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Short Communication

Surface changes in *Pseudomonas aeruginosa* exposed to chlorhexidine diacetate and benzalkonium chloride

Bahgat M.A. El-Falaha, D.T. Rogers, J.R. Furr and A.D. Russell

Welsh School of Pharmacy, University of Wales Institute of Science and Technology, Cardiff CF1 3XF, Wales (U.K.)

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Summary

Pseudomonas aeruginosa strains 799 (wild-type) and 799/61 (envelope mutant) were exposed to chlorhexidine diacetate and benzalkonium chloride, and their hydrophobicity measured in a hydrocarbon (xylene) system. Both drugs induced changes in surface hydrophobic properties at concentrations well below those that inhibited cellular growth. A comparison of these findings has been made with a wild-type and an envelope mutant of *Escherichia coli*, both of which strains are more sensitive than *Ps. aeruginosa* to these drugs.

Pseudomonas aeruginosa is an important pathogen that frequently shows above-average resistance to many antibacterial agents (Stickler and Thomas, 1982). These inhibitors include cationic-active substances such as cetrimide, benzalkonium chloride (BZK) and chlorhexidine diacetate (CA) (Hugo and Russell, 1982).

In a previous study, we compared the sensitivities of a wild-type strain (799) of *Ps. aeruginosa* with its envelope mutant, 799/61, to a range of disinfectants, antiseptics and preservatives and found that 799/61 was generally more sensitive (El Falaha et al., 1983). It has subsequently been found (manuscript in preparation) that cells of both strains take up approximately equal amounts of CA or of BZK. It has also been observed (El-Falaha et al., 1984) that CA and BZK will increase the hydrophobicity of cells of wild-type and envelope mutant strains of *Escherichia coli*.

Correspondence: A.D. Russell, Welsh School of Pharmacy, University of Wales Institute of Science and Technology, P.O. Box 13, Cardiff CF1 3XF, Wales, U.K.

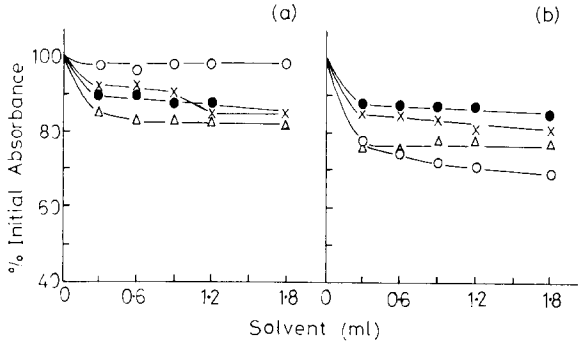

Fig. 1. Hydrophobic nature of *Ps. aeruginosa* strains exposed to different solvent systems. (a) strain 799; (b) strain 799/61. Xylene, O——O; *n*-hexadecane, ●——●; cyclohexane, △——△; isopropyl myristate, ×——×.

It therefore seemed of value to examine the effects of these inhibitors on the more resistant strains of *Ps. aeruginosa.*

Ps. aeruginosa 799 and 799/61 were grown for 18 h at 37°C on the surface of Isosensitest (IST) agar (Oxoid, London), and harvested with PUM buffer, pH 7.1, containing (g/l): $K_2HPO_4 \cdot 3H_2O$ 22.2, KH_2PO_4 7.26, urea 1.8, $MgSO_4 \cdot 7H_2O$ 0.2, glass-distilled water to 1 litre. The resultant suspension was centrifuged at 5000 rpm for 15 min and washed twice in PUM buffer and then resuspended in buffer to an absorbance of 1.0 at 400 nm in a Unicam SP600 series 2 spectrophotometer (Pye Unicam, Cambridge).

Chlorhexidine diacetate (CA) was purchased from ICI Pharmaceuticals, Macclesfield and benzalkonium chloride (BZK) from Berk Pharmaceuticals, Shalford, Surrey. 1.25 ml volumes of various concentrations of CA or BZK were added to 23.75 ml volumes of cell suspension at 20°C to give the final desired drug concentrations. Thorough mixing was achieved on a vortex mixer. After 15 min contact at 20°C, the cells were centrifuged at 5000 rpm and the cell pellet washed twice with, and resuspended in, 25 ml PUM buffer. The absorbance at 400 nm was determined, and the hydrophobic properties of the cells monitored as described below. Cells which had not been pretreated with CA or BZK served as controls.

Five test organic solvents were studied: xylene, *n*-hexadecane, *n*-octanol (octan-1-ol), cyclohexane and isopropyl myristate. To 4.8 ml volumes of control (untreated) or pretreated cell suspensions in PUM buffer were added various volumes (0.3–1.8 ml) of test solvents and the mixture vortexed for 2 min. After 15 min to allow phase separation to occur, the aqueous phase was carefully pipetted and the absorbance at 400 nm determined and compared with that of the cell suspension before addition of solvent.

Cells of the envelope mutant, 799/61, showed a slightly greater surface hydrophobicity than those of the wild-type strain, 799 (Fig. 1), possibly because of the increased exposure of the inner core of the lipopolysaccharide (LPS; Rosenberg et al., 1980) or because of differences in the site of phospholipids in the outer and inner leaflets of the outer membrane (Rosenberg et al., 1982). When octanol was studied

(not shown) volumes of 1.8–3.0 ml had to be used before any increase in hydrophobicity became apparent, because of problems of phase separation.

Subsequent experiments were devoted towards the effects of CA and BZK on surface hydrophobicity in the two *Ps. aeruginosa* strains, as evidenced by their response to the hydrocarbon, xylene. In a previous study (El-Falaha et al., 1983), the following minimal inhibitory concentrations (MICs, μg/ml) of these agents were obtained: (1) *Ps. aeruginosa* 799 and BZK: low inoculum 150, high inoculum 450; (2) *Ps. aeruginosa* 799/61 and BZK: low inoculum 100, high inoculum 450; (3) 799 and CA: low inoculum 10, high inoculum > 50; (4) 799/61 and CA: low inoculum 4, high inoculum 15. Thus, the MIC ratios for 799 : 799/61 are 1.5–1 for BZK, and 2.5 to > 3.3 for CA. These MIC values are higher than those found for *E. coli* (El-Falaha et al., 1983; see also Table 1).

Depending on concentration, both CA and BZK increased the hydrophobicity of the two *Ps. aeruginosa* strains. Results with CA are presented in Fig. 2a and b from which it may be seen that 2 or 10 μg CA/ml and 10 μg/ml, respectively, had some effect on the hydrophobicity of 799 and 799/61, whereas concentrations of 25 or 50 μg/ml were necessary to increase the hydrophobicity markedly. These findings are to be compared with those obtained with a wild-type and an envelope mutant of *E. coli* where CA at 2 μg/ml, and especially 10 μg/ml, induced a marked increase in hydrophobicity (El-Falaha et al., 1984). A comparison of the MIC value of CA and the lowest concentration deemed to induce an increase in hydrophobicity (hydrophobicity-increasing concentration, HIC) of the cell surface for the *Ps. aeruginosa* and *E. coli* strains is presented in Table 1. In all instances, the HIC is less than the MIC, and this is particularly so for the most resistant strain, *Ps. aeruginosa* 799, where the HIC is considerably below the MIC. Thus, CA affects the cell surface of

TABLE 1

RELATIONSHIP BETWEEN MINIMAL INHIBITORY CONCENTRATION (MIC) AND HYDROPHOBICITY-INCREASING CONCENTRATION (HIC) FOR CHLORHEXIDINE DIACETATE AND BENZALKONIUM CHLORIDE

Organism	Drug [a]	HIC [b] (μg/ml)	MIC [c] (μg/ml)	Ratio $\frac{\text{MIC}}{\text{HIC}}$
Ps. aeruginosa 799	CA	2–10	> 50	> 25 to > 5
Ps. aeruginosa 799/61	CA	10	15	1.5
E. coli DC0	CA	2	4	2
E. coli DC2	CA	2	2	1
Ps. aeruginosa 799	BZK	25	450	18
Ps. aeruginosa 799/61	BZK	25	450	18
E. coli DC0	BZK	< 25	100	> 4
E. coli DC2	BZK	< 25	20	> 0.8

[a] CA, chlorhexidine diacetate; BZK, benzalkonium chloride.

[b] HIC, lowest concentration that induces an increase in hydrophobicity.

[c] MIC against large inocula.

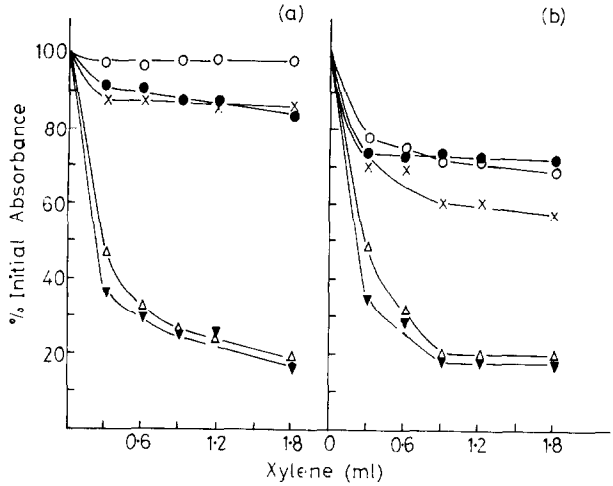

Fig. 2. Effect of pretreatment of *Ps. aeruginosa* strains with different concentrations of chlorhexidine diacetate (CA) and their subsequent response to xylene. (a) strain 799; (b) strain 799/61. CA concentrations (μg/ml); 0, O——O; 2, ●——●; 10, ×——×; 25, △——△; 50, ▼——▼.

this organism at a concentration much less than the MIC value. Whether the resistance presented to CA in this strain results from an outer membrane barrier or from a different inner membrane must await the outcome of further experiments.

The effects of BZK on the hydrophobic properties of *Ps. aeruginosa* 799 and 799/61 are depicted in Fig. 3a and b. A low concentration, 2 μg/ml, had little

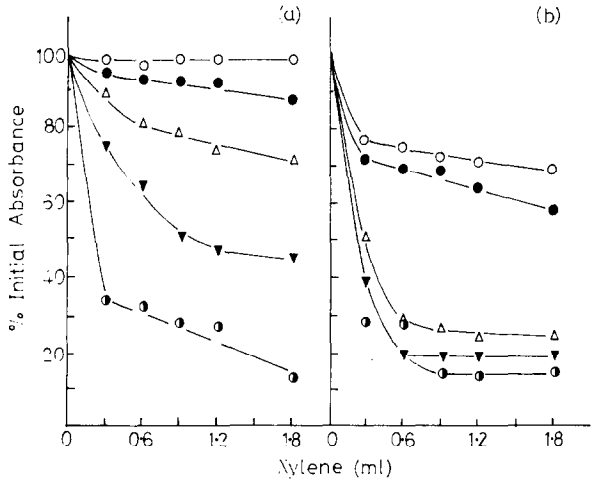

Fig. 3. Effect of pretreatment of *Ps. aeruginosa* strains with different concentrations of benzalkonium chloride (BZK) and their subsequent response to xylene. (a) strain 799; (b) 799/61. BZK concentrations (μg/ml): 0, O——O; 2, ●——●; 25, △——△; 50, ▼——▼; 100, ◑——◑.

effect, whereas 25 μg/ml and above increased the hydrophobicity of the mutant, with 50, and especially 100 μg/ml, having a marked effect on the wild-type strain. With the two *E. coli* strains, a BZK concentration of between 2 and 25 μg/ml was required to produce a significant increased in hydrophobicity (El-Falaha et al., 1984). A comparison of MICs and HICs is presented in Table 1, from which it may be observed that HIC values are considerably less than MIC values for both *Ps. aeruginosa* strains, which show a similar, but not identical, pattern of response to BZK. Richard and Cavill (1976) found that pretreatment of *Ps. aeruginosa* with BZK produced cells which were sensitive to polysorbate 80, and Hoffman et al. (1973) noted ultrastructural changes in BZK-resistant *Ps. aeruginosa* grown in the presence of BZK.

The results presented in this paper demonstrate that sub-inhibitory concentrations of CA and BZK can influence the cell surface of *Ps. aeruginosa* as well as the more sensitive *E. coli*. Hydrophobicity measurements will thus provide rapid information about these changes, and although they do not indicate how a drug acts, they can be invaluable when related to other observations.

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