Composition of the outer membrane of *Proteus mirabilis* in relation to serum sensitivity in progressive stages of cell form defectiveness

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A serum-resistant strain of *Proteus mirabilis* was used to determine whether changes in the composition of surface components could be detected following induction of progressive stages of cell form defectiveness by β -lactam antibiotics. The critical stage was the conversion from filaments to the spheroplast form, which was accompanied by increased susceptibility to the bactericidal action of human serum. Inner and outer membranes of the bacterium, its filament form and its spheroplast form were separated by sucrose density-gradient centrifugation after digestion of peptidoglycan, followed by osmotic lysis of the cells. Outer membranes of the bacterial and the filament forms sedimented at the same density, whilst the outer membrane fraction of the spheroplast form sedimented in a region of lesser density. In addition, the amounts of two major outer-membrane proteins as well as the O-polysaccharide content of the lipopolysaccharide were reduced in the spheroplast form. These results indicate a general disorganization in structure and assembly of components in regard to their interactions with one another in the outer membrane of the spheroplast form.

Introduction

Invading bacteria are primarily recognized and attacked by the immune system through cell surface structures, and any changes in surface components could affect immune processes. This is especially relevant for the bactericidal action of serum complement on Gram-negative bacteria, as components of the outer membrane of these micro-organisms can act as a barrier to the killing effects of complement (Taylor, 1983; Goldman *et al.*, 1984). Of particular importance in this regard are the effects of antibiotics on cell surfaces of bacteria.

It has been well established that treatment of *Escherichia coli* with β -lactam antibiotics affects biosynthesis of peptidoglycan, leading to functional disorganization and morphological changes. Although peptidoglycan synthesis is the primary target, secondary effects on Gram-negative bacteria could be expected, such as destabilization of the association of peptidoglycan with the outer membrane, which in turn might lead to disorganization of assembly and loss of outer membrane components.

The effects of β -lactam antibiotics on the composition

of the outer membrane of Gram-negative bacteria have not been studied systematically. There are, however, a number of reports documenting changes in outer membrane or surface components after treatment of these bacteria with various β -lactam antibiotics (James, 1975; Taylor *et al.*, 1982; Nixdorff *et al.*, 1985; Suerbaum *et al.*, 1987). As shown first in *E. coli* and later in related enterobacteria, progressive stages of defective cell shape and cell wall structure are caused by the action of β -lactam antibiotics binding preferentially to individual penicillin-binding proteins (PBPs) which perform essential functions in different stages of peptidoglycan synthesis and morphogenesis. Depending on their different binding selectivities, the antibiotics either cause uniform cell wall defects or induce progressive defects at increasing concentrations as the result of successive binding to additional PBP targets (Spratt, 1975, 1977; Curtiss *et al.*, 1979; Ohya *et al.*, 1979; Georgopapadakou, 1988).

For the present studies a model system derived from the serum-resistant strain *Proteus mirabilis* VI was used. The system includes the bacterial form, its filament form induced by cefuroxime and its spheroplast form induced by cefoxitin. The decisive advantage of working with the *P. mirabilis* system is the ability of these organisms to survive and multiply extensively in all different stages of

Abbreviations: LPS, lipopolysaccharide; PBP, penicillin-binding protein.

cell wall damage in the presence of β -lactams, a condition that has been designated the L-form state (Dienes & Weinberger, 1951). Thus, the requirement for growing cultures in bactericidal assay systems (Taylor, 1983) can be met, and uniform populations of the wall-defective states can easily be obtained for biochemical characterization (Martin & Gmeiner, 1979; Martin, 1983). This is in striking contrast to the transient nature of filament and spheroplast states of *E. coli* and closely related enterobacteria. With these organisms, the inactivation of essential PBPs is lethal, and sufficient uniform cell material for analyses cannot be obtained.

In the present investigation, comparative biochemical analyses were carried out on the outer-membrane fractions of the bacterial, the filament and the spheroplast forms of *P. mirabilis* VI. Also, the susceptibility of all three cell forms to the bactericidal action of human serum was examined.

Methods

Bacteria. *P. mirabilis* VI was from the collection of H. H. Martin.

β -Lactam antibiotics. Cefuroxime was purchased from Hoechst and cefoxitin was purchased from Merck, Sharp & Dohme.

Cultivation of the bacterial form. An overnight culture of *P. mirabilis* VI in 50 ml L-medium (Martin, 1964), incubated at 37 °C with shaking at 120 r.p.m., was added to 1 litre L-medium to an OD₅₇₈ of 0.3. The culture was incubated as above to late exponential phase (OD₅₇₈ = 0.8).

Induction and cultivation of the filament form. *P. mirabilis* VI was induced to filament formation 1 d before use in an experiment by addition of 0.1 µg cefuroxime (ml L-medium)⁻¹, and cultivated as described in the bacterial form.

Induction and cultivation of the spheroplast form. *P. mirabilis* VI was induced to spheroplast formation 2 d before use by addition of 60 µg cefoxitin (ml L-medium)⁻¹. An overnight culture of the bacteria was inoculated into L-medium and incubated at 37 °C with increasing degrees of shaking during the day (70 and 90 r.p.m.) and finally at 120 r.p.m. overnight. A second culture was inoculated from this material and incubated overnight at 37 °C with shaking at 120 r.p.m. This overnight culture was added to 1 litre L-medium to an OD₅₇₈ of 0.3 and incubated at 37 °C with shaking at 120 r.p.m. to late exponential phase. The medium always contained 60 µg cefoxitin ml⁻¹. Complete spheroplast formation in *P. mirabilis* VI was also obtained in the same manner by increasing the concentration of cefuroxime from the filament-forming concentration of 0.1 µg ml⁻¹ to 10 µg ml⁻¹ (Huber, 1985). At these concentrations of antibiotics, growth of the spheroplasts was not inhibited.

Preparation of inner and outer membranes. Isolation of inner and outer membranes on sucrose density gradients was done according to Taylor & Kroll (1984). Briefly, cells were cultivated to late exponential phase in L-medium; one portion was labelled with 0.33 µCi [2-³H]glycerol ml⁻¹ (specific activity, 1 Ci mmol⁻¹, 37 GBq mmol⁻¹; Amersham) for incorporation into phospholipids, and another with 0.05 µCi *N*-acetyl-D-[1-¹⁴C]glucosamine ml⁻¹ (specific activity, 40–50 mCi mmol⁻¹, 1.48–1.85 GBq mmol⁻¹; Commissariat à l'Energie Atomique, Gif-sur-Yvette, France) for incorporation into lipopolysaccharide (LPS).

Peptidoglycan was digested with lysozyme and the cells were ruptured by osmotic shock. If the osmolysis was not effective, the cells were lysed by shaking with glass beads in a cell mill for 5–10 min. Membrane material was harvested by ultracentrifugation and separated into inner and outer membranes on a linear sucrose density gradient (15–65%, w/w) by ultracentrifugation. Fractions containing 20 drops were collected from the bottom of the centrifuge tube. Absorbance was monitored at 278 nm; sucrose concentrations were measured by refractometry.

Analytical methods. Protein content was determined by the method of Markwell *et al.* (1978). Fatty acids of phospholipids and LPS were analysed by gas-liquid chromatography according to Gmeiner & Martin (1976). SDS-PAGE of proteins in membrane fractions was carried out in a slab gel apparatus (GE4; Pharmacia LKB) according to Lugtenberg *et al.* (1975) as previously described (Nixdorff *et al.*, 1977). Estimation of the O-polysaccharide chain length of LPS was made by amino acid analysis of lysine and *N*-acetyl-D-glucosamine as described by Gmeiner (1975). LPS was extracted from whole cells of the bacterial form and the spheroplast form by the phenol/water method (Westphal *et al.*, 1952).

Bactericidal assay. A modification of the photometric assay of Muschel & Treffers (1955) was used. Normal human serum in amounts ranging from 0.03 to 2.25 ml was brought to 2.25 ml with L-medium. Corresponding amounts of heat-inactivated serum were included as controls. Thereafter, 0.75 ml of a late exponential phase culture was added and the contents were mixed. At time 0, 0.8 ml was removed for measuring OD₅₇₈. The tubes were incubated at 37 °C in a shaker water bath for 60 min (kill period). An aliquot (0.8 ml) was removed and 1.0 ml L-medium was added to each tube. The tubes were incubated for an additional 90 min (outgrowth of the surviving organisms) and a final OD₅₇₈ measurement was made. The percentage survival was calculated by dividing the OD₅₇₈ of tubes containing active human serum by the OD₅₇₈ of tubes containing the respective concentration of heat-inactivated human serum and multiplying by 100.

Normal human serum. Blood was collected aseptically from healthy volunteers and allowed to clot at room temperature for 1 h and 3–4 h thereafter at 4 °C. Clots were removed and the serum was collected by centrifugation and sterilized by filtration. Sera were stored in aliquots of 1.0 ml at –80 °C until use. Pooled human serum was also obtained from the German Red Cross (Hagen/Westfalen, Germany).

Results

Separation of membranes

Centrifugation of membranes of the bacterial, filament and spheroplast forms of *P. mirabilis* VI on sucrose density gradients produced the profiles presented in Fig. 1. The separation profile of membranes of the bacterial form (Fig. 1a) revealed essentially four fractions with peak densities of 1.27, 1.25, 1.19 and 1.16 g ml⁻¹ (peaks 1–4, respectively). Incorporation of radioactive marker molecules into the membrane fractions gave a first indication of the location of inner and outer membranes in the gradient. Incorporation of *N*-acetyl-D-[1-¹⁴C]-glucosamine into mainly LPS and peptidoglycan would be expected. It serves as a specific marker of the outer membrane after removal of peptidoglycan by lysozyme

Fig. 1. Separation of membranes from the bacterial (a), filament (b) and spheroplast (c) forms of *P. mirabilis* VI in linear (15–65%, w/w) sucrose density gradients. Numbers at positions indicated by arrows are peak densities (g ml^{-1}). ---, Sucrose concentration; —, A_{278} ; incorporation of $[2\text{-}^3\text{H}]\text{glycerol}$ (---) and $N\text{-acetyl-D-[1-}^{14}\text{C}]\text{glucosamine}$ (.....) is given as c.p.m.

digestion during the membrane separation procedure. Any remaining peptidoglycan fragments would tend to associate with outer membrane material upon separation in sucrose density gradients (Osborn *et al.*, 1972). $[2\text{-}^3\text{H}]\text{Glycerol}$ was used as a marker for phospholipids. The greater portion of $N\text{-acetyl-D-glucosamine}$ incorporated into the membrane fractions of the bacterial form was found in the material contained in the first two peaks. Accumulation of $[2\text{-}^3\text{H}]\text{glycerol}$ in phospholipids was also greatest in these two fractions. Some $[2\text{-}^3\text{H}]\text{glycerol}$, far less than expected, was incorporated into the material of the third peak, whilst almost none was present in the fourth peak. These results tentatively located outer membrane material in the area of the first two peaks, and cytoplasmic membranes in the third and possibly the fourth peaks. An additional fraction with a peak density of 1.21 g ml^{-1} (peak 3a) was seen in the profile of the filament form (Fig. 1b). It should be noted that the gradient profiles reported in Fig. 1 were highly reproducible in repeated experiments and were characteristic for the different cell forms of *P. mirabilis* VI.

Analyses of proteins in the gradient fractions

Analyses of the proteins contained in the membrane fractions by SDS-PAGE (Fig. 2) showed very similar patterns for the bacterial and the filament forms, in that the four major outer-membrane proteins of *P. mirabilis* VI detectable by SDS-PAGE were concentrated in peak 1 (membranes with a buoyant density of 1.27 g ml^{-1}). They represent proteins with approximate molecular masses of 39, 36, 19 and 15 kDa (Gmeiner, 1981; Nixdorff *et al.*, 1977). The proteins in peaks 3 and 4 represent other, primarily cytoplasmic membrane proteins, which show distinctly characteristic patterns of mobility in SDS-PAGE (Hasin *et al.*, 1974; Kroll *et al.*, 1980; Van Heerikhuizen *et al.*, 1975).

The protein pattern of the spheroplast form (Fig. 2c) was decidedly different. The major outer-membrane proteins were most prominent in peak 2. Furthermore, there was an apparent reduction in the amounts of the 19 kDa and possibly the 15 kDa outer-membrane proteins. This was also observed previously in cell wall fragments isolated from the spheroplast form (Nixdorff *et al.*, 1985).

The protein patterns represented in Fig. 2 were highly reproducible.

Comparative analyses of LPS and phospholipids in membrane fractions isolated from the bacterial, filament and spheroplast forms of *P. mirabilis* VI

Analyses of the amounts of LPS and phospholipids in the membrane fractions of the various forms (Table 1)

Fig. 2. SDS-PAGE of proteins in the outer and inner membranes of *P. mirabilis* VI bacterial form (a), filament form (b) and spheroplast form (c). Lanes: S, standard proteins; 1–4, 45 µg protein from sucrose density-gradient fractions harvested at peak densities of 1.27 (lane 1), 1.25 (lane 2), 1.19 (lane 3) and 1.16 (lane 4) g ml⁻¹.

Table 1. *Quantitative analyses of amounts of phospholipids and LPS in membrane fractions of the bacterial form, the filament form and the spheroplast form of P. mirabilis* VI

Values in the table represent the means ± SE from two determinations each on two to five separate samples.

Gradient sample	Phospholipids [nmol 16:0* (mg protein) ⁻¹]	LPS [nmol 3-OH-14:0† (mg protein) ⁻¹]
Bacterial form, peak 1	411.92 ± 30.1	165.75 ± 9.9
Filament form, peak 1	343.40 ± 69.1	265.10 ± 50.5
Spheroplast form, peak 1	140.83 ± 46.3	35.55 ± 10.7
Spheroplast form, peak 2	247.76 ± 49.7	154.71 ± 32.9

* 16:0 = palmitic acid.

† 3-OH-14:0 = 3-hydroxytetradecanoic acid (3-hydroxymyristic acid).

confirmed the presumption that outer membranes of the bacterial and the filament forms were located in gradient peak 1, while the outer membrane of the spheroplast form was located primarily in gradient peak 2. As a measurement of LPS content, the amount of 3-hydroxytetradecanoic acid (3-OH-14:0) in the sample was determined and reported per mg protein. Phospholipids were measured as total content of hexadecanoic acid (16:0) minus the content of 16:0 in LPS in the sample. LPS and phospholipids were present in peak 1 gradient fractions of both the bacterial and the filament forms in ratios similar to those reported for the outer membrane of *E. coli* (Nakamura & Mizushima, 1975). In comparison, the material in peak 1 of the spheroplast membranes contained much less LPS. However, peak 2 of the spheroplast form contained LPS and phospholipids in amounts similar to those found in peak 1 of the other forms.

Analyses of the fatty acid composition of the phospholipids in the outer membrane fractions isolated from the three forms of *P. mirabilis* VI showed no detectable differences (data not shown).

Comparison of the O-polysaccharide content of LPS from the bacterial form and the spheroplast form of P. mirabilis VI

It was of particular interest to determine whether any change in the type of LPS could be detected in the spheroplast form of *P. mirabilis* VI, especially because of the changes in outer membrane composition of the spheroplasts reported above.

LPS from *P. mirabilis* VI contains, as strain-specific components, lysine and galactosamine in the O-polysaccharide chain, which can be measured by amino acid analysis (Gmeiner, 1975). Therefore, determination of the amounts of these two components per mole of 3-OH-14:0 (lipid A) gives an estimation of the relative length of the O-polysaccharide chain. In Table 2, the results show that the ratio of lysine to galactosamine in

Table 2. *Content of lysine and galactosamine in LPS from the bacterial form and the spheroplast form of P. mirabilis* VI

LPS source	Galactosamine [nmol 3-OH-14:0* ⁻¹]	Lysine [nmol 3-OH-14:0* ⁻¹]
Bacterial form	2.150	0.937
Spheroplast form	1.026	0.469

* 3-OH-14:0 = 3-hydroxytetradecanoic acid (3-hydroxymyristic acid).

Table 3. Serum sensitivity of different cell forms of *P. mirabilis* VI

Percentage survival is reported as means $\pm$ SE of values taken from three to five separate experiments.

Normal human serum (% v/v)	Percentage survival			
	Bacteria	Filaments (0.1 μ g cefuroxime ml ⁻¹)	Spheroplasts (60 μ g cefoxitin ml ⁻¹)	Spheroplasts (10 μ g cefuroxime ml ⁻¹)
75	89.4 $\pm$ 6.3	76.5 $\pm$ 8.6	8.9 $\pm$ 2.2	22.9 $\pm$ 0.8
60	92.2 $\pm$ 4.5	82.9 $\pm$ 4.3	13.1 $\pm$ 3.4	39.8 $\pm$ 2.6
50	97.2 $\pm$ 2.8	86.7 $\pm$ 7.6	38.4 $\pm$ 2.6	58.2 $\pm$ 4.2
40	90.8 $\pm$ 6.5	87.2 $\pm$ 2.1	62.9 $\pm$ 3.1	77.0 $\pm$ 21.0
25	96.8 $\pm$ 3.2	98.0 $\pm$ 1.1	88.3 $\pm$ 3.3	91.5 $\pm$ 12.9
10	97.5 $\pm$ 2.2	97.4 $\pm$ 2.6	98.1 $\pm$ 1.3	94.6 $\pm$ 10.1
3	100.0 $\pm$ 0	100.0 $\pm$ 0	100.0 $\pm$ 0	85.3 $\pm$ 32.4

the LPS of both the bacterial and the spheroplast forms is constant, as would be expected. However, the bacterial form contains twice as much lysine and galactosamine per mole of 3-OH-14:0 as the spheroplast form. Thus, the O-polysaccharide content of the LPS in the spheroplast form is approximately half of that found in the LPS of the bacterial form.

Serum sensitivity of spheroplast forms of *P. mirabilis* VI

Conversion to serum sensitivity occurred after induction of *P. mirabilis* VI to the spheroplast form with cefoxitin (Table 3). Similar results were obtained previously in a preliminary investigation (Siegmond-Schultze *et al.*, 1985). *P. mirabilis* VI was also converted into uniform spheroplasts by applying a concentration of cefuroxime higher than that used to specifically induce filament formation. These spheroplasts were only slightly less sensitive to the effect of complement than the spheroplasts induced by cefoxitin.

Discussion

The results of these studies show that the outer membrane of the spheroplast form of *P. mirabilis* VI induced by the β -lactam antibiotic cefoxitin has a composition and character different from the outer membrane of the bacterial form, or of its filament form induced by a low concentration of cefuroxime.

The regulation of the synthesis or translocation of outer membrane components of the spheroplast form may be disturbed in some way (Rogers & Thurman, 1983). This is particularly indicated by the changes in protein composition and the amount of O-polysaccharide in LPS. It is possible that subtle changes in the qualitative or quantitative composition of outer membrane components could lead to changes in outer membrane density, as observed in the spheroplast form.

Alternatively, a defect in the organization of peptidoglycan might lead to an instability, a reorganization or redistribution of components in regard to their interactions with one another in the outer membrane. This could cause a stronger tendency of components of the inner and outer membranes to associate, particularly during attempts at isolation.

The observed disorganization of the outer membrane of the spheroplast form occurred in parallel with at least a partial loss of serum resistance. Induction to the spheroplast form was therefore a very critical event with regard to alteration of serum sensitivity. This was accentuated with the conversion of the resistant bacterial form to serum-sensitive spheroplasts by two different β -lactam antibiotics. These results are consistent with the view that various β -lactams use different lines of attack on a system consisting of multiple targets to initiate a series of events leading to functional disorganization of the cell envelope, expressed as cell form defects in rod-shaped bacteria (Martin, 1983). Rather extensive disorganization of the outer membrane can accompany spheroplast formation, as elaborated by the analyses of the surface components of the cefoxitin-induced spheroplasts discussed above.

The interaction of peptidoglycan with the outer membrane surely contributes to the stability of the cell envelope. Electron micrographs of spheroplasts of *P. mirabilis* show extensive loss of outer membrane fragments in the form of spherical particles which slough off the surface (Hofschneider & Martin, 1968). Thin sections of penicillin-induced spheroplasts of *E. coli* viewed in the electron microscope show occasional large spaces between inner and outer membranes (Schnaitman, 1970) as well as areas of total detachment of the outer membrane from the cell surface (Gumpert *et al.*, 1971). These exposed areas of cytoplasmic membrane might represent more accessible targets for the binding and activation of complement components. Alternatively, or in addition, peptidoglycan-free areas might

allow more extensive interaction of inner and outer membranes, which would reduce the distance between them and promote destruction by complement (Bhakdi *et al.*, 1987).

One factor that apparently plays a role in resistance of Gram-negative organisms to the bactericidal action of complement is the amount of O-polysaccharide in LPS (Goldman *et al.*, 1984; Joiner *et al.*, 1984; Reynolds & Pruul, 1971). Whether the observed reduction in the amount of O-polysaccharide in the outer membrane of the spheroplast form was alone decisive is difficult to assess. Taylor *et al.* (1981) have reported that treatment of several clinical isolates of *E. coli* with subinhibitory concentrations of mecillinam led to the formation of spherical (round) cells along with increased sensitivity to the bactericidal action of serum. Further studies (Suerbaum *et al.*, 1987) showed that LPS in the outer membrane of these spherical cells had shorter O-polysaccharide chains. Despite differences in bacterial strains and antibiotics employed, there are definite parallels between the results of these studies and those reported here.

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