Fractionation of the *Providencia stuartii* Cell Envelope

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Cell envelopes (i.e. unfractionated inner and outer membranes) were obtained from *Providencia stuartii* by following procedures previously applied to the isolation of envelopes from *Escherichia coli*. The *P. stuartii* envelopes contained known inner membrane enzymes that included a variety of dehydrogenases and ATPase. The catalytic activity of the ATPase depended upon the concentration of magnesium ions, the substrate (ATP) level and the ratio of magnesium ions to ATP. Cell envelopes from *P. stuartii* were further fractionated to recover inner and outer membrane polypeptides by treatment with the detergent Sarkosyl. Proteins from the periplasmic region were recovered by a simple osmotic shock procedure also previously applied to *E. coli*. The purity of the various *P. stuartii* cell envelope fractions was assessed by a combination of techniques that included one- and two-dimensional gel electrophoresis of proteins, enzyme assays and detection of penicillin-binding proteins.

INTRODUCTION

Many recent investigations have been directed towards elucidation of cell envelope structure in Gram-negative bacteria (Owen *et al.*, 1982; Lugtenberg & Van Alphen, 1983). These studies arise in part from the need to understand the role of cell envelopes in the pathogenesis of bacterial infections and the manner by which cell envelope composition influences antibiotic susceptibility (Chopra & Ball, 1982; Lugtenberg & Van Alphen, 1983; Nikaido & Vaara, 1985). Although extensive genetic and biochemical studies have been conducted on cell envelopes from a wide variety of Gram-negative bacteria (Owen *et al.*, 1982; Lugtenberg & Van Alphen, 1983), there are notable exceptions. The Gram-negative bacterium *Providencia stuartii* is one such exception.

In this paper we describe methods for the fractionation of the *P. stuartii* cell envelope. These methods may provide the starting point for future studies on pathogenicity and antibiotic resistance in this organism which is an important cause of urinary tract infection, particularly in long-term catheterized patients (Hawkey, 1984; Warren, 1986).

METHODS

Bacterial strains and growth conditions. Experiments were done with *P. stuartii* strains P308 and P306 (Hawkey *et al.*, 1985; Chopra *et al.*, 1987). The latter carries the plasmid pUB2660, which encodes a TEM2-type β -lactamase (Hawkey *et al.*, 1985). Bacteria were grown aerobically at 37 °C either in a nutrient broth (Lab M broth; London Analytical and Bacteriological Media) or on nutrient agar (Lab M agar; London Analytical and Bacteriological Media).

Preparation of cell envelopes and fractionation to yield inner and outer membrane polypeptides. Total cell envelopes were prepared from *P. stuartii* by following a procedure based on that of Tsuchiya & Rosen (1975*a*), originally developed for isolation of envelopes from *E. coli*. The method was modified slightly in that bacteria were disintegrated by sonication instead of by passage through a French press, mercaptoethanol was replaced by

Abbreviation: PBP, penicillin-binding protein.

dithiothreitol (2 mM) and membranes were stored at -70°C in small portions. Inner and outer membrane polypeptides were fractionated after treating cell envelopes with the detergent Sarkosyl (Filip *et al.*, 1973; Chopra & Shales, 1980).

Preparation of periplasmic and cytoplasmic fractions. Periplasmic proteins were obtained from *P. stuartii* by an osmotic shock procedure (Heppel, 1968). Cytoplasmic fractions were obtained by sonication of osmotically shocked bacteria that had been washed to remove released periplasmic proteins. After sonication, cell envelopes (and unlysed cells) were removed by centrifugation (85000 g, 4°C , 3 h) and the supernatant containing cytoplasmic contents was retained.

Protein determinations. Protein was determined using the Folin-Ciocalteu reagent according to Herbert *et al.* (1971).

Electrophoresis of polypeptides and determinations of isoelectric point and molecular mass. One- and two-dimensional electrophoresis of both radio-labelled and non-labelled proteins was done as previously described (Dixon & Chopra, 1986). The isoelectric points of uncharacterized proteins were determined as described by Ames & Nikaido (1976). Molecular masses of uncharacterized proteins were calculated by constructing calibration curves of log molecular mass versus distance migrated for polypeptides of known molecular mass (Hames & Rickwood, 1981). A computer program for regression analysis was used to process the data. Scanning densitometry of separated polypeptides was done with a Joyce-Loebl Chromoscan 3 equipped with automated peak integration facilities.

Detection of penicillin-binding proteins (PBPs). Cell envelopes from *P. stuartii* were treated for 10 min with benzyl[^{14}C]penicillin [potassium salt, 50 mCi mmol $^{-1}$ (1.85 GBq mmol $^{-1}$), Amersham] as described by Spratt (1977). The reaction was terminated by the addition of non-radioactive benzylpenicillin and Sarkosyl (Spratt, 1977). PBPs were resolved by slab gel electrophoresis according to Suzuki *et al.* (1978) and detected by fluorography (Chamberlain, 1979).

Enzyme assays. β -Lactamase, ATPase, succinic-, NADH-, lactate-, malate-, and glycerol-3-phosphate-dehydrogenases were assayed at 37°C following previously published procedures (Tsuchiya & Rosen, 1975*b*; Shohayeb & Chopra, 1985; Dixon & Chopra, 1986). With the exception of ATPase, the rates of the enzymic reactions were monitored continuously in a Pye Unicam PU8800 spectrophotometer, which was coupled to a micro-computer equipped with Pye Unicam kinetics software, permitting rate calculations by least squares fit (see Chopra, 1986).

RESULTS

Preparation of cell envelopes and assays for known membrane enzymes

Cell envelopes (i.e. material containing both inner and outer membranes) provide the usual starting material for biochemical studies on Gram-negative membrane structure (Owen *et al.*, 1982). It was therefore important to establish that such material could be prepared from *P. stuartii*. Cell envelopes were easily obtained from *P. stuartii* by following the procedure of Tsuchiya & Rosen (1975*a*), a method originally devised for use with *E. coli*. Experiments involving the β -lactamase producing *P. stuartii* strain PV306 showed that the final washed envelope preparation contained $<0.5\%$ of the total cellular β -lactamase content. This indicates negligible contamination of *P. stuartii* envelopes with soluble protein.

In order to establish that the envelopes retained biological activity a number of enzymes known to be envelope associated in other Gram-negative bacteria were assayed in *P. stuartii* cell envelope preparations. The following enzymes were detected [nmol substrate utilized min $^{-1}$ (mg envelope protein) $^{-1} \pm \text{SD}$, $n = 3$]: ATPase, 285.8 ± 7.1 (in the presence of 1 mM-Mg $^{2+}$ and 6 mM-ATP); glycerol-3-phosphate dehydrogenase, 14.2 ± 0.7 ; lactate dehydrogenase, 7.9 ± 0.3 ; malate dehydrogenase, 3.6 ± 0.1 ; NADH dehydrogenase, 134.2 ± 5.4 ; and succinate dehydrogenase, 103.5 ± 0.7 . It is well known that the catalytic activity of bacterial ATPases are influenced by the substrate (ATP) level and the concentration of magnesium ions (Downie *et al.*, 1979). The activity of the *P. stuartii* ATPase was also dependent upon these parameters (Fig. 1). These data concerning ATPase catalytic activity provide further evidence for the authenticity of the *P. stuartii* cell envelope material.

Treatment of P. stuartii cell envelopes to recover outer and inner membrane polypeptides

One of the simplest methods for separating inner and outer membrane polypeptides in bacteria such as *E. coli* depends upon the selective solubilization of inner membranes by the detergent Sarkosyl (Chopra & Shales, 1980; Owen *et al.*, 1982). *P. stuartii* cell envelopes were

Fig. 1

Fig. 1. Effect of substrate and magnesium ion concentrations on the activity of ATPase from *P. stuartii* PV308. ATPase assays were performed as described in Methods using ATP concentrations of 2 mM (○) and 6 mM (●). The absolute value corresponding to the relative activity of 1 is 285.8 nmol ATP utilized min⁻¹ (mg envelope protein)⁻¹ at 37 °C. Error bars denote $\pm$ SD, values are from triplicate determinations on a single envelope preparation.

Fig. 2

Fig. 2. Scanning densitometer profiles of polypeptides in various fractions derived from *P. stuartii* PV308. Peaks to the left have higher molecular masses than those to the right, the range being approximately 200 kDa (left) to 10 kDa (right). (a) Sarkosyl-insoluble material derived from cell envelopes; (b) material released from osmotically shocked bacteria; (c) Sarkosyl-soluble material derived from cell envelopes; (d) soluble material obtained after removal of fractions (a)–(c) above.

extracted with Sarkosyl and detergent-soluble and detergent-insoluble proteins recovered. Electrophoretic analysis of these fractions demonstrated that their polypeptide composition differed fundamentally (Fig. 2*a,c*). In particular, the Sarkosyl-insoluble material contained three major polypeptides in the 30–40 kDa region (polypeptides 12–14, in Fig. 3*a*) not found in the Sarkosyl-soluble material (cf Fig. 2*a,c*). Two-dimensional electrophoresis of Sarkosyl-insoluble material demonstrated that the major polypeptides (i.e. 12–14) were acidic (Fig. 3*b*) with isoelectric points in the range 4.8–5.1. In all bacteria so far examined, PBPs are located in the cytoplasmic membrane (i.e. the Sarkosyl-soluble fraction). Several PBPs were detected in the cell envelope of *P. stuartii* PV308 (Fig. 4).

Fig. 3

Fig. 4

Fig. 3. Separation of polypeptides from the outer membrane of *P. stuartii* PV308. Outer membranes were prepared by treatment of cell envelopes with Sarkosyl and proteins were analysed by electrophoresis. (a) One-dimensional electrophoresis; those polypeptides (1–21) reproducibly identified are labelled to the right of the profile. The molecular masses of the resolved polypeptides fall between 120 kDa (top) and 10 kDa (bottom). (b) Two-dimensional electrophoresis. The *P. stuartii* outer membrane polypeptides 12, 13 and 14 are shown, which have pI values of 4.8, 4.9 and 5.1 respectively.

Fig. 4. Separation of PBPs from the envelope of *P. stuartii* PV308. Envelopes were prepared and treated with benzyl[^{14}C]penicillin as described in Methods. PBPs were detected by fluorography after separation of membrane proteins by electrophoresis (see Methods). The PBPs are numbered from the highest molecular mass (PBP 1, 88 kDa) to the lowest (PBP 7, 42 kDa).

The molecular masses of the PBPs were as follows: PBP 1, 88 kDa; PBP 2, 78 kDa; PBP 3, 64 kDa; PBP 4, 58 kDa; PBP 5, 47 kDa; PBP 6, 43 kDa; and PBP 7, 42 kDa. These molecular masses were calculated by direct comparison (i.e. co-electrophoresis) with *E. coli* PBPs, and using the molecular masses quoted for *E. coli* PBPs by Boulton & Orr (1983). The *P. stuartii* PBPs all separated into the Sarkosyl-soluble fraction with no detectable quantities in the Sarkosyl-insoluble fraction (data not shown). These experiments suggest that treatment of *P. stuartii* cell envelopes with Sarkosyl achieves inner and outer membrane protein separation with an efficiency comparable to that in *E. coli*.

Recovery of periplasmic proteins from P. stuartii

Proteins were released from *P. stuartii* by the osmotic shock procedure of Heppel (1968). One-dimensional electrophoresis showed that the proteins released (Fig. 2b) differed from those in

Fig. 5. Two-dimensional electrophoretic separation of polypeptides released by osmotic shock from *P. stuartii* PV308. Acidic polypeptides are located to the left and basic polypeptides to the right, the range of pI values being 4.8 (left) to 7.1 (right). High molecular mass polypeptides are located at the top, and low molecular mass components at the bottom, the range being 180 kDa (top) to 10 kDa (bottom).

the outer and inner membrane fractions (Fig. 2*a,c*) and also from the proteins in the fraction defined as cytoplasmic contents (Fig. 2*d*). Furthermore, when the β -lactamase-producing strain P306 was subjected to osmotic shock, the material released contained a high enzyme specific activity (data not shown). The proteins released by osmotic shock from *P. stuartii* therefore appear to be derived principally from the periplasmic region. Further evidence for this was provided by two-dimensional gel electrophoresis of proteins released by osmotic shock (Fig. 5). The polypeptide profile of the material released by osmotic shock differed from the profiles obtained with other cell fractions (data not shown). In particular, the osmotic shock procedure did not appear to cause substantial release of outer membrane proteins because the characteristic major acidic polypeptides of the outer membrane (12–14, Fig. 3*b*) were not detected in the proteins released by osmotic shock (Fig. 5).

DISCUSSION

Several aspects of the biology of *P. stuartii* have been examined in detail, but not, apparently, the nature of its cell envelope (Hawkey, 1984; Warren, 1986). This paper has presented data concerned with the latter.

Since *P. stuartii* is a Gram-negative rod, we anticipated that methods applicable to cell envelope fractionation in *E. coli* might also be successful when applied to *P. stuartii*. As noted by Owen *et al.* (1982), procedures for bacterial membrane isolation should be designed in such a way as to retain the membrane in its 'native' state and to remove as far as possible material originating from other cell compartments. We have demonstrated that it is possible to obtain *P. stuartii* cell envelopes that retain membrane enzyme activity and that are not contaminated with soluble proteins by following the procedures of Tsuchiya & Rosen (1975*a*), originally devised for

E. coli. Apart from demonstrating that envelopes contained characteristic membrane enzymes, we were able further to demonstrate that the activity of the *P. stuartii* ATPase was dependent on the concentration of magnesium ions, the ATP level, and the ratio of magnesium ions to ATP, features that are typical of bacterial membrane ATPases (Nelson *et al.*, 1974; Downie *et al.*, 1979). In addition we have established that other well-proven techniques for the separation of envelope fractions in *E. coli* can also be applied to *P. stuartii* i.e. the use of Sarkosyl for inner and outer membrane polypeptide separations and osmotic shock for recovery of periplasmic proteins.

However, some procedures devised for the study of *E. coli* membranes cannot be used directly for *P. stuartii*. For instance, we have not yet been able to separate inner and outer membrane vesicles from *P. stuartii* cell envelope preparations by isopycnic centrifugation on sucrose gradients (Owen *et al.*, 1982), and a method used to release (solubilize) ATPase from *E. coli* membranes (Rosen & Alder, 1975) was not successful when applied to *P. stuartii* cell envelopes (data not shown).

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