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Sequential loss of outer membrane lipopolysaccharide and sensitivity of *Escherichia coli* to antibacterial agents

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Summary

The effects of 3 phenolics and of a homologous series of esters of *para*-hydroxybenzoic acid (the parabens) on *Escherichia coli* with known deletions in outer membrane lipopolysaccharide (LPS) are described. Of the phenolics, chlorocresol was the most active and phenol the least, with butyl *p*-hydroxybenzoate the most effective paraben. The most sensitive strain to the various inhibitors was usually a deep rough mutant (heptoseless LPS). Studies were also made with a chelating agent, ethylenediamine tetraacetate (EDTA) in combination with some parabens. EDTA potentiated, to some extent, the activity of the methyl and butyl esters, its activity depending upon the concentration at which it was employed.

Introduction

We recently described the comparative sensitivities to a homologous series of esters of *para*-hydroxybenzoic acid (the parabens) of wild-type, lipopolysaccharide (LPS)-deficient and porin-deficient strains of *Escherichia coli* (Russell et al., 1985). Apart from one rough strain, D21, the LPS-deficient mutants were deep rough, heptoseless strains. It was found that the deep rough strains were the most sensitive organisms to the methyl (Me) and ethyl (Et) esters and especially to the propyl (Pr) and butyl (Bu) esters. Changes in cell sensitivity were related to the LPS deficiency, to outer membrane protein deficiency and especially to likely surface exposure to phospholipid on the one hand and to paraben hydrophobicity on the other. Studies have also been performed on strain sensitivity to cationic-type inhibitors (Russell and Furr, 1986a and b).

The entire LPS molecule (Fig. 1) consists of O-polysaccharide, an R-core and, innermost, linked to the R-core via 2-ketodeoxyoctonate (KDO), lipid A. In this report, we compare the sensitivities to parabens and some phenolics of a wild-type (LPS entire) strain and of LPS strains which become progressively rougher and we attempt to relate these findings to the known hydrophobic properties of these esters. In addition, we

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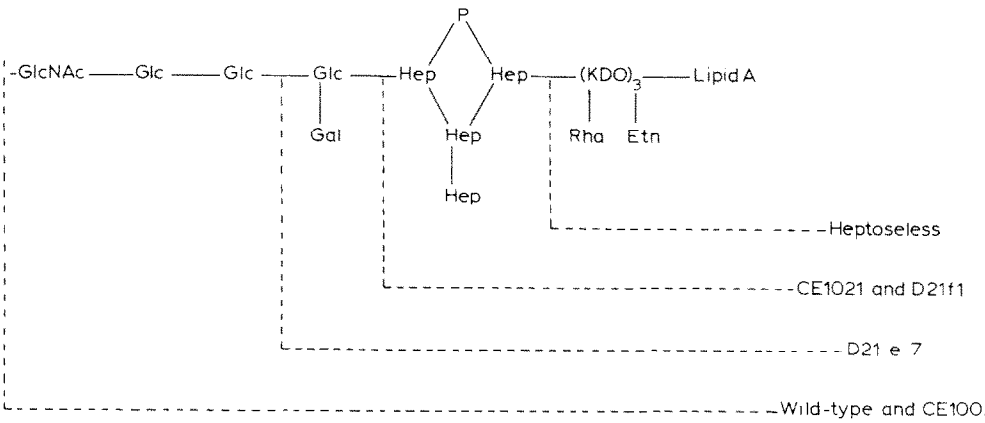

Fig. 1. Composition of lipopolysaccharide (LPS) in the various *E. coli* strains used in this investigation (based on Van Alphen et al., 1977). GlcNAc, N-acetylglucosamine; Glc, glucose; Gal, galactose; Etn, ethanolamine; P, phosphate. Lack of a particular component in a strain is shown alongside the LPS structure.

have examined the effects of the chelating agent, ethylenediamine tetraacetate (EDTA) on the sensitivity of the various strains to the parabens. Several workers have demonstrated that EDTA removes Mg^{2+} and a considerable amount of LPS from the outer membrane of Gram-negative bacteria (Russell 1971; Leive 1974; Wilkinson 1975). Kabara and Wernette (1982) and Hart (1984) have demonstrated that EDTA appears to potentiate the activity of the Me ester against *Ps. aeruginosa* and *E. coli*.

Materials and Methods

Bacterial strains

These were *E. coli* strains PC 1349 (wild-type) and the following rough strains: CE 1002, CE 1021, D21, D21e7, D21f1 and D21f2. Their characteristics are described in Table 1. Strains were grown in Nutrient Broth (Oxoid, London) at 37°C, and used in the following experiments. The solid medium was Nutrient Agar (Oxoid).

Sensitivity to inhibitors

Sensitivity to inhibitors was measured in two ways.

(a) Antibiotic disc sensitivity testing. Overnight broth cultures grown at 37°C were diluted 1 in 10 in broth, and 0.2 ml vols. were mixed with 20 ml molten agar at about 45°C. Plates were poured, and antibiotic discs placed onto the surface of the solidified agar. After 1 h at 20°C for prediffusion, plates were incubated at 37°C for 24 h and zones of inhibition were measured.

(b) Determination of minimum inhibitory concentrations (MICs). Overnight, 37°C broth cultures were diluted 1 in 100 in broth, and 1 μl volumes placed by a multipoint inoculator (Denley, Billingshurst) onto the surface of overdried (37°C, 2 h) agar plates containing the desired drug concentration. Plates were incubated at 37°C for 24 h, the presence or absence of growth noted, and the MIC determined.

Effect of EDTA

MICs of parabens were determined, by a method identical to that described above, in agar containing EDTA (final concentrations 5×10^{-4} M and 10^{-3} M). Suitable controls were also included, viz. drug concentrations alone (EDTA absent), EDTA alone (drug absent) and plain agar (both drug and EDTA absent).

Results and Discussion

Table 1 provides a summary of the properties and characteristics of the various *E. coli* strains used in this investigation. Wild-type strain, CE

TABLE 1

Escherichia coli strains employed in this study

Strain	Protein deficiency	LPS deficiency
PC 1349	None	None
CE 1002	None	Galactose-deficient
CE 1021	OmpF (50% loss)	Glucose-deficient
D21	None	Slight rough
D21e7	OmpF (75% loss)	Galactose-deficient and heptose-bound phosphate
D21f1		Glucose-deficient
D21f2		Deep rough (heptoseless)

LPS, lipopolysaccharide (for further details, see Fig. 1); Omp, outer membrane protein.

1002, CE 1021 and D21 were resistant to hydrophobic antibiotics (rifampicin, novobiocin, erythromycin) whereas the heptoseless mutant D21f2 was sensitive.

The effects of the Me, Et, Pr and Bu parabens are described in Table 2. All 7 strains showed a similar pattern of response to the Me ester. However, as the homologous series is ascended, there is a gradual increase in sensitivity in relation to the wild-type strain (PC 1349). The rough strain D21 is as resistant to, or even more resistant than, PC 1349; CE 1002 has a 'normal' type of LPS apart from the absence of galactose and gives an identical response to the wild-type strain. Strain D21e7 lacks galactose and heptose-bound phosphate, and strains CE 1021 and D21f1 lack glucose, and it is noticeable that all strains show an increased sensitivity in comparison to the wild-type strain, PC 1349. However, the most sensitive is the deep rough (heptoseless) mutant, D21f2. A more meaningful comparison is provided in Table 3, where molar ratios of MICs of Me paraben:test paraben are listed; the higher the ratio, the more effective is the test paraben. The results suggest that slight depletions in LPS can make a considerable difference in increasing sensitivity to hydrophobic inhibitors (order of hydrophobicity is Bu > Pr > Et > Me). A similar conclusion is apparent from the graphical presentation made in Fig. 2, where differences in slopes of lines for (a) PC 1349 and CE 1002, (b) CE 1021, D21e7 and D21f1, and (c) D21f2, can be observed. However, these make no allowance for any possible depletion of porins; porin or other protein loss, as well as LPS depletion, could play a role in exposing deeper-seated molecules (phospholipids?) which may act as a pathway for the entry of hydrophobic inhibitors; see also Hancock (1984) and Nikaido and Vaara (1985).

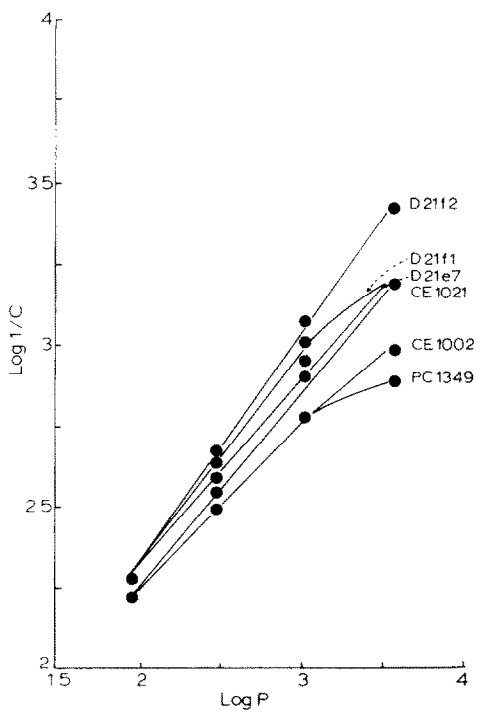

Fig. 2. Plot of the reciprocal of $\log_{10}$ molar inhibitory concentrations ($\log 1/C$ values) vs partition coefficients ($\log P$) for Me, Et, Pr and Bu parabens. Figures indicate the test strain.

The effects of two concentrations of EDTA (10^{-3} M and 5×10^{-4} M) on bacterial sensitivity to the parabens are summarised in Table 4. Strains CE 1002 and CE 1021 did not always grow on media containing 10^{-3} M EDTA (paraben absent) and thus the value given for the effect of EDTA at this concentration on the Me or Bu paraben against these two strains has not always been confirmed. Generally, the effects of EDTA were not startling: potentiation of activity of a paraben increased with increasing EDTA concentration (ratio of MIC in the absence of EDTA/presence of EDTA), and the extent of the increased activity was of a similar order with all the strains (ratio of MIC vs PC 1349/MIC vs test strain). The findings suggest

TABLE 2

MICs of parabens against Escherichia coli strains

Strain	Me ester			Et ester			Pr ester			Bu ester		
	(a)	(b)	Ratio	(a)	(b)	Ratio	(a)	(b)	Ratio	(a)	(b)	Ratio
PC 1349	900	5.92×10^{-3}	1	500	3.012×10^{-3}	1	300	1.668×10^{-3}	1	250	1.289×10^{-3}	1
CE 1002	900	5.92×10^{-3}	1	500	3.012×10^{-3}	1	300	1.668×10^{-3}	1	250	1.289×10^{-3}	1
CE 1021	900	5.92×10^{-3}	1	450	2.71×10^{-3}	1.1	225	1.25×10^{-3}	1.33	125	6.444×10^{-4}	2
D21	900	5.92×10^{-3}	1	nd	nd	nd	nd	nd	nd	> 250	$>1.289\times10^{-3}$	> 1
D21e7	800	5.26×10^{-3}	1.1	425	2.56×10^{-3}	1.18	200	1.111×10^{-3}	1.5	125	6.444×10^{-4}	2
D21f1	800	5.26×10^{-3}	1.1	375	2.26×10^{-3}	1.33	175	9.722×10^{-4}	1.71	125	6.444×10^{-4}	2
D21f2	800	5.26×10^{-3}	1.1	350	2.108×10^{-3}	1.43	150	8.333×10^{-4}	2	75	3.866×10^{-4}	3.3

Columns (a), MIC expressed in μg/ml; columns (b), MIC expressed in molar terms; ratio denotes ratio of (MIC vs PC 1349:MIC vs test strain); nd, not done.

TABLE 3

Comparative efficacy of various paraben esters against Escherichia coli strains

Strain	MIC ratios (molar basis)		
	Me : Et	Me : Pr	Me : Bu
PC 1349	1.97	3.55	4.59
CE 1002	1.97	3.55	4.59
CE 1021	2.18	4.74	9.19
D21	nd	nd	< 4.59
D21e7	2.05	4.73	8.16
D21f1	2.33	5.41	8.16
D21f2	2.5	6.31	13.61

MIC ratios are calculated from the data presented in Table 2; nd, not done.

that EDTA has a similar effect on all the strains, e.g. in LPS and/or Mg^{2+} removal, or that the parabens are themselves so weakly active that a more delicate and sophisticated method than the one used here is needed to measure differences between the strains. Similar types of responses have been observed with smooth and rough strains of *Salmonella typhimurium* (Russell and Furr, 1986c) but it is noticeable with both the *E. coli* strains (Russell and Furr, 1986b) and the *S. typhimurium* strains (Russell and Furr, 1986c) that the effect of cetylpyridinium chloride is markedly affected by EDTA.

When 3 phenolics (phenol itself, cresol and chlorocresol) were examined, the results in Table 5 were obtained. These findings were unexpected, but we have no reason to doubt their validity since they have been confirmed on several occasions. The strains tended to show the same order of sensitivity to an individual phenol, with cresol being only slightly more active than phenol, but chlorocresol several-fold more active (Table 6). In the light of these results, we decided to investigate the sensitivity to these 3 inhibitors of 3 other heptoseless mutants (CE 1055, CE 1057, CE 1059) described in our previous studies (Russell et al., 1985; Russell and Furr, 1986a). These 3 organisms gave the same order of response to an individual phenol as the heptoseless mutant D21f2. There appear, then, to be considerable differences in the manner in which the strains respond to parabens on the one hand and phenolics on the other (although an alternative explanation could be that the MIC method is not sufficiently sensitive to

TABLE 4

Effect of EDTA on the activity of parabens against Escherichia coli

Strain	EDTA concn. (M)	Me ester				Bu ester			
		(a)	(b)	PC 1349 / Test strain	MIC (−EDTA) / MIC (+EDTA)	(a)	(b)	PC 1349 / Test strain	MIC (−EDTA) / MIC (+EDTA)
PC 1349	5×10^{-4}	700	4.61×10^{-3}	1	1.29	175	9.02×10^{-4}	1	1.43
	10^{-3}	400	2.63×10^{-3}	1	2.25	75	3.87×10^{-4}	1	3.33
CE 1002	5×10^{-4}	700	4.61×10^{-3}	1	1.29	225	1.16×10^{-3}	0.78	1.11
	10^{-3}	300	1.97×10^{-3}	1.33	3	75	3.87×10^{-4}	1	3.33
CE 1021	5×10^{-4}	900	5.92×10^{-3}	0.78	1	150	7.73×10^{-4}	1.17	0.83
	10^{-3}	300	1.97×10^{-3}	1.33	3	50	2.58×10^{-4}	1.5	2.5
D21	5×10^{-4}	800	5.26×10^{-3}	0.9	1.13	150	7.73×10^{-3}	1.17	> 1.67
	10^{-3}	400	4.61×10^{-3}	1	2.25	125	6.44×10^{-3}	0.6	> 2.0
D21e7	5×10^{-4}	700	4.61×10^{-3}	1	1.14	125	6.44×10^{-4}	1.4	1
	10^{-3}	400	2.63×10^{-3}	1	2.0	75	3.87×10^{-4}	1	1.67
D21f1	5×10^{-4}	700	4.61×10^{-3}	1	1.14	150	7.73×10^{-3}	1.17	0.83
	10^{-3}	400	2.63×10^{-3}	1	2.0	50	2.58×10^{-4}	1.5	2.5
D21f2	5×10^{-4}	600	3.95×10^{-3}	1.2	1.33	75	3.87×10^{-4}	2.33	1
	10^{-3}	300	1.97×10^{-3}	1.33	2.67	50	2.58×10^{-4}	1.5	1.5

Columns (a), MIC in μg/ml; columns (b), MIC in molar terms. MIC (−EDTA) and MIC (+EDTA): MIC in presence and absence, respectively, of EDTA of stated concentration. The higher the ratio, the greater is the effect of EDTA. Strains CE 1002 and CE 1021 grew on media containing 5×10^{-4}M EDTA but not always on media containing 10^{-3} M (in both instances, test drugs absent). All other strains were not inhibited by either concentration of EDTA.

TABLE 5

MICs of phenolics against Escherichia coli strains

Strain	Phenol			Cresol			Chlorocresol		
	(a)	(b)	Ratio	(a)	(b)	Ratio	(a)	(b)	Ratio
PC 1349	1 500	1.595×10^{-2}	1	875	8.094×10^{-3}	1	150	1.052×10^{-3}	1
CE 1002	1 375	1.462×10^{-2}	1.09	625	5.782×10^{-3}	1.4	125	8.766×10^{-4}	1.2
CE 1021	1 250	1.33×10^{-2}	1.2	625	5.782×10^{-3}	1.4	125	8.766×10^{-4}	1.2
D21	1 750	1.86×10^{-2}	0.86	1 000	9.25×10^{-3}	0.875	150	1.052×10^{-3}	1
D21e7	1 750	1.86×10^{-2}	0.86	875	8.094×10^{-3}	1	100	7.013×10^{-4}	1.5
D21f1	1 375	1.462×10^{-2}	1.09	875	8.094×10^{-3}	1	125	8.766×10^{-4}	1.2
D21f2	1 500	1.595×10^{-2}	1	875	8.094×10^{-3}	1	100	7.013×10^{-4}	1.5

(a), Expressed in μg/ml; (b), expressed in molar terms. Ratio is ratio of MIC vs PC 1349:MIC vs test strain.

detect significant effects in the different strains). The $\log_{10}$ partition coefficient (log P) values in octanol/water for the 3 phenolics range from 1.46–3.10 and for the 4 parabens from 1.96–3.57 (Hansch and Leo, 1979) and it is difficult, therefore, to ascribe different effects to differences in the hydrophobic nature of the phenolic inhibitors. Reference to our studies with *Salmonella typhimurium* strains (Russell and Furr, 1986b) shows that the slopes of the lines for plots of log $1/C$ (where C is the MIC value) vs log P for the 3 phenols against those strains were not dissimilar for the smooth, Ra, Rd_1 and Re strains whereas there were differences between slopes for the smooth and Ra strains on the one hand, and Rd_1 and Re on the other when similar plots were made for the parabens.

The effects of the 4 parabens on the various *E. coli* strains have been reconsidered on the basis of a simple mathematical approach. This assumes that the response (R) of an organism to a paraben depends on ability of the antimicrobial agent to penetrate both components in LPS; in our simple model, these components are described as being sugar (variable) and lipid (assumed to be constant in all strains). R will thus depend on two factors:

(i) partition coefficient (P). The effect of P will vary in each compartment, thus

$$R \propto P^n P^m \tag{1}$$

where n is the response factor in lipid, and m in sugar;

(ii) sugar content, in which there should be an inverse relationship, thus

$$R \propto {}^1/_{S^\ell} \tag{2}$$

in which S represents the sugar and ℓ the 'response coefficient' to sugar. Hence

$$R = \frac{k \cdot P^n P^m}{S^\ell} \tag{3}$$

from which

$$\log R = \log k + (n + m) \log P - \ell \times \log S \tag{4}$$

In the wild-type strains, a value of unity has been assumed for the sugar content. Thus,

$$\log R = \log k + (n + m) \log P \tag{5}$$

TABLE 6

Comparative efficacy of various phenolics against Escherichia coli strains

Strain	MIC ratios	
	Phenol : Cresol	Phenol : Chlorocresol
PC 1349	1.97	15.16
CE 1002	2.53	16.68
CE 1021	2.3	15.16
D21	2.01	17.68
D21e7	2.3	26.52
D21f1	1.81	16.68
D21f2	1.97	22.74

Calculated from data presented in Table 4.

TABLE 7

Calculation of $\ell \times \log S$ and Δm values

Strain	$\ell \times \log S$	Δm (increase from PC 1039)
PC 1039	0	0
CE 1002	0	0
CE 1021	0.382	0.182
D21e7	0.282	0.158
D21f1	0.262	0.158
D21f2	0.542	0.288

A plot of log R (i.e. log of MIC value) against log P gives a straight line from which log $k = -0.768$, and $(n + m) = 0.415$.

Plots for the other strains also produce reasonably straight lines which enable calculation of values for ℓ log S and of m (n is considered to remain constant). These values are listed in Table 7, from which two conclusions may be reached: (a) higher values of m with the sugar-deficient strains demonstrate greater ease of penetration by the higher parabens; (b) ℓ log S has been calculated from the intercept of plots of $\log_{10}$ response (R) (ratio of molar MIC of Me paraben vs PC 1349: molar MIC of a paraben vs test strain) against log P. This value represents penetration by hypothetical antibacterial agents of log $P = 0$, or $P = 1$, i.e., they would be much more hydrophilic than the 'real' parabens. Wild-type strains are consequently less susceptible to parabens, apparently because the high sugar content masks the underlying phospholipid molecules which may be responsible for aiding these inhibitors across the outermost parts of the bacterial cell. The mathematical term, ℓ log S, is thus a measure of sugar deficiency and is zero value for wild-type strains with whole LPS. The most sensitive strains to a paraben are those possessing a heptoseless LPS, with the Bu ester being the most effective inhibitor.

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