipoprotein (Braun and Rehn, 1969; Braun, 1975), which is present in about 7 X 10^5 copies in *E. coli* (Nikaido and Vaara, 1985). About one-third of the lipoprotein (DiRenzo *et al.* 1978) is covalently attached to the peptidoglycan by the amino group at the C-terminal lysine (Braun, 1975; Inouye *et al.* 1972). The sulfhydryl group of the N-terminal cysteine residue is substituted with a diglyceride, and the amino group of the N-terminal cysteine is

substituted with a fatty acid residue through an amide linkage (Braun, 1975). The lipoprotein probably plays a structural role in maintaining the integrity of the cell envelope, by holding the outer membrane onto the peptidoglycan. A lipoprotein deletion mutant (lpo) of *E. coli* K12 shows evagination of the outer membrane a division sites, increased release of outer membrane in the form of blebs, increased sensitivity to EDTA, and changes in the barrier function of the outer membrane (Hirota *et al.* 1977)

Outer membranes of gram-negative bacteria are porous to molecules smaller than 600D (for review see Nikaido and Vaara, 1985). The major outer membrane proteins, or porins, are responsible for the 'molecular sieve' properties of the outer membrane. Rosenbusch (1974) was the first to characterize the matrix protein, or porins, of *E. coli*. The porins of *E. coli* share similar properties and are referred to by names given to their corresponding structural genes, OmpC, OmpD, OmpF and PhoE (Osborn and Wu, 1980; Nikaido and Vaara, 1985). OmpC, OmpF and PhoE are the porins of *E. coli* K-12, whereas OmpD is found in the outer membrane of *Salmonella typhimurium* LT2 (Nurimen *et al.* 1976). OmpC, OmpD and OmpF are responsible for the permeability of outer membranes to small polar substances such as mono- and disaccharides (Nakae *et al.* 1979), amino acids (Nakae *et al.* 1979) and inorganic ions (Benz *et al.* 1978; Benz *et al.* 1979; Schindler and Rosenbusch, 1978).

The porins form general hydrophilic pores which allow nonspecific diffusion of solutes across the outer membrane (Osborn and Wu, 1980). Trimers of porin proteins cross the outer membrane and function as sieves with a cutoff point of about 600 to 700 daltons (Nakae *et al.*

1979). Studies involving reconstitution of pores in liposomes suggest the channel diameter for the porins to be between 1.1 and 1.2 nm (Nikaido and Rosenberg, 1981).

Extraction of *E. coli* in 2% SDS at 70°C solubilizes all cellular proteins except the lipoprotein and the porin proteins (including OmpC, D, and F proteins, LamB). The porins retain their native structure (Rosenbusch *et al.* 1980) and remain non-covalently attached to the peptidoglycan, unless heated to 90°C in 2% SDS (Rosenbusch, 1974). The porin proteins share several properties including: resistance to proteolysis, a high content of β structure in the native state, strong self-association to form trimers, and a strong association with peptidoglycan after extraction with SDS (Benz *et al.* 1978; Benz *et al.* 1979; Nakae *et al.* 1979; Rosenbusch, 1974).

After extraction of cell envelopes of *E. coli* with 2% SDS at 70°C, the porins can be seen by electron microscopy (Steven *et al.* 1977). The porins are attached to the peptidoglycan as a periodic monolayer in three-fold symmetry on a hexagonal lattice with a 7.7 nm repeat spacing (Steven *et al.* 1977). Two types of porin lattice can be formed by reconstitution of purified porin trimers with dimyristoyl phosphatidylcholine (Engel *et al.* 1985). Reconstitution with high protein concentrations (final protein-to-lipid weight ratios of 6) yields a rectangular lattice (unit cell dimensions a=13.7 nm, b=7.9 nm) and a hexagonal lattice (a=7.9 nm). Three-dimensional reconstructions of such reconstituted porin lattices indicated three separate pores, on the outer surface of the outer membrane, merge into a single pore on the periplasmic side (Engel *et al.* 1985).

The major "non-porin" outer membrane protein of gram-negative enteric bacteria is the OmpA protein. A characteristic feature of the OmpA protein is its "heat-modifiability" (Nakamura and Mizushima, 1976; Schnaitman, 1973), or the decrease in mobility of the protein in SDS polyacrylamide gels upon heating of the protein in SDS. Similar behaviour has been noted for other outer membrane proteins such as protein II or opacity protein of *Neisseria gonorrhoeae* (Heckels, 1977; Swanson, 1978) and group III protein of *Brucella spp.* (Verstreate *et al.* 1982). Three envelope proteins of *Bordetella pertussis* display heat-modifiable migration in SDS polyacrylamide gels (Armstrong and Parker, 1986).

While the outer membrane proteins of *E. coli* and *Salmonella typhimurium* have been studied in detail, the outer membrane proteins of most other gram-negative bacteria are poorly characterized. The outer membrane proteins of *Neisseria gonorrhoeae* form a disulfide linked complex (Newhall *et al.* 1980). Porins of *Pseudomonas aeruginosa* are attached to the peptidoglycan, but not in the same way as those of enterobacteria (Hancock *et al.* 1981). Also, several porins of non-enterobacterial origin often do not form SDS resistant oligomers (Douglas *et al.* 1981; Hancock *et al.* 1979), and have larger pore sizes. For example, protein F of *P. aeruginosa* forms a pore of diameter 2.2 nm (Benz and Hancock, 1981).

The major outer membrane protein of *Aeromonas salmonicida* is a 42 K protein that is similar to the porins of enterobacteria. It is noncovalently associated with the peptidoglycan, it is released from the peptidoglycan by 0.1M NaCl and SDS, and its electrophoretic mobility is dependent upon the solubilization temperature (Darveau *et al.* 1983).

When added to a planar lipid bilayer the 42 K protein caused the conductance of the bilayer to increase by several orders of magnitude (Darveau *et al.* 1983) indicating that it is a porin-type protein. The outer membrane of *Aeromonas hydrophila* contains a 43 K pore forming protein, with a conductance similar to that of *Aeromonas salmonicida* (Darveau *et al.* 1983). While *Aquaspirillum serpens* contains two peptidoglycan-associated proteins, with molecular weights of 32K and 33 K (Koval and Murray, 1981), it is was not determined if they are pore-forming proteins.

Outer membrane protein profiles are dependent upon growth conditions (Sterkenburg *et al.* 1984; Brown and Williams, 1985). Growth of *E. coli* with limiting amounts of phosphate, sulphate, or iron results in the induction of new outer membrane proteins (Lugtenberg and van Alphen, 1983). Growth of *P. aeruginosa* under magnesium limitation results in the synthesis of outer membrane protein H1 (Nicas and Hancock, 1980). When gram-negative bacteria are grown under iron-limited conditions, several new proteins appear in the outer membrane (Gilleland, 1988). Some of the proteins induced by iron-limiting conditions function as receptors for iron siderophores (Nielands, 1982). Growth of plant pathogenic *Pseudomonas* sp. under iron limited conditions induces several outer membrane proteins with molecular weights between 70 K and 90 K (deWeger *et al.* 1986; Magazin *et al.* 1986; Meyer *et al.* 1979). Although siderophore receptors are commonly high molecular weight proteins, ^{59}Fe-labelled pyochelin (an iron siderophore) was shown to bind to an outer membrane protein of molecular mass 14 K in *P. aeruginosa* (Sokol and Woods, 1983). At least four additional envelope proteins are synthesized by *Mycobacterium*

smegmatis when grown under iron limitation (Hall *et al.* 1987). Bacteria growing *in vivo* are also under iron limitation, and synthesize iron-regulated envelope proteins (Griffiths *et al.* 1983).

1.2.5.2 Lipopolysaccharide

Immunoelectron microscopy (Muhlradt and Golecki, 1975; Munford and Osborn, 1983) and enzymatic digestion of whole cells (Funahara and Nikaido, 1980) have been used to show that lipopolysaccharide is found only in the outer leaflet of the outer membrane. Indeed, the outer leaflet of the outer membrane appears to contain exclusively lipopolysaccharide, while the inner leaflet contains only exclusively phospholipid (Smit et al. 1975; Kamio and Nikaido, 1976). This assymetry of the outer membranes of enteric organisms may be required for the resistance of enterics to bile salts and endogenous phospholipases found in the gut (Nikaido and Vaara, 1985).

The chemistry and structure of LPS has been the subject of several reviews (Luderitz et al. 1982). LPS is an amphipathic molecule composed of three distinct regions. The hydrophobic lipid A portion, consisting of six or seven saturated fatty acid chains linked to glucosamine, anchors the LPS molecule into the outer membrane. The core region consists of a short carbohydrate chain containing 3-deoxy-D-*manno*-octulosonic acid (KDO) and heptose. The sugars of the R core are similar in many different species, whereas the sugars in the hydrophilic O-side chain (ie. O-antigen) differ in composition and structure, often within the same species. The LPS molecule is oriented in the outer leaflet of the outer membrane with the fatty acid side

chains anchored in the membrane with the hydrophilic O-side chain exposed on the outer surface.

The heterogeneity of LPS is a result of alterations to the lipid A and core polysaccharides, and of variations in the number of repeat units comprising the O-antigen (Goldman and Lieve, 1980; Munford *et al.* 1980; Palva and Makela, 1980; Peterson and McGroarty, 1985). SDS polyacrylamide gel electrophoresis and gel filtration have been the most often used methods of separation of the subclasses of LPS (Goldman and Lieve, 1980; Hitchcock and Brown, 1983; Morrison and Lieve, 1975; Munford *et al.* 1980; Palva and Makela, 1980; Peterson and McGroarty, 1985).

1.3 Regularly Structured Surface (S) Layers

1.3.1 Introduction

The outermost layer of many bacteria consists of a regular macromolecular array of proteins arranged in either a tetragonal, hexagonal, or oblique pattern. These layers are referred to as surface (or structured) layers or S-layers. Sleytr *et al.* (1988) have defined "S-layer" as "two-dimensional crystalline arrays of proteinaceous subunits forming surface layers on procaryotic cells." These layers are of considerable interest because often they consist of the highest molecular weight protein in cells possessing such layers, and that protein is present in higher amounts than any other cellular protein, an energy investment indicating their importance to the cell. In cells possessing an S-layer, at least 5% of the total cell protein is present as S-layer protein (Howard and Tipper, 1973; Thornley *et al.* 1974; Smit

et al. 1981). Since S-layer proteins are present in such large amounts, they should make excellent models for the study of biosynthesis, transport and assembly of cell structures. They must be functional and are thought to perform important protective functions, possibly related to their forming a semipermeable barrier enclosing and protective to the cell (Sara *et al.* 1988; Manigley *et al.* 1988). They are found on a wide variety of eubacteria and archaeobacteria (for reviews see Sleytr and Messner, 1983, 1988; Smit, 1986), and are arranged in p2, p4 or p6 symmetry with lattice spacings ranging from 2.8 nm, for *Methanospirillum hungatei* (Stewart *et al.* 1985; Beveridge *et al.* 1985), to 35 nm for *Chromatium buderi* (Remsen *et al.* 1970; Baumeister and Engelhardt, 1987). S-layers are composed of protein or glycoprotein subunits with molecular weights ranging from 13 K to 560 K (Sleytr and Messner, 1983; Wildhaber *et al.* 1988), and assembled into multimeric units to form symmetrical and paracrystalline arrays, usually held together by non-covalent bonds including hydrogen bonds, hydrophobic bonds and ionic bonds (Sleytr and Messner, 1983; 1988).

The first S-layer to be observed was that of an *Aquaspirillum* sp. (Houwink, 1953). S-layers were later observed on the surfaces of *Halobacterium halobium* (Houwink, 1956), *Rhodospirillum rubrum* (Salton and Williams, 1954), *Deinococcus radiodurans* (Glauert, 1962), again on *Aquaspirillum serpens* (Murray, 1963), and on *L. hyalina* (Murray, 1963, Chapman *et al.* 1963). S-layers may be more common than previously believed. At the time of publishing of the review by Sleytr and Messner (1983), S-layers had been demonstrated on the surfaces of 142 species in 58 genera of Eubacteria and Archaeobacteria (Koval and Murray, 1986). By 1988 the numbers had increased to 188 species in

58 genera of eubacteria and 54 species in 25 genera of archaeobacteria (Sleytr and Messner, 1988). Crystalline surface layers have also been observed on some green algae (Roberts *et al.* 1982, 1985). The study of S-layers seems to have been neglected, perhaps because they are not found on the enterobacteria, on which most of the detailed study of the cell envelope has been done. Also, S-layers may be often overlooked, as negative stains of cells, or envelope fragments, may not show evidence of an S-layer. For example, the S-layer of *Azotobacter vinelandii* is disrupted not only by uranyl acetate and phosphotungstic acid, but is also removed by suspension of cells or envelopes in distilled water (Bingle *et al.* 1987). S-layers can be demonstrated on these cells by chemical fixation before negative staining, or by metal shadowing (with or without freeze-etching) of whole cells (Messner *et al.* 1984; Koval and Jarrell, 1987).

When present, S-layers are not always the outermost layer of the cell. Many cells with S-layers possess an additional layer of exopolysaccharide external to the protein array (Costerton *et al.* 1981). Specific monoclonal antibodies have been used to demonstrate the penetration of the S-layer of *Aeromonas salmonicida* by the carbohydrate chains of lipopolysaccharide (Chart *et al.* 1984).

1.3.2 Structure

Several methods of image reconstruction have been used in the past. These include linear integration or direct superimposition (Markham *et al.* 1963), optical filtering (Klug and DeRosier, 1966) and digital filtering by computer (Aebi *et al.* 1973). All the methods of

image processing involve taking the average of several images of the same object, in the case of S-layers, the object being the unit cell.

Repeating features of a crystalline lattice give rise to diffraction spots, when optical methods are used. These diffraction spots are analogous to the maxima arrived at by Fourier transforms of crystalline data, and are geometrically related to the crystal structure of the image (Stewart, 1986).

The problems encountered in imperfect crystalline structures, and small and irregularly shaped crystal fragments may be negated by using correlation averaging (Saxton and Baumeister, 1982). Correlation averaging involves moving a reference image (usually one unit cell) around a large patch of the lattice, and comparisons between the reference image and the local image are made by cross-correlation. Local correlation peaks are then taken to be the centres of mass of the unit cells. An early use of correlation averaging was the study of the tetragonal lattice of *Sporosarcina ureae* (Baumeister *et al.* 1981).

Most S-layers completely cover the cell surface, and may be arranged in p2 (oblique), p4 (square), or p6 (hexagonal) symmetry with varying arrangements of delicate linking structures between the units of the array (Sleytr and Messner, 1983). Thus is formed a complex network of structure with spaces penetrating the full thickness of the layer.

Three-dimensional reconstructions have been made from several negatively stained S-layers and these have been discussed in detail in several reviews (Hovmoller et al. 1989; Baumeister et al. 1989; Baumeister and Engelhardt, 1987). The S-layer of *Methanospirillum hungatei* is unusual, as it consists of four approximately spherical

polypeptides approximately 2.5 nm in diameter arranged in a row (Stewart *et al.* 1985; Shaw *et al.* 1985).

The lattice spacing of the hexagonally arranged units of the S-layer of *Sulfolobus acidocaldarius* is 22 nm (Deatherage *et al.* 1983; Taylor *et al.* 1982). The subunit molecular weight of the Sulfolobus S-layer is 140,000 to 170,000. A large space 18.5 nm across at the bottom, and 8 nm deep, opens with a 5 nm pore at the outer surface of the S-layer.

The subunits of the S-layer of *Chlamydia trachomatis* (Chang *et al.* 1982) have a molecular weight of only 39,500, and join to form a central ring of six subunits around a central depression 8 nm deep and 10 nm in diameter. The depression opens toward the inner surface of the S-layer.

The S-layer of *Synechocystis* sp. (Karlsson *et al.* 1983) consists of hexamers arranged around a central pore that is 10 nm deep and 2.5 nm in diameter. The end of the pore facing the cell is closed and the other end is open. The hexamers are arranged in six-fold symmetry, with a 15.2 nm lattice spacing. The linking elements between the hexamers are along two-fold symmetry axes and are located in the middle of the array. The molecular weight of the monomer is approximately 100 K.

The three-dimensional structure of the S-layer of *Aquaspirillum serpens* VHA has been reconstructed to a resolution of 1.6 nm (Dickson *et al.* 1986). The subunits have a molecular weight of 140 K (Glaeser *et al.* 1979), and are arranged, with a lattice spacing of 15.5 nm, as hexamers around a central 2.5 nm pore, which is closed at the bottom of the hexamer. In side-views of frozen hydrated specimens, the

hexamers are 15.5 nm high (Glaeser *et al.* 1979), while the three-dimensional reconstruction from tilt series of negatively stained arrays gave a height of 11.0 nm (Dickson *et al.* 1986). The linking elements are of the delta type, and connect the central hexamers at the outer surface of the array.

The S-layer, or T-layer (Aebi *et al.* 1974; Kistler *et al.* 1977; Smith and Kistler, 1977), of *Bacillus sphaericus* T-1 has p4 symmetry, a lattice spacing of 13 ± 0.2 nm, and a thickness of 8 nm (Lepault *et al.* 1986). Three-dimensional reconstructions of negatively stained and tilted specimens (Lepault *et al.* 1986) support the conclusion that the T-layer structure can be divided into three domains: a major domain, a minor domain, and an arm (Aebi *et al.* 1974), and that these domains are located in different planes. The minor domain is made of cone-like structures 3 nm high. The arm domain is thicker than the major domain (ca. 4 nm). The major domain protrudes about 4 or 5 nm from the arm domain (Lepault *et al.* 1986).

The structure of the S-layer of *Bacillus sphaericus* P-1 is similar to that of *Sporosarcina ureae* (Lepault *et al.* 1986; Engelhardt *et al.* 1986). The S-layer of the latter is arranged in four-fold symmetry (Beveridge, 1979; Engelhardt *et al.* 1986) with a lattice constant of 12.9 nm and a minimum thickness of 6.6 nm. The tetragonal unit cell contains a massive core at the major four-fold axis of symmetry [similar to the major tetramer of Aebi et al (1974)], arms between adjacent unit cells on the two-fold axes, and spurs connecting substructures of neighboring unit cells at the minor fourfold axes of symmetry [minor tetramers of Aebi et al (1974)] (Engelhardt *et al.* 1986).

Like the S-layers of *Sporosarcina ureae* and *Bacillus sphaericus*, the S-layers of *Azotobacter vinelandii* (Bingle *et al.* 1987) and *Aeromonas salmonicida* (Dooley *et al.* 1989) are arranged with tetragonal symmetry, and the three-dimensional structure has been determined. Both of these S-layers consist of 'funnel-shaped' subunits situated and one fourfold-symmetry axis and connected at the other fourfold-symmetry axis to form 'cruciform' linking structures (Bingle *et al.* 1987b; Dooley *et al.* 1989). The opening of the funnel formed by the subunits is exposed to the environment, while the apex of the funnel faces the outer membrane and the cruciform linking elements are located at the outermost surface of the S-layer. The primary difference between the S-layers of *A. vinelandii* and *A. salmonicida* is the apparently greater amount of protein mass in the linkers of *A. vinelandii*, as compared to *A. salmonicida* (Dooley *et al.* 1989). The S-layers of *Azotobacter vinelandii* and *Aeromonas salmonicida* lack the domain-type structure (major and minor tetramers) seen in the S-layers of *Sporosarcina ureae* (Beveridge, 1979; Engelhardt *et al.* 1986) and *Bacillus sphaericus* (Aebi *et al.* 1974; Lepault *et al.* 1986), and *Bacillus polymyxa* (Burley and Murray, 1983).

1.3.3 Isolation

Several methods have been used to isolate S-layers or their component proteins. S-layers of gram-positive organisms are easily isolated as extensive sheets by solubilization of the underlying peptidoglycan of wall fragments by lysozyme or autolysis (Sleytr, 1976; Beveridge, 1979; Kawata *et al.* 1984). The sheets may then be made soluble in guanidine HCl or urea prior to biochemical studies of the

component polypeptides (Masuda and Kawata, 1980). Alternatively, the S-layer may be solubilized from the peptidoglycan by urea, and the soluble protein may be further purified by ammonium sulphate precipitation (Lewis *et al.* 1987) or repeated cycles of assembly and disassembly (Sleytr *et al.* 1986).

Isolation of S-layers from envelopes of gram-negative bacteria often proves more difficult than simple solubilization of cytoplasmic membranes and digestion of peptidoglycan. The S-layers of gram-negative bacteria range from being delicate and easily lost, to resistant to mechanical and chemical treatments. The latter are often easily isolated by solubilization of all envelope components, except the S-layer and the peptidoglycan, by detergents such as SDS. The peptidoglycan may be digested with lysozyme, leaving the S-layer. An example of this type of S-layer is that of *Deinococcus radiodurans* (Thompson *et al.* 1982). The layer is washed clean of all attached membranes and protein by extraction in SDS.

S-layers of intermediate fragility are the most common type found on gram-negative bacteria. These S-layers are usually isolated from cell envelopes obtained from cells which have been ruptured in a French press. The cytoplasmic and outer membranes are usually selectively solubilized by extraction with detergents such as Triton X-100 (Evenberg and Lugtenberg, 1982; Kist and Murray, 1984), sodium lauryl sulphate (Phipps *et al.* 1983) or sodium deoxycholate (Phipps *et al.* 1983). The S-layer is then extracted from the cell wall preparation with guanidine HCl (Buckmire and Murray, 1970; Phipps *et al.* 1983), urea (Kist and Murray, 1984), EDTA or EGTA (Thornley *et al.* 1974), and may be further purified by gel filtration, ion exchange or other

techniques used in protein purification. A simple purification scheme was used to isolate the S-layer of *Aeromonas salmonicida,* based upon the resistance of the layer to sodium deoxycholate. Whole cells were lysed and directly extracted in buffer containing deoxycholate, the S-layer was then extracted from the residue with guanidine HCl and desalted on Sephadex G-25 (Kay *et al.* 1984). The S-layer of *Caulobacter crescentus* is shed into the growth medium attached to red-pigment aggregates, where it can be isolated by differential centrifugation (Smit *et al.* 1981). These aggregates shed by C. crescentus can treated with SDS and the soluble S-layer can be isolated by gel filtration chromatography (Smit and Agabian, 1984).

Some S-layers of gram-negative bacteria prove to be delicate, and easily lost. An example is that of *Flexibacter polymorphus* (Ridgway and Lewin, 1983). Cells which have been made into spheroplasts and osmotically lysed, are treated with Triton X-100 and the S-layer is released by sonication. The soluble S-layer is then further isolated by cesium chloride density gradient centrifugation. Some S-layer proteins are released from the cell surface in a relatively pure form without prior disruption of the cell envelope. For example, the S-layer protein of *Rhodospirillum salexigens* is released from intact cells by washing cells in 30% sucrose to remove proteins attached to the cell surface by ionic bonds (Evers *et al.* 1984). The protein comprising the S-layer of *Aeromonas salmonicida* can be extracted from whole cells by treatment with 0.2 M glycine hydrochloride (pH 3.0) (Dooley *et al.* 1988). The S-layer of *Azotobacter vinelandii* is unusual, in that it is removed from whole cells simply by washing with distilled water (Bingle *et al.* 1984).

Repeated extraction of cell envelopes of *Halobacterium salinarium* with chloroform/methanol, 2/1 has been used to obtain lipid-free cell envelopes (Mescher *et al.* 1974). The S-layer protein of *H. salinarium* can be partially purified from lipid-free envelopes by extraction in aqueous phenol (Mescher and Strominger, 1976). Most envelope proteins were found in the phenol phase, while the S-layer protein, its degradation products, and nucleic acids were found in the aqueous phase. The S-layer protein was then separated from the residual phenol and nucleic acids by chromatography on DEAE-cellulose. This procedure results in a yield of greater than 30% of the total S-layer protein (Mescher and Strominger, 1976).

1.3.4 Chemistry

While S-layers consist of polypeptides with a wide range of molecular weights, most S-layers studied so far are similar in amino acid content. S-layer proteins contain a high proportion of acidic and hydrophobic amino acids and few sulfur-containing amino acids (Buckmire and Murray, 1973; Messner *et al.* 1984; Beveridge, 1979; Kay *et al.* 1981, 1984; Schenk and Earhart, 1981; Mescher and Strominger, 1976; Sleytr *et al.* 1986; Kist, 1986; Lewis *et al.* 1987). When the ratio of the number of polar amino acids to the number of nonpolar amino acids (Barrantes, 1975; Cantor and Schimmel, 1980) is calculated from the amino acid composition, the values are typical for extrinsic membrane proteins (Sleytr and Messner, 1983), and resemble the values obtained for spinin (Easterbrook and Coombs, 1976), pilin (Brinton, 1965) and the membrane glycoprotein of *Myxococcus xanthus* (Maeba, 1986). The S-layer of *Sulfolobus acidocaldarius* is exceptional among

S-layer proteins, containing few charged amino acids, but high levels of hydroxyl-containing amino acids (Michel *et al.* 1980). The *S. acidocaldarius* S-layer is resistant to detergents and high temperatures, perhaps not unusual for an organism that grows at pH 0.9 and 90°C.

Studies of secondary structure of S-layer proteins using infrared spectroscopy and circular dichroism show a high proportion of aperiodic structure with about one-third β structure and short stretches of α-helix (Baumeister *et al.* 1982; Phipps *et al.* 1983; Bingle *et al.* 1986; 1987).

It is becoming increasingly evident that several S-layers are composed of glycoproteins. The bacteria possessing glycosylated S-layers, and their sugars (in parentheses) are: *Bacillus stearothermophilus* NS2004/3a (repeating rhamnose trisaccharide and a repeating glucose tetrasaccharide) (Messner *et al.* 1986; Christian *et al.* 1986), *Bacillus stearothermophilus* E4-65 (glucose, mannose and galactose) (Sleytr *et al.* 1986), *Clostridium thermohydrosulfuricum* L111-69 (disaccharide repeating unit of rhamnose and mannose) (Sleytr and Messner, 1988), *Desulfotomaculum nigrificans* (mannose, glucose, rhamnose and galactose) (Sleytr *et al.* 1986), *Bacillus sphaericus* mosquito pathogenic strains (< 2% by weight) (Lewis *et al.* 1987), *Methanothrix concilii* (mannose, glucose, ribose and rhamnose) (Patel *et al.* 1986), *Methanospirillum hungatei* JF1 (arabinose) (Patel *et al.* 1986), *Methanospirillum hungatei* GP1 (glucose, rhamnose, ribose, galactose) (Patel *et al.* 1986), *Halobacterium halobium* (galactose, glucuronic acid, galacturonic acid, N-acetylglucosamine and 3-O-methylgalacturonic acid) (Mescher and Strominger, 1976). The S-layer glycoprotein of *Bacillus*

stearothermophilus contains two types of glycan chains, one chain is composed of rhamnose residues only. Using ^{1}H and ^{13}C-NMR spectroscopy, the structure of the rhamnose containing chain was found to be [->2)-α-L-Rha*p*-(1->2)-α-L-Rha*p*-(1->3)-β-L-Rha*p*-(1->]$_{n\sim 50}$ (Messner *et al.* 1986; Christian *et al.* 1986). This carbohydrate chain has been proposed to have a helical structure with a diameter of 3.1 nm and a length of 10-15 nm (Christian *et al.* 1986). Glycosylated S-layer proteins have been demonstrated in *Clostridium, Desulfotomaculum* (Messner and Sleytr, 1988; Sleytr *et al.* 1986) and *Bacillus stearothermophilus* (Kupcu *et al.* 1984; Christian *et al.* 1986).

The S-layer protein of *Halobacterium salinarium* is a 200 K glycoprotein with 34 to 38 O-linked di- and trisaccharides, and a large amino sugar-containing heterosaccharide. The O-linked di- and trisaccharides are attached to the protein via O-glycosidic bonds between galactose at the reducing end of the saccharide and the hydroxyl group of threonine. The amino sugar-containing heterosaccharide is attached to the protein by an N-glycosylamine bond to the amide group of asparagine (Mescher and Strominger, 1976).

1.3.5 Forces Holding S-layers Together and to the Underlying Surface

Depending on the the particular organism, an S-layer can be in contact with lipopolysaccharide and outer membrane proteins, peptidoglycan and techoic acids, or pseudomurein. Some S-layers appear to penetrate into the outer membrane (Sleytr and Messner, 1988; S. Smith, personal communication) or, in the case of spirochaetes, penetrate into the outer sheath (Masuda and Kawata, 1982; Hovind-Hougen, 1976). The only reported case of a specific interaction of an

S-layer with an underlying structure is the interaction of the D. radiodurans S-layer with the underlying 5'-3'-exonuclease (Peters *et al.* 1988).

Subunits of S-layers interact with each other, and with the underlying layer, through non-covalent interactions. Most S-layers can be disassembled by hydrogen bond breaking agents such as urea, guanidine HCl and formamide, or by extremes of pH (Nermut and Murray, 1967; Buckmire and Murray, 1970; Buckmire and Murray, 1973; Sleytr, 1975; Beveridge and Murray, 1976). The S-layers of *Aquaspirillum putridiconchylium* and *Acinetobacter sp.* are removed by cationic substitution (Beveridge and Murray, 1976; Thorne *et al.* 1975), a process in which the divalent cations holding the layer together are replaced by monovalent cations such as Na or Li. Interestingly, the S-layer of *Sporosarcina ureae* requires magnesium for the integrity of the array (Beveridge, 1979). The array is not disrupted by the sodium, but is disrupted by several divalent cations, such as $CaCl_2$, $SrCl_2$, and $BaCl_2$ (Beveridge, 1979). The disruption of the array by these divalent cations could be reduced by adding equimolar amounts of $MgCl_2$.

An important distinction must be made between intersubunit contacts and contacts between the S-layer protein and the underlying layer. Two types of bonds must be present: one type of bond holds the subunits of the S-layer together to form the array, the other type holds the subunits onto the cell surface. Disruption of intersubunit contacts alone will not release the S-layer protein from the cell envelope, but will disorganize the array. An example of this is the disorganization of the S-layer of *Clostridium thermosaccharolyticum* and

C. thermohydrosulfuricum by acidification with a buffer at a pH less than three. No protein is solubilized by this low pH treatment, however, and the native array reforms upon raising the pH to neutrality (Sleytr, 1975). The S-layers of *Aquaspirillum* "Ordal" are disorganized, but not released from the outer membrane, by homogenization of whole cells in 0.1M sodium acetate buffer at pH 4.6 (Beveridge and Murray, 1976). The S-layer is disorganized by a combination of the substitution of calcium with sodium, and the low pH. Disruption of the contacts between the S-layer protein and the underlying layer may release the S-layer as an intact array (if intersubunit contacts are not broken), or may release the S-layer in the form of monomers (if the intersubunit contacts are broken).

Inter-subunit contacts are responsible for maintenance of the array of S-layers. These contacts range from weak, being broken by low concentrations of metal chelators or detergents, to extremely strong. An example of the latter are the sheaths of *Methanospirillum hungatei* and *Methanothrix concilii*. Both of these structures are not solubilized by strong base at room temperature, or by boiling SDS, but are soluble in boiling 1N NaOH (Patel *et al.* 1986; Beveridge *et al.* 1985). The S-layer of *Thermoproteus tenax* is also resistant to solubilization under harsh conditions (Sleytr, Baumeister). The resistance of these S-layers to solubilization is similar to that of the spore coat of *Bacillus subtilis*. The *B. subtilis* spore coat is refractory to solubilization in strong base, ethanol, acetone, formic acid, dioxane, chloroform, acetic acid, LiBr, guanidine HCl, β-mercaptoethanol, and dithiothreitol (Hiragi, 1972). The only reagent capable of bringing about solubilization was 90% dichloroacetic acid.

Most S-layer proteins are not solubilized by nonionic detergents. This is not surprising, as nonionic detergents are not efficient at breaking protein-protein interactions (Helenius and Simons, 1975). For example, the S-layer of *Acinetobacter* sp. is not detached, nor made soluble, by Tween 80 or sodium deoxycholate (Thorne *et al.* 1975). Generalizations cannot be made however, as Triton X-100 causes the release of the S-layer of *Acinetobacter* when 80% of the envelope lipid was previously removed (Thorne *et al.* 1975). Also, the S-layer of *Methanococcus voltae,* which is unlike the S-layers of *Methanothrix* and *Methanospirillum,* is solubilized by Nonidet P-40, Triton X-100, Triton N-101, Brij 58, Triton X-165, Triton X-114 and Tween 20 (Koval and Jarrell, 1987), all of which are nonionic detergents and do not usually have an effect on S-layers (Sleytr and Messner, 1983). The S-layer of *Methanococcus voltae* is also exceptional, in that is made soluble by 50 mM dithiothreitol (Koval and Jarrell, 1987), perhaps suggesting that disulfide bonds are important in holding the layer together. This is not without precedent however, as the units of the S-layer of *Chlamydia trachomatis* are held together by disulfide bonds (Newhall and Jones, 1983; Bavoil *et al.* 1984).

Chalcroft *et al.* (1986) suggest the contacts in the S-layer of *Pseudomonas acidovorans* are weak, and rely on an intact outer membrane to retain the proper structure. This was suggested because solubilization of the outer membrane by SDS also solubilized the S-layer. However, many S-layers are solubilized by SDS, a strongly denaturing detergent. Clearly, the solubility of an S-layer in several agents, denaturing and non-denaturing, must be examined before

conclusions regarding the nature of the intersubunit contacts can be drawn.

1.3.6 Self Assembly

Several S-layers require divalent cations for assembly onto the cell surface or self-assembly *in vitro*. The S-layer of *Aquaspirillum serpens* requires Ca^{2+} for assembly (Buckmire and Murray, 1970; Beveridge and Murray, 1976; Buckmire and Murray, 1976; Kist and Murray, 1985; Koval and Murray, 1985), and is removed from the cell surface by extensive dialysis against EDTA or EGTA (Koval and Murray, 1983). The S-layer of *Aquaspirillum putridiconchylium* will assemble onto the cell surface during growth of the organism in medium containing 1 mM $CaCl_2$ or 1 mM $SrCl_2$ (Beveridge and Murray, 1976), whereas addition of equal concentrations of lithium, sodium, potassium, magnesium, manganese, iron, or three polyamines produces a surface without an S-layer.

The dependence of cations for reassembly of the *Azotobacter vinelandii* S-layer has been examined in detail. Reattachment of distilled-water-extracted S-layer protein to outer membrane vesicles of *A. vinelandii* is dependent upon both the ionic strength of the suspending medium and the type of cation present (Bingle *et al.* 1984). Both monovalent and divalent cations promoted attachment to OMV at an ionic strength of 5×10^{-3}, while only divalent ions promoted attachment to OMV at an ionic strength of 5×10^{-4}. Monovalent ions are able to promote attachment of the S-layer protein to OMV only, and are unable to do so with whole cells suggesting differences between the surfaces of OMV's and whole cells (Bingle *et al.* 1984).

S-layer assembly may occur *in vitro* either with or without a template. Assembly in vivo, on the cell surface, always occurs on a template, either the outer membrane, in gram-negative bacteria, or the external surface of the peptidoglycan layer, in gram-positive bacteria. Assembly of S-layers may occur on a previously synthesized, underlying, S-layer (see next section). Cross-linking studies have been used to demonstrate the interaction of S-layer proteins with underlying proteins in the gram-negative outer membrane (Koval and Murray, 1981; Doran *et al.* 1987). In both studies, the S-layer proteins could not be cross-linked to outer membrane proteins, perhaps reflecting a paucity of reactive lysine groups in the S-layer proteins, or an absence of interactions between the S-layer proteins and outer membrane proteins.

S-layers of *Aquaspirillum serpens* VHA (Chester and Murray, 1978) and *Aeromonas salmonicida* (Belland and Trust, 1985) have been demonstrated to interact with LPS. In *Acinetobacter* sp., inhibition of synthesis of the O-side chain by bacitracin causes the release of LPS, outer membrane proteins and S-layer protein into the culture supernatant (Thorne *et al.* 1976). This does not suggest, however, that the S-layer interacts directly with the LPS, as the S-layer protein did not attach to isolated lipopolysaccharide. Outer membrane proteins were released when O-side chain synthesis was inhibited, and the S-layer protein of *Acinetobacter* sp. has been suggested to interact with the outer membrane proteins through carboxyl groups bridged by a divalent cation (Thorne *et al.* 1975).

The type of lattice of all S-layers is determined by inter-subunit contacts, and not by interaction with the underlying template (Sleytr, 1976). This can be seen by the self-assembly of subunits *in vitro*. Self-assembly of S-layers *in vitro* occurs when the agent used to disrupt the native array is removed and replaced with a buffer with the proper pH, ionic strength and ion requirements (Brinton *et al.* 1969; Aebi *et al.* 1973; Sleytr, 1976, Hastie and Brinton, 1979, Masuda and Kawata, 1980; Tsuboi *et al.* 1982). Perhaps the best illustration that S-layer proteins, and not the supporting layer, contain the information regarding the symmetry and lattice spacing of the array is the heterologous reattachment and reassembly of soluble S-layer protein onto naked cell walls. Incubation of cell walls of *Clostridium thermosaccharolyticum* with soluble S-layer protein from *C. thermosaccharolyticum* and *C. thermohydrosulfuricum* results in assembly of both the tetragonal S-layer of *C. thermosaccharolyticum,* and the hexagonal S-layer of *C. thermohydrosulfuricum* to the same cell wall (Sleytr, 1975). Possible exceptions to this rule of S-layer proteins containing all the information for the symmetry and lattice spacing of the layer, are the proteins comprising the outer tier of double-layered S-layers. The outer layer of *Aquaspirillum serpens* MW5 will not assemble without the inner layer present as a template (Kist and Murray, 1984), likewise, the outer layer of *Bacillus brevis* (Tsuboi ?) will not assemble without the inner layer. It appears, in these cases, that the protein comprising the outer array does not contain all the information required to assemble into the native array.

Isolated S-layer proteins usually assemble into flat sheets, open-ended cylinders, or closed vesicles (Aebi *et al.* 1973; Hastie and Brinton, 1979; Masuda and Kawata, 1980; Sleytr, 1976; Sleytr *et al.* 1986). The soluble S-layer of *B. stearothermophilus* NS 2004/3a reassembles into cylinders of varying sizes when dialyzed against low concentrations (2.5 to 10 mM) of Ca^{2+} or Mg^{2+}. Dialysis against 2.5 to 10 mM Na^{+} or Li^{+} resulted in smaller assemblies. Increasing the concentration of cations to 50 to 500 mM reduces the size and order of the self-assembly products (Messner *et al.* 1986). Reassembly of most S-layer proteins requires the presence of divalent cations. Only the S-layer of *Acinetobacter* sp. has been reported to require an anion for reassembly (Thorne *et al.* 1975), with anions of increasing valence reducing the amount of self assembly. Interestingly, the S-layer of *Acinetobacter* sp. is released from the outer membrane by EDTA and by replacing Mg^{2+} with Na^{+} (Thorne *et al.* 1975).

Self-assembly of the S-layer protein of *Bacillus stearothermophilus* follows multiphasic kinetics. A rapid initial assembly was formed, consisting of oligomeric precursors of 12 to 16 subunits ($Mr > 10^6$). These "small crystallites" then fuse and slowly assemble into the native S-layer (Jaenicke *et al.* 1985).

Although calcium is not required for attachment of the S-layer of *Azotobacter vinelandii* to the cell surface, it is necessary for the organization of surface located S-layer protein to change from a disordered arrangement into a regular tetragonal arrangement (Doran *et al.* 1987). No calcium induced change in conformation of either S-layer protein monomers (Bingle *et al.* 1987), or tetramers (Bingle *et al.* 1986) from *Azotobacter vinelandii* could be detected by circular

dichroism. Doran *et al.* (1987) suggest the change in protein conformation induced by calcium binding may be as subtle as the calcium induced conformational change in the regularly arranged gap junction protein from eukaryotic cells (Unwin and Ennis, 1984).

1.3.7 Multilayered and Complex S-layers

Most bacteria with S-layers possess only a single layer. The bacteria with double S-layers are: *Aquaspirillum metamorphum* (Beveridge and Murray, 1975), *Aquaspirillum* "Ordal" (Beveridge and Murray, 1976), *Aquaspirillum serpens* MW5 (Buckmire, 1970; Stewart and Murray, 1982; Kist and Murray, 1984), *Aquaspirillum sinuosum* (S. Smith personal communication, *L. hyalina* (Chapman *et al.* 1963; Murray, 1963; Pangborn and Starr, 1965; Austin and Murray, 1987; 1988), *Nitrocystis oceanus* (Watson and Remsen, 1970), *Bacillus brevis* (Tsuboi *et al.* 1982; Yamada *et al.* 1981), *Bacillus macroides* (Holt and Leadbetter, 1969) and *Corynebacterium diptheriae* (Kawata and Masuda, 1972). A most interesting organism, Walsby's "Square Bacterium" is blessed with, not two, but three S-layers (Javor *et al.* 1982; Parkes and Walsby, 1981; Walsby, 1980; Stoeckenius, 1981). The superficial wall structure of *Aquaspirillum* "Ordal" consists of five layers, including an inner S-layer arranged in tetragonal symmetry and an outer hexagonal S-layer (Beveridge and Murray, 1976).

All organisms with multiple S-layers, whose layers have been characterized biochemically, have different polypeptide subunits in each layer. The outer layer of *Aquaspirillum serpens* MW5 consists of a 125 K protein, while the inner layer consists of a 150 K protein (Kist and Murray, 1984). The double S-layer of *Bacillus brevis* consists of two

different proteins, a 130 K protein and a 150 K protein (Tsuboi *et al.* 1982; Yamada *et al.* 1981). Assembly of S-layers sometimes occurs on the inner surface of the peptidoglycan of gram positive organisms (Tsuboi *et al.* 1982; Yamada *et al.* 1981), and even on both sides of the peptidoglycan (Wahlberg *et al.* 1987).

Periodic structure has been found in the outer membrane of *Bacteroides buccae*, in addition to the hexagonally arranged surface layer present on the external surface (Kerosuo *et al.* 1987). Although regularly arranged proteins have been demonstrated in *C. trachomatis* (Chang *et al.* 1982) and *B. nodosus* (Every and Skerman, 1983), this is the first report of a regularly arranged protein in the outer membrane, combined with an S-layer.

"Goblet-shaped particles" have been described on the surface of the heterotrophic marine gliding bacterium *Flexibacter polymorphus* (Ridgway and Lewin, 1983) and unusual "wineglass-shaped" wall units have been observed on the surfaces of the marine photosynthetic bacteria *Chromatium buderi* (Cohen-Bazire *et al.* 1969; Remsen *et al.* 1970) and *Amoebobacter bacillosus* (Cohen-Bazire *et al.* 1969), and the methane-utilizing bacterium *Methylomonas albus* (Jefferies and Wilkinson, 1978; Remsen *et al.* 1970). In *Chromatium buderi*, the outer layer of the cell envelope is composed of five-sided, cup-shaped structures, 35 nm in diameter, whereas a sculptured layer consisting of hexagonally arrayed 5 nm particles was observed on the surface of *Chromatium gracile*. The fresh-water photosynthetic bacteria, *Chromatium weissei* and *C. okenii*, possess hexagonal S-layers made up of hollow cone-shaped units 25 nm long and 13 nm in diameter, with a centre-to-centre spacing of 19 nm (Hageage and Gherna, 1971).

At least one of the morphological units contains multiple subunits. Goblet-shaped particles from *Flexibacter polymorphus,* isolated by cesium-chloride density gradients have been shown to consist of four major polypeptides (Ridgway and Lewin, 1983). Several functions have been proposed for the goblet-shaped particles of *Flexibacter polymorphus,* including attachment of the cells to an underlying substrate, a requirement for gliding motility (Burchard, 1981; Humphrey *et al.* 1979; Ridgway, 1976a; Ridgway *et al.* 1975). Pate and Chang (1979) have also suggested that the goblets might be responsible for generating the force required for gliding motility.

Attachment of an S-layer by fine fibres, as in the attachment of the perforate layer to the outer membrane of *L. hyalina,* has been reported for a strain of *Synechocystis* (Lounatmaa *et al.* 1980). The hexagonally arranged S-layer of the cyanobacterium, *Synechocystis,* has a repeat spacing of 15.5 nm, and is connected to the outer membrane by fine fibers revealed by ruthenium red staining.

1.3.8 Synthesis and Turnover

A regulatory mechanism to govern the amount of S-layer protein synthesis has not yet been found. The S-layer protein of an *Acinetobacter* sp. is assembled onto the outer membrane; when the cell is covered, S-layer protein monomers continue to be synthesized and excreted into the culture medium (Thorne *et al.* 1975). In the case of the gram-positive *Clostridium thermosaccharolyticum* and *C. thermohydrosulfuricum,* the S-layer protein subunits form small areas of pattern in the region of the septum, where they are synthesized

(Sleytr and Glauert, 1975; Sleytr, 1975). The subunits then rearrange to form large areas which eventually cover the entire cell surface.

The biogenesis of the *Caulobacter crescentus* S-layer was followed using immunoelectron microscopy (Smit and Agabian, 1982). Newly synthesized subunits appear to inserted randomly over the surface of the organism except at the stalk. Growth of the stalk is accompanied by a *de novo* synthesis of S-layer protein to cover this area of the cell.

1.3.9 Genetics

The genes coding for some S-layer proteins are now being identified and cloned. The genes for the *Caulobacter crescentus* (Smit and Agabian, 1984), *Bacillus brevis* 47 (Tsukagoshi *et al.* 1984), and *Deinococcus radiodurans* (Peters and Baumeister, 1986) have all been cloned into *E. coli*, and detected by expression assays. The genes for the S-layer proteins of *B. brevis* and *D. radiodurans* were inserted into plasmids pBR322 and pUC8, respectively. The gene coding for the 130K S-layer protein of *C. crescentus* was cloned into a lambda 1059 library and transcription of the cloned gene was initiated by a lambda promoter (Smit and Agabian, 1984).

The gene coding for the S-layer of *Deinococcus radiodurans* was cloned into *E. coli* using the plasmid pUC8, expressed under control of a *Deinococcus* promoter (Peters *et al.* 1988). From the sequence, it was deduced that the *D. radiodurans* S-layer polypeptide contains six cysteine residues, four of which contribute to disulphide bridges (Peters *et al.* 1988). Secondary structure determinations suggest a content of 30% β structure and virtually no α helix (Peters *et al.* 1988).

The gene coding for the 90 K S-layer of *Halobacterium halobium* was cloned and sequenced (Lechner and Sumper, 1987) and the sites of glycosylation have been related to the primary structure (Lechner and Sumper, 1988). Twelve possible N-glycosylation sites (Asn-X-Ser(Thr); X=Asp, Pro) exist throughout the sequence of the H. halobium S-layer gene. From chemical evidence, about 10 sulfated saccharides of the type (hexuronic acid 1-4)$_{2-3}$Glc are linked through N-glycosidic linkages to the polypeptide (Wieland *et al.* 1983), suggesting most of the potential N-glycosylation sites are glycosylated. All of the O-linked Glc-Gal disaccharides can be recovered in a single pronase-generated fragment (Wieland *et al.*, 1983), suggesting a cluster of O-linked disaccharides. DNA sequencing indicated 14 Thr residues are clustered within 20 amino acids (positions 755 to 774). Since the S-layer polypeptide contains about 20 Glc-Gal disaccharides, all of the 14 Thr residues within the cluster are probably glycosylated (Lechner and Sumper, 1988).

Sequencing of the *H. halobium* S-layer polypeptide also indicates the presence of a leader sequence, and a hydrophobic stretch of 21 amino acids (positions 795 to 815) beginning three amino acids from the C-terminus (Lechner and Sumper, 1988). An arrangement has been proposed (Lechner and Sumper, 1988) for the insertion of the *H. halobium* glycoprotein into the cytoplasmic membrane via the hydrophobic stretch of amino acids, with the cluster of O-linked disaccharides located near the external surface of the cytoplasmic membrane, and the N-linked polysaccharides are randomly located from positions 305 to 781.

The synthesis, export, and assembly of the S-layer of *Aeromonas salmonicida* has been analyzed by transposon mutagenesis (Belland and Trust, 1985). The S-layer protein is produced by a single chromosomal gene. The subunits pass through the cell envelope and assemble on the surface of cells possessing O polysaccharide chains on the lipopolysaccharide. One insertion, in a 9.2-kbp *Eco*RI fragment, produced a mutant completely lacking the ability to produce the S-layer protein (Belland and Trust, 1985).

1.3.10 Function

Although S-layers have been proposed to have many functions, a general purpose for all S-layers has yet to be found. Whatever the function is, it must be crucial to survival of the cell. A typical S-layer may account for up to 15% of the total cell protein. When the selective pressures are removed and strains are maintained in the laboratory, the capacity to form an S-layer may be lost upon prolonged subculture. This has occurred with both *Aquaspirillum serpens* and *Bacillus stearothermophilus* (Messner *et al.* 1984), and suggests that S-layers are synthesized (at great energy expense to the organism) for a reason important to the cell while in the environment, but not necessarily important while in the laboratory.

Compared to lipopolysaccharide and peptidoglycan, S-layers may be considered non-conservative structures which have changed during evolution of bacteria and in response to the environment (Messner *et al.* 1984). However, an interesting observation was made by Smit and Agabian (1984) that approximately 20 isolates of marine *Caulobacter* strains all showed significant hybridization when probed with DNA from

a cloned S-layer gene probe from *Caulobacter crescentus*. This indicates that all 20 isolates contain DNA coding for a protein similar to the 130 K S-layer protein of *C. crescentus* and also suggests that all the marine isolates may have similar surface layer structures (Smit and Agabian, 1984). As more genes coding for S-layers are cloned, and subsequent gene probes become available, this inter-species or inter-strain similarity may be found to be more common.

Because of their surface location, S-layers represent the principal interface between the bacterial cell and its environment. The most likely function of S-layers is that of a permeability barrier to large molecules and predators. Sleytr et al have shown that S-layers of gram positive organisms have selective permeabilities for proteins with certain molecular weights. The pore size of 6 nm determined for *Thermoproteus tenax* and *Thermoproteus neutrophilus* is in accordance with the nominal molecular weight cutoff of the surface layer of 67K (Sara and Sleytr, 1985). The S-layer of *Methanospirillum hungatei* has a subunit spacing of only 2.8 nm (Stewart *et al.* 1985). The porosity of this S-layer would allow only small molecules through the layer. This is completely in line with the nutritional requirements of this methanogen, namely carbon dioxide, hydrogen, nitrogen salts and minerals.

Some strains of *Aquaspirillum serpens, A. sinuosum* and *L. hyalina* which lack S-layers are susceptible to attack by *Bdellovibrio bacteriovorus,* while the covered strains are resistant to attack (Buckmire, 1971; S. Lanys, M. Sc. Thesis, U.W.O., 1971; Koval, 1989). Further indication of a protective function of S-layers is seen in the

resistance to proteolysis of some intact layers (Rachel *et al.* 1983; Beveridge *et al.* 1985).

In bacteria which do not possess a peptidoglycan layer however, S-layers may impart shape to the cell. In *Halobacterium,* a change in shape from rod to spheres occurs if glycosylation of the S-layer protein is inhibited by bacitracin (Mescher and Strominger, 1975, 1976). The thermophilic Archaeobacteria, *Thermoproteus tenax* and *Thermoproteus neutrophilus* are both rod-shaped organisms, enclosed by an S-layer. The S-layer is the only rigid part of the cell envelope of these organisms. Messner *et al.* (1986) propose that the shape of the main cylindrical portion of the S-layer is determined by the "mass distribution and bonding properties of the protomers." Lattice disinclinations were observed at the poles of the cells, and these faults were required to close off the cylinder.

Probably more functions are known for the S-layers of the aeromonads than for all other S-layers studied. Without its S-layer (or A layer), *Aeromonas salmonicida* is avirulent to fish, and does not autoaggregate (Kay *et al.* 1981; Ishiguro *et al.* 1981; Evenberg *et al.* 1982). Cells with an S-layer demonstrate enhanced binding to in vitro-cultivated fish and mouse macrophages (Trust *et al.* 1983). The presence of an S-layer protects *Aeromonas salmonicida* from serum complement (Munn *et al.* 1982), a large number of bacteriophage (Ishiguro *et al.* 1981), as well as several proteases (Phipps *et al.* 1983). Recently, the S-layer of Aeromonas salmonicida has been shown to non-specifically bind Congo Red, this binding was enhanced by high salt concentrations (Ishiguro *et al.* 1985).

Several strains of S-layer containing isolates of *Aeromonas hydrophila* and *A. sobria* have been found to share several characteristics, clumping in static broth culture (autoagglutination, autoaggregation), a common somatic antigen, resistant to lysis by bacteriophage Aeh1, resistance to complement-mediated lysis, and pathogenicity in an animal model system (Dooley and Trust, 1988; Dooley *et al.* 1988; Paula *et al.* 1988). Both *Aeromonas hydrophila* and *A. sobria* have been implicated as common pathogens in both fish and humans. In humans, O:11 S-layer-containing strains have been preferentially associated with invasive infections rather than localized disease (Janda *et al.* 1987)

The S-layer of *Bacillus sphaericus* P-1 has been postulated to be a bacteriophage receptor (Howard and Tipper, 1973). Among the insect pathogenic *Bacillus sphaericus* strains, the chemical composition of the S-layers has been found to vary among different phage types of the strains (Lewis *et al.* 1987), suggesting that the S-layer proteins may be the receptor for some of the bacteriophages in the phage typing scheme. It has been suggested that the arrangement and molecular weight differences of S-layers be considered as taxonomic characters to distinguish between closely related species (Hollaus and Sleytr, 1972; Messner and Sleytr, 1984).

1.4 Surface Appendages

Bacteria produce surface appendages of simple structure for motility, adhesion and cell-cell contact.

1.4.1 Flagella

As defined by DePamphilis and Adler (1971) the 'intact flagellum' consists of the external filament and the hook-basal body (HBB) complex. The basal body comprises the 'motor', while the hook is the link between the basal body and the filament. The basal body was first observed by Murray and Birch-Andersen (1963) in thin sections of *A. serpens*. The HBB complex of *Salmonella typhimurium* has been isolated and shown to consist of several different polypeptides ranging in molecular weights from 27K to 65K (Aizawa *et al.* 1985). Coulton and Murray (1977) described "concentric membrane rings" on the underside of the outer membrane where the flagella insert into the cell envelope of *A. serpens*. Brief digestion of whole cells with a Myxobacter protease demonstrated that these concentric membrane rings were associated, as a collar, around the base of the flagellar filament. Using *A. serpens* as a subject, Coulton and Murray (1978) examined the relationship of the basal body with the cell envelope and, by measuring the dimensions of the basal complex, the disks of the basal complex, and the perforations of the envelope layers at the flagellar insertion sites, derived a model for the insertion of the basal body into the complementary regions of the cell envelope. These, and other authors (Coulton and Murray, 1978; Swan, 1985) have noted finger-like processes extending from the bottom of the M ring into the cytoplasm. These extensions may be an example of components which are easily lost during preparation of specimens for electron microscopy.

Bacteria of the order *Spirochaetales* possess periplasmic flagella (also called axial filaments, periplasmic filaments, endoflagella, or flagella [Smibert, 1984]) which, as the name suggests, are located between the outer membrane and the cell cylinder (Canale-Parola, 1978; Holt, 1978). While the filaments of most bacterial flagella are composed of a single polypeptide, the periplasmic flagellar filaments of *Spirochaeta* (Brahamsha and Greenberg, 1988; Greenberg *et al.* 1985; Joseph and Canale-Parola, 1972), *Leptospira* (Naumann, 1969), and *Treponema* (Norris *et al.* 1988; Limberger and Charon, 1986; Cockayne *et al.* 1987) consist of between two and six major species of polypeptides within the same filament. *Borrelia*, in contrast, is a spirochete with periplasmic flagellar filaments of only a single polypeptide (Barbour *et al.* 1986).

1.4.2 Fimbriae and Pili

Bacterial fimbriae were first described by Anderson (1949) and Houwink and van Iterson (1950). These thin hair-like structures were named "fimbriae", Latin for "fibres or fringe" by Duguid *et al.* (1955). Brinton (1959) later referred to identical structures as "pili" taken from Latin for "hair or fur." Fimbriae generally are homopolymers of a low molecular weight, hydrophobic protein, pilin (Brinton, 1965; Salit and Gotschlich, 1977). *E. coli* type 1 fimbriae are composed of subunits of 158 amino acids (Abraham and Beachey, 1987). Brinton (1965) was close when he suggested type 1 fimbriae consist of monomers of protein subunits of about 170 amino acids (15.7 to 17 K) assembled into a right-handed helix that is 7 nm in diameter.

Several fimbriae types are produced among Eubacteria (reviewed by Jones and Isaacson, 1984). Members of the Enterobacteriaceae produce fimbriae to allow attachment to eukaryotic cells via specific receptors (Beachey, 1981; Ofek and Beachey, 1978; Salit and Gotschlich, 1977). Many kinds of fimbriae have been described for *E. coli*, and some of these are virulence factors. The P fimbriae of *E. coli* serve as adhesins for strains that cause urinary tract infections (Korhonen *et al.* 1982; Vaisanen-Rhen *et al.* 1984), tne K88 (Jones and Rutter, 1972; Stirm *et al.* 1967), K99 (Moon *et al.* 1977; Orskov *et al.* 1975), and 987 (Isaacson *et al.* 1977) fimbriae of animal enterotoxigenic *E. coli* strains allow attachment of *E. coli* to intestinal epithelial cells. Conjugative pili are required for bringing about cell-to-cell contact during bacterial conjugation (Achtman, 1975).

Type 1 fimbriae are resistant to solubilization by urea (Korhonen *et al.* 1980; Salit and Gotschlich, 1977; Karch *et al.* 1985), cold SDS (McMichael and Ou, 1979), octyl glucoside (Karch *et al.* 1985) and 6M guanidine hydrochloride (Eshdat *et al.* 1981), but they are solubilized by saturated (8 M) guanidine hydrochloride (Eshdat *et al.* 1981). Fimbriae subunits, dissociated by guanidine hydrochloride, will reassemble by dialysis against 10 mM $MgCl_2$ (Eshdat *et al.* 1981; Karch *et al.* 1985). A synthetic peptide containing residues 23 to 35 of type 1 fimbriae has recently been shown to assemble into structures resembling the native fimbriae except that they appear less rigid and rodlike (Abraham and Beachey, 1987).

Strains of *E. coli* may produce type P fimbriae, or "Pap pili" (pili associated with pyelonephritis) (Korhonen *et al.* 1982). Although morphologically similar, they are immunologically and structurally distinct from type I fimbriae.

Conjugative pili are composed of a single pilin type arranged in a helix to give thin tubular filaments 8 nm wide and about 1 µm long (Folkard *et al.* 1979).

1.4.3 Spinae

Flagella and fimbriae are standard non-prosthecate appendages, and their structure and chemistry are well studied. A less well known bacterial appendage are spinae (Easterbrook and Coombs, 1976; McGregor-Shaw *et al.* 1973; Easterbrook *et al.* 1973). Spinae are hollow shaft-like structures 1-3 µM in length and 65 nm in diameter (Easterbrook and Coombs, 1976). The shaft and base are made up of a helically wound filament. The spinae are attached at the base, to the outer leaflet of the outer membrane, by a pronase sensitive protein (Willison *et al.* 1977). Spinae are detached from the surface of the cell by shear forces and may be isolated by differential centrifugation (Easterbrook and Coombs, 1976). Spinae are not dissociated by chelating agents, extremes of pH, guanidine HCl, organic solvents, or cold SDS and are also resistant to digestion by proteases. Hot SDS will solubilize spinae to a 37 K subunit (spinin) (Easterbrook and Coombs, 1976).

Chapter 2

MATERIALS AND METHODS

2.1 Organism and Growth Conditions

Lampropedia hyalina UWO 884 (originally obtained from R. E. Hungate, University of California, Davis) was grown at 30°C on solid medium containing 0.3% each of yeast extract and Bacto peptone (Difco, Detroit, MI), 0.05% sodium acetate pH 7.3 and solidified with 1.5% Bacto agar (Difco, Detroit, MI) (YPA agar). After three days the cells formed an uninterrupted lawn across the surface of the agar. Cells were also grown in YPA broth, with shaking at 150 rpm, at 30°C (Psychrotherm incubator-shaker, New Brunswick Scientific, New Brunswick, NJ.). A spontaneous variant of *L. hyalina* UWO 884 (U.W.O. 440), which had lost the property of forming sheets, was grown in YPA broth supplemented with 10 mM $CaCl_2$ (Fisher Scientific, Fairlawn, NJ). Stock cultures of both strains of *L. hyalina* were maintained on slants of YPA agar.

2.2 Cell Fractionation

2.2.1 Preparation of Cell Envelopes

All steps were carried out at 4°C unless otherwise indicated. Three litre broth cultures in early stationary phase (24 h) were centrifuged (3,500 *g*, 30 min). The pellet was washed once in 50 mM HEPES (Sigma, St. Louis, MO) pH 7.5 containing 10 mM $CaCl_2$, and resuspended in 25 ml of the same buffer. The cells were disrupted by two passages through a French pressure cell (Aminco) at 16,000 lbs/in^2 (1 psi = 6.895 kPa). Cell envelopes were sedimented by centrifugation (48,000 *g*, 30 min) and washed in 50 mM HEPES containing 1% Triton X-100 (Sigma, St. Louis, MO) to remove traces of cytoplasmic membrane.

2.2.2 Isolation of the Perforate Layer

Cell envelopes were resuspended in 2% SDS (Bio-Rad Laboratories, Richmond, CA.), and incubated for 5 minutes at room temperature. The envelopes were then centrifuged (48,000 *g*, 30 min) at room temperature. The SDS extraction was repeated, and the extracted envelopes were then washed with 50 mM HEPES pH 7.5 until the $OD_{280\ nm}$ of the wash fluids was negligible. The extract was incubated in 50 mM HEPES pH 7.5 containing 200 µg/ml lysozyme (Sigma, St.Louis, MO) for two hours at room temperature, washed once with 2% SDS, and again with 50 mM HEPES pH 7.5.

2.2.3 Isolation of the Punctate Layer

Cell envelopes (30 mg dry weight) were resuspended, by homogenization, in 30 ml 100 mM Tris (Bio-Rad, Richmond, CA) buffer pH 8.0, containing 150 mM NaCl and 2% Na deoxycholate (Sigma, St. Louis, MO) (Tris-NaCl-DOC) and incubated for 15 minutes at room temperature. The envelopes were then centrifuged (48,000 *g*, 30 min). The Tris-NaCl-DOC extraction was repeated, and the extracted envelopes were washed with 100 mM Tris pH 8.0 until the OD_{280nm} approached zero. The punctate layer was solubilized by incubation in 10 ml of unbuffered 3M guanidine HCl (Schwartz-Mann, Canadian Scientific Products, Hyde Park, Ont.) at room temperature for 30 minutes with periodic mixing. The suspension was centrifuged (150,000 *g*, 60 min), and the resultant supernatant was dialyzed against 1L of 10 mM sodium phosphate buffer pH 7.5. The soluble protein was applied to a hydroxyapatite (Bio-Rad, Richmond, CA) column (5 ml bed volume) and eluted with a 10 mM to 200 mM gradient of sodium phosphate buffer pH 7.5. Thirty drop (1.4ml) fractions were collected and protein was detected by the Bio-rad protein assay (Bradford, 1976).

When a mixture of the soluble components of the punctate layer were required, Tris-NaCl-DOC extracted envelopes were incubated in 3M guanidine HCl and the soluble punctate layer was separated from the envelopes by centrifugation (40,000 rpm 70.1Ti 1h). The guanidine HCl was then removed by dialysis against 50 mM HEPES buffered at pH 7. Protein prepared this way remained soluble.

2.2.4 Isolation of the Composite Layers (intact S-layer)

Cells grown on YPA agar (48 hours, 30°C) were removed from the agar surface by washing in Tris-NaCl-DOC. The suspension, containing cells and pieces of the S-layer shed from the cells, was centrifuged (3,500 *g*, 15 min) to remove most of the intact cells. The lysed cells and S-layer were removed by further centrifugation (48,000 *g*, 30 min) and washed again in Tris-NaCl-DOC to remove attached membranes and contaminating proteins.

2.3 Preparation of Antisera and Purification of IgG

Samples from fractions obtained after hydroxyapatite column chromatography, and containing the 240K or 60K polypeptide, were applied to SDS PAGE gels and the polypeptides were separated by electrophoresis. Gels (1.5 mm) were stained for one hour in Coomassie blue R-250 and destained for approximately one hour. Bands containing the 240K and 60K polypeptides were excised from the gel, equilibrated in several changes of 300 mM sodium phosphate buffer pH 7.5 and frozen at -70°C until required for injection. Prior to injection, the gel slices were homogenized in a Luer-lock syringe with 500 µl of 0.9% NaCl, checked for neutral pH, and mixed with and equal volume of complete Freund's adjuvant. Samples containing approximately 20 µg of each protein were injected subcutaneously into the shoulders of New Zealand white rabbits (two rabbits for each antigen). Four weeks after the initial injection, the rabbits were boosted with approximately 20 µg of each protein mixed with complete Freud's complete adjuvant by intramuscular injection into the hind legs. The boost was repeated, in

the same manner, twelve weeks after the initial injection. Eight days after the final boost, the rabbits were bled by cardiac puncture and sera were collected.

IgG was purified from sera by affinity chromatography on protein A-Sepharose affinity columns (Smith-Gill *et al.* 1985). Serum, diluted with one-half of a volume of 0.1 M sodium phosphate buffer pH 8.0, was applied to a protein A-Sepharose affinity column which was pre-equilibrated with 0.1 M sodium phosphate buffer pH 8.0. The column was washed with 0.1 M sodium phosphate buffer pH 8.0 until the $O.D._{280nm}$ was ≤ 0.05 units. The bound IgG was then eluted with 0.2 M glycine HCl pH 3.0. Fractions (500 µL) containing the eluted IgG were collected into equal volumes of 0.1 M Tris buffer pH 8.0. The fractions containing protein were pooled and concentrated (typically 3.0 ml, representing three fractions, were concentrated to 500 µL) using Ultracent-30 cartridges (Bio-Rad, Richmond, CA). Concentrated IgG was dispersed into 20 µL aliquots and stored at -80°C.

2.4 Analytical Techniques

2.4.1 Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate gel electrophoresis was done by the method of Laemmli (1970). Samples were diluted 1:1 in sample buffer containing 2% (w/v) SDS, 1% (v/v) β-mercaptoethanol, 10% glycerol and 0.05% (w/v) bromphenol blue in 60 mM Tris buffer pH 6.8 and boiled for 5 minutes. When 16 cm slabs were used, samples were stacked in a 4% gel at 15 ma per slab, and separated in various per cent acrylamide gels (5% to 15% acrylamide) at 30 ma per slab. When a miniature gel

electrophoresis apparatus was used (Hoefer Mighty Small, Hoefer Scientific, San Francisco, CA), samples were run at 15 ma for 0.75 mm thick gels and 30 ma for 1.5 mm thick gels. All reagents were electrophoresis grade from Bio-Rad Laboratories (Richmond, CA.).

2.4.2 Fixation and Staining of Electrophoresis Gels

For protein staining with Coomassie Blue G-250, gels were fixed and stained in 7% (v/v)acetic acid, 25% (v/v) methanol and 0.1% (w/v) Coomassie Blue G-250. Gels were also stained for protein using the Bio-Rad silver stain for proteins. Glycoproteins, in polyacrylamide gels, were stained using several methods. Periodic acid-Schiff's stain for glycoproteins (Beeley, 1985) was used with fetuin included as a positive glycoprotein control. The gels were fixed overnight in a solution of 10% acetic acid and 15% isopropanol and were then incubated for one hour in a solution of 10% acetic acid and 25% isopropanol. The carbohydrate was oxidized by incubating the gels in a solution of 0.5% periodic acid (BDH, Toronto, Ont.) for two hours and the gels were then washed twice in 0.1% sodium arsenite. Following a ten minute incubation in 5% acetic acid, the gels were incubated in Schiff's reagent (BDH, Toronto, Ont.) overnight. The colour was developed by rinsing gels in a solution of 0.6% sodium metabisulphite in 0.01 M HCl.

Alternatively, glycoproteins were detected by staining with Alcian Blue 8GX (Sigma, St. Louis, MO) using a method based on that of Wardi and Michos (1972). Gels were fixed in 7% (v/v) acetic acid overnight, rinsed in distilled water, and oxidized with 1% (w/v) periodic acid (in 3% acetic acid) for 50 minutes at room temperature. Excess periodic

acid was removed by repeated washing in distilled water and the gels were incubated in 0.5% sodium metabisulphite (Fisher Scientific, Fairlawn, NJ) for 30 minutes to reduce any excess periodic acid. The sodium metabisulphite was washed away with distilled water and the oxidized glycoproteins were stained overnight with 0.5% (w/v) Alcian Blue 8GX in 3% (v/v) acetic acid.

Glycoproteins were detected in polyacrylamide gels using the cationic dye "Stains-all" (Green *et al.* 1973). The gels were washed and fixed overnight in 7% acetic acid and 25% (v/v) methanol. This solution was changed three times, as traces of the running buffer precipitated in the staining solution. The gels were rinsed well with distilled water and stained in 0.005% (w/v) Stains-all (Eastman-Kodak, Rochester, NY) in 10% formamide (Fisher Scientific, Fairlawn, NJ), 25% isopropanol in 15 mM Tris buffer pH 8.8 (Green *et al.* 1973). Gels were destained by exposure to light before photography. Gels were photographed through an orange filter to enhance the contrast.

The final method used to detect glycoproteins in polyacrylamide gels was the thymol-H_2SO_4 method of Racusen (1979). Gels were fixed overnight in 25% isopropanol and 10% acetic acid, followed by two washes (30 min each) in the same solution. The gels were then incubated in the same solution containing 0.2% (w/v) thymol (Sigma, St. Louis, MO) for two hours. The gels were finally incubated in a solution of 80% H_2SO_4 and 20% ethanol. The gels would turn opalescent upon incubation in the 80% H_2SO_4 and 20% ethanol solution. After approximately one hour the opalescence would disappear and the bands containing glycoprotein would appear orange-brown in colour.

2.4.3 Electrophoretic Transfers and Immunodetection of Transfered Proteins (Western Blots)

Soluble proteins from whole cell envelopes were separated by SDS PAGE using 7% acrylamide gels in the usual manner. The separated proteins were transferred to nitrocellulose using a modification of the method of Towbin *et al.* (1979). Proteins were transferred in buffer containing 0.025 M Trizma base and 192 mM glycine. Unlike the traditional buffer (Towbin *et al.* 1979), the transfer buffer did not contain methanol. The inclusion of methanol in transfer buffer often prevents the efficient transfer of high molecular weight proteins. Proteins were transferred to nitrocellulose sheets (Schleicher and Schuell, Mandel Scientific, Rockwood, Ont.) at 70 V for 3.5 hours. Transfers were done in a cold room (4°C) with stirring, however temperatures during transfers often reached 50°C. The efficiency of transfer was checked by cutting of strips from the transfer and staining these strips with 1% Amido black in 25% methanol and 10% acetic acid for five minutes. The strips were destained in 10% methanol.

Nitrocellulose sheets containing transferred proteins were incubated overnight in 100 mM Tris buffer pH 7.5 containing 150 mM NaCl (Fisher Chemical, Fairlawn, NJ), 0.1% bovine serum albumin (BSA) (Sigma, St. Louis, MO), 0.05% Tween 20 (Sigma, St. Louis, NJ) and 0.01% sodium azide (Tris-NaCl-BSA-Tween 20). All further incubation and washing steps were in this buffer, unless noted otherwise. After blocking overnight, the sheets were incubated with dilutions (1:50 to 1:1,000) of primary (anti-60K or anti-240K) IgG in Tris-NaCl-BSA-Tween 20 for 1.5 hours. After washing (three times for 5 min each wash), the

transferred proteins were incubated with the secondary IgG-horse radish peroxidase conjugate (Bio-Rad, Richmond, CA) (1:3,000 dilution of stock anti-rabbit IgG conjugated to horse radish peroxidase diluted in Tris-NaCl-BSA-Tween 20) for 1.5 hours. The nitrocellulose sheets were then washed (two washes of 5 min each) in Tris-NaCl-BSA without Tween 20. IgG-horse radish peroxidase conjugates were detected by the addition of 4-chloro-1-napthol (Sigma, St. Louis, MO) in 100 mM Tris pH 7.5 containing 150 mM NaCl and a trace amount of H_2O_2 (5 to 30 min incubation depending upon the intensity of the signal desired).

2.4.4 Protein Determination

Soluble protein was determined using the method of Bradford (1976).

2.4.5 Amino Acid Analysis

Purified perforate layer was hydrolyzed (1 mg in 6 N HCl), sealed under vacuum, at 110°C for 18 h and the percent composition of amino acids was determined in a Beckman Model 120C amino acid analyzer.

2.5 Electron Microscopy

2.5.1 Preparation of the S-layers

The perforate layer was isolated by the method of Austin and Murray (1987). Cells from a two day culture of *L. hyalina* strain UWO 884 were washed off a slant of YPA agar with 50 mM HEPES pH 7.5. The suspended cells were then disrupted by sonication for 20 seconds at 4°C. The insoluble material was washed twice in 2% SDS in distilled

water, followed by several washes in 50 mM HEPES pH 7.5. Material prepared in this manner consisted of the perforate layer and small amounts of peptidoglycan.

The punctate layer could not be isolated as intact pieces from the perforate layer. Preparations composed of the combined layers were prepared by suspension of cells in distilled water by repeated aspiration in a Pasteur pipette, leading to shedding of the S-layers.

2.5.2 Electron Microscopy

Micrographs were routinely taken with a Philips EM300 or Philips EM400T electron microscope operated at an accelerating voltage of 60 kV. The EM400T was fitted with a low dose beam deflection unit and a -60° to +60° goniometer stage.

2.5.3 Thin Sections

For routine preparation for thin sectioning, cells were fixed by the method of Burdett and Murray (1974). Fixed samples were washed with cacodylate buffer, enrobed in 2% Noble agar (Difco, Detroit, MI), and stained with 1.0% uranyl acetate (BDH Chemicals, Toronto, Ont.) in distilled water for 2 hours. The samples were dehydrated through a 30 to 100% ethanol series and embedded in Spurr's (Marivac Ltd., Halifax, N.S.) or Vestopal W (Martin Jaeger Co., Geneva, Switzerland) embedding resin. Thin sections were cut on a Reichert OMU2 Ultra Microtome with glass knives and stained with lead citrate and uranyl acetate (Reynolds, 1963).

Samples for immunoelectron microscopy were fixed in 10 mM sodium phosphate buffer containing 2.5% glutaraldehyde (Polysciences, Warrington, PA) and 1% paraformaldehyde (BDH, Toronto, Ont.) for 1.5 h. Cells to be used in immunolabeling experiments were usually not post-fixed in OsO_4. Fixed cells were enrobed in 2% Noble agar and washed several times with 10 mM sodium phosphate buffer pH 7.5. The agar blocks were dehydrated through a graded series of ethanol to 95% ethanol, and were infiltrated with L. R. White resin (Bio-Rad, Richmond, CA) overnight at room temperature. After several changes of the resin, blocks were polymerized at 60°C for 20 hours.

2.5.4 Freeze Fractures

Cells were scraped off YPA agar, placed on the centre of a gold grid, and quickly plunged into liquid Freon 22, cooled by liquid nitrogen. The frozen cells were placed in a Balzers freeze-fracture device (model BA510M; Balzers AG, Liechtenstein) and fractured at -196°C. Fractured samples were deep-etched for varying times (10 to 60 sec) at -100°C and shadowed with platinum and carbon. The replicas were floated off onto full strength commercial bleach (Javex), and remaining organic material was dissolved overnight.

2.5.5 Freeze-drying and Low Angle Shadowing

Sheets of perforate layer isolated by SDS extraction were washed several times by suspension in glass distilled water followed by centrifugation (15,000 *g*). The washed sheets of perforate layer were adsorbed to a glow-discharged grid. Specimens were washed several times by floating the grid on drops of distilled water. The grid was

plunged into a mixture of 67% propane and 33% isopentane cooled with liquid nitrogen and placed into a Balzers freeze etch device which was precooled to -170°C and continually flushed with nitrogen gas while the chamber was open. The frozen specimens were dried *in vacuo* at -80°C and platinum-carbon shadowed at an angle of 25° at the same temperature (Kistler *et al.* 1978).

2.5.6 Negative Stains

To prepare the combined punctate and perforate layers for negative staining, a small amount of cells, grown on YPA agar, was vigorously suspended in a drop of water by aspirating the cells in a Pasteur pipette; this appeared to release more of the combined layers. To prepare the isolated perforate layer for negative staining, perforate layer isolated by extraction with SDS was suspended in a drop of distilled water. A glow-discharged, carbon-coated grid covered with a Formvar or Parlodion film was floated on the suspensions for approximately two minutes. The grid was removed from the drop and floated on a drop of 1% ammonium molybdate and 0.1% glycerol at pH 7.5 (combined punctate and perforate layers) or 1% sodium tungstosilicate (isolated perforate layer) for approximately two minutes. Excess stain was removed by touching the edge of the grid to a torn edge of Whatman No. 1 filter paper.

2.5.7 Electron Microscopy of Negatively Stained Samples and Selection of Micrographs for Processing

Only grids containing a high density of S-layers were used, such that focusing on areas adjacent to a specimen was possible. After defocusing the second condenser lens to give the required current density, the specimen was moved into the beam and the image was recorded immediately, without additional focusing. This procedure reduced exposure of the specimen to the electron beam.

Electron micrographs, judged visually to have the proper focus, staining intensity, and long range order, were screened on an optical diffraction bench. Diffraction patterns of micrographs showing third order, or better, diffraction spots were photographed on 70 mm film. The diffraction patterns were inspected for lack of astigmatism, lack of drift, proper focus, and lack of spreading of the diffraction spots. The corresponding micrographs were digitized on an Optronics P-1000 rolling drum densitometer with a raster spacing of 25 μm corresponding to 0.5 nm at the sample.

2.5.8 Image Processing

All image processing was done using the SEMPER image processing system (Saxton *et al.* 1979) operating on a Digital VAX 8200 computer. A high resolution interactive raster graphics display (Metheus Omega 440) was used to display the digitized micrographs and the corresponding power spectra and averages. 512 by 512 pixel areas were selected from digitized micrographs and gradients due to uneven accumulation of negative stain were removed. These 'selected areas'

were used for subsequent processing by quasi-optical filtration and correlation averaging.

Quasi-optical filtration of a selected area was done by computing its Fourier transform and the power spectrum. The latter was multiplied by a radial gradient to give greater weight to the higher order spots. The base vectors of the reciprocal lattice were fitted to the positions of diffraction peaks to calculate a mask with windows centred at the reciprocal lattice points which had unity transmission and a diameter of 0.2 of the lattice spacing. The unweighted transform was then filtered by multiplication with the mask and the image was reconstructed by calculation of the inverse Fourier transform.

Correlation averages were calculated following several strategies (Saxton and Baumeister, 1982; Dorset *et al.* 1983; Engel and Massalski, 1984; Baumeister *et al.* 1986). First, a randomly selected patch containing 3 to 7 unit cells was used as a reference to calculate a cross-correlation function. A band-pass filter was applied to remove low frequency contributions that can disturb the peak search procedure and high frequencies which do not carry structural information. Correlation functions were scanned repeatedly with successively smaller thresholds, until the number of peaks detected represented at least 50% of the unit cells in the field. Subframes centred at those peak positions were then averaged to yield a correlation averaged projection of the lattice. The first average thus calculated was then used as a reference for a refinement run. Second, a reference was selected from quasi-optical filtered images which exhibited structural homogeneity, high contrast and distinct six-fold symmetry. Again, two correlation runs were accomplished to obtain the

final average. Third, the strain field analysis was applied to exclude severely distorted unit cells (Saxton and Baumeister, 1982).

2.5.9 Immunoelectron Microscopy

For negative stain immunolabeling, sheets of S-layer released by cells growing on YPA agar were suspended in 50 µL of 50 mM HEPES pH 7.5. A carbon coated grid was floated on the drop of suspended S-layer for approximately 5 minutes. The volumes of all drops were 50 µL and the excess buffer was removed by touching to the torn edge of filter paper for each transfer. The grid was incubated on a 50 µL drop of 100 mM Tris buffer pH 7.5 containing 150 mM NaCl, 0.1% BSA and 0.05% Tween 20 (Tris-NaCl-BSA-Tween 20) for 15 minutes. All subsequent incubation and washing steps used this buffer unless stated otherwise. The grid was floated on a 1:10 dilution of stock IgG (stock IgG had an $O.D._{280nm}$ of approximately 2.5) for 15 minutes and was washed twice with Tris-NaCl-BSA-Tween 20 for three minutes per wash. The grid was then incubated on a 1:10 dilution of stock protein A coated 5 nm colloidal gold (Polysciences, Warrington, PA) in Tris-NaCl-BSA-Tween 20 for 20 minutes. The excess colloidal gold was washed away with three one minute washes in Tris-NaCl-BSA-Tween 20 followed by three thirty second washes in distilled water. The grids were then floated on 1% ammonium molybdate containing 0.1% glycerol as per usual for negative staining.

For immunolabeling of thin sections, sections of cells embedded in L.R. White resin and mounted on nickel grids were incubated on a drop of Tris-NaCl-BSA-Tween 20 for 15 minutes, then incubated on a drop of a 1:10 dilution of stock IgG in Tris-NaCl-BSA-Tween 20 for two hours.

The sections were washed with two incubations of 3 minutes each on drops of Tris-NaCl-BSA-Tween 20 followed by two hours incubation on a 1:20 dilution of protein A-colloidal gold in Tris-NaCl-BSA-Tween 20. The sections were then washed three times for 3 minutes each wash on Tris-NaCl-BSA-Tween 20 to remove non-specifically bound protein A-gold particles. The final three washes were in distilled water for one minute each wash. The sections were then stained for 10 minutes with uranyl acetate and 3 minutes with lead citrate (Reynolds, 1963).

2.5.10 Silver Methenamine Staining of Sections

Sections of cells embedded in Spurr's resin were floated on a drop of 1% aqueous periodic acid solution for 15 minutes at room temperature. The sections were then washed on three drops of distilled water and floated on a freshly prepared silver methenamine solution in an oven for about one hour at 60°C in the dark. Completion of staining was usually accompanied by a slight yellow tinge to the silver methenamine solution. The sections were washed several times with distilled water and floated on a drop of 3% sodium thiosulfate for 5 minutes at room temperature and washed thoroughly with distilled water.

The entire procedure was done by transferring sections from drop to drop, rather than attaching them to a grid and transferring the grid. Copper grids were oxidized by the staining solution and silver precipitated out of solution onto the grid. The procedure was optimized by covering the stain at all times and reducing the staining time in the silver methenamine solution to 30 minutes. Staining for

longer than 30 minutes resulted in coarse grains and binding of silver to the cytoplasm.

2.6 Chemical Treatments

2.6.1 Cell Envelopes

Cell envelopes were resuspended in 100 mM HEPES pH 7.5 to an $O.D._{600nm}$ of 1.0. An aliquot of this suspension was added to an equal volume of dissociating agent, mixed, and incubated for various times at room temperature. Envelope suspensions were centrifuged (15,000*g*, 15 min) and the supernatants were removed. Residual pellets were washed once in 50 mM HEPES pH 7.5, and the state of the S-layers was examined by electron microscopy after negative staining, and by SDS polyacrylamide gel electrophoresis.

2.6.2 Isolated Perforate Layer

Isolated sheets of perforate layer were suspended in distilled water to an absorbance reading of 0.2 O.D. units at 600nm. A 500 µL aliquot of this suspension was added to an equal volume of chemical extractant. The suspension was thoroughly mixed and incubated for periods of one or 12 hours at room temperature. Following incubation, the turbidity of the suspension was measured by its absorbance at 600nm. The suspension was then centrifuged (15,000*g*, 15 min) and soluble protein in the supernatant was determined by the procedure of Bradford (1976).

The pH stability of the perforate layer was examined using citric acid-Na_2HPO_4 buffer from pH 2.6 to 7.4, and Na_2HPO_4-NaOH buffer from pH 9.7 to 12.9. After incubation at room temperature for one hour, the turbidity of the suspensions was measured by OD_{550nm}. The suspensions were then centrifuged (15,000*g*, 15 min) and the soluble protein in the supernatants was measured by the method of Bradford (1976).

2.6.3 Reassembled Punctate Layer

A 200µL aliquot of reassembled punctate layer ($O.D._{600nm}$=0.5) was centrifuged (15,000g 10min) and the pellet was resuspended in 200µL of 50 mM HEPES pH 7.5 containing 10 mM $CaCl_2$ and either 2% Triton X-100, 150 mM NaCl, or both Triton X-100 and NaCl. The same procedure was used to examine the effects of 50 mM Tris pH 8.0 containing 10 mM EDTA or 10 mM EGTA, 50 mM Tris pH 8.0 alone, or 50 mM HEPES pH 7.5 containing 150 mM NaCl and 10 mM $CaCl_2$

2.6.4 Protease Digestion of the Perforate Layer

Isolated sheets of perforate layer were heated to 60°C in 6M guanidine HCl to solubilize a portion of the layer. Following removal of the guanidine HCl by dialysis against 50 mM HEPES pH 7.5, the resultant mixture of monomer and high molecular weight assemblies was incubated with various proteases (100 µg/ml, two hours at room temperature). The samples were then boiled in SDS polyacrylamide gel electrophoresis sample buffer for one minute, and separated by SDS PAGE on a 15% acrylamide gel. An alternative procedure entailed digestion of sheets of the perforate layer with the same proteases,

under identical conditions, followed by electrophoresis on 5% acrylamide gels. All proteases were obtained from Sigma (St. Louis, MO).

2.7 Reassembly of the Punctate Layer

Approximately 1 mg of cell envelopes were resuspended in 10 ml of 3M guanidine HCl at room temperature. After 30 minutes of incubation, the suspension was centrifuged (150,000 *g*) for one hour and the supernatant was removed and dialysed overnight at 4°C against 50 mM HEPES pH 7.5 buffer containing various cations or chelating agents.

For reassembly of the punctate layer onto the perforate layer, the punctate layer was separated from the cell envelopes, added back to the cell envelopes, and dialysed to remove the guanidine HCl. The punctate layer was removed from DOC-extracted cell envelopes by incubation in 3M guanidine HCl. After separation of the soluble punctate layer from the cell envelopes by centrifugation (150,000 *g*, one hour), the soluble punctate was added back to the cell envelopes and dialysed against 50 mM HEPES pH 7.5 with, or without, 10 mM $CaCl_2$. Reassembly was assayed by negative staining.

The above procedure was refined for SDS PAGE analysis of reassembly onto the perforate layer. Sheets of the perforate layer were isolated by SDS extraction of cell envelopes as described and were suspended to an OD_{600nm} of approximately 0.5. The soluble components of the punctate layer were obtained by extraction of DOC-extracted envelopes with 6 M urea and were adjusted to 100µg/ml by concentration with an Ultracent-30 filter. Two-hundred microlitres of a

suspension of perforate layer were added to 1.8 ml of the soluble punctate layer and the mixture was dialysed overnight at 4°C against 50 mM HEPES pH 7.5 or 50 mM HEPES pH 7.5 containing 10 mM $CaCl_2$. The same amount of soluble punctate layer (1.8 ml of a 100 µg/ml solution) was added to 200 µl of HEPES buffer pH 7.5 and dialysed under the same conditions. A final control was composed of 1.8 ml of HEPES buffer pH 7.5 and 200 µl of the suspension of purified perforate layer. This latter mixture was also dialysed against 50 mM HEPES and 50 mM HEPES containing 10 mM $CaCl_2$.

Chapter 3

RESULTS

3.1 Observations of Whole Cells

Light microscopy of growing cells of *L. hyalina* UWO 884 showed square to rectangular organized colonies (Fig. 1a). The edges of the colonies often displayed ruffled edges, probably a result of buckling of the sheet due to imperfections in the division pattern or slight differences in local growth rates of the attached cells. Electron microscopy of thin sectioned (Fig. 1b,c) or freeze fractured preparations (Fig. 1d) also showed the cells to form a continuous layer, a single cell in thickness. Although it is difficult to follow the S-layer over the surface of a tablet of cells at low magnification (Fig. 1b) the freeze-etched tablet shows how the S-layer covers the entire surface of the tablet, without penetrating between adjacent cells (Fig. 1d).

The envelope of *L. hyalina* was composed of several layers. From the cytoplasm outward, the envelope consisted of a cytoplasmic membrane, a thin peptidoglycan layer, an outer membrane, a fibrous intercalated layer, the perforate layer, and the outermost layer, the punctate layer (Fig. 2a). These two outermost layers together composed the S-layer. The S-layer covered, not individual cells, but entire tablets consisting of several cells. *L. hyalina* UWO 884 produced

the composite punctate layer and perforate layers in excess and large pieces, often larger than 1µm across, were shed during growth of the organism on solid media, allowing visualization of the layers by negative stain (Fig. 2b).

3.2 The Cell Envelope

3.2.1 Extraction With Detergents

Envelopes incubated for periods of up to 16 hours with various nonionic detergents or bile salts still possessed a well ordered punctate and perforate layer (Fig. 3a). The small pieces of membrane, which were obvious in the non-treated samples, disappeared after detergent extraction of the envelopes. While nonionic detergents and bile salts had no effect on the punctate layer, anionic and cationic detergents were more prone to disrupt the punctate layer. The weakly anionic detergent, N-lauroylsarcosine did not produce obvious disruption of the punctate layer (Fig. 3b). Exposure of cell envelopes to 1% SDS led to solubilization of all envelope components except the perforate and peptidoglycan layers (Fig. 7a,b), while 0.1% SDS dissolved attached membrane vesicles but did not affect the integrity of either of the S-layers (Fig. 3c). Several cationic detergents, including CTAB, DTAB, TDTAB, selectively disrupted the punctate layer, causing it to form long fibres about 3 nm in thickness (Fig. 3d).

Detergent extraction of cell envelopes was assayed by SDS PAGE of extracted cell envelopes (Fig. 4). Few of the envelope proteins were solubilized by nonionic detergents or bile salts. Sodium dodecyl sulphate solubilized all of the envelope polypeptides except the 32K

polypeptide. The cationic detergents were intermediate in their efficacy of solubilization, releasing predominantly lower molecular weight polypeptides (less than 66K) from the envelopes. Cationic detergents did not affect the perforate layer.

3.2.2 Extraction With Urea and Guandine HCl

Extraction of cell envelopes with urea or guanidine HCl caused a concentration dependent release of protein from the cell envelopes (Fig. 5a). Extraction of cell envelope proteins was directly proportional to the concentration of guanidine HCl from 0 to 4.5 M. Above 4.5 M guanidine HCl extraction of cell envelope proteins was more efficient than at the lower concentrations, reaching maximium extraction at 5.0 M guanidine HCl. Urea was much less efficient at extracting proteins from cell envelopes than was guanidine HCl, with 6M urea extracting approximately one-third of the protein extracted by an equal concentration of guanidine HCl. SDS PAGE of the soluble protein from urea and guanidine extractions showed a concentration dependent solubilization of several polypeptides (Fig. 5b) from the cell envelopes. The predominant polypeptides solubilized by urea and guanidine HCl had apparent molecular mass values of 32K, 60K, 66K and 240K. Electron microscopy of negatively stained envelopes indicated that extraction of cell envelopes with 3M guanidine HCl or 6M urea caused complete release of the punctate layer, and partial solubilization of the perforate layer. The insoluble residue, remaining after extraction with 3M guanidine HCl, contained pieces of perforate layer and outer membrane pieces with fibres, perhaps from the intercalated layer,

radiating outwards (Fig. 5c). Extraction with 3M urea led to partial dissolution of the punctate layer (Fig. 5d).

3.3 Perforate Layer

3.3.1 Isolation and Appearance of the Isolated Layer

The perforate layer was isolated, from cell envelopes, as large sheets by dissolution of the adherent membranes and punctate layer by repeated washing of the envelopes with 2% SDS at room temperature. This was followed by digestion of the peptidoglycan by 200µg/ml of lysozyme. Observation of the material by negative staining showed the presence of the perforate layer only (Fig. 6a), while examination by SDS PAGE indicated the presence of a 32K polypeptide accompanied by small amounts of a 66K polypeptide (Fig. 6b).

3.3.2 Structure

The perforate layer was not visible beneath the punctate layer in the comp· site layers sloughed off the cells (Fig. 2b). This is most likely due to the heavy stain uptake by the rel•tively thick punctate layer, thus obscuring most of the detail of the underlying perforate layer. Isolated pieces of perforate layer were easily obtained by washing cell wall fragments with 2% SDS at room temperature to remove the attached punctate layer and associated proteins and lipids. When this material was negatively stained, the arrangement of stain-filled "pores" on a hexagonal lattice became evident (Fig. 7a). The lattice constant was 14.6 ± 0.4 nm. The surface texture of the perforate layer became evident in freeze-dried preparations after metal sha• wing (Fig.

7b). The perforate layer appeared to be rigid, with little lattice deformation. Diffraction patterns of micrographs of negatively stained perforate layer revealed spots extending to hexagonal diffraction orders of 5,1 indicating approximately 2 nm resolution (Fig. 7c). As may be deduced from correlation averaged images (Fig. 7d), the perforate layer appeared to consist of block-shaped units arranged as trimers, which further associated into a two-dimensional hexagonal lattice. Each of the block-shaped units was centred about a two-fold axis of symmetry, suggesting that each was a dimer of the 32 K polypeptide.

3.3.3 Chemical Stability

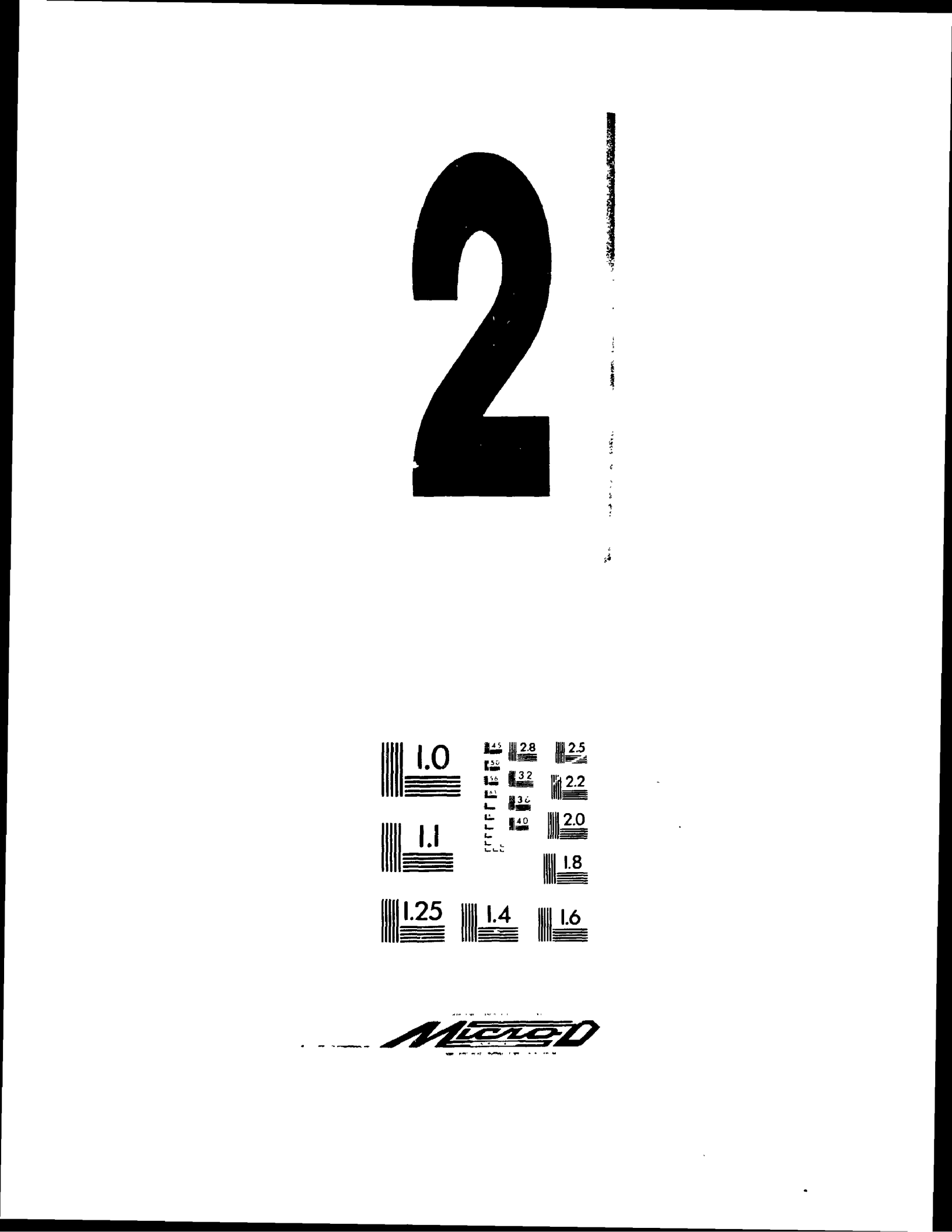
One hour incubation, at room temperature, of cell envelopes with nonionic detergents (Triton X-100, Brij 58, Tween 20, Tween 80), cationic detergents (cetyltrimethylammonium bromide and dodecyltrimethylammonium bromide), anionic detergents (SDS), bile salts (sodium deoxycholate and cholic acid), or the zwitterionic derivative of cholic acid, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) (Lichtenberg *et al.* 1983), did not attain solution of the perforate layer (Fig. 4 and Table 1). Reducing agents (β-mercaptoethanol and dithiothreitol) and chelating agents (EDTA and EGTA) also did not solubilize the perforate layer (Table 1).

Isolated sheets of perforate layer were dissociated in buffers below pH 4, and above pH 11. Dissociation was measured as an increase in soluble protein (Fig. 8) of a suspension of the perforate layer. An increase in turbidity, due to aggregation of sheets of the perforate layer, was noted at basic pH values (data not shown).

3.3.4 Component Polypeptide

SDS polyacrylamide gel electrophoresis of the solubilized perforate layer resulted in the appearance of a single band, migrating at a position corresponding to 32K (Fig. 6b). High molecular weight aggregates remained at the top of the separating gel, but their amounts decreased with longer solubilization times. Amino acid analysis of the component polypeptide revealed a high proportion of glutamic and aspartic acid (Table 2). When the ratio 3 (Barrantes, 1975) was calculated, a value of 1.16 was obtained, typical of an extrinsic protein.

The proportion of free monomers to intact sheets of perforate layer was indicated by the proportion of the low molecular weight (32K) form to the high molecular weight assemblies at the top of SDS polyacrylamide gel electrophoresis gels. Five percent acrylamide gels were used to follow proteolysis of the high molecular weight assemblies. The assembled form of the 32K polypeptide, as it exists in the intact perforate layer, was not hydrolysed by endoproteinase glu-C from *Staphylococcus aureus,* but was degraded by trypsin, thermolysin, proteinase K, and pronase (Fig. 9a). The 32K monomeric subunits of the perforate layer, obtained by guanidine HCl solubilization, were sensitive to all the proteases used, including endoproteinase glu-C (Fig. 9b). The migration of the monomer was not affected by incubation with sialidase or ß-glucosidase (Fig. 9b).


2
1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
MicroD

3.4 Punctate Layer

3.4.1 Structure

In fixed and sectioned cells of *L. hyalina* the units of the punctate layer appear tapered toward the outside to produce spiny units with closed tips (Fig. 10a). This contrasts with the image provided by side views of the punctate layer. Periodically, a piece of the combined layers could be found that was lying on its edge (Fig. 10b). These side views showed the units of the punctate layer to be shaped like hollow goblets, with the outer aspect of the units tapered to form a small open tube. Freeze-etched preparations of whole cells also showed the punctate layer to consist of tubes open at the external aspect (data not shown). Negatively stained sheets of the composite S-layer show that the tips of the spines extending from the stain terminate in an open pore approximately 7 nm in diameter (Fig. 10c). From thin sections, and negatively stained side views, the punctate layer was estimated to be approximately 45 nm deep.

Large pieces of the S-layer, often more than 1µm across, were shed during growth of the organism on solid media, thereby allowing easy preparation and visualization of the layers by negative staining. Most of these pieces consisted of both the perforate and the punctate layer (Fig. 11a). Occasionally, a piece of punctate layer was revealed without the perforate layer being present as a backing layer (Fig. 11b), but these isolated pieces of punctate layer were too few, and small, to be used for image processing.

Several negative stains were used to stain the composite punctate and perforate layers. Sodium phosphotungstate and ammonium molybdate filled the area between the tips of the tubular units of the punctate layer, obscuring the linking elements, whereas 1% uranyl acetate disrupted the structure of the punctate layer. After trying several negative stains, the best combination was found to be 1% ammonium molybdate pH 7.0, containing 0.1% glycerol. Ammonium molybdate at neutral pH did not cause disruption of the structure and yet produced sufficient contrast. Glycerol was included to dilute the stain and support the fragile structure. The punctate layer appeared as central hexagonal rings, with a diameter of 8 nm, connected in a hexagonal arrangement by Y-shaped linking elements to give a lattice constant of 25.6±0.5 nm (Fig. 11a). Typical diffraction patterns of the composite layers revealed six-fold symmetric spots extending to (5,1) and, occasionally (6,1), indicating a resolution of approximately 2 nm (Fig. 11c). Correlation averaged images of the composite layers revealed the punctate layer, but the perforate layer remained obscured by the overlying punctate layer (Fig. 11d). The central rings of the punctate layer were located about six-fold symmetry axes, while the linking elements were about three-fold symmetry axes. According to the notation proposed by Saxton and Baumeister (1986), the punctate layer can thus be described as M_6C_3. With centres of mass located about six-fold symmetry axes, and connecting elements about three-fold symmetry axes.

Occasionally, a piece of the composite S-layers could be found where the punctate layer did not completely cover the perforate layer. These pieces would show the relationship between the lattice spacings of the two layers (Fig. 12). It cannot be determined if the punctate layer is arranged specifically on the perforate layer but not completely covering the underlying layer or if the punctate layer is in the process of detaching from the perforate layer. If the punctate layer has separated from the perforate layer it may have shifted in position changing the relative lattice orientations from the native state. By digitally manipulating filtered images, a graded composite of the two layers was created. This graded composite shows a possible relative arrangement of the two layers (Fig. 12, inset).

3.4.2 Solubility

Several protein denaturants, ion chelators and detergents were tested for their effect on the structure and integrity of the punctate layer (Table 3). The punctate layer was made soluble by 3 M guanidine HCl or concentrations of urea greater than 3 M (Fig. 5). Urea and guanidine selectively extracted three polypeptides from cell envelope preparations. These polypeptides migrated in SDS PAGE gels with molecular weights of 60K, 66K and 240K. Concomitant with solubilization of these three polypeptides, was the disappearance of the punctate layer from the insoluble fraction of the cell envelopes.

Incubation of the S-layer in 100 mM Tris pH 8.0 containing 10 mM EDTA or 10 mM EGTA disorganized, but did not appreciably solubilize, the punctate layer (Fig. 13a and b). Incubation in Tris buffer pH 8.0 without addition of EDTA or EGTA did not disrupt the structure of the

punctate layer (Fig. 13c). The punctate layer could not be stained with 1% uranyl acetate, as this stain caused disorganization of the layer.

Several nonionic detergents and bile salts, and one zwitterionic bile salt, were tested for their effect on the punctate layer (Table 3). Triton X-100, Tween 80, sodium cholate, sodium deoxycholate and CHAPS, all at 2% (v/v) concentration, had no effect on the structure or stability of the native punctate layer (as it exists on the perforate layer). The cationic detergents cetyltrimethylammonium bromide (CTAB), dodecyltrimethylammonium bromide (DTAB) and tetradecyltrimethylammonium bromide (TDTAB) showed interesting effects on the punctate layer. The layer was disorganized, and induced to form extensive filaments in the presence of of these cationic detergents (Fig. 3d). Incubation in 1% SDS solubilized the punctate layer, whereas 0.1% SDS caused minimal disruption to the layer.

3.4.3 Fractionation

The punctate layer was made soluble by incubating sodium deoxycholate extracted cell envelopes in guanidine HCl or urea for 15 minutes at room temperature. Cell envelopes incubated in 3M urea showed small patches of intact punctate layer, while preparations incubated in 3M guanidine HCl completely lacked the punctate layer. The soluble punctate layer was dialyzed overnight versus 10 mM sodium phosphate buffer pH 7.5 and fractionated on a hydroxyapatite column.

Elution of the bound protein from the hydroxyapatite column with a linear gradient of sodium phosphate typically produced three peaks (Fig. 14a). Examination of these peaks by SDS PAGE indicated two of the peaks contained the 66K and 60K polypeptides (Fig. 14b). The polypeptides always eluted in the same order: 66K followed by 60K. The 240K polypeptide eluted separately from the 66K and 60K polypeptides. When examined by negative stain these fractions contained various components of the punctate layer. The isolated 240K polypeptide could be observed as long, slender and slightly curved polypeptides (Fig. 15a). The isolated 60K polypeptide formed what appeared to be small multimers sometimes appearing as ring-shaped units (Fig. 15b), while the 66K fraction appeared as an amorphous background when viewed in negative stain (Fig. 15c). When the fractions containing the 60K and 240K polypeptides were examined by negative stain, complete assemblies of the native punctate layer were observed (Fig. 15d).

Attempts were made to isolate the punctate layer by successive assembly and disassembly. Although this was useful for concentration of the layer, preparations that went through two or three successive reassemblies showed the same multitude of bands when examined by SDS PAGE. Two-times reassembled punctate layer appeared similar to native punctate layer when viewed in negative stain.

Reassembled sheets of the punctate layer could be washed in 50 mM HEPES pH 7.5 containing either 150 mM NaCl (HEPES-NaCl) or 2% Triton X-100 (HEPES-TX-100), or both (HEPES-NaCl-TX-100). Washing reassemblies in HEPES-NaCl left the reassemblies intact, while HEPES-TX-100 and HEPES-NaCl-TX-100 disorganized the reassemblies. When

examined by SDS PAGE, the reassemblies washed in HEPES-TX-100, HEPES-NaCl, or HEPES-NaCl-TX-100 all contained the same polypeptides, in the same amounts, as the reassemblies washed in HEPES-$CaCl_2$. While Triton X-100 disrupted the reassemblies, and NaCl did not, neither treatment reduced the number of polypeptides in the reassemblies (data not shown).

3.4.4 Immunolabeling

3.4.4.1 Western blots and detection of 60K and 240K with antibodies.

Protein A-Sepharose columns were used to separate IgG from other serum proteins. When the whole serum was applied to the protein A-Sepharose column, a large peak of serum protein eluted from the column. As the column was washed with phosphate buffer, the serum protein peak gradually tailed off to give an $O.D._{280nm}$ of zero. A steep peak of IgG eluted from the protein A-Sepharose column when eluted with low pH glycine buffer (Fig. 17a).

Dilutions of anti-240K IgG, equivalent to 1:1,000 of the concentration in whole serum, labelled the 240K polypeptide sufficiently to detect with HRP-labelled anti-rabbit IgG on Western blots of cell envelopes. Higher concentrations of anti-240K IgG (1:200) also specifically labelled only the 240K polypeptide (Fig. 17b). Low dilutions (1:50) of the anti-60K IgG failed to produce detectable label of the 60K polypeptide in Western blots.

3.4.4.2 Immunoelectron microscopy

When the S-layer was incubated successively with anti-60K or anti-240K IgG and protein A-colloidal gold, gold particles were bound to sheets of the punctate layer (Fig. 17a,b). Little difference was observed in either the localization, or the amount, of the label bound to the S-layer. Labelling with antibodies to either protein was specific for the punctate layer, and very few gold particles were found randomly on the grid. No gold particles were found bound to fimbriae, or other cell components.

When cells fixed in 2.5% glutaraldehyde and 1% paraformaldelyde, dehydrated in graded ethanol, and infiltrated in L.R. White resin, were labelled with anti-240K or anti-60K IgG the antibody bound specifically to the punctate layer (Fig. 17c,d). All dilutions of IgG used (1:20 to 1:100 of the serum IgG concentration) could be used without nonspecific binding to the resin or other cell constituents. Some label was found in the cytoplasm and clusters of gold particles were often observed in the area of the cytoplasmic membrane, or periplasm. Often these clusters appeared to completely cross the cytoplasmic and outer membranes. For both section and negative stain immunolabelling, no labelling was obtained when preimmune sera were used in place of immune sera, indicating the absence of non-specific binding.

Since fixation in aldehydes, without post-fixation in OsO_4 gave poor contrast for the cell envelope layers, cells were processed for section immunolabeling with an osmium post-fixation. When post-fixation of cells in 1% OsO_4 in 10 mM sodium phosphate buffer pH 7.5 was used, the cell envelope layers became visible after sections were

stained with lead citrate and uranyl acetate. Cells fixed in 1% OsO_4 labelled intensely with anti-240K IgG, but did not label with anti-60K IgG (data not shown), suggesting that the epitopes on the 60K polypeptide were destroyed by osmium tetroxide.

3.4.5 Assembly

3.4.5.1 Reassembly onto the perforate layer

Incubation of cell envelopes in guanidine HCl, or urea, for short periods of time (15 minutes) at room temperature selectively solubilized the punctate layer. When this mixture, of soluble punctate protein and intact perforate layer, was dialyzed versus 50 mM HEPES pH 7.5 containing 10 mM $CaCl_2$ or 10 mM $SrCl_2$ the punctate layer reassembled onto the perforate layer (Fig. 18a,b) in a manner resembling the "crazy paving" described by Sleytr (1975) for *Bacillus stearothermophilus.* The attachment between perforate and punctate layers was evident in profile views, where the negatively stained perforate layer appeared as a thin layer and the punctate layer appearing in profile in negative stains, much as it does in thin sections stained with heavy metals (see Fig. 10b). Dialysis against 50 mM HEPES pH 7.5 without the addition of divalent cations resulted in limited reassembly, in the form of small patches (Fig. 18c). Dialysis against either distilled water or 100 mM KCl caused the punctate layer to reassemble, although not as extensively as with $CaCl_2$ or $SrCl_2$. Dialysis against 50 mM tetrasodium EDTA in 50 mM HEPES buffer pH 7.5 did not allow any assembly of the punctate layer (Fig. 18d).

3.4.5.2 Reassembly without a template

The punctate layer could be routinely reassembled, without the perforate layer present as a template, by dialysis of the soluble proteins against 50 mM HEPES pH 7.5 containing 10 mM $CaCl_2$ (Fig. 19a). Addition of 150 mM NaCl to the HEPES-Ca^{2+} appeared to increase both the amount, and the order, of the reassemblies (Fig. 19b). Reassembled punctate layer could be negatively stained with 1% ammonium molybdate or 1% phosphotungstate. Uranyl acetate denatured the reassemblies, likely a result of low pH.

Limited reassembly could be obtained by dialysis of the guanidine HCl soluble extract against 50 mM HEPES pH 7.5 without added $CaCl_2$, probably a result of small amounts of calcium remaining in the extract. This calcium may have remained bound to the protein, or present at trace levels in a soluble state. Addition of EDTA to 50 mM HEPES prevented all reassembly.

Reassemblies formed in 50 mM HEPES containing 150 mM NaCl and 10 mM $CaCl_2$ were incubated for 15 minutes in various chelators to check for their effect on the structure of the reassemblies. Incubation in 50 mM Tris pH 8.0 containing 10 mM EDTA or 10 mM EGTA disrupted the structure of the reassembled punctate layer (Fig. 20a,b), while incubation in 50 mM Tris pH 8.0 without the chelators did not affect the structure of the reassemblies (Fig. 20c). Incubation in 50 mM HEPES pH 7.5 or 50 mM HEPES pH 7.5 containing 150 mM NaCl and 10 mM $CaCl_2$ did also did not affect the structure of the reassemblies (Fig. 20d).

3.4.5.3 Protein composition of the reassemblies.

When the guanidine HCl soluble portion of cell envelopes was dialysed against HEPES buffer pH 7.5, no assemblies large enough to be sedimented by a short centrifugation (15,000 *g* for 5 min.) were formed. Addition of 10 mM $CaCl_2$ to the HEPES buffer resulted in reassemblies large enough to be removed by centrifugation. These reassemblies consisted predominantly of the 240K and 60K polypeptides (Fig. 21). Addition of purified sheets of the perforate layer to the guanidine HCl soluble portion of cell envelopes resulted in the inclusion of the 66K polypeptide in the reassemblies (Fig. 21) and caused a corresponding decrease in the amount of the 66K polypeptide in the soluble fraction. The 66K polypeptide is included in the assemblies formed on the perforate layer, but is not a component of the assemblies formed without the perforate layer. The 66K polypeptide was not a contaminant of the perforate layer preparation as the perforate layer alone did not contain the 66K polypeptide.

3.4.6 Evidence for a Polyanionic Nature

Several lines of evidence suggest that the punctate layer, if not a glycoprotein, has a polyanionic nature. When ruthenium red was incorporated into the primary and secondary fixatives the central core of the punctate layer stained heavily, suggesting it contained acidic polysaccharide (Fig. 22a). The punctate layer also stained intensely when cells were fixed in the presence of Alcian blue (Fig. 22b). When used to stain SDS polyacrylamide gels, Alcian blue bound mostly to the 240K polypeptide (Fig. 23) with some labeling of the 32K polypeptide.

Several glycoprotein stains specifically bound to the 240K polypeptide. Stains-all, a cationic carbocyanine dye, stained the 240K polypeptide blue (Fig. 23) suggesting the presence of sialic acid, while the 240K polypeptide could also be detected by the thymol-H_2SO_4 stain for glycoproteins (Fig. 23). This suggests the 240K polypeptide has a polyanionic nature, and may be a glycoprotein or sulphated glycoprotein. The 240K polypeptide did not stain well with Coomassie blue when it was prepared by directly solubilizing cell envelopes in SDS PAGE sample buffer (Fig. 23), but stained with Coomassie blue if it was previously solubilized in guanidine HCl or urea before preparation for SDS PAGE (Fig. 5).

While ruthenium red and Alcian blue may show nonspecific binding, other, more specific, methods also suggest the glycoprotein nature of the punctate layer. When thin sections of *L. hyalina* are reacted with the silver methenamine technique, silver is deposited in the outer membrane, and the perforate and punctate layers (Fig. 24). The deposition of silver grains in these areas demonstrates the presence of periodate induced aldehyde groups indicating the presence of carbohydrate. When envelope proteins are separated on SDS polyacrylamide gels and stained with the periodic acid-Schiff stain (PAS) the 240K polypeptide stained positive, suggesting that it contains carbohydrate (Fig. 24).

Chapter 4

DISCUSSION

S-layers form regular arrays on the external surfaces of many bacteria. These layers often consist of the highest molecular weight protein in cells possessing such layers, and that protein is usually present in higher amounts than any other cellular protein. They may perform important protective functions in the bacteria, possibly related to their forming a semipermeable barrier (Koval and Murray, 1986). S-layers have been suggested to form specific cell-cell connections involving alignment of the pores to form continuous channels between cells (Baumeister and Hegerl, 1986). Regardless of any function that they may have to benefit bacteria, S-layers should also make excellent models to study the export and assembly of proteins.

4.1 Arrangement of Cells

When grown in a stationary fluid medium *L. hyalina* grew as a thin film which covered the surface of the broth and extended up onto the glass several centimetres. On solid medium the cells grew as square to rectangular colonies which became ruffled as they grew larger. Murray (1963) has noted that cells of sheeting strains of *L. hyalina* have a cuboidal shape, while cells of non-sheeting strains have

a spherical shape and are larger than the sheeting cells. This might be a result of a "corset-like effect upon the tablet" imparted by the S-layer (Murray, 1963). The cells were arranged in groups or tablets of 64 or 128 cells, with the tablets being distinguished by the slightly increased spacing between them than the usual inter-cell distance (Chapman *et al.* 1963). As Chapman *et al* (1963) noticed, the sheets were easily disrupted to produce separate tablets of cells. The cell-cell connections within a tablet were quite strong and were resistant to breakage by all treatments except those which disrupted the cells. As the culture matured, however, tablets began to break up and disorganized aggregates of cells became common. It has been reported that overnight treatment of *L. hyalina* in Schweitzer's reagent (cupra-ammonium sulphate) can break down tablets into single cells (Chapman *et al.* 1963). Cell division alternated in two planes and did not appear to be perfectly synchronized. As noted by Murray (1963) cells within a tablet are in various stages of cell division.

4.2 The Cell Wall of *Lampropedia hyalina*. General Comments

The observed structure of the S-layer of *L. hyalina* is identical to that described (Murray, 1963: Chapman *et al.* 1963) and the lattice spacings measured in this study are within 0.4 nm to those previously reported (Murray, 1963; Chapman *et al.* 1963). The S-layer was attached to the cells by a fibrous intercalated layer of varying thickness. A similar structure exists between the outer membrane and the S-layer of *Bacteroides buccae* (Sjogren *et al.* 1985), although its presence was not noted by the authors.

The strain used in this study (*L. hyalina* UWO 884) possessed a complete S-layer, with both punctate and perforate layers. An additional laboratory strain (*L. hyalina* 2283), which had only "isolated islands" of the punctate layer, did not grow as sheets unless the growth medium was supplemented with $CaCl_2$, in which case it grew as extensive sheets similar to those produced by *L. hyalina* UWO 884. It is interesting to note that addition of $CaCl_2$ to the non-sheeting strain not only caused it to form sheets, but also caused it to become covered in a complete punctate layer. This supports Murray's (1963) observation that non-sheeting strains lack a complete punctate layer, and shows that a non-sheeting strain can be induced to form sheets when the complete punctate layer is restored.

The S-layer of *L. hyalina* proved amenable to detailed study of its structure by image processing of negatively stained fragments. The combined punctate and perforate layers were shed from the cell surface during growth on solid media; this shedding was enhanced by gentle mechanical forces during preparation for negative staining. The perforate layer was resistant to repeated washing in sodium dodecyl sulphate at room temperature (Austin and Murray, 1987), affording a simple method to provide clean preparations of this layer.

4.2.1 Disruption of the Cells and Isolation of the Envelope Components

Extraction of whole cells with Tris buffer pH 8 containing 2% sodium deoxycholate was used to obtain a fraction enriched for the perforate and punctate layers, along with peptidoglycan and its associated proteins. This chemical extraction procedure was used for cells grown on solid YPA agar, as the sheets of S-layer shed by the

cells when grown on solid medium were not disrupted by this treatment. To isolate the perforate layer, *L. hyalina* was broken by passage through a French pressure cell operated at 16 000 lbs/in2. Cells disrupted in this manner were usually grown to high density in broth cultures, yielding large quantities of cells.

4.2.2 General Complexity of the Cell Envelope

Thin sections of *L. hyalina* show the presence of a complex cell envelope. The cells possess a cytoplasmic membrane, peptidoglycan layer and outer membrane which are typical in appearance for gram-negative bacteria. All similarity to the 'typical' gram-negative envelope stops at the outer membrane. The fine fibres comprising the intercalated layer extend from the outer membrane to the first S-layer, or perforate layer. The punctate layer is assembled onto the perforate layer, and appears to constitute the outermost surface of the cells.

The nature of the intercalated layer remains elusive. Pangborn and Starr (1966) observed that the fibres appeared as extensions of the cell wall that had been "pulled apart" to give a "tacky appearance". While the fibres are extensions of the cell wall, it is more likely that they are linear molecules, perhaps proteins, inserted into the outer membrane. Extraction of cell envelopes with 3M guanidine HCl stripped the S-layer from the envelopes and exposed the fibres, allowing them to be seen in negatively stained preparations. The straight fibres appeared to extend away from the outer membrane, and could be seen radiating from outer membrane vesicles.

The protein profile of isolated cell envelopes reflects the complexity seen in the profile of the cell envelope by thin sectioning. Envelopes of most gram-negative bacteria usually contain less than a dozen polypeptides (Lugtenberg and VanAlphen, 1983) whereas the envelope of *L. hyaiina* contains many more polypeptides than usual for a gram-negative bacterium. It is not unusual that nonionic detergents solubilized few of the envelope polypeptides, as these detergents do not usually affect protein-protein interactions (Helenius and Simons, 1975). Triton X-100 treatment of human erythrocytes extracts only a subset of membrane proteins, leaving the erythrocyte cytoskeleton, which includes spectrin, actin and ankyrin, morphologically intact (Yu *et al.* 1973).

4.3 The Perforate Layer

4.3.1 Isolation

One criterion used for purity of an S-layer is the presence of a single band when the material is run on SDS PAGE gels. This criterion must follow the identification of the S-layer in a preparation by electron microscopy. These two criteria were met for the preparation of the perforate layer prepared by SDS extraction of cell envelopes.

The perforate layer was isolated from cell envelopes of *L. hyalina* by dissolution of the attached lipid and protein with SDS, leaving a residue of peptidoglycan with the perforate layer still attached. The peptidoglycan and its associated proteins were then dissolved with lysozyme, resulting in a homogeneous preparation of perforate layer. Analysis of the isolated sheets of perforate layer by SDS PAGE

indicated that they were composed entirely of a polypeptide with an apparent molecular mass of 32K.

4.3.2 Structure of the Perforate Layer

4.3.2.1 Structure as determined by CTEM and image enhancement.

Since the signal-to-noise ratio increases with the square root of the number of unit cells (Unwin and Henderson, 1975) used for image analysis, the largest assemblies of evenly stained S-layers were used for reconstructions. A large enough specimen may contain enough unit cells that staining is not required (Henderson and Unwin, 1975). Also, molecules held within a large crystalline lattice may be less vulnerable to beam and high vacuum damage, than individual molecules or molecules within a smaller lattice.

Correlation averaging (Saxton *et al.* 1979) was used to digitally process the negatively stained arrays. Correlation averaging involves moving a reference, preferably a single unit cell, around the entire array and comparing the reference with local image detail by cross-correlation. Local correlation peaks indicate the rough molecular positions and are refined by peak centre-of-mass determinations and averages are then produced by superimposition of regions centered on the final peak positions. Correlation averaging has the advantage over Fourier filtering of micrographs because it does not suffer from the loss of resolution when the image is not perfectly crystalline and only the unit cells with a correlation over a preset threshold are included in the average, thereby discarding the distorted, or imperfect, unit cells (Baumeister *et al.* 1986).

The perforate layer is a homopolymer of a 32K polypeptide assembled in six-fold symmetry. The regular six-fold symmetry of the perforate layer led Chapman *et al.* (1963) to suggest that the perforate layer may be "built up from three sets of fibrillar units, each set making angles of 120° with the other two and with the units interwoven to preserve a symmetrical arrangement". The lattice spacing of 14.6 nm was almost identical to that reported previously (Murray, 1963; Chapman *et al.* 1963) but was 1 nm greater than that reported by Pangborn and Starr (1966). Reconstructions of electron micrographs of negatively stained sheets of the perforate layer indicate the placement of a block-shaped unit on an axis of two-fold symmetry, suggesting that this unit is actually a dimer. Supporting this conclusion is the appearance of a stain-filled line running across the middle of the block, perhaps the actual interface between monomers. This deposit of stain would be located in a cleft at the interface of the two complementary surfaces. The negative stain would not penetrate between the two monomers, as protein-protein interfaces have a packing density that is as high as in the protein interior (Schulz and Schirmer, 1979).

By examining the computed diffraction pattern it can be seen that the data extend to approximately 2 nm, the limit of resolution of the reconstructions of the perforate layer. Most larger proteins are divided into structural domains of 100 to 140 residues, which corresponds to a globule about 2.5 nm in diameter (Schulz and Schirmer, 1979). With resolution of 2 nm, the disposition of any domains in the protein should be visible. The domain structure of the 32K polypeptide may have been evident as the monomer did not appear

simply as a globular protein, but appeared to be expanded where it contacted the adjacent protein at the two-fold axis. The perforate protein is large enough to contain two domains of 100 to 140 residues each. Maintenance of protein-protein contacts may be the only function of the domains.

The structure of the perforate layer does not fit the classification scheme of Saxton and Baumeister (1986). In this scheme, monomers are simplistically described as composed of a heavy domain (M) and a light domain (C). The centre of mass of the morphological units is denoted as M, while the regions connecting the centres of mass are denoted as C. The centres of mass are described as having p2, p4 or p6 symmetry, while the connectivity regions are described as having p2, p3 or p4 symmetry. The centres of mass of the perforate layer lie on a two-fold symmetric axis (M_2) but it is difficult to determine the connectivity of the perforate layer.

Using the image obtained from correlation averaging, and the centre to centre spacing of 14.6 nm, the size of the monomer can be obtained. The dimer measures 85Å by 44Å, for an area of 3,740Å^2. The thickness of the perforate layer can be measured, from micrographs of edge views of the perforate and punctate layers. When this is done, the perforate layer can be found to be about 40Å thick. Thus the volume of the dimeric unit of the perforate layer is 3,740Å^2 * 40Å, which equals 149.6 nm^3. The volume of the morphological monomeric unit is the volume of the dimer divided by two, or 74.8 nm3. The mass per unit volume of other S-layer polypeptides have been calculated and found to be 430 to 480 daltons/nm^3 for *Clostridium* (Crowther and Sleytr, 1977) and 450 daltons/nm^3 for *Aquaspirillum putridiconchylium*

(Stewart *et al.*, 1980). If a value of 450 daltons/nm^3 is used for the perforate polypeptide, a molecular mass of the monomeric unit can be obtained. This value works out to be 74.8 nm^3 * 450 daltons/nm^3, or 33,660 daltons. This is an indirect way of determining mass, and involves estimations of the dimensions of the polypeptide from the correlation average, and also involves presumptions of the packing density of the polypeptide. It does, however, give a mass value very close to that obtained from SDS PAGE (32,000 daltons).

Calculations of the molecular mass from dimensions obtained from three-dimensional reconstructions usually are underestimated by 50 to 80% when compared to the values obtained by SDS gel electrophoresis or STEM mass mapping (Baumeister and Engelhardt, 1987; Dickson *et al.* 1986; Engelhardt *et al.* 1986). This discrepancy has been suggested (Dickson *et al.* 1986) to result from disorder in the specimen caused by radiation damage and the effects of flattening during negative staining. While the error caused by radiation damage could not be controlled, the artifacts introduced by flattening of structures in the z-direction were negated by obtaining the value for the thickness of the perforate layer from side views of the layer.

The perforate layer bears a strong resemblance, structurally and chemically, to the S-layer of *Chlamydia trachomatis.* The 39,500 K subunits of the S-layer of C. trachomatis are resistant to dissociation by sodium deoxycholate (Chang *et al.* 1982), as are the 32K subunits of the perforate layer (Austin and Murray, 1987). The subunits of the S-layer of *C. trachomatis* are packed such that subunits in adjacent rings are in contact across two-fold symmetry axes (Chang *et al.* 1982);

precisely the arrangement formed by the subunits of the perforate layer.

Freeze-dried and shadowed preparations of the perforate layer demonstrated that the two sides of the layer were not identical. This may explain the observation of Chapman *et al.* (1963) that the hexagonal pattern of the perforate layer was not visible on some fragments of the layer. These authors suggested that the perforate layer was obscured by a 'superincumbent structureless layer'. A proportion of the isolated sheets of perforate layer lacked the usual honey comb pattern, when viewed after freeze drying and shadowing. These sheets were prepared by SDS extraction, a method which results in a preparation consisting of only a single polypeptide.

4.3.3 Chemistry of the Perforate Layer

Most S-layers studied to date contain a high proportion of acidic amino acids (Sleytr and Messner, 1983; Messner *et al.* 1984; Beveridge, 1979; Kay *et al.* 1981; Mescher and Strominger, 1976). A good indication of whether a protein is considered hydrophobic or hydrophilic is the "ratio 3" (Barrantes, 1975; Cantor and Schimmel, 1980). This is simply the ratio of polar or charged amino acids (Arg, Lys, His, Gly, Glu, Asp, Asn, and His) to nonpolar amino acids (Ile, Tyr, Phe, Leu, Val, and Met). A value greater than unity indicates an excess of polar or charged amino acids over nonpolar amino acids. When the "ratio 3" (Barrantes, 1975) was calculated from the amino acid composition of the perforate layer, a value of 1.16 was obtained. This value is indicative of a non-membrane or extrinsic membrane protein, and is similar to those calculated for several other S-layer proteins (Sleytr and

Messner, 1983). In the case of the perforate layer, it is not surprising that the value for ratio 3 suggests an extrinsic membrane protein, as the S-layer is not located either in, or on, a membrane, but is assembled several nanometers external to the outer membrane.

4.3.3.1 Intersubunit contacts in the perforate layer

The detailed nature of the intersubunit contacts remains unknown, but the bonds appear to be stronger than the intersubunit contacts of most other S-layers. None of the detergents tested, including anionic, cationic, and nonionic detergents, and bile salts, were capable of solubilizing the perforate layer. The insolublity of the perforate layer led Chapman *et al.* (1963) to suggest that the perforate layer is composed of a high-molecular-weight polysaccharide. A comparable resistance to solubilization by detergents has been observed for the S-layers of *Deinococcus radiodurans* (Thompson *et al.* 1982; Kubler *et al.* 1980), *Sulfolobus acidocaldarius* (Michel *et al.* 1980) and *Methanospirillum hungatei* (Beveridge *et al.* 1985), and also for the spore coat of *Bacillus subtilis* (Hiragi, 1972). The S-layer of *Pseudomonas* strain EU2 is also insoluble in SDS (unpublished results).

The subunits of the perforate layer were solubilized by prolonged incubation in high concentrations of urea, guanidine HCl, formamide or formic acid, demonstrating that the subunits interact with each other through non-covalent interactions. With few exceptions, most S-layers can be disassembled by chaotropic agents such as urea, guanidine HCl and formamide, or by extremes of pH (Sleytr and Messner, 1983). The perforate layer was solubilized at pH values corresponding to the pK_a of the R groups of aspartic acid, glutamic acid, lysine and arginine.

This suggests that protonation, and deprotonation, of the carboxyl, amino, and guanidinyl groups of the respective acidic and basic amino acids are involved in the breaking of intersubunit contacts, leading to solubility of the monomers.

4.3.3.2 Possible disposition of amino acids in the intact perforate layer

Monomers of the perforate polypeptide were hydrolysed into numerous low molecular weight fragments by endoproteinase glu-C from *Staphylococcus aureus,* however the same protease did not digest the protein when it was assembled into sheets. This suggests the acidic amino acids, glutamic acid and aspartic acid, which are the sites for endoproteinase glu-C, were not accessible to the protease while the protein was in its assembled state. As a result of their charged nature, glutamic acid and aspartic acid are most often found on the surface of a protein. A possible explanation for the absence of surface located acidic amino acids, is the participation of glutamic and aspartic acid in the intersubunit contacts, thereby making them inaccessible to endoproteinase glu-C. Trypsin, specific for the basic amino acids lysine and arginine, hydrolysed the 32K protein when it was in the assembled perforate layer, suggesting the presence of one or more external, exposed, basic amino acids in the assembled layer.

The pK_a of the guanidinyl group of arginine is pH 12.5, while in a protein molecule this is reduced to approximately pH 12. At pH 12 the aggregates of the perforate layer dispersed, as indicated by a precipitous drop in the OD_{550nm}, and the sheets of the perforate layer dissolved, as indicated by a sharp increase in soluble protein. It appears that, as the guanidinyl group of arginine looses its proton,

thereby loosing its charge, the perforate layer dissociates into soluble polypeptides.

A similar situation, of resistance to proteases while in the native state, is seen in the resistance of the S-layer of *Aquaspirillum serpens* VHA to both pronase and trypsin (Buckmire and Murray, 1970). Porin protein from the outer membrane of *E. coli* is also resistant to proteases while in the native state. Trimers of porin protein are resistant to proteolysis by trypsin, pronase and proteinase K in the absence or presence of 1% SDS (Rosenbusch *et al.* 1980), whereas the unfolded polypeptide is sensitive to proteolysis (Rosenbusch, 1974). The aggregates of porin protein released after SDS extraction of *E. coli* are also resistant to the above proteases (Rosenbusch, 1974). The close packing and high degree of secondary structure of the porins may be responsible for the protease resistance and insolubility of the porins in chaotropes, detergents and inorganic solvents (Rosenbusch *et al.* 1980).

Accessibility of the S-layer proteins of *Azotobacter vinelandii* to various proteases has been examined with the S-layer both on and off the cell surface (Doran *et al.* 1987). Trypsin, thermolysin and *Staphylococcus* endoproteinase glu-C, all at 10 to 100 µg/ml, had no effect on the protein while it was on the cell surface. Increasing the concentration of trypsin and endoproteinase glu-C to 1 mg/ml hydrolysed surface located S-layer protein, but the fragments remained associated with the outer membrane until extracted with Sarkosyl (Doran *et al.* 1987). A similar phenomenon has been observed with the intact S-layer of *Deinococcus radiodurans* (Rachel *et al.* 1983). Like the polypeptide comprising the perforate layer, the S-layer protein of

Azotobacter vinelandii is degraded when exposed to trypsin, thermolysin or endoproteinase glu-C while in the soluble state (Doran *et al.* 1987).

4.4 The Punctate Layer

4.4.1 Appearance of the Punctate Layer on the Cell Envelope

The punctate layer consisted of 40 nm high spines with apparently hollow centres, joined at the middle of each spine to give a centre-to-centre spacing of 25.6 nm. Negative stains of sheets of the punctate layer released from the cells growing on agar media, demonstrated the hexagonal arrangement of the subunits joined by Y-shaped linking elements.

The structure of the linking arms of the punctate layer is similar to the linking arms found in other S-layers with Y-shaped linking arms. These include *Aquaspirillum serpens* VHA (Murray, 1963; Buckmire and Murray, 1970, 1973, 1976; Glaeser *et al.* 1979; Dickson *et al.* 1986) and *Thermoproteus tenax* (Wildhaber *et al.* 1987; Messner *et al.* 1986). The punctate layer is, however, of a higher order of complexity and may consist of more than a single polypeptide component.

4.4.2 Components of the Punctate Layer

The usual methods of identification of the S-layer in a preparation by electron microscopy, followed by analysis of the polypeptides in the preparation by SDS PAGE led to unusual results in the case of the punctate layer. The punctate layer could be extracted from cell envelopes by urea or guanidine HCl. SDS polyacrylamide gel

electrophoresis of the soluble protein indicated the presence of three polypeptides with molecular weights of 60K, 66K and 240K. These three polypeptides could be reassembled (or successively assembled, disassembled and reassembled) to form the punctate layer. Analysis of the reassemblies by SDS PAGE always indicated the presence of at least these three polypeptides.

Few S-layers consist of more than a single polypeptide. An exception is the S-layer of *Flexibacter polymorphus* (Ridgway *et al.* 1975; Ridgway, 1977) which has been reported to consist of four different polypeptides (Ridgway and Lewin, 1983). In eukaryotic cells, several protein assemblies consist of multiple types of polypeptides. For example, clathrin trimers isolated from coated vesicles consist of three heavy chains and three light chains (Kirchhausen and Harrison, 1981). The heavy chains have a molecular weight of 180,000, the two types of light chains have molecular weights of 36,000 and 33,000 (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981). Each heavy chain has a single closely associated light chain (Kirchhausen and Harrison, 1981). Purified trimers can reassemble *in vitro* into coat-like structures referred to as cages (Ungewickell and Branton, 1981; Crowther and Pearse, 1981). It is interesting to note that the reassembled clathrin cages possess more than a slight resemblance to the punctate layer.

The 240K polypeptide isolated by hydroxyapatite chromatography appeared, by negative stain, to be a long slightly curved polypeptide. Often limited assemblies of two or three unit cells would form from the 240K polypeptide. These reassemblies occurred in the sodium phosphate buffer that the protein was eluted in. As no exogenous

calcium was added to the buffer, the calcium required for assembly of the polypeptides may have been tightly bound to the polypeptides throughout extraction and chromatography, or small amounts of calcium may have leached from the hydroxyapatite into the phosphate buffer. The soluble punctate layer applied to the HTP column did not reassemble before application to the column. This is likely a result of the low concentration of the protein in the sample applied to the column, as compared to the concentrated state of the protein after elution (eluted protein was usually 5 times more concentrated than that applied). This ability of hydroxyapatite columns to concentrate protein has been used for several other applications (Tiselius *et al.* 1956; Smith and Lee, 1978).

The 240K polypeptide was unusual in its staining behaviour. When whole cells or envelopes were solubilized and separated by SDS PAGE, the 240K polypeptide did not stain well with Coomassie blue G-250 or silver stains. The polypeptide did bind Alcian blue 8GX and Stains-all. Both of these cationic dyes bind to acidic groups, with Alcian blue binding to sulphate and carboxyl groups (Jones and Reid, 1973), and Stains-all binding to phosphoproteins, glycosaminoglycans, proteoglycans and nucleic acids (Green and Pastewka, 1975). Incorporation of Alcian blue into the fixative for thin sections increased the staining of the punctate layer, and demonstrated amorphous material extending 20 to 40 nm outward from the punctate layer. This binding of Alcian blue by the intact punctate layer, and by the isolated 240K polypeptide suggests that this polypeptide has a polyanionic nature, and may be a glycoprotein or sulfated glycoprotein. Supporting this conclusion is the staining of the 240K polypeptide with

the periodic acid-Schiff's stain and the thymol-sulphuric acid stain (Racusen, 1979) for glycoproteins. It must be noted that conclusive evidence that a particular protein is a glycoprotein may only be obtained when a glycopeptide is obtained and the linkage region characterized. If the 240K polypeptide is a glycoprotein, and is not merely contaminated with tightly bound lipopolysaccharide, it would be the first glycosylated S-layer protein found in a gram-negative bacterium (Sleytr and Messner, 1988).

While the 240K polypeptide is an extended (approx. 70 nm long) molecule and is obvious in negatively stained preparations, the 66K and 60K polypeptides are not immediately recognizable in negative stain. The 60K protein appears to form oligomers approximately 5 nm across, and is required for proper reassembly of the punctate layer. All reassemblies of the punctate layer include both the 240K linkers and the 60K polypeptide. Small assemblies of the linkers, without the 60K polypeptide, appear to lack the defined central pore in the centre of mass centred on the six-fold symmetry axis. This suggests that the 60K polypeptide is somehow involved in forming the structure of the main unit of the layer. The 66K polypeptide is not included in self-assemblies of the punctate layer formed without the perforate layer. It is, however, included in reassemblies formed on the perforate layer, suggesting that it attaches to the perforate layer and may form a bridge between the punctate and perforate layers.

Immunocytochemical studies with antibodies to both the 60K and 240K polypeptides indicate that the punctate layer contains both of these polypeptides. The soluble components of the punctate layer were separated by chromatography on a hydroxyapaptite column. Fractions

containing the 240K and 60K polypeptides were applied to SDS PAGE gels and the proteins were separated by electrophoresis. Bands containing the 240K and 60K polypeptides were excised and antibodies to these proteins were raised in rabbits. IgG was purified from sera containing anti-60K and anti-240K antibodies and was used for Western blots and immunoelectron microscopy studies.

Anti-240K IgG bound specifically to the 240K polypeptide after transfer to nitrocellulose by Western blotting. The binding of anti-240K IgG was absolutely specific for the 240K band, even at low dilutions of the IgG. Anti-60K did not detect the 60K polypeptide. The 60K polypeptide was transferred to the nitrocellulose, as it could be stained with Amido black. The 60K polypeptide may have been washed off the nitrocellulose during the overnight blocking stage, or during the several washes in Tween 20-containing blocking buffer. Loss of transferred proteins has been observed in other cases. After transfer to Schleicher and Schuell nitrocellulose, only 6% of the ϵ subunit of the *E. coli* F_1-ATPase remains attached to the nitrocellulose after the blocking and incubation steps (Dunn, 1986). Another nonionic detergent, NP-40, has been demonstrated to remove transferred proteins from nitrocellulose (Lin and Kasamatsu, 1983). Alternatively, the epitopes of the 60K polypeptide may be denatured after transfer such that they are not recognized by the anti-60K IgG. The preparation of anti-60K IgG labelled the punctate layer in both negative stains and post-embedding labeling of sections and thus contained antibody to the 60K protein.

Incubation of S-layer fragments in dilutions of antibody to either the 60K or the 240K polypeptides resulted in specific labeling of the punctate layer. No label was found attached to fimbriae or other cellular structures. Sections of *L. hyalina* embedded in L.R. White resin and incubated with anti-60K or anti-240K IgG demonstrated binding mostly to the punctate layer. Some label was found in the intercalated layer and through the cell envelope. The label found in the cell envelope layers was usually a line of several gold particles extending from the cytoplasmic membrane across to the intercalated layer. As actively growing, logarithmic phase cells were added directly to the fixative, it is tempting to speculate that the linear colloidal gold label crossing the envelope layers represents protein being actively secreted for insertion into the growing S-layer. This possibility could be further examined by plasmolysing cells in sucrose-containing fixative and looking for a correspondence between membrane adhesion sites and the presence of label. This would indicate that the antibody is labeling a protein being secreted for incorporation into the S-layer. Similarly, the few colloidal gold particles observed in the intercalated layer may be demonstrating the transfer of the proteins from the outer membrane to the S-layer.

4.4.3 Reassembly of the Punctate Layer

Assembly of S-layers is an example of a self-directed organization of a biological system. The process of self-organization involves the transformation of information encoded by the DNA into a three-dimensional structural form. Goel and Thompson (1986) regard self-organization as being "information driven", or controlled by information

contained within the system itself. Denaturation of the punctate layer in guanidine HCl or urea led to solubilization of the components of the layer. Removal of the denaturant by dialysis resulted in reassembly of the native layer.

Self-assembly of S-layers without a template has been reported for S-layers from several bacteria (Brinton *et al.* 1969; Glauert and Thornley, 1974; Sleytr, 1976; Masuda and Kawata, 1980; Michel *et al.* 1980; Sleytr and Plohberger, 1980; Tsuboi *et al.* 1982; Word *et al.* 1983). Often the self-assembly products are formed from oligomeric precursors (Jaenicke *et al.* 1985; M. Kist, Ph.D. Thesis). The S-layer of *Bacillus stearothermophilus* reassembles by first forming oligomeric precursors of 12 to 16 monomers which then assemble to form the final S-layer structure (Jaenicke *et al.* 1985). As the punctate layer consists of more than one type of polypeptide, it is difficult to propose an assembly mechanism for this structure. The isolated 240K polypeptide appears to self-assemble into arrays resembling the native punctate layer, but the centres of mass centred on the six-fold symmetry axes appear different. Occasionally star-shaped units with six bent arms radiating from a central core can be observed (data not shown), suggesting that these are the oligomeric precursors from which the punctate layer is assembled. Structures similar to these star-shaped units arise from the *Caulobacter crescentus* S-layer (Smit *et al.* 1981).

Dialysis of the soluble components of the punctate layer against HEPES buffer containing $CaCl_2$ produced limited self-assembly of the layer. The reassemblies were approximately 300 nm wide. Addition of the perforate layer as a template increased the overall size of the reassemblies. Incubation of the perforate and punctate layers in 3 M

guanidine HCl for short periods of time at room temperature selectively solubilized the punctate layer. When this mixture, of the soluble components of the punctate layer and the intact perforate layer, was dialyzed versus HEPES buffer pH 7.5 containing $CaCl_2$ or $SrCl_2$ the punctate layer reassembled onto the perforate layer. Dialysis against HEPES buffer without addition of divalent cations resulted in limited reassembly onto the perforate layer, in the form of small patches with poor order. While addition of the perforate layer as a template improved the reassembly of the punctate layer, the outer layers of both *Aquaspirillum serpens* MW5 and *Bacillus brevis* fail to reassemble into even small patches without the inner layer being present as a template (Kist and Murray, 1984; Tsuboi *et al.* 1982; Yamada *et al.* 1981).

The presence of Ca^{2+} has been found to be essential for the assembly of the S-layers of *Aquaspirillum serpens* (Buckmire and Murray, 1970; Buckmire and Murray, 1973; Kist and Murray, 1984; Koval and Murray, 1985), *A. putridiconchylium* (Beveridge and Murray, 1974; 1976), *A. metamorphum* (Beveridge and Murray, 1975), *Aquaspirillum* "Ordal" (Beveridge and Murray, 1976) and *Azotobacter vinelandii* (Bingle *et al.* 1984; Doran *et al.* 1987). The S-layer can be extracted from the surface of *A. putridiconchylium* and *Aquaspirillum* "Ordal" by substitution of Ca^{2+} with Na^{+} (Beveridge and Murray, 1986). It is not surprising that Ca^{2+} is also required for assembly of the punctate layer.

Assembly of the punctate layer from its soluble components occurred in several buffers with, and without, addition of $CaCl_2$. Based on this evidence alone, it appears that Ca^{2+} was not required for reassembly of the layer. However, it is likely that enough Ca^{2+}

remained bound to the soluble polypeptides to induce reassembly. This appears to be the case, as dialysis of the soluble polypeptides against buffers containing EDTA or EGTA prevented reassembly of the punctate layer. Also, incubation of self-assembled punctate layer in EDTA or EGTA disorganized the structure within minutes. The incorporation of 150 mM NaCl actually seemed to enhance the assembly of the punctate layer, indicating that ionic strength is important for reassembly. Assembly in Na^+-containing buffers appears to be contrary to the situation involving the S-layers of several species of *Aquaspirillum* where Na^+ causes disruption of the S-layer, however Na^+ is combined with low pH buffers to disorganize or remove the *Aquaspirillum* layers (Beveridge and Murray, 1976). The combination of low pH and cationic substitution may be required to remove the Ca^{2+} from the protein.

A summary of the isolation of the components of the punctate layer and their reassembly, both with the perforate layer and without the perforate layer, is presented in Figure 25.

4.4.4 Structure of the Punctate Layer

The combined punctate and perforate layers make up the complex S-layer of *L. hyalina*. The punctate layer alone (ie. without the underlying perforate layer) was not often observed, thus a reconstruction of the isolated punctate layer was not possible. Cells tended to shed fragments of S-layer and this material was used for reconstruction of the combined layers. Material prepared in this way has been used for image processing of the S-layers of several gram-negative eubacteria, including *Caulobacter crescentus* (Smit *et al.*, 1981), *Aeromonas hydrophila* (Murray *et al.* 1988) and *Azotobacter*

vinelandii (Bingle *et al.* 1987). S-layer fragments shed from *L. hyalina* were not contaminated with membrane pieces or vesicles of lipopolysaccharide, as is common with other gram-negative bacteria (Smit *et al.* 1981; Murray *et al.* 1988; Austin unpublished data). This is most likely because the S-layer of *L. hyalina* is not directly associated with the outer membrane, but is located several nanometers from the outer membrane.

The structure of the S-layer of *L. hyalina* determined here, closely matches that found in the initial studies on this structure (Murray, 1963; Chapman et al., 1963; Pangborn and Starr, 1966). Fixed, dehydrated and sectioned preparations of *L. hyalina* showed the outer, punctate layer to be composed of long, spine-shaped units closed at the tips. These spines were connected, about two-thirds of the way down the spine, to adjacent spines. When viewed face-on (ie. perpendicular to the array plane) in negative stain composed of dilute ammonium molybdate containing glycerol, the tips of the spines were open to the negative stain and appeared as small rings. This view was supported by the appearance of the open-ended tubes observed in freeze-etched cells and in side-views of the punctate layer. Side-views of other S-layers obtained by negative stain or frozen-hydrated specimens, show the outer surface to be flat, with an inner sculpted surface, as is the case with *Caulobacter crescentus* and *Aquaspirillum serpens* VHA (Smit *et al.* 1981; Glaeser *et al.* 1979; Dickson *et al.* 1986). The punctate layer is unusual in that the outer aspect of the layer is not flat, but tapers to a fine tip.

The units of the punctate layer were visible in freeze etched preparations of *L. hyalina*, as tubular units oriented at right angles to the surface of the cell. The eutectic obscured the fine linking arms which radiate from the tubular units. Preservation of the delicate punctate layer was not as good when freeze-drying was used to preserve the structure. This is similar to the disappearance of the characteristic S-layer topography seen on freeze-etched cells of *Thermoproteus neutrophilus* during freeze-drying (Messner *et al.* 1986). The punctate layer is an extended assembly of proteins and is, therefore, porous to water and ions. Water-containing structures are susceptible to collapse upon removal of the water (Kellenberger, 1987), this is demonstrated by the collapse of the bacterial capsule upon dehydration (Bayer *et al.* 1985) or the collapse of bacteriophage T4 tail fibres (Kellenberger and Kistler, 1979).

The arrangement of the linking arms of the punctate layer is similar to that found in other S-layers with Y-form linkages, or M_6C_3, according to the classification scheme of Saxton and Baumeister (1986). That is, the heavy domains are grouped around the sixfold axis to form the "core" while the lighter domains of the monomers provide connectivity about the threefold axis. Other bacterial S-layers displaying this general arrangement include *Aquaspirillum serpens* VHA (Murray, 1963; Buckmire and Murray, 1970, 1973, 1976; Glaeser *et al.* 1979; Dickson *et al.* 1986), *Clostridium thermohydrosulfuricum* (Sleytr, 1975; Crowther and Sleytr, 1977; Cejka *et al.* 1986) and *Caulobacter crescentus* (Smit *et al.* 1981).

The punctate layer is superficially similar to the S-layer found on the archaebacteria *T. tenax* and *T. neutrophilus* (Messner *et al.* 1986; Wildhaber and Baumeister, 1987; Wildhaber *et al.* 1988). The S-layers of *T. tenax* and *T. neutrophilus* consist of an open lattice with most of the mass located around the threefold and sixfold axes, a similar arrangement to that seen for the punctate layer. The stain exclusion patterns of the S-layers of *T. tenax* and *T. neutrophilus* suggest the linking elements arise from a single, curved proteinaceous subunit (Messner *et al.* 1986). Much the same arrangement of linking arms can be proposed for the punctate layer of *L. hyalina*. Also like the punctate layer, the S-layers of *T. tenax* and *T. neutrophilus* have extended regions centered on the sixfold axes. Unlike the punctate layer, these extensions do not contain easily observable pores (Messner *et al.* 1986). In *T. tenax* and *T. neutrophilus* the centers of mass on the sixfold axes extend toward the cell surface producing a rough inner surface and a smooth outer surface (Messner *et al.* 1986; Wildhaber and Baumeister, 1987; Wildhaber *et al.* 1988), while the centers of mass on the sixfold axis of the punctate layer extend both toward the cell surface (to attach to the underlying perforate layer) and away from the cell to produce a 'spiny' surface.

Determination of the three-dimensional structure of S-layers is becoming increasingly common (Hovmoller *et al.* 1988), and reveals a high degree of variability of structure on the outer surface, whereas the inner surfaces of S-layers tend to be conserved (Baumeister and Engelhardt, 1987). Both surfaces of the punctate layer appear unusually complex when compared to other bacterial S-layers. The

composite S-layer of *L. hyalina* should make an interesting specimen for three-dimensional reconstruction.

An especially useful application of three-dimensional structure analysis would be to determine the arrangement of the punctate layer as it is situated on the underlying perforate layer. The spatial relationship of the two layers has been suggested (Chapman *et al.* 1963) such that each spine of the punctate layer covers a hole in the perforate layer, much as each Y-shaped link between spines is also situated over a hole in the perforate layer. Digital manipulation of processed images of the perforate layer and the composite S-layer presented in this thesis illustrate this relationship. Chapman *et al* (1963) suggest that it is difficult to observe the perforate layer in negative stains of the composite layers because the two layers are in perfect register in the manner described. This appears to be correct, as diffraction patterns of the composite layers indicate perfect superposition of the two layers. Out of register lattices should display two sets of lattices in Fourier space, this clearly was not the case.

4.5 Localization of Anionic Sites in the Cell Envelope

Several stains may used to show the presence of capsular material. Ruthenium red (Bayer and Thurow, 1977; Luft, 1971; Woolcock and Mutimer, 1978; Jensen and Bertram, 1986), Alcian blue (Brooker and Fuller, 1975; de Hormaeche *et al.* 1978; Jensen and Bertram, 1986), polycationic ferritin (Weiss *et al.* 1979) and phosphotungstic acid (Glick, 1970; Pease, 1970) and related compounds (Rambourg, 1967) have been used to visualize extracellular carbohydrate layers.

The Alcian blue method of pre-fixation for electron microscopy was originally applied to mammalian tissues (Shea, 1971). The basis for staining is the reaction of Alcian blue, a cationic dye, with polyanionic substances, via sulphate and carboxyl groups (Jones and Reid, 1973). Contrast may be enhanced by post staining with lanthanum (Shea, 1971) or by using Alcian blue as the primary fixative, followed by ruthenium red as the secondary fixative (Dykstra and Aldrich, 1978). Ruthenium red, the most commonly used stain for exopolysaccharide, is a polycationic dye with six positive charges per dye molecule (Carrondo *et al.* 1980), giving it an affinity for anionic polymers, especially acidic polysaccharides (Luft, 1971).

Incorporation of Alcian blue into the fixative for thin sections increased the staining of the punctate layer, and demonstrated amorphous material extending outward from the punctate layer. This material is presumed to be acidic exopolysaccharide. The binding of Alcian blue to the punctate layer in vivo, as seen by thin sectioning, and *in vitro*, as seen by the binding to the 240K band in SDS PAGE gels, suggests that the punctate layer has a polyanionic nature. Ruthenium red resulted in a more discrete localization of anionic material in the punctate layer. Fixation of cells in the presence of ruthenium red indicated the presence of ruthenium red-positive material in the centres of the spines of the punctate layer. Usual fixation, without ruthenium red, results in a non-staining area in the centre of each spine.

Several examples exist in which exopolysaccharide has been demonstrated as an adhesive, connecting bacteria to a substrate. In a study of lactobacilli in the chicken crop using the Alcian blue-lanthanum method, Brooker and Fuller (1975, 1976) noted extracellular layers and connecting strands between the bacterial cells and between the bacteria and the chicken crop epithelial cells. Using Alcian blue alone, Fletcher and Floodgate (1973) observed connecting strands between marine bacteria adhering to a Millipore filter. These authors described two morphological types of exopolysaccharide, a dense layer (primary exopolysaccharide) at the cell surface and a more extended fibrous layer (secondary exopolysaccharide) external to the primary layer. Alcian blue has been used to show capsular material and connecting strands between cells of gonococci grown *in vivo* (de Hormaeche *et al.* 1978). Inclusion of Alcian blue in aldehyde fixatives has been demonstrated to help preserve the "cell coat" of several mammalian cell types (Behnke and Zelander, 1970), and was used to preserve the extracellular matrix of a marine bacterium (Fletcher and Floodgate, 1973). The capsule of *Haemophilus pleuropneumoniae* is removed by washing in cacodylate buffer or distilled water (Jensen and Bertram, 1986). Capsules were observed on Alcian blue stained cells of *H. pleuropneumoniae* when the cells were fixed before removal from the agar medium. Alcian blue staining material extended 20 to 100 nm outside the outer membrane of the cells (Jensen and Bertram, 1986).

Bacteria which are, in nature, normally found attached to a substrate may be attached by fimbriae, or, in the case of the stalked caulobacters, by holdfasts. Several bacteria appear to be held to a substrate by interactions mediated through exopolysaccharide

(Costerton, 1981). In particular, a marine pseudomonad has been shown to adhere to surfaces by an acidic exopolysaccharide which it produces (Fletcher and Floodgate, 1976). *L. hyalina* may use exopolysaccharide to adhere to substances. Growth of *L. hyalina* in stationary liquid culture, or on solid media, results in sheets of cells which adhere to the glass culture vessel and grow for several centimeters up the sides of the flask. When grown on solid medium, within a flat culture flask, the sheets will grow up the sides of the vessel and, eventually, cover the upper surface of the flask with a monolayer of cells.

4.6 Localization of Carbohydrate in the Cell Envelope

While cationic dyes can be used to locate anionic sites in the cell envelope, they are not necessarily specific for carbohydrate. To locate carbohydrate containing regions of the cell envelope, the periodic acid-Schiff's base reaction was used. Oxidation of free hydroxyl groups or amino groups on adjacent carbons of a sugar by periodic acid produces free aldehyde groups. The aldehyde groups may be detected by the reduction of silver ions to elemental silver at these sites. The silver methenamine technique was first used by Rambourg (1967), and DeMartino and Zamboni (1967), for localization of carbohydrate by electron microscopy. The technique is similar to the silver proteinate technique (Seligman *et al.* 1965) and is also referred to as the silver hexamine or silver hexamethylenetetramine technique.

When stained using the silver methenamine technique, walls of *L. hyalina* show three layers of silver deposition: the outer membrane, the perforate layer and the punctate layer. The carbohydrate of the lipopolysaccharide is likely to be the major carbohydrate of the outer membrane. The binding of silver to the perforate and punctate layers suggests the presence of sugars in these two layers. The silver deposition outside the cell is further evidence of the extracellular polysaccharide produced by *L. hyalina*.

A low level of nonspecific binding of silver occurred in the cytoplasm, this is not unusual for this technique (Erdos, 1986). The lack of an appreciable amount of silver deposition in the cytoplasm suggests that glycosylation of the polypeptides probably occurs at the cytoplasmic membrane level. Attempts were made to localize the silver grains to morphological units, but this was not possible because of the rather large size of the grains. The silver grains were approximately 10 nm in diameter, and attempts to further reduce the size of the grains resulted in reduced labeling of the surface layers.

Chapter 5

CONCLUSION

Both the arrangement of cells within a colony and the S-layer of *Lampropedia hyalina* are unusual. The S-layer surrounds, not individual cells, but entire tablets of cells and is a structure of considerable complexity.

The perforate layer was easily isolated because of its insolubility in detergents. It appears to be a simply constructed, rigid component of the S-layer. The perforate layer was an excellent subject for high resolution electron microscopical examination of its structure because of this ease in isolation and rigid nature with negligible lattice distortion.

The elaborate and delicate punctate contrasts with the simple perforate layer. The complexity of the punctate layer is reflected in its polypeptide composition. Incubation of the S-layer in 3 M guanidine HCl selectively solubilizes the punctate layer, leaving the perforate layer mostly intact, and releasing three major polypeptides with molecular weights of 60K, 66K and 240K. Reassemblies of similar structure to the native punctate layer are formed if the soluble punctate layer is dialyzed against buffer containing 10mM $CaCl_2$ or 10mM $SrCl_2$. These reassemblies contain both the 240K polypeptide and the 60K polypeptide. Antibodies to these two polypeptides demonstrate that they are both present in the native punctate layer. The only

other S-layer reported to consist of more than a single type of polypeptide is the equally elaborate layer produced by *Flexibacter polymorphus* (Ridgway and Lewin, 1983).

While the resolution obtained from studies involving three-dimensional structure determination is not usually as good as that obtained for studies of two-dimensional structure, the depth and complexity of this S-layer should make it an excellent candidate for this type of study. An especially interesting application would be to determine precisely how the perforate and punctate layers are situated relative to each other. The graded montage of the filtered images presented in this thesis supports the arrangement that Chapman *et al.* (1963) proposed twenty-six years ago. A three-dimensional study would answer this question conclusively.

The function of this complex structure remains unknown. Variants of *L. hyalina* which lack the S-layer grow as individual cells rather than in tablets (Murray, 1963), suggesting that the S-layer somehow holds the cells together within a tablet. These naked variants are susceptible to predation by *Bdellovibrio bacteriovorus,* while the covered strains are resistant to *B. bacteriovorus* (S. Lanys, M.Sc. thesis, U.W.O.). While the S-layer is not required for growth in culture, the layer must have an important function in nature, especially when one considers the large investment in energy required to synthesize such a complex S-layer.

Table 1: Effect of various treatments upon the integrity of the perforate layer

Treatment	Result
8 M urea	soluble
6 M guanidine HCl	soluble
100% formamide	soluble
90% formic acid	soluble
2% SDS (room temp.)	intact
2% SDS (100°C)	soluble
2% LIS	intact
95% ethanol	intact
100% CCl_4	intact
2% β-mercaptoethanol	intact
2% dithiothreitol	intact
2% CTAB	intact
2% DTAB	intact
distilled water	intact
10 mM HEPES pH 7.5	intact

Table 2: Amino acid composition of the perforate layer

Amino acid	Mole per cent
ARG	4.05
GLX	9.99
LYS	2.48
ASX	14.69
HIS	1.65
SER	5.32
THR	7.52
PRO	3.56
TRP	N.D.
TYR	4.24
ALA	12.29
GLY	10.16
PHE	5.09
CYS	N.D.
LEU	6.48
MET	N.D.
VAL	9.46

N.D. Not determined

Table 3: Effect of various treatments upon the integrity of the punctate layer

Treatment	Result
distilled water	intact
50 mM HEPES pH 7.5	intact
50 mM HEPES pH 7.7 + 10 mM $CaCl_2$	intact
2% Tween 20	intact
2% Tween 80	intact
2% Brij 58	intact
2% CHAPS	intact
2% Na deoxycholate	intact
2% cholic acid	intact
2% sodium dodecyl sulphate	soluble
2% DTAB	filament formation
2% CTAB	filament formation
2% TDTAB	filament formation
5% LIS	soluble
6M guanidine HCl	soluble
6M urea	soluble
40 mM EDTA	intact
40 mM EGTA	partially disorganized
1N NaOH	soluble

Envelopes were incubated 1 hour at room temperature and examined by electron microscopy after negative staining. Further (overnight) incubation showed no change.

Figure 1: Arrangement of *Lampropedia hyalina* within a sheet

a). Light micrograph of cells growing on an agar surface. Note the regular arrangement of cells and the division of the sheet into tablets.

b). Electron micrograph of a thin section perpendicular to the plane of the sheet showing the elongated shape of the cells.

c). Electron micrograph of a thin section through the plane of a sheet of cells.

d). Electron micrograph of a freeze fractured and etched tablet of cells showing the punctate layer arranged in p6 symmetry.

Bars = 500 nm

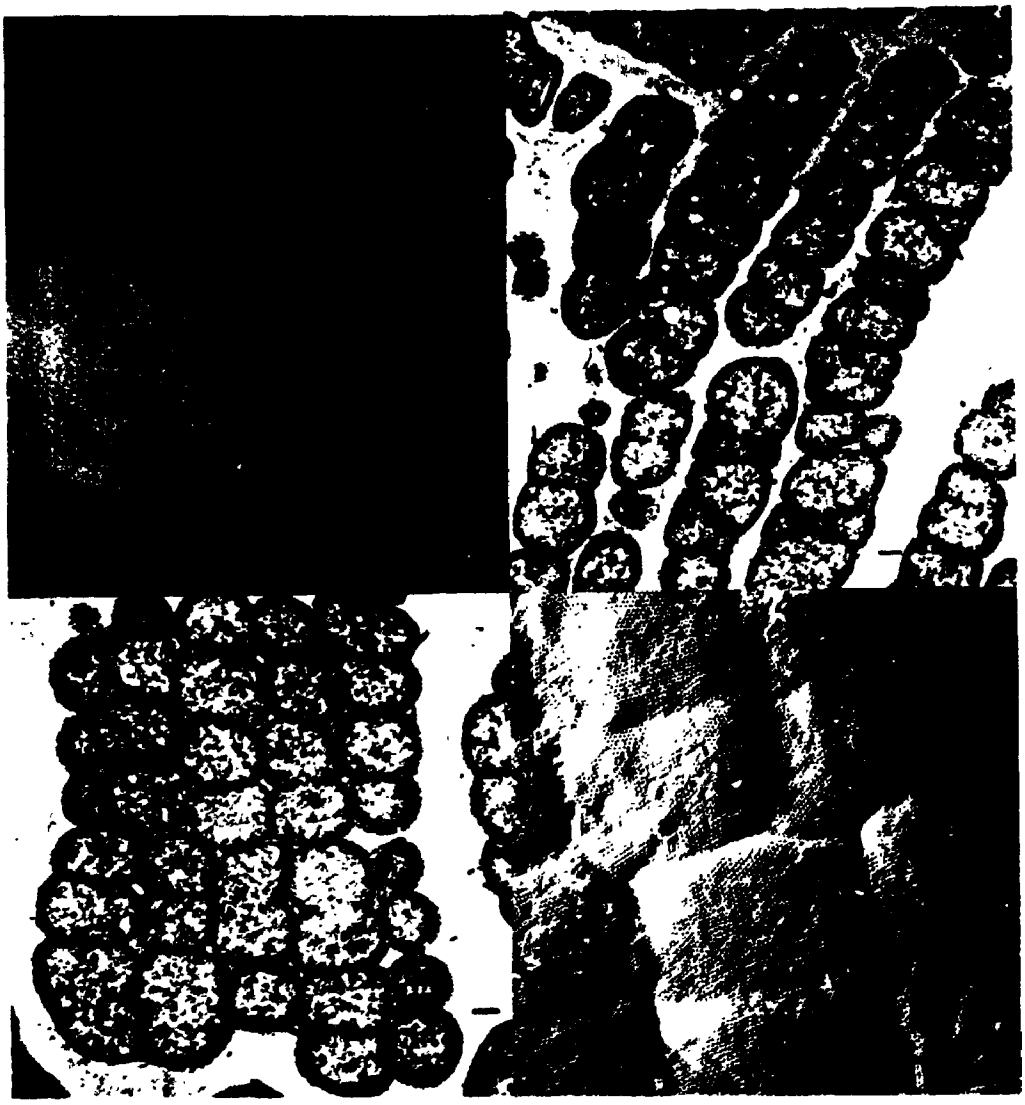


Figure 2: Structure and arrangement of layers of the cell envelope

a). Electron micrograph of a thin section showing the cell envelope layers. pu, punctate layer; pe, perforate layer; in, intercalated layer.

b). Electron micrograph of a negative stain of the composite layers shed from cells during growth on solid medium.

Bars = 100 nm

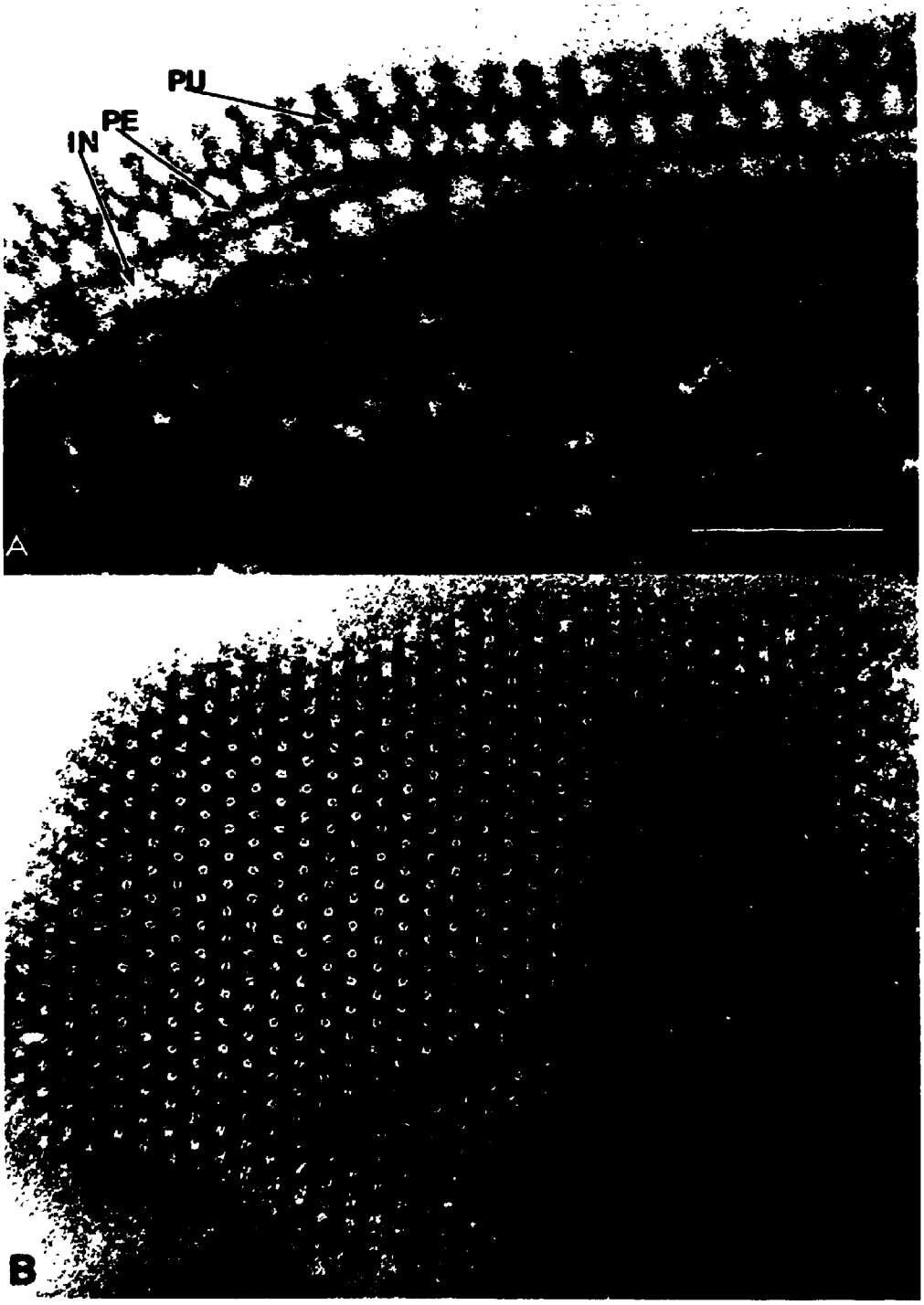

PU
PE
IN
A
B

Figure 3: Effect of detergents on the structure of cell envelopes

Cell envelopes were incubated with various detergents, then negatively stained and examined by electron microscopy.

a). 2% sodium deoxycholate. The punctate layer is not disturbed. This is typical of the bile salts and the nonionic detergents.

b). 2% N-lauroylsarcosine.

c). 0.1% SDS.

d). 2% CTAB. The punctate layer was induced to form linear filaments by CTAB, DTAB and TDTAB.

Bars = 100 nm


A
B

Figure 4: Effect of detergents on the protein content of cell envelopes

SDS PAGE of cell envelopes after extraction with various detergents. All detergents were used at a concentration of 1% (w/v or v/v) in 50 mM HEPES buffer pH 7.5. Lanes: 1, 50 mM HEPES pH 7.5; 2, Triton X-100; 3, Tween 80; 4, CHAPS; 5, cholic acid; 6, sodium deoxycholate; 7, CTAB; 8, DTAB; 9, TDTAB. The positions of the 32K (perforate layer), 60K and 66K polypeptides are indicated with arrows. The 240K polypeptide is not stained. Gel was stained with Coomassie blue G-250.

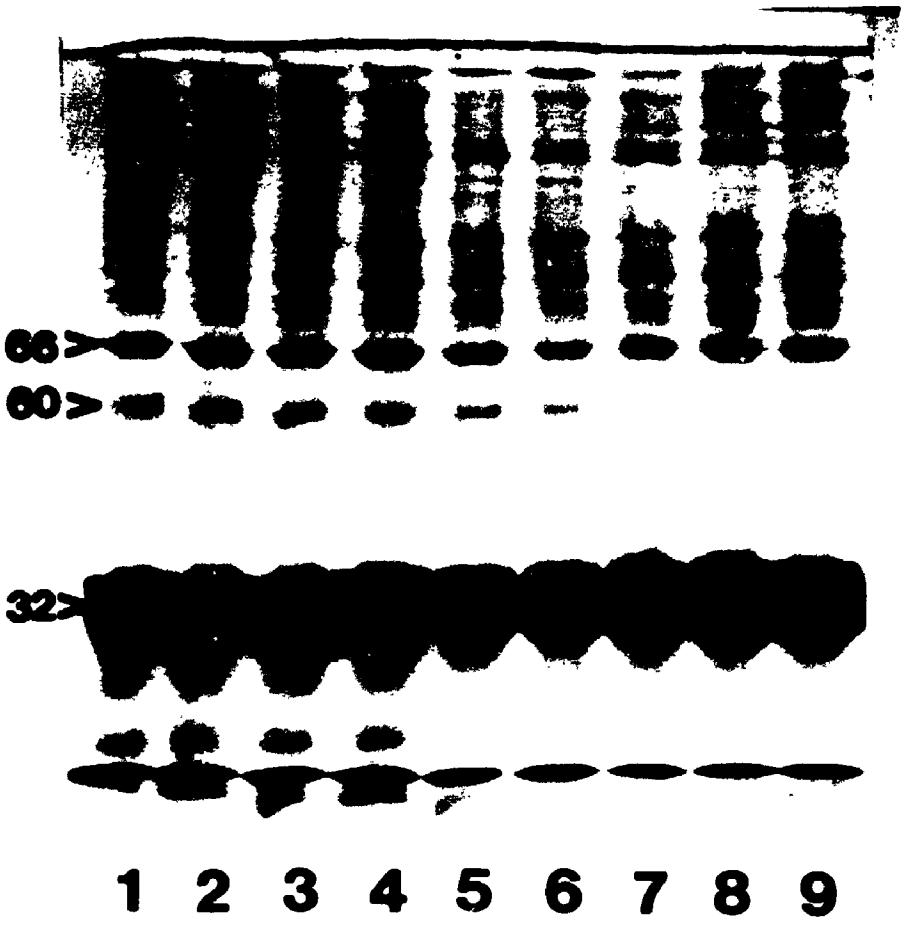

66>
60>
32>
1 2 3 4 5 6 7 8 9

Figure 5: Effect of urea and guanidine HCl on cell envelopes

a). Graph showing the release of protein from cell envelopes extracted with urea or guanidine HCl.

b). SDS PAGE of soluble proteins released from cell envelopes after extraction with guanidine HCl and urea. Lanes: 1, distilled water; 2, 1.5M GHCl; 3, 3M GHCl; 4, 6M GHCl; 5, 1.5M urea; 6, 3M urea; 7, 6M urea; 8, 8M urea. The 32K, 60K, 66K and 240K polypeptides are labeled. Gel was stained with Coomassie blue G-250.

c). Electron micrograph of a negatively stained preparation of cell envelopes after extraction with 3M guanidine HCl. Note the fine fibres extending from the cell envelope surface.

d). Electron micrograph of a negatively stained preparation of cell envelopes after extraction with 3M urea. Note the partial extraction of the punctate layer.

Bars = 100 nm

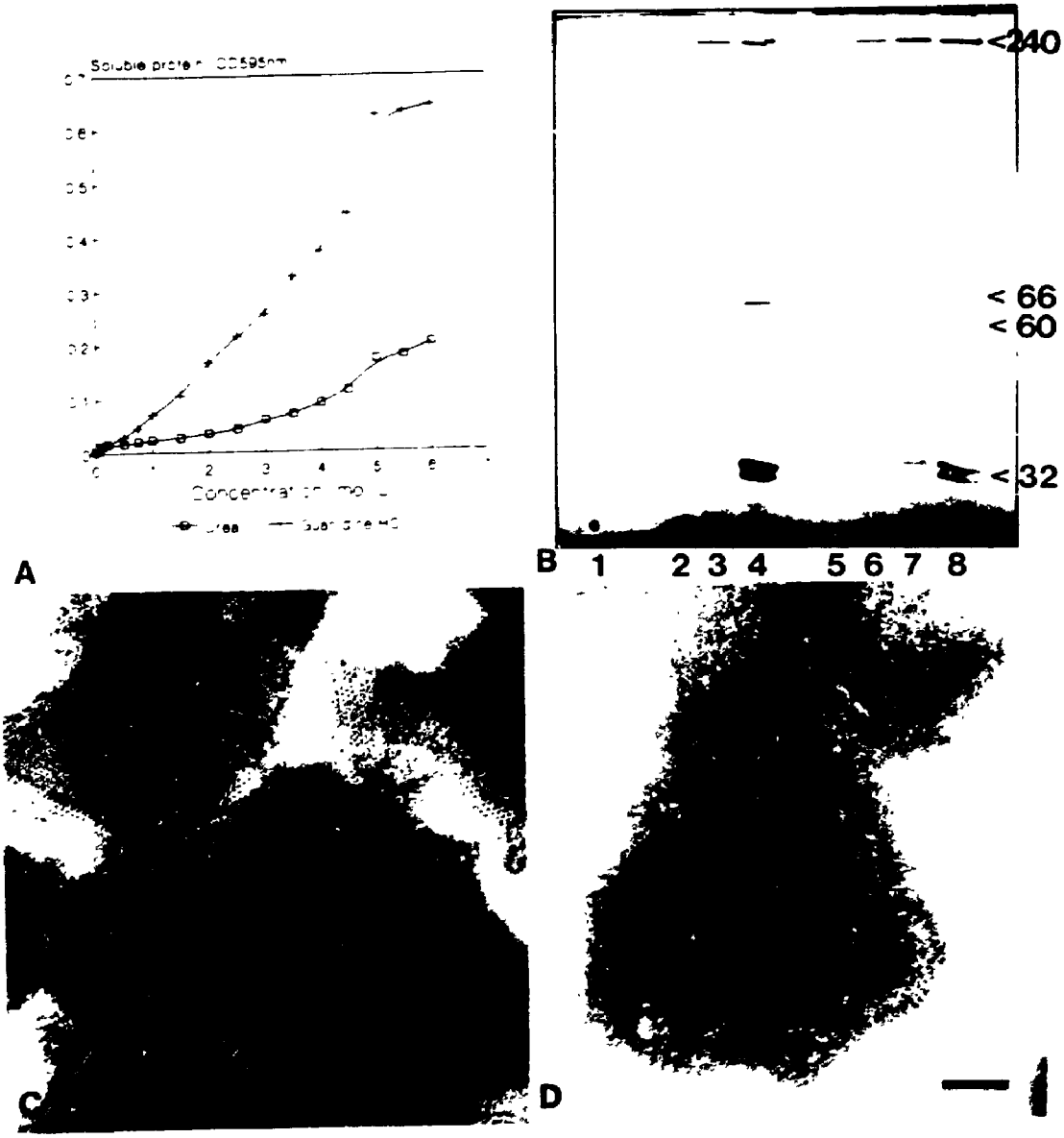

A
B
C
D
1
2 3 4
5 6 7 8
<240
< 66
< 60
< 32

Figure 6: Appearance and composition of the isolated perforate layer

a). Electron micrograph of the perforate layer negatively stained with 1% sodium tungstosilicate. Bar = 100 nm

b). SDS PAGE of the isolated perforate layer. Gel was stained with Coomassie blue G-250.

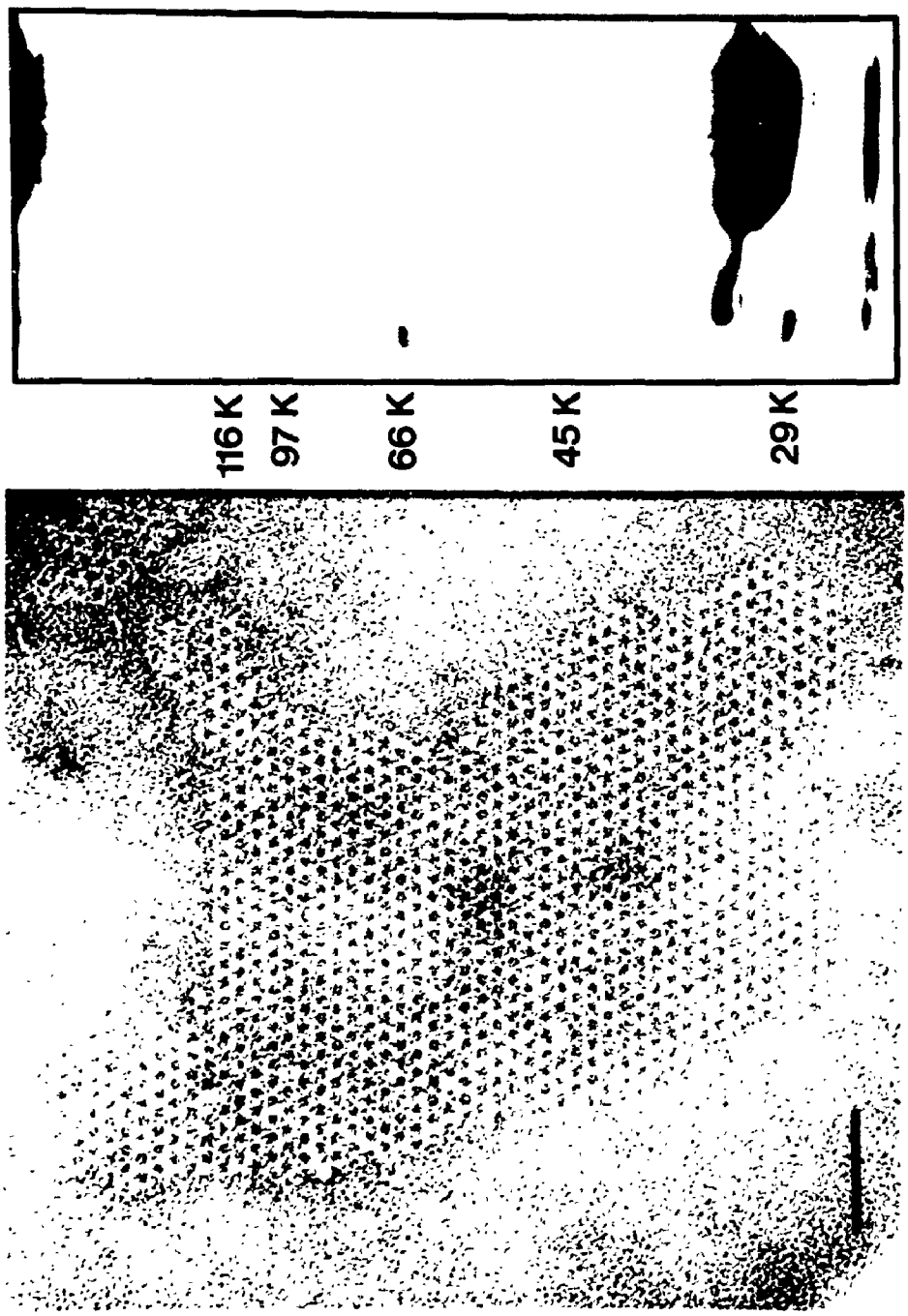

116 K
97 K
66 K
45 K
29 K

Figure 7: Detailed structure of the perforate layer and processed images

a). Electron micrograph of sheets of the perforate layer negatively stained with 1% sodium tungstosilicate.

b). Electron micrograph of freeze-dried and shadowed sheets of the perforate layer.

c). Fourier transform of negatively stained perforate layer. The diffraction spots at Miller indices 5,1 are circled, and indicate resolution of approximately 20 nm.

d). Correlation averaged image of the perforate layer.

Bars = 100 nm

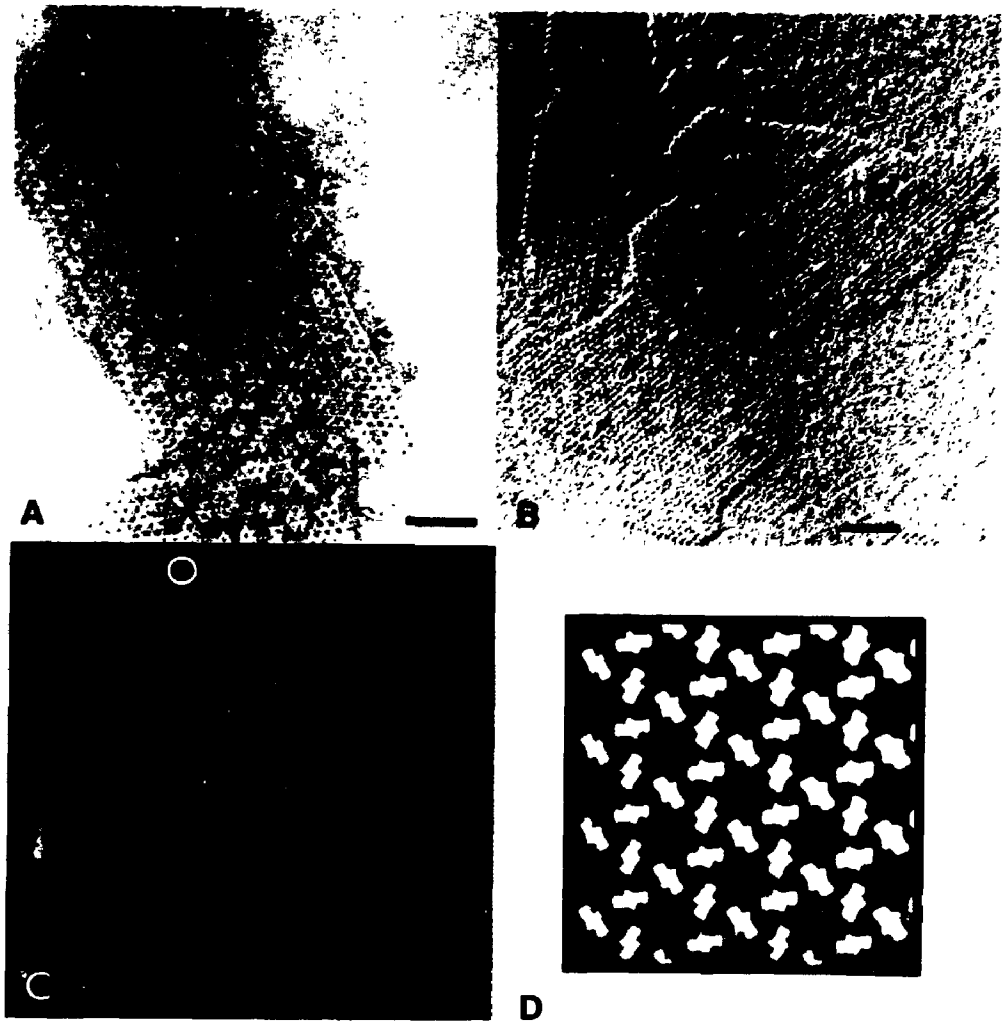

A
B
C
D

Figure 8: pH dissociation of the isolated perforate layer

Solubility of the perforate layer as a function of pH. The degree of solubility of the perforate layer was assayed by the appearance of soluble protein after removal of the intact layer by centrifugation.

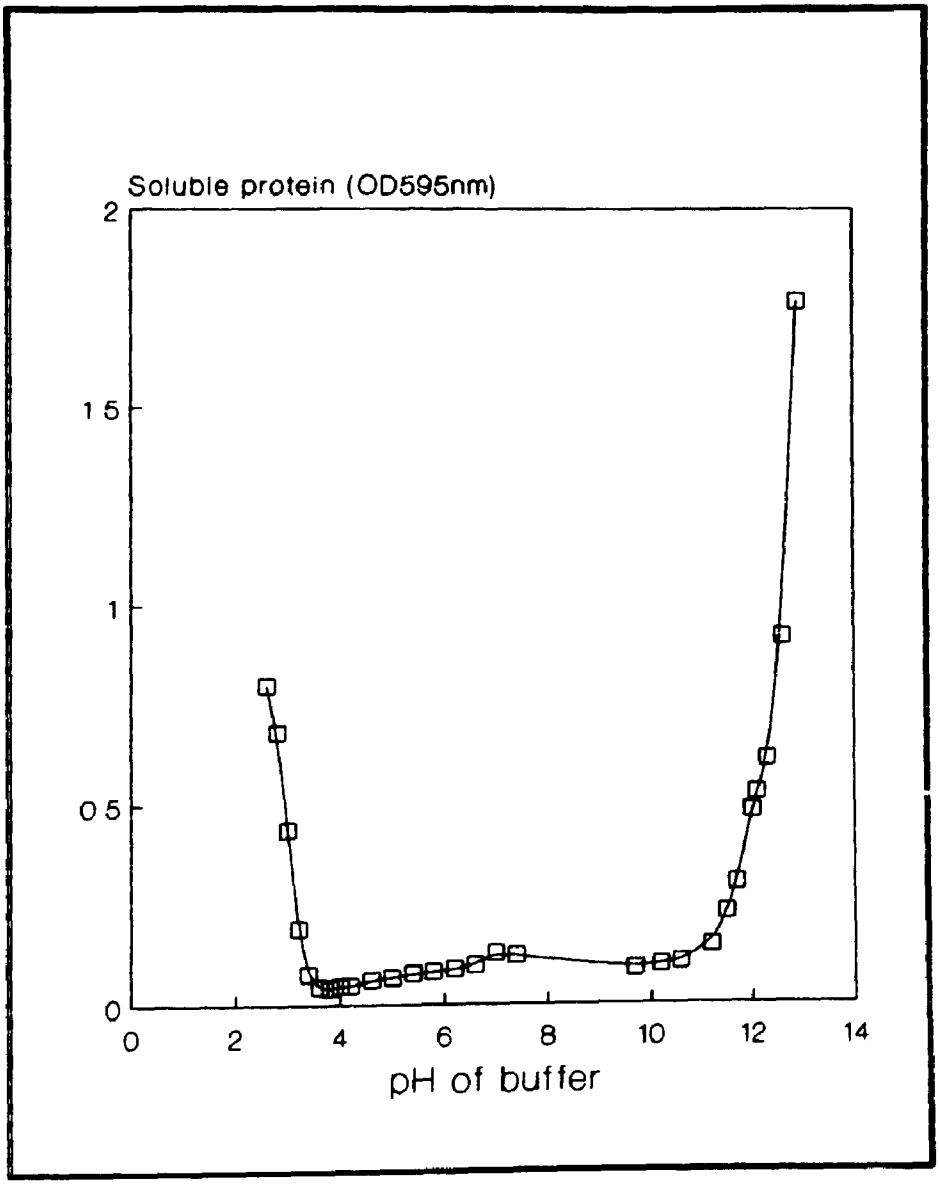

Soluble protein (OD595nm)
2
1 5
1
0 5
0
0
2
4
6
8
10
12
14
pH of buffer

Figure 9: Proteolysis of the assembled and monomeric forms of the 32K protein

Differential degradation of the assembled and monomeric forms of the 32K protein by various proteases.

a). SDS PAGE (5% acrylamide) of the perforate layer after limited digestion of assembled sheets with various proteases. Lane 1, no protease; 2, trypsin; 3, thermolysin; 4, endoproteinase glu-C; 5, papain; 6, proteinase K. Gel was stained with a silver stain.

b). SDS PAGE (15% acrylamide) of partially dissociated perforate layer after digestion with various enzymes. Lane 1, no enzyme; 2, trypsin; 3, thermolysin; 4, endoproteinase glu-C; 5, papain; 6, proteinase K; 7, sialidase; 8, β-glucosidase; 9, molecular weight standards. Gel was stained with Coomassie blue G-250.

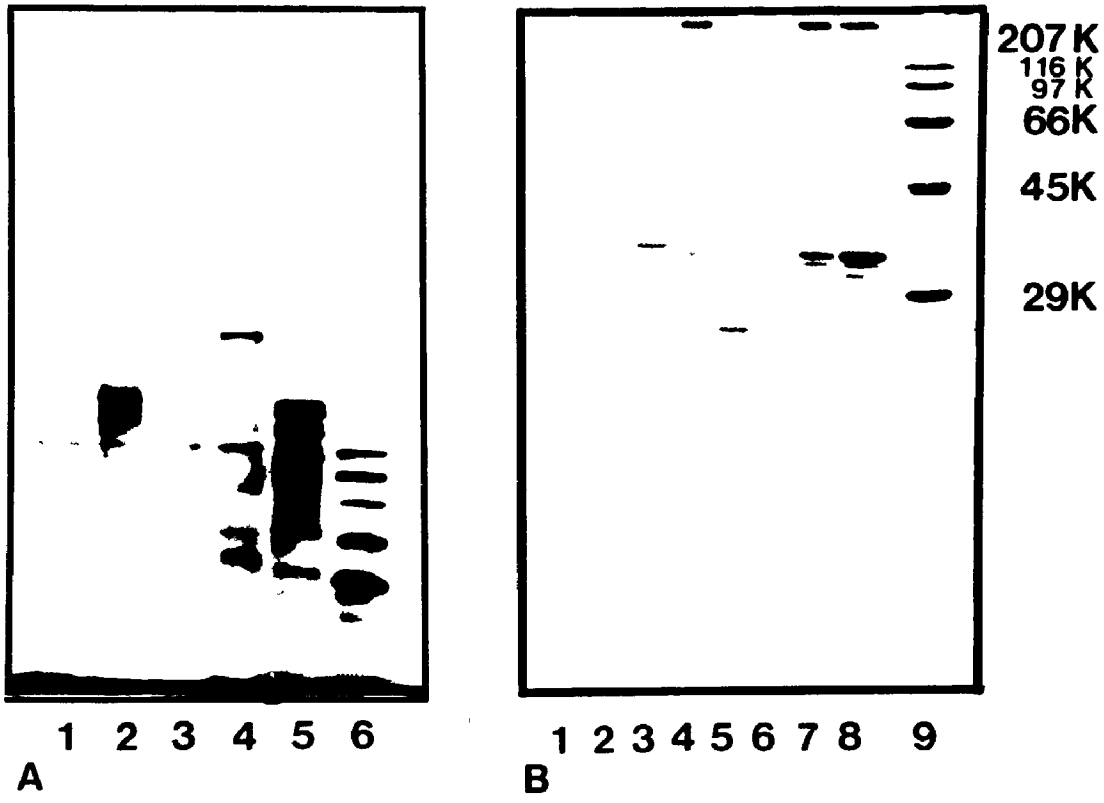

207K
116K
97K
66K
45K
29K
1 2 3 4 5 6
A
1 2 3 4 5 6 7 8 9
B

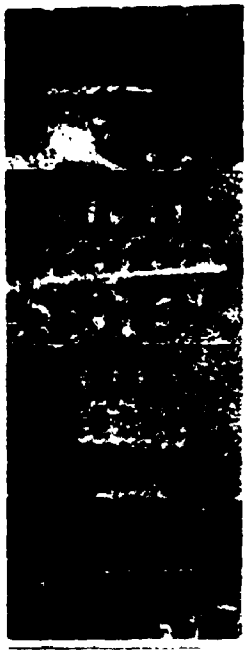
Figure 10: Structure of the punctate layer

a). Electron micrograph of a thin section showing the cell envelope and S-layers.

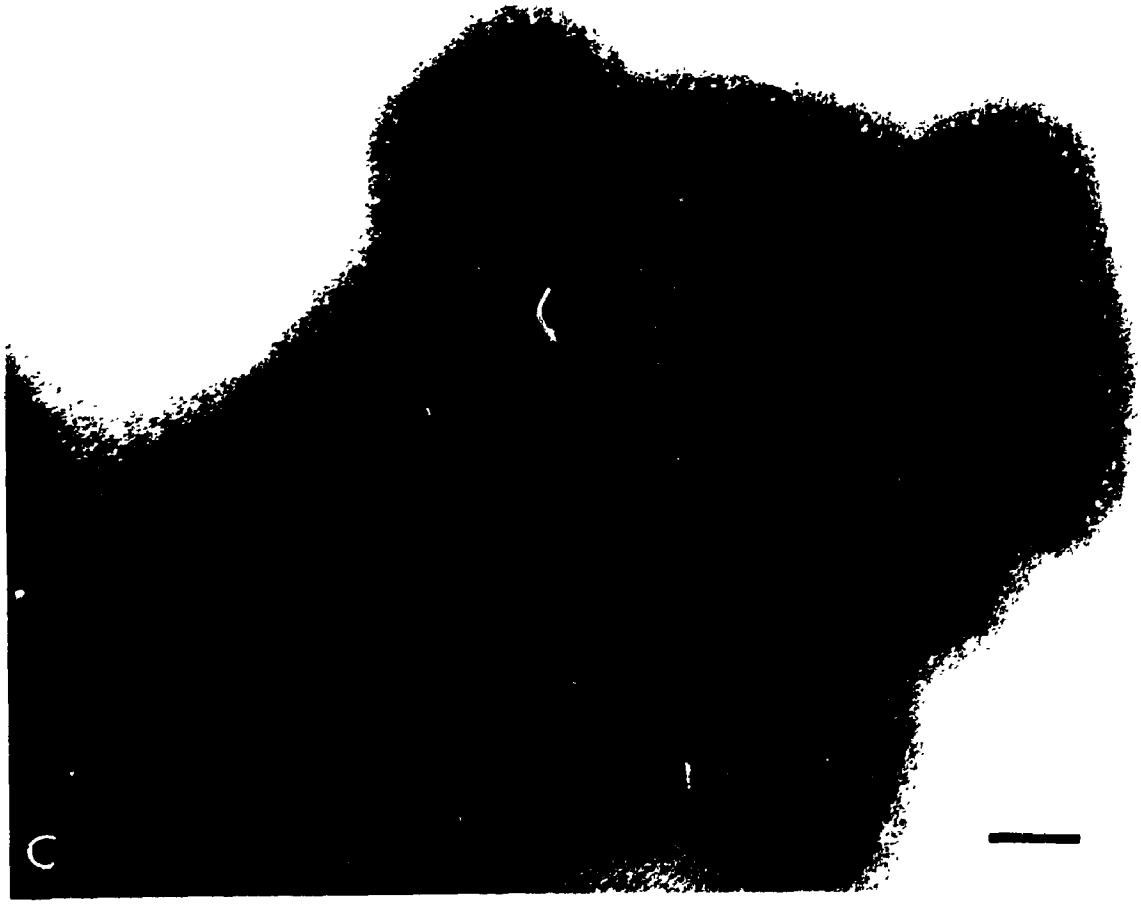
b). Electron micrograph of several side views of the combined punctate and perforate layers in negative stain.

c). Electron micrograph of a negatively stained piece of the composite S-layer.

Bars = 100 nm



B



C

Figure 11: Detailed structure of the composite S-layer and processed images

a). Electron micrograph of the composite S-layer sloughed off the cells and negatively stained with 1% ammonium molybdate and 0.1% glycerol. The perforate layer is obscured by the punctate layer.

b). Electron micrograph of a small piece of the punctate layer without the perforate layer present as a backing layer.

c). Computed diffraction pattern of negatively stained composite layers. The diffraction spots at Miller indices 6,1 are circled, and indicate resolution of approximately 20 nm.

d). Correlation averaged image of the composite perforate and punctate layers after imposing six-fold symmetry.

Bars = 100 nm

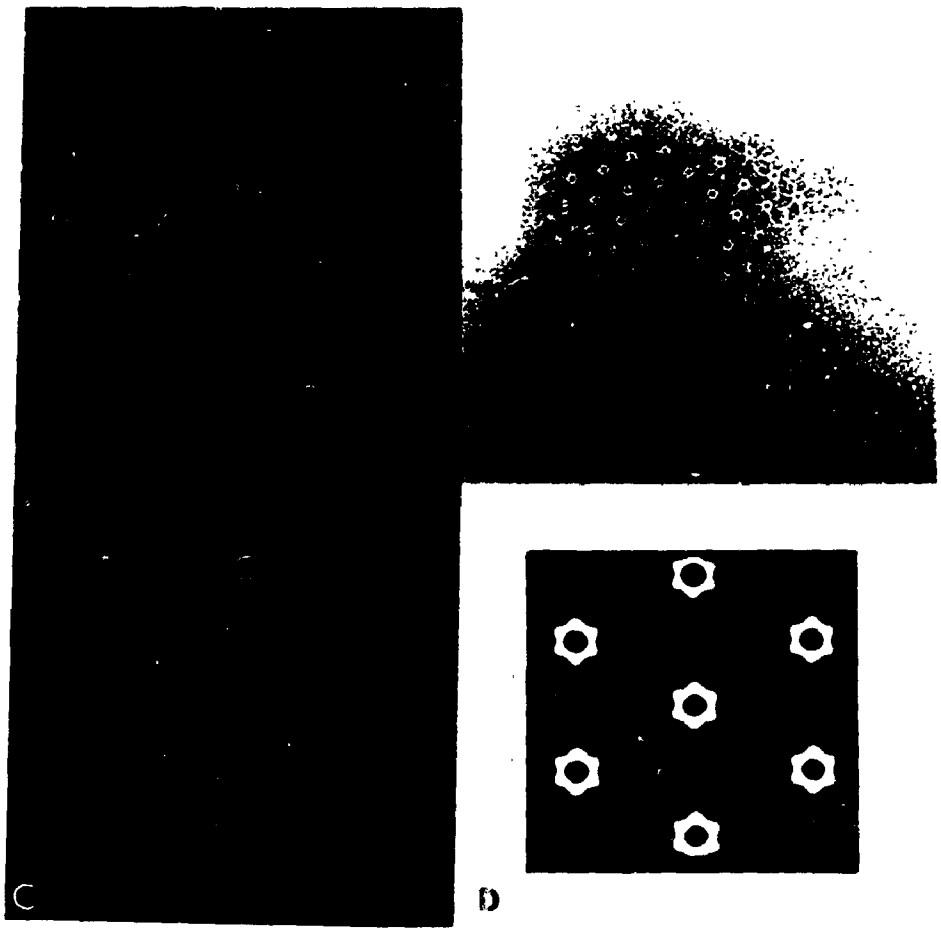

C
D

Figure 12: Relationship between lattices of the perforate and punctate layers

Composite of perforate and punctate layers as viewed in negative stain, showing the relative lattice spacings of the two layers. The punctate layer may be in the process of detaching from the underlying perforate layer. Inset shows a graded composite of an overlay of the punctate layer on the perforate layer.

Bar = 100 nm

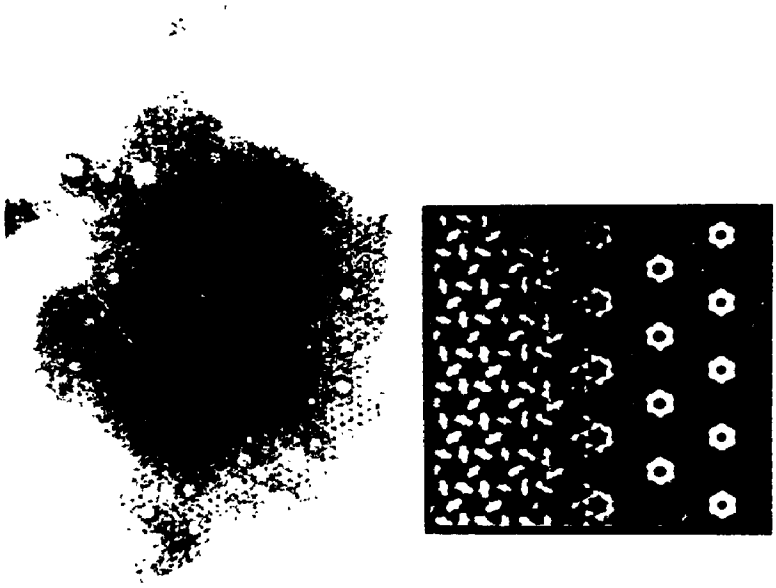


Figure 13: Effect of EDTA and EGTA on the structure of the punctate layer

Cell envelopes were incubated with EDTA and EGTA, then negatively stained and examined by electron microscopy.

a). 100 mM Tris pH 8.0 containing 10mM EDTA.

b). 100 mM Tris pH 8.0 containing 10mM EGTA.

c). 100 mM Tris pH 8.0.

Bars = 100 nm

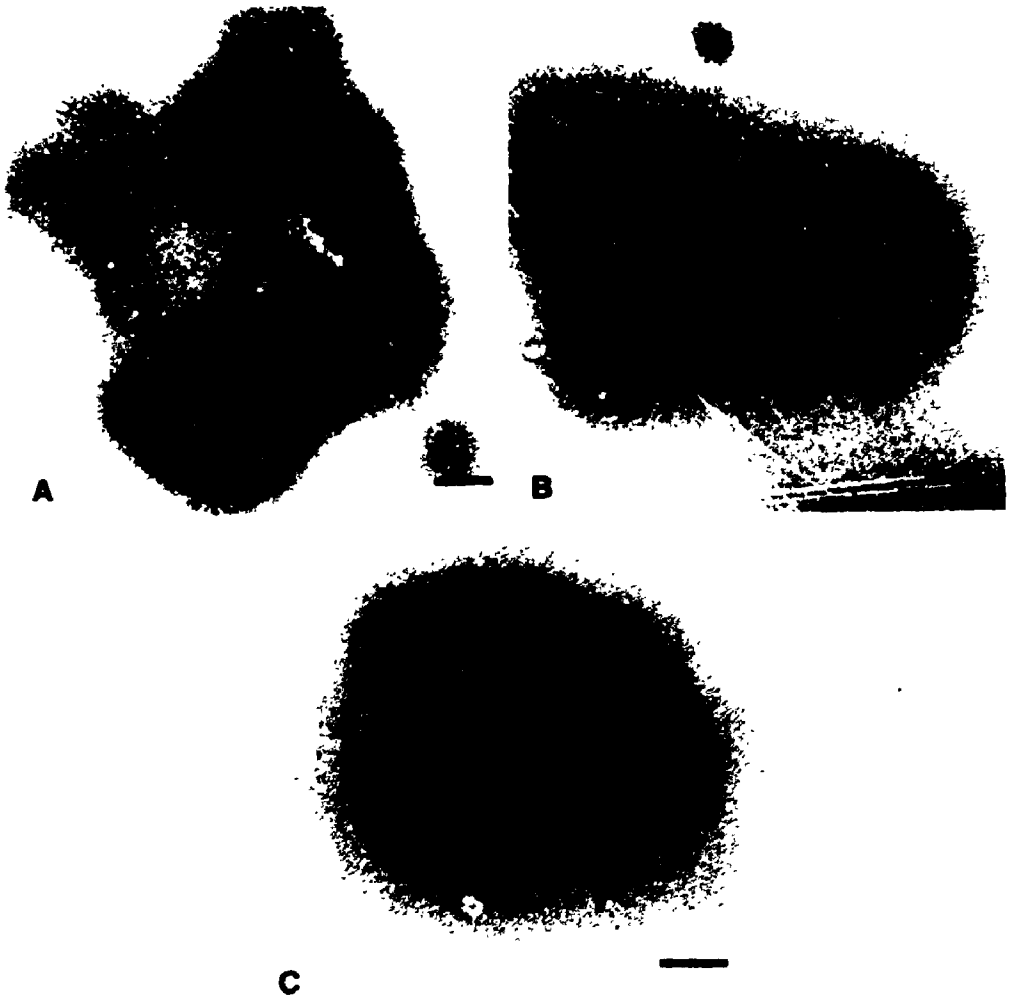
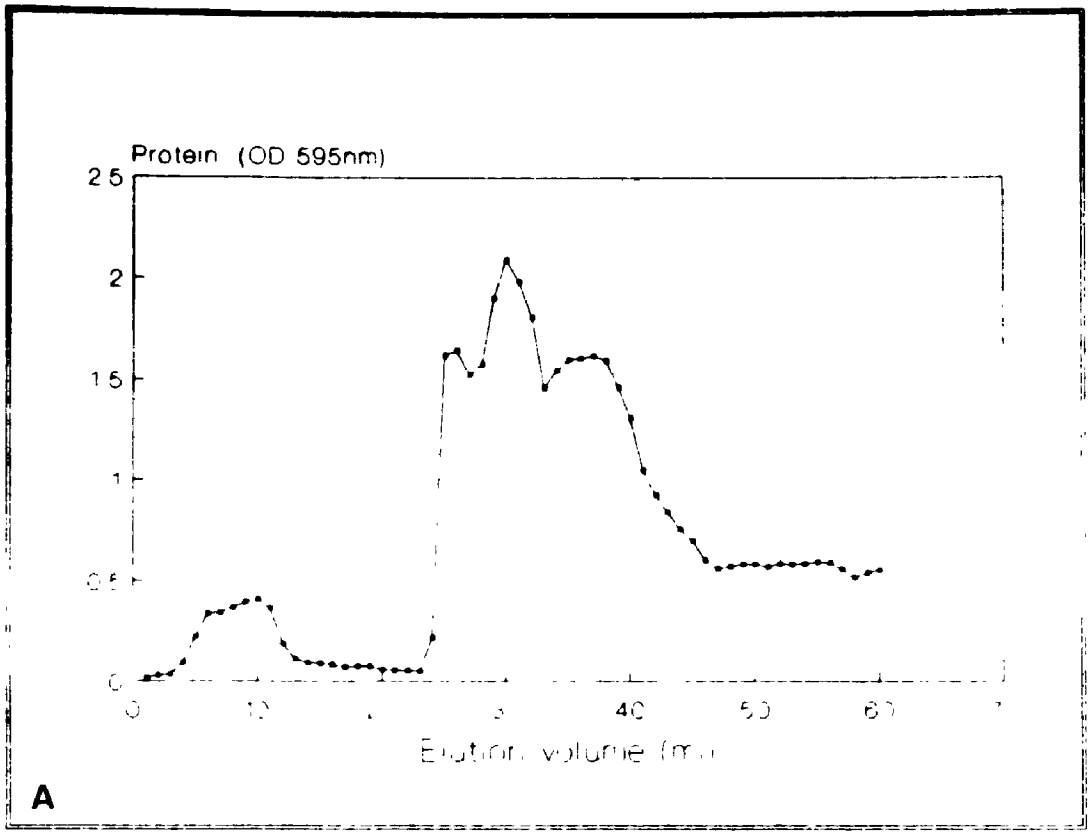

A
B
C

Figure 14: Separation of the soluble components of the punctate layer by column chromatography on hydroxylapatite.

a). column elution profile. Protein was detected with the Coomassie blue dye binding assay as described in the Materials and Methods section.

b). SDS PAGE of the fractions containing protein. Lanes: 1, sample applied to column; 2 and 3, fractions 10 and 11; lanes 4 to 20, fractions 25 to 40. Gel was stained with Coomassie blue G-250.


Protein (OD 595nm)
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60
Elution volume (ml)
A

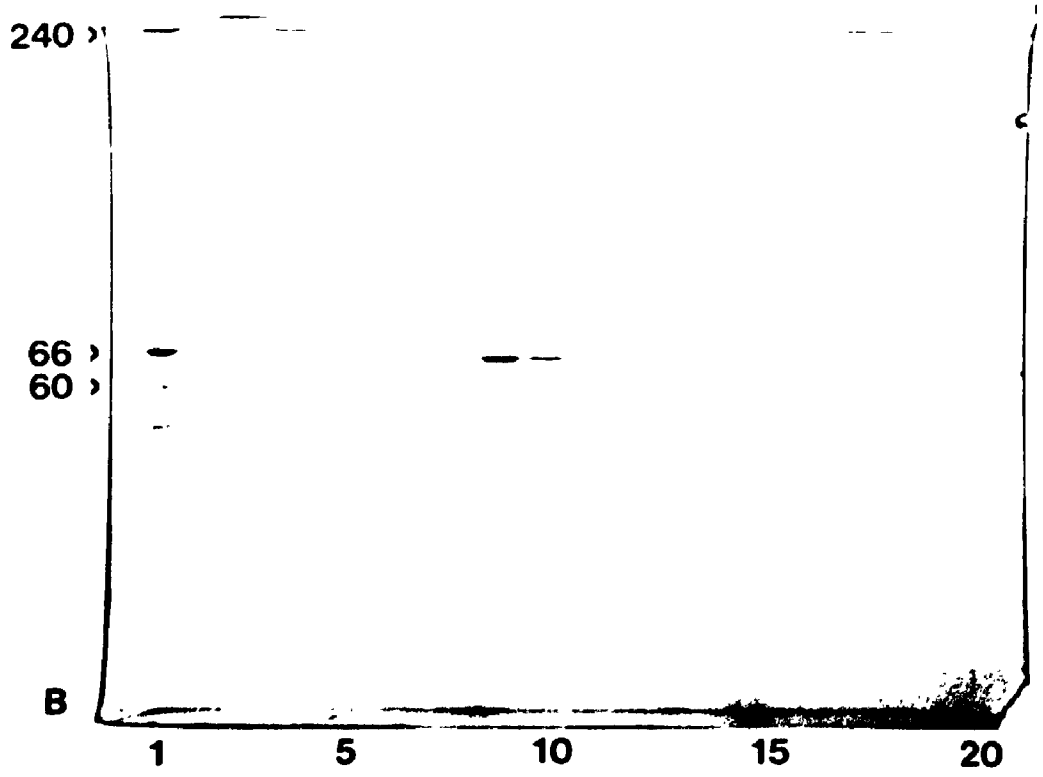

240 »
66 »
60 »
B
1
5
10
15
20

Figure 15: Structure of the isolated components of the punctate layer

Fractions obtained from hydroxylapatite column chromatography were negatively stained and examined by electron microscopy.

a). Isolated 240K showing the slender, curved protein molecules and assemblies of the 240K protein. Single molecules are visible in the background stain.

b). Isolated 60K.

c). Isolated 66K visible only as an amorphous background on the grid.

d). Mixture of 240K and 60K assembled to form structures resembling the native punctate layer.

Bars = 100 nm

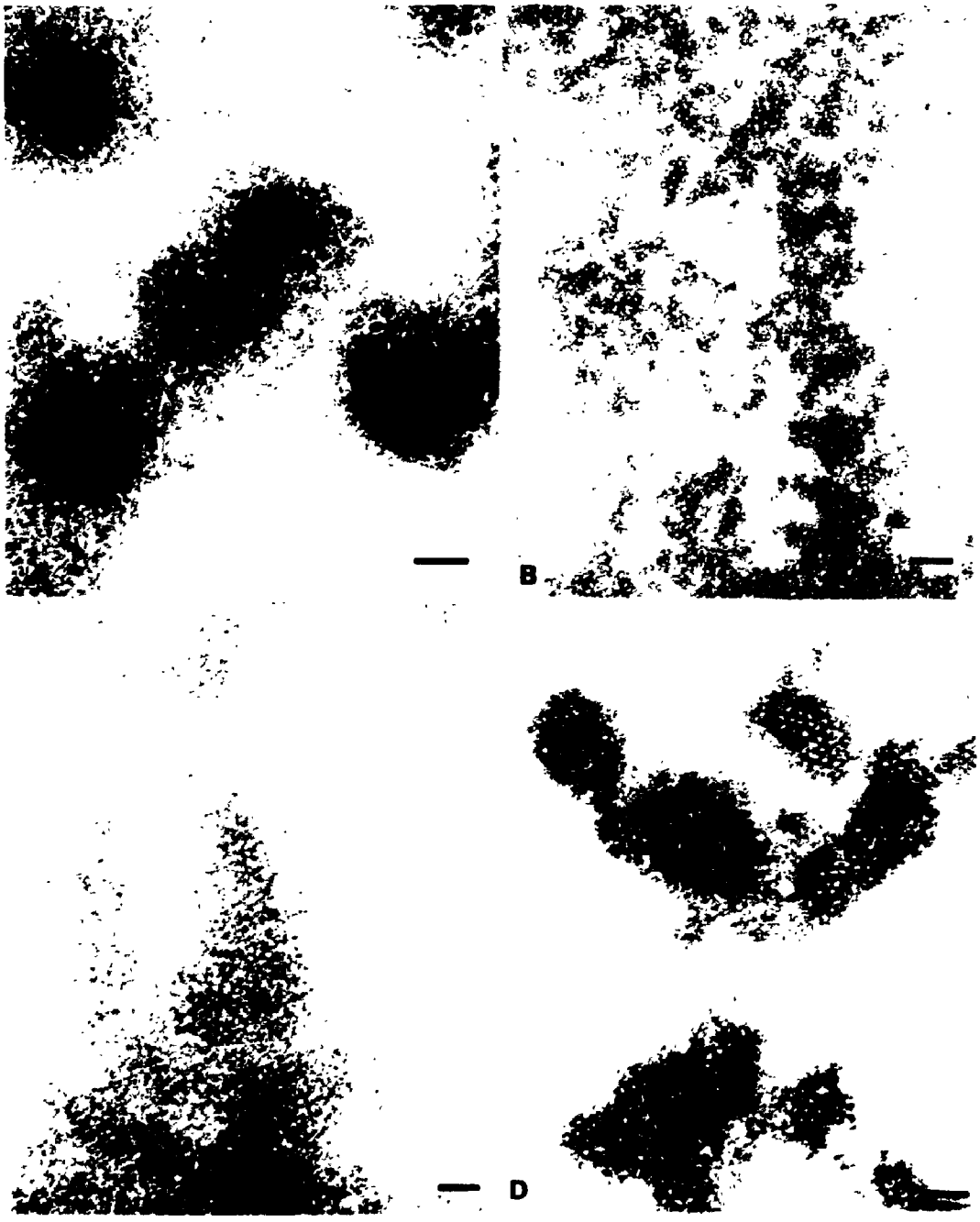
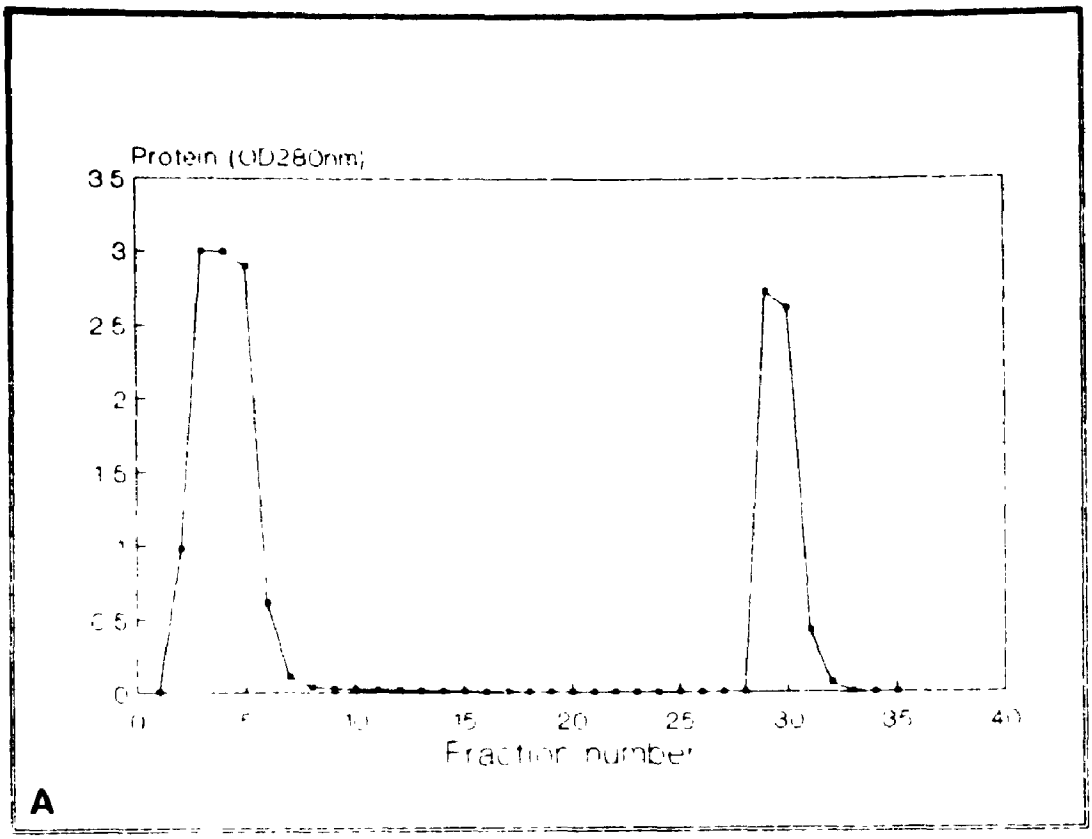

B
D

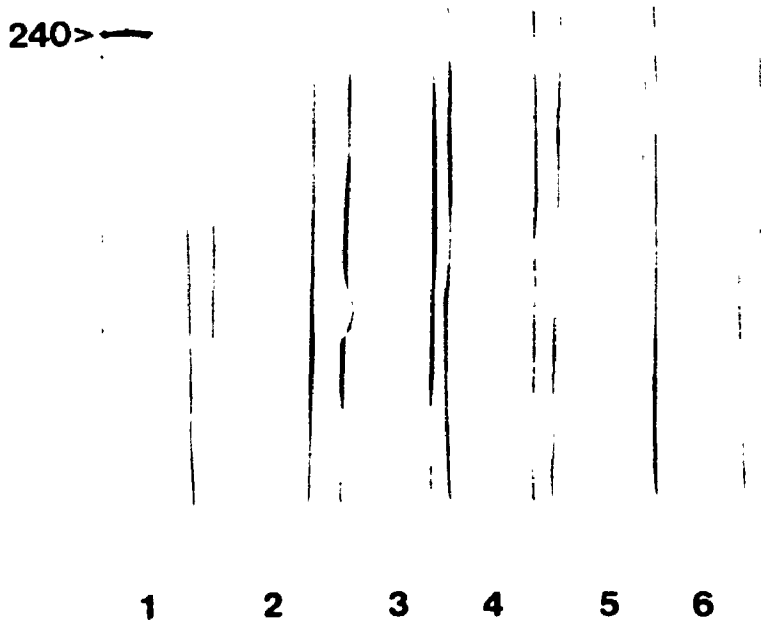
Figure 16: Isolation of IgG from serum and detection of 240K with western blots

a). A typical protein A column profile for IgG purification from whole rabbit serum.

b). Western blot of cell envelopes preparation. The 240K polypeptide was detected with anti-240K IgG. Antibodies to the 60K polypeptide did not detect the 60K polypeptide transferred onto nitrocellulose by Western blotting. Lane 1, 1:200 dilution of anti-240K IgG; lane 2, 1:400 dilution of anti-240K; lane 3, 1:800 dilution of anti-240K; lane 4, 1:1000 dilution of anti-240K; lane 5, 1:50 dilution of anti-60K IgG; lane 6, 1:100 dilution of anti-60K.


Protein (OD280nm)
3.5
3
2.5
2
1.5
0.5
0
0
5
10
15
20
25
30
35
40
Fraction number

A


240>

1 2 3 4 5 6

B

Figure 17: Immunolabel electron microscopy of the punctate layer

The punctate layer was labeled with antibodies (IgG) to both the 60K and 240K polypeptides. The IgG was then detected with protein A complexed to 5 nm colloidal gold particles.

a). anti-60K negative stain.

b). anti-240K negative stain.

c). anti-60K thin section.

d). anti-240K thin section.


A
B
D

Figure 18: Reassembly of the punctate layer using the perforate layer as a template

The punctate layer was made soluble in 3M guanidine HCl, added to the intact perforate layer and the mixture was dialysed against various buffers. Preparations were checked by electron microscopy after negative staining. Complete reassembly occurred only in buffer containing $CaCl_2$ or $SrCl_2$. Addition of EDTA inhibited reassembly.

a). 50 mM HEPES with addition of 10mM $CaCl_2$.

b). 50 mM HEPES with addition of 10mM $SrCl_2$.

c). 50 mM HEPES without addition of divalent cations. Note the assembly of small patches of the punctate layer (pu) on the perforate layer (pe).

d). 50 mM HEPES with addition of 10mM EDTA.

Bars = 100 nm

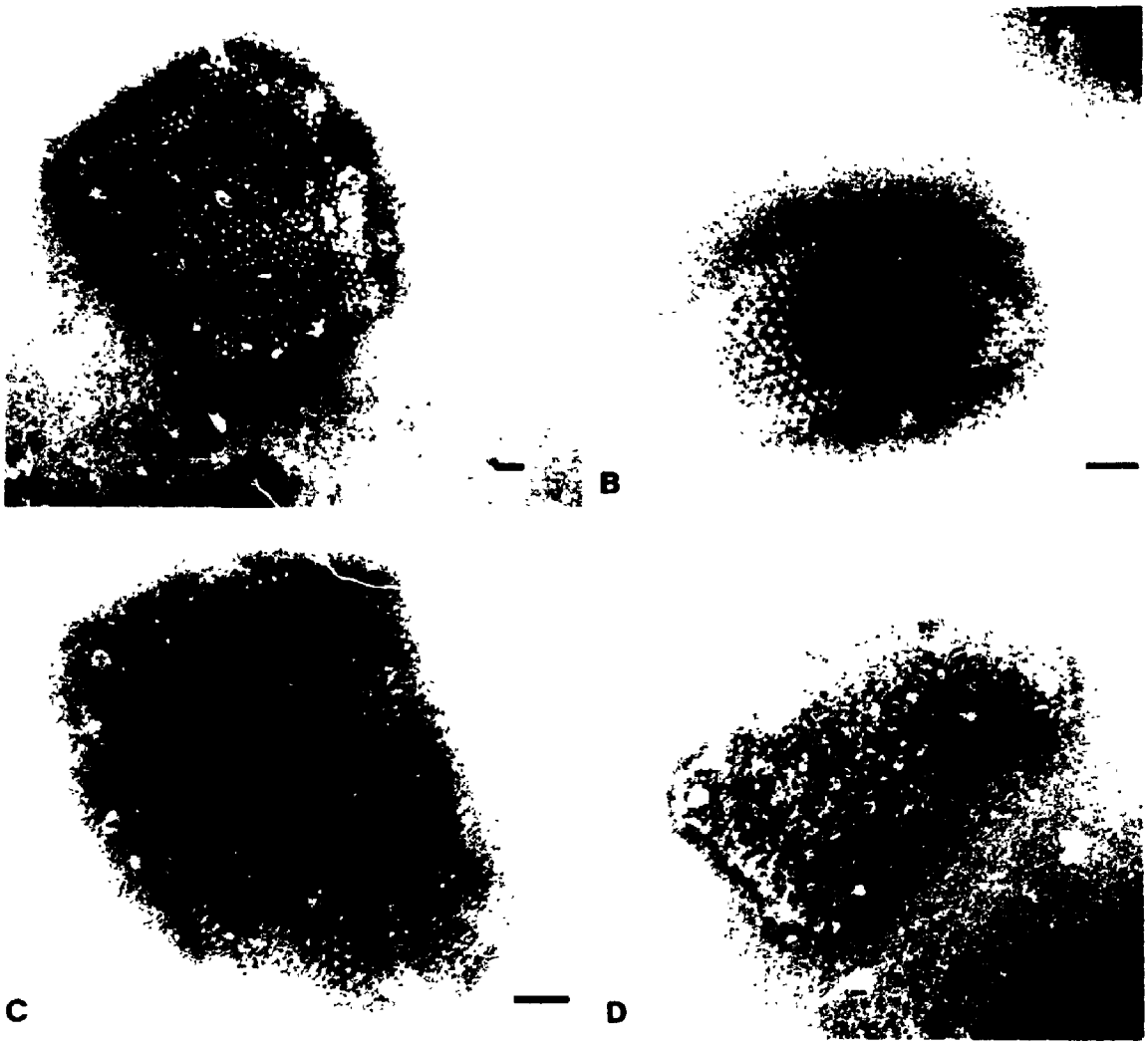

B
C
D

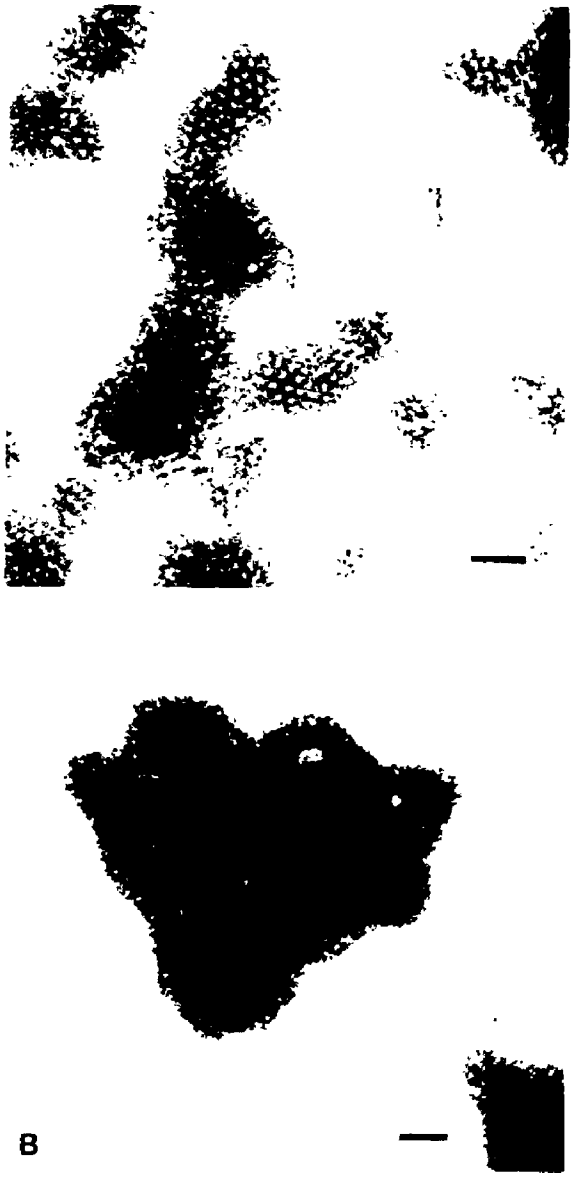
Figure 19: Reassembly of the punctate layer without a template

The punctate layer was made soluble in 3M guanidine HCl, removed from the insoluble perforate layer by centrifugation, and dialysed against the following buffers.

a). 50 mM HEPES with addition of 10mM $CaCl_2$

b). 50 mM HEPES with addition of 150mM NaCl and 10mM $CaCl_2$

Bars = 100 nm



B

Figure 20: Effect of EDTA and EGTA on pre-formed assemblies of the punctate layer

The reassemblies were formed by dialysis of guanidine HCl soluble punctate layer against 50 mM HEPES pH 7.5 containing 150 mM NaCl and 10 mM $CaCl_2$. Aliquots of the reassemblies were then incubated for 15 minutes in the following solutions and examined by electron microscopy after negative staining.

a). 50 mM Tris pH 8.0 containing 10 mM EDTA

b). 50 mM Tris pH 8.0 containing 10 mM EGTA

c). 50 mM Tris pH 8.0

d). 50 mM HEPES pH 7.5 containing 150 mM NaCl and 10 mM $CaCl_2$

Bars = 100 nm

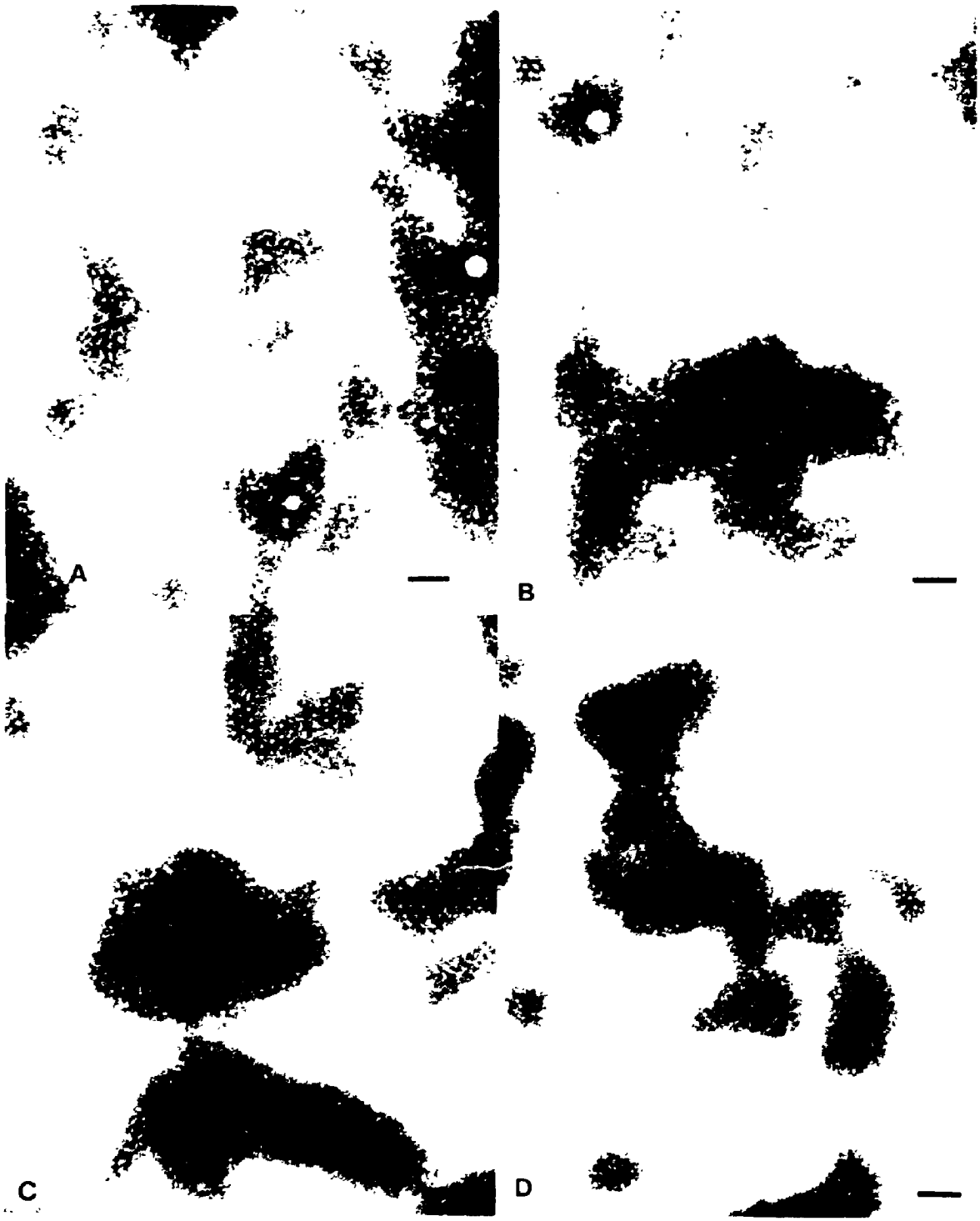

A
B
C
D

Figure 21: Effect of $CaCl_2$ and the perforate layer on the protein composition of reassemblies of the punctate layer

Sheets of the perforate layer were isolated by SDS extraction of cell envelopes. The soluble components of the punctate layer were obtained by extraction of DOC-extracted envelopes with 6 M urea. A suspension of perforate layer was added to the soluble punctate layer and the mixture was dialysed overnight at 4°C against 50 mM HEPES pH 7.5 or 50 mM HEPES pH 7.5 containing 10 mM $CaCl_2$. The same amount of soluble punctate layer was added to HEPES buffer pH 7.5 and dialysed under the same conditions. The samples were then removed from the dialysis tubing, centrifuged (15,000 *g* for 5 min) to remove reassemblies and material adhering to the perforate layer, and these pellets were analyzed by SDS PAGE. Gel was stained with Coomassie blue G-250.

a). HEPES.

b). HEPES + $CaCl_2$.

c). HEPES + perforate layer.

d). HEPES + $CaCl_2$ + perforate layer.

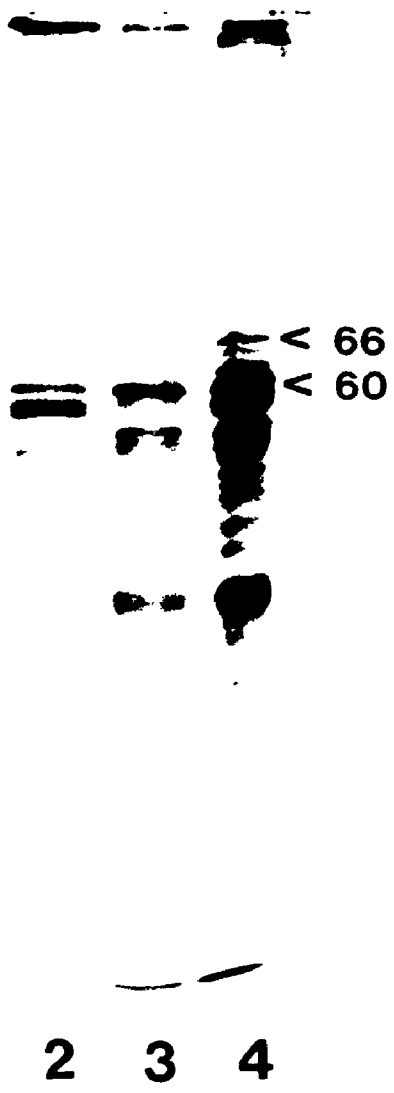

< 66
< 60
1
2
3
4

Figure 22: Binding of cationic dyes to the punctate layer

a). Thin section electron micrograph of cells fixed with glutaraldehyde and osmium tetroxide with the inclusion of 0.05% ruthenium red. Inset shows how ruthenium red enhanced the staining of the centres of of the units of the punctate layer. Note also how the punctate layer has assembled onto both sides of a single sheet of the perforate layer.

b). Thin section electron micrograph of cells fixed in glutaraldehyde containing 1% Alcian blue. Alcian blue enhanced the staining of the punctate layer and demonstrated amorphous material external to the cell envelope.

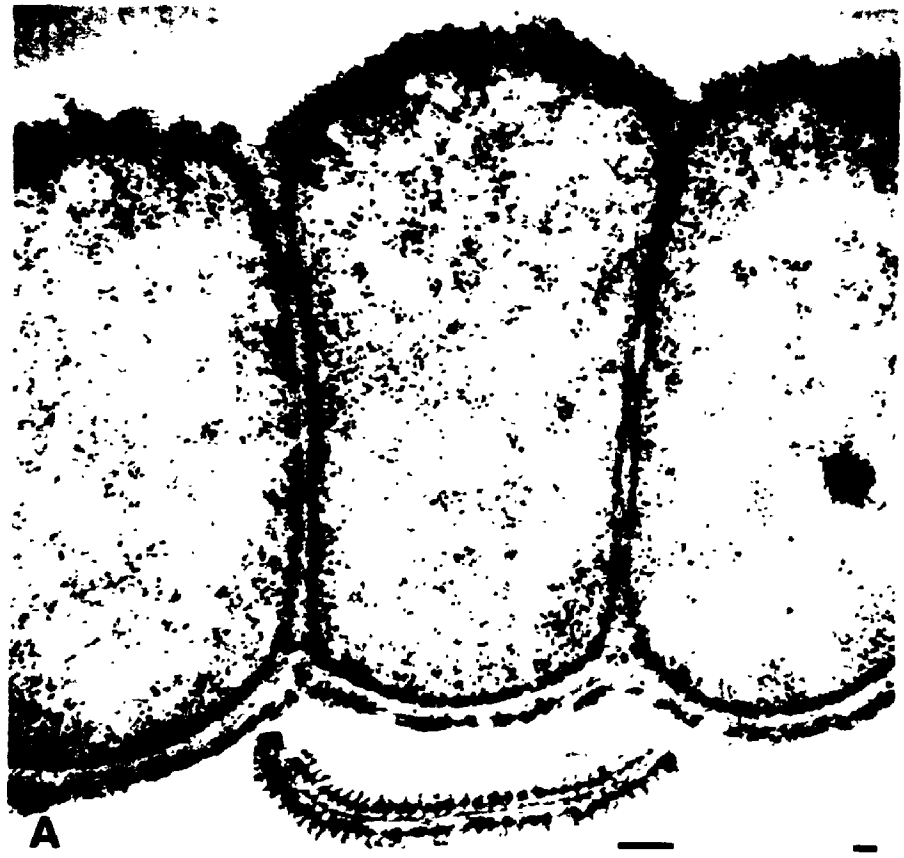

A

Figure 23: Detection of the 240K polypeptide with various glycoprotein stains

Cell envelopes were directly made soluble in SDS PAGE sample buffer and envelope proteins were separated by SDS PAGE. The separated proteins were then stained with various glycoprotein stains and with Coomassie blue. Lane 1, thymol-H_2SO_4; 2, Alcian blue; 3, Stains-all; 4, Coomassie blue.

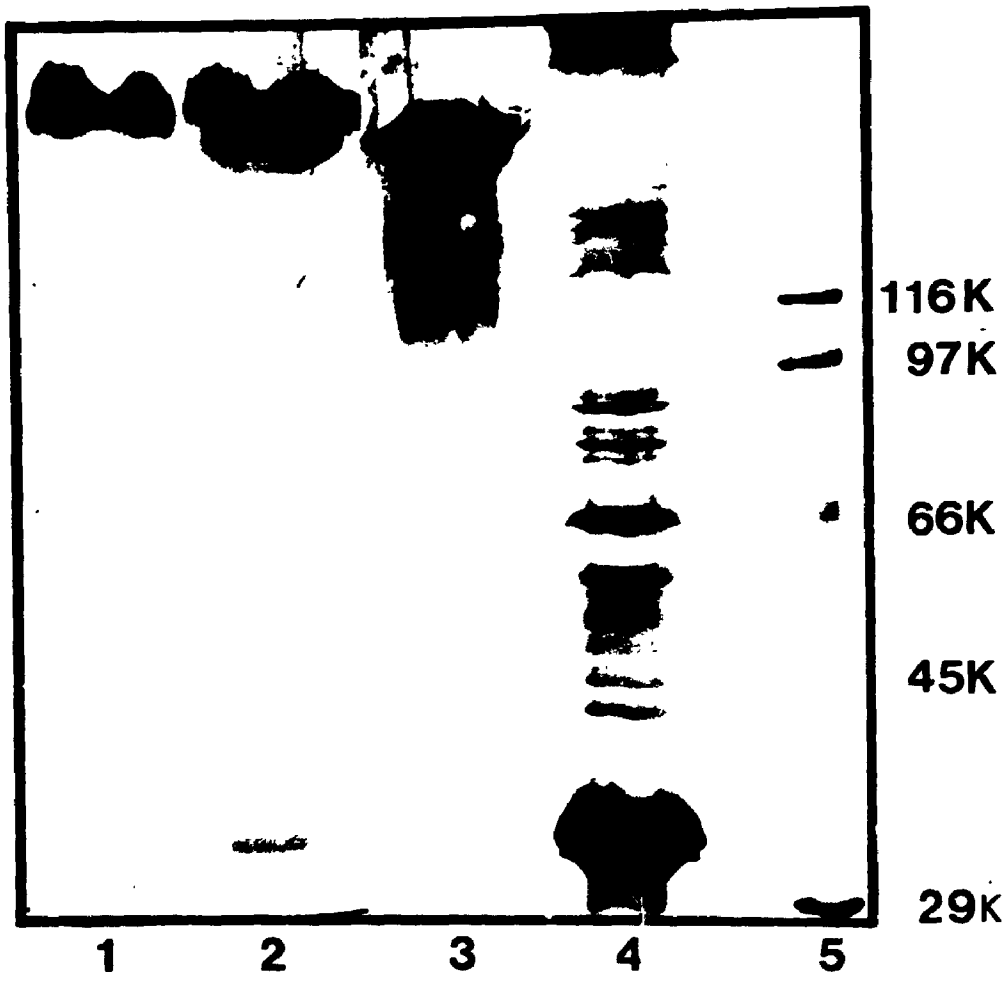

116K
97K
66K
45K
29K
1
2
3
4
5

Figure 24: Periodic acid Schiff's base reaction of cell envelopes and the 240K polypeptide

Thin section electron micrograph of cells labelled with silver methenamine after oxidation of sugars with periodic acid. Silver grains have developed over the outer membrane, perforate layer and punctate layer.

Cell envelope proteins were separated by SDS PAGE and stained with Coomassie blue and periodic acid-Schiff's (PAS) stain to detect carbohydrate. The 240K polypeptide was strongly PAS positive (arrow) but did not stain with Coomassie blue.

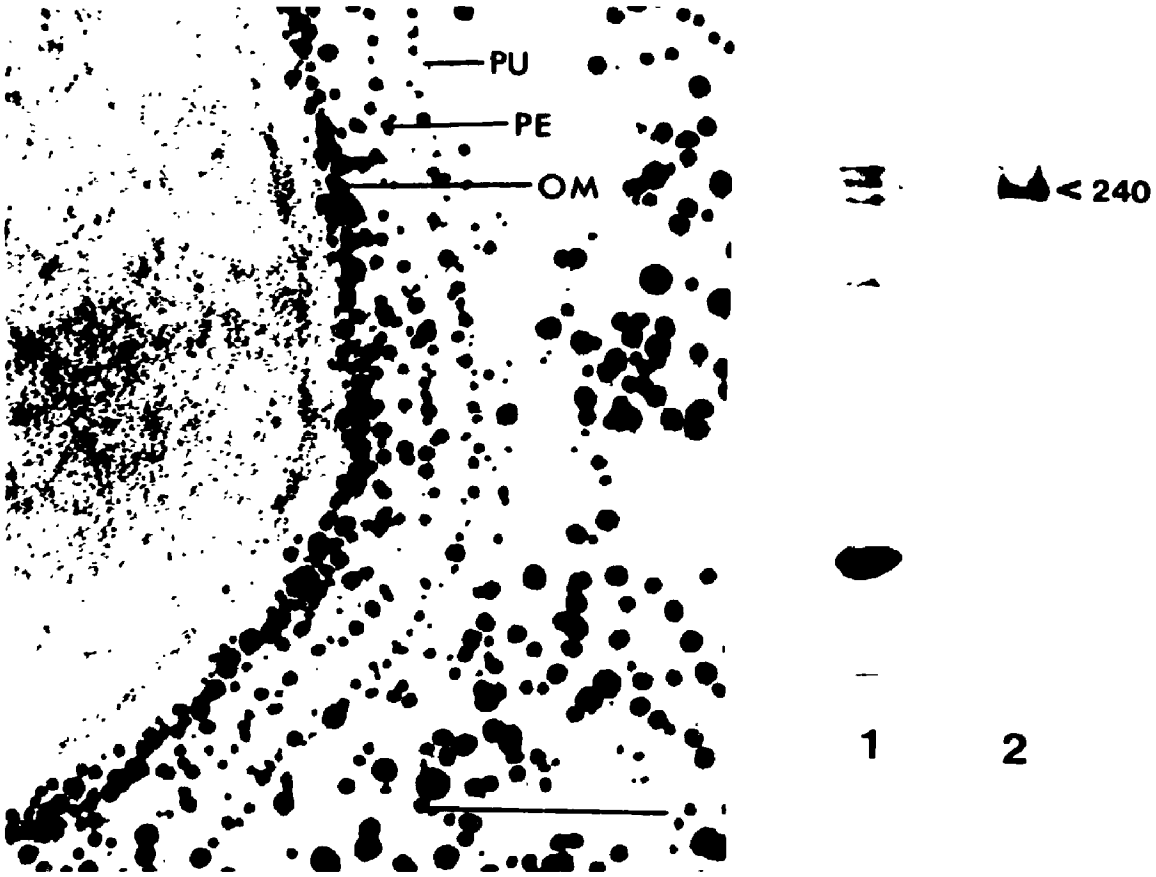
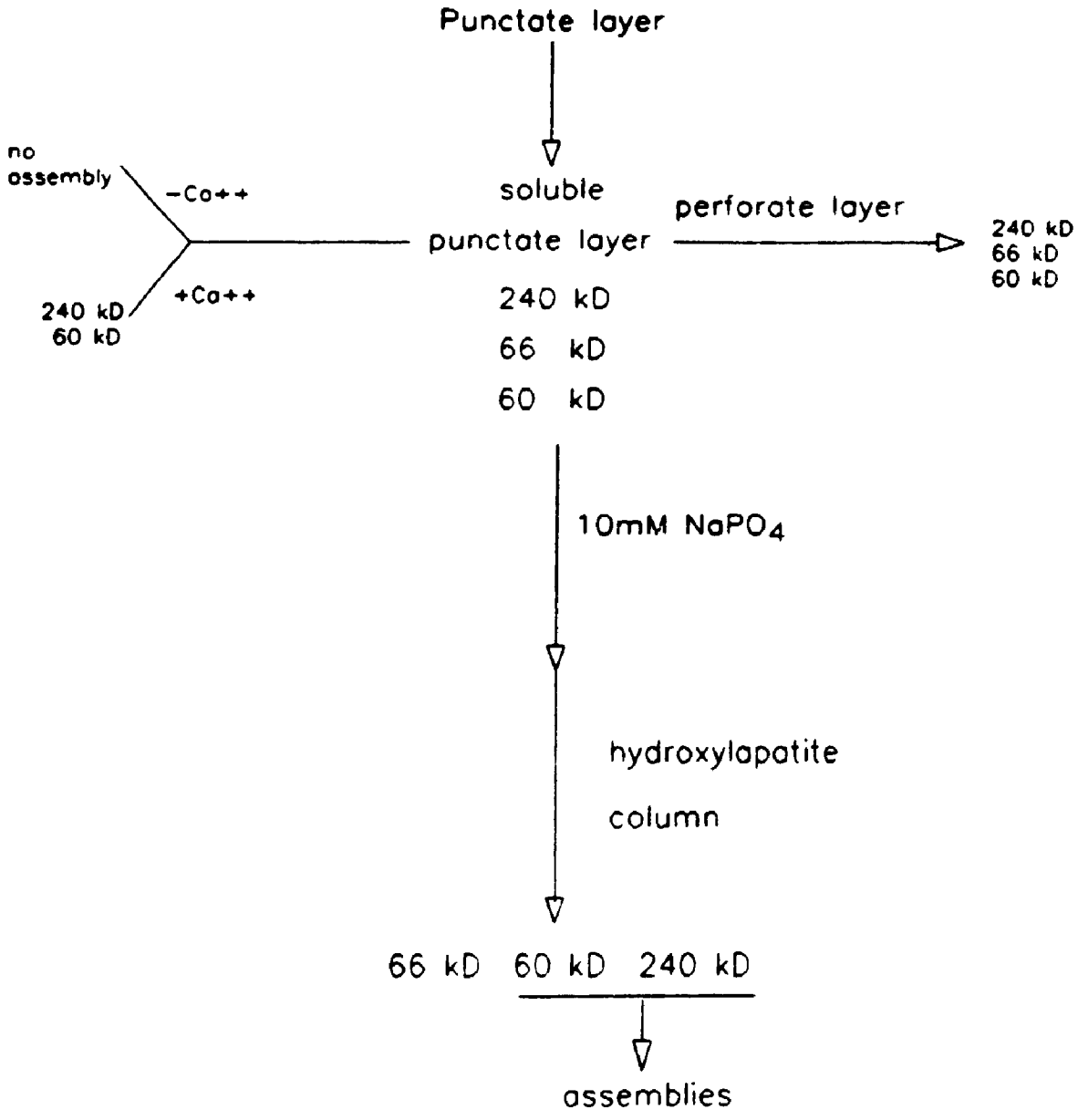

PU
PE
OM
< 240
1
2

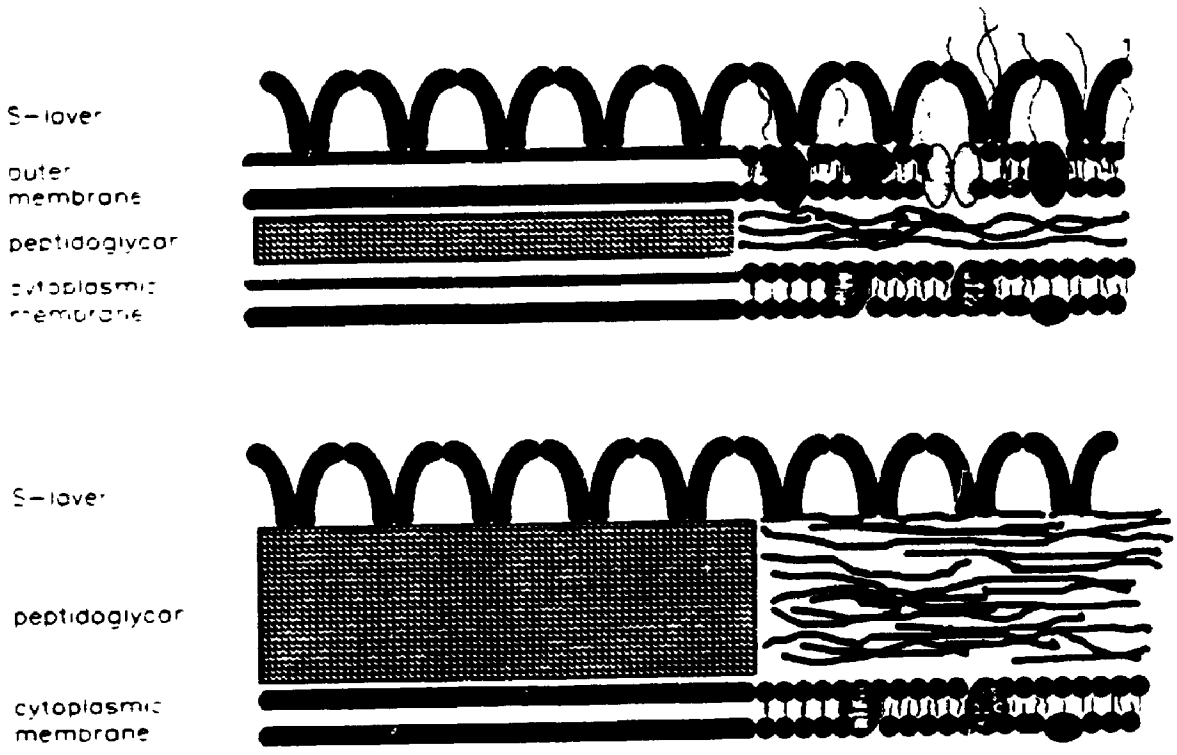
Figure 25: Summary of the isolation of the components of the punctate layer and their reassembly


Punctate layer
soluble
punctate layer
240 kD
66 kD
60 kD
no
assembly
-Ca++
+Ca++
240 kD
60 kD
perforate layer
240 kD
66 kD
60 kD
10mM NaPO4
hydroxylapatite
column
66 kD
60 kD
240 kD
assemblies

Appendix A

Architecture of the gram negative and gram positive cell envelopes

Schematic drawing of gram negative (top) and gram positive (bottom) cell envelopes.


S-layer
outer membrane
peptidoglycan
cytoplasmic membrane
S-layer
peptidoglycan
cytoplasmic membrane
S-layer protein
Membrane protein
Phospholipid
Porin complex
Lipopolysaccharide
Peptidoglycan

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