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Comparative sensitivity of smooth, rough and deep rough strains of *Escherichia coli* to chlorhexidine, quaternary ammonium compounds and dibromopropamidine isethionate

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Summary

The effects of chlorhexidine diacetate (CHA) and some quaternary ammonium compounds (QACs) on wild-type strains of *Escherichia coli* and on mutants (slightly rough, moderately rough and deep rough) are described. CHA was almost equally active in low concentrations against all the strains, whereas the three QACs, cetylpyridinium chloride (CPC), domiphen bromide and benzethonium chloride were most active against the deep rough strains, and least against the wild type and slightly rough strains. Moderately rough strains were consistently found to be slightly less sensitive to the QACs than the deep rough ones. The inhibitory concentration of CPC for any strain was much less in broth than in agar. Studies with ethylenediamine tetraacetate (EDTA) demonstrated that the chelating agent had only a marginal, if any, effect on the activity of CHA, whereas in the presence of EDTA, the activity of CPC against strains with complete or almost complete lipopolysaccharide was enhanced considerably.

Introduction

In a previous paper (Russell and Furr, 1986a), we described the effects of chlorhexidine acetate (CHA) and two quaternary ammonium compounds (QACs) on wild-type, heptoseless mutants and porin-deficient mutants of *Escherichia coli*. It was observed that all strains were almost equally sensitive to CHA, whereas the lipopolysaccharide (LPS)-defective strains were considerably more sensitive to benzalkonium chloride (BZK) and cetylpyridinium chloride (CPC) than were wild-type organisms, with porin-deficient strains occupying an intermediate position. It was postulated that the exposure of phospholipid at the cell surface of these mutants presented a typical membrane structure through which the two QACs could diffuse.

Hydrophobic antibiotics such as cloxacillin appear unable to enter readily cells of Gram-negative bacteria until a certain degree of roughness (decreasing amount of the R-core: see Fig. 1) of the LPS becomes apparent (Hancock, 1984;

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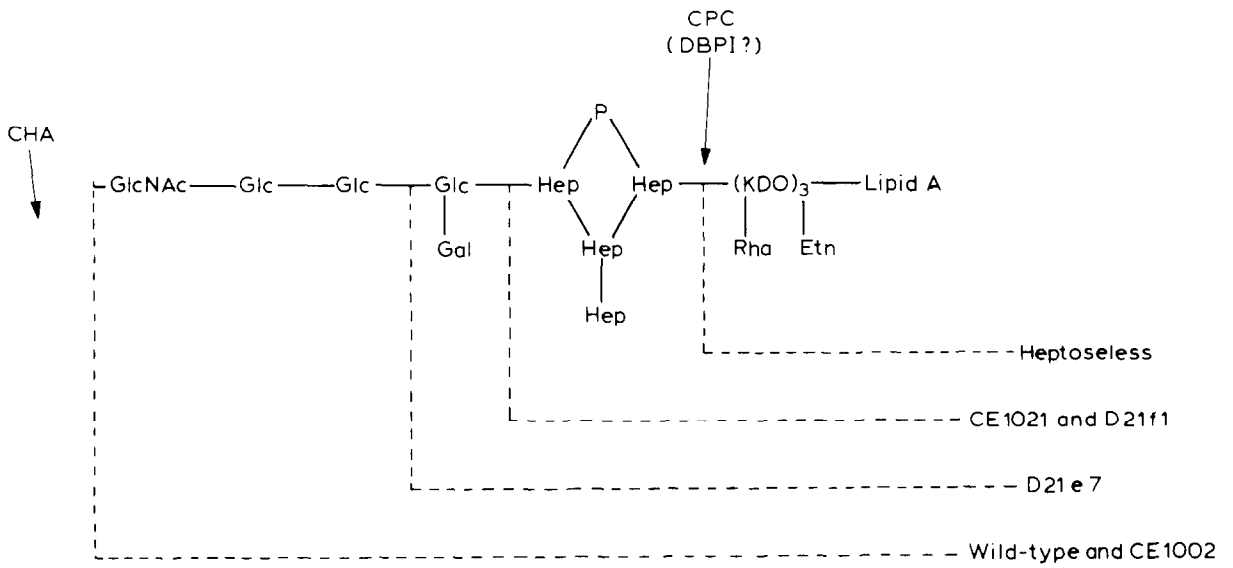

Fig. 1. Chemical composition of the R core of the lipopolysaccharide (LPS) of E. coli strains and LPS loss necessary for adequate penetration of inhibitors. This is depicted as being to the left of each arrow, e.g. with chlorhexidine diacetate (CHA) no loss of LPS is considered necessary. Other abbreviations: CPC, cetylpyridinium chloride; DBPI, dibromopropamidine isethionate; GlcNAc, N-acetylglucosamine; Glc, glucose; Hep, L-glycero-D-mannoheptose; KDO, 2-ketodeoxyoctonate; Rha, rhamnose; Etn, ethanolamine; P, phosphate. (The chemical structure of LPS is based on Van Alphen et al 1977).

Nikaido and Vaara, 1985). Very little work to date has been carried out on the effect of antiseptics, disinfectants and preservatives on rough and deep rough mutants, and in this paper we have compared the effects of CHA and some QACs on strains of *E. coli* with known lesions in their LPS. In addition, the effects of ethylenediamine tetraacetate (EDTA) on the sensitivity of the strains to CHA and CPC have been studied. EDTA was chosen because of its known effects on the outer membrane of Gram-negative bacteria, in particular its binding to Mg^{2+} and the release of some 30–50% of the LPS (Russell, 1971; Leive, 1974; Wilkinson, 1975).

Materials and Methods

Bacterial strains

The bacterial strains and their properties are listed in Table 1. Table 3 also provides information about their antibiotic sensitivity, which will be discussed later.

Antimicrobial susceptibility

(1) Sensitivity to hydrophobic antibiotics. Bacterial cultures were grown overnight at 37 °C in BHI broth, and were diluted 1 : 10 in this medium. 0.2 ml vols. of diluted cultures were inoculated into 20 ml BHI agar and plates poured. When these had set, antibiotic discs (erythromycin 30 μg, novobiocin 30 μg, fusidic acid 30 μg, cloxacillin 10 μg and rifampicin 2 μg) were placed on the surface. After a 1 h period at room temperature for diffusion to occur, plates were incubated for 24 h at 37 °C and diameters of inhibition zones recorded. In other experiments, cultures grown in nutrient broth or in BHI broth for 24 h at 37 °C were diluted 1 : 10 in the appropriate broth and 0.2 ml vols. added to 20 ml molten nutrient agar or BHI agar, as appropriate, containing EDTA (5×10^{-4} M or 10^{-3} M), and plates poured. Discs were placed on the surface (including the large mol. wt. hydrophilic antibiotic, vancomycin, as a 30 μg disc) and the above procedure repeated. The same experiment was carried out in which 0.1-ml vols.

of undiluted culture were inoculated into agar.

(2) MIC determinations. Minimum inhibitory concentrations (MICs) were determined by spotting 1 μl drops from a Denley multipoint inoculator of 1 : 1000 dilutions of overnight 37 °C BHI or nutrient broth cultures on to the surface of overdried (3 h, 37 °C) BHI or nutrient (as appropriate) agar plates containing the desired drug concentration. Plates were incubated for 24 h and the presence or absence of growth noted. The MIC was the lowest drug concentration that completely prevented growth. In other experiments, the effect of EDTA, as the disodium salt, on the MIC values of CHA and CPC was studied in BHI agar and in nutrient agar. The effect of different EDTA concentrations, in the absence of antiseptic, was also determined.

(3) Agar diffusion procedure. Overnight 37 °C cultures in broth were diluted 1 : 10 and 0.2 ml vols. mixed with 20 ml molten agar at about 45 °C. Plates were poured and allowed to set and harden. Two cups, diameter 8 mm, were cut out in each of two sectors. In the top sector, the distance between the two cups was 10 mm, in the lower sector 30 mm. To one cup in each sector was added 0.1 ml of CPC (500 μg/ml), to the other 0.1 ml of EDTA (10^{-2} M). After 1 h at room temperature for diffusion to occur, the plates were incubated at 37 °C and zones of inhibition measured after 24 h.

Results and Discussion

Bacterial strains

The characteristics of the strains are summarised in Table 1, whilst Fig. 1 provides a diagrammatic representation of the chemical composition of the R core of the LPS of these strains. PC0479 and PC1349 are wild-type strains, D21 a rough strain and D21f2 a deep rough (heptose-less) mutant. The other mutants have the following deletions in their R-core (Fig. 1): CE1022 lacks galactose, D21e7 lacks galactose and heptose-bound phosphate and CE1021 and D21f1 lack glucose.

These mutants may lack certain outer membrane proteins. Other deep rough mutants (CE1059, CE1057 and CE1059: Russell et al., 1985) were also employed when required.

TABLE 1

Characteristics of Escherichia coli strains [c]

Strain	Protein deficiency [b]	LPS [a] deficiency
PC0479	None	None
PC1349	None	None
D21	None	Slight rough
CE1002	None	Galactose-deficient
CE1021	OmpF (50% loss)	Glucose-less
D21e7	OmpF (75% loss)	Galactose-deficient and heptose-bound phosphate
D21f1		Glucose-deficient
D21f2		Deep rough
CE1055	OmpA OmpF	Deep rough
CE1057	OmpC OmpF	Deep rough
CE1059	OmpA OmpC OmpF	Deep rough

[a] LPS, lipopolysaccharide.
[b] Omp, outer membrane protein.
[c] Van Alphen et al., (1977).

Experiments on the sensitivity or resistance of the various strains to some hydrophobic antibiotics showed that the deep rough strains were considerably more sensitive to these inhibitors (novobiocin, erythromycin and rifampicin) than were the wild-type strains.

Susceptibility to chlorhexidine and QACs

The responses of the various strains, as determined by using the BHI agar method, to CHA and QACs are considered in Table 2. It is clear that all of the strains were inhibited by low CHA concentrations. CHA appears to enter *E. coli* cells readily, a conclusion also reached in earlier studies (Russell and Furr, 1986).

Considerable variation existed in the sensitivity of the *E. coli* strains to the QACs (CPC, BC and DB: Table 2). The wild-type strains (PC1349, PC0479), the rough strain (D21) and the mutant (CE1022) with only galactose lacking from the core LPS are the least susceptible to all three QACs. The most sensitive are the deep rough mutants (D21f2, CE1055, CE1057 and CE1059), with those strains (CE1021, D21f1, D21e7) having a slightly more complete LPS (Fig. 1, Table 1)

TABLE 2

Sensitivity [a] of test strains to antiseptics

Strain	CHA [b]		CPC		BC	DB	DBPI
	N. agar	BHI [c] agar	N. agar	BHI [c] agar	BHI [c] agar	BHI [c] agar	N. agar
PC0479	ND	2	ND	125	100	100	10 –20
PC1349	2	ND	> 250	ND	> 100	80	10 –15
D21	2.5	2	> 250	100–150	> 100	80	10 –15
CE1002	2	ND	> 250	ND	> 100	15–20	2.5– 5
CE1021	2	ND	7.5	ND	50	5	2.5– 5
D21e7	2.5	ND	12.5	ND	20	5	5 – 7.5
D21f1	2.5	ND	10	ND	20	5	7.5–10
D21f2	2.5	2	7.5	5	5	2	2.5– 5
CE1055	ND	1	ND	2.5–5	2	2	5 –10
CE1057	ND	2	ND	5	2.5	2	10 –15
CE1059	ND	1	ND	2.5–5	1	1	2.5– 5

[a] Figures are MIC values (μg/ml); ND, not done.

[b] CHA, chlorhexidine diacetate; CPC, cetylpyridinium chloride; BC, benzethonium chloride; DB, domiphen bromide; DBPI, dibromopropamidine isethionate.

[c] Data for CHA, CPC and BHI agar taken from Russell and Furr (1986). Results for DBPI varied from experiment to experiment (5 experiments made); the range of MICs is thus quoted.

being only marginally less sensitive; this has been confirmed on various occasions.

MIC values for the QACs in BHI agar tend to be high (Table 2). BHI medium is a complex, nutritionally rich medium, and might be expected to reduce the activity of QACs (Hugo and Russell, 1982). A re-assessment of the effects of some QACs in nutrient agar (Table 2), however, again demonstrated that the smooth strain (PC1349) was the most resistant to these agents and the deep rough strain (D21f2) the most sensitive.

Effect of EDTA

The rationale for studying EDTA was that it removes not only a considerable amount of envelope cations (Mg^{2+} and Ca^{2+}) but also some 30–50% of the LPS (Russell, 1971; Leive, 1974; Wilkinson, 1975). Techniques for studying EDTA action have basically been of two types (Russell, 1982): (a) simultaneous use of EDTA with test agent in a nutrient medium; (b) pretreatment with EDTA, followed by exposure to test antibacterial. Method (a) suffers from the possible disadvantage that it can sequester metal ions from the culture medium which would either influence the growth of the organism or the activity of the inhibitor or

TABLE 3

Effect of EDTA on sensitivity of test strains to hydrophobic antibiotics and to vancomycin in nutrient agar

Strain	EDTA conc. (M)	Inhibition zone diameter (mm)			
		Rif [a] (2)	Eryth. (30)	NV. (30)	Vanc (30)
PC1349	0	– [b]	13	–	–
	5×10^{-4}	9	15	–	11
	10^{-3}	20	18	14	23
CE1002	0	–	12	–	12
	5×10^{-4}	–	15	–	16
	10^{-3}	–	18	19	20
CE1021	0	–	13	14	–
	5×10^{-4}	–	10	16	13
	10^{-3}	19	19	26	20
D21	0	–	11	–	–
	5×10^{-4}	11	14	–	10
	10^{-3}	20	18	13	23
D21f2	0	18	21	15	18
	5×10^{-4}	17	21	13	14
	10^{-3}	21	19	25	17

[a] Antibiotics (in brackets, μg per disc): Rif, rifampicin; Eryth, erythromycin; NV, novobiocin; Vanc, vancomycin.

[b] –, no zone of inhibition.

both. Nevertheless, conclusions based on this type of method, indicating barriers in Gram-negative bacteria to vancomycin, rifampicin, novobiocin and coumermycin (Cleeland et al., 1970), have been substantiated by other procedures. A rapid variation of method (b) has been described for *E. coli* and *Neisseria gonorrhoea* (Scudamore et al., 1979a and b). Rifampicin, novobiocin, actinomycin D and erythromycin were found to be excluded in normal (untreated) but not in EDTA-exposed cells.

In the present work, only method (a) has been examined. No enhancement by EDTA of CPC or CHA against any strain was observed in BHI agar. No zones of inhibition around cups containing CPC (0.1 ml of 500 μg/ml solution) or EDTA (0.1 ml of 10^{-2} M solution) in close proximity or at a distance were observed in BHI agar (although in some instances, a zone of inhibition appeared around EDTA cups in nutrient agar). These findings could reflect a lack of effect of EDTA on bacteria or be related to the relative ineffectiveness of CPC in BHI agar, including poor diffusibility, or a lack of effect of EDTA in nutrient or especially BHI agar.

When antibiotic-impregnated discs were placed on the surface of inoculated agar plates containing EDTA, the zones of inhibition depended on: (i) the type of agar used; (ii) the concentration of EDTA; and (iii) the type of antibiotic present. This aspect was thus examined in greater detail, using both types of agar, and some typical results are presented in Table 3. The final number of cfu/ml of agar was ca. 5×10^5. Inhibition zones

TABLE 4

Effect of EDTA on antibacterial sensitivity of test strains in nutrient agar

Strain	EDTA conc. (M)	CHA [a]		CPC [a]	
		MIC (μg/ml)	Ratio [b]	MIC (μg/ml)	Ratio [b]
PC1349	0	2.5	1	> 250	1
	5×10^{-4}	2.5	1	250	> 1
	10^{-3}	2	1.25	10	> 25
CE1002	0	2.5	1	> 250	1
	5×10^{-4}	2.5	1	125	> 2
	10^{-3}	2	1.25	– [c]	–
CE1021	0	2.5	1	7.5	1
	5×10^{-4}	2.5	1	5–10	1.5–0.75
	10^{-3}	2	1.25	–	–
D21	0	2.5	1	> 250	1
	5×10^{4}	2.5	1	75–100	> 3.33– > 2.5
	10^{-3}	2	1.25	7.5	> 3.33
D21e7	0	2.5–5	1	12.5	1
	$5–0^{-4}$	2.5	2	10–12.5	1.25–1
	10^{-3}	2	1.25–2.5	5	2.5
D21f1	0	2.5–5	1	10	1
	5×10^{-4}	2.5	1–2	10	1
	10^{-3}	2.5	1–2.5	5	2
D21f2	0	2.5–5	1	7.5	1
	5×10^{-4}	2.5	1–2	5	1.5
	10^{-3}	2	1.25–2.5	5	1.5

[a] CHA, chlorhexidine diacetate; CPC, cetylpyridinium chloride.

[b] Ratio of MIC in absence of EDTA: MIC in presence of EDTA (the greater the ratio, the greater the influence of EDTA).

[c] Organism did not grow in presence of EDTA (10^{-3} M), CPC absent.

in BHI agar were consistently less than those in nutrient agar, and thus only the latter findings are described. Inoculum size appears to be fairly critical, since the magnitude of the results is reduced when increasing numbers of cfu are used. It is apparent that EDTA increased the inhibitory effects of the hydrophobic antibiotics and of the large mol.wt. hydrophilic antibiotic, vancomycin.

The final experiment with EDTA involved a comparison of MIC values of CHA and of one QAC (CPC) in the presence and absence of EDTA using nutrient agar as test medium (Table 4). Suitable controls demonstrated that EDTA at this concentration usually had no effect on colony formation, although occasionally CE1002 and CE1021 did not grow. The presence of EDTA generally had little effect on the inhibitory concentration of CHA, which implies that this drug freely enters the cells. This conclusion was previously reached by El-Falaha et al. (1985) and by Russell and Furr (1986). In contrast, the activity of CPC against cells with whole LPS was increased markedly in the presence of the chelating agent (10^{-3} M). EDTA removes a considerable amount of LPS with a consequent exposure of phospholipid bilayer regions thereby allowing the rapid penetration of hydrophobic molecules (Nikaido, 1976).

Experiments were also made with EDTA at a lower concentration (5×10^{-4} M). As would be expected, this concentration of chelator potentiated activity of CPC to a lesser extent.

Dibromopropamidine isethionate (DBPI)

DBPI is a diamidine (Hugo, 1971) which shows greater activity against Gram-positive than Gram-negative bacteria. Like other cationic bactericides, it is more effective at alkaline pH. It was examined here for three reasons: first it is a useful, although not widely used, antibacterial agent (Hugo and Russell 1982); second, it is a different type of cationic agent, and consequently it was deemed of interest to study its activity against the various test strains; and third, resistance to DBPI has recently been found to occur in some multiple-antibiotic resistant strains of *Staphylococcus aureus* (Townsend et al., 1984). Results (Table 2) have not been clear cut, a fairly wide variation in MIC values being noted from one experiment to another. Further studies are needed with DBPI before valid conclusions are reached.

Conclusions

Ru et al. (1985) showed that activity of esters *p*-hydroxybenzoic acid (the parabens) increased when deep rough (heptoseless) mutants of *E. coli* were tested as opposed to wild-type strains. The effect was subsequently found to depend, at least in part, on the amount of LPS present (Russell et al., 1987). The same conclusion is true with the QACs but the overall effect is much more dramatic. In contrast, CHA appears to enter most types of cell readily, although further experiments involving leakage of intracellular constituents and intracellular damage may be needed to substantiate this claim.

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