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Activity of benzalkonium chloride and chlorhexidine diacetate against wild-type and envelope mutants of *Escherichia coli* and *Pseudomonas aeruginosa*

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Summary

Whole cells of wild-type (DCO) and envelope mutant (DC2) strains of *Escherichia coli* took up approximately equal amounts of the cationic surfactant, benzalkonium chloride, although the mutant was considerably more sensitive to this bactericide. Lower concentrations of benzalkonium were needed to induce K^+ leakage from the mutant than from the parent cells. DCO and DC2 showed the same order of sensitivity to chlorhexidine diacetate (minimum inhibitory concentrations against single cell inocula, 1.5 and 0.4 μg/ml, respectively) and took up approximately equal amounts of this antiseptic. K^+ leakage was much greater from DC2 exposed to benzalkonium and slightly higher from chlorhexidine-treated DC2 than from drug-treated DCO. Similar studies with wild type (799) and envelope mutant (799/61) strains of *Pseudomonas aeruginosa* showed that they took up similar amounts of benzalkonium or of chlorhexidine from solution. However, somewhat greater leakage of K^+ occurred from 799/61 than from 799. Lysozyme-EDTA spheroplasts of a wild-type strain and its envelope mutant were equally sensitive to an antibacterial agent.

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Introduction

Benzalkonium chloride and chlorhexidine diacetate are important preservatives, disinfectants and antiseptics (Hugo and Russell, 1982) which are believed to damage the bacterial cytoplasmic membrane (Russell, 1983). In a previous report (El-Falaha et al., 1983) we compared the sensitivity of wild-type and envelope mutant strains of *Escherichia coli* and *Pseudomonas aeruginosa* to these compounds and to other antibacterial agents and showed that the mutants were more sensitive to the quaternary ammonium compound than were the parent strains, although the reason for this has yet to be fully elucidated. In contrast, the two strains of *E. coli* showed a similar order of sensitivity to chlorhexidine diacetate. The mutants have also been found to be considerably more sensitive to many different types of antibiotics (Zimmermann, 1979; Russell and Furr, 1982; Clark, 1984).

In the present report, we describe the uptake of benzalkonium and chlorhexidine by whole cells of the two *E. coli* strains and examine this uptake in relation to potassium (K^+) leakage, an early indication of membrane damage (Lambert and Hammond, 1973). For comparison, similar experiments have been made with *Ps. aeruginosa.*

Materials and Methods

Bacterial strains

These consisted of *E. coli* DCO and *Ps. aeruginosa* 779 and their respective envelope mutants DC2 and 799/61. Cells were grown on the surface of Isosensitest (IST) agar in Roux flasks at 37°C for 24 h. The growth was washed off the surface and washed three times in sterile glass distilled water, and resuspended in one of the following: (i) for drug uptake and K^+ leakage, in glass distilled water; (ii) for lysozyme-EDTA-Tris, in 0.02 M Tris buffer, pH 7.8.

Drug uptake

Microelectrophoretic technique. Uptake of benzalkonium chloride and chlorhexidine diacetate was determined by a means of a novel microelectrophoresis technique (Furr et al., 1981). Bacterial suspensions in water were adjusted to a cell density of 2 mg dry wt./ml. 5 ml was added to 5 ml of benzalkonium chloride or chlorhexidine diacetate solutions. After 15 min at 20°C, the suspension was centrifuged and the concentration of drug remaining in the supernatant fluid assayed by measuring the effect on the electrophoretic mobility of *E. coli* NCTC 9001 (Furr et al., 1981).

Spectrophotometric procedure. Uptake of chlorhexidine diacetate was also measured by means of a chloroform extraction method. After reaction of 5 ml of bacterial suspension (2 mg dry wt./ml) with 5 ml of drug solution and centrifugation, 5 ml of the supernatant fluid was transferred to a 50 ml separating funnel. 5 ml of 0.1 N NaOH was added, admixed and then extracted 4 times with 5 ml chloroform. The pooled chloroform extract was evaporated to dryness under reduced

pressure at 40°C in a Buchi Rotavapor R110 (Orme Scientific, Middleton, Manchester). The free base was extracted with 5 ml 0.1% v/v acetic acid. The chlorhexidine content of the solution was determined by measuring the absorbance at 252 nm in a Cecil CE 292 Digital UV spectrophotometer (Cambridge). This extraction method was not subject to interference by intracellular materials released from drug-treated cells.

K^+ leakage

5 ml of a washed cell suspension (2 mg dry wt./ml) in water and 5 ml of an aqueous drug solution were mixed and held at 20°C for 15 min. The suspension was centrifuged, and the supernatant fluid assayed for K^+ content by means of a flame photometer (EEL, Harlow, Essex).

Lysozyme-EDTA spheroplasts

Spheroplasts of the four strains were prepared by treating cells with lysozyme in the presence of ethylenediamine tetraacetic acid (EDTA) and Tris buffer (Repaske, 1958). 1 ml of a triple-washed bacterial suspension in 0.02 M Tris buffer, pH 7.8, was added to 99 ml of 0.02 M Tris pH 9 containing 0.01 M EDTA, lysozyme (100 μg/ml) and sucrose (0.5 M). The system was kept at 20°C for up to 1 h, and samples removed at 15 min intervals and examined microscopically (Watson Microsystem 70 phase contrast microscope, Micro Instruments, Oxford) until there was complete conversion to spheroplasts.

9 ml aliquots were then removed and added to 1-ml volumes in 0.5 M sucrose of various chlorhexidine diacetate and benzalkonium chloride solutions. Changes in optical density at 500 nm in a Unicam SP600 spectrophotometer were determined after 10 and 60 min at 20°C.

Results and Discussion

In terms of minimal inhibitory concentrations (MICs) the envelope mutant *E. coli* DC2 was considerably more sensitive to benzalkonium chloride than the parent organism the respective MICs being 45 and 2 μg/ml against small inocula, a ratio of 22.5 : 1. It might thus be expected that the quaternary ammonium compound would be bound to a greater extent to the cells of the former organism. Such, however, was not the case and the results presented in Fig. 1 demonstrate that cells of the two organisms took up the drug to approximately the same degree. Since sorption to the surface layers does not necessarily correlate well with the bactericidal effect of a drug, membrane damage as determined by K^+ leakage was measured. Lower concentrations of bactericide were needed to induce leakage from the envelope mutant DC2 than from DCO (Fig. 2a; Table 1).

MIC values for chlorhexidine against single cell inocula of DCO and DC2 were 1.5 and 0.4 μg/ml, respectively, giving a ratio of 3.75 : 1. Cells of both strains took up almost identical amounts of the antiseptic. Actual uptake recorded depended upon the method of assay. Membrane damage, as measured by K^+ leakage, was also

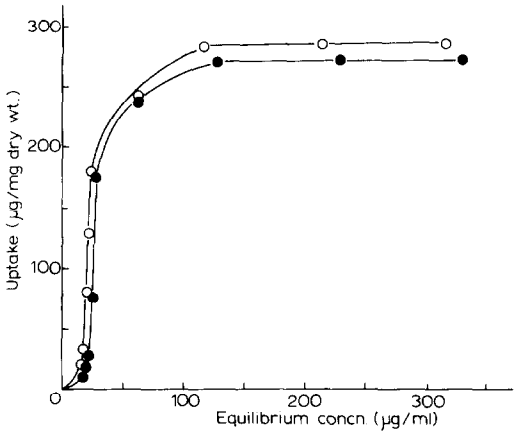

Fig. 1. Uptake of benzalkonium chloride by *Escherichia coli*. ●———●, wild-type strain (DCO); ○———○, envelope mutant (DC2).

determined. The results (Fig. 2b) indicated that only slightly higher concentrations of K^+ were released from the chlorhexidine-treated mutant than from wild-type cells.

Studies on drug uptake can provide information about the nature of its binding to cells (Hugo, 1982), although in view of the large number of cells involved, the technique must be considered as being a rather crude procedure. Furthermore, drug uptake does not necessarily correlate with cellular sensitivity, as evinced by benzalkonium sorption. From the results presented, it seems feasible to propose that in DCO less of this compound penetrates the outer membrane to reach the presumed target site, the inner membrane, than with DC2 (Table 1). With chlorhexidine,

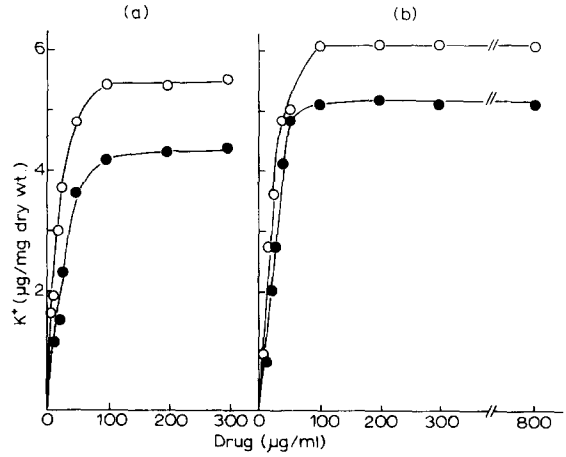

Fig. 2. Leakage of K^+ induced by: (a) benzalkonium chloride; and (b) chlorhexidine diacetate. ●———●, *E. coli* DCO; ○———○, *E. coli* DC2.

TABLE 1

COMPARISON OF K^+ RELEASE FROM DRUG-TREATED *E. COLI* STRAINS DCO and DC2

Benzalkonium chloride conc. (μg/ml)	Ratio of K^+ release (μg/mg dry wt.) DC2 : DCO	Chlorhexidine diacetate conc. (μg/ml)	Ratio of K^+ release (μg/mg dry wt.) DC2 : DCO
0.125	3	10	1.1
0.25	2.6	20	1.35
0.5	2.6	25	1.33
1	2.6	40	1.17
2.5	2.2	50	1.04
5	2	100	1.2
10	1.7	150–800	1.17
20	2		
25	1.6		
50	1.33		
100	1.3		
250	1.25		
300	1.28		

however, the ratio of K^+ released from DC2 to that from DCO is only slightly greater than 1 (Table 1), which implies that chlorhexidine passes through the outer membrane of both types of cells almost equally.

A possible alternative explanation is that differences exist in the inner (cytoplasmic) membrane between DCO and DC2. This has been subjected to indirect assessment by exposing lysozyme-Tris-EDTA spheroplasts suspended in 0.5 M sucrose to the two agents and measuring lysis spectrophotometrically and microscopically. The findings (contact 10 min; Fig. 3a and b) indicated little difference in susceptibility of spheroplasts of the two strains to an antibacterial drug from which it has been inferred that the inner membrane has no major role to play in determining resistance to chlorhexidine or benzalkonium in these two strains. Little

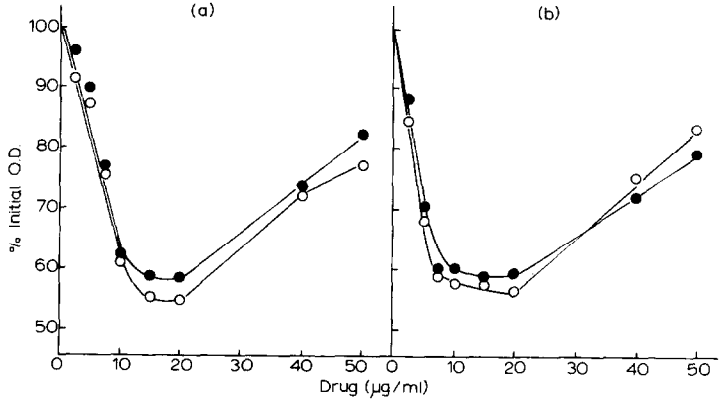

Fig. 3. Lysis of lysozyme-Tris-EDTA spheroplasts of *E. coli* by: (a) benzalkonium chloride; and (b) chlorhexidine diacetate. ●———●, DCO; O ——— O, DC2.

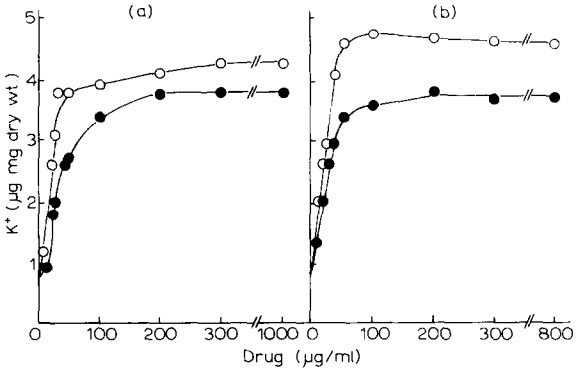

Fig. 4. Leakage of K^+ induced by: (a) benzalkonium chloride; and (b) chlorhexidine diacetate. ●———●, *Ps. aeruginosa* 799; ○———○, *Ps. aeruginosa* 799/61.

additional lysis resulted from a contact time of 60 min. At high drug concentrations, it appeared that precipitation or coagulation of cell contents occurred (Fig. 3a and b). Thus, in the wild-type strain, the outer membrane appears to act as a barrier to the entry of benzalkonium, but less so to that of chlorhexidine.

Clark (1984) compared the envelope composition of DCO and DC2, and could find no significant differences in phospholipid, peptidoglycan cross-linking and fatty acid composition. Minor differences in protein composition were noted but were not described further. The lipopolysaccharide (LPS) content of the two strains was similar, but it was postulated that the mutant contained extra ethanolamine residue(s) in the LPS core, the net negative charge of which was consequently decreased, and that the ethanolamine might be involved in an increase of envelope permeability towards antibacterial agents.

The envelope mutant of *Ps. aeruginosa,* 799/61, was more sensitive to some antibacterial agents than the parent strain, 799 (El-Falaha et al., 1983). MIC values of benzalkonium chloride against single cell inocula were 100 and 150 µg/ml, respectively, and of chlorhexidine 4 and 10 µg/ml, respectively. Cells of strains 799/61 and 799 took up similar amounts of a test disinfectant. The uptake patterns also followed closely upon those found for *E. coli* (Fig. 1); these findings again demonstrate that uptake provides little information about the site of action of a substance or even about the sensitivity of cells to that agent. Recent studies (El-Falaha et al., 1985) have shown that quite low drug concentrations will increase the hydrophobicity of *E. coli* and *Ps. aeruginosa.*

K^+ leakage tended to be rather higher from drug-treated 799/61 than from 799 cells (Fig. 4a and b). However, the ratio of K^+ leaked from mutant: wild-type was generally not much greater than 1 (Table 2). In view of the rather higher resistance of *Ps. aeruginosa* than *E. coli* to the test chemicals, and especially to benzalkonium, it is conceivable that the outer membrane plays a significant role in the resistance of *Ps. aeruginosa.* Added weight for this contention was obtained by comparing the sensivity of lysozyme-EDTA spheroplasts of strains 799 and 799/61 to the two drugs (Fig. 5a and b). Spheroplasts of the two strains were lyzed equally well by

TABLE 2

COMPARISON OF K^+ RELEASE FROM DRUG-TREATED *PS. AERUGINOSA* STRAINS 799 AND 799/61.

Benzalkonium chloride conc. (μg/ml)	Ratio of K^+ release (μg/mg dry wt.) 799/61 : 799	Chlorhexidine diacetate conc. (μg/ml)	Ratio of K^+ release (μg/mg dry wt) 799/61 : 799
5	1	5	N.D. *
10	1.33	10	1.54
20	1.44	20	1.3
25	1.55	25	1.12
40	1.46	40	1.41
50	1.41	50	1.35
100	1.15	100	1.25
150	1.14	150	1.3
200	1.07	200–800	1.27
300	1.13		
400	1.13		
500	1.16		
600	1.13		
800	1.18		
1000	1.13		

* N.D. = not done.

exposure for 10 min (or, not shown, for 60 min) to benzalkonium chloride or chlorhexidine diacetate. These results imply that, in the absence of the outer cell layers, the inner membrane of *Ps. aeruginosa* is not implicated in resistance to

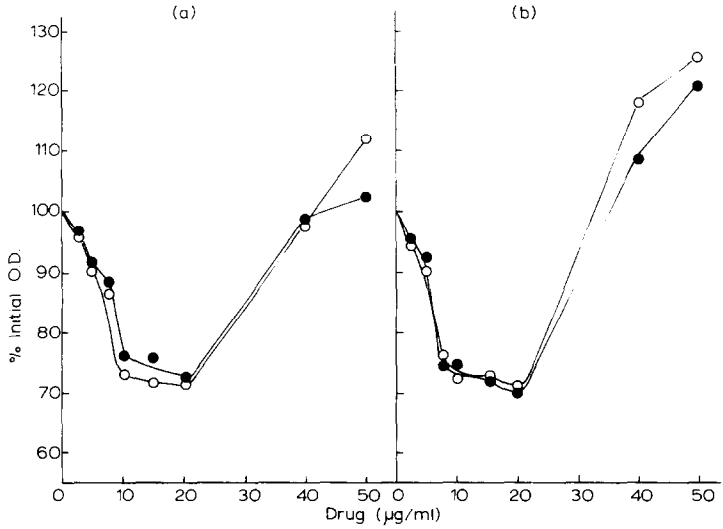

Fig. 5. Lysis of lysozyme-Tris-EDTA spheroplasts of *Ps. aeruginosa* after contact for 10 min with: (a) benzalkonium chloride; and (b) chlorhexidine diacetate. ●——●, strain 799; ○——○, strain 799/61.

chlorhexidine or benzalkonium. A rather different conclusion has recently been reached from studies with *Serratia marcescens,* where it was shown that spheroplasts of a chlorhexidine-resistant strain were considerably more resistant to chlorhexidine than were those from a chlorhexidine-sensitive strain (Lannigan and Bryan, 1985). Less lysis was induced by chlorhexidine or benzalkonium in spheroplasts of *Ps. aeruginosa* than in those of *E. coli*; this might imply that differences exist in the chemical nature and/or structure of the inner membrane of the two types of organisms, with a consequent difference in sensitivity to cationic agents.

Zimmermann (1979, 1980) showed that penetration of β-lactam antibiotics into *Ps. aeruginosa* 799/61 was much greater than into strain 799. The mutant is, in fact, considerably more sensitive to many other antibiotics and to various antiseptics and disinfectants (El-Falaha et al., 1983). The reason for this increased sensitivity is believed to be related to the composition of the outer membrane of the two organisms. Darveau and Hancock (1983) found an LPS alteration in 799/61 as compared to 799; they further demonstrated that size heterogeneity of LPS can exist in a single organism and that the LPS of 799/61 contained only a small proportion of O-antigenic side-chains. Kropinski et al. (1982) proposed that the state of the LPS could influence the number of open functional protein F pores in the outer membrane of *Ps. aeruginosa.* The low outer membrane permeability of strain 799 caused by the small proportion of open functional porins may be the reason for the high resistance of this strain to many antibiotics (Angus et al., 1982) or indeed to some other types of antibacterial agents.

It is not known how chlorhexidine and benzalkonium chloride enter Gram-negative bacterial cells, or indeed whether they share a common pathway. Further studies using strains with known deletions in porins and/or LPS might yield valuable information about these pharmaceutically important antimicrobial agents.

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