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Examination of the cell envelope of a Spina-producing marine pseudomonad

by

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ABSTRACT

Marine pseudomonad D71 (MPD71) (NCMB 2018; McGregor-Shaw et al., 1973) was grown in conditions favourable or unfavourable for the production of appendages termed spinae (McGregor-Shaw et al., 1973). Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and immunoblot analyses revealed a 42 kilodalton (kD) protein only in the outer membrane (OM) of spined cells. This protein was concluded to be the spina protomer (spinin; Easterbrook and Coombs, 1976). Spined, unspined, and "despined" OMs contained immunodetectable 65kD and 21-24kD ("low MWr") proteins using hyperimmune antispinin antiserum absorbed of cross-reactive antibodies using Escherichia coli K12 and Pseudomonas aeruginosa PAO envelope. Further absorption with unspined cells eliminated the 65kD species from immunoblots. The relatedness of the 65kD and "low MWr" species to spinin was not resolved.

Envelope perturbants affected spination. However, the action of procaine and phenethyl alcohol was different than that of carbonyl cyanide m-chlorophenyl hydrazone (CCCP). The inhibition of spination by CCCP was coincident with the immunological detecti of a protein slightly higher in MWr (2kD) than spinin.

Finally, SDS-PAGE comparison of the proteinase K insoluble material from unspined and spined samples indicated a rough type of structure for the MPD71 LPS.

SYMBOLS AND ABBREVIATIONS

M.....molar

mM.....millimolar

uM.....micromolar

L.....litre

mL.....millilitre

uL.....microlitre

m.....metre

cm.....centimetre

mm.....millimetre

um.....micrometre

nm.....nanometre

A.....Angstrom

g.....gram

mg.....milligram

ug.....microgram

kD.....kilodalton

w/w.....weight/weight

w/v.....weight/volume

v/v.....volume/volume

sec.....seconds

min.....minutes

h.....hours

°C.....degrees Centrigrade

p.....density

pH.....$\log_{10}$ of the inverse hydrogen ion concentration

pI.....isoelectric point

MWr.....relative molecular weight

%.....percent

SDS.....sodium dodecyl sulfate

OM.....outer membrane

CM.....cytoplasmic membrane

PG.....peptidoglycan

m-Dpm.....meso-diaminopimelic acid

LPS.....lipopolysaccharide

CCCF.....Carbonyl cyanide m-chlorophenyl hydrazone

PEA.....Phenethyl alcohol

ß .....beta

α .....alpha

ε .....epsilon

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INTRODUCTION

Aims of this study

The focus of this study is the marine pseudomonad MPD71 (NCMB 2018; McGregor-Shaw et al., 1973). MPD71 populations whose members lack or possess proteinaceous appendages termed spinae (McGregor-Shaw et al., 1973) can be generated (Easterbrook and Sperker, 1982). The structure of spina and its protomer (spinin; Easterbrook and Coombs, 1976) have been intensively investigated. However, the response of the cell envelope to spina-permissive conditions has not been investigated. It is the cell response which was investigated in the present study. Specifically, three areas were examined: 1) alterations of the cell envelope during the unspined to spined morphological transition, 2) the nature of the unspined and spined states of the cell, and 3) the trans-envelope passage of spinin.

To undertake these investigations, knowledge of the MPD71 spina protomer, the structure of the bacterial cell envelope, and protein secretory mechanisms is essential. A large body of evidence has been gathered on spina and the spinin protomer; a summary of pertinent information is presented in the first section of the Introduction. In contrast, information concerning the structure of the MPD71 envelope is contemporous with this thesis. Therefore, a detailed consideration of envelope structure (presented in

the Introduction) must rely on other Gram-negative bacteria.

Finally, the past decade has witnessed an explosion of research activity on the mechanism(s) of protein secretion. In the Introduction, I have attempted to summarize those features of the secretory process which have proven useful in examining the mechanism of the trans-envelope passage of spinin.

Spinae - Definition and Structure

Spinae (s. spina) are bacterial appendages. As defined by Easterbrook, spina are "pericellular, nonprosthecate, rigid, tubular, appendages that are produced by certain Gram-negative bacteria" (Easterbrook et al., 1976). A nonprosthecate appendage is not bounded by an extension of the cell boundary layers (Staley, 1968; Stefanov and Nikitin, 1965).

Reports describing similar-appearing appendages on in situ samples have included fresh water (Leifson, 1960; Staley, 1968), soil (Stefanov and Nikitin, 1965), and marine organisms (Haubold, 1978; Moll and Ahrens, 1970; Suzina and Fikhte, 1977; McGregor-Shaw et al., 1973; Perkins et al., 1981). Such a geographic and metabolic diversity of spined bacteria argues for their general, widespread natural occurence.

The most intensively studied spina to date is that produced by the marine pseudomonad designated by

McGregor-Shaw et al. (1973) as MPD71. MPD71 spinae are constructed of a shaft, 65 nm in diameter, surmounting a coniform base with a maximum diameter of 120 nm (Easterbrook et al., 1973). Striations, 11.75 nm in width with a low helical pitch relative to the short axis of the shaft (Easterbrook and Coombs, 1976; Easterbrook et al., 1976) are apparent along the entire structure (Easterbrook et al., 1973, 1976; Willison et al., 1977). The striations have a "beaded" appearance (Easterbrook et al., 1976), with a centre-to-centre spacing along a row of 5.6 nm and a spacing between rows of approximately 12 nm (i.e., the width of a striation) (Easterbrook et al., 1976). Each "bead" is proposed (Easterbrook et al., 1976) to represent an oligomer of the spina protomer[1].

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1 As defined by Klotz et al. (1975), an oligomer is an associated state containing a number of protomers. A protomer is the minimum-sized subunit which, on association with a number of identical subunits, will generate the quaternary structure. The spinin protomer is also considered to represent the monomer form of the protein. A spinin monomer is thus either the product of translation, or the "mature" protein following secretory processing.

The protomer, obtained by dissociation of spina in SDS at 90-100°C, is termed spinin (Easterbrook and Coombs, 1976). Rigourous physicochemical and immunological examination have established the homogeneity of spinin (Coombs et al., 1978). At dissociation temperatures below about 80°C, the exogenous heat energy is insufficient to disrupt the secondary structure of spinin (Coombs et al., 1976). At temperatures above 80°C, structural breakdown of spina is coincident with a transition of spinin from an antiparallel ß-pleated sheet to an α-helical/unordered secondary structure (Coombs et al., 1976).

Estimates of the molecular weight ratio (MWr) of spinin have proven to be variable. Electrophoresis in the presence of sodium dodecyl sulfate (SDS) yields MWr values ranging from 32 kilodaltons (kD) to 42 kD (Easterbrook and Coombs, 1976; Coombs et al., 1978; and this thesis). This variation may be a partial consequence of the different electrophoretic parameters employed. More significantly, an estimate of 19 kD has been obtained by sedimentation equilibrium analysis in the presence of 6M guanidine hydrochloride, and by cross-linking studies using glutaraldehyde and dimethylsuberimidate (Coombs et al., 1978). The 19 kD value is similar to that derived based on the amino acid composition of spinin (approximately 22 kD; Coombs et al., 1978).

The amino acid composition of spinin is similar to that of other nonprosthecae appendage proteins, flagellin and

pilin, being high in non-polar residues (Easterbrook and Coombs, 1976). However, spinin is much lower in basic amino acid residues. Tryptophan, proline, and cysteine are absent (Easterbrook and Coombs, 1976). The observed stability of spinae to chemical treatments which dissociate flagella and pili are likely due to the high amount of antiparallel β -sheet within spinin. Flagellin, in contrast, is predominantly in the α-helical conformation. Since spinae, flagella, and pili are self-assembled structures, the structure of these appendages depends on the associations established by their protomers (Beveridge, 1981). While spinae exhibit some variation in design (Easterbrook and Subba Rao, 1984) they are structurally dissimilar from flagella and pili.

Surface association and distribution of spinae

The spina base is attached to the external surface of the cell. This conclusion results from the following observations: a) The outer membrane (OM) is continuous beneath the base (Easterbrook et al., 1973; Willison et al., 1977); b) Intact spinae can be detached from stationary growth phase cells by the use of shear force (Easterbrook and Alexander, 1983) and from mid-logarithmic growth phase cells by the sequential application of proteolytic enzymes and shear force (Easterbrook and Alexander, 1983; Willison et al., 1977). This "despining" procedure does not perturb the OM of mid-log phase cells

(Easterbrook and Alexander, 1983); c) A spina first appears at the surface as a circle or a shorter arc of filament which extends to form the overlapping basal ring structure (Easterbrook and Alexander, 1983). The diameter of this ring is the same as the maximum diameter of the base as measured from passively released spinae; d) The short arcs of filament have never been observed to extend outward from the cell (Easterbrook and Alexander, 1983).

The effectiveness of enzymes such as trypsin and pronase in releasing spina argues for an association between the base protomers and surface exposed OM protein(s). The identity of the latter species is unknown.

Since the kinetics of spination have been established (Easterbrook and Alexander, 1983), MPD71 cultures can be examined in the early stages of spina assembly. The initial appearance of the appendage has just been described. They are initially evident at the newly-formed pole of recently divided cells or in the equatorial region of the cell where a division event is imminent (Easterbrook and Alexander, 1983). In other Gram-negative bacteria, these regions are the sites of maximum incorporation of newly synthesized envelope material (Koch, 1982; Smit and Nikaido, 1978; Schwarz et al., 1975; ; Koch et al., 1982). Assuming a similar mode of envelope expansion in MPD71, these regions of the cell may constitute a region of looser-knit fabric (Koch, 1985). Thus, the OM traversal of a large number of spinin molecules may be accomplished more easily. The

generalized surface assembly of spinae in stationary-phase cells may reflect the "loosening" of the envelope fabric due to autolysis or decreasing quality of the external environment (i.e., nutrient depletion, accumulation of metabolic waste products).

Physiological parameters affecting protein production

The Gram-negative envelope is not a static structure but must be responsive to the external milieu. A cell must coordinate its growth and regulatory processes in an environment of fluctuating nutritional, ionic, and temperature parameters (Bayer, 1981; Koch, 1985), while being responsive towards the negative influences of (as examples) bacteriocins, detergent-like compounds, and antibiotics (Costerton, 1977; Bayer, 1981; Lugtenberg and Van Alphen, 1983; Parr et al., 1987).

The OM, being the layer in immediate contact with the external environment (at least in the laboratory environment) has had to assume a responsive capacity. The importance of this response has been emphasized by Bayer (1981) and Brown and Williams (1985).

Since the in situ nutrient supply and temperature can fluctuate, it is not unexpected that several OM polypeptides display a transcriptionally-regulated response to these parameters (Manning and Reeves, 1977; Scott and Harwood, 1980; Lundrigan and Earhart, 1984; Lugtenberg et al., 1976). It has been known for some time that the

content of two E. coli OM polypeptides (Omps F and C by the designation of Reeves, 1979) is altered in response to the exogenous levels of mono-, di-, and tri-saccharides, NaCl, and KCl (osmotic regulation; Van Alphen and Lugtenberg, 1977; Lugtenberg et al., 1977; Kawaji et al., 1979). These alterations are compensatory (i.e., as the content of one of the species is decreased, the content of the other is increased), thus maintaining a constant OmpF + C content (Van Alphen and Lugtenberg, 1977). Since these initial investigations, other transcriptionally osmoregulated OM and periplasmic polypeptides have been identified in E. coli and S. typhimurium (Reeves, 1979; Csonka, 1982; Gowrishankar, 1985; Hall and Silhavy, 1981; Ozawa and Mizushima, 1983; Perroud and LeRudulier, 1985; Villarejo et al., 1983, Garrett et al., 1983; Mizuno et al., 1984; Cairney et al., 1985; Barron et al., 1986).

The collective observations of the environmental regulation of cell envelope polypeptides underscores the intimate sensory behaviour of a bacterium. It is not surprising that several of these species have been shown to form transmembrane pores in vivo (Lugtenberg and Van Alphen, 1983; Poole et al., 1986). This would permit a rapid temporal control by the cell over the entry of osmolytes and various nutrients at the cell surface-environmental interface.

MPD71 is also an environmentally responsive bacterium with respect to the production of spina (Easterbrook and

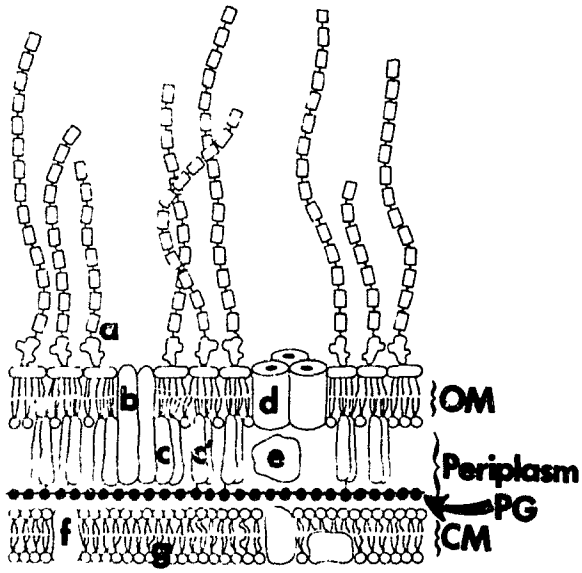
Sperker, 1982). While the nutrient concentration is not a controlling factor in spination, the parameters of pH, temperature, and ionic concentration are. As described by Easterbrook and Sperker (1982): "At optimal or lower temperatures of growth, it is possible to maintain organisms growing actively in the unspined state, provided their metabolic activities do not increase the pH of their environment much above levels optimal for growth. At higher than optimal temperatures spinae are produced at lower pH values, and for all temperature values the percentage of spined organisms in the population increases as either the pH is increased or the salt concentration is decreased". The physiological conditions necessary to generate totally unspined or spined MPD71 populations have been defined (Easterbrook and Sperker, 1982). Briefly, growth at 24°C, pH 6.8, 0.2M NaCl, represses spination, while the simultaneous alteration of temperature (to 34°C) and pH (to 7.4) stimulates production of the appendage by the entire population. Altering an individual parameter in vitro results in the production of spinae by a portion of the MPD71 population (Easterbrook and Sperker, 1982) in an apparent adaptive manner (Easterbrook and Sperker, 1982). This variation in population response is considered further in the Discussion.

The Gram-negative envelope

Introduction

Fig 1. Diagram of the cell envelope of Gram-negative bacteria, modified from that of Lugtenberg and Van Alphen (1983). The symbols used designate the following: a=lipopolysaccharide, b=transmembrane, peptidoglycan associated protein, c=lipoprotein, d="porin" protein, e=periplasmic protein, f=cytoplasmic membrane protein, g=phospholipid, OM=Outer Membrane, PG=Peptidoglycan, CM=Cytoplasmic Membrane

Fig 1.



Being a Gram-negative bacterium (McGregor-Shaw et al., 1972), MPD71 possesses an envelope. The envelope is the cells "skin". It consists of four layers which differ physicochemically from one another. A simplified depiction of the envelope has been shown in Fig 1. The two envelope membranes are amphipathic, whether due to a bilayer of phospholipids (as in the cytoplasmic membrane, CM) or to the apposition of leaflets of phospholipid and lipopolysaccharide (LPS) (as in the outer membrane,OM). Proteins are dispersed integrally and peripherally throughout both bilayers.

Ultrastructurally, both membranes present a "double-track" cross-sectional appearance (DePetris, 1967; Steed and Murray, 1966; Murray et al., 1965), resulting from the binding of metal cations to the hydrophilic, anionic leaflets of the bilayers. The interior, hydrophobic domains do not bind the cations and appear electron transparent (Braun, 1975; Hirashima et al., 1973). The structural asymmetry of the OM can be demonstrated by use of more specialized staining protocols (Shands, 1971; Frehel and Leduc, 1987).

The cytoplasmic membrane (CM)

The hydrophobic interior of the CM is comprised of the fatty acid chains of phosphatidylcholine and phosphatidylethanolamine. These phospholipids are arranged symmetrically (White et al., 1972). CM proteins, dispersed

throughout the bilayer, have varied functions, including transport, synthesis, and oxidative phosphorylation (Rodgers, 1970; Fox, 1972; Michaelis and Beckwith, 1982; Bell et al., 1971; White et al., 1972; Hinckley et al., 1972; Osborn et al., 1972_a; Osborn et al., 1972_b).

The periplasm

The region between the CM and OM is the periplasmic space. The material contained within is the periplasm (Beveridge, 1981). Since isoosmotic conditions produce a separation between the membranes, the periplasmic space is functional and not artifactual (Beveridge, 1981). The periplasmic volume fluctuates; in a hypertonic medium the volume increases as the cell plasmolyses. A hypo tonic medium produces a decrease in periplasmic volume (Beveridge, 1981). Under normal physiological conditions, the volume approximates 20-40% of the total cell volume (Beveridge, 1981). Since the periplasm and cytoplasm are isoosmotic (Stock et al., 1977), the periplasm acts as a physiological buffer between the cytoplasm and the exterior of the cell. This buffering capacity permits the bacterium to monitor the external osmolarity without exposing the cytoplasm to its rigors. A further consequence of this isoosmolarity is the development of turgour pressure, a necessity for both the mechanical support of the cell wall (Beveridge, 1981) and growth (Koch, 1983, 1985). Finally, a related physiological function of the periplasm is a

consequence of the establishment of a Donnan equilibrium between the periplasm and exogenous small MW molecules (Stock et al., 1977). The resultant electrical potential across the OM (approximately 30mV; Hazelbauer, 1979) might be utilized by the OM in the gating response of certain polypeptides (Schindler and Rosenbusch, 1978), although this is debatable (Hancock, 1987).

The peptidoglycan (PG)

In Gram-negative cells the PG resides within the periplasmic space. It is closely associated with the OM via ionic and covalent associations with certain integral OM polypeptides (Lugtenberg and Van Alphen, 1983). The OM, by virtue of these associations, assists in the maintainance of the cells' morphology (Amano and Williams, 1984; Henning and Haller, 1975). The PG is, however, the primary shape-maintaining structure (Gmeiner, 1979; Weidel and Pelezar, 1964; Weidel et al., 1960; Braun et al., 1976). The PG is constructed of a grating of linear glycan strands composed of alternating residues of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc, a lactyl ether derivative of GlcNAc) (Beveridge, 1981). The saccharides are linked β1-4 (Schleifer and Kandler, 1972). MurNAc is also amide linked through its lactyl carboxyl group to an L-alanine residue of a short peptide "stem". The peptides cross-link the glycan strands. In many Gram-negative bacteria this linkage is between the carboxyl group of the

C-terminal D-alanine residue of one peptide stem and the ε -amino group of the diamino acid (meso-diaminopimelic acid; m-Dpm) of an adjacent peptide. The linkage efficiency varies among different bacteria (Takabe, 1965; Amano and Williams, 1983; Forsberg et al., 1972). The PG is thus viewed as a covalently-linked macromolecule (Weidel and Pelzer, 1964) whose flexibility and susceptibility to degradative action is a consequence of the glycan cross-linkage efficiency (Amano and Williams, 1983).

X-ray powder diagrams of nonhydrated PG reveals lattice constants of 1.03 x 0.44 nm (Formanek et al., 1974); that is, the disaccharide repeat along a glycan strand is 1.03 nm, and adjacent strands are separated by 0.44 nm. Hydration of PG expands the lattice to 1.90 x 0.98 nm (Burge et al., 1977). Measurements of the surface area occupied by the hydrated PG fabric (Braun et al., 1973) indicate that sufficient material is available to cover the cell surface. In fact the Gram-negative PG can be theoretically arranged as a three layered structure (Braun et al., 1976). In this arrangement, however, the peptides would be completely extended and the resultant strain would not permit efficient cross-linkage. It is more likely that the PG is arranged as a monolayer approximately 3.0 nm in thickness (Beveridge, 1981). Modelling studies indicate the peptide stem can assume a fully extended length of 4.2 nm and a folded, spherical form with a diameter of about 1.0 nm (Braun et al., 1973). In light of the afore-mentioned

hydrated lattice constant, it becomes difficult to accomodate the peptides in the same plane as the glycan chains. Instead, the peptides may extend outward from the glycan plane (Braun et al., 1973; Formanek et al., 1974) in a common direction (Formanek et al., 1974), or in various directions off each glycan "cylinder" (Labischinski et al., 1979). Both arrangements would be amenable to cross-linkage (Formanek et al., 1974; Burge et al., 1977).

The separate biosynthesis of the disaccharides and peptides and their subsequent union, occurs at the CM (Mirelman, 1974). Incorporation of the disaccharide pentapeptide complex into the pre-existing PG fabric occurs in the periplasm. For this incorporation, selective hydrolysis of the pre-existing PG fabric must occur. This must be a delicately coordinated process, in order to maintain the structural support afforded by the PG fabric while permitting growth of the sacculus (Koch, 1982, 1983, 1985; Goodell and Schwarz, 1974).

The outer membrane (OM)

Introduction

The OM represents the interface between a bacterial cell and the external environment (at least in the laboratory setting). It is through this bilayer that nutrients and metabolic waste products must pass, from which harmful agents must be excluded, and which (along with the periplasm) functions as an environmental "sensor".

The OM is composed of phospholipid (20% by weight), LPS (45%), and protein (35%) (Lugtenberg and Van Alphen, 1983). The OM is asymmetrically constructed, with phospholipid confined to the inner leaflet and LPS at the outer (Funahara and Nikaido, 1980). This construction is reflected in the difficulty in generating a fracture plane through the OM interior (Beveridge, 1981) and the tolerance of cells to SDS concentrations which solubilize phospholipid bilayers (Lugtenberg and Van Alphen, 1983).

The phospholipid content of the cell is essentially restricted to the envelope (Cronan and Vagelos, 1972), consisting of phosphatidylethanolamine (PE), phosphatidylcholine, phosphatidylglycerol, and diphosphatidylglycerol (cardiolipin). PE predominates, particularly in the OM (Cronan and Vagelos, 1972; Lugtenberg and Peters, 1976; Osborn et al.,1972$_b$; White et al., 1972), accounting for 70-80% of the total envelope phosphorus (Cronan and Vagelos, 1972). The enrichment of PE in the OM may be a consequence of its participation with LPS in the formation of stable bilayers (Fried and Rothfield, 1978). Since phospholipids are common to both the CM and the OM, it is not suprising that they have been implicated in regions of OM-CM structural blending. These "adhesion zones" were originally proposed to be permanent envelope structures (Bayer, 1979) based on their electron microscopic detection and functional significance (Bayer,

1968a,b, 1981; Bayer and Bayer, 1985). Both their permanence and existance is still a topic of debate.

It has been suggested (DeLeij et al., 1978, 1979) that regions of membrane commonality exist transiently. If a vesicle is released from the CM and fuses with the inner leaflet of the OM, it is conceivable that an intermediate structure reminescent to an adhesion zone could be detected. In another model the membranes form domains of inverted phospholipid micelles (Cullis and Hope, 1978; Lugtenberg and Van Alphen, 1983). This model is attractive in light of the observation that the binding of Ca^{2+} leads to a localized conformational change of phospholipid bilayers, generating inverted micelles (Cullis and Hope, 1978). This might in turn induce an OM-CM fusion, establishing a functional adhesion region (Ohki, 1982; Wilschut et al., 1982). Since the fusion event involves only phospholipid (Jones and Osborn, 1977 a,b) such adhesion sites could be randomly located over the cell. The pattern of surface appearance of newly synthesized LPS (Leive, 1977; Muhlradt and Golecki, 1975; Muhlradt et al., 1973) is consistent with this pattern . Secondly, the bidirectional movement of phospholipid from one membrane to another (Langley et al., 1982) would allow the cell to ensure OM integrity when that integrity has been compromised (Nikaido, 1976; Muhlradt and Golecki, 1975). The functional relevance of this model has not yet been established.

On a less contentious note, a correlation has been established between the phospholipid fatty acid constituent and membrane fluidity (Cronan and Vagelos, 1972). The proportion of unsaturated fatty acids increases as the growth temperature decreases. The double bond(s) in the fatty acid chains would enable maintainance of some membrane fluidity at the lower temperature (Cronan and Vagelos, 1972). In contrast, possession of saturated fatty acid chains would reinforce the ordered quasi-crystalline state of the bilayer at elevated temperatures. Thus, incorporation of fatty acyl chains of various structure would allow the bacterium to maintain the membrane fluidity necessary for proper physiologic function (Halegoua and Inouye, $1979_{a,b}$). External factors function in this process as well; exogenous Mg^{2+} or Ca^{2+} interact with the polar domain of phospholipid, promoting establishment of a quasi-crystalline structure. The temperature required for the phase transition (i.e., transition from an ordered to an unordered state) is increased as a result. Monovalent cations such as Na^{+} and K^{+} decrease the phase transition temperature (Trauble and Eibl, 1974). Since both totally unordered and para-crystalline OM phases are detrimental to the bacterium (Halegoua and Inouye, $1979_{a,b}$), the importance of phospholipids in the physiologic response of the bacterium must be considerable.

Lipopolysaccharide (LPS)

LPS is a macromolecule unique to the Gram-negative OM (Inouye, 1979). It is localized exclusively in the outer leaflet of the membrane (Funahara and Nikaido, 1980) where it constitutes a physical/chemical barrier for the cell (Szmelcman and Hofnung, 1975; van der Ley et al., 1986a, b; Grossman et al., 1987).

Two general structural forms of LPS exist. The "complete" or "smooth" form consists of a hydrophobic portion (lipid A), a core consisting of a largely invariant arrangement of saccharides, and polysaccharide chains whose length and composition is variable (Chester and Meadow, 1975; Goldman and Leive, 1986). Lipid A can be extracted by mild acid hydrolysis of the bacterial cell (Adams and Singh, 1970) or by treatment with a phenol-chloroform-petroleum ether mixture (Galanos et al., 1969). The lipid A of enterobacteria is a glycolipid consisting of a short chain of β1-6 linked glucosamine disaccharides to which are linked seven fatty acyl chains (Luderitz et al., 1973). In E. coli, βhydroxymyristic acid accounts for 60% of the total lipid A fatty acyl content (Burton and Carter, 1964). Lipid A is linked to the core region of the LPS via a glucosamine hydroxyl moiety and the 2-keto-3-deoxyoctonate (KDO) core sugar. The remaining lipid A constituent (the fatty acyl chains) selectivly partitions into the lipophilic domain of

the OM (Galanos et al., 1969), where it functions in stabilizing the bilayer (Luderitz et al., 1973; Labischinski et al., 1985).

The core region of enterobacterial LPS contains KDO as well as glucose, galactose, and N-acetyl-D-glucosamine (Lugtenberg and Van Alphen, 1983). This composition is reasonably invariant among members of the Enterobacteriaceae, while a variation is encountered among other bacteria (Chester and Murray, 1975; Adams et al., 1968).

The O-side chains constitute the most variable region of the LPS. They display a ladder-like pattern following electrophoresis and silver staining (Tsai and Frasch, 1982; Hitchcock and Brown, 1983; Mumford et al., 1980) due to variation in chain polymerization (Carlson, 1984; Goldman and Leive, 1986; Palva and Makela, 1980; Hitchcock and Brown, 1983; Jann et al., 1975; Mumford et al., 1980). These chains can extend outward up to 150 nm from the surface (Shands, 1965), although this orientation may artifactually reflect the antigenic stabilization of the chains. X-ray diffraction analysis using oriented LPS multilayers are more reconcilable with a heavily coiled polysaccharide chain in the immediate proximity of the cell surface (Labischinski et al., 1985). Whether in an extended (Shands, 1965) or contracted (Labischinski et al., 1985) configuration, the polysaccharide represents the initial contact between the bacterium and the exterior milieu.

Their implication in the barrier function of the cell (Grossman et al., 1987) is thus not unexpected.

"Rough" forms of LPS lack the O-polysaccharide chain portion as well as increasing lengths of the core. Both forms possess the "inner core" and the lipid A portions of the macromolecule; their absence is lethal (Osborn, 1979; Gmeiner and Schlecht, 1979; Rick et al., 1977).

An in vivo role for divalent cations (particularly Ca^{2+} and Mg^{2+}) in stabilization of the LPS and in the salt-bridging of LPS macromolecules and adjacent proteins has been proposed (Ferris and Beveridge, 1984, 1985, 1986; Coughlin et al., 1981; Strain et al., 1983). The resultant screening of charged groups within the LPS would permit a more compact KDO-core (Ferris and Beveridge, 1985). Evidence to support this has been obtained (Labischinski et al., 1985). Indeed, EDTA-chelation of ionic species releases of up to 50% of the LPS (Leive, 1977; Voll and Leive, 1970; Bayer and Leive, 1977; Shands and Chun, 1980; Leive et al., 1968; Stinnett et al., 1973; Van Alphen et al., 1978). OM architecture is altered as a result (Gilleland et al., 1973). Labischinski et al. (1985) have suggested that the LPS of S. abortus-equis (a complete structure) and S. minnesota R595 (a "deep-rough" structure) exhibit a high degree of structural (bilayer) order in in vitro LPS - phospholipid systems. Both this and other observations (Kato et al., 1985) are consistent with a paracrystalline LPS outer leaflet of the OM which possesses

long-range order. In vivo this matrix must accommodate proteins.

Enterobacterial Common Antigen (ECA)

Of the several hundred Gram-negative strains examined, ECA has been detected only in members of the Enterobacteriaceae and two Aeromonas sp. (Mayer and Schmidt, 1979). ECA is a polymer of N-acetyl-D-glucosamine and D-mannosaminuronic acid, which is partially esterified by palmitic acid (Lugtenberg and Van Alphen, 1983).

Ultrastructural (Acker et al., 1986) and immunofluorescence studies (Aoki et al., 1966) have demonstrated the OM localization of the ECA. There would appear to be scant in vivo association between the antigen and OM proteins, since the ECA content remains constant in E. coli mutants lacking one or more of these proteins (Lugtenberg and Van Alphen, 1983). ECA also may be associated with the core regions of "rough" LPS (Mayer and Schmidt, 1979).

OM polypeptides

The majority of proteins in the OM do not have an enzymatic function. The bulk of the enzymatic activity in the cell envelope is associated with the CM. As will be described subsequently, OM polypeptides serve other, varied functional roles.

Lipoprotein

This 15kD species is the most abundant protein in the OM (DiRienzo et al., 1978). It exists as either a "free" form (approximately 5 x 10^5 copies/cell) or a "bound" form (approximately 2.5 x 10^5 copies/cell). The latter is covalently linked to every 10-12th m-Dpm residue of the PG (Braun, 1975; Braun and Rehn, 1969; Braun and Sieglin, 1970), maintaining an intimate association between the OM and the PG (Sonntag et al., 1978). A related function, stabilization of the OM, seems likely as well (Suzuki et al., 1970; DeMartini et al., 1976); the copious "blebbing" of the Neisseria gonorrhoeae OM is consistent with the absence of lipoprotein (Hebeler et al., 1978).

Outer membrane protein (Omp)A

OmpA is present in E. coli in approximately 10^5 copies/cell (Lugtenberg and Van Alphen, 1983). In SDS-PAGE systems, its MWr is variable (28-35kD) due to the heat-modifiable behaviour of the protein (Schnaitman, 1973; Mizushima, 1974; Reithmeier and Bragg, 1974). This variation reflects incomplete denaturation of the protein at lower temperatures (Lugtenberg and Van Alphen, 1983), due perhaps to its high content of ß-sheet secondary structure (Heller, 1978). OmpA has been judged to be a transmembrane protein, by virtue of its apparent covalent linkage to the PG (Overbeeke and Lugtenberg, 1980),

reaction with exogenous antibody, and utilization by bacteriophages, sex pili, and colicins as a surface receptor (Lugtenberg and Van Alphen, 1983; Datta et al., 1977; Van Alphen et al., 1977). However, PG linkage may not be direct, but may rather be in concert with the bound form of lipoprotein (Morona et al., 1984). OmpA does not function as a "porin".

PG-associated proteins with a "pore" function

To date, forty-four "porins" have been identified and characterized in 32 species of Gram-negative bacteria (Hancock, 1987). As described by Hancock (1987) porins are a class of OM protein responsible for the molecular sieving property of the bacterium. Sieving is achieved by an aggregation of the protein to form aqueous channels. These allow nonspecific diffusion of a variety of compounds which are below a certain critical size. Molecules which exceed this limit are physically excluded. The pore diameter and the number and position of charged moieties within the channel determines the exclusion limit (Hancock, 1987). Certain porins do, however, demonstrate a pronounced substrate specificity (Overbeeke and Lugtenberg, 1982; Poole and Hancock, 1986; Ferenci et al., 1980; Poole et al., 1986).

The sieving function appears to be due solely to the protein (Hancock, 1987) with no contribution from phospholipid or LPS (Parr et al., 1986). A proposed sieving

mechanism is voltage-gating (i.e., the opening and closing of a channel in response to the voltage potential across the channel). Voltage-gating may not operate *in vivo*, however (Hancock, 1987). While voltages in the order of 120V do indeed promote channel closure (Schindler and Rosenbusch, 1978; Dargent *et al.*, 1986), this level is far in excess of the Donnan (physiological) potential across the OM (30-80V; Hazelbauer, 1979; Stock *et al.*, 1977). Thus, porin channels may not be voltage-regulated *in vivo*. A recent study (Martinac *et al.*, 1987) indicates that pressure-regulated ion channels are present in the *E. coli* OM. These may have significance with respect to an osmosensory and regulatory capacity by the bacterium.

Many porins examined to date share similarities in their pI values (acidic), and predominance of B-sheet order, although exceptions have been identified (Benz, 1985). In *E. coli* the similarities extend to the amino acid sequence, particularly for the OmpF, OmpC, and PhoE proteins and less so (but still significantly) for LamB (Mizuno *et al.*, 1983). Finally, many porin species aggregate in the membrane as a biologically active trimer; however, a *Paracoccus* sp. and two *Rhodopseudomonas* species are unique in that they appear to have a dimer as the biologically active pore form (Flammann and Weckesser, 1984; Zalman and Nikaido, 1985; Weckesser *et al.*, 1984). Even given a trimeric aggregation of the porin species, ultrastructural

and voltage-induced channel closure experiments have revealed different channel arrangements (Hancock, 1987).

An important consideration concerning this barrier capacity is that the exclusion limit established by the porin channels may well reflect the external milieu of the bacterium. For example, members of the Enterobacteriaceae have an exclusion limit of approximately 660 daltons (Nakae and Nikaido, 1975; Decad and Nikaido, 1976). Their natural habitat is the colon, thus the cells must cope with a variety of degradative enzymes. Additionally the CM must be shielded from the saponification by bile salts, fatty acids, and glycerides (Lugtenberg and Van Alphen, 1983). The physical exclusion of such compounds may be achieved by the presence of small OM entry pores and a charged membrane surface. In contrast, P. aeruginosa displays an exclusion limit of upwards of 6,000 daltons (Hancock et al., 1979; Woodruff et al., 1986). The bacterium resides in soil and aqueous environments where nutrient concentration would not approach that in the gut milieu of the enterobacteria. Pseudomonas is able to degrade surrounding proteins through elaboration of surface and excreted proteases (Hancock and Nikaido, 1978). Possession of large OM pores would allow access of resultant large peptides or proteolytic break-down products into the periplasm, where further peptidolysis could occur. As well, such pores might permit entry of hydrophobic solutes encased in a shell of water molecules (Hancock and Nikaido, 1978). Since P. aeruginosa

is able to utilize lipids, entry of these hydrophobic compounds would be advantageous.

P. aeruginosa is also of concern medically, being a frequent isolate from wounds, burns, and urinary tract infections. The high intrinsic antibiotic resistance of the bacterium (Benz and Hancock, 1981) reflects the low permeation of antibiotic compounds through the OM bilayer (Angus et al., 1982; Parr et al., 1987). Upon initial consideration, the low penetrability of the OM seems difficult to reconcile with the possession of large hydrophilic pores. However, Woodruff and co-workers (1986) have demonstrated that the nonspecific channels formed in P. aeruginosa are heterogeneous in size. The smaller diameter channels are predominant (Benz and Hancock, 1981; Woodruff et al., 1986). The diameter of the latter (< 0.2nm) might sterically retard antibiotic penetrability, although the small MW values of many of the commonly employed hydrophilic antibotics (350-500) indicates that the Pseudomonas OM should present no more of a barrier than does the OM of E. coli. The increased resistance of Pseudomonas is likely a function of a reduction in the number of utilizable (large enough diameter) surface channels as well as both the presence of degradative periplasmic enzymes and a relatively impermeable CM (Hancock and Nikaido, 1978)

"Minor" OM polypeptides

The designation minor refers to the lower levels of several other resident OM proteins. In the presence of certain compounds, the content of some of these proteins can increase dramatically. Their physiological importance as receptors or in substrate transport under conditions of limiting substrate concentration has been demonstrated (DiRienzo et al., 1978). Table 1 summarizes these species.

Regularly structured surface layers

Regularly structured (RS) layers are present on most bacteria obtained from natural settings (Beveridge, 1981). The selective pressure for their production is lost in the noncompetitive environment of the laboratory (Beveridge, 1981).

RS layers are proteinaceous arrays. Each array is constructed of identical subunits (protomers) (Sleytr, 1978), results from the self-assembly of the excreted protomers. All the information and energy for this assembly is contained within the protein subunits themselves (Sleytr, 1978; Beveridge, 1981; Sara and Sleytr, 1987a). The different array formats which have been observed depend on the nature of the binding between adjacent protomers (Sleytr, 1978; Beveridge, 1981). Binding can involve divalent cations, which salt-bridge anionic residues on

Table 1 "Minor" OM proteins of E. coli K12

PROTEIN; MWr(kD); COPIES/CELL; FUNCTION AND COMMENTS
FepA (FeuB) - 81kD; ?; uptake of Fe^{3+}-enterochelin
FhA (TonA) - 79kD; ?; uptake of Fe^{3+}-ferrichrome
Fec (Cit) - 78kD; ?; uptake of Fe^{3+}-citrate
BtuB - 60kD; 200-300; uptake of Vitamin B12
Nmp - 38kD; 2×10^5; produced to replace Omps F and C
Lc - 2×10^5; replaces Omps F and C in lysogenized cells
TsxA - 26kD; 10^4; uptake of nucleosides and deoxynucleosides
Protein G - 15kD; ?; possible role in coordination of cell elongation and DNA replication

? unknown

References James, 1975; Braun and Hantke, 1977; Lundrigen and Earhart, 1981; Braun et al., 1976; Hancock et al., 1976; DiRienzo et al., 1978

adjacent protomers (Beveridge, 1981) and couple the array to the underlying anionic cell surface (Beveridge, 1981).

While RS layers represent the predominant interface between the cell and its environment, little is known of their function. The A-layer of Aeromonas salmonicida is so far the only layer with a demonstrated function, namely a virulence function in salmonids (Ishiguro et al., 1981; Kay et al., 1981). Other proposed functions are protective (i.e., exclusion of enzymes, antibiotics, heavy metals, and protection from endoparasitic digestion; Beveridge, 1981; Sleytr, 1983; Sara and Sleytr, 1987_b). The molecular sieving capacity of the RS layer of Bacillus stearothermophilus (Sara and Sleytr, 1987_b) excludes molecules in excess of 30kD. A similar exclusion limit of Gram-negative layers would not hinder the accessibility of lower MW compounds to the surface pores. Of course, the exclusion limit will depend on the construction of the array, and so on the nature of the protomer.

Like the RS protomers, spinin also undergoes self-assembly. Alternate assembly products are possible in both systems (Sletyr, 1978; Easterbrook and Coombs, 1983; Sleytr and Messner, 1983). Whether the analogies between these protomers extends to their mechanism of excretion is unknown. However, similarities may exist. Since neither array is apparent inside the cell, a conformational change presumably occurs as the protein subunits are externalized.

The altered conformation would drive the self-assembly process.

RS proteins may become surface-localized at the sites of OM-CM continuity (Sleytr and Messner, 1983). Each site would be a nucleation site for the crystallization of the protomers. Enlargement of these crystalline regions would result upon the lateral movement of newly excreted protein to the edge of the array (Sleytr and Messner, 1983; Sara and Sleytr, 1987a). Lattice discontinuities would ensure surface coverage. Indeed, it is the observation of lattice defects over the surface of the cell which prompted the suggestion of protomer insertion at multiple sites (Sleytr and Messner, 1983). Other alternatives are possible. For example, the protomers may be extruded from one exit site. Self-assembly during the exit would result in the "flowing" of the array over the cell surface. This process could also involve an OM-CM adhesion region.

It is not surprising that the excretion pathway of the RS proteins (and of spinin) has remained unclear. The RS layers and spina have been the subject of ultrastructural, rather than genetic, study. In order to gain insight into the mechanism of excretion of either of these protomers, it is necessary to examine other systems.

Protein secretion

Protein secretion is defined as the process by which a protein crosses a membrane. In Gram-negative bacteria

proteins which are resident in the periplasm and the OM are secreted proteins. Species which entirely traverse the envelope are considered to be excreted proteins.

Numerous investigations have been concerned with the mechanism by which a protein destined for secretion is able to negotiate a single membrane bilayer. The protein must of necessity pass across hydrophilic and hydrophobic domains (Beveridge, 1981). In many procaryotic and eucaryotic proteins the membrane traversal is accomplished using an amino-terminal, hydrophobic "signal sequence" (Michaelis and Beckwith, 1982; Silhavy et al., 1983). This sequence contains a central hydrophobic stretch of amino acids flanked by charged stretchs (von Heijne, 1984, 1985_a). This charge character allows the partitioning of the sequence into the lipid domain of the bilayer (Blobel, 1980; Oliver, 1985; Spirin, 1985; von Heijne, 1984, 1985_a; Blobel and Dobberstein, 1975; Davis and Tai, 1980; Bassford et al., 1981; Koshland et al., 1982; Silhavy et al., 1983; Freudl et al., 1987). A region of the sequence becomes exposed to the periplasm, where it is recognized and cleaved by a periplasmic enzyme (Michaelis and Beckwith, 1983). Continued translation acts to extrude the remainder of the polypeptide into the periplasm. A conformational change in the "mature" protein as it emerges into the periplasm renders the translocation irreversible (Michaelis and Beckwith, 1983).

This process is co-translational; i.e., the translation and secretion events are coupled. However, some proteins can be efficiently secreted following the completion of translation (Wickner, 1979, 1980; Randall, 1983). Additionally, certain of these species lack an amino-terminal, "cleavable" signal sequence. To explain the secretion of these proteins, the conformation of the mature protein has been proposed to have a central role in the translocation step (Randall, 1983; Wickner, 1979, 1980; Joseffson and Randall, 1981; Zimmermann and Wickner, 1983; Randall and Hardy, 1984; Felmlee et al., 1975; Goodman et al., 1981; Ito et al., 1981; Wu et al., 1983). So-called insertion sequences have been implicated in this conformational "membrane trigger" hypothesis (Wickner, 1979; Blobel, 1980; Oxender et al., 1980). These sequences, which can be at the amino-terminus of the polypeptide or more internalized, are signal sequence-like in their hydrophobic character (Blobel, 1980). Repositioning of such a sequence to the carboxyl terminus of the protein stops translocation (Blobel, 1980; Davis and Model, 1985; Coleman et al., 1985). Thus the sequence is able to alternately facilitate or stop translocation, depending upon its location in the polypeptide.

In this light, the signal sequence and membrane trigger hypotheses may be more closely related mechanistically than has been traditionally perceived. Thus secretion, rather than consisting of several fundamentally distinct

mechanisms, may in fact represent modifications of an underlying common mechanistic process (Crooke and Wickner, 1987; Briggs et al., 1986; Singer et al., 1987).

Factors which perturb protein secretion

The importance of membrane fluidity to the protein secretion process has been recognized (Halegoua and Inouye, 1979; Ito et al., 1977). If membrane fluidity perturbances are pronounced the secretory process is disrupted, leading to the accumulation of secretory precursors (Oliver and Beckwith, 1982; Ito et al., 1977, 1981). A comparable effect is achieved by "jamming" a common site or step in the secretion process (Michaelis and Beckwith, 1982; Moreno et al., 1980; Ito et al., 1981). This focal point is likely the CM translocation event (Bassford and Beckwith, 1979; Emr and Bassford, 1982; Silhavy et al., 1983).

Envelope perturbants such as procaine and phenethyl alcohol (PEA) also produce comparable accumulation of proteins destined for secretion (Pages and Lazdunski, 1982a; Lazdunski et al., 1979; Silhavy et al., 1983; Matsuyama et al., 1986). Their effects are also likely the result of an alteration of membrane fluidity (Shanes, 1960; Papahadjopoulos, 1972) due to an increase in

the packing density of the envelope membranes (Halegoua and Inouye, 1979). Fluidity changes may perturb the functioning of an envelope protein important in the secretory process (Taylor et al., 1983) although the nature of this blockage remains unclear. This protein has been termed EnvZ by Hall and Silhavy (1981).

EnvZ is postulated to sense the external environment and, via transduction of a signal into the cell by an as yet unknown mechanism, shift the equilibrium state of a cytoplasmic protein (OmpR) towards either a mono- or multimeric form (Hall and Silhavy, 1981). One result of this may be the expression of either ompF (OmpR monomer) or ompC (OmpR multimer). The gene products, OmpF and Ompc, are OM proteins which function as aqueous pores (Lugtenberg and Van Alphen, 1983). Interruption of the EnvZ signal by perturbance of the envelope membranes would thus be predicted to exert pleiotrophic effects (Hall and Silhavy, 1981). Both the manufacture of proteins (i.e., transcription or translation) and processing of proteins destined for secretion would be effected. These effects have been confirmed (Silhavy et al., 1983).

Secretory interruptions are also observed following the dissolution of the proton motive force (pmf) normally operative across membranes such as the bacterial CM (Michaelis and Beckwith, 1983; Date et al., 1980; Daniels et al., 1981; Enequist et al., 1981; Geller et al., 1986). An example of a pmf-disruptive agent is carbonyl cyanide

m-chlorophenyl hydrazone (CCCP) (McLaughlin, 1982). CCCP is a protonophore (McLaughlin, 1982), and as such allows the free flow of hydrogen ions across a membrane which is otherwise impermeable to the ion. The normal impermeability of the bilayer permits the establishment of a proton gradient, which drives the phosphorylation of ADP to ATP via F1-ATPase (Lehninger, 1975). Hence CCCP uncouples phosphorylation from the electron transport chain (Lehninger, 1975). The pmf, which consists of both proton and electrochemical gradients, is collapsed.

The exact role of the pmf in the secretory process has not been fully elucidated. Since ATP appears to be essential for the in vitro post-translational (and probably co-translational) translocation of certain E.coli polypeptides (Tai and Chen, 1986; Chen and Tai, 1987) the pmf may be involved in the replenishment of ATP via H^+-ATPase. Another role, involving the electrochemical gradient, may be to maintain a functional topology of relevant CM secretory machinery (Rhoads et al., 1984), or to facilitate the "electrophoresis" of the secretory signal domain across the bilayer (Wickner, 1980).

The excretion of proteins

If a bacterial protein is synthesized with a precursor region in order to cross the CM, then upon extrusion from the CM into the periplasmic space a conformational change would occur. This would reflect the transition of the

protein from the lipophilic interior of the CM to the hydrophilic surroundings of the periplasm. Alternatively, even if a hydrophilic, proteinaceous CM pore was utilized for membrane traversal, a conformational change would likely result upon exit of the protein from the pore (Singer, 1987). In both cases, a second membrane translocation event is required in order to externalize the protein. It is the negotiation of the second bilayer (the OM) which has produced much speculation. That it occurs is clear, since the excreted proteins of several Gram-negative bacteria appear to be normally routed through the periplasm (Howard and Buckley, 1983; Hirst and Holmgren, 1987; Wagner et al., 1983; Oudega et al., 1982, 1984; Felmlee et al., 1985; Pohlner et al., 1987).

OM translocation may involve the stabilization of a periplasmic conformation of the secreted protein (Crooke and Wickner, 1987) so that subsequent recognition and utilization of a proteinaceous OM pore can occur (Mooi and DeGraaf, 1985; Dougan et al., 1982, 1983; Mooi et al., 1982). Alternatively a signal sequence-like region near the carboxyl terminus of the mature protein may act to maintain the protein in a translocation-competent conformation, or to form an OM pore through which the remainder of the protein can exit the cell (Pohlner et al., 1987). It is worth mentioning that the latter mechanism can be thought of as a membrane-trigger type of secretion. Thus, the two "types" of secretory mechanisms (signal sequence and

membrane trigger) may operate at different stages in the export pathway of the same protein.

It is also conceivable that proteins could be routed directly from the cytoplasm to the cell surface via regions of OM-CM continuity. A signal sequence would not be required for this process, although it could be accommodated (Lory et al., 1983). The proposed use of the adhesion sites by RS proteins has been mentioned earlier. Other molecules, such as LPS, may also use adhesion sites (Osborn, 1979). Direct involvement of adhesion sites in the secretion process has not been demonstrated as yet, however.

If the interior of the adhesion sites were hydrophilic, leakage of cytoplasmic contents might occur. Hence it seems reasonable to suppose that the interior would retain the hydrophobic character of the lipid domains of the CM and OM. This would have implications for the conformation of proteins being secreted through the conduit, and may impose restrictions on the type of molecule which could utilize such adhesion sites.

Summary

MPD71 is a Gram-negative bacterium (McGregor-Shaw et al., 1972; Easterbrook et al., 1973) and so, by definition, possesses an envelope. The observations that a transition of MPD71 from an unspined to a spined form indicates that the spina protomer must negogiate the envelope. The pathway

of this traversal is not known. Neither is it clear whether change occurs in the structure of the envelope during the phenotypic transition.

The elucidation of spina-restrictive and permissive cultural conditions (Easterbrook and Sperker, 1982), and knowledge of the temporal kinetics of spination following a shift from spina-restrictive to permissive conditions (Easterbrook and Alexander, 1983) has laid the groundwork for the present study. In this study, populations have been analyzed at various times following the shift (i.e., throughout the period of the morphological transition, during the period when spina assembly is commencing, and during the maximal assembly of spina). This has permitted the investigation of both envelope alterations and the effect of secretory perturbants on the spinin excretion pathway. These investigations comprise the remainder of the thesis.

MATERIALS AND METHODS

Organisms

Marine pseudomonad D71 NCMB 2018 (McGregor-Shaw *et al.*, 1972), *Escherichia coli* K12 strain AB264, and *Pseudomonas aeruginosa* PAO1 were maintained as liquid stock cultures at 4°C. The complex medium used is detailed below. For long-term storage mid-logarithmic growth phase cultures were diluted 1:1 with 50% v/v glycerol and stored at -70°C.

Clinical isolates of *E. coli* required for one experiment were obtained from the Victoria General Hospital. They were supplied as primary cultures on nutrient agar slopes. These were used directly, with no intervening subculture.

Chemicals and reagents

Electrophoretic materials and molecular weight calibration standards were purchased from Bio-Rad (Richmond, CA, USA). ^{14}C calibration standards (5 $uCimL^{-1}$, specific activity 50 $uCimg^{-1}$) were obtained from New England Nuclear (Boston, MA, USA) and Amersham (Oakville, ONT, CAN), as was ^{35}S methionine (5 $mCimL^{-1}$, >800Ci $mmol^{-1}$). Purified lipopolysaccharide from *Salmonella minnesota* and *E. coli* 0111B4 were from, respectively, List Biologicals (Campbell, CA, USA) and Sigma Chemical Co. (St. Louis, MO, USA).

Buffering capacity for most media and solutions was provided by Hydroxyethylpiperazine ethanesulfonic acid (HEPES). Frequent use was made of 10 mM HEPES pH 6.8, 1.0 mM $MgCl_2$, referred to hereafter as "Buffer".

Media and growth conditions

The media and physiological growth parameters utilized for MPD71 have been described in detail elsewhere (Easterbrook and Sperker, 1982), and so are only briefly outlined here. The complex medium used for the majority of experimental procedures was comprised of salts (0.2M NaCl, 0.01M $CaCl_2$, 0.01M KCl, 0.05M Mg_2SO_4), Bacto-peptone (0.5% w/v), and Bacto-yeast extract (0.1% w/v,). $CaCl_2$ was prepared separately as an aqueous 1.0M stock solution. These were sterilized by autoclaving. The medium was buffered using HEPES (final concentration 50 mM); the pH was adjusted with 10N NaOH prior to sterilization. The resultant alteration in Na^+ concentration was negligible.

As required, the defined medium developed by Michael Coughlin (Easterbrook and Sperker, 1982) was used. Physiological parameters demonstrated (Easterbrook and Sperker, 1982) to be permissive (pH 7.4, 34°C, constant agitation), and restrictive (pH 6.8, 24°C, with or without agitation) for spination were used. E. coli and P. aeruginosa were cultured in the complex medium of lower pH at 34°C with constant agitation.

Population densities were monitored optically (540 nm) using a Bausch and Lomb Spectronic 20 spectrophotometer. Samples were contained in glass tubes having a 1.0 cm light path. The spectrophotometer was zeroed with respect to absorbance prior to each set of measurements using uninoculated growth medium.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

An electrophoretic system similar to that described by Laemmli (1970) was utilized. The pH of the stacking gel was 6.8 or 8.8 and that of the separating gel was 8.8; Tris provided buffering capacity. Separating slab gels 14 cm in length were cast using a vertical assembly apparatus (Bio-Rad). As required, linear acrylamide gradients of 7.5-15% or 10-15% were cast using a gradient former (BRL, Gaithersburg, MD, USA). With the exception of the 15% gels, which were 0.75 mm thick, all gels were 1.5 mm in thickness. The composition of the stacking/separation gels is given in Table 2. Samples were dissociated at 100°C for 5 min prior to electrophoresis in a buffer whose composition is given in Table 3.

Table 2 Composition of the stacking and separating polyacrylamide gels

COMPONENT	STOCK SOLUTION	FINAL CONCENTRATION	
		1	2
Acrylamide/N,N'-Methylene-bis-acrylamide	30%/0.8%[1]	6%	10,12, 15%
Ammonium persulfate	10%	0.001%	0.001%
Sodium dodecyl sulfate (SDS)	10%	0.03%	0.2%
NaCl	0.4%	0.15%	0.28%
N,N,N',N'-Tetramethyl-ethylene-diamine (TEMED)	100%v/v	0.0003% v/v	0.0004% v/v
Triethylaminomethane sulfonic acid (Tris)	1.0M, pH 6.8 or 8.8	0.13M	0.37M

1 w/v unless otherwise noted, 2 Stacking gel, 3 Separating gel

Table 3 Composition of the electrophoretic dissociation buffer

COMPONENT	STOCK SOLUTION	FINAL CONCENTRATION
Tris	0.25M, pH 6.8	0.08M
Glycerol	50%v/v	12.5%v/v
2-mercaptoethanol	100%v/v	0.05%v/v
SDS	10%w/v	2.5%w/v
Bromphenol Blue	0.5%v/v	0.025%v/v

Samples were individually dispensed into the wells by use of a microsyringe (Hamilton Co., Reno NV, USA) and the assembled gel unit was submerged in 3 L of a buffer consisting of 0.03M Tris, 0.19M Glycine, and 0.1% w/v SDS. Buffer of identical composition was added to the upper reservoir and electrophoresis was performed at 10°C using 5 milliamperes/gel . Electrophoresis was stopped either when the bromophenol blue tracking dye was within 5 mm of the bottom of the gel or, in the case of 15% separation gels, 60 min after the dye front had migrated off of the gel. Protein bands were detected through staining with Coomassie Brilliant Blue R-250 or by silver staining (Tsai and Frasch, 1982). For the Coomassie Blue stain, the gels were washed to remove SDS and stained with Coomassie Brilliant Blue R250 (0.2%w/v). Both operations were done at 45°C for 60 min in an aqueous solution of 25%v/v isopropanol and 10%v/v acetic acid. Destaining was performed at 45°C in 7%v/v acetic acid.

Immunoblot protocol

The protocol used was essentially that of Towbin et al. (1979). Samples were electrophoresed in duplicate; one set was then stained with Coomassie Brilliant Blue R-250, while the other set was used for the immunoblot. Comparison of the resultant patterns permitted

the localization of immunodetectable species in the corresponding stained sample.

Following SDS-PAGE, polypeptides were electrically transferred (50V, 90 min, 10°C) to a pre-wet (in Phosphate buffered saline, PBS) nitrocellulose support . The nitrocellulose was then washed using blot buffer [150mM NaCl, 50mM Tris, 5.0mM Ethylenediaminetetraacetic acid, 0.05% v/v Nonidet P-40, 1.0% w/v Bovine serum albumin (BSA); final pH 7.4]. This and all subsequent operations were performed for 12 h at room temperature with the nitrocellulose enclosed in a heat-sealed plastic sleeve. Even distribution of liquid over the surface of the nitrocellulose was ensured by use of a rotatory platform. The following steps were then done sequentially: replacement of the wash fluid with a 1:1000 dilution in blot buffer of absorbed hyperimmune antispinin antiserum; blot buffer wash; replacement of wash fluid with a 1:1000 dilution in blot buffer of a goat antirabbit IgG-horseradish peroxidase conjugate; blot buffer wash. After the final wash, immunoreactive species were visualized in a horseradish peroxidase-catalyzed reaction utilizing 4-Chloro-1-Napthol as the substrate. The reaction was terminated by transfer of the nitrocellulose to PBS. The blot was photographed immediately using Contrast Process Ortho film.

Preparation of hyperimmune antispinin antisera

Mature white rabbits of approximately 1.0 kg body weight were used. Prior to the injection series a small volume of blood was collected from each rabbit. The preimmune serum was separated and stored at -20°C. Each rabbit was injected at four sites (flank and shoulder) with a total of 200 ug sonicated spinae in 0.85% v/v saline (diluted 3:1 using Complete Freunds' adjuvant). After 30, 68, and 120 days the rabbits were injected at two sites (shoulder and groin) with a total of 100 ug spinae in 0.85% v/v saline. One week following each injection the rabbits were bled, and the serum antispinin titer established by ELISA (see below). For the final serum collection the rabbits were exsanguinated.

Absorption of cross-reactive serum antibodies

Envelopes of E. coli K12 strain AB264 and P. aeruginosa PAO1 were added to a 1:100 PBS dilution of hyperimmune antispinin antiserum. The final protein concentration of each envelope preparation was approximately 30 ug.mL^{-1}. In most procedures intact, unspined MPD71 cells were also added to an equal protein concentration (such antiserum is here after described as fully absorbed). The suspensions were incubated at 34°C for 60 min with periodic mixing after which the envelopes and cells were recovered by

centrifugation (12,000 x g, 10 min, 24°C). This cycle was repeated three more times. Following the final wash the supernatant was removed and centrifuged at 40,000 x g, 20 min, 4°C. The resulting supernatant was stored at -20°C.

Enzyme-linked immunosorbant assay (ELISA)

The assay was performed in 96 well tissue culture plates. Wells were coated with approximately 1.0 ug of spinin by the application of 200 uL of a sonicated spinae suspension (5 ug protein.mL^{-1}) in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, 3mM NaN_3, final pH 9.6). The antigen adsorbed to the wells during an overnight incubation at 4°C. (In this and subsequent incubations, the plates were in a moistened chamber in order to minimize evaporation). Unbound antigen was removed by washing; this and subsequent washes were done three times, 3 min each time, using PBS containing 0.001% (v/v) Tween-20 (PBST; final pH 7.4). Following the final wash 200 uL of diluted antiserum (in PBS- 1% w/v BSA) was added to each well. The initial dilution was 1:50, with 1:5 serial dilutions thereafter. Blank wells received only the diluent. A 2 h incubation period preceeded washing. All the wells, including the blanks, next received 200 uL of a 1:1000 dilution (in PBST) of a goat antirabbit IgG-alkaline phosphatase conjugate. Unbound conjugate was removed by washing after 2.5 h. An alkaline phosphatase-catalyzed reaction was initiated by the addition to each well of 200 uL of p-nitrophenyl

phosphate (1.0 mg.mL^{-1} in 3mM NaN_3 - 10% v/v diethanolamine, final pH 9.8). The absorbance (405 nm) of each well was recorded at 5 min intervals for a maximum of 30 min using a Titertek plate scanner. The end point of each sample dilution series was considered to be the lowest dilution whose absorbance approximated those of the background. The inverse of this value represented the antibody titer.

Purification of spinae

The purification regimen of Easterbrook and Coombs (1976) was employed without modification, or was modified as described subsequently. Overnight (14-16 h) cultures in spinae-permissive conditions (Easterbrook and Sperker, 1982) were harvested (6,000 x g, 15 min, 4°C) and the cells were washed and resuspended in 0.15M NaCl. Spinae were detached by blending in two 30 sec pulses with an intervening 30 sec period. The cells were removed by centrifugation (as above); the supernatant was recovered and centrifuged twice more as above. The final supernatant was then centrifuged at 65,000 x g for 30 min at 4°C. The sediment was resuspended in 0.15M NaCl and centrifuged at 65,000 x g for 60 min. The pellet was retrieved and resuspended in deionized distilled water, sodium azide was added to a final concentration of 1% (w/v) and the suspension stored at 4°C. The final preparations were free of contaminating polypeptides and lipopolysaccharide, as

judged by electron microscopic and SDS-PAGE analyses, and by the absence of 2-keto-3-deoxyoctonate (Weissbach and Hurwitz, 1959; see below).

Negative contrasting, enumeration of MPD71 populations, and electron microscopy

Samples were negatively-contrasted following their adsorption to carbon-shadowed, Formvar-coated 200 mesh copper grids by the application of two drops of aqueous 2% w/v uranyl acetate containing 50 ug·mL^{-1} of bacitracin as a spreading agent. This solution was removed by blotting each grid to a piece of Whatman #1 filter paper, and the grids were examined.

In those experiments in which the extent of spination of a population was determined, the following was performed. A small volume of the particular sample was withdrawn, a carbon-shadowed, Formvar-coated 200 mesh copper grid was floated on the drop for 5 min to allow for the adsorption of the cells to the support film, and negative-contrast was provided. At least 300 cells were examined in each sample, with cells scored as either unspined or spined. Those cells possessing only the base structure of the spina, as judged by the appearance of a ring on the cell surface (Easterbrook and Alexander, 1983), were scored as spined.

All samples were examined using a Philips EM300 electron microscope operating at 60 kV. Images were recorded on Fine Grain Positive film (Eastman Kodak, Rochester, NY).

Morphological transition of MPD71

The physiological parameters that are permissive and restrictive for spination are known (Easterbrook and Sperker, 1982). Overnight MPD71 cultures in complex media were generated under conditions restrictive for spination. These cultures were enumerated (as described in the preceeding section) before use in order to ensure that each population was totally unspined. Inocula from such cultures were shifted by dilution (1:3 with respect to volume, Easterbrook and Alexander, 1983) into pre-warmed (34°C) complex medium, pH 7.4 and incubation continued under spina-permissive conditions (Easterbrook and Sperker, 1982). The cultures were monitored electron microscopically for their extent of spination at frequent intervals there after.

In one experiment, the effect of envelope perturbants on the growth, spination, and polypeptide profile of MPD71 was assessed. Overnight cultures were grown in the complex medium under spinae-restrictive conditions. After enumeration to ensure that this culture was totally unspined, aliquots were diluted (1:3) in pre-warmed (34°C) complex medium and incubated under spina-permissive conditions (section 3.3). One culture was monitored electron microscopically at intervals thereafter to determine the percentage of cells possessing spinae. When approximately 40% of the cells in this culture possessed

spinae, the envelope perturbants were added to the media to the following final concentrations: Phenethyl alcohol (PEA; 0.01, 0.05, 0.1, 0.5% v/v), Procaine (2, 5, 10, 20uM), Carbonyl cyanide m-chlorophenyl hydrazone (CCCP; 1, 5, 10, 20uM), Absolute ethanol (0.001, 0.005, 0.01, 0.1% v/v). These compounds were prepared as stock solutions just prior to use: PEA (1.0% v/v in the complex medium pH 7.4), Procaine (10mM in the same type of medium), CCCP (0.1mM in absolute ethanol; the resultant maximum ethanol concentration in treated samples was 0.001% v/v), Absolute ethanol (Consolidated Alcohols, Toronto, ONT).

The culture which had been enumerated was left untreated. Both it and the remaining treated cultures were incubated under spina-permissive cultural conditions for a further 60 min. Throughout the experiment the cultures were monitored spectrophotometrically (540 nm) and enumerated electron microscopically for the percentage of spined cells.

In another experiment the morphological transition of MPD71 populations in the presence of CCCP, and the absence or presence of chloramphenicol following removal of the CCCP-imposed spination block was assessed. Overnight, unspined MPD71 populations were shifted by dilution (1:3) into six volumes of complex medium pH 7.4 and incubated under spina-permissive conditions. One of the cultures was enumerated electron microscopically frequently thereafter to determine the percentage of cells possessing spinae.

When approximately 10% of this population was spined, the remaining cultures received CCCP to final concentrations of 1,2,2,2, and 5uM. Incubation continued until the untreated population was totally spined. All the cultures were then harvested and "Buffer"-washed two times. The untreated cells, and those treated with 1,2, and 5uM CCCP were resuspended in 500 uL "Buffer" and saved. The remaining two 2uM CCCP-treated cultures were resuspended in 4 mL of the complex medium, pH 7.4. In one, chloramphenicol was added to a final concentration of 20 $ug \cdot mL^{-1}$. Both cultures were incubated under spina-permissive conditions for 2h.

All the cultures were monitored with respect to their growth (as judged by their optical density measurements at 540nm) and enumerated electron microscopically to determine the percentage of spined cells.

SDS-PAGE of MPD71 during the morphological transition

Unspined cells were shifted to spina-permissive conditions as described previously. 1.0 mL aliquots were withdrawn at regular frequent intervals thereafter. A portion was negatively stained and the proportion of cells which possessed spinae determined by electron microscopic enumeration. The remainder of each suspension was centrifuged to recover the cells (10,000 x g, 5 min, room temperature using an Eppendorf microcentrifuge). Each pellet was resuspended in 500uL Buffer; 100 uL provided material for determination of total protein (Lowry et al.,

1951). Volumes containing approximately 30 ug protein were centrifuged (as above), the cells were disrupted by suspension in 20 uL electrophoretic disruption buffer at 100°C for 5 min, and SDS-PAGE was performed. Samples were electrophoresed in duplicate sets; one set was visualized using Coomassie Brilliant Blue R-250 while the other was immunoprobed with hyperimmune antispinin antiserum.

SDS-PAGE of PEA-treated MPD71 populations

An overnight MPD71 culture was generated under conditions restrictive for spination. After being enumerated to ensure the absence of spinae, aliquots of this population were diluted (1:3) into pre-warmed (34°C) complex medium, pH 7.4. One of the cultures was left untreated. The remaining three cultures immediately received PEA to final concentrations of 0.02, 0.04, or 0.08% v/v. The PEA was prepared as a stock 1% v/v solution in the complex medium just prior to use. All the cultures were then incubated under spina-permissive conditions for 3 h. They were harvested and the OMs isolated using Sarkosyl and lysozyme as described subsequently. The OMs were assayed for total protein (Lowry et al., 1951) and amounts containing approximately 30 ug protein were electrophoresed in duplicate sets on a 12% w/v acrylamide gel. One set was stained using Coomassie Brilliant Blue R250 and the other was electrically transferred to nitrocellulose and probed using fully absorbed hyperimmune antispinin antiserum.

Effect of CCCP on the polypeptide profile of E. coli

Three cultures, grown to equal cell densities (0.80 at 540 nm) were each separated into four aliquots. These were harvested, washed, and resuspended in 500 uL pre-warmed (34°C) complex medium, pH 6.8. To each suspension ^{35}S-Methionine was added to a final activity of 20 uCi.mL^{-1}. After 5 min at 34°C, three suspensions from each set received CCCP to final concentrations of 1,2,or 5uM. The remaining suspension remained untreated. Incubation continued for a further 30 min at which time all the samples were harvested, "Buffer"-washed twice, and resuspended in 200 uL of "Buffer". 20 uL of each suspension was added to 0.5 mL of a protein solubilizer (Protosol, New England Nuclear) and 10 mL of an aqueous scintillation counting fluid (Aquasol-2, New England Nuclear) was added. Samples were quantified for incorporated radiolabel and volumes containing 100,000 cpm were electrophoresed. The gels were soaked for 60 min in a fluorographic enhancer (EnHance, New England Nuclear), washed with distilled water, and vaccum-dried. The dried gels were exposed to X-ray film (XAR-5, Kodak) for 24 h at -70°C prior to development and photography.

Transient, nonlethal nature of the CCCP effect on spina assembly

Unspined MPD71 cultures were shifted to conditions permissive for spination, and treated with CCCP (5uM) as described previously. Incubation continued for a further 4 h; absorbance measurements at 540 nm and electron microscopic enumeration of the extent of spination were conducted throughout the procedure.

In another procedure, unspined cultures were generated by overnight (15 h) growth in complex medium pH 6.8 supplemented with 1,2,or 5uM CCCP. Absorbance readings were recorded at 0 h and 15 h. The cultures were harvested and washed twice using pre-warmed complex medium, pH 7.4. Each final pellet was resuspended in 4mL of complex medium, pH 7.4 and incubated under conditions permissive for spination. Cultures were frequently monitored thereafter for their extent of spination as described previously.

The immediacy of the CCCP effect on spination

Triplicate aliquots of an overnight, unspined MPD71 culture were shifted to spina-permissive conditions. Following the observed onset of spination, CCCP concentrations of 5uM were established at three times post-shift. The extent of spination at the time of CCCP

addition, and up to two hours thereafter, was determined by electron microscopic enumeration.

Chloramphenicol cessation of protein synthesis

Aliquots from an overnight, unspined D71 culture were shifted by dilution into nine portions of the complex medium, pH 7.4 (total volume, 6 mL). Incubation under spina-permissive conditions followed. One of the cultures was enumerated for the percentage of spination at frequent intervals thereafter. When approximately 20% of the cells in each culture possessed spina, ^{35}S-Methionine was added to each culture to a final activity of 20 uCi.mL^{-1}. Incubation resumed for 15 min prior to the addition of chloramphenicol (prepared as a 5 mg.mL^{-1} stock solution in absolute ethanol) as follows: sets of three cultures received final concentrations of 20 and 100 ug.mL^{-1}. The remaining set did not receive chloramphenicol. All the cultures were reincubated and harvested after 1,5,and 15 min; the untreated cultures were harvested after either 1,5, or 15 min. Thus, at every time point an untreated sample was recovered as well as samples treated with both concentrations of chloramphenicol. Samples were "Buffer"-washed twice and solubilized by suspension in 500 uL Protosol. Duplicate 20 uL volumes were added to 10 mL of aqueous scintillation fluid and incorporated radiolabel was quantified.

Extraction of the cell envelope

A procedure similar to that detailed by Beveridge and Kovál (1981) was used. Cultures were harvested (6,000 x g, 15 min, 4°C) at an optical density (540 nm) corresponding to a mid logarithmic phase of growth (approximately $2x10^9$ cells.mL^{-1}). Each sample was "Buffer"-washed by centrifugation (as above) and resuspended in 19.4 mL of Buffer. Deoxyribonuclease (1930 Kunitz units.mg^{-1} solid), ribonuclease-A (72 Kunitz units.mg^{-1} solid), and phenyl methylsulfonyl fluoride were added to final concentrations of 50 ug.mL^{-1}, 100 ug.mL^{-1}, and 1.0mM respectively (0.2 mL each of 100x stock solutions in "Buffer"). Cells were broken by passage twice through a cold French Pressure Cell operating at 15,000 $lb.in^2$. The absence of intact cells was verified by electron microscopic examination. Following a 60 min incubation at room temperature, digested nucleic acid was removed by four "Buffer" washes (40,000 x g, 20 min, 4°C). Each wash supernatant was monitored for nucleic acid at 260 nm. The final pellet was resuspended in 5 mL of "Buffer" and stored at -20°C.

Outer membrane (OM) isolation

A) Treatment of cell envelopes with detergent and lysozyme

The protocol was adapted from those described by Schnaitman (1971) and Filip et al. (1973). Envelopes were suspended to a final protein concentration of approximately 8 $mg.mL^{-1}$ in 2-3 mL of 0.5% w/v Sodium lauroyl sarcosinate (Sarkosyl)-10mM HEPES pH 6.8. The suspension was agitated for 30 min at 30°C and the Sarkosyl-insoluble material recovered at 40,000 x g, 20 min, 4°C; the supernatant was either retained or discarded. The insoluble material was suspended in 5.0 mL of a lysozyme solution (100 $ug.mL^{-1}$ in 10mM HEPES pH 6.4) and gently stirred at room temperature for 60 min. Lysozyme-insoluble material was recovered by centrifugation (as above but for 60 min), "Buffer"-washed, resuspended in 2-3mL "Buffer", and stored at -20°C.

B) Use of the regimen of Osborn et al. (1972_b)

The procedure was performed essentially as described by Osborn et al. (1972_b) and so is only briefly described here. Lysozyme and EDTA were added to an iso-osmotic suspension of cells to generate spheroplasts. These were lysed osmotically and membraneous material recovered at 40,000 x g, 30 min, 4°C. Ultracentrifugation (100,000 x g, 14 h, 4°C) employing a linear 40 - 60% w/v sucrose gradient achieved separation of membrane fractions of varying densities. Gradients were constructed by layering volumes of 60,55,50,45,and 40% w/v sucrose over a 1/4 volume cushion of 65% w/v sucrose. Linearity developed by diffusion during a 4°C, 20 h storage prior to use. Bands

were visualized by refracted light and collected by side puncture of the tube. A series of deionized distilled water washes (80,000 x g, 60 min, 4°C) removed the sucrose from each sample. Each sample was resuspended in 500 uL "Buffer" and stored at -20°C. SDS-PAGE of volumes containing approximately 30 ug protein was performed as described previously. The separated proteins were stained using Coomassie Brilliant Blue R250.

In one experiment, 0.25 mL fractions were collected following puncture of the bottom of the tube, and each was monitored at 280 and 260 nm.

The effect of detergents on the MPD71 OM protein profile

A) Sarkosyl-lysozyme insoluble material (containing approximately 20 ug protein) was suspended in 25% w/v sucrose and layered onto a sucrose gradient identical to that described above. A similarly prepared gradient received only sucrose. Ultracentrifugation (as above) was performed and 20 drop fractions were collected by bottom puncture of the tube. The density of the control fractions was determined by refractive index measurements. The protein content of sample fractions was determined spectrophotometrically (Warburg and Christian, 1942).

B) MPD71 envelope suspensions, each containing approximately 100 mg protein, were centrifuged (40,000 x g, 30 min, 4°C) and resuspended in 10 mL of the following:

- 10mM HEPES pH 6.8 (A)
- 10mM HEPES pH 6.8 - 1.0mM $MgCl_2$ (B)

- 0.5% w/v Sarkosyl in (A)
- 0.5% w/v Sarkosyl in (B)
- 0.5% w/v Sarkosyl in (A) containing 1.0mM EDTA
- 2.0% v/v Triton X-100 in (A)
- 2.0% v/v Triton X-100 in (B)

Each suspension was treated with lysozyme and the insoluble material was recovered as described above, resuspended in 200 uL Buffer and assayed for total protein (Lowry et al., 1951). Amounts containing 30 ug protein were electrophoresed and the separated proteins stained using Coomassie Brilliant Blue R250.

Removal of spinae from MPD71 cells

Willison et al. (1977) and Steven Alexander (MSc thesis, Dalhousie University, 1982) described a procedure in which spina were amenable to mechanical removal following application of pronase. While effective, the procedure can promote lysis of a substantial portion of the population. In order to preserve the integrity of as many cells as possible for subsequent isolation and SDS-PAGE of the "despined" OM, the following regimen was applied.

MPD71 populations obtained by an overnight culture under spina-permissive conditions were recovered at 6,000 x g, 15 min, 4°C. Resuspension in 0.5% w/v paraformaldehyde (in "Buffer") for 45 min at room temperature was followed by a low-force, short-time (1,000 x g, 60 s) centrifugation to remove suspended particles. A higher force centrifugation

(6,000 x g, 15 min, 4°C) recovered the cells and a further two "Buffer" washes removed excess fixative. Cells from the final wash were resuspended in 2.0 mL of "Buffer" containing 1%w/v BSA for 30 min at room temperature in order to block accessible paraformaldehyde residues. Excess albumin was removed by two "Buffer" washes (as above) and the cells were resuspended in 2.0 mL of pre-warmed (34°C) "Buffer" containing Pronase (final concentration of 1.0 $mg.mL^{-1}$). Suspensions were maintained at 34°C for 20 min and periodically vortexed. The cells were recovered by centrifugation (6,000 x g, 15 min, 4°C) and resuspended in 1 mL of "Buffer".

SDS-PAGE of unspined, spined, and "despined" cells and OMs

Sarkosyl-lysozyme insoluble fractions were obtained from unspined cells as well as from cells prior to, and following removal of the spinae. The cells and OM fractions (approximately 30 ug total protein) were electrophoresed using a 12% w/v acrylamide slab gel. Samples were either stained using Coomassie Blue or probed in an immunoblot using fully absorbed hyperimmune anti-spinin serum.

Immuncytochemistry of whole cells

A regimen modified from that described by Roth (1981) was used. MPD71 cultures were shifted by dilution from spina-restrictive to permissive conditions. At intervals after the shift aliquots were withdrawn, negatively

contrasted, and examined electron microscopically to ascertain the percentage of the enumerated population which was spined. When the proportion of spined cells in the cultures was approximately 20%, each culture was harvested (6,000 x g, 15 min, 4°C) and resuspended in 1-3 mL "Buffer" containing 0.5% w/v paraformaldehyde. Samples were incubated at 24°C for 45 min and washed free of excess fixative in a series of three "Buffer" washes (these and subsequent washes were performed at 6,000 x g, 10 min, 24°C). The final pellet was resuspended in 100 uL of 1:100 or 1:500 dilutions in "Buffer" of unabsorbed preimmune serum or fully absorbed hyperimmune antispinin antiserum, respectively. Following a 60 min incubation period at 24°C, unbound antibody was removed by three "Buffer" washes. The final pellet was resuspended in 100 uL of a 1:500 dilution in "Buffer" of a 5 nm diameter colloidal gold bead-goat anti-rabbit IgG conjugate (hereafter referred to as probe). Incubation and washes were conducted as described above and the final pellet was resuspended in 200 uL "Buffer". Carbon-reinforced, Formvar-coated 200 mesh copper grids were floated on drops of each suspension to allow for adsorption of the cells to the support film. Each grid was then washed under a stream of deionized distilled water. The cells were negatively-contrasted and excess stain was blotted off. In certain control samples the cells were exposed only to the probe.

Immunocytochemistry of MPD71 ultrathin sections

Aliquots from the same MPD71 populations described in the preceeding section were collected following the paraformaldehyde and washing steps. These samples were post-fixed in osmium tetroxide (1% v/v in 10mM HEPES pH 6.8) for 60 min at room temperature, dehydrated using a graded ethanol series (also at room temperature), embedded in Epon 812, and polymerized at 45°C.

In another procedure cells from unspined and spined populations were embedded in Lowicryl K4M. The regimen employed was modified from of Armbruster et al. (1982). Cultures received paraformaldehyde (1% v/v in sodium cacodylate pH 7.4) to a final concentration of 0.05% v/v at the time of harvest. Harvested cells (6,000 x g, 15 min, 4°C) were resuspended in the stock fixative and maintained for 30 min at room temperature. The cells were recovered by centrifugation at 5,000 x g, 5 min, room temperature (using an Eppendorf microfuge in this and subsequent centrifugations in this section), resuspended in cacodylate buffer for 2 min, again recovered by centrifugation, and resuspended in the cacodylate buffer in two 15 min changes at 4°C. Dehydration and infiltration of the samples were carried out as described subsequently. The cells were recovered by centrifugation in between the steps. The steps were:

30% v/v ethanol at 4°C for 5min; 50% v/v ethanol, (same temperature and time); 70% v/v ethanol, (same temperature and time); 90% v/v ethanol, (same temperature, 30 min); 90% v/v ethanol-Lowicryl K4M (1:1)- 60 min at -10°C; 90% v/v ethanol-Lowicryl K4M (1:2)- 60 min at -10°C; 100% Lowicryl K4M, 60 min at -15°C; 100% Lowicryl K4M overnight at -15°C.

The infiltrated samples were transferred to a Beam capsule, filled with fresh resin, and capped immediately in order to minimize exposure of the resin to the air. The resin was polymerized by ultraviolet radiation (260 nm) at -20°C for approximately 72 hours.

Ultrathin sections (approximately 50 nm) were cut using a Porter-Blum ultramicrotome equipped with either a glass knife (for the Epon-embedded samples) or a diamond knife (for the Lowicryl-embedded samples). Sections were collected on uncoated 400 mesh nickel grids. Each grid was floated on a drop of 1% w/v BSA-0.1% v/v Triton X-100 in PBS pH 7.4 for 10 min. This and all subsequent operations were done at room temperature under a petri dish lid. The presence of a piece of moistened filter paper minimized evaporation-related concentration change in antibody or probe. The grids were briefly transferred to a drop of PBS and then to a drop of diluted unabsorbed preimmune serum or fully absorbed hyperimmune antispinin antiserum (1:100-1000 or 1:500-5000, respectively, in PBS). Certain grids were floated on a drop of PBS. All the grids were incubated for 60 min as described above. Unbound antibody was then

removed in the following wash series (all subsequent wash series were performed this way as well): "jet wash" under a stream of PBS, float grid on a drop of PBS for 3 min and "jet wash" using PBS. After the final wash each grid was transferred to a drop of a 1:100-4000 dilution of the probe and incubated for 60 min. Another wash series removed unbound probe molecules. Each grid was "jet washed" using deionized distilled water and contrasted by application of two drops of aqueous 1% w/v uranyl acetate. Excess stain was removed using filter paper and the samples were examined electron microscopically.

Extraction of lipopolysaccharide

MPD71 and E. coli cells were resuspended in 10mM HEPES pH 6.8 to an absorbance (540 nm) of 0.20. 1.0 mL of each suspension (approximately 2 x 10^9 cells) was centrifuged in an Eppendorf microfuge (10,000 x g, 5 min) and the supernatants were discarded. Each pellet was solubilized at 100°C in 50 uL of electrophoretic dissociation buffer containing 50mM dithiothreitol. Proteinase K was added to a final concentration of 200 $ng.uL^{-1}$ (10 uL of a 1.0 mg.mL-1 aqueous stock). Digestion occurred during a 2 h incubation at 60°C and was terminated by boiling. Aliquots of 10 uL were analyzed by SDS-PAGE, employing a 15% acrylamide slab gel. Proteinase K-insoluble material was visualized by silver chloride deposition (Tsai and Frasch, 1981).

Assay for 2-keto, 3-deoxyoctonic acid (KDO)

The procedure described by Weissbach and Hurwitz (1959) was employed. A KDO stock solution (0.08mM in 0.018N H_2SO_4) was used to construct a standard concentration series in water (0-0.8 umol KDO.mL^{-1}) (total volume 100 uL). Aliquots (100uL) of each sample were assayed; an equal amount was likewise assayed for total protein cencentration (Lowry et al., 1951). Thus, regardless of the initial sample concentration, KDO concentrations were expressed as umol KDO.mg^{-1} protein. As a positive control, E. coli K12 strain AB264 envelopes were used.

RESULTS

Evaluation of the antisera

Many experiments in this investigation utilized antispinin antiserum. Thus, it was necessary to demonstrate the specificity of the antiserum towards spinin. Preimmune serum was not immunoreactive towards the envelope of morphologically spined cells (Fig 2B, lane 1) or purified spinin (lane 4 in panel B). In contrast, several envelope proteins from E. coli K12 strain AB264 and Pseudomonas aeruginosa PAO1 were immunodetectable (Fig 2B, lanes 2 and 3). Use of hyperimmune antispinin antiserum (prepared in the same rabbit against sonically-dissociated spina) with comparable samples produced the immunoblot pattern shown in Fig 2A. Spinin was readily immunodetectable (lane 4). A species of similar electrophoretic mobility was evident in the spined envelope sample (lane 1), as were two other species. The MWr's of these two species were not estimated. In the lanes containing E. coli and P. aeruginosa envelope material, numerous immunoreactive proteins were evident. These E. coli and Pseudomonas species were eliminated from subsequent immunoblots by absorption of the antiserum using envelopes from the same strains (not shown).

Comparable antiserum was evaluated using the enzyme-linked immunosorbant assay (ELISA) regimen. Table 4 shows the antispinin antibody titers obtained with the pre- and hyperimmune sera. The preimmune serum had a negligible

Fig 2. Immunoblot examination of purified spinin and the cell envelopes of Escherichia coli K12 strain AB264, Pseudomonas aeruginosa PAO1, and morphologically spined MPD71 cells. The samples were examined using hyperimmune antispinin serum (panel A) and preimmune serum (panel B) from the same rabbit. The antiserum had been prepared to sonically-disrupted spina. Lanes in both panels contain: (1) MPD71 envelope; (2) AB264 envelope; (3, PAO1 envelope; (4) Spinin. Envelopes were applied as 30ug total protein and spinin as 5ug protein. A 12%w/v acrylamide gel was used.

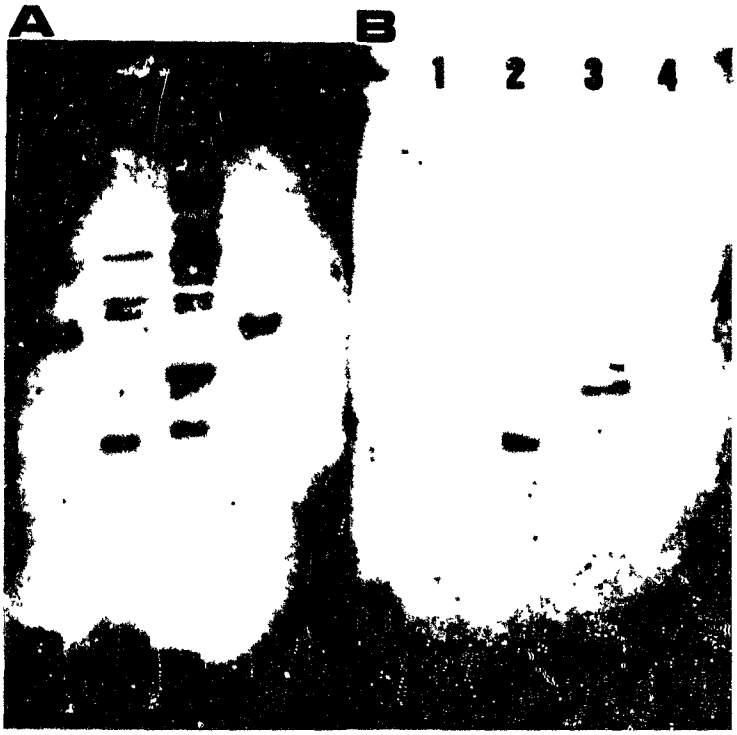

A
B
1 2 3 4

Table 4 Antispinin antibody titer as determined by the enzyme-linked immunosorbant assay[a,b]

SAMPLE	TITER (x 10^6)
Preimmune serum	0.003
Hyperimmune antispinin antiserum	
- unabsorbed	3.9
- absorbed with K12 and PAO1 envelope	3.9
- absorbed with K12 and PAO1 envelope and MPD71 unspined cells	0.78
- absorbed with MPD71 unspined cells	3.9

For each absorption procedure, the antiserum was incubated at 34°C for 60 min in the presence of 30 $ug.mL^{-1}$ of the envelopes and/or unspined cells. The absorption process was repeated four times. The supernatant from the final treatment, following removal of the envelopes and/or cells, was used in the ELISA determinations.

a titer is the reciprocal of the highest dilution greater in absorbance than the blank control

b the data represents the average of three separate determinations for each sample

antispinin antibody titer while the hyperimmune antiserum had a high titer. Absorption of cross-reactive antibodies from the hyperimmune antiserum using E. coli and P. aeruginosa envelope did not alter the titer. Inclusion of D71 unspined cells in the absorption mixture reduced the titer only slightly (Table 4). Thus, cross-reactive antigens could be removed from the antiserum without appreciably affecting the antispinin antibody titer.

Physiological parameters influencing spination

Production of certain OM polypeptides is influenced by pH (Heyde and Portalier, 1987; Taglicht et al., 1987), temperature (Craig and Jacobsen, 1985; Kropinski et al., 1987), and salinity (Van Alphen and Lugtenberg, 1977; Kawaji et al, 1979; Barron et al, 1986). The effect of these physiological parameters on spination was previously examined (Easterbrook and Sperker, 1982) in a morphological study. These observations were confirmed and extended in the present study. It was confirmed that extremes of pH and salinity determined whether the population was spined or not (Table 5). Alteration of individual parameters resulted in a variable response of the populations with respect to spination (Table 5). SDS-PAGE of the same samples demonstrated that while the overall polypeptide profiles were similar, there were certain changes in staining intensity (Fig 3). In particular a species which migrated with the same electrophoretic mobility as purified spinin

Table 5 Percentage of enumerated MPD71 populations which possessed spina when cultured under varying parameters of pH, temperature, and salinity

GROWTH PARAMETERS		#[1] UNSPINED CELLS	# SPINED CELLS	TOTAL #	% SPINED
pH 6.8, 22°C					
NaCl	0.1M	230	20	250	8
	0.2M	290	10	300	3
	0.3M	293	0	293	0
	0.4M	287	0	287	0
pH 6.8, 34°C					
NaCl	0.1M	32	268	300	89
	0.2M	152	148	300	49
	0.3M	268	32	300	11
	0.4M	291	9	300	3
pH 7.4, 22°C					
NaCl	0.1M	113	187	300	62
	0.2M	263	32	300	11
	0.3M	300	0	300	0
	0.4M	288	12	300	4
pH 7.4, 34°C					
NaCl	0.1M	3	297	300	99
	0.2M	48	252	300	84
	0.3M	155	145	300	48
	0.4M	166	144	300	48

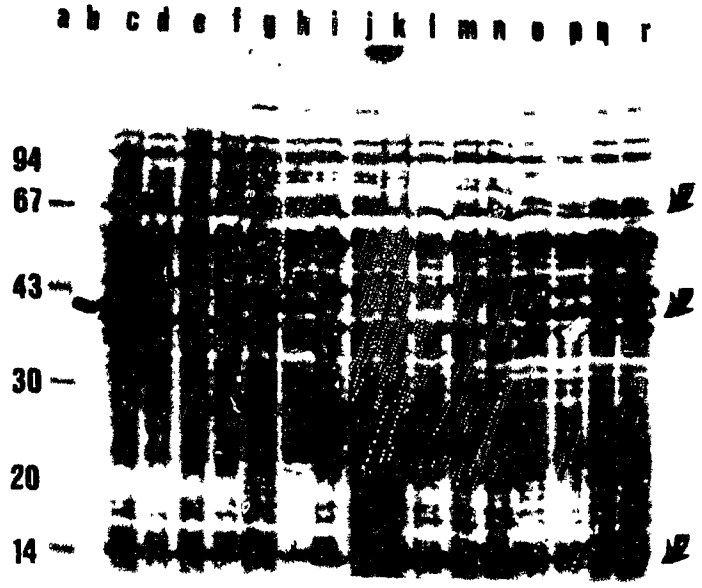
1 #=number
The percentage of the populations which possessed spina were determined by electron microscopy of negatively-contrasted cells. Cells which possessed spina or the base structure of nascent spina were scored as spined. At least 250 cells were examined in each sample.

Fig 3. Electropherogram of MPD71 whole cell lysates following growth in a defined medium (Easterbrook and Sperker, 1982) under different physiological parameters. A 10-14%w/v acrylamide gradient gel was used. All samples were applied as 30ug total protein, except for spinin which was applied as 5ug total protein. Lane a contains MWr standards whose values (in kilodaltons, kD) are shown to the left of the figure. In this and all subsequent gels the standards used were: phosphorylase b (94kD), bovine serum albumin (67kD), ovalbumin (43kD), carbonic anhydrase (30kD), soybean trypsin inhibitor (20kD), and lactalbumin (14kD).

Samples: (a) MWr standards; (b) Spinin; Lanes c-f contain lysates from cultures grown at 22°C, pH 6.8, and 0.1M (c) 0.2M (d) 0.3M (e) 0.4M NaCl (f); Lanes g-j contain lysates from cultures grown at 34°C, pH 6.8, and 0.1M (g) 0.2M (h) 0.3M (i) 0.4M NaCl (j); Lanes k-n contain lysates from cultures grown at 22°C, pH 7.4, and 0.1M (k) 0.2M (l) 0.3M (m) 0.4M NaCl (n); Lanes o-r contain lysates from cultures grown at 34°C, pH 7.4, 0.1M (o) 0.2M (p) 0.3M (q) 0.4M NaCl (r).

The polypeptides were stained using Coomassie Brilliant Blue R250.

Fig 3.



(approximately 42kD), and polypeptides of about 65kD and 14kD were affected (Fig 3). The content (based on the staining intensity) of the 65kD and 14kD species was increased when the level of the 42kD species was reduced. The 42kD species was detectable in all lysates. However, since staining of this protein in the unspined samples was weak, it was presumably present in a low amount (Fig 4, compare lanes d,f,and g).

Thus the physiologic response by MPD71 affected the content of at least the 65, 42, and 14kD polypeptides. In subsequent experiments only pH and temperature were altered. Maintaining a constant osmotic pressure (Na+ concentration) assured a total population transition from the unspined to the spined phenotype. Only occasionally was a less than complete phenotypic transition observed.

Analyses of MPD71 populations during the phenotypic transition

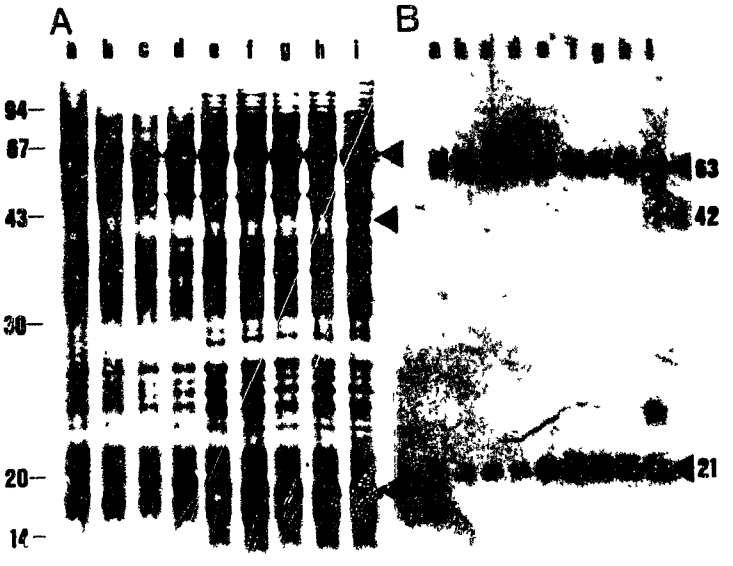
The behaviour of the 65, 42, and 14kD species during the unspined to spined phenotypic transition was investigated next. Cultures grown overnight under spina-restrictive conditions were shifted by dilution (Easterbrook and Alexander,1983) to conditions permissive for spination. Cells obtained at frequent intervals during the transition were dissociated and electrophoresed. The polypeptides were then either stained using Coomassie Brilliant Blue R250 or probed with antispinin antiserum in an immunoblot. The

Fig 4. SDS-PAGE protein profile and immunoblot pattern of MPD71 lysates following a shift from spina-restrictive to permissive conditions. A 10-14%w/v acrylamide gel was used. Panel A displays the protein profile and panel B displays the immunoblot pattern. The position of the MWr standards are shown to the left of the gel, and the positions of the 65, 42, and "low MWr" species are indicated by the arrowheads to the left and right of the figure.

Samples in both panels: (a) Unspined cell lysate; (b) 0.5h after the shift to spina-permissive conditions {post-shift}; (c) 1.0h post-shift; (d) 1.5h post-shift; (e) 2.0h post-shift; (f) 2.5h post-shift; (g) 3.0h post-shift; (h) 4.0h post-shift; (i) 15h post-shift.

The percentage of the enumerated populations which were spined were: Unspined lysate (0%); 0.5h (0%); 1.0h (0%); 1.5h (0%); 2.0h (7%); 2.5h (17%); 3.0h (33%); 4.0h (45%); 15h (100%). Although the samples should have been 30ug total protein, the resultant staining intensities indicated that equal amounts of protein were not applied to each lane.

Fig 4.



antiserum used in this experiment was absorbed with E. coli K12 and P. aeruginosa PAO1 envelope.

The results from this experiment is shown in figure 4. The 65kD species noted in the preceeding section was detectable throughout the transition period (Fig 4A and B) as was a species with a MWr of about 21kD. The apparent increase in the immunodetectable level of the 21kD species may be due to the inadvertant use of unequal sample concentrations. The MWr estimate of the 21kD species exhibited variation (21-25kD) throughout the study, perhaps due to minor variations in an electrophoretic parameter such as pH. For ease of referral this species will be here after designated as the "low MWr" species. The MWr variation of this species will be considered further in the Discussion.

The MPD71 population used in figure 4 displayed a prolonged unspined to spined transition period for undefined reasons. This was fortuitous since it demonstrated the visual (staining) and immunological detection of the 42kD species in the cell lysates only when a majority (in this case 100%) of the population possessed spina (Figs 4A and B). Although a weakly stained band with a similar mobility was evident in phenotypically unspined samples (Fig 4A,lane a), it was not immunologically detectable (Fig 4B,lane a).

The morphological appearance of cells during the unspined to spined transition was investigated next. MPD71

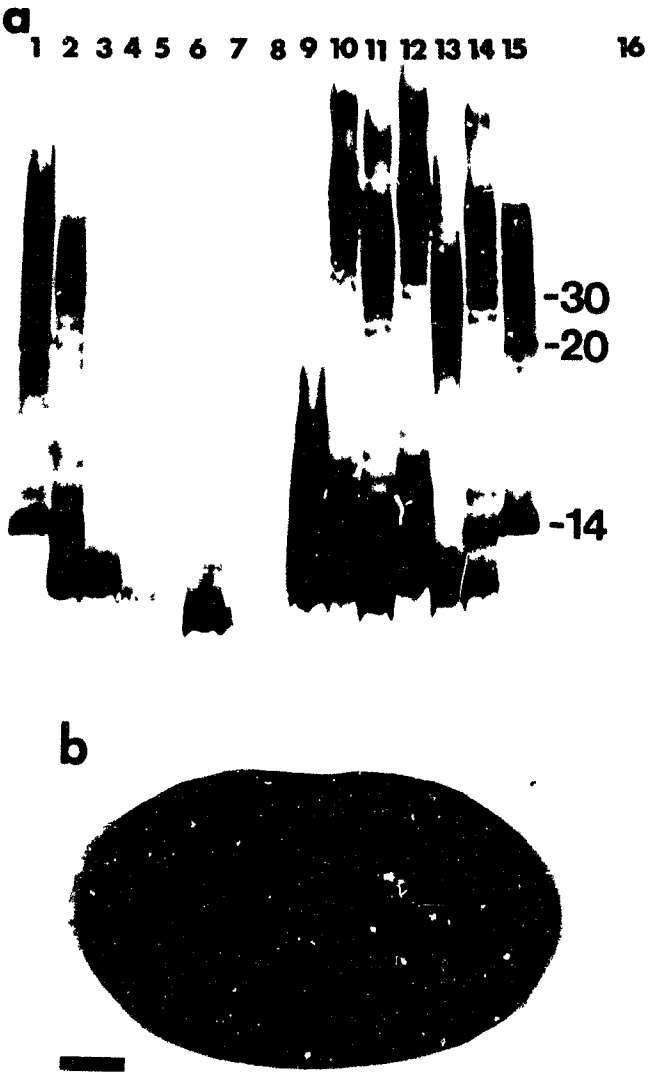
populations were harvested when approximately 40% of the cells in an enumerated sample possessed spinae. Of the remaining 60% of the cells, it is reasonable to suppose that some of them would have commenced production of spinin. These cells would still be, by definition, unspined (using the presence of spina as the criterion). This subset of the population would not be evident in the electrophoretic analysis, since the entire population was sampled in the SDS-PAGE procedure. Electron microscopy (specifically immunoelectron microscopy), might detect such a subset of cells.

A small diameter (5nm) goat antirabbit IgG-colloidal gold bead conjugate (probe) was used for the immunocytochemical procedures. The small diameter of the probe minimized the chances of steric hinderance of probe binding, especially in regions of the spina base. Despite its small diameter the probe was readily discernable when applied to a carbon-shadowed, Formvar-coated 200 mesh copper grid (Fig 5). The probe did not appear prone to aggregation. The possibility remained, however, that nonspecific aggregation or binding of the probe could occur due to the presence of a charged cell surface or appendages other than spina. To test these possibilies E. coli K12 strain AB264 and five E. coli clinical isolates obtained from the Victoria General Hospital were treated with probe. Strain AB264 has a "rough" LPS structure. In contrast, clinical isolates invariably possess "smooth" LPS

Fig 5. Electron micrograph of the 5nm diameter colloidal gold bead-goat antirabbit IgG probe. A carbon-reinforced, Formvar-coated 200 mesh copper grid was floated on a drop of a 1:500 dilution (in Phosphate buffered saline pH 7.4) of the probe and left for 5min to permit adsorption of the probe to the Formvar film. Unbound probe was washed off using deionized distilled water, the water was removed by blotting the grid on filter paper, and the grid was examined. No contrasting agent was applied. Scale bar = 100 nm.

Fig 5.

Fig 6. SDS-PAGE and immunoelectron microscopic analyses of E.coli O111B4, K12 strain AB264, and E.coli clinical isolates. Panel a shows a silver stained (Tsai and Frasch, 1982) 15%w/v acrylamide gel containing: (1 and 15) MWr standards whose values (kD) are shown to the right of lane 15; (2) LPS from E.coli O111B4 (10ug); (3-7) LPS species from S.minnesota, (3) Ra type, (4) Rb type, (5) Rc type, (6) Rd type, (7) Re type {Each of these LPS samples was applied as 1ug}; (8) Blank lane; (9) Proteinase K-insoluble material from E.coli K12 strain AB264.; (10-14) Proteinase K-insoluble material from clinical isolates 1-5, respectively; (16) Proteinase K in an amount (2ug) equivalent to that present in the digestion mixtures. The cell shown in Panel b was treated with a 1:100 dilution (in Phosphate buffered saline, pH 7.4) of probe. Panel b: A cell from the culture of clinical isolate 3. Scale bars = 100 nm.


a
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
-30
-20
-14
b

(Hitchcock et al, 1986). These LPS structures were confirmed by SDS-PAGE of the proteinase K-insoluble material (Hitchcock and Brown, 1983) from each sample (Fig 6a). The clinical isolates displayed the ladder-like arrangement (Fig 6a, lanes 10-14) given by O-polysaccharide side chains (Hitchcock et al, 1986). K12 strain AB264 displayed the pattern of a "rough" LPS (Fig 6a,compare lane 9 with the LPS standards in lanes 2 to 7).

Possession of "rough" LPS tends to increase the hydrophobic character of the cell surface (Nikaido, 1979). "Smooth" strains, in contrast, retain more of an anionic surface character (Beveridge, 1981). Following treatment with the probe, all the cells examined were devoid of the probe (an example is a cell shown in Fig 6b). Thus, cell surface charge had no influence on the binding of the probe. Fimbriae and flagella, when present, did not influence binding of the probe either (not shown).

Having demonstrated that the probe did not bind or aggregate in a nonspecific fashion, immunocytochemical analyses were conducted using MPD71 whole cells. The cells were obtained from populations in which about 40% of the cells possessed spina. As mentioned earlier, it is probable that some of the unspined cells in such populations would have already started to manufacture spinin. They had not yet begun to assemble spina, however.

All the cells remained undecorated in populations treated either with unabsorbed preimmune serum plus the

probe or solely with the probe (Figs 7 and 8). The cells shown in Fig 7 were not contrasted with uranium, in order to more unambiguously assess the presence or absence of the small diameter probe (Use of a larger diameter probe would have made this assessment easier. However, such a probe might have masked sites of spina base assembly). To confirm that the cell surface did indeed remain undecorated, some of these cells were embedded in Epon 812 and sectioned. As shown in Fig 9 the surface of both spined (panels a-c) and unspined (panel d) cells were either devoid of immunodecoration or only sparingly decorated.

Other cells from the same populations were treated with hyperimmune antispinin serum (which had been absorbed using E. coli and P. aeruginosa envelopes and unspined cells) plus the probe. Morphologically unspined cells remained undecorated (not shown). Morphologically spined cells in the same populations were, however, immunodecorated (Fig 10). Decoration of the spina shaft was extensive. The spina base was also decorated, but not as extensively (Fig 10). The remainder of the cell surface was not decorated. The specificity of the spina decoration was emphasized in those cells which had lost their cytoplasmic contents (Fig 11) and at higher magnifications (Fig 12). The lack of surface decoration is also emphasized in figures 11 and 12. In a few instances, a probe moiety was observed in association with an area of a spina where unwinding of the protomer helix had occurred (Fig 12a).

Fig 7. Electron micrographs of morphologically unspined cells obtained from a population where approximately 40% of the cells were spined. The cells were treated with dilutions (1:100 in Phosphate buffered saline pH 7.4) of unabsorbed preimmune serum and the probe (Panel a), or with the probe alone (Panel b). Negative contrast was not provided. The contrast observed was due to the samples. The arrows in both panels indicate probe moieties. Scale bars = 100 nm.

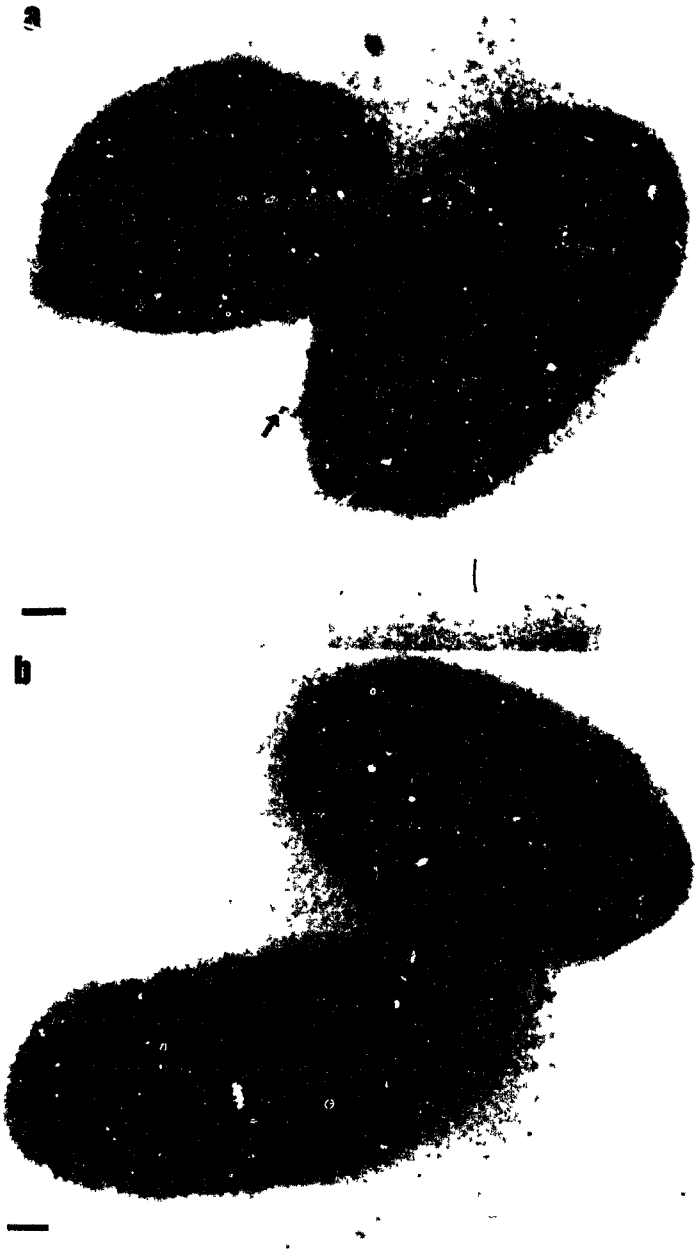

a
b

Fig 8. Electron micrographs of three morphologically spined cells resident in the 40% spined, 60% unspined populations following treatment with 1:100 dilutions (in Phosphate buffered saline pH 7.4) of unabsorbed preimmune serum plus probe (Panels a and b) or with an equivalent dilution of probe (Panel c). These samples, and all other negatively-contrasted samples were contrasted using aqueous 2%w/v uranyl acetate containbacitracin ($50ug \cdot mL^{-1}$) as a spreading agent. The arrowheads in Panel C indicate probe moieties. Scale bars = 100 nm.

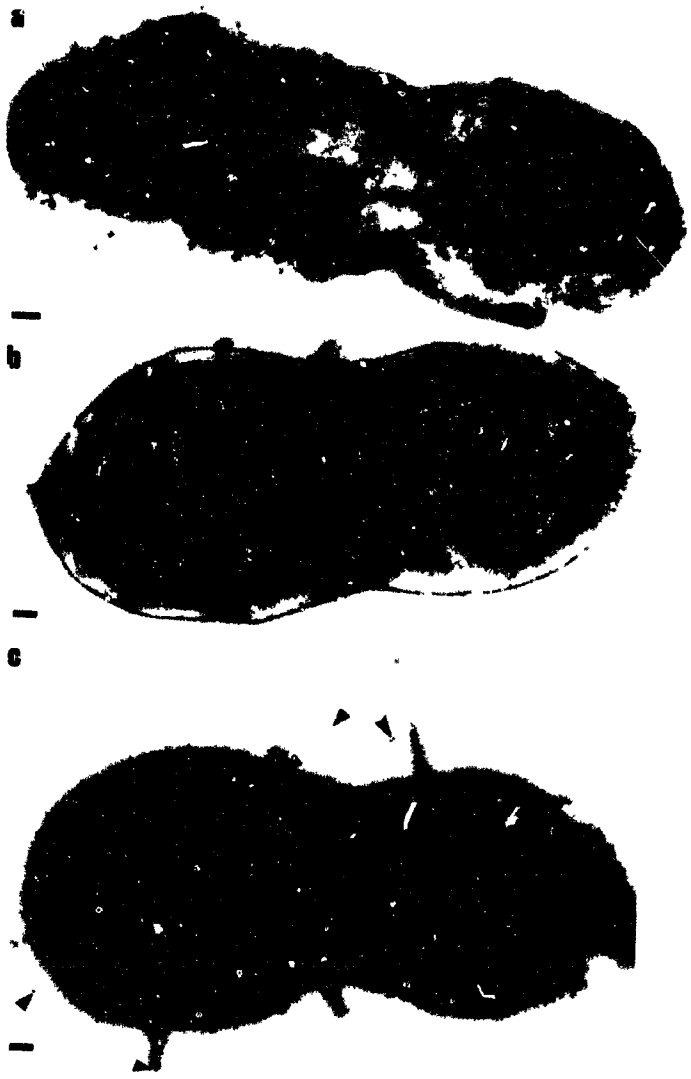

a
b
c

Fig 9. Electron micrographs of ultrathin Epon 812 sections of MPD71 cells obtained from a 40% spined/60% unspined population following the application of fully absorbed hyperimmune antispinin serum and probe (i.e., cells were immunodecorated prior to embedding). Panel a shows a spined cell with a spina apparent in cross-section (indicated by the arrow). Panel b shows a similar cell with a spina partially revealed in a longitudinal section plane. Panel c shows a morphologically spined and unspined cell, while Panel d shows a pair of unspined cells. In this and all subsequent thin sections contrast was provided by 2% aqueous uranyl acetate. Scale bars = 100 nm.

Fig 9.

Fig 10. Electron micrographs of morphologically spined cells resident in a population consisting of about 40% spined cells, which had been treated with a dilution (1:500, in Phosphate buffered saline pH 7.4) of fully absorbed hyperimmune antispinin serum and probe. These cells were contrasted with uranyl acetate. The cells in Panels a and b were from the same population, but were treated in separately-conducted experiments. Scale bars = 100 nm.

Fig 11. Electron micrograph of a morphologically spined cell from a comparable population as described in the legend to the previous figure. The population from which this cell was observed was treated with hyper-immune serum and probe as described in the previous figure legend. This cell had lost a majority of its cytoplasm and had adhered more flatly to the grid. As a result, probe moieties can be visualized more easily. Spina are indicated by the solid circles and spina base structures are indicated by the arrows. Scale bar = 100 nm.

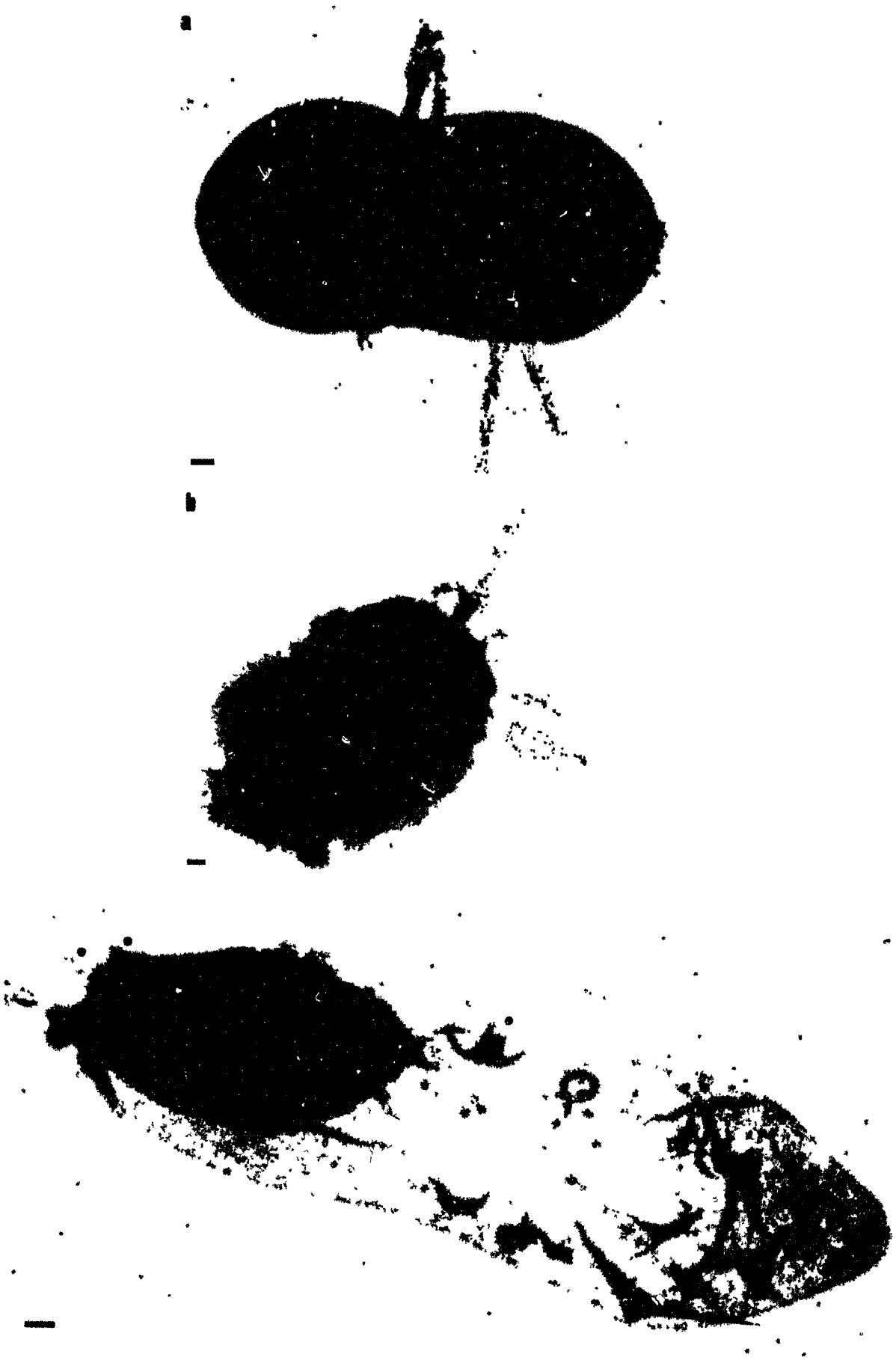
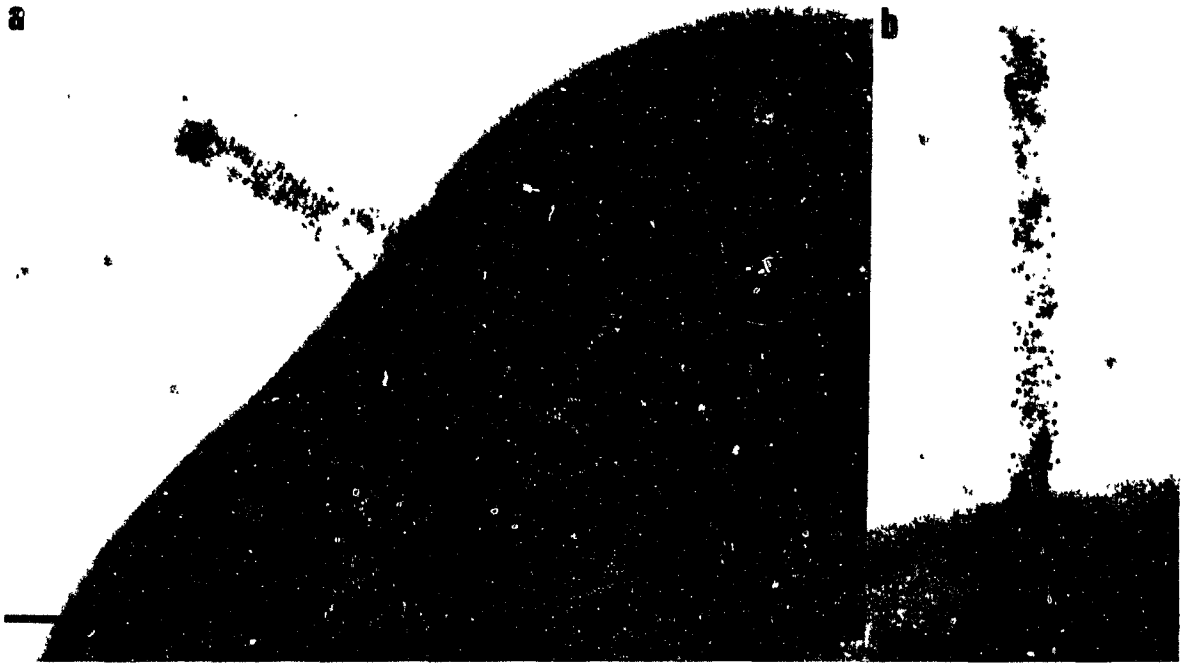


Fig 12. Higher magnification images of spina and surrounding surface of two morphologically spined cells. The cell populations were treated with 1:500 dilutions of fully absorbed hyperimmune antiserum and probe. Note the association of a probe moiety with a distal region of spina structural derangement in Panel a, and the difference in the electron-scattering behaviour and size of the probe versus the particles apparent in the background of Panels a and b. Scale bars = 100 nm.

Fig 12.



The probe itself was strongly electron-opaque (see Fig 12 for example). Particles distributed over the surface of the support film in figures 10-12 were, in contrast, less electron-opaque (see Fig 12). Additionally, the diameter of these particles (14nm; an average of 20 separate measurements) were larger than the probe diameter. These observations suggested that the particles were not probe moieties. They may have been deposited upon negative-contrasting (i.e., bacitracin) or during the immunocytochemical regimen (i.e., aggregates of BSA).

Immunocytochemistry of ultrathin sections

In the preceeding immunocytochemical procedure, spinin which was not surface-accessible would not have been detected. Assuming that such "buried" spinin was present in an antigenically-recognizable conformation, the protein might be detected immunologically in ultrathin sections. To test this idea, ultrathin sections were obtained from Epon-812 embedded cells. The cells used in this experiment were obtained from the same populations (i.e., 40% spined, 60% unspined) as used in the immunocytochemical procedure described in the preceeding section. Use of the same populations in both immunocytochemical procedures allowed comparison of surface and interior immunolabelling patterns.

Cells were obtained prior to treatment with the antiserum and the probe. These samples were processed through to Epon 812, sectioned, and then treated with the

antiserum and the probe. In sections treated only with the probe or with preimmune serum plus probe, the Epon resin displayed a random, nonspecific decoration pattern (Fig 13). This behaviour has been described in other immunocytochemical studies (Roth, 1982). In contrast, treatment of sections using hyperimmune antispinin antiserum (absorbed with E. coli and P. aeruginosa envelopes and unspined cells) and the probe resulted in the specific immunodecoration of spina (Figs 14 and 15). When the walls of the spina tube or base were exposed in cross-section, immunodecoration was evident (Fig 15). It was difficult to assess whether this decoration was of the spina wall, the lumen, or both. Spina exposed in longitudinal section were not always immundecorated uniformly along their length (Fig 15a and b). This may reflect the exposure of both the interior and exterior surfaces of the spina in the plane of the section. The nondecorated regions may represent the interior surface, since this surface is hydrophobic (R.W. Coombs, PhD Thesis, 1977, Dalhousie University). There was no immunodecoration of the cytoplasm or envelope of either morphologically unspined or spined cells (Figs 14 and 15). The lack of cell surface decoration was consistent with that obtained with whole cells.

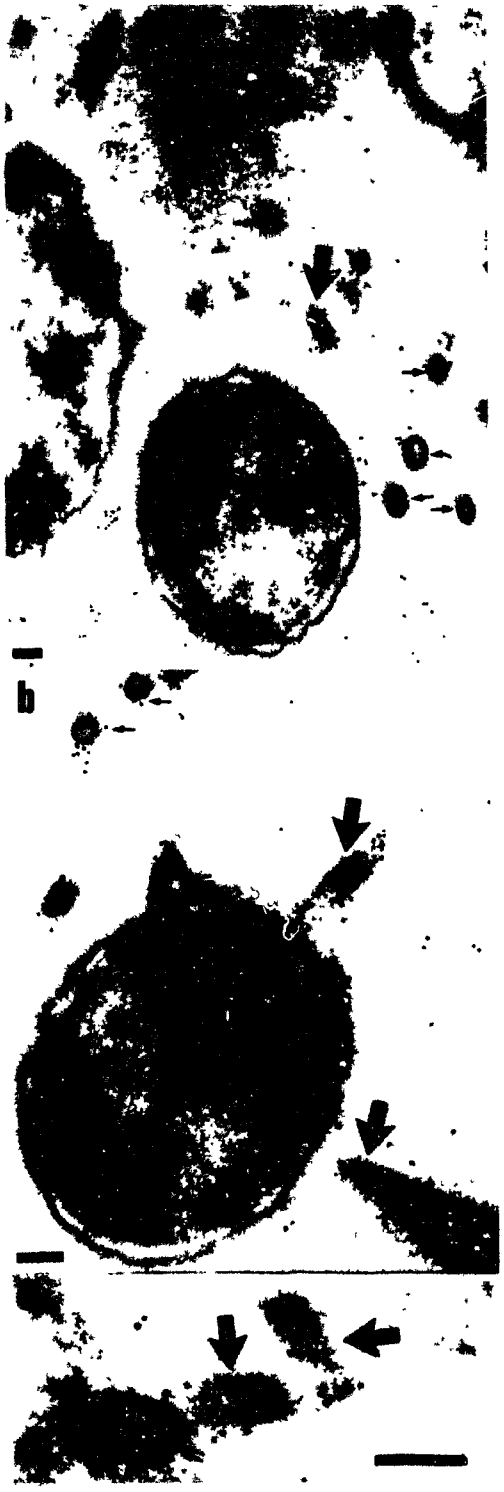
The immunocytochemical protocol which was used in this procedure can disrupt antigenicty (Roth, 1982). Thus, it was necessary to try another regimen which was less

Fig 13. Electron micrographs of ultrathin sections of Epon 812 embedded MPD71 from a 40% spined/60% unspined population. The sections were treated with either a 1:1000 dilution (in Phosphate buffered saline pH 7.4) of the probe (Panels a and b) or with a 1:100 dilution of unabsorbed preimmune serum and a 1:1000 dilution of the probe (Panels c and d). Scale bars = 100 nm.

Fig 13.

Fig 14. Electron micrograph of an ultrathin section of an Epon 812 embedded spined cell from a 40% spined/ 60% unspined population. The section was treated with a 1:500 dilution (in Phosphate buffered saline pH 7.4) of fully absorbed hyperimmune antispinin serum and a 1:1000 dilution of the probe. Scale bars = 100 nm.

Fig 15. Electron micrographs of Epon 812 embedded samples obtained from a comparable 40% spined/ 60% unspined cell population. Panel a shows a morphologically unspined cell and spina. The spina lie horizontally (large arrows) and perpendicularly (small arrows) to the plane of the section. Panel b shows a morphologically spined cell and several spina. The large and small arrows denote the same features as in panel a; the double small arrow denotes a region of a spina base where immunodecoration appears quite specific. Panel c shows a higher magnification image of a spina. The shaft is indicated by the large arrows and the base by the two small arrows. Scale bars = 100 nm.


b

disruptive to antigenicity. Cells were processed and embedded in Lowicryl K4M using a regimen similar to that described by Armbruster et al. (1982). This protocol has been demonstrated to be less disruptive to epitopes (Roth, 1982). Cells obtained from populations grown in spina-restrictive or permissive conditions were used. Electron microscopy of negatively-contrasted samples revealed that all the cells grown under spina-restrictive conditions were unspined (0 spined cells out of 330 cells examined). The cultures growing in spina-permissive conditions were harvested during the phenotypic transition when approximately 40% of the cells were spined (125 spined cells out of 340 cells examined). This was done to try and detect the subset of unspined cells which might still be present in the population. Some of the resulting ultrathin sections were treated with 1:100 and 1:1000 dilutions of, respectively, unabsorbed preimmune serum and probe or with probe alone. In neither instance was immunodecoration evident (Figs 16 and 17). Other sections were treated with 1:4000 and 1:1000 dilutions of, respectively hyperimmune antispinin serum (absorbed with E. coli and P. aeruginosa envelopes and unspined cells), and probe. The results of these treatments are shown in Figs 18 and 19. The periphery of unspined cells was extensively immunodecorated (Fig 18). It was the OM which appeared to be decorated. The periplasmic space, CM, and the cell interior was relatively undecorated. Examination of a population in which

Fig 16. Electron micrographs of ultrathin sections of Lowicryl K4M embedded cells obtained from populations in which approximately 40% of the enumerated cells were spined. The cells shown in the two panels were from separately embedded preparations of this population. Sections were treated with a 1:100 dilution of unabsorbed preimmune serum and a 1:1000 dilution of the probe. The dilutions were in phosphate buffered saline pH 7.4. Scale bars = 100 nm.

Fig 17. Electron micrograph of an ultrathin section of Lowicryl K4M embedded cells which were obtained from the same population described in the legend to figure 16. The section was treated with a 1:1000 dilution (in phosphate buffered saline, pH 7.4) of the probe. Scale bar = 100 nm.

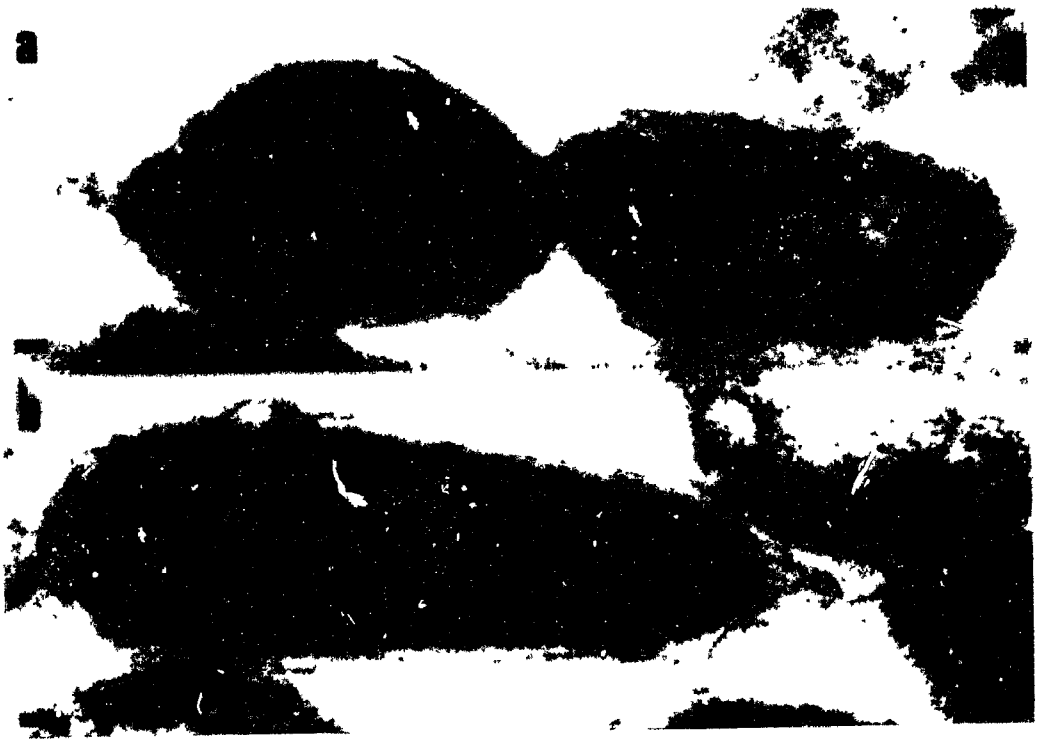
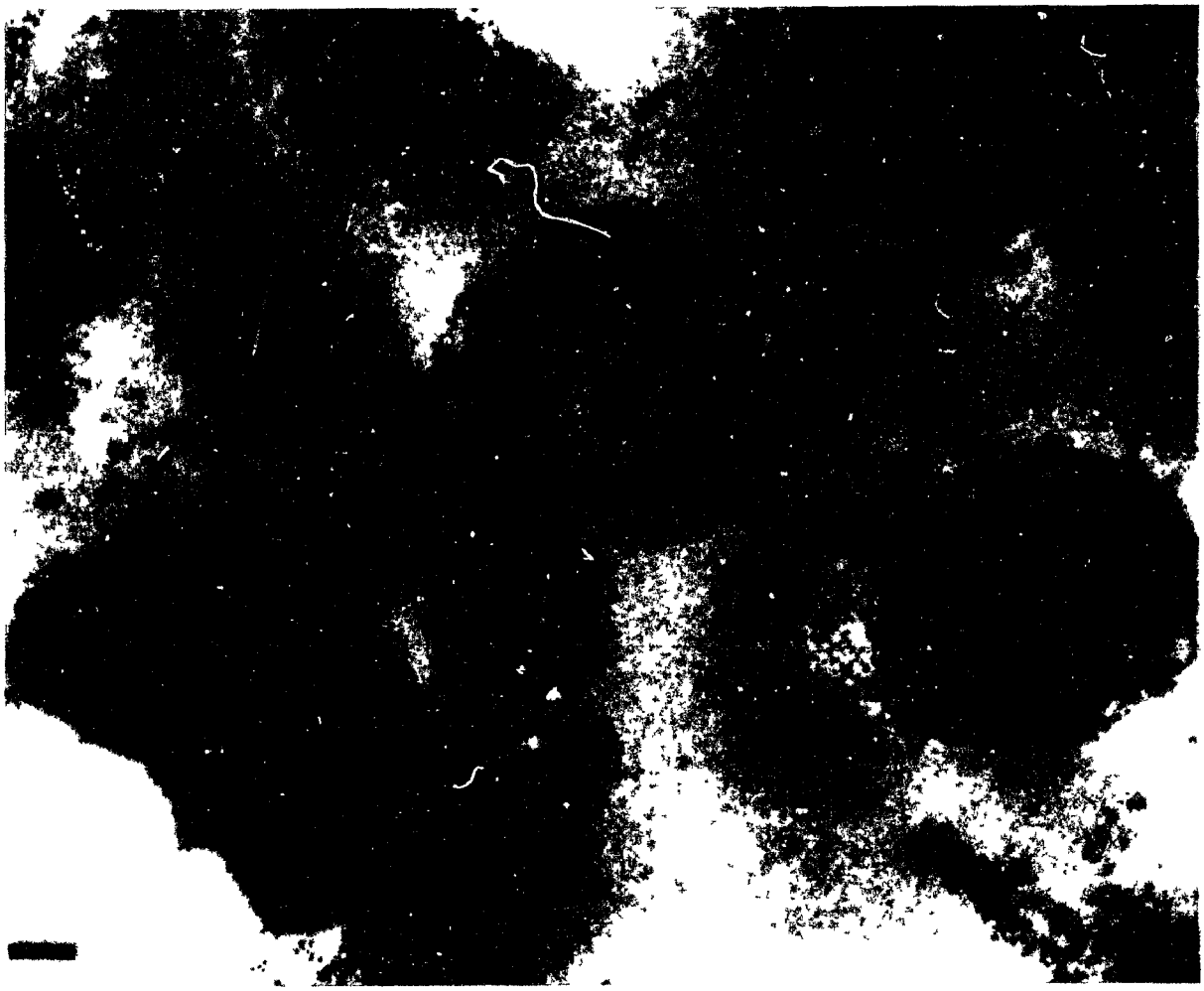


Fig 18. Electron micrograph of an ultrathin section of Lowicryl K4M embedded cells obtained from a population cultured under spina-restrictive physiological parameters. Enumeration of a sample volume showed that the culture was totally unspined. The section was treated with 1:4000 dilutions (in Phosphate buffered saline pH, pH 7.4) of fully absorbed antispinin serum and the probe. Scale bar = 100 nm.

Fig 19. Electron micrograph of an ultrathin section of Lowicryl K4M embedded cells obtained from the same 40% spined population used in figure 16. Although only about 40% of the cells in an enumerated sample possessed spina, all the cells visible in this section are spined. The section was treated before examination with 1:4000 dilutions (in Phosphate buffered saline pH 7.4) of fully absorbed hyper-immune antispinin serum and the probe. Scale bar = 100 nm.

Figs 18 19.



approximately 40% of the enumerated cells possessed spina revealed the immunodecoration pattern shown in Fig 19. Only spined cells were observed, perhaps due to aggregation of these cells during specimen preparation. The tendency for spined cells to aggregate has been noted before (Easterbrook and Sperker, 1982). The periphery of the cells (the OM) was extensively immunodecorated, as were the spina. As with unspined cells, the periplasmic space, CM, and the cell interior remained relatively undecorated.

Effect of selected envelope perturbants on the growth, spination, and polypeptide profile of MPD71

Spina assembly can be disrupted by phenethyl alcohol (PEA) (Easterbrook, in press). As described in the Introduction, PEA is an envelope perturbant which can disrupt the secretory process. To further characterize the effects of PEA and several other envelope perturbants on spina assembly, it was necessary to establish their effect on MPD71 growth. These results are shown in Tables 6 and 7. PEA concentrations of 0.01 and 0.05% v/v did not affect cell growth (as judged by an increase in the optical density throughout the experiment). At 0.1% v/v PEA growth was less, while 0.5% v/v PEA inhibited growth (Table 6). Electron microscopy of the same cultures revealed that spination was affected only at the growth-inhibiting concentration. It must be emphasized, however, that the perturbants were introduced into the cultures when

Table 6 Absorbance (540nm) measurements of MPD71 cultures treated with PEA, procaine, or Absolute ethanol

SAMPLE		$T=0_1$	$T=2_2$	$T=3_3$
Untreated		0.15(0.01)	0.50(0.01)	0.56(0.02)
PEA	0.01%v/v	0.10(0.01)	0.50(0.01)	0.56(0.01)
	0.05%	0.10(0.02)	0.51(0.01)	0.56(0.02)
	0.10%	0.10(0.01)	0.49(0.01)	0.54(0.01)
	0.50%	0.10(0.02)	0.49(0.02)	0.46(0.03)
Procaine	2uM	0.15(0.03)	0.49(0.02)	0.54(0.01)
	5	0.14(0.02)	0.49(0.01)	0.55(0.02)
	10	0.15(0.03)	0.49(0.02)	0.50(0.02)
	20	0.15(0.01)	0.50(0.01)	0.47(0.01)
Ethanol	0.001%v/v	0.16(0.02)	0.50(0.02)	0.59(0.01)
	0.005%	0.16(0.01)	0.50(0.01)	0.64(0.03)
	0.01%	0.15(0.02)	0.49(0.03)	0.65(0.01)
	0.1%	0.15(0.03)	0.50(0.02)	0.37(0.04)

The number in the brackets is the standard deviation. The data represent the average of three separately conducted experiments. Following addition of the perturbant, incubation under spina-permissive conditions was continued until the untreated control culture was 100 per cent spined. The percentage of the cells which possessed spina was determined as summarized in the legend to Table 5.

1 - at the time of shift of an aliquot of the unspined culture into fresh medium and incubation under spina-permissive conditions. T is in hours.
2 - at the time of addition of the inhibitor to the culture (when approximately 10% of the cells in an enumerated population possessed spina).
3 - at the time of harvesting of the culture (when all the cells in an enumerated population possessed spina).

Table 7 Determination of the percentage of enumerated populations which are spined in MPD71 cultures treated with PEA, Procaine, CCCP, or Absolute ethanol

SAMPLE		A	B	C	D
Untreated	1	195	120	315	38
	2	34	291	325	90
PEA 0.01%v/v		72	333	405	82
0.05%		63	264	327	81
0.1%		66	315	381	83
0.5%		117	234	351	67
Procaine 2uM		----------			a
5		24	300	324	93
10		66	315	381	83
20		117	234	351	67
CCCP 1uM		222	180	402	45
2		204	169	373	45
10		215	174	389	45
20		204	180	384	47
Ethanol 0.001%v/v		24	300	324	93
0.005%		105	330	435	76
0.01%		240	177	417	42
0.1%		----------			b

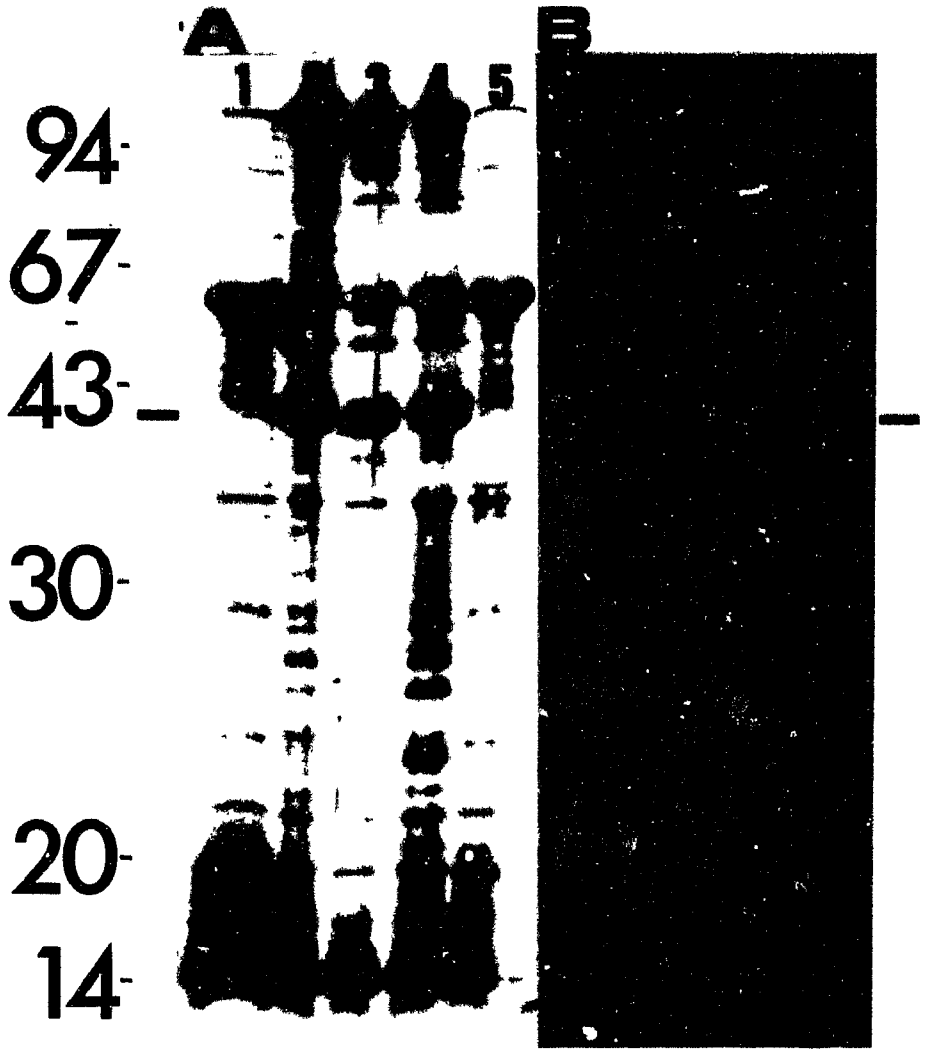
A - number of unspined cells
B - number of spined cells
C - total number of cells counted
D - percentage of the total population which possessed spinae
1 - population enumerated for the percentage of spined cells at the time of the addition of the inhibitors to the treated cultures (T=2 from Table 6)
2 - population enumerated at the conclusion of the experiment (T=3 from Table 6)
a - not enumerated
b - not enumerated due to extensive lysis of the cells in the population

The percentage of an enumerated population which possessed spina was determined by electron microscopy of negatively-contrasted cells.

approximately 10% of the cells in the enumerated sample possessed spinae. The lack of an effect on production of spinin by PEA was not surprising. It has been shown (Easterbrook, in press) that the introduction of PEA following the initiation of spina assembly does not inhibit further assembly. The PEA results shown in Table 7 are consistent with this observation. However, introduction of PEA at the time of the shift from spina-restrictive to permissive conditions totally inhibits spina assembly (Easterbrook, in press). In order to extend this morphological observation, PEA was introduced to final concentrations of 0-0.08% v/v into MPD71 cultures at the time of shift to spina-permissive conditions. Following growth, the cultures were harvested and the envelope was obtained by French Pressure Cell explosion of the cells. The envelopes were examined by SDS-PAGE. At PEA concentrations of 0.02 and 0.04% v/v, the cultures were comparable in their growth and spination to the untreated (0% PEA) culture. The 42kD species was present in all three envelope fractions (Fig 20A, lanes 2-4). It was readily immunodetectable (Fig 20B, lanes 2-4). A 14kD species was immunodetectable in the untreated (0% PEA) and the 0.04% PEA-treated envelope, but was only weakly immunologically detected in the 0.02% PEA-treated envelope. The envelope from unspined cells and from the 0.08% v/v PEA-treated population (which was unspined) lacked the 42kD species and a variety of high MWr (> 67kD), mid-range MWr (42-67kD),

Fig 20. Effect of phenethyl alcohol on the protein profile of the MPD71 envelope. The procedure used is detailed in the Materials and Methods. A 12% w/v acrylamide gel was used. Separated proteins were stained using Coomassie Brilliant Blue R250 (Panel A) or probed with fully absorbed hyperimmune antispinin serum following their immobilization to nitrocellulose (Panel B). The samples in both panels are: (1) Unspined envelope; (2) Envelope from an untreated (0% v/v PEA) cell population; (3-5) Envelope from cell populations treated with PEA. The concentrations of PEA used were, respectively, 0.02, 0.04, and 0.08% v/v. The lines to either side of the figure indicate the position of spinin. MWr values, in kilodaltons, are shown to the left of the figure.

Fig 20.



and lower MWr (20-30kD) species (Fig 20A and B, compare lanes 1 and 5 with lanes 2-4). The 14kD species was immunodetectable, although the envelope of unspined cells had a greater content of the protein than did the 0.08% PEA-envelope (Fig 20A and B). Thus the envelope of 0.08% PEA-treated cells had the same protein profile (qualitatively) as the unspined OM, even though the PEA-treated cells had been grown under spina-permissive conditions.

Three other envelope perturbants (procaine, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), and ethanol) were tested. Procaine was detrimental to growth and spination at concentrations of 10 and 20uM (Tables 6 and 7). CCCP, however, halted growth and spination at all concentrations (Table 7). The CCCP effects must have occured very shortly after its addition, since the per cent spination at the time of CCCP addition and at the time of harvest were similar (Table 7). The discrepency in the per cent spination values between the untreated culture (38%) and the CCCP-treated cultures (45%) at T=2 was a consequence of the time lag while the untreated sample was being enumerated. Since the cultures destined to receive CCCP were still untreated during this interval, the percentage of cells possessing spinae would continue to increase.

Ethanol inhibited growth only at the highest concentration employed (Table 6). In fact ethanol concentrations of 0.001-0.01% v/v stimulated growth,

perhaps due to utilization of the compound as a carbon substrate. Spination was, however, progressively restricted (Table 7), until lysis occured. In this regard it must be noted that an ethanol concentration equivalent to that in the CCCP solutions (0.001% v/v) did not affect either cell growth or spination (Tables 6 and 7).

Comparably-treated MPD71 populations were analyzed electrophoretically. The immunoblot pattern was distinctive (Fig 21B). Samples treated with CCCP displayed an immunoreactive species migrating at slightly larger MWr than spinin (Fig 21B, compare lanes o-q with purified spinin in lane a). This larger MWr species was not evident in the other samples. Comparison of the mobilities of several other MPD71 polypeptides (Fig 21A) indicated a difference of approximately 2kD between the immunoreactive species in the CCCP-treated samples.

Effect of CCCP on the radiolabeling profile of E.coli

Given the effects of CCCP noted above, as well as the known perturbance of the compound on E.coli K12 (Silhavy et al., 1983) it was appropriate to confirm the effectiveness, on K12, of the CCCP concentrations used for MPD71. A CCCP concentration of 2uM was selected since it was the intermediate concentration used with MPD71. The resultant radiolabeling patterns from three separately conducted experiments with E. coli K12 strain AB264 are shown in figure 22.

Fig 21. SDS-PAGE examination of MPD71 populations which were treated with phenethyl alcohol (PEA), procaine, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), or absolute ethanol. The cultures were treated when approximately 20% of the cells in an enumerated sample possessed spina. Cell lysates were obtained and subsequently electrophoresed on duplicate 12%w/v acrylamide separation gels and either stained using Coomassie Brilliant Blue R250 Panel A) or probed with fully absorbed hyperimmune antispinin serum following immobilization on nitrocellulose (Panel B).
The samples in both panels are: (a) Spinin (5ug); (b and n) Lysate from untreated populations (these and the remaining samples were applied as 30ug total protein); (c-f) PEA for 4h at final respective concentrations of 0.01, 0.05, 0.1, 0.5%v/v; (g-j) PEA for 60 min at the same final respective concentrations; (k-m) Procaine for 60 min at final respective concentrations of 5, 10, and 20uM; (o-q) CCCP for 60 min at final respective concentrations of 1, 2, and 5uM; (r-t) Absolute ethanol for 60 min at final respective concentrations of 0.001, 0.005, and 0.01%v/v. The position of the MWr standards are shown to the left of panel A.

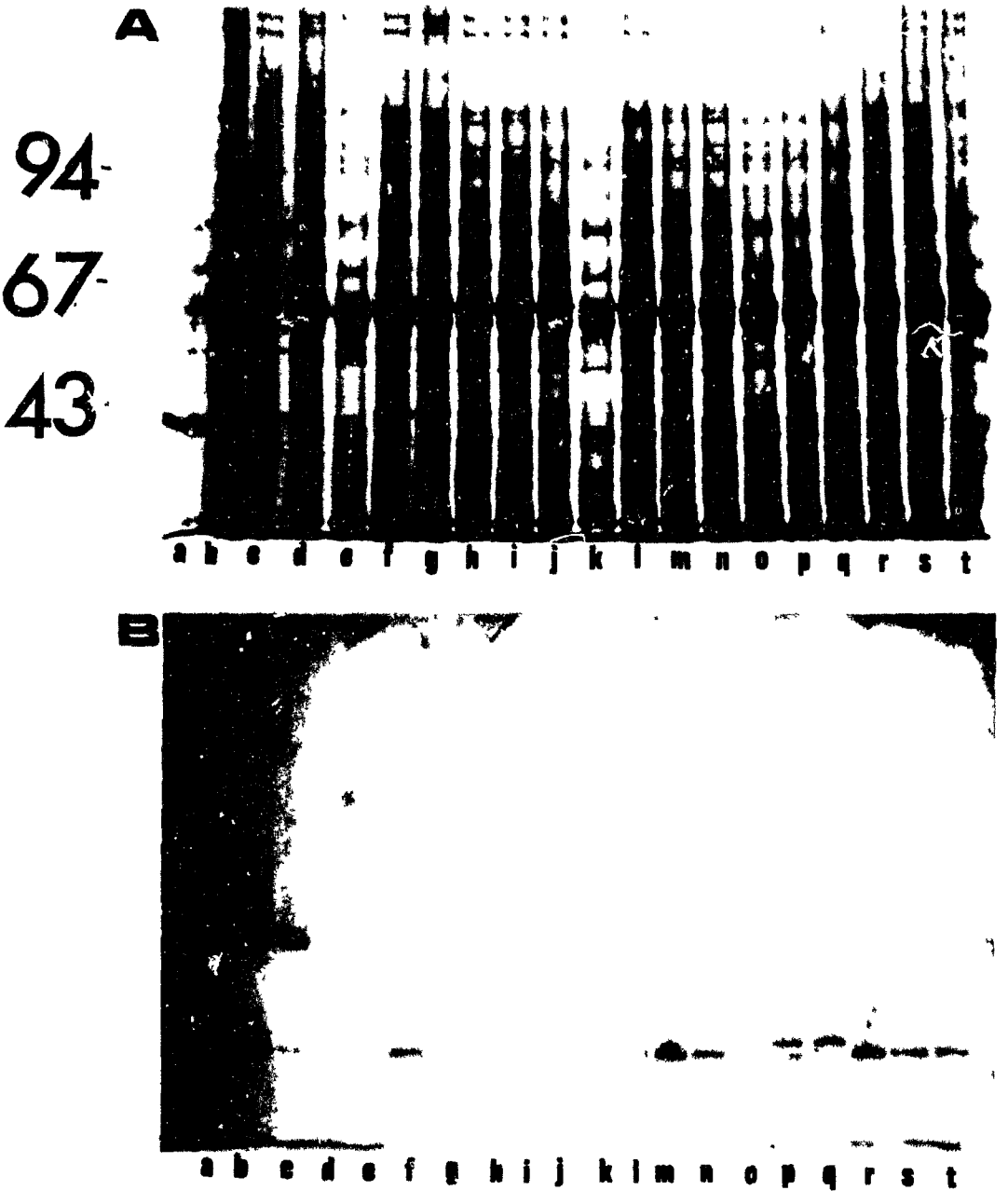
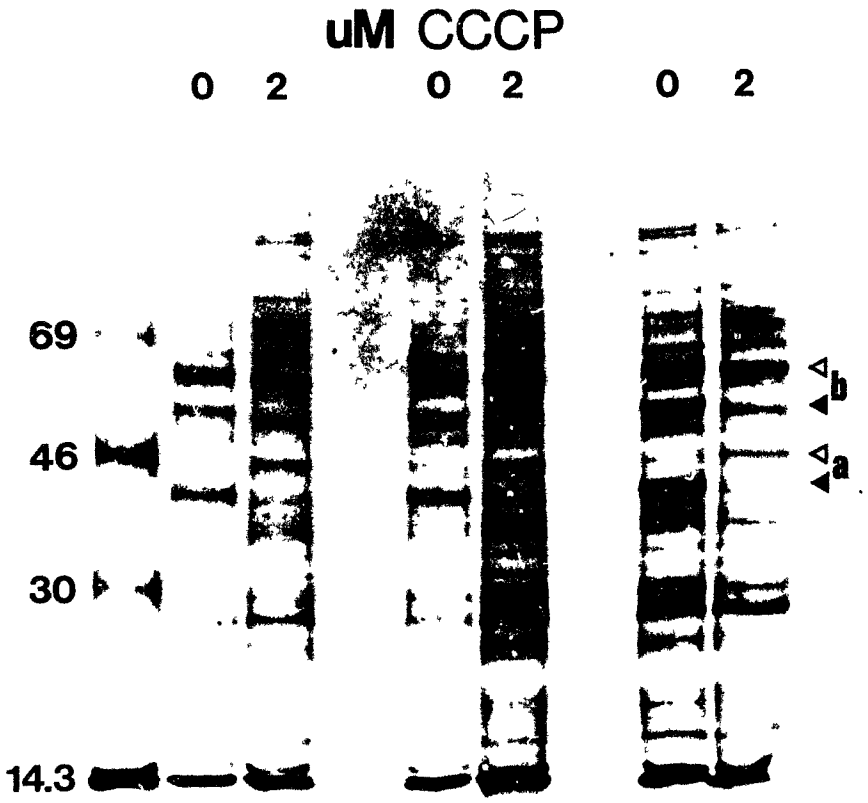

A
94
67
43
a b c d e f g h i j k l m n o p q r s t
B
a b c d e f g h i j k l m n o p q r s t

Fig 22. The effect of CCCP on the incorporation of ^{35}S-methionine into polypeptides of E.coli K12 strain AB264. Samples from three independently conducted experiments are shown in this figure. ^{14}C-labeled MWr standards are in the far left lane of the gel and their values (kD) are shown to the left of the figure. Samples were treated with 2u'] CCCP and 20uCi ^{35}-S methionine prior to SDS-PAGE. The gel was dried and exposed to X-ray film for 24 h at -70°C prior to development. The closed triangles show two intensively-labeled species (a and b) in the untreated cell lysates. The open triangles show two species (a and b) which were labeled in the CCCP-treated cell lysates.

Fig 22.



Two polypeptides were more prominently labeled in the untreated samples. These species were weakly labeled in the CCCP-treated samples. The CCCP-treated samples displayed instead two slightly higher MWr species. These "before and after" species have been arbitrarily paired in figure 22 (a-47 and 49kD; b-52 and 60kD). Other investigators have identified a similar MWr pattern for the "a" pair as representing "mature" and "precursor" species of LamB (Emr and Bassford, 1981), although in the absence of immunoprecipitation experiments the identity of both the "a" and "b" pairs were not established. Irrespective of the identity of these species it is evident that CCCP concentrations which affected MPD71 spination also affected the protein profile of E. coli.

The immediacy and reversibility of the CCCP-block in MPD71

The cessation of spination by CCCP seemed to be rapid. This was confirmed in another experiment in which CCCP was introduced to MPD71 populations at various points during the unspined to spined phenotypic transition. CCCP arrested spination regardless of when it was added (Fig 23). This effect differed from the PEA effect, where spination was not inhibited if PEA was added after assembly of spina had commenced (Table 7; and Easterbrook, in press).
The permanence of the CCCP-spination block or the possibility of an immediate lethal effect of the compound

Fig 23. Confirmation of the immediacy of the CCCP-induced spination block. MPD71 overnight unspined cultures were shifted from conditions restrictive for spination to spina-permissive conditions; CCCP was added to a final concentration of 5uM at various times after the shift. The extent of spination was evaluated at the time of CCCP addition and for two hours thereafter by electron microscopy.
The circles represent the mean values for three untreated cultures. CCCP was added to comparable cultures at the times indicated by the arrows. The squares represent the mean values for the three cultures used in each CCCP determination.

Fig 23.

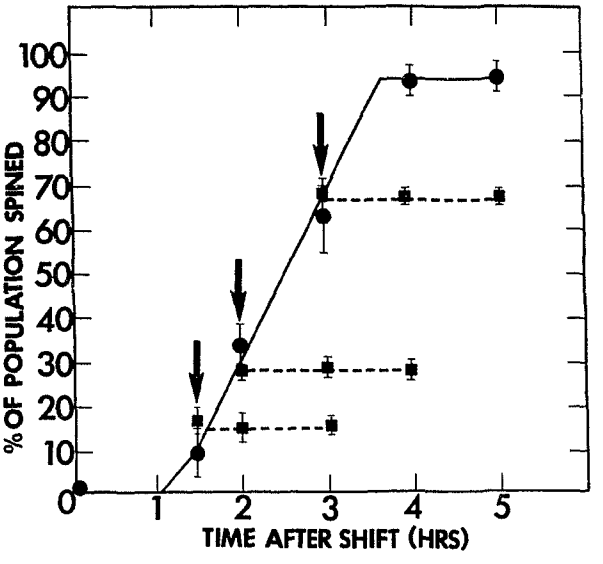
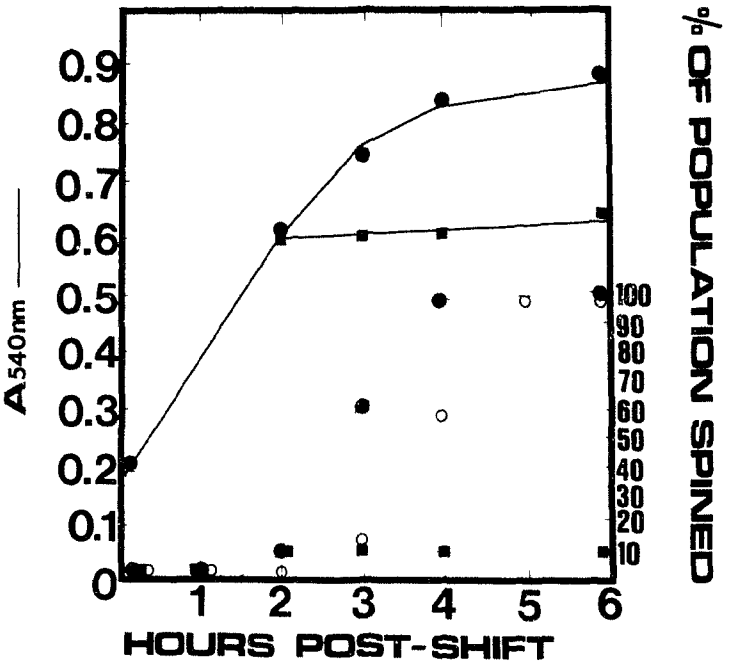

100
90
80
70
60
50
40
30
20
10
0
% OF POPULATION SPINED
1
2
3
4
5
TIME AFTER SHIFT (HRS)

Fig 24. Reversibility of the CCCP-induced spination block. The optical density measurements are indicated by the solid lines and the percentage of enumerated populations are indicated by the dashed lines. The results shown represent the mean of three separately conducted experiments. The solid circles represent the untreated samples and the solid square represent samples which received CCCP (final concentration 5uM). CCCP was added when an enumerated untreated culture was approximately 10% spined (about 2h after the shift to spina-permissive conditions). The open circles indicate the percent spined values for a set of three cultures which had been grown overnight (15h) in spina-restrictive conditions in the presence of 1uM CCCP prior to washing and resuspension in fresh complex medium in spina-permissive conditions.

Fig 24.


A540nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
HOURS POST-SHIFT
100
90
80
70
60
50
40
30
20
10
% OF POPULATION SPINED

could not be determined in this experiment. The resumption of spination following the removal of the CCCP block was monitored electron microscopically in another experiment. The results are summarized in Table 8 and figure 24. The addition of CCCP (final concentration 1, 2, or 5uM) halted growth (Fig 24) and spination (Table 8). The untreated culture became 100 percent spined during the same time interval (Table 8). When the CCCP-treated cells were washed and resuspended in fresh medium under spina-permissive conditions the block to spina assembly was quickly overcome (Table 8). The increase in optical density of these cultures (not shown) indicated that the cells had remained viable during their exposure to CCCP. Even cells exposed to 1uM CCCP for 15 h remained viable (Fig 24), as long as they were cultured under spina-restrictive conditions. Following removal of CCCP, the lag period prior to the visual detection of spina was slightly longer than that of the untreated control culture. The temporal kinetics of spination in both cultures were identical, however (Fig 24).

The requirement for protein synthesis for the continued production of spina was confirmed by the inclusion of chloramphenicol in the fresh medium following the removal of CCCP (Table 8). Inclusion of chloramphenicol maintained the cell population at the enumerated per cent spination present prior to the washing step (Table 8). This result is in contrast to that found for the pilin of E. coli K12

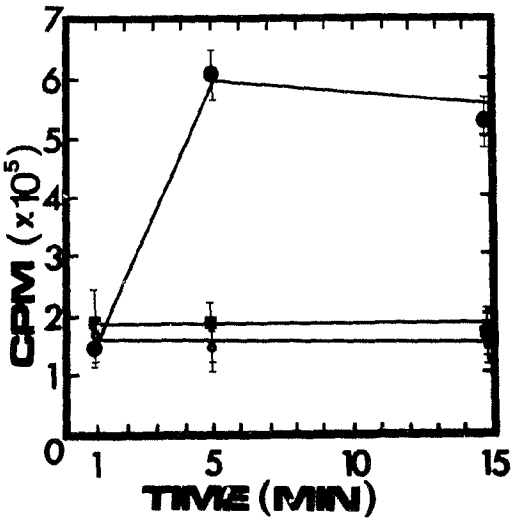
Table 8 Percentage of the population possessing spina in the presence of CCCP, and following its removal

SAMPLE	A	B	C	D
Unspined culture used as the inoculum	300	0	300	0
Untreated culture at the time of CCCP addition	272	31	303	10
Untreated culture 90min following the addition of CCCP to the other cultures	24	285	309	92
Culture treated with 1uM CCCP for 90min	260	40	300	13
Culture treated with 2uM CCCP for 90min	265	38	306	12
Culture treated with 5uM CCCP for 90min	255	35	290	12
2uM-treated culture 90min following resuspension in fresh medium	30	273	303	90
2uM-treated culture 90min following resuspension in fresh medium containing chloramphenicol	283	44	327	13

A - number of unspined cells counted
B - number of spined cells counted
C - total number of cell counted
D - percentage of the enumerated population which possessed spina. The method used to determine this value is summarized in the legends to Table 5.

Fig 25. Demonstration of the cessation of protein synthesis in MPD71 cells upon the addition of chloramphenicol. The regimen used is detailed in Materials and Methods. The solid circles represent the mean counts (from three separately performed experiments) of the incorporated radiolabel in the untreated cultures. The solid squares and small, open circles represent the mean counts (from three separately performed experiments) of the incorporated radiolabel in those cultures which received chloramphenicol at time=0 min to final respective concentrations of 20 and 100 $ug \cdot mL^{-1}$.

Fig 25.



(Beard and Connolly, 1975) where the depletion of a pilin pool allowed the continued assembly of pili in the presence of chloramphenicol. The efficacy of chloramphenicol (cessation of protein synthesis) for MPD71 was demonstrated (Fig 25). Radiolabel incorporation into total cell protein was inhibited by the addition of chloramphenicol (Fig 25).

The experiment summarized in Tables 7 and 8 was also monitored electron microscopically using the length of the spina as a marker of the effect of CCCP. Spina-restrictive conditions produced unspined populations (Fig 26). All the cells became spined within 3-4 h following the shift to spina-permissive conditions. CCCP was introduced into other comparably-shifted cultures when the populations appeared as shown in figure 27. Cells scored as spined typically possessed short spinae or the base structure of nascent spina (Fig 27a and b); i.e., they had recently commenced spina assembly (Easterbrook and Alexander, 1983). The remaining cells (about 80% of the population) were morphologically unspined (Fig 27a and b). Populations treated with CCCP appeared the same as these cells, even 90 min after CCCP addition (compare Figs 27 and 28). This was consistent with the observed immediacy of the CCCP spination block (Fig 23).

When the CCCP-treated cells were washed and resuspended in fresh medium under spina-permissive conditions for 90 min, the resulting population appeared as in figure 29. The cells were indistinguishable from control (untreated) cells

Fig 26. Electron micrograph of negatively-contrasted MPD71 cells from a population grown under spina-permissive conditions. Aliquots of this culture were subsequently shifted to conditions favourable for spination. Scale bar = 100 nm.

Fig 27. Morphological appearance of MPD71 populations at the time of addition of CCCP (2uM). Approximately 10% of the cells in each enumerated sample possessed spina at the time of the addition of CCCP. The cells shown in Panels a and b were obtained from separate populations. Scale bar = 100 nm.

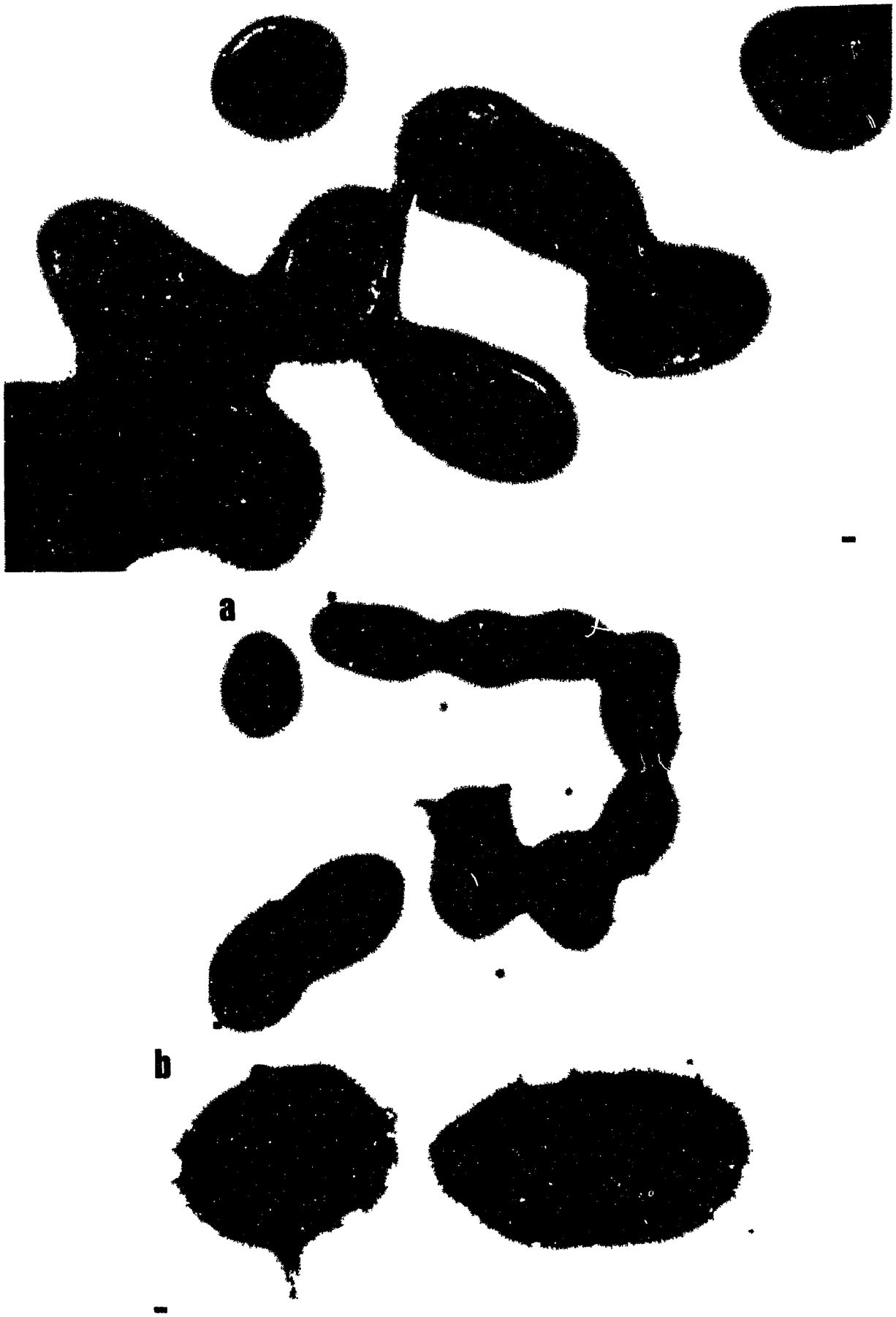
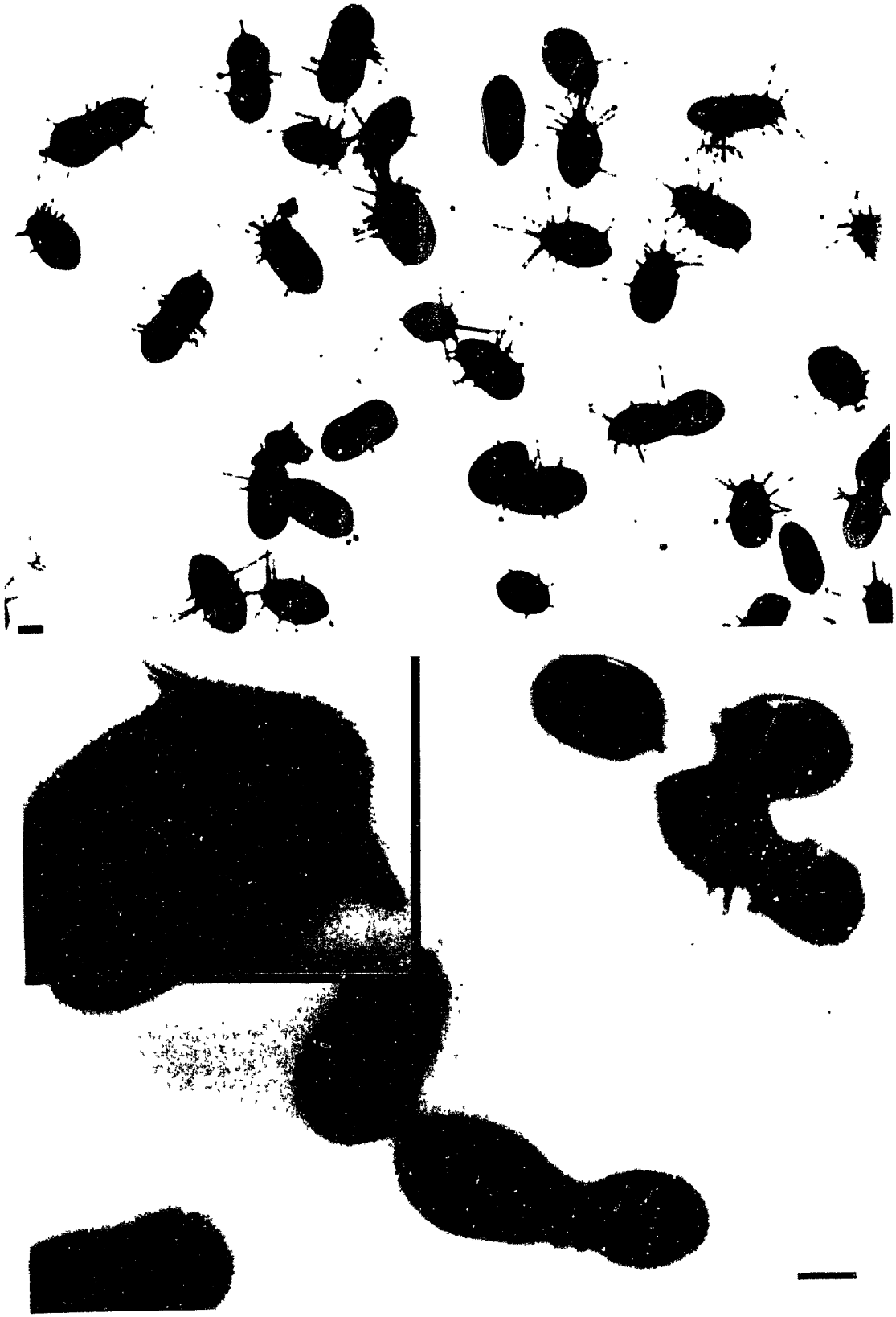

a
b

Fig 28. Morphological appearance of the cells in MPD71 populations 90 min after the addition of CCCP. Panels a and b show cells from a population treated with 1uM CCCP. Panels c and d show cells from a population treated with 2uM CCCP. Panels e-g show cells from a population treated with 5uM CCCP. The arrowheads in Panel b indicate the base structures of nascent spina. Scale bars = 100 nm.

Fig 28.

Fig 29. Electron micrograph of negatively-contrasted cells from a MPD71 population which had been harvested from 5uM CCCP-containing medium, washed, and resuspended in fresh complex medium under spina-permissive conditions for 90 min. Scale bar = 100 nm.

Fig 30. Electron micrographs of negatively-contrasted cells from a population treated as described in the legend to the previous figure, with the inclusion of chloramphenicol (20ug·mL^{-1}) in the fresh medium. The spina observed were usually short; the cell shown in the insert is a typical example. Scale bar = 100 nm in the insert. The other scale bar represents 500 nm.



from a totally spined population. Inclusion of chloramphenicol in the fresh medium maintained the cells as they were when CCCP was present (Fig 30). The cells appeared the same as those sampled at the time of CCCP addition (Fig 27), and 90 min later (Fig 28).

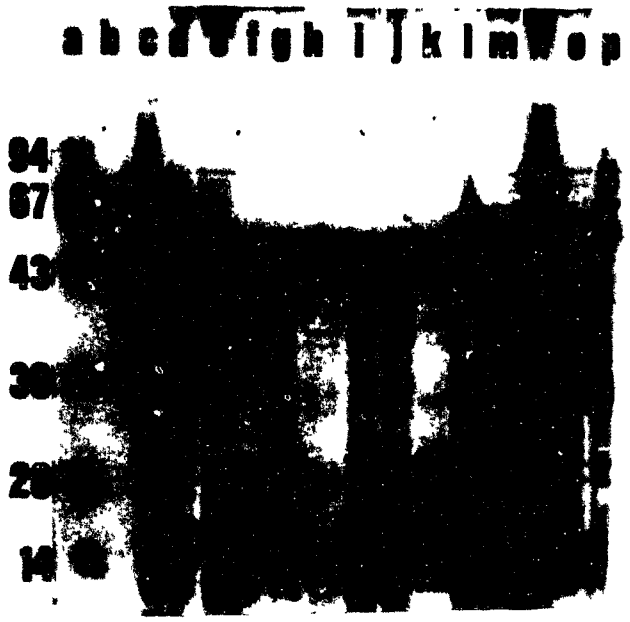
Isolation of the MPD71 OM

In order to further characterize the effects of the envelope perturbants on MPD71, and to investigate the structure of the cell envelope, it was neccesary to examine the MPD71 outer membrane (OM). Detergents such as sodium lauroyl sarcosinate (Sarkosyl) and Triton X-100 have been used along with lysozyme to extract the OM from other Gram-negative bacteria (Schnaitman, 1981; Filip et al., 1973). Preliminary OM isolation experiments in the present study tried both detergents. Triton X-100 was unsatisfactory due to the extensive contamination of the final detergent-lysozyme insoluble fraction (Fig 31, lanes m - p). Replacing Triton X-100 by Sarkosyl gave a final insoluble fraction enriched for approximately 15 proteins (Fig 31, compare lanes g,i, and k with lanes m and o). Treatment of unspined envelope solely with lysozyme produced a comparable fraction except for the inclusion of a species having a MWr identical to lysozyme (Fig 31, lanes d and e).

Inclusion of EDTA in the Sarkosyl treatment affected the resultant protein profile, as observed by the absence of

Fig 31. SDS-PAGE examination of MPD71 populations treated with Sarkosyl, Sarkosyl-EDTA, or with Triton X-100. Lane a contains the MWr standards whose value (kD) are shown to the left of the gel. A 10-15%w/v acrylamide gel was used and the proteins were stained using Coomassie Brilliant Blue R250. All samples were applied as 30ug total protein except for spinin (5ug). Samples: (a) MWr standards; (b) Spinin; (c) Unspined cell lysate; (d) Unspined envelope; (e) Treatment of the envelope with lysozyme ($100ug \cdot mL^{-1}$); (f) Supernatant from the previous treatment; (g) Spined Sarkosyl-lysozyme insoluble material, no Mg^{2+}; (h) Sarkosyl soluble fraction from the previous treatment; (i) as in lane g, with Mg^{2+}; (j) Sarkosyl-soluble fraction from the previous treatment; (k) Spined Sarkosyl-lysozyme insoluble material, no Mg^{2+}, with 1uM EDTA; (l) Sarkosyl-soluble material from the previous treatment; (m) Spined Triton X-100 lysozyme insoluble fraction, no Mg^{2+}; (n) Soluble fraction from the previous treatment; (o) As in lane m, with Mg^{2+}; (p) Soluble fraction from the previous treatment.

Fig 31.

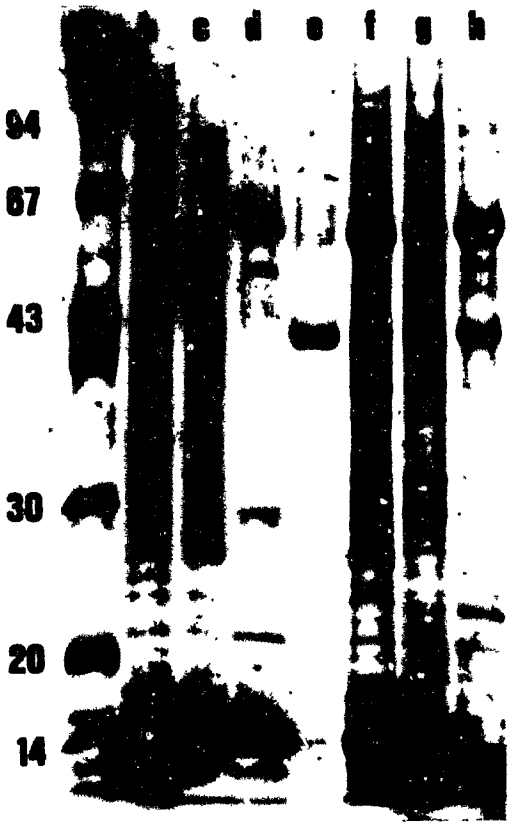


24, 30, and 35kD proteins in the EDTA-treated fraction (Fig 31, compare lanes i and k). Inclusion of Mg^{2+} (supplied as $MgCl_2$, final concentration 1.0mM) in a Sarkosyl-lysozyme procedure had no effect on the protein profile of the resulting insoluble fraction (Fig 31, lane k). In spite of this observation, Mg^{2+} was omitted from subsequent Sarkosyl-extraction procedures to conform with previous investigations (Filip et al., 1973).

The Sarkosyl supernatant contained approximately 7 proteins, including the 65kD and 42kD species. In fact, the 42kD species was present solely in the Sarkosyl supernatant. This result was peculiar to this experiment. A comparable Sarkosyl-lysozyme extraction procedure, performed later in the study, is shown in figure 32. Unspined and spined whole cell, French Press-derived envelope, and Sarkosyl-lysozyme insoluble fractions obtained during the procedure are shown. The Sarkosyl-lysozyme insoluble fractions contained 16-20 proteins ranging in MWr from about 14 to 90kD (Fig 32, lanes d and h). The spined fraction had much more (based on staining intensity) of the 42kD polypeptide than did the unspined fraction (Fig 32, compare lanes d and h). Both Sarkosyl-lysozyme insoluble fractions contained a considerable amount of a 14kD species. This species was not as prominant in the envelope fractions (i.e., prior to the application of lysozyme).

Fig 32. SDS-PAGE monitoring of the OM isolation procedure. A 12%w/v acrylamide gel was used to separate the proteins. Samples: (a) MWr standards, whose value in kilodaltons is shown to the left of this lane; (b) Unspined cell lysate; (c) French Pressure Cell-derived envelope fraction from the unspined cell lysate; (d) Sarkosyl-lysozyme insoluble material obtained from the unspined envelope; (e) Spinin (5ug); (f) Spined cell lysate; (g) Envelope fraction from the spined cell lysate; (h) Sarkosyl-lysozyme insoluble material obtained from the spined envelope. The MPD71 samples were each applied as 30ug total protein.

Fig 32.



Comparable envelope and Sarkosyl-lysozyme insoluble fractions were negatively-contrasted and examined electron microscopically. The samples shown in panel A of figures 33 and 34 were obtained by French Pressure Cell breakage of MPD71 cells. By analogy with the results of Schnaitman (1981), these are likely vesicularized envelope material. Further treatment of these vesicles with Sarkosyl and lysozyme generated spherical vesicles which were smaller than the envelope vesicles (Figs 33B and C, Fig 34B).

When using detergents for membrane solubilization, consideration must be given to the potential extraction of protein from membranes (Helenius and Simons, 1975; Chopra and Shales, 1980). As well, the potential exists for the nonspecific association of solubilized proteins with the insoluble membrane fraction. If these effects are pronounced, the presumed OM could not be considered as representing OM. In order to establish if the MPD71 Sarkosyl-lysozyme fraction was OM, the protocol of Osborn et al. (1972_b) was applied. Protein from spheroplast lysates was detected spectrophotometrically at three bands following centrifugation (100,000 x g, 15h, 4°C) in a linear 40-60% w/v sucrose gradient (Fig 35). Comparison with similarly conducted procedures with other Gram-negative bacteria (Osborn et al, 1972_b) suggested that the least dense MPD71 band represented CM and the mid band a mixture of CM and OM. The white-appearing densest band (1.23 $g.cc^{-1}$ for unspined samples, 1.25 $g.cc^{-1}$ for spined

Fig 33. Electron micrographs of the envelope (Panel A) and the Sarkosyl-lysozyme insoluble (Panels B and C) fractions obtained from a morphologically unspined population. The samples were negatively contrasted with 2% aqueous uranyl acetate containing bacitracin as a spreading agent. Scale bars = 100 nm.

Fig 34. Electron micrographs of the envelope (Panel A) and the Sarkosyl-lysozyme insoluble (Panel B) fractions obtained from a morphologically spined population. The samples were negatively contrasted with uranyl acetate. Scale bars = 100 nm.

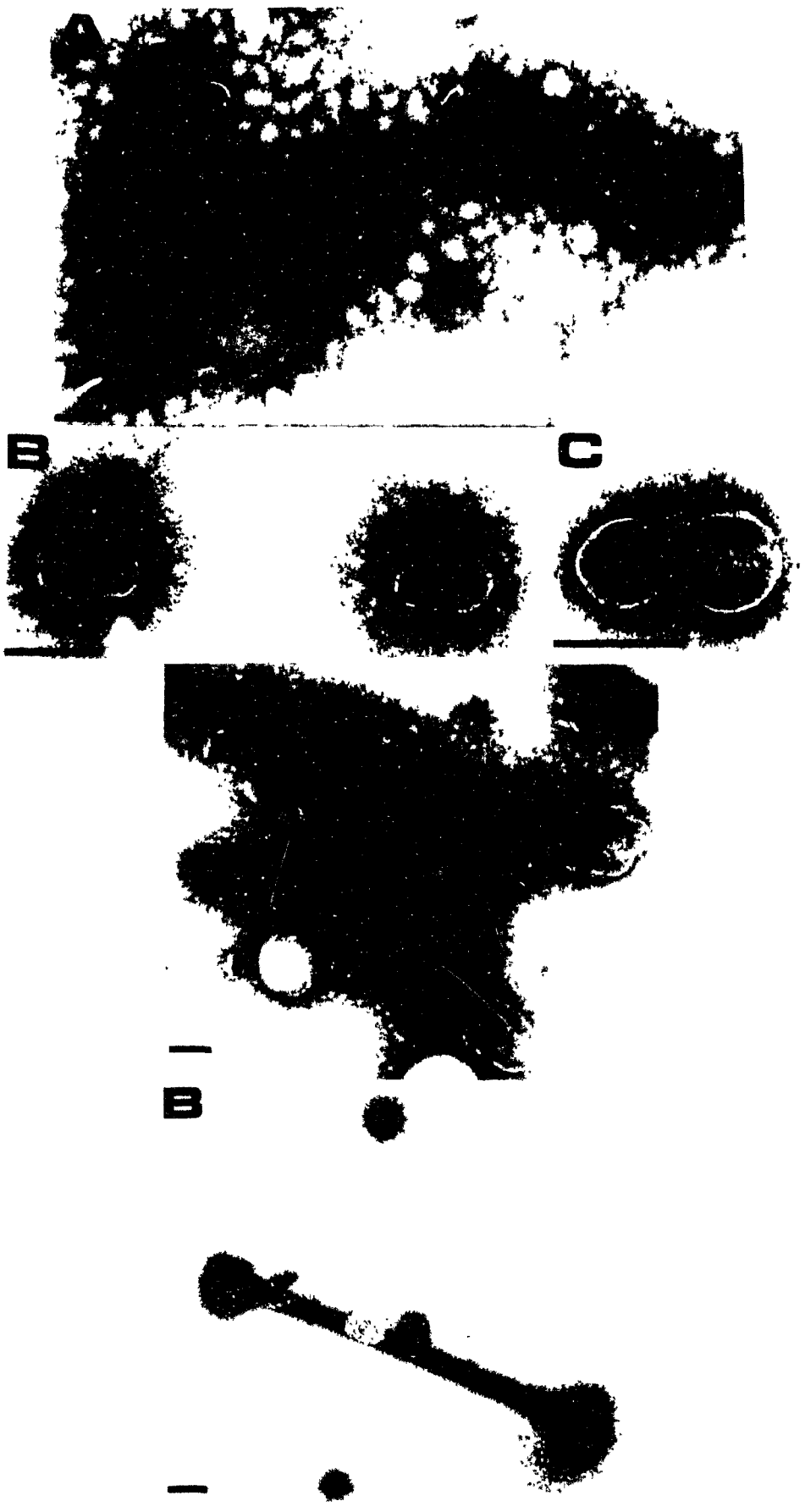
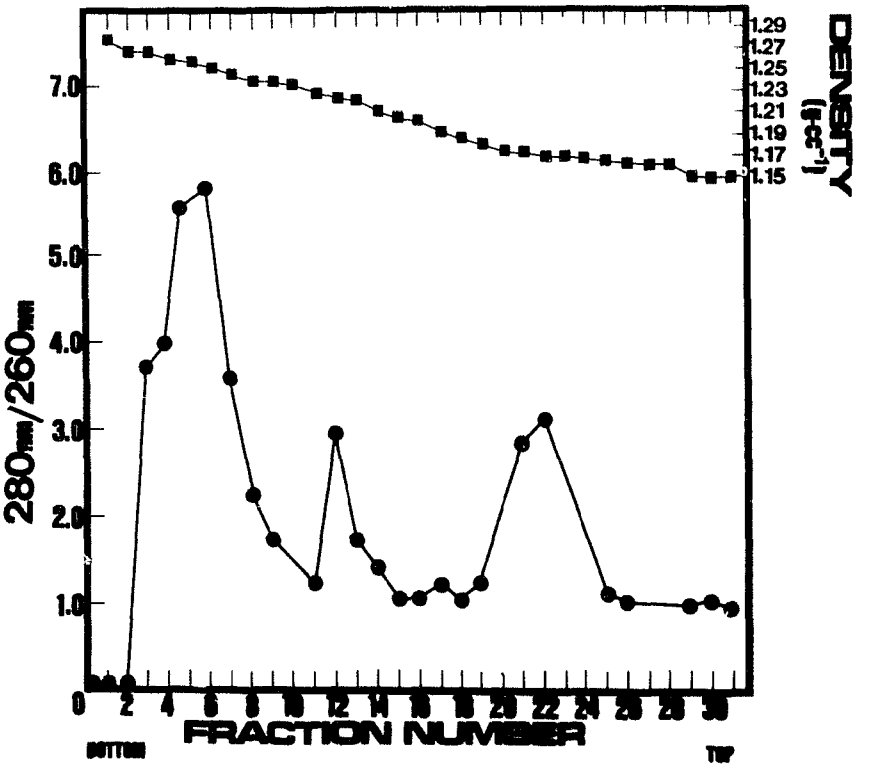

A
B
C
B

Fig 35. Spectrophotometric protein profile of 0.5mL fractions collected by bottom puncture of a 40-60%w/v sucrose gradient following ultracentrifugation (100,000 x g, 14h, 4°C) of an unspined spheroplast lysate. Each fraction was monitored at 280 nm and 260 nm and this ratio was plotted against the fraction number. This ratio determination forms the basis of a spectrophotometric protein determination protocol (Cooper, 1977) although in this figure the necessary manipulations required to express the protein concentration as $mg \cdot mL^{-1}$ were not performed. A similarly prepared gradient was centrifuged as described above and the 0.5mL fractions were monitored for their refractive index value. The density profile of this gradient is displayed at the top of the figure.

Fig 35.


1.29
1.27
1.25
1.23
1.21
1.19
1.17
1.15
DENSITY
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
2
4
FRACTION NUMBER
BOTTOM
TOP

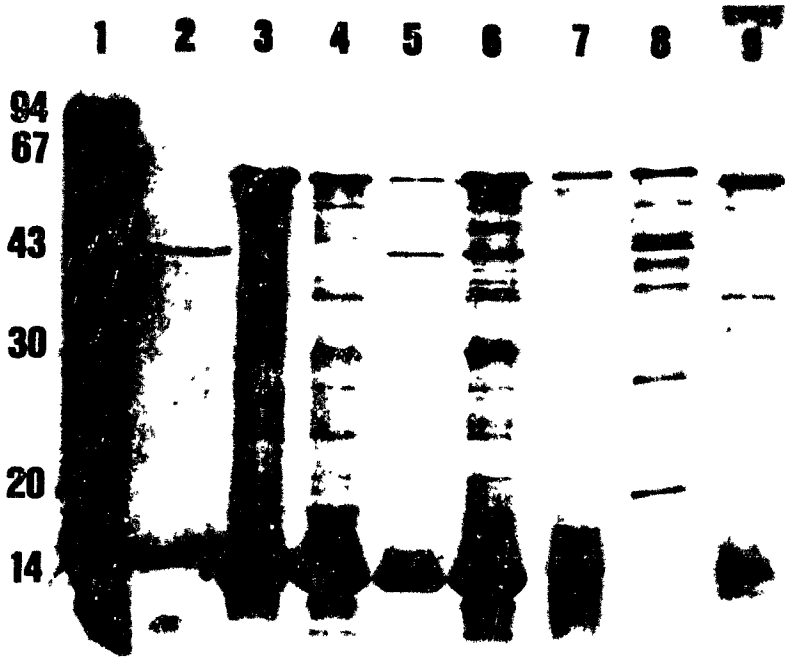
samples) would, by elimination, consist of OM. Attempts to demonstrate the presence of an OM marker (2-keto, 3-deoxyoctonic acid) and a CM marker (succinate dehydrogenase) were unsuccessful.

Similar gradient bands were obtained from unspined and spined spheroplast lysates in comparably conducted procedures. These were washed free of sucrose using deionized distilled water, and analyzed by SDS-PAGE. The polypeptide profiles of the unspined and spined OMs were similar to the comparable Sarkosyl-lysozyme insoluble material (Fig 36; compare lane 3 with 4 and lane 5 with 6). In this gel, polypeptides in excess of 65kD were not stained for unknown reasons. Differences were apparent between the unspined and spined OMs. A species of about 18kD was present only in the unspined OMs. As noted previously (Fig 33), the 42kD species was more prominant in the spined OM. Furthermore, this species was not present in the least dense (CM) band from a comparable spined spheroplast lysate (Fig 36, lane 9). The protein was present in those lysate fractions expected to contain OM (Fig 36, lanes 7 and 8). Finally, the OMs obtained using both regimens contained a substantial amount of the 14kD species (Fig 36, lanes 3-6). Based on its staining intensity, it was the most abundant protein in the OM. The absence of a similar MWr species in the samples in lanes 7-9 is curious, since lysozyme was also employed in the preparation of the spheroplasts from which this material

Fig 36. SDS-PAGE comparison of the Sarkosyl-lysozyme insoluble material and the OM derived using the regimen of Osborn et al. (1972). The MPD71 samples were applied as 30ug total protein with the exception of that in Lane 5, which was inadvertantly underloaded. A 12%w/v acrylamide gel was used and the proteins were stained using Coomassie Brilliant Blue R250.

Samples: (1) MWr standards, whose values (kD) are given to the left of the gel; (2) Spinin (5ug); (3) Sarkosyl-lysozyme insoluble fraction obtained from an unspined cell population; (4) Unspined OM obtained using the regimen of Osborn et al. (1972); (5) Sarkosyl-lysozyme insoluble fraction obtained from a spined cell population; (6) Spined OM obtained using the regimen of Osborn et al. (1972); (7) The densest band recovered from a sucrose gradient following separation of the membraneous material from a comparably prepared spined spheroplast lysate; (8) The mid-density band obtained from the same gradient as the previous sample; (9) The least dense band recovered from the same gradient.

Fig 36.



was derived. This may reflect a lack of vesicularization of lysozyme (Uemura and Mizushima, 1975) during the preparation of this particular sample.

As a final confirmation of its identity as OM, spined Sarkosyl-lysozyme insoluble material obtained in a separately conducted procedure was applied to a linear 40-60% w/v sucrose gradient and centrifuged (100,000 x g, 13 h, 4°C). Protein (280nm-absorbing) was present solely at a sucrose density of 1.25 $g.cc^{-1}$.

The prominance of the 14kD species in fractions obtained following the application of lysozyme has been noted. The quantity of lysozyme necessary to produce a similar staining intensity (5 ug; Fig 37) was present in the suspensions used to digest the peptidoglycan. However this band is not composed exclusively of lysozyme, since a 14kD band was present in cell lysates (Fig 4) and envelope (Fig 33) which had not been exposed to lysozyme.

Finally, it was of interest to determine if the 65kD and "low MWr" species, which were immunoreactive in blots of whole cells (Fig 4), were OM species. Sarkosyl-lysozyme insoluble material was probed in an immunoblot with hyperimmune antispinin serum (absorbed with E. coli and P. aeruginosa envelope and unspined cells). Purified spinin was immunodetectable (lane 3 of panel 38B). The 42kD species was stained prominently in the spined OM and much less so in the unspined OM (Fig 38, lanes 2 and 4 of panel A). It was immunodetectable solely in the spined OM

Fig 37. Coomassie Brilliant Blue R250 staining intensities of the Sarkosyl-lysozyme insoluble fractions from unspined and spined MPD71 cell populations, and of purified lysozyme. A 12%w/v acrylamide gel was used. Samples: (a) MWr standards, whose values (kD) are shown to the left of the gel; (b) The insoluble fraction from an unspined cell population; (c) The insoluble fraction from a spined cell population; (d) Spinin; (e) Hen egg white lysozyme.
The MPD71 samples were applied as 30ug total protein, spinin as 2.5ug, and lysozyme as 5ug.

Fig 37.

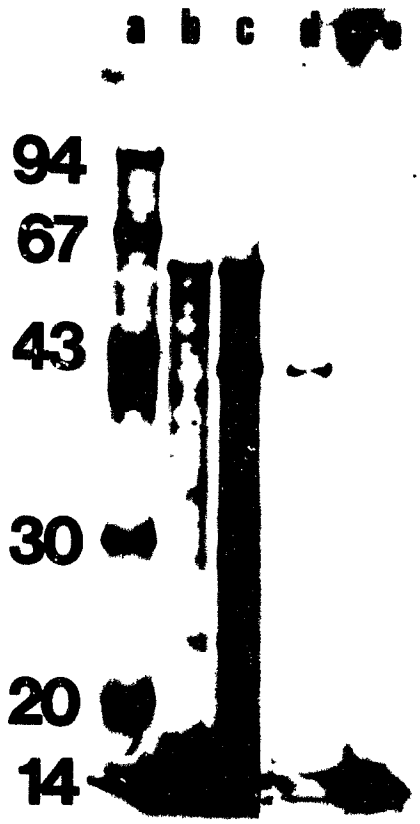
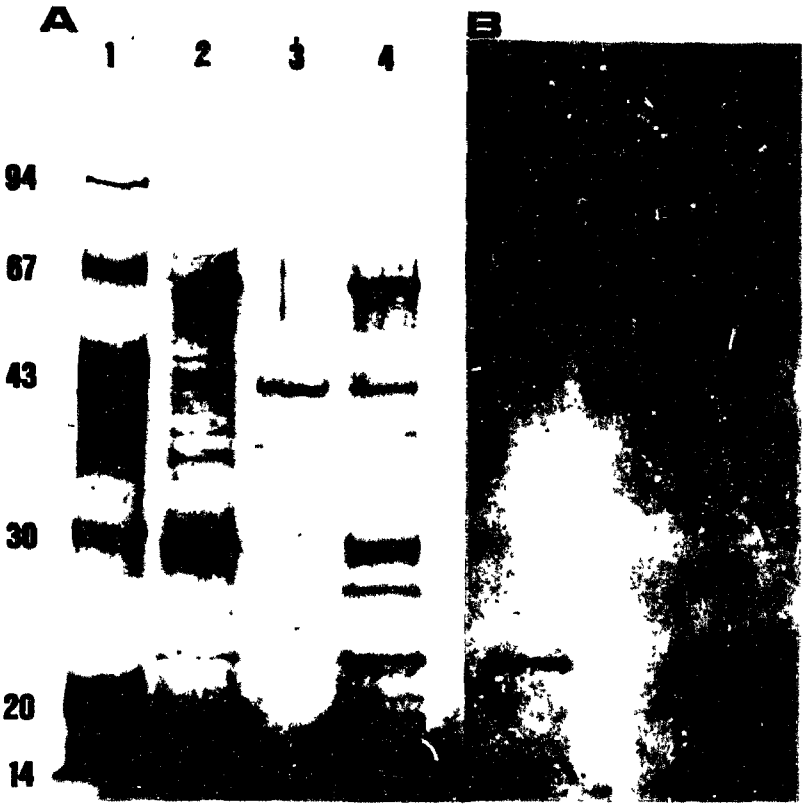


Fig 38. SDS-PAGE examination of the Sarkosyl-lysozyme insoluble fractions obtained from one of the OM isolation procedures employed in this study. A 12%w/v acrylamide gel was used to separate the polypeptides, which were then either stained using Coomassie Brilliant Blue R250 (Panel A) or probed with fully absorbed hyperimmune antispinin serum following their immobilization on nitrocellulose (Panel B).

Lane 1 of Panel A contains the MWr standards whose kilodalton values are displayed to the left of the gel. The remaining lanes in both panels contain: (2) Sarkosyl-lysozyme insoluble material obtained from an unspined MPD71 cell population; (3) Spinin; (4) Sarkosyl-lysozyme insoluble material obtained from a spined MPD71 cell population. The MPD71 samples were applied as 30 ug total protein and spinin as 5ug total protein.

Fig 38.



(compare lanes 2 and 4 of Fig 38B). In contrast, both OMs contained an immunoreactive species which migrated with a similar MWr to that of the "low MWr" species (Fig 38B). The content of this species appeared to be greater in the spined OM. The prominant 14kD OM species was not immunoreactive.

Removal of spinae from MPD71 cells, and SDS-PAGE examination of unspined, spined, and "despined" cells and OMs

If the 42kD OM protein was spinin, then it might no longer be detectable following the removal of the spina. In order to investigate this, the "despining" protocal of Willison et al. (1977) was tried. Separation of the spina from the cells was accomplished by repeated cycles of low speed (6,000 x g, 10min, room temperature) centrifugation. Since this proved disruptive to many cells (not shown), the cells were lightly fixed using paraformaldehyde (0.1% v/v final concentration) prior to the despining procedure. Reactive fixative groups were blocked by BSA to ensure pronase activity. Examination of cell populations before and after the despining procedure confirmed its success in removing the spina (Fig 39).

The despined OM was obtained (as were the analogous fractions from unspined and spined cell populations) as the Sarkosyl-lysozyme insoluble fraction. SDS-PAGE of these OMs and whole cells was followed either by Coomassie Brilliant

Fig 39. Electron micrographs of negatively-contrasted MPD71 cells prior to (Panel a), and following (Panel b), the removal of spina. Cells were lightly fixed using 0.1% w/v paraformaldehyde prior to the application of pronase (100 $ug.mL^{-1}$) and mechanical shear force. Scale bars = 100 nm.

Fig 39.

Blue R250 staining or an immunoblot using hyperimmune antispinin serum (which had been absorbed of cross-reactive antibodies using E. coli and P. aeruginosa envelopes). The results are shown in figure 40. A species co-migrating with purified spinin was apparent in the spined cell and OM fractions (Fig 40A, compare lane 4 with lanes 5 and 6). This species was not apparent in the "despined" cell and OM fractions (Fig 40A, compare lanes 2 and 3 with lane 4). OMs from a comparable experiment gave the same results (Fig 40B, lanes b and e). As well, the despined OM did not contain any stainable 42kD material (Fig 40B, lane c). When the same OMs were examined immunologically, the 42kD species was detected only in the spined OM (Fig 40C, lane e). All three OMs displayed the 65kD and "low MWr" species (Fig 40C, lanes b, c, and e), although the "low MWr" species was less prominant in the despined OM.

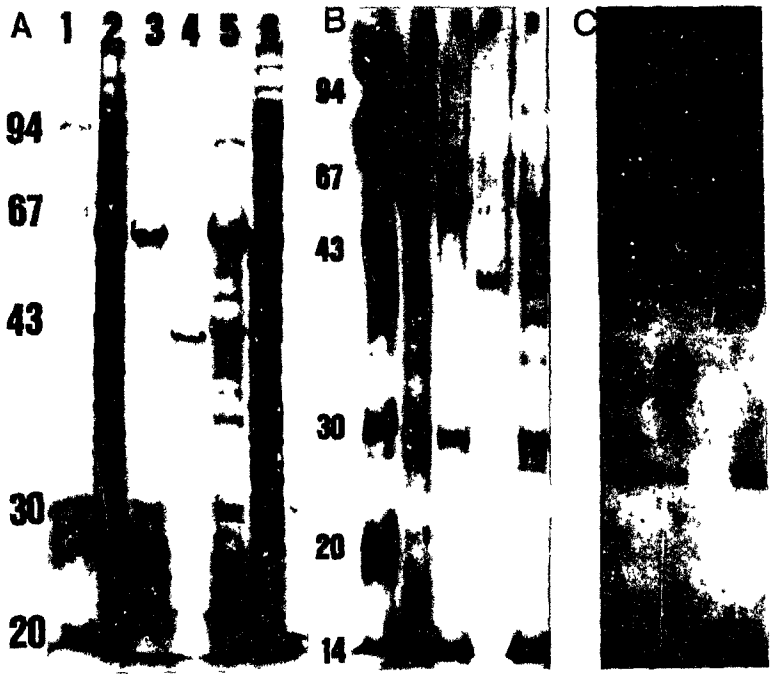
Extraction and SDS-PAGE of the MPD7 lipopolysaccharide (LPS)

The results presented thus far have focussed on the polypeptides of pseudomonad D71. Being a Gram-negative bacterium (McGregor-Shaw et al, 1972) MPD71 should, by definition, possess LPS as an OM constituent (Beveridge, 1981).

Initial attempts to demonstrate the presence of LPS in whole cell, envelope, or OM fractions by colourimetric detection of the KDO constituent (Weissback and Hurwitz,

Fig 40. SDS-PAGE examination of unspined, spined, and "despined" whole cell lysate and OM fractions. Panel A shows the Coomassie Brilliant Blue R250 stained protein profiles of "despined" and spined whole cell lysate (Lanes 2 and 6, respectively) and the Sarkosyl-lysozyme insoluble material obtained from "despined" and spined populations (Lanes 3 and 5, respectively). These samples were applied as 30ug total protein. Purified spinin (5ug) is in Lane 4. Lane 1 contains the MWr standards. The Coomassie Brilliant Blue R250 stained proteins of the Sarkosyl-lysozyme insoluble material obtained from separate populations is displayed in Panel B, and the corresponding immunoblot pattern (using partially absorbed hyperimmune antispinin serum) is shown in Panel C. Panels B and C contain Sarkosyl-lysozyme material obtained from unspined, spined, and "despined" populations. Lane a of Panel B contains the MWr standards, whose values (kD) is displayed to the left of the panel. Lanes b-e of Panels B and C contain: (b) Sarkosyl-lysozyme insoluble material obtained from an unspined MPD71 cell population; (c) Sarkosyl-lysozyme insoluble material obtained from a "despined" sample; (d) Spinin (5ug); (e) Sarkosyl-lysozyme insoluble material obtained from a spined MPD71 cell population.

Fig 40.

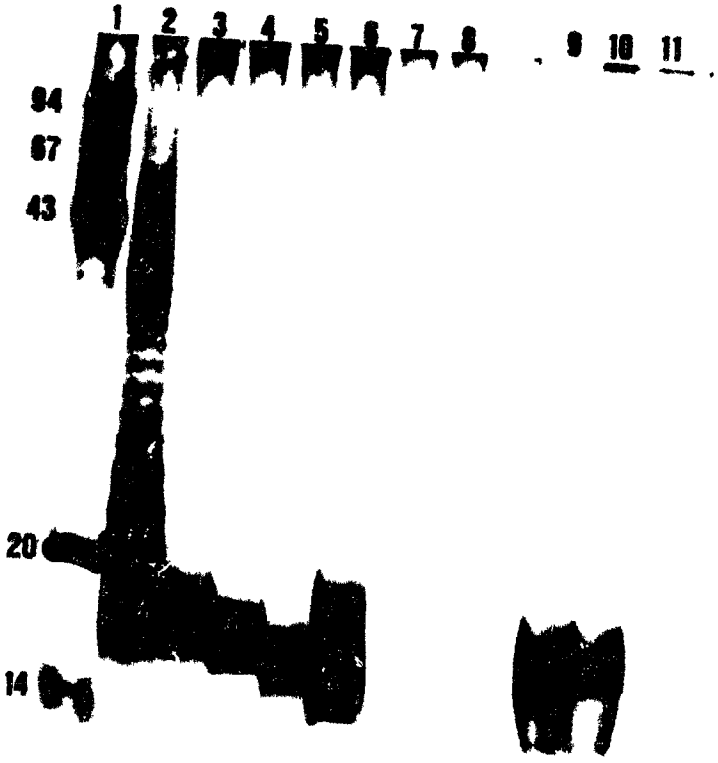


1959) were unsuccessful. However, the material obtained from 500mg wet wt of MPD71 cells (unspined and spined) using the LPS extraction regimen of Darveau and Hancock (1983) was KDO positive. Comparison of the intensity of the colour (as measured by the absorbance at 548nm) produced with that given by a similar wet wt of E. coli K12 strain AB264 (with a KDO content of 0.35umol.mg^{-1} protein; F.G. Ferris, PhD Thesis, University of Guelph, 1985) indicated a KDO content for MPD71 of approximately 0.20umol.mg^{-1} protein.

Although LPS is a universal constituent of the OM, structural variation (particularly in the polysaccharide side chains) can be considerable (Hitchcock et al, 1986). To elucidate the structure of the MPD71 LPS, cells were treated with proteinase K. The regimen utilized has been demonstrated as effective and exclusive in its LPS extraction ability (Hitchcock and Brown, 1983). The resulting fractions were electrophoresed and silver stained (Tsai and Frasch, 1982) (Fig 41). No differences were apparent in the fractions from unspined and spined cell populations (Fig 41, compare lanes 9 and 10). Comparison of the MPD71 profiles with those of the "smooth" LPS of E. coli O111B4 (lane 2) and the "rough" species of Salmonella minnesota indicated a Rc rough type of structure for the MPD71 LPS (Fig 41). Further related experiments (not shown) revealed no appreciable differences in either the content or structure of the LPS in cells grown in defined and

Fig 41. Electrophoretic detection of lipopolysaccharide (LPS). MPD71 samples were digested with proteinase K as detailed in the Materials and Methods. 10 uL aliquots were electrophoresed on a 15% w/v acrylamide SDS gel, and the LPS was visualized by silver staining using the protocol of Tsai and Frasch (1982). Samples: (1) MWr standards, whose kilodaltons values are shown to the left of the gel; (2) LPS from E. coli 0111B4 (5 ug); (3-8) LPS from S. minnesota: Ra type (3), Rb type (4), Rc type (5), Rd type (6), Re type (7), Lipid A (8); (9 and 10) MPD71 proteinase K-insoluble material from cells grown in the complex medium under, respectively spina-restrictive and permissive conditions; (11) Proteinase K in an amount (2 ug) equivalent to that used in the digestion mixture.

Fig 41.



complex media, in complex medium supplemented with 1% w/v glucose, or in the LPS fraction obtained using the regimen of Darveau and Hancock (1983).

DISCUSSION

The focus of the present investigation has been the unspined to spined morphological transition of marine pseudomonad D71 (MPD71). A morphological transition is, by definition, a change in the morphology of the cell. In the case of MPD71 this change is the assembly of spina. Preceeding spina assembly, the cell must presumably detect the physiological alteration, undertake the necessary transcriptional and/or translational events required to manufacture the spina monomer (spinin), and route the protein across the cell envelope.

The mechanism of these pre-assembly events has not been addressed prior to the present investigation. Elucidation of spina-restrictive and permissive conditions (Easterbrook and Sperker, 1982) and the temporal kinetics of spination (Easterbrook and Alexander, 1983) laid the groundwork for this study. Examination of MPD71 populations at various periods during the morphological transition allowed the following questions to be addressed: How does a cell perceive the physiological alterations? How does spinin traverse the cell envelope? Is spinin localized exclusively in the spina? Does the cell envelope of a spined cell differ compositionally from the envelope of an unspined cell?

Several conclusions can be drawn from the present investigation. These are summarized here and in Table 9. The unspined to spined phenotypic transition can be

Table 9. Conclusions from the present investigation

1. The 42kD protein present in the OM fraction of morphologically spined cells is spinin.

2. The membrane material obtained by the sequential application of Sarkosyl and lysozyme is OM.

3. Two OM species of MWr 65kD and 21-24kD are immunodetected in both MPD71 morphovars.

4. Spinin (42kD) is present only in the spined OM exclusively as the structural constituent of spina.

disturbed by the use of envelope perturbants (procaine and PEA) and by dissipating the proton motive force (CCCP). Furthermore, the synthesis of protein is required for the subsequent assembly of spina following the removal of such perturbants.

The outer membrane (OM) of MPD71 can be obtained using sequential application of Sarkosyl and lysozyme, but is not specifically extracted by the nonionic detergent Triton X-100. The unspined to spined phenotypic transition is manifest electrophoretically by an increased content of some 10 outer membrane (OM) proteins. In particular, the content of a 42kD protein is increased. This protein has the same MWr as purified spinin, its increase in concentration coincides with the production of spinae by the majority of the cell population, and it is immunodetectable using antispinin serum. Hence it is concluded to be spinin. A comparable 42kD protein is not detectable upon immunoblot examination of the unspined or despined OM. However, the unspined, spined, and despined OMs contain immunodetectable proteins having MWrs of 65kD and 14-24kD ("low MWr" species). The relatedness, if any, of these species to spinin has not been established. Since the reactivity of the antiserum towards the 65kD species can be removed by absorption (of the antiserum) with unspined whole cells, the epitope of this protein is presumably surface-accessible. The "low MWr" species remains immunodetectable using such fully absorbed sera and

so is likely not surface-accessible. Use of comparably-absorbed serum in immunocytochemical procedures specifically labels spinae in both whole cells and thin sections of Epon 812-embedded cells. However, the OM of Lowicryl K4M-embedded cells are labeled in both unspined and spined cells.

Finally, the lipopolysaccharide (LPS) component of the MPD71 OM is a "rough" Rc type, as judged by electrophoretic comparison with smooth LPS of E. coli 0111B4 and rough types of S. minnesota. The remainder of the Discussion will expand on these conclusions.

Envelope and OM structure

Since one of the aims of the present study was to examine the composition of the OM during the morphological transition, it was necessary to obtain the OM. The physicochemistry of the OM will markedly influence the efficacy of an extraction regimen. Thus, analysis of the OM extraction regimens tried with MPD71 provides information on the structure of this membrane. Two OM extraction regimens were tried. In one of these, envelopes were treated with a detergent to selectively dissolve the cytoplasmic membrane (CM). The MPD71 cell envelope was obtained by pressure explosion of the cells. The resulting vesicles retained an element of the cell shape, suggestive of the presence of PG. However, the presence of PG was not demonstrated. Since the envelope was invariably an

intermediate in the OM isolation regimen, confirmation of this component was not a priority. These vesicles were subsequently treated with either a nonionic detergent (Triton X-100) or an ionic detergent (Sarkosyl). With E. coli, Triton X-100 selectively dissolves the CM in the presence of Mg^{2+} (Schnaitman, 1971; DePamphilis and Adler, 1971), while Sarkosyl incompletely solubilizes the CM when Mg^{2+} is present (Filip et al., 1973). With MPD71, however, the presence or absence of Mg^{2+} had no apparent effect on the extraction process. Rather, the detergent used was the critical factor. Thus, the application of Triton X-100 and lysozyme was ineffective. The protein profiles of the Triton-soluble and Triton-lysozyme insoluble fractions were similar to each other and to the untreated envelope, except for the presence of a prominant 14kD species in the insoluble fraction. Since this species was also present in envelopes treated solely with lysozyme, it is reasonable to conclude that a majority of the 14kD species represents contaminating lysozyme trapped in the vesicularized OM. A prominant 14kD species was similarly present in the Sarkosyl-lysozyme insoluble fraction and in the spheroplast lysate OM recovered from a linear sucrose density gradient (Osborn et al., 1972b); both of these procedures utilize lysozyme. However, a 14kD band is observed in the electropherograms of both untreated cell lysates and cell envelopes so at least some of this material must be comprised of MPD71 protein.

The simplest interpretation of the results obtained using Triton X-100 is that the detergent is nonselective and incomplete in solubilizing the MPD71 envelope membranes. If so, then treatment of the envelope with Triton X-100 should generate a suspension consisting of extracted OM and CM proteins and partially solubilized envelope vesicles. Since the envelopes would be recovered at lower centrifugal force conditions than the solubilized proteins, both fractions would appear similar electrophoretically. If Triton had no effect, then the envelope proteins would have been detected only in the Triton-lysozyme insoluble fraction. This was not observed. Furthermore, lysozyme itself was not efficient in solubilizing the envelope.

The action of Triton on both the OM and the CM could be explained by the presence of phospholipid bilayers in both membranes. Binding of Mg^{2+} (whether supplied exogenously, or resident in the membranes) to the polar head groups of the phospholipids would mask their charge and intercalation of nonpolar compounds, such as Triton, could result. The CM contains regions of phospholipid bilayer (Beveridge, 1981). However, these regions are less frequent in the OM. In the OM, the fatty acyl chains of the LPS contribute to the hydrophobic character of the membrane. This architecture can be altered in the OM of "deep-rough" mutants, where a rearrangement of phospholipid from the inner leaflet of the membrane to the outer leaflet occurs (Nikaido, 1979; Smit

et al., 1975). In these "deep-rough" OMs, intercalation of Triton might occur.

Since electropherograms of the LPS of MPD71 reveals a pattern comparable to the Rc type of S. minnesota rough LPS, it is conceivable that the MPD71 OM may have regions of phospholipid in its outer leaflet.

Clearly the selection of the detergent is important, since the use of Sarkosyl produced a different electrophoretic pattern. Furthermore, the Sarkosyl-lysozyme insoluble MPD71 material represents OM, as judged by the identity in protein profiles between this material and the densest (OM) band of a spheroplast lysate recovered as described by Osborn et al. (1972_b). The observation that Sarkosyl-lysozyme insoluble material banded at the same sucrose density as the densest lysate band in a comparable gradient provides additional support for the OM nature of this material. The inability to demonstrate the presence of markers for the OM (KDO) and the CM (succinate dehydrogenase) does make this conclusion equivocal, however. Since these assay procedures were successful using E. coli K12, other reasons must be responsible for the failure of the assays with MPD71. With respect to the KDO assay, and to the differing effects of the detergents, this may reflect an increased tenacity of association of OM components for one another, relative to the CM. The extent of these associations is emphasized by the difficulty in demonstrating the KDO constituent of the LPS when the LPS

is in an incorporated state, be that in the whole cell, envelope, or the OM. Once extracted, however, LPS readily yields a detectable chromogen in the KDO assay. Thus, proteinase K digestion of the fractions obtained from the sucrose gradient might have permitted detection of KDO in the subsequent assay procedure. With respect to the CM marker chosen, it is conceivable that succinate dehydrogenase is not present in MPD71.

Nevertheless, until further biochemical data is available, the Sarkosyl-lysozyme insoluble material is concluded to contain OM.

The equivalence of the LPS staining intensities and KDO content in both MPD71 morphovars suggests there is a similar LPS content in both morphovars. This is not surprising. A spina base 120nm in diameter (Easterbrook et al., 1976) encloses a surface area of 0.011um^2 ($\pi r^2 = 3.142 \times (0.06)^2$; r=radius of the base). Assuming 17+/-5 spinae per cell (Easterbrook and Alexander, 1983), the total surface area enclosed would be 0.13-0.24um^2. This is only about 2% of the total surface area of a cell[1].

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1 The cell is assumed to have average dimensions of 1 x 3um (MacGregor-Shaw et al., 1973). To estimate the surface area the cell poles are combined to form a sphere with a diameter of 1um. The surface area of the sphere is $4\pi r^2 = 4(3.142)(0.5)^2 = 3.142$um^2. The remainder of the cell is regarded as a cylinder approximately 2um in length, thus its' surface area is $2\pi rl = 2(3.142)(0.5)(2) = 6.28$um^2. The total cell surface area is 3.142 + 6.28 = 9.42um^2.

Alteration of only 2% of the surface during the phenotypic transition should not drastically decrease the LPS content, since LPS is probably more generally distributed (Lugtenberg and Van Alphen, 1983). However, since spinae are first detected at regions of the cell where cell division has recently occured or is imminent (Easterbrook and Alexander, 1983), and since the envelope in these regions may not be as stabilized as in other regions (Koch, 1985), LPS in these regions may be displaced by the "flow" of spinin during the period of maximal assembly of spinae. If so, no decrease in the quantity of LPS can be detected by SDS-PAGE. While the LPS content of MPD71 appears to be invariant during the phenotypic transition, the content of an OM protein increases considerably (based on the intensity of staining). This protein migrates with a MWr (42kD) equivalent to that of the species obtained from heat-dissociated spinae, and both species are immunodetected when immobilized on nitrocellulose and probed with an antiserum raised against heat or sonically-dissociated spina. Thus, it is concluded that the 42kD OM protein is comprised of spinin. MWr estimates of spinin include 32kD (R.W. Coombs, PhD Thesis, Dalhousie University, 1977) and 37kD (Easterbrook and Coombs, 1977), probably due to minor alterations in the physiological pH or the electrophoretic parameters (Coombs et al., 1976, 1978). Indeed, during the course of the present study, spinin was occasionally observed to migrate

as a "doublet" with MWr values of 42kD and 43kD. This effect was apparently influenced by the pH of the gel system, since in one experiment where the pH of the stacking gel was reduced to 6.8 from 8.8, spinin migrated as a single 42kD species. Use of other MW determination techniques which do not use SDS yields a value of 19-22 kD for spinin (Coombs et al., 1978). The higher MWr values in the presence of SDS may be due to the formation of a spinin dimer, due to hydrophobic association. Since SDS also associates hydrophobically with polypeptides, its binding capacity would be reduced. This has in fact been observed with spinin (R.W. Coombs, PhD Thesis, 1977, Dalhousie University). If the spinin dimer was stable to the temperature employed for dissociation, then upon SDS-PAGE the dimer would migrate with a MWr of (19-22) x 2, or 38-44kD. The MWr observed in this study (42kD) is consistent with this interpretation. Additionally, there is precedent for the formation of SDS-stable dimers in the pilin of P. aeruginosa PAO1 (Frost and Paranchych, 1977) and in the coat protein of bacteriophage f1 (Makino et al., 1975).

Another consequence of the reduced binding of SDS can be an inefficient masking of charge, which can cause anomalous electrophoretic migration of highly charged proteins such as histone F1 (Weber and Osborn, 1975). However, this effect produces only up to a 50% variation in MWr. The 220% difference in the observed MW estimates for spinin (19

versus 42kD) is too great to be accounted for by the inefficient masking of charge.

Thus, the 42kD spinin species may well represent a dimer of a 19-22kD spinin monomer. This conclusion has relevance in light of the OM immunoblot and thin section immunocytochemical results obtained in this study. Immunoblot examination of unspined and spined OM detects species of 65kD and 19-24kD ("low MWr"), using as a probe antispinin antiserum absorbed of cross-reactive antibodies using envelopes of E. coli K12 and P. aeruginosa PAO1. Since the 65kD species is eliminated from subsequent immunoblots by the further absorption of the antiserum with unspined whole cells, the 65kD species likely represents a protein unrelated to spinin but having a similar, surface exposed epitope. The continued immunodetection of the "low MWr" species using this "fully absorbed" antiserum warrants further comment.

Assuming that the antiserum was free from contamination, it is appropriate to suggest that the "low MWr" species represents the spinin monomer (the variation in the MWr of the "low MWr" species observed during this study may be due to the conformation of the polypeptide; Weber and Osborn, 1975). If so, it is present as an OM species in both MPD71 morphovars. In the unspined OM, spinin would be an integral or peripheral protein since, by definition, unspined cells lack spinae. Thus, the difference in the OM immunodecoration patterns between the thin sections of Epon

812 and Lowicryl K4M-embedded cells could be explained on the basis of conformational alterations between the OM-localized and polymerized (as the spina protomer) forms of spinin. Presumably the fixation steps in the Epon regimen disrupted the spinin epitope, rendering the OM-incorporated spinin antigenically undetectable. In contrast, the Lowicryl regimen preserves antigenicity (Roth, 1981).

Clearly spinin can undergo conformational alterations, which might facilitate either membrane intercalation or polymerization to form a spina (Easterbrook and Coombs, 1983). Thus, as proposed by Easterbrook and Alexander (1983) spinin could be considered to be an OM protein which, when present in excess or if excreted, will self-assemble to generate spinae. If this is so, the function(s) of the OM-localized and polymerized forms of spinin are probably different. While a unique function has not yet been demonstrated for spinae, proposed roles include the protection of the cell from protozoan ingestion and the promotion of aggregation (Easterbrook and Sperker, 1982). Since these roles require the presence of a projecting appendage, a similar role(s) for the OM-incorporated spinin is difficult to envision.

The preceeding speculation assumes the purity of the spina preparation used as the immunogen. Spina can be obtained free of contaminating protein and LPS (Easterbrook and Coombs, 1976). Care must be exercised, however, to

avoid contamination of the spina preparation. Indeed, examination of "isolated" spina does occasionally reveal membrane blebs adhering to the spina base (as an example see figure 11 of Willison et al., 1977). If membrane material is present, then LPS certainly would be. LPS is a potent immunogen, even when present in small quantities (Hitchcock et al., 1986), so its presence would be problematical in subsequent immunoblot or immunocytochemical procedures. I cannot exclude the possibility of LPS contamination of the spina preparations used as immunogens. While KDO was not detected in these preparations, this does not necessarily mean it is absent. Rather, as with the OM, KDO may need to be freed of its association with spinae before it can be detected. Additionally the possibility of protein contamination of the spina preparations cannot be excluded, although it seems unlikely. The antiserum used in this study was raised against a spina preparation comprised solely of the 42kD species (as judged by Coomassie Brilliant Blue R250 and high-performance liquid chromatographic analyses; K.B. Easterbrook, personal communication, and by silver staining of SDS-PAGE gels; not shown). If the "low MWr" species was a contaminating protein, it must have been tenaciously associated with the interior and/or the exterior of the spina. However, the presumed low level of this contaminating protein relative to spinin is hard to

reconcile with the strong immunodetection of the "low MWr" species in the unspined and spined OMs.

Clearly the resolution of the identity and relatedness of the immunoreactive species to the spina protomer will require the development of antisera directed towards specific, distinct epitopes and a comparative analysis of the immunoreactive proteins.

Perturbance of the envelope during spination

The ability of a cell to "sense" an environmental alteration is required for the unspined to spined transition. This sensory capacity is not uniform for all the cells in a population, since alteration of the individual parameters of pH, temperature, and salinity results in a variation in the proportion of the cells which produce spinae (Easterbrook and Sperker, 1982; and Table 5 of this thesis). This variation in the MPD71 population response brings to mind the response of E.coli to osmolarity (Van Alphen and Lugtenberg, 1977), pH (Heyde and Portalier, 1987; Taglicht et al., 1987) and temperature (Ludrigan and Earhart, 1984). A component of these environmental responses is the fluctuation in the OM content of two E. coli proteins (OmpF and OmpC) at the level of transcription (Lugtenberg and Van Alphen, 1983). An envelope protein (EnvZ) and a cytoplasmic protein (OmpR) are proposed to have a central role in this regulatory

process (Hall and Silhavy, 1981; Taylor et al., 1983), the details of which have been presented in the Introduction.

If MPD71 cells use a protein functionally analogous to EnvZ as an environmental sensor, then compounds which disrupt membrane fluidity and perturb an "EnvZ-like" protein might perturb spination. The observation that procaine and PEA (which are similar in composition and effect; Halegoua and Inouye, 1979) perturbed the MPD71 spination process is consistent with the involvement, in spination, of an EnvZ-like protein. Procaine concentrations of 1-20uM progressively inhibit the assembly of spinae. The procaine concentrations used were at least 50-fold lower than those which perturbed the transcription and co-translational processing of E.coli proteins (Lazdunski et al., 1979; Pugsley et al., 1980). Perhaps comparable concentrations would produce a more demonstrable effect on the production and/or processing of spinin. At the concentrations which were used, procaine may have specifically perturbed unspined cells. In the experiments described in this thesis procaine was added when approximately 10% of the enumerated cells possessed spinae. It is thus not unexpected that some unspined cells may have already produced the mRNA for spinin. Once the mRNA had been produced, and translation of spinin had begun, perturbation of an Env Z-like protein by compounds such as procaine and PEA may be ineffective in inhibiting spina assembly by these cells.

Electropherograms of the OMs from unspined cells and 0.08% v/v PEA-treated cells (in which spination was repressed) revealed an identity in their protein profiles. The repression of spination did not affect cellular viability (see Table 6, 0.01-0.1% v/v PEA) provided the envelope perturbant is present from the time of shift to spina-permissive conditions. Thus, the cells were maintained in an unspined "state" even in spina-permissive conditions. In the case of PEA, its introduction after spina assembly has begun, or the spination response is "turned on", does not affect the subsequent assembly of spinae (Table 7). Thus, 0.08% PEA-treated cell populations were either unaffected by the membrane perturbant (they "sensed" the environmental alteration and produced spinae) or were susceptible to the perturbant (they did not "sense" the environmental alteration). The observation that the OM obtained from spined cells contained up to 10 polypeptides which were absent in the OM of cells in which spination had been suppressed by the membrane perturbants is evidence of the pleiotrophic nature of the envelope perturbance. An EnvZ-like protein, although unidentified as yet, is a candidate for these effects.

Another inhibitor of spination, CCCP, acts in a different fashion from envelope perturbants such as procaine and PEA. Addition of CCCP (1-5uM) uniformly arrests MPD71 cells in "suspended animation". Inhibition of growth and spination occurs immediately after the addition

of CCCP, regardless of when it is introduced following the shift of a population to spina-permissive conditions. Furthermore the presence of CCCP results in the accumulation of an immunodetectable protein about 2kD greater than spinin. In other bacteria CCCP causes an accumulation of secretory precursors due to a CCCP-induced dissipation of the proton motive force (Michaelis and Beckwith, 1982; Zimmermann and Wickner, 1983; Bakker and Randall, 1984). By analogy with these bacteria it is appropriate to suggest that this higher MWr species may well represent a "pre-spinin" secretory precursor. The identity of the 44kD species as a spinin secretory precursor is premature given the paucity of data. Experiments designed to detect a precursor were unsuccessful. Attempts to separate the 42 and 44kD species using gels of varying acrylamide concentration could not adequately separate these species from the background of other proteins of similar MWr. Attempts were made to radiolabel possible precursors in the presence of CCCP, however, use of a complex medium proved fruitless due to the presence of a relatively large concentration of unlabeled amino acids. This necessitated the use of defined media, which were unsuccessful due to the poor growth and spination of the cultures.

In another experimental avenue, the predicted difference in the pI for a "pre" and "mature" spinin suggested the possibility that a precursor species could be distinguished

by isoelectric focussing. In one preliminary and frustratingly suggestive experiment, spinin from heat-dissociated, purified spinae migrated as two species with pIs of 3.35 and 3.4 (data not shown), comparable to the results of Coombs et al (1978). These species were immunodetectable in the lysate of an untreated (spined) population. In contrast, a CCCP-treated lysate displayed instead an immunodetectable species with a pI of 3.6. The increased positive charge of this species relative to the spina protomer would be consistent with the presence of a signal sequence (von Heijne, 1984, 1985a). Unfortunately it was not possible to duplicate these results The spina system is not alone in producing such results. The RS proteins of Aeromonas and Methanobacterium species are translated as secretory precursors, but these precursors cannot be detected upon immunoprecipitation (T.J. Trust and S.F. Koval, personal communication). Thus, at this time the details of the spinin excretion pathway remains unclear. Presumably this pathway shares similarities with the mechanisms presented elsewhere in this thesis. Clearly the elucidation of the DNA sequence encoding spinin would resolve the outstanding questions concerning the presence of secretory competent domains within the protein, as well as the size of the protein. Obviously production of MPD71 mutants defective in spina assembly would shed light on those envelope proteins involved in the trans-envelope passage of spinin and/or its surface anchorage. The

independent reconstitution of the two immunodetectable OM species (65kD and "low MWr") and spinin into phospholipid vesicles or black lipid bilayers (Schindler and Rosenbusch, 1978) would represent a useful experimental avenue. Spina assembly on the surface of translationally-competent vesicles containing one or more of these proteins (or other OM species) would be highly suggestive of a role of these proteins in the spination event *in vivo*.

The unspined and spined "states" of MPD71

The unspined to spined morphological transition of MPD71 has been the focus of the present investigation. The experiments described in this thesis have examined MPD71 populations following a shift from spina-restrictive to permissive conditions. While the population as a whole becomes spined in a relatively short time, the transition is not synchronous. Thus, cells in the same population will be unspined and spined. As discussed earlier, this may reflect a difference in the ability of cells to perceive the environmental alteration. The nature of this difference(s) remains unclear, but may involve a sensory protein. Sensing an altered environment triggers a series of events which encompass the morphological transition. Thus, it seems reasonable to suppose that there is an intermediate form of the cell, rather than simply unspined and spined forms. The features of this intermediate form are not clear. Examination of the protein profiles of

PEA-treated OMs does not reveal an obvious transition pattern from unspined to spined. Rather, the transition from an unspined to a spined OM protein profile is more abrupt. This may reflect the examination methods used. Perhaps subtler changes in membrane architecture remained undetected.

Although the picture which has emerged concerning the nature of the MPD71 spination process is by no means complete, elucidation of the OM protein profile in the unspined and spined morphovars, and their alteration during the phenotypic transition provides a firmer foundation on which future experimentation can be based.

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APPENDIX PUBLICATION

Hoyle, B.D., and K.B. Easterbrook. 1986. Electrophoretic studies on the cell envelope of a spina-producing marine pseudomonad. Can. J. Microbiol. 32:901-908.

Electrophoretic studies on the cell envelope of a spina-producing marine pseudomonad

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The effect of physiological parameters known to determine the transition from unspined to spined phenotype in marine pseudomonad D71 has been evaluated using electrophoretic techniques. The levels of at least four protein species (65, 42, 21, and 14 kilodalton (kDa)) in whole-cell lysates were affected. The 65-, 42-, and 14-kDa species were shown to be the major constituents of the outer membrane isolated by gradient separation of spheroplast lysate or detergent dissociation. Both the 42- and 21 kDa species were detected in immunoblots of outer membrane using antispinin antiserum exhaustively preabsorbed with unspined cells. The 21-kDa species was detected in both unspined and spined outer membrane, whereas the 42-kDa species, which migrated with the same mobility as purified spinin, was detected only in spined outer membrane. The 65-kDa species was no longer immunodetected and so likely represented a surface-exposed polypeptide common to both phenotypes. The structure and content of the lipopolysaccharide as determined by electrophoretic analysis was unchanged during the transition in phenotype. Lipopolysaccharide could not be detected in untreated whole-cell lysates, but could be detected chemically and electrophoretically after digestion of the cells. Comparison with LPS species from *Salmonella minnesota* indicated a deep-rough structure.

HOYLE, B. D., et K. B. EASTERBROOK. 1986. Electrophoretic studies on the cell envelope of a spina-producing marine pseudomonad. Can. J. Microbiol. **32**: 901–908.

Les effets de paramètres physiologiques qui sont connus comme déterminant la transition d'un phénotype sans spinae vers un phénotype avec spinae ont été évalués à l'aide de techniques électrophorétiques chez la pseudomonade D71. Les niveaux d'au moins quatre espèces de protéines (65, 42, 21 et 14 kiloDaltons (kDa)) ont été effectés dans des lysats de cellules entières. Les espèces à 65, 42 et 14 kDa se sont avérées les constituants majeurs de la membrane externe et furent isolées par séparation de gradients de lysats de sphéroplastes ou par dissociation avec détergents. Les espèces à 42 et 21 kDa furent décelées dans des immunobuvardages de la membrane externe à l'aide d'un antisérum qui fut dirigé contre la spinine et préabsorbé de façon exhaustive par des cellules sans spinae. L'espèce à 21 kDa a été décelée tant dans les membranes externe des cellules avec spinae que dans celles des cellules sans spinae, alors que l'espèce à 42 kDa, qui migre avec la même mobilité que la spinine purifiée, ne fut décelée que dans les membranes externe des cellules avec spinae. L'espèce à 65 kDa n'a pas été immunodécelée et, donc, représente possiblement un polypeptide de surface commun aux deux phénotypes. La structure et la teneur en lipopolysaccharides, telles que déterminées par des analyses électrophorétiques, sont demeurées inchangées au cours de la transition des phénotypes. Les lipopolysaccharides n'ont pu être décelés dans les lysats de cellules entières non traitées mais, après digestion des cellules, ils purent être détectés chimiquement et par électrophorèse. Une comparaison avec les espèces de lipopolysaccharides dérivées de *Salmonella minnesota* indique l'existence d'une structure fortement ruqueuse.

[Traduit par la revue]

Introduction

Two nonprosthecate bacterial appendages, flagella and pili (fimbriae), have been long recognized and extensively studied. The rigid, helically organized protein appendages termed spinae by Easterbrook and Coombs (1976) must now be added as a third class. It has been proposed (Easterbrook and Subba Rao 1984) that spinae constitute a group of appendages with characteristic architecture possessing slightly variable morphology.

The prototype spinate organism is the marine pseudomonad D71 (NCMB 2018; MPD71) described by McGregor-Shaw *et al.* (1973). For this bacterium, both the structure and development of spinae have been intensively studied (Coombs *et al.* 1976; Easterbrook *et al.* 1976; Willison *et al.* 1977) and their production has been shown to be controlled by physiological parameters (Easterbrook and Sperker 1982; Easterbrook and Alexander 1983). Populations that are specifically unspined or spined can be readily obtained (Easterbrook and Sperker 1982).

Both the structure and the control of the protomer spinin resemble that of outer membrane structural proteins in other Gram-negative bacteria, suggesting the possibilities that a phenotypically unspined organism might contain spinin in its surface in lower amount, in an altered conformation, or perhaps associated with other proteins. To investigate these possibilities and simultaneously characterize the envelope protein composition of D71, we isolated the outer membrane from unspined and spined cultures and analyzed them electrophoretically in a sodium dodecyl sulfate (SDS) – polyacrylamide gel system and immunologically by immunoblotting with antisera raised against purified spinae. We found alterations in the levels of two immunologically reactive proteins during the transition from the unspined to spined phenotype. One of these proteins was detected in both morphological phenotypes. As part of the envelope characterization we also extracted the lipopolysaccharide (LPS) and compare its electrophoretic pattern to that of LPSs of other Gram-negative bacteria.

Materials and methods

Chemicals

Unless otherwise noted, chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Organisms and reagents

Marine pseudomonad D71 (NCMB 2018) (McGregor Shaw *et al* 1973), *Pseudomonas aeruginosa* PAO-1, and *Escherichia coli* K12 strain AB264 were obtained from our laboratory culture collection. Purified lipopolysaccharide from *E. coli* 0111B4 was purchased from Sigma Chemical Co.; purified lipopolysaccharide from *Salmonella minnesota* was purchased from List Biologicals (Campbell, CA, U.S.A.).

Media

Generally, a complex medium similar to that described previously (Baumann *et al.* 1971) was used. The medium, unbuffered or buffered using 50 m*M* HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) or Trizma base, was adjusted to a pH of 6.9 or 7.5 using 10 *N* NaOH prior to autoclaving. For one experiment a defined medium developed by Michael Coughlin (Easterbrook and Sperker 1982) was used.

SDS polyacrylamide gel electrophoresis (PAGE)

SDS PAGE was performed essentially as described by Laemmli (1970) using stacking–separation gel systems of 4:12 and 7:15%, with respect to acrylamide concentration. For the gel shown in Fig. 1, a 7–17% acrylamide gradient was used. Following electrophoresis, all gels were washed to remove SDS and stained with Coomassie brilliant blue R250 (0.2%, v/v). Both operations were done at 45°C in an aqueous solution of 25% (v/v) isopropanol and 10% (v/v) acetic acid. Destaining was performed at 45°C in 7% (v/v) acetic acid. Molecular weight markers (14–94 kilodalton (kDa); Bio-Rad) were used to calibrate the gels.

Immunoblotting

The "Western blot" procedure described by Towbin *et al.* (1979) was employed using a 1:1000 dilution of preabsorbed antiserum (see below for details of the absorption procedure) and a 1:1000 dilution of goat antirabbit horseradish peroxidase conjugated IgG (Miles Laboratories, Rexdale, Ont.). Labelled species were visualized as a grey product in a horseradish peroxidase catalyzed reaction using 4-chloro-1-napthol as the substrate. Electrical transfer conditions of 60 V for 70 min were established as optimal from gel to nitrocellulose of ^{14}C labelled polypeptides (Amersham, Oakville, Ont.) ranging in molecular mass from 70 to 15 kDa.

Antisera

Mature white rabbits, approximately 1 kg in weight, were injected intramuscularly at the shoulder and flank with a total of 1.0 mg purified spinae in complete Freund's adjuvant. After 30 days the rabbits were boosted by similar injection with 100 μg purified spinae in 0.85% saline. The rabbits were bled by cardiac puncture 1 week later.

Absorption of nonspecific IgG from antisera

Nonspecific IgGs were removed from immune sera by dilution (1:1000) in blotting buffer containing 30 $\mu g \cdot mL^{-1}$ of *E. coli* K12 strain AB264 and *P. aeruginosa* PAO envelope vesicles and incubation for 60 min at 34°C with periodic agitation. Following the incubation period envelope material was removed by centrifugation at maximum speed in an Eppendorf microfuge for 10 min. This procedure was repeated three times and the final supernatant, representing absorbed antiserum, was collected and used in subsequent immunoblots. In one experiment 30 $\mu g \cdot mL^{-1}$ of D71 unspined cells were also included in the absorption mixture.

Effect of physiological alterations on spination

To investigate the effect of salinity, pH, and temperature on the electrophoretic profile and spination, D71 cells were cultured in the defined medium at either pH 6.8 or 7.4 with a NaCl concentration ranging from 0.1 to 0.4 M. The specific conditions of pH 6.8, 24°C, 0.2 M NaCl, and of pH 7.4,34°C, 0.2 M NaCl represent those parameters which generate, respectively, unspined and spined phenotypes of D71 (Easterbrook and Sperker 1982) and have been included for continuity. Mid to late logarithmic growth phase cultures (defined in preliminary growth experiments) were harvested at 6000 × g, 15 min,4°C, and the cells were washed once using 10 mM HEPES (pH 6.8), then lysed by resuspension in a small volume of 4% (w/v) SDS for 20 min at room temperature. The lysates were diluted 10-fold in electrophoretic dissociation buffer, boiled for 5 min, and amounts containing 30 μg protein (as determined by the method of Lowry *et al.* 1951) were subjected to electrophoresis in a 10—14% acrylamide gradient gel. As was observed by Markwell *et al.* (1978), the presence of 0.4% (w/v) SDS in the Lowry assay volume did not interfere with spectrophotometric determination of protein. A portion of each sample was withdrawn prior to SDS lysis for negative contrasting and electron microscopy as described below. At least 300 cells were evaluated in all samples and scored as either unspined or spined.

Isolation of the cell envelope

Escherichia coli AB264, *P. aeruginosa* PAO-1, or D71 cells were harvested by centrifugation (6000 × g, 15 min, 4°C) at an optical density (660 nm) which was determined in preliminary experiments to correspond to a late logarithmic phase of cultural growth (1.0 for D71 cultures, 1.2 for *E. coli* and *P. aeruginosa*; approximately 2 × 10^9 cells·mL^{-1}) and washed twice with 10 m*M* HEPES (pH 6.8)–0.1 m*M* $MgCl_2$ using the same centrifugation conditions. The final pellet was resuspended at a population density of 5 × 10^8 cells·mL^{-1} in the above buffer containing deoxyribonuclease (50 $\mu g \cdot mL^{-1}$), ribonuclease (100 $\mu g \cdot mL^{-1}$), and phenylmethyl sulfonyl fluoride (PMSF, a protease inhibitor; final concentration, 1.0 m*M*). The suspension was passed twice through a cold French pressure cell (American Instrument Co., Silver Springs, MD) at 15 000 psi (1 psi = 6.895 KPa) and the lysate was incubated at room temperature for 1–2 h to allow for digestion of nucleic acid. Repeated washes (40 000 × g, 30 min, 4°C) using the above buffer removed residual nucleic acid from the envelope preparation; the final pellet was resuspended to an approximate protein concentration of 10 $\mu g \cdot mL^{-1}$ in the above buffer and either used immediately or stored at −20°C.

Isolation of the outer membrane (OM) as a detergent —lysozyme membrane fraction

Envelope material was resuspended in 0.5% (w/v) Sarkosyl buffered with 10 m*M* HEPES (pH 6.8) to a concentration of approximately 8 mg protein·mL^{-1} and incubated at 30°C for 30 min with periodic agitation. Sarkosyl-insoluble material was recovered at 40 000 × *g*, 30 min, 4°C, and the supernatant was discarded. The pellet was resuspended in 5 mL of a lysozyme solution (100 $\mu g \cdot mL^{-1}$ lysozyme in 10 m*M* HEPES (pH 6.4), incubated at room temperature for 60 min with constant agitation, and the detergent–lysozyme insoluble membrane material was recovered by centrifugation (40 000 × *g*, 60 min, 4°C). This material was washed once with 10 m*M* HEPES (pH 6.8) – 1.0 m*M* $MgCl_2$ using the same centrifugation conditions, resuspended in a small volume of the same buffer, and either used immediately or stored at −20°C.

Gradient isolation of OM

Spheroplasts were prepared from D71 cells as detailed by Osborn *et al.* (1972) and were lysed osmotically by dilution in ice-cold deionized distilled water. Membraneous material was collected by centrifugation (40 000 × *g*, 60 min, 4°C) and resuspended in 25% (w/v) sucrose. An aliquot of this suspension (representing about 20—25 mg protein) was layered onto a sucrose gradient constructed by layering equal volumes of 55, 50, 45 and 40% (w/v) sucrose solutions over a cushion of 60% sucrose. The gradients were stored overnight at 4°C prior to use to eliminate gradient discontinuities by diffusion. In other experiments a similar amount of the Sarkosyl—lysozyme insoluble membrane material was layered onto similarly constructed gradients. All gradients were centrifuged (150 000 × *g*, for 13–15 h, 4°C) and 0.5-mL fractions were collected from the bottom of each tube. Residual sucrose was removed by washing (40 000 × *g*, 30 min, 4°C) four or five times using 10 m*M* HEPES (pH 6.8) – 1.0 m*M* $MgCl_2$. The final pellet was resuspended in a small volume of the same buffer and used immediately.

Preparation and SDS—PAGE of D71 lipopolysaccharide (LPS)

The procedure of Hitchcock and Brown (1983) was modified somewhat with respect to proteinase K digestion conditions. D71 cells were suspended in 10 m*M* HEPES (pH 6.8) – 1.0 m*M* $MgCl_2$ and a volume containing approximately 2 × 10^8 cells was centrifuged at maximum speed in an Eppendorf microfuge for 5 min and the pellet was resuspended in 40 μL of Laemmli dissociation buffer. The suspension was boiled for 5 min, allowed to cool, 10 μL of a 1.0 mg·mL^{-1} proteinase K solution was added, and the mixture was incubated at 60°C for 3 h with periodic agitation. After boiling for 5 min, sample aliquots of 10 μL were electrophoresed using a 15% acrylamide slab gel and LPS was visualized using the silver stain

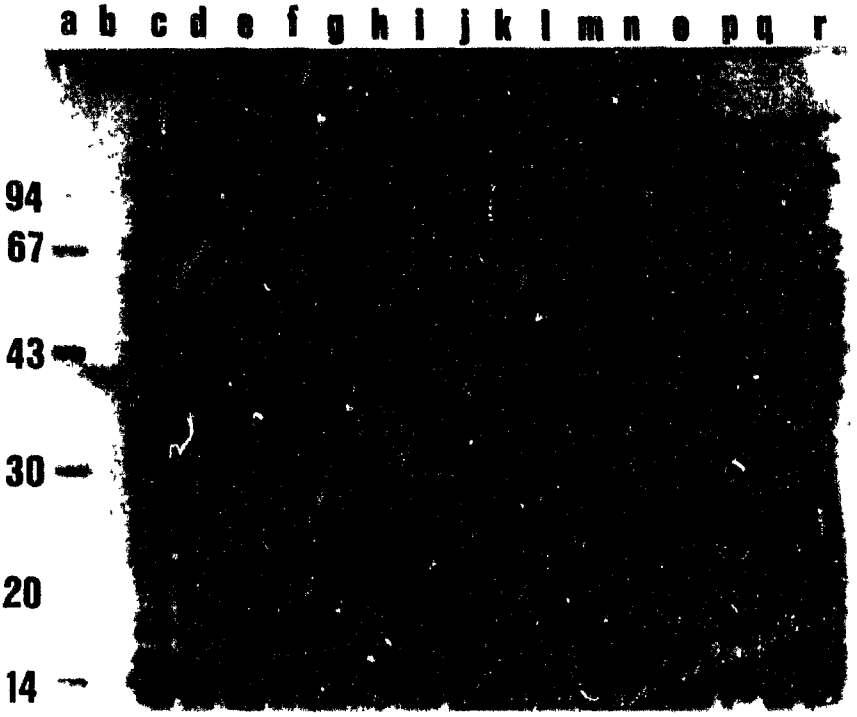

FIG. 1. SDS–PAGE protein profile of D71 whole-cell lysates following growth in a defined medium under several different physiological parameters. The preparation of the gradient gel and electrophoresis procedure are described in Materials and methods. All D71 samples in this and subsequent gels were run as 30 μg total protein. The molecular mass, in kilodaltons, of protein standards is indicated to the left of the gel. In this and subsequent figures the protein standards used were as follows (molecular weight): phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000), and α-lactalbumin (14 000). Samples: lane *a*, molecular weight standards; *b*, spinin (5 μg); lanes *c–f* contain cell lysates from cultures grown at 22°C, pH 6.8; *c*, 0.1 *M* NaCl; *d*, 0.2 *M* NaCl; *e*, 0.3 *M* NaCl; *f*, 0.4 *M* NaCl; lanes *g–j* contain cell lysates from cultures grown at 34°C, pH 6.8; *g*, 0.1 *M* NaCl; *h*, 0.2 *M* NaCl; *i*, 0.3 *M* NaCl; *j*, 0.4 *M* NaCl; lanes *k–n* contain cell lysates from cultures grown at 22°C, pH 7.4; *k*, 0.1 *M* NaCl; *l*, 0.2 *M* NaCl; *m*, 0.3 *M* NaCl; *n*, 0.4 *M* NaCl; lanes *o–r* contain cell lysates from cultures grown at 34°C, pH 7.4; *o*, 0.1 *M* NaCl; *p*, 0.2 *M* NaCl; *q*, 0.3 *M* NaCl; *r*, 0.4 *M* NaCl.

procedure of Tsai and Frasch (1982). LPS was also isolated using the procedure of Darveau and Hancock (1983) and characterized by SDS–PAGE.

Electron microscopy

Whole cells, envelope fractions or OM vesicles were negatively contrasted with 1% (w/v) aqueous uranyl acetate containing 50 $\mu g \cdot mL^{-1}$ bacitracin. Samples were examined using a Philips EM300 electron microscope operating at 60 kV.

Results

Physiological parameters influencing spination

The effect of various physiological parameters on D71 spination has been analyzed previously in a morphological study (Easterbrook and Sperker 1982). Since the production of structural–functional proteins is known to be influenced by such parameters as pH and salinity, SDS—PAGE of cell lysates was carried out to investigate possible induced changes in cellular protein composition. As observed previously (Easterbrook and Sperker 1982) extremes of pH and Na^+ concentration determined whether the cell population was spined or not, while alterations in individual parameters resulted in variation in the proportion of the population which was spinate. SDS—PAGE of the same samples (Fig. 1) demonstrated that while the overall protein profiles were similar there were some changes in band position and intensity; in particular a species which migrated with the same mobility as purified spinin at 42 kDa, and polypeptides of about 65 and 14 kDa (Fig. 1) were affected. There appeared to be an increased content (based on staining intensity) of the 65- and 14-kDa polypeptides when the level of the 42-kDa species was reduced. A 42-kDa species was present in all lysates; however, since staining in unspined samples was very low, it was presumably present in a low amount (Fig. 1; compare lanes *d*, *f*, and *g*). Thus, it appeared that a physiologic response in D71 was manifest, electrophoretically, as an alteration in the content of three polypeptides. In subsequent experiments only temperature and pH were altered to control spination. Maintaining a constant osmotic pressure (i.e., Na^+ concentration) allowed an easier transition from the unspined to the spined phenotype; only occasionally was a less than complete phenotypic transition observed.

SDS–PAGE and immunoblot analyses of whole-cell lysates during the phenotypic transition

To investigate the electrophoretic mobility and content of the three polypeptides noted in Fig. 1 during the transition period, cultures grown overnight under spina restrictive conditions were shifted by dilution (Easterbrook and Alexander 1983) to spina permissive conditions. Samples of cells taken during the transition were dissociated, electrophoresed, and the separated polypeptides were either visualized directly using Coomassie brilliant blue R250 or probed with antispinin antiserum in an immunoblot procedure. The 65-kDa species noted previously was visually and immunologically detectable throughout the transition period (Figs. 2A and 2B) as was additionally a polypeptide of about 21 kDa. The 14-kDa protein noted in Fig. 1 was not immunodetected. Re-examination of Fig. 1 shows the presence of a 20- to 21-kDa species whose content also was

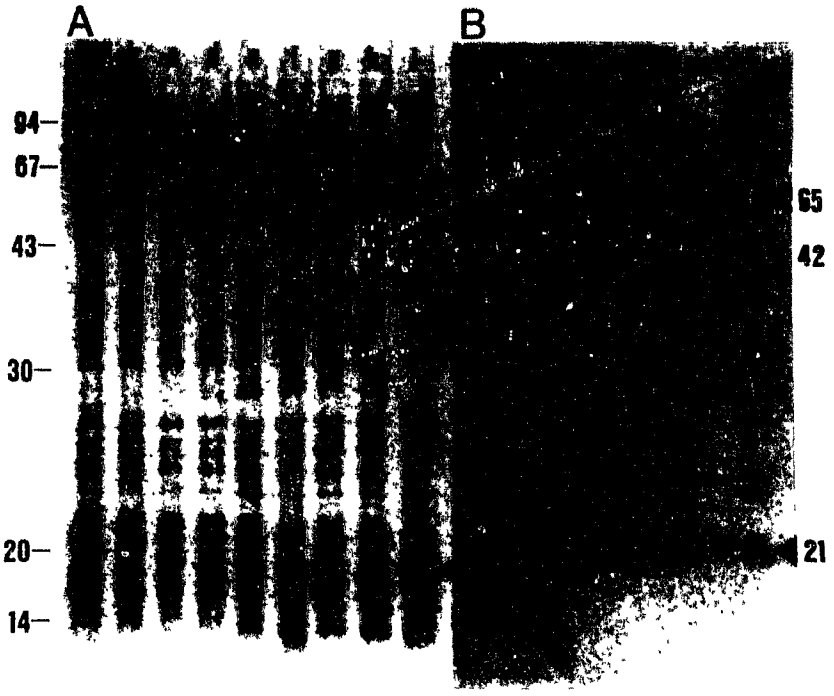

FIG. 2. SDS–PAGE protein profile and immune blot pattern of D71 whole-cell lysates prior to, and following, a shift from spina restrictive to spina permissive cultural conditions. A 10–15% acrylamide gradient gel was prepared and electrophoresis performed as described in Materials and methods. Figure 2A shows the protein profile of whole-cell lysates and Fig. 2B shows the corresponding immune pattern. The molecular mass values of the protein standards used (see Fig. 1) are given in kilodaltons to the left of the figure. The positions of the standards are indicated. The arrowheads to the left of A and B indicate the position of the 65-, 42-, and 21-kDa species. Samples (in both A and B): *a*, unspined cells; *b*, 0.5 h following a shift to spina permissive conditions (postshift); *c*, 1.0 h postshift; *d*, 1.5 h postshift; *e*, 2.0 h postshift; *f*, 2.5 h postshift; *g*, 3.0 h postshift; *h*, 4.0 h postshift; *i*, 15 h postshift. The percentage of enumerated cells which were spined, as determined by electron microscopy (see Materials and methods), in the samples are as follows: unspined culture, 0%; 0.5 h, 0%; 1.0 h, 0%; 1.5 h, 0%; 2.0 h, 7%; 2.5 h, 17%; 3.0 h, 33%; 4.0 h, 45%; 15 h, 100%.

altered in response to physiological conditions (Fig. 1, compare lanes *c–f* with *g* and lane *n* with *o–r*), although not as dramatically as were the 65-, 42-, and 14-kDa polypeptides. Although the spination kinetics of D71 populations routinally display some variability, the spination period of the population used in the shift experiment summarized in Fig. 2 was quite prolonged. This was fortuitous since it permitted an unambiguous assessment of the coincident visual and immunological detection of spinin. As shown in Fig. 2, the 42-kDa protein was clearly evident (visually and immunologically) only when a majority of the sample population was spined (in this particular case 100%) (Figs. 2A and 2B). Although a weakly stained band of similar mobility was present in phenotypically unspined samples (Fig. 2A, lane a), it was not detected using antispinin antibody (Fig. 2B, lane a). The 42-kDa species likely represented spinin since its immunological detection in Fig. 2B was correlated with the presence of spinae on the cell and since it migrated with the same electrophoretic mobility as purified spinin.

Isolation of the outer membrane of D71

Since the base of an intact spina (which is comprised of spinin) is intimately associated with the OM layer of pseudomonad D71 (Willison *et al.* 1977), it was appropriate to determine if the immunologically cross-reactive 65- and 21-kDa polypeptides were also constituents of this membrane. The OM was isolated by sequential application of detergent and lysozyme or by use of the procedure described by Osborn *et al.* (1972). In preliminary detergent–lysozyme OM isolation experiments, the use of Triton X-100 was found to be unsatisfactory owing to extensive contamination of the OM fraction with other cellular proteins (data not shown), so sodium lauroylsarcosinate (Sarkosyl) became the detergent of choice for subsequent OM isolations. Whole cell, envelope, and OM fractions obtained during such an isolation procedure are compared in Fig. 3. While comparison of the whole-cell lysates was difficult owing to overloading of the spined sample, the different polypeptide patterns given by the envelope and OM fractions were clearly evident. The Sarkosyl–lysozyme insoluble OM, whether from unspined or spined cells, contained 16 to 20 peptides ranging in molecular mass from approximately 14 to 90 kDa (Fig. 3, lanes 4 and 8). There was, as expected, a greatly increased content of the 42-kDa species in the spined OM. To ensure that the use of Sarkosyl had not significantly modified the OM, we used as a control the prodecure detailed by Osborn *et al.* (1972) for *Salmonella typhimurium* to separate membraneous material from D71 into three distinct bands in a sucrose gradient. By comparison with fractionation of other Gram–negative envelopes (Osborn *et al*, 1972) and morphological evaluation, we concluded that the lightest band (in terms of density) likely represented cytoplasmic membrane, and the mid band contained both cytoplasmic and OMs. The white–appearing, densest OM band (1.23 $g \cdot cm^{-3}$ for unspined samples, 1.25 $g \cdot cm^{-3}$ for spined) was recovered, washed free of sucrose, and subjected to electrophoresis. Comparison of these OM protein profiles with those of the Sarkosyl–lysozyme derived OM (Fig. 4) revealed commonality in 18 polypeptides between fractions from the two isolation procedures indicating that the

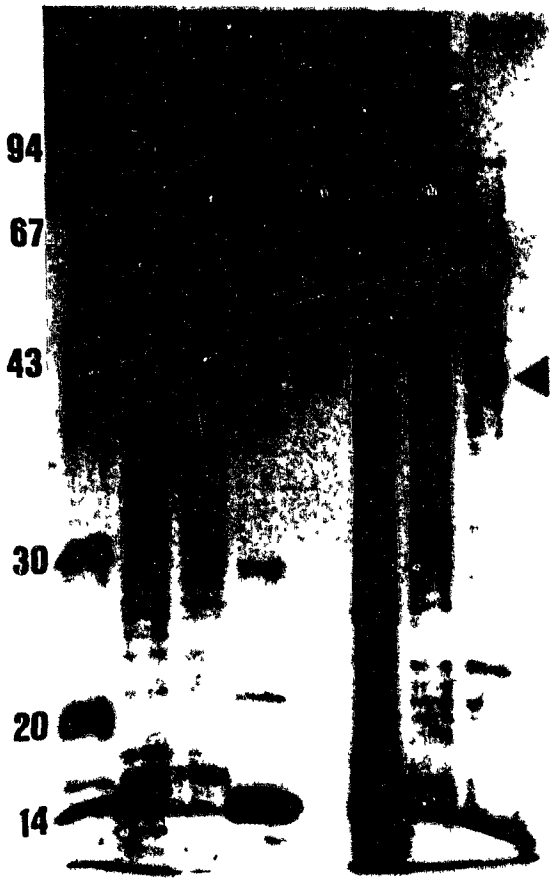

FIG. 3. SDS–PAGE monitoring of the OM isolation procedure. A 12% acrylamide gel was constructed and electrophoresis was performed as described in Materials and methods. The molecular mass values, in kilodaltons, of proteins standards are shown on the left of the gel. Samples: lane 1, molecular weight standards (see Fig. 1); 2, unspined whole-cell lysate; 3, unspined envelope; 4, unspined Sarkosyl–lysozyme insoluble fraction; 5, spinin (5 μg); 6, spined whole-cell lysate; 7, spined envelope; 8, spined Sarkosyl–lysozyme insoluble fraction. The arrowhead to the right of the gel indicates the position of spinin.

detergent–lysozyme insoluble material was in fact OM. (These particular OMs lacked electrophoretically detectable proteins greater than 65 kDa. We have no explanation for this finding.) In a confirmatory experiment Sarkosyl–lysozyme insoluble OM isolated from spined cells was also centrifuged in a sucrose gradient; proteinaceous material absorbing at 280 nm was detected only at a position corresponding to a density of $1.25\ g \cdot cm^{-3}$.

As shown in Fig. 3 and 4A, OMs contained a relatively high amount of the 65- and 14-kDa species. Since lysozyme was used in the isolation procedures and the potential exists for it to be trapped in vesicles (Uemura and Mizushima 1975), some of the intense staining of the 14-kDa band may be due to that. However, as discussed later, a D71 polypeptide is also present. The presence of a greatly increased amount of the 42-kDa species in the spined fractions has already been noted. OMs from both morphological forms of D71 contained the 21-kDa polypeptide. Three of these polypeptides (65, 42, 21 kDa) could be immunologically detected in similarly prepared OMs (only Sarkosyl–lysozyme insoluble Om was examined), although the immune pattern displayed some variation depending on the antiserum preparation used (compare Figs. 4B and 4C). The 42-kDa species was immunologically detectable only in the spined OM, whereas the 65- and 21-kDa species were immunologically detectable in both OMs. Since these same proteins were immune labelled in whole-cell lysates (Fig. 2B), they appear to be constituents of, or associated with, the D71 OM. The possible relation of the cross-reactive proteins to spinin will be considered in the discussion.

Electron microscopy of the OM isolates

As part of the monitoring of the detergent–lysozyme OM isolation procedure, envelope and OM fractions collected during the procedure were negatively contrasted and examined in the electron microscope. The envelope vesicles shown in Figs 5A and 5B retained elements of the cell shape, presumably owing to the presence of peptidoglycan. Treatment of envelopes with Sarkosyl and lysozyme generated vesicles which appeared spherical and smaller than envelope vesicles (Figs. 5C and 5D), and which were bounded by only a single membrane. In some cases intact spinae or fragments were observed in association with OM vesicles and these associations could be nonspecific since vesicles could be dispersed along the entire length of intact spinae (Fig. 5D).

SDS–PAGE of D71 LPS

Gram-negative bacteria usually possess LPS as an OM constituent (Beveridge 1981; Lugtenberg and van Alphen 1983) and this may be of varying side chain complexity. Initial attempts to detect the 2-keto-3-deoxyoctonate (KDO) component of D71 LPS using the thiobarbituric acid procedure of Weissback and Hurwitz (1959) were unsuccessful when whole-cell, envelope, or OM fractions were used. However, the fraction from approximately 500 mg wet weight (wt) whole cells using the LPS isolation procedure of Darveau and Hancock (1983) was KDO positive. Comparison of the color produced in the KDO assay procedure with that given by either intact cells or an extract from a similiar wet wt of *E. coli* K12 strain AB264 (with a KDO content of 0.35 μmol · mg dry wt. cells $^{-1}$) indicated a KDO content of approximately 0.20 μmol · mg dry wt $^{-1}$. Since the KDO assay works satisfactorily for *E. coli*, it is concluded that as with certain other Gram-negative bacteria (Adams *et al.* 1969; Tornabene *et al.* 1982; Volk *et al.* 1972), the LPS of D71 remained unreactive in the KDO assay when in the OM owing associations with other components of the membrane. To identify the type of LPS (i.e., smooth or rough), material obtained by proteinase K digestion of whole cells was electrophoresed and visualized by silver staining (Hitchcock and Brown 1983). No differences were observed in the electrophoretic profiles of the LPS fractions (Fig. 6). Comparison with various LPS species from *Salmonella minnesota* indicated that the LPS from both morphologic forms of D71 was of the deep-rough type (Fig 6). In related experiments there was similarly no difference observed in either the amount or type of LPS in cells grown in defined as compared with complex media, or in the LPS isolated using the protocol of Darveau and Hancock (1983).

Discussion

As is the case with other Gram-negative bacteria (DiRienzo *et al.* 1978), the OM of marine pseudomonad D71 has proven to be made up of only a small number of polypeptide species. Two major approaches were used to isolate the OM layer. One of these utilized detergents, either in the presence of Mg^{2+} (i.e.,

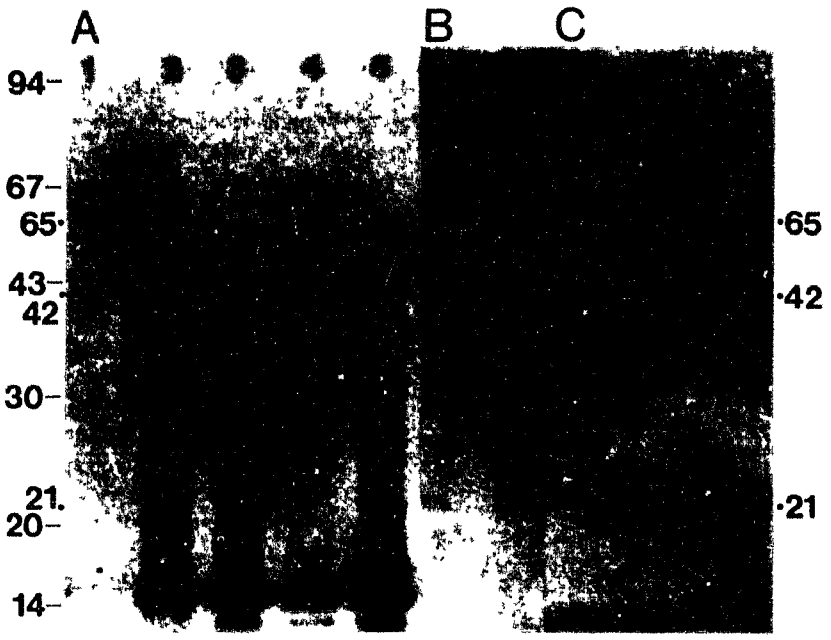

FIG. 4. SDS–PAGE comparison of the Sarkosyl–lysozyme insoluble fractions and the OM derived using the procedure of Osborn *et al.* (1972) (A) and immune blot pattern of similarly prepared Sarkosyl–lysozyme insoluble fractions from unspined and spined forms of D71 (B and C). The positions of the protein standards used (see Fig. 1) are shown to the left of the figure. The molecular mass values are in kilodaltons. (A) Lane 1, spinin (5 μg); 2, unspined Sarkosyl–lysozyme insoluble fraction; 3, unspined OM derived as per Osborn *et al.* (1972); 4, spined Sarkosyl–lysozyme insoluble fraction; 5, spined OM derived as per Osborn *et al.* (1972). (B and C) Immunoblot analyses of unspined and spined Sarkosyl–lysozyme insoluble OMs using absorbed antisera (see Materials and methods for details of the absorption procedure). (B) Immunoblot pattern given by antiserum absorbed with K12 and PAO envelopes. Lane 1 contains unspined OM, lane 2 contains spinin (5 μg), and lane 3 contains spined OM. (C) Immunoblot pattern given by antiserum absorbed with K12 and PAO envelopes and D71 unspined cells. Lane 1 contains unspined OM, lane 2 contains spinin (5 μg), and lane 3 contains spined OM. The molecular mass, in kilodaltons, of the species detected using the antisera are indicated to the left and right of the figure.

FIG. 5. Electron microscopic examination of envelope and OM preparations obtained from unspined and spined populations of D71. Samples obtained as described in Materials and methods were negatively contrasted using uranyl acetate. (A) Unspined envelope; (B) spined envelope; (C) unspined OM; (D) spined OM. In D the solid arrows indicate vesicles which were associated with a spina. All bars 100 nm.

Triton X-100; Schaitman 1971) or in the absence of this cation (i.e., sodium lauroylsarcosinate; Filip *et al.* 1973), to selectively dissociate the cytoplasmic membrane prior to lysozyme digestion of the peptidoglycan (Schnaitman 1971). The second OM isolation technique employed sucrose gradient centrifugation to separate membraneous material from a spheroplast lysate (Osborn *et al.* 1972). The detergent–lysozyme insoluble fraction produced could be contaminated with solubilized OM peptides extracted by the detergent, by lysozyme entrapped in membrane vesicles (Uemura and Ishimoto 1975), or might even have had exposed OM peptides extracted by the detergent. OM isolation experiments which utilize detergents need to be interpreted with caution. In this study both OM isolation techniques yielded comparable unspined and spined fractions

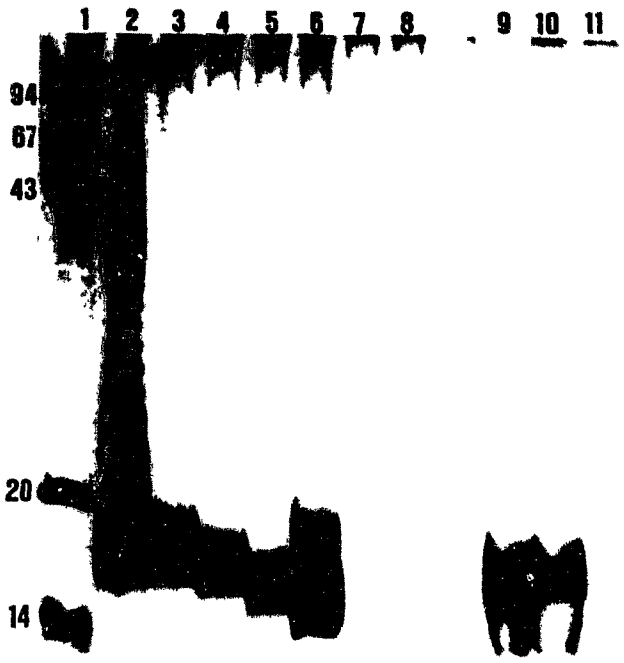

FIG. 6. Electrophoretic detection of LPS. The LPS isolation and electrophoretic procedures are detailed in Materials and methods. Samples were visualized using the silver stain procedure of Hitchcock and Brown (1983). Lane 1, molecular weight standards (see Fig. 1; molecular mass values in kilodaltons are shown to the left of the figure); 2, LPS from *Escherichia coli* 0111B4 (5 μg); lanes 3–7 contained purified LPS species from *Salmonella minnesota*; 3, Ra type; 4, Rb type; 5, Rc type; 6, Rd type; 7, Re type; 8, lipid A; lanes 9 and 10 contain D71 LPS which was obtained using the proteinase K digestion procedure detailed in Materials and methods; 9, LPS from cells grown in the complex medium under unspined conditions (24°C, pH 6.8, 0.2 *M* NaCl); 10, LPS from cells grown in the complex medium under spined conditions (34°C, pH 7.4, 0.2 *M* NaCl). Lane 11 contains proteinase K at a concentration equivalent to that in the digestion mixtures (2 μg).

(Fig. 4A), indicating that the Sarkosyl–lysozyme insoluble fraction indeed represented OM. There was an equivalent amount of stained material of apparent molecular mass of 14 kDa in the OMs isolated using both procedures, although as has been commented on earlier, vesicularization may occur during the Sarkosyl–lysozyme isolation procedure. Additionally, since the amount of purified lysozyme required for equal staining intensity exceeds by fivefold that which could have theoretically become vesicularized (B.D. Hoyle, unpublished observation), we must conclude that a D71 polypeptide with a mobility equal to that of lysozyme is also present. This is confirmed by electrophoresis of lysozyme-free whole-cell lysates (Fig. 1).

The 14-kDa polypeptide and a 65-kDa species were the major proteins (as judged by staining intensity in the unspined OM). The spined OM also contained a significant amount of a 42-kDa polypeptide. This latter protein has the same apparent mobility as that of dissociated spinae in the electrophoretic system used and reacts, after immobilization on nitrocellulose, with antisera prepared against purified spinin; it is concluded to be spinin. The mobility of spinin observed in this study differs somewhat from that reported earlier (Easterbrook and Coombs 1976). Such variable behaviour has been consistently noticed with spinin in response to slight pH differences or to electrophoretic conditions, and is no doubt a consequence of its unusual amino acid composition (Coombs *et al.* 1976).

Since electron microscopic examination of OM preparations from spined cells indicates the presence of a large number of associated spinae, it is surprising that spinin is only represented in electropherograms in apparently low levels. It has, however, been noted (K.B. Easterbrook, unpublished observation) that the amount of stained material subsequently observed in gels can be increased by a factor of some five-fold if spinae are heated at alkaline pH or in the presence of urea prior to dissociation in SDS. This suggests that dissociation is either then more efficient or some configurational change occurs. Unfortunately, such manipulations appear ineffective when attempted with cell lysates and the low level of detection for spinin in consequence prevents an unambiguous assessment of whether spinin is also present in small amounts in unspined cells. Electrophoresis of unspined cell lysate, envelope, and OM reveals a component of similar mobility as spinin, but since we have never been able to detect an immunologically reactive 42-kDa component regardless of the concentration of either protein or antibody, we must conclude that its presence is fortuitious.

We have attempted to provide a more valid comparison of the levels of the 42-kDa protein in unspined and spined cell lysates by preparing OM fractions from spined cells after deliberate removal of their spinae by pronase treatment followed by mechanical stress. Such processing should eliminate polymeric spinin (Willison *et al.* 1977). Electrophoretic examination of the "despined" OM showed the level of both stained and immunologically reactive 42 kDa in the treated OM is drastically reduced (data not shown). The apparent absence of an immunologically reactive 42-kDa protein (spinin) from unspined cell lysates or OM preparations is consistent with the view that this species represents spinin generated by dissociation of spinae in the presence of SDS (since such structures are by definition absent in unspined cells) rather than a protein integrated in the OM. The model of spinin–spinae assembly we support assumes that dimerization creates an initial symmetrical unit that can subsequently polymerize, and dissociation of such a structure in the presence of SDS would likely generate a stable population of dimers. Such dimerization has been shown to occur with other proteins.

Two other OM polypeptides (21 and 65 kDa) also react with antispinin sera. What, if any, is their relation to spinin? Since sedimentation equilibrium analysis of heat-dissociated spinae in guanidine hydrochloride indicates a molecular mass of 19 kDa for the protomer, it is obviously appropriate to suggest that a polypeptide with a comparable mobility in PAGE might represent spinin present in a conformation or association different from that it normally assumes in the assembled spina. If this is the case, then such a species is present in unspined cells as well as spined, and since cross-reactive antibodies, such as those directed towards the 65-kDa species, cannot be removed from sera by absorption with intact unspined cells, it is likely not exposed on the outer surface of OM. Association of this protein with the inner leaflet of the OM or with other envelope layers (i.e., periplasmic space and cytoplasmic membrane) would have to be concluded.

Since the immune reactivity against the 65-kDa protein can be removed from antispinin sera by absorption with unspined cells, it is probable that this represents either a cross-reactive species sharing epitopes with spinin or a highly immunogenic contaminant protein present in the spina preparation used as immunogen. Cross-reactive antigens are common among the

OM proteins of Gram-negative bacteria (Mayer and Schmidt 1979), but contamination cannot be excluded in view of the method of isolation of spinae. Thus healthy, logarithmically growing cells resist the shearing used to detach spinae and suffer no loss of viability (Easterbrook and Alexander 1983), but older cells are more sensitive and are damaged. SDS–PAGE evaluation of some spinae preparations does in fact indicate the presence of both 65-kDa and approximately 20-kDa components, even though the amount of contaminant membrane visualized by electron microscopy appears far too little to equate with the intensity of protein staining observed. The problematic proteins could be firmly associated with the base of the spinae, or perhaps could be adsorbed to the outside or inside of spinae and thus not recognizable in the electron microscope, or might, unlike spinin, stain intensely. The explanation invoking cross-reactive antigens is supported by the fact that an antiserum raised against a spina preparation shown to be single component by both PAGE and high-performance liquid chromatography still reacts with both approximately 20- and 65-kDa proteins (K. B. Easterbrook, unpublished result). The question of the identity of the 21- and 65-kDa proteins and their relation, if any, to spinin perhaps can only be answered when the proteins have been biochemically characterized or tested against antisera specific for a single epitope.

Regardless of the explanation for the immunologically reactive components in the envelope, the extent of interaction between D71 OM components in emphasized by the difficulty in demonstrating LPS in the incorporated state, be it whole cell, envelope, or OM. Once extracted the LPS readily yields a detectable chromogen in KDO assay procedure and appears electrophoretically similar to that in other Gram-negative bacteria. Capsular polysaccharide is produced by D71 under certain growth conditions (K.B. Easterbrook, unpublished observations), but the nature of the LPS (deep-rough) remains constant, as does its quantity.

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