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Disruptive Effects of Tris and Sodium Lauroyl Sarcosinate on the Outer Membrane of *Pseudomonas cepacia* Shown by Fluorescent Probes

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The disruptive effects of Tris buffer and sodium lauroyl sarcosinate (Sarkosyl) on the outer membrane (OM) of *Pseudomonas cepacia* were investigated with several fluorescent probes. Tris increased the permeability of the OM to 6-anilino-1-naphthalenesulphonic acid and 2-*p*-toluidinylnaphthalene-6-sulphonate. The degree of damage to the OM was enhanced when the pH was decreased. 3-(*N*-morpholino)propanesulphonic acid buffer had a small but significant effect at acid pH, while citrate/phosphate buffer showed insignificant effects. Sarkosyl released 3,3′-dipentyloxacarbocyanine iodide (CC_5) from CC_5-labelled OM or whole cells and altered OM fluidity as studied by fluorescence polarization.

INTRODUCTION

The outer membrane (OM) of Gram-negative bacteria acts as a permeability barrier for the penetration of drugs (Brown, 1975; Nikaido & Nakae, 1979). Hydrophobic compounds are unable to penetrate the OM of some strains, because the outer leaflet is covered with hydrophilic, closely stacked and highly charged lipopolysaccharide (Nikaido & Nakae, 1979; Shales & Chopra, 1982). Hydrophilic compounds penetrate the OM by diffusion through the pore-forming proteins (porins) embedded in the membrane. However, there is a size limit for the penetration through the porins. Molecules larger than about 600 daltons are unable to diffuse through the porins in *Enterobacteriaceae* (Nikaido & Nakae, 1979). Treatment of bacteria with EDTA has been shown to increase permeability to a large number of compounds (Leive, 1968). This is probably due to the removal of some of the lipopolysaccharide from the outer leaflet of the OM resulting in the redistribution of the phospholipids from the inner leaflet to the outer leaflet of the membrane (Nikaido & Nakae, 1979).

Fluorescence probing has been widely accepted as a tool for the investigation of membrane structure and function (Radda, 1975; Azzi, 1975). Much information on the properties of the biological membranes such as polarity, microviscosity and lipid-phase transition has been obtained through this technique (Trauble & Overath, 1973; Cheng *et al.*, 1974). The mechanisms of action of antibiotics such as polymyxin (Hartmann *et al.*, 1978; Newton, 1954) and colicins (Helgerson *et al.*, 1974) have also been studied with fluorescent probes. Tris has been reported to damage the OM (Voss, 1967; Irvin *et al.*, 1981). Sodium lauroyl sarcosinate (Sarkosyl) is a detergent that is commonly used in the preparation of the OM of Gram-negative bacteria, since it selectively solubilizes the cytoplasmic membrane (Filip *et al.*, 1973). Recently, Sarkosyl was reported to remove several minor OM proteins from the OM of *Escherichia coli*

Abbreviations: OM, outer membrane; ANS, 6-anilino-1-naphthalenesulphonic acid; CC_5, 3,3′-dipentyloxacarbocyanine iodide; DPH, 1,6-diphenyl-1,3,5-hexatriene; MOPS, 3-(*N*-morpholino)propanesulphonic acid; TNS, 2-*p*-toluidinylnaphthalene-6-sulphonate.

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(Chopra & Shales, 1980). This study shows the disruptive effects of both Tris and Sarkosyl on the cell envelope of *Pseudomonas cepacia* by the use of several fluorescent probes.

METHODS

Bacteria and culture conditions. The organism used throughout this study was *Pseudomonas cepacia* NCTC 10661. Organisms were grown at 37 °C in 5-l conical flasks containing 2 l nutrient broth or chemically defined medium consisting of: glucose, 20 mM; KCl, 3 mM; NaCl, 3 mM; $(NH_4)_2SO_4$, 12 mM; $MgSO_4$, 3·2 mM; K_2HPO_4, 1·2 mM; $FeSO_4$, 0·02 mM; 3-(*N*-morpholino)propanesulphonic acid (MOPS), 50 mM; the pH was adjusted to 7·4 with NaOH. All chemicals were Analar grade (BDH). Vigorous aeration of the culture was achieved with a magnetic stirrer.

Chemicals. 2-*p*-Toluidinylnaphthalene-6-sulphonate (potassium salt) (TNS) and 6-anilino-1-naphthalene-sulphonic acid (ANS) were obtained from Sigma. 2-Methylanthracene and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Aldrich. The fluorescent properties of the probes used in this study have been described previously (Azzi, 1975; Radda, 1975; Shinitzky & Barenholz, 1978).

Preparation of the OM of P. cepacia. The OM was prepared by sucrose density gradient centrifugation as described by Anwar *et al.* (1983).

Effects of pH and buffer systems on the fluorescence of TNS or ANS. Several buffer systems such as Tris/maleate buffer (Gomori, 1948), citrate/phosphate buffer (McIlvaine, 1921) and MOPS buffer were used. MOPS buffer (final concentration, 50 mM) was adjusted to an appropriate pH with 1 M-NaOH.

The cells were grown in chemically defined medium and harvested at mid-exponential phase (optical density at 470 nm = 0·5) by centrifugation at 5000 *g* for 15 min, washed twice with saline then resuspended in an appropriate buffer to give an OD_{470} of 0·25. The cell suspension (4·9 ml) was transferred to a test tube and 0·1 ml of TNS or ANS (in ethanol) was added (final concentration, 10 μM). The cells were then incubated at 37 °C for 15 min and the fluorescent spectra taken, using an Aminco-Bowman Spectrofluorimeter and a Hewlett Packard 7035B X-Y recorder.

Effects of Sarkosyl on the fluorescence of 3,3'-dipentyloxacarbocyanine iodide (CC_5) in the OM and whole cells of P. cepacia. The OM isolated as above or the whole cells (grown in nutrient broth) were treated with CC_5 (in ethanol; final concentration, 2 μM), incubated for 15 min, centrifuged at 38000 *g* for 1 h, and washed once with distilled water. They were treated with Sarkosyl (final concentration, 2%, w/v) or left untreated (control) for 30 min at room temperature, and then centrifuged at 38000 *g* for 1 h. The supernate was collected and the pellet was resuspended in the same volume of distilled water (10 ml). Fluorescent spectra of the supernate (Sarkosyl-soluble material) and pellet (Sarkosyl-insoluble material) of both samples were taken.

Effect of Sarkosyl on the fluorescence polarization of the OM of P. cepacia. OM suspensions treated with Sarkosyl (final concentration, 2%) as described above or untreated were resuspended in distilled water and adjusted to an OD_{470} of 0·2. An appropriate fluorescent probe solution (0·25 ml) was added to 24·75 ml of the cell suspension (final concentration of probe, 5 μM), which was then incubated for 15 min at 37 °C, centrifuged at 38000 *g* for 60 min, washed once with distilled water and resuspended in 25 ml of distilled water. Fluorescence polarization was measured as described by Gratzel & Thomas (1973). Microviscosity was calculated from the fluorescence polarization measurement of DPH as described by Shinitzky & Barenholz (1978).

RESULTS AND DISCUSSION

Effects of pH and buffer systems on the fluorescence of TNS or ANS in P. cepacia

It was reported by Ballard *et al.* (1972), using Tris, that fluorescence of ANS in the presence of *E. coli* increased as the pH was decreased. We also found that the fluorescence of TNS or ANS increased as the pH decreased from 7·4 to 5·2 (Fig. 1). However, a large increase in the fluorescence intensity of ANS could only be observed when Tris buffer was used. Similar results were obtained with TNS (data not shown). MOPS had a small but significant effect at acid pH, while citrate/phosphate buffer showed insignificant increases in the fluorescence intensity (data not shown). This study confirms the observations of other workers (Voss, 1967; Irvin *et al.*, 1981) that Tris damages the OM, probably by breaking the salt-bridges formed between divalent metal cations such as magnesium and structural components such as lipopolysaccharide and releases these components as has been suggested by Voss (1967). This effect of Tris is enhanced when the pH is decreased. There are no previous reports of MOPS acting in this way.

Effect of Sarkosyl on the cell envelope of P. cepacia

Filip *et al.* (1973) found that Sarkosyl specifically solubilized the cytoplasmic membrane of *E. coli*, leaving the OM relatively intact. Their conclusion was based on the similarity of the OM

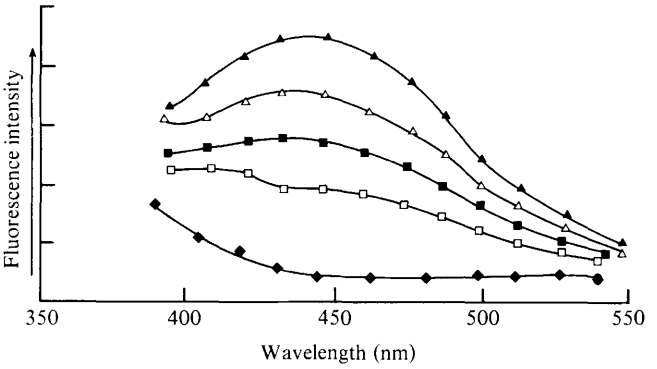

Fig. 1. Effects of pH in Tris/maleate buffer on fluorescence of ANS in *P. cepacia* NCTC 10661. ▲, pH 5·2; △, pH 6·2; ■, pH 6·6; □, pH 7·0; ◆, pH 7·4. Excitation wavelength of ANS at 355 nm.

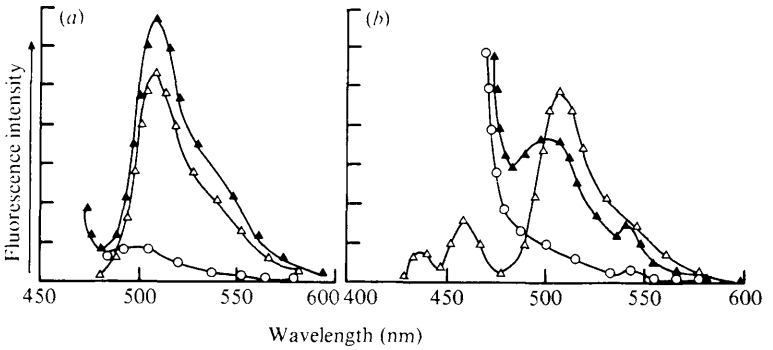

Fig. 2. Effects of Sarkosyl on fluorescence of CC_5 in (*a*) the OM and (*b*) whole cells of *P. cepacia* NCTC 10661. ▲, OM (in *a*) or whole cells (in *b*); △, Sarkosyl-soluble material; ○, Sarkosyl-insoluble material. Excitation wavelength of CC_5 at 470 nm.

protein patterns obtained by the sucrose density gradient centrifugation and Sarkosyl methods. However, Chopra & Shales (1980) found that several minor OM proteins were extracted by Sarkosyl treatment. In the present study, both OM and whole cells of *P. cepacia* were treated with CC_5, and these samples were then extracted with Sarkosyl. The results showed that CC_5 was released from both the OM and the whole cells by Sarkosyl (Fig. 2). If Sarkosyl had no effect on the OM, then the complete disappearance of the fluorescence of CC_5 from the OM or whole cells would not be observed. The location of CC_5 in the OM or whole cell is thus accessible to Sarkosyl. The mechanism probably involves penetration of Sarkosyl into the OM or whole cells, followed by partitioning of solubilized CC_5 into the aqueous phase. The effect of Sarkosyl on the OM was also studied by fluorescence polarization (Table 1). The fluorescence polarization of the OM increased about twofold following Sarkosyl treatment as studied with CC_5 and 2-methylanthracene, and the microviscosity of the OM increased by approximately 0·5 poise (0·05 Pa·s) following Sarkosyl treatment as studied with DPH.

We conclude that Tris damages the OM of *P. cepacia*, and increases its permeability to ANS or TNS. The degree of damage can be enhanced by decreasing the pH. In this study, Sarkosyl removed CC_5 from the OM or intact cells of *P. cepacia* and caused changes in the fluidity of the OM. Sarkosyl should not be used in the preparation of the OM for the study of physical properties. If it is used in chemical studies of OM preparations, it is likely to remove some proteins from the OM, and great care in interpretation of results is required (Chopra & Shales, 1980).

Table 1. *Effects of Sarkosyl on fluorescence polarization and microviscosity of the OM from P. cepacia NCTC 10661 grown in nutrient broth*

The data are means of three determinations ±S.D. Microviscosity is expressed in poise (1 poise = 0·1 Pa·s). Excitation and measured emission were: 470 nm and 505 nm for CC_5, 365 nm and 430 nm for DPH, and 375 nm and 435 nm for 2-methylanthracene.

	Sarkosyl-treated OM		Untreated OM	
Probe	Fluorescence polarization	Microviscosity	Fluorescence polarization	Microviscosity
CC_5	0·22 ± 0·01	—	0·12 ± 0·02	—
DPH	0·37 ± 0·02	2·06 ± 0·07	0·30 ± 0·02	1·55 ± 0·09
2-Methylanthracene	0·22 ± 0·03	—	0·13 ± 0·01	—

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