Effect of permeabilizing agents on antibacterial activity against a simple *Pseudomonas aeruginosa* biofilm

H.M. Ayres, D.N. Payne[1], J.R. Furr and A.D. Russell

Welsh School of Pharmacy, University of Wales Cardiff, Cardiff, and [1]Reckitt & Colman Products Ltd, Hull UK

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H.M. AYRES, D.N. PAYNE, J.R. FURR AND A.D. RUSSELL. 1998. A simple *Pseudomonas aeruginosa* G48 biofilm on stainless steel discs provided a useful primary screen of the potentiating effects of various permeabilizing agents on antibacterial agents. Experiments with *Ps. aeruginosa* suspensions could not be used to predict the effects of biocides and permeabilizers on biofilms. Although antibacterial activity against biofilms was less than demonstrated in suspension tests, potentiation by some permeabilizers was still observed.

INTRODUCTION

It has been suggested by several authors that bacterial growth under laboratory conditions may not be an accurate reflection of natural bacterial ecosystems (Brown and Williams 1985; Costerton *et al*. 1987; Gilbert *et al*. 1993). Zobell (1943) described the ability of marine bacteria to attach themselves to a variety of inert surfaces. Bacteria can colonize many different types of surfaces to produce adherent biofilms. Such bacteria are enmeshed within a hydrated mucoexopolysaccharide glycocalyx (Brown and Gilbert 1993).

The glycocalyx is polyanionic in nature and might bind with cationic biocides, the actions of which are thereby quenched (Hoyle *et al*. 1990). Bacteria present as biofilms show greater resistance to antibacterial agents than do sessile bacteria produced under laboratory static growth systems (Nichols *et al*. 1989). It is therefore important to devise methods for overcoming this resistance and in this paper, the effects of permeabilizing agents (Vaara 1992; Ayres *et al*. 1993) in conjunction with antibacterial compounds on a simple biofilm of a strain of *Pseudomonas aeruginosa* are described. The Malthus AT system has been shown (Ayres *et al*. 1998) to provide a satisfactory system for examining the effects of permeabilizing agents on the activity of biocides; consequently, this procedure was also employed in this study.

MATERIALS AND METHODS

Test organism

Pseudomonas aeruginosa G48 was grown overnight in 100 ml of Nutrient broth (Oxoid) in a shaking water-bath at 37 °C. The cells were triple-washed and resuspended in sterile phosphate buffer (100 mmol l^{-1}, pH 7·0). This suspension was further diluted with butter to produce a suspension containing about 10^8 cfu ml^{-1}.

Correspondence to: Prof. A.D. Russell, Welsh School of Pharmacy, University of Wales Cardiff, Cardiff CF1 3XF, UK.

Chemicals and media

Chlorhexidine diacetate (CHA) was purchased from Zeneca Pharmaceuticals, triclosan from Ciba-Geigy, cetylpyridinium chloride (CPC), disodium edetate (EDTA) from BDH, the trisodium salt of nitrilotriacetic acid (NTA), the trisodium salt of citric acid dihydrate, disodium tartrate and benzalkonium chloride (BZK) from Sigma, and sodium polyphosphate (SPP) from Aldrich.

Chloroxylenol (*p*-chloro-*m*-xylenol, PCMX) was used as the formulation Dettol, containing 4·8% w/v PCMX and provided by Reckitt and Colman. Triclosan was dissolved in a few drops of 95% v/v ethanol and then made up to volume with distilled water. Preliminary experiments demonstrated that the small amount of ethanol had no effect on cell viability.

The culture medium used in the Malthus analyser was Special Peptone Yeast Extract (SPYE) which consisted of Special peptone (Oxoid) 30 g, yeast extract (Oxoid) 2·5 g, distilled water to 1 litre.

Neutralizing solution consisted of the European Suspension Test (EST) inactivation fluid of the following composition: phosphate buffer (50 mmol l^{-1}) pH 7·2 10 ml, L-histidine 1 g, sodium thiosulphate 5 g, lecithin 3 g, Tween-80 30 ml, distilled water 900 ml. This was sterilized by autoclaving.

Preparation of biofilms

Stainless steel discs were soaked for 60 min in 5% v/v Decon 90. They were washed thoroughly under running purified

water to remove all detergent. Discs were then covered in 95% v/v ethanol, left to soak overnight and dried by evaporation in a laminar flow unit. Discs so prepared were shown to be sterile; they were placed well spaced in Petri dishes, covered with the bacterial suspension and incubated for 60 min at 25 °C to allow adherence. The discs were then immersed in SPYE broth and incubated for a further 4 h at 25 °C to allow biofilm development. This method produced biofilms with about 10^7 cfu cm^{-2}.

Test procedure

At all times, the discs were handled with great care to avoid disruption of the biofilm. Biofilm discs were placed in sterile solutions in distilled water of the antibacterial agent and/or permeabilizers for 5 min at 20 °C. Each disc was transferred to EST neutralization fluid for 5 min and then into a 10 ml Malthus tube containing 2 ml SPYE broth. Control (untreated) biofilm discs were held for 5 min in sterile glass-distilled water and 5 min in EST neutralization fluid before transfer to a Malthus tube. Tubes were connected to the Malthus-AT apparatus and incubated at 37 °C for 40 h as described previously (Ayres *et al.* 1998).

Up to eight tests were carried out per system (Tables 1 and 2).

RESULTS

Antibacterial agent used alone

Sub-inhibitory concentrations of antibacterial agents, except triclosan, were employed against biofilms of *Ps. aeruginosa* G48. In view of solubility problems, triclosan was used at a non-inhibitory concentration. An antibacterial compound at concentrations used to inhibit biofilms caused no growth to be detected when used against the organism in suspension tests, except for triclosan where the detection time (DT) was delayed.

Permeabilizers used alone

Both EDTA and SPP used alone caused a slight delay of about 2 h in DT. This must be borne in mind in any instances where biocide potentiation is observed.

Chlorhexidine plus permeabilizer

EDTA enhanced the activity of CHA against biofilms (Table 1). A marginal increase in activity was also observed with NTA and citrate (both of which showed good potentiation against the organism in suspension tests; Ayres *et al.* 1998) and with tartrate. SPP antagonized the activity of CHA.

Benzalkonium chloride plus permeabilizer

EDTA, citrate and SPP potentiated the activity of BZK, NTA had a slight effect but tartrate had none.

Cetylpyridinium chloride plus permeabilizer

EDTA was the only permeabilizer to demonstrate any significant potentiating effect with CPC (Table 1).

Chloroxylenol plus permeabilizer

SPP had the greatest potentiating effect on PCMX activity (Table 2), with no growth being detected in four out of six tests. EDTA and tartrate produced similar delays in mean DTs to SPP but with no growth being detected in only one of six tests.

Triclosan plus permeabilizer

Good potentiation was observed with EDTA, citrate and SPP. The triclosan solubilizing system had no effect on biofilm growth.

DISCUSSION

The method used to produce biofilms was a rapid and simple one. Biofilms were produced in closed growth, static conditions on submerged surfaces and thus, no allowances can be made for the effects of growth rate. The technique does, however, serve as a useful primary screen of the effects of an antibacterial chemical in the presence and absence of a permeabilizing agent on a bacterial biofilm, although it would be interesting to study these combinations on more established biofilms.

It is of interest to note that higher concentrations of an antibacterial agent had to be used in the present study to prevent subsequent microbial growth than those in suspension tests with the Malthus (Ayres *et al.* 1998). Concentrations against biofilms of CHA and PCMX were doubled, BZK was used at five times its concentration and CPC at 10 times its concentration in suspension tests. Quaternary ammonium compounds and other antiseptics have been shown to be much less effective against surface-attached organisms than planktonic cells (Stickler *et al.* 1991; Dhaliwal *et al.* 1992). Problems occurred with triclosan because of its poor solubility.

Taken as a whole, the results provide examples of increased DTs when permeabilizers are used in conjunction with biocides. In several instances, however, improvements were marginal and occasionally antagonism was found, for example, and as expected, with CHA and SPP. The increased DTs, however, do not necessarily provide evidence of a lethal

Table 1 Effect of permeabilizing agents on the activity of cationic biocides against *Pseudomonas aeruginosa* G48 as a biofilm

Antibacterial agent*	Permeabilizer*†	No. of tests‡	Mean detection time (h)	Range of detection times (h)	No. of tests where no growth (NG) detected
CHA 180 μg ml^{-1}	None	8	16·8	14·3–19·6	0
(0·018% w/v)	EDTA, 10 mmol l^{-1}	6	22·4	18·0–NG	1
	NTA, 0·005	6	17·8	15·9–23·4	0
	Citrate, 0·5	6	18·1	13·5–21·7	0
	Tartrate, 0·25	6	17·8	14·3–20·5	0
	SPP, 0·25	6	11·6	9·4–13·2	0
BZK 50 μg ml^{-1}	None	8	16·9	13·0–23·2	0
(0·005% w/v)	EDTA, 10 mmol l^{-1}	6	24·8	17·8–NG	2
	NTA, 0·005	6	17·8	15·5–23·0	0
	Citrate, 0·5	6	22·0	13·7–36·6	0
	Tartrate, 0·5	6	16·0	9·2–23·0	0
	SPP, 0·25	6	24·5	18·2–NG	2
CPC 1 mg ml^{-1}	None	8	17·2	15·3–18·9	0
(0·1% w/v)	EDTA, 10 mmol l^{-1}	6	22·2	19·7–24·2	0
	NTA, 0·005	6	19·2	14·9–22·9	0
	Citrate, 0·5	6	17·4	15·4–18·8	0
	Tartrate, 0·5	5	16·6	14·8–19·6	0
	SPP, 0·25	6	18·6	14·3–19·5	0

* CHA, chlorhexidine diacetate ; BZK, benzalkonium chloride ; CPC cetylpyridinium chloride.
† As % w/v, except for EDTA. NTA, nitrilotriacetic acid ; SPP, sodium polyphosphate.
‡ Using Malthus AT system.
NG, no growth.

Table 2 Effect of permeabilizing agents on the activity of phenolic-type biocides against *Pseudomonas aeruginosa* G48 as a biofilm

Antibacterial agent*	Permeabilizer†	No. of tests‡	Mean detection time (h)	Range of detection times (h)	No. of tests where no growth (NG) detected
PCMX 192 μg ml^{-1}	None	8	18·3	15·8–NG	1
(0·0192% w/v)	EDTA, 10 mmol l^{-1}	6	22·9	20·0–NG	1
	NTA, 0·005	6	19·1	12·4–31·6	0
	Citrate, 0·5	6	20·6	16·6–NG	1
	Tartrate, 0·25	6	22·6	15·0–NG	1
	SPP, 0·25	6	22·9	21·6–NG	4
Triclosan	None	5	8·5	8·1–9·2	0
100 μg ml^{-1}	EDTA, 10 mmol l^{-1}	4	20·9	16·7–28·3	0
(0·01% w/v)	NTA, 0·005	4	8·2	7·1–8·9	0
	Citrate, 0·5	4	17·6	16·3–19·7	0
	Tartrate, 0·5	4	7·9	5·6–11·1	0
	SPP, 0·25	4	19·1	18·0–22·6	0

* PCMX, *p*-chloro-*m*-xylenol.
† As % w/v, except for EDTA. NTA, nitrilotriacetic acid ; SPP, sodium polyphosphate.
‡ Using Malthus AT system.
NG, no growth.

effect; they could merely reflect an extended lag phase of damaged or stressed organisms which tend to have longer DTs than equivalent numbers of untreated (undamaged) cells.

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