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# Development of bacterial resistance to several biocides and effects on antibiotic susceptibility

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**KEYWORDS**
Biocides; Antibiotic susceptibility; Thymol; Eugenol; Triclocarban; DDDMAC; ADMAO

**Summary** The aims of this study were to investigate the development of bacterial resistance to eugenol, thymol, trichlorocarbanalide (TCC), didecyldimethylammonium chloride (DDDMAC) and C10-16-alkyldimethyl, N-oxides (ADMAO) and subsequent effects on antibiotic susceptibility. An agar minimum inhibitory concentration (MIC) method was used to assess the activity of the biocides against standard bacterial strains and laboratory mutants. A range of techniques including disk diffusion and gradient plate experiments were used to attempt to develop bacterial 'resistance' or tolerance to the biocides. The mutants produced were examined for cross-resistance to the other biocides and to antibiotics via disk diffusion and gradient plate MIC methods. Outer membrane proteins of the mutants were extracted and examined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). *Escherichia coli* triclosan-resistant mutants were not cross-resistant to eugenol, thymol, TCC, DDDMAC and ADMAO. Mutants with elevated MICs to DDDMAC (*E. coli* and *Pseudomonas aeruginosa*), thymol (*E. coli*) and eugenol (*E. coli*) were isolated, but all remained sensitive to higher concentrations of the agents. Bacteria with elevated MICs to TCC and ADMAO were not obtained. Some low-level cross-resistance between DDDMAC, eugenol and thymol was observed with the *E. coli* gradient plate mutants, as well as reduced susceptibility to antibiotics, most notably chloramphenicol. The lack of cross-resistance of the triclosan mutants suggested that the mode of action of triclosan is not shared with the other biocides studied. SDS–PAGE results indicated that the DDDMAC *P. aeruginosa* mutant had a reduced amount (or absence) of one outer membrane protein in comparison with the standard strain. In conclusion, under laboratory conditions, bacterial exposure to thymol, eugenol and DDDMAC can lead to reduced susceptibility between selected biocidal agents and antibiotics, more specifically, chloramphenicol. However, further studies are required to determine if this is of clinical significance.


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## Introduction

The development of bacterial resistance to eugenol, thymol, triclocarban (TCC), didecyldimethylammonium chloride (DDDMAC) and C10-16-alkyldimethyl, N-oxides (ADMAO), and subsequent effects on antibiotic susceptibility were investigated. Eugenol, a phenolic essential oil found in cloves, has antimicrobial properties.[1,2] It is used in dentistry as a root canal sealer and has been shown to inhibit the growth of a range of organisms, including facultative anaerobes,[3–6] *Escherichia coli* 0157:H7,[1] *Penicillium citrinium*[2] and human herpes virus in vitro.[7] Thymol, an essential oil found in thyme, is used in mouthwashes.[8] Thymol has bacteristatic or bactericidal activity against a range of bacteria including *E. coli* and *Staphylococcus aureus*,[9] but not *Pseudomonas aeruginosa*.[10] Triclocarban (3,4,4′-trichlorocarbanilide; TCC) is a component of some antibacterial soaps.[11] TCC is a bacteristatic agent with activity against methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE),[12] but not Gram-negative organisms.[13] DDDMAC is a bactericidal quaternary ammonium compound (QAC), which has been used for surface disinfection of cattle-sheds, wood preservation,[14,15] and cellulosic string protection.[16] The surfactant ADMAO possesses significant antimicrobial activity and is active against *S. aureus* and *Saccharomyces cerevisiae*.[17,18] Activity increases with chain length up to 14[19] and is linked to interaction with the cell membrane.[17,20]

Triclosan (also known as Irgasan) is a broad-spectrum biocide used in a large number of healthcare and consumer products.[21,22] For many years, it was thought that its mode of action was via non-specific disruption of the bacterial cell,[22] but it has now been shown to function, in part, by disrupting the synthesis of lipids in *E. coli* via inhibition of FabI, an enoyl reductase.[23] This has increased worries of possible cross-resistance between biocides and antibiotics due to potential similarities in mechanism.[24,25] Indeed, multiple antibiotic resistant (MAR) mutants of *E. coli* have been produced by exposure to the disinfectant pine oil.[26]

The present study examined the potential for development of bacterial resistance to the antimicrobial agents described above using a range of mutant production techniques. Possible reductions in susceptibility to antibiotics in the mutants were investigated using antibiotic disk diffusion tests and antibiotic gradient plates. The possibility of cross-resistance to other biocides was also explored.

## Materials and methods

### Biocides

Eugenol, thymol and TCC were obtained from Sigma, Poole, UK. ADMAO and DDDMAC from Procter and Gamble, Cincinnati, Ohio, USA. Due to the restricted solubility of eugenol, thymol and TCC in water, stock solutions were prepared with absolute ethanol (Fisher, Loughborough, UK). Final concentrations of ethanol in the presence of bacteria did not exceed 2.5% (v/v). ADMAO and DDDMAC stock solutions were prepared with sterile, deionized water.

### Micro-organisms

*E. coli* ATCC 10536, *S. aureus* ATCC 9518 and *P. aeruginosa* ATCC 15442 (European Standard BSEN 1276: 1997, suspension test strains), plus four triclosan-resistant *E. coli* and their parent strain *E. coli* ATCC 8739 were used in this study. The minimum inhibitory concentrations (MICs) of all of the triclosan mutants is above 250 μg/mL compared with 0.1 μg/mL for the parent strain, and is stable after 15 subcultures. Bacteria were inoculated into 10 mL nutrient broth (Oxoid, London, UK) and grown for 18 h at 37°C in a shaking water-bath operating at 75 rev/min, to give approx. $1 \times 10^9$ cfu/mL.

### MICs

The activity of the biocides against the triclosan-resistant mutants was examined using agar MICs. Petri dishes (Sterilin triple vented; Fisher) were prepared using double-strength nutrient agar (Oxoid), biocide and sterile, deionized water to give a specific final concentration of biocide in single strength agar. The maximum concentrations of biocide used were as follows: 1060 μg/mL eugenol, 1000 μg/mL thymol, 5 μg/mL TCC, 20 000 μg/mL DDDMAC and 8% (v/v) ADMAO. Final concentrations of ethanol in the Petri dishes did not exceed 2.5% (v/v), and control agar containing 2.5% ethanol was tested for bacteriostatic activity during each experiment. Control plates of single-strength nutrient agar with no biocide were also prepared. The Petri dishes were over-dried and a Denley multipoint inoculator (Thermo Life Sciences, Basingstoke, UK) was used to place 1 μL spots (containing approx. $1 \times 10^5$ cfu) of each culture on to the agar surface. All plates were incubated overnight at 37°C. Any growth was

recorded and compared with the growth of the controls.

## Development of bacterial resistance

Methods used for resistant mutant production were as follows:

1. Paper disk diffusion: paper discs soaked in biocide (1060 μg/mL eugenol, 1000 μg/mL thymol, 5 μg/mL TCC, 20 000 μg/mL DDDMAC and 8% v/v ADMAO) were placed on the surface of nutrient agar swabbed with a bacterial suspension. Colonies growing in a second inner zone after incubation were collected and tested for increased resistance using the agar MIC method.
2. Cup plate diffusion: the biocide (1060 μg/mL eugenol, 1000 μg/mL thymol, 5 μg/mL TCC, 20 000 μg/mL DDDMAC) was placed in a well in the bacteria-swabbed agar and colonies growing within the main inhibition zone after incubation were tested for increased resistance.
3. Stepwise training in broth: bacteria were subcultured in increasing concentrations of biocide in nutrient broth and tested for stable increases in MIC.
4. Repeated exposure to sub-lethal biocide concentrations in nutrient broth, followed by MIC determination.
5. Growth in basal medium plus biocide. The basal medium consisted of (g/L in water): 0.0653 g/L $K_2HPO_43H_2O$, 0.0260 g/L $KH_2PO_4$, 0.0531 g/L $Na_2HPO_4$, 0.0051 g/L $NH_4Cl$, 0.0329 g/L $MgSO_47$-$H_2O$, 0.0825 g/L $CaCl_2$ 0.00045 g/L $FeCl_3$. This was followed by agar MICs to determine whether the susceptibility had been altered.
6. Biocide gradient agar plates: Petri dishes were prepared to give specific final concentrations of biocide in 20 mL of single-strength nutrient agar set on an angle. Once set, 20 mL of single-strength nutrient agar was pipetted on top of the first agar layer. After biocide diffusion, 18 h cultures of the organisms were swabbed along the gradient and incubated for 24 h at 37°C. The distance of growth of each streak across the Petri dish was measured and the gradient plate MIC calculated from distance of growth of streak × concentration of biocide in the first 20 mL agar/width of the Petri dish at this point. The bacteria with the highest MIC in each experiment were used to inoculate the next set of gradient plates. Gradient subculturing was stopped after six subcultures because increases in MIC had reached a maximum (Table II).

## Mutant MIC stability

Mutant MIC stability was confirmed by subculturing the isolates 15 times in 10 mL nutrient broth in the absence of biocide. Gradient plates were used to compare the MICs of mutant and parent strains after one, five, 10 and 15 subcultures. Petri dishes containing a gradient of biocide were prepared and inoculated, and MICs calculated as described above. Agar MICs were used to test the stability of the disk diffusion isolates, as described above.

## Antibiotic and biocide susceptibility

The mutant and standard strains were tested for antibiotic susceptibility using antibiotic disk diffusion tests based on the BSAC standardized disk susceptibility testing method.[27] Diagnostic sensitivity test (DST; Oxoid) agar plates were dried and inoculated from stock cultures containing approx. $1 \times 10^7$ cfu/mL with sterilized cotton-wool swabs (Fisher). Disks were applied to the agar surface within 15 min of inoculation and incubated (within 15 min) at 37°C for 24 h. Inhibition zone sizes of the standard and mutant strains were recorded and compared.

The antibiotics tested and their concentrations in the disks were as follows (μg, except where stated), penicillin G 10 units, vancomycin 30, tetracycline 30, cefuroxime 30, gentamicin 10, erythromycin 15, polymyxin B 300 units, ciprofloxacin 5, chloramphenicol 30 (Difco), carbenicillin 100, tobramycin 10, amikacin 30, neomycin 30, kanamycin 30, ceftazidime 30, piperacillin 100, ampicillin 10 (Becton-Dickinson). A one-tailed, two-sample *t*-test assuming unequal variances (99% confidence interval) was used to assess possible differences in zone size and where possible, results were compared with antibiotic zone break points.[27]

The gradient plate method was adapted to obtain MICs of chloramphenicol (Sigma) for the *E. coli* mutants. A one-tailed, two-sample *t*-test assuming unequal variances (99% confidence interval) was used to test the significance of differences in MIC. The possibility of the mutants having cross-resistance to other biocides was examined using the gradient plate MIC method.

## Outer membrane proteins

The outer membrane proteins (Omp) of the six mutants and their three parent strains were examined using SDS-PAGE. Omp were prepared using the sarkosyl method.[28,29] Overnight nutrient broth cultures (200 mL) at 37°C were harvested,

**Table I** MICs of the triclosan-resistant mutant

| Biocide | Average (mode) MIC and range of MIC values | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *E. coli* ATCC 8739 | | Tm1 | | Tm2 | | Tm3 | | Tm4 | |
| | Mode | Range | Mode | Range | Mode | Range | Mode | Range | Mode | Range |
| Eugenol (μg/mL) | 530 | - | 1060 | 530-1060 | 1060 | 530-1060 | 1060 | 530-1060 | 1060 | 530-1060 |
| Thymol (μg/mL) | 500 | - | 500 | - | 500 | - | 500 | - | 500 | - |
| Triclocarban (μg/mL) | >5 | - | >5 | - | >5 | - | >5 | - | >5 | - |
| DDDMAC (μg/mL) | 50 | 10-50 | 10 | - | 50 | 10-50 | 10 | - | 50 | 10-50 |
| ADMAO% (v/v) | 4 | - | 8 | - | 8 | - | 8 | - | 8 | 4-8 |

Three to four repeat experiments were performed. Tm, triclosan mutant; -, No variation occurred.

washed once and resuspended in 10 mL of ultra pure water. Cells were sonicated while on ice (10 × 30 s, with a 60 s cooling period between each burst) using a MSE ultrasonic sonicator (Sanyo-Gallenkamp, Loughborough, UK). The sonicate was centrifuged at 3500 ***g*** for 20 min at 4°C to remove debris and unbroken cells. The supernatant was then added to 1 mL of 20% sodium *N*-lauroyl sarcosinate (Sarkosyl; Sigma). The mixture was incubated at room temperature and shaken intermittently for 30 min to solubilize the cytoplasmic membrane. The suspension was centrifuged three times at 58 500 ***g*** for 60 min at 4 °C in a Sorvall ultracentrifuge (Dupont, Stevenage, UK). Between centrifugations, cell pellets were washed with 2 mL ultra-pure water, with final resuspension in 0.05 mL ultra-pure water containing 1 mm phenylmethylsulphonyl fluoride (PMSF; Sigma). The Omps were stored at −80°C until use.

## Protein determination

Protein concentration was determined using the Lowry assay. The following reagents were prepared: (1) reagent A: 2% (w/v) $Na_2CO_3$ (BDH, Poole, UK) in 0.1 N NaOH (Sigma); (2) reagent B: 0.5% (w/v) $CuSO_45H_2O$ (BDH) in 1% (w/v) sodium tartrate (Fisher); (3) reagent C: 50 mL reagent A and 1 mL reagent B; (4) reagent E: 50% (v/v) folin and Ciocalteu's phenol reagent (Sigma).

One millilitre of reagent C was added to 0.2 mL Omp or bovine serum albumin (Sigma) standard, mixed well and allowed to stand for at least 10 min. Reagent E (0.1 mL) was added and mixed within 1-2 s. After 20-30 min the absorbance of the solution was read at 750 nm. A standard curve was prepared for 0-200 μg/mL bovine serum albumin and used to calculate the protein content of the samples.

## SDS-PAGE

Samples were diluted in ultra-pure water to give a protein concentration of 10 μg per 4 μL. Ten microlitres of the diluted sample was added to 10 μL cracking buffer [50 mM tris-HCl, pH 6.8 (BDH); 5% 2-mercaptoethanol (Sigma); 2% lauryl sulphate (SDS; Sigma); 0.1% bromphenol blue (Sigma); 10% glycerol (BDH)] and heated to 100°C for 3 min.

Four microlitres samples (containing 5 μg protein) were loaded into the PhastSystem (Amersham Pharmacia Biotech, Little Chalfont, UK) and electrophoresed with PhastGel 12.5 and 20% homologous gels (Amersham Pharmacia Biotech). A broad-range molecular weight standard (BioRad) was diluted 1:50 (3 μL plus 147 μL ultra-pure water) and processed and electrophoresed with the samples. Gels were stained with the PhastSystem protein silver-staining kit (Amersham Pharmacia Biotech) and scanned as jpeg files.

# Results

## MICs

The MICs of the compounds used in this study for the triclosan mutants were similar to that for the parent strain (*E. coli* ATCC 8739; Table I). Thus cross-resistance to eugenol, thymol, TCC, DDDMAC or the ADMAO was not demonstrated with respect to triclosan.

## Development of bacterial resistance

Resistant mutants were difficult to produce, with only two of the six methods producing bacteria with stable increases in MICs (Table II). One stable mutant was produced using the paper disk diffusion method (DDDMAC-*E. coli*; from ATCC 10536).

**Table II** Minimum and maximum increases in MIC observed during the development of bacterial resistance stability studies

| Biocide tested (units) | Parent strain | Mean MIC of parent strain (range) | Isolated strain (method used) | Mean MIC of isolated strain (range) | Mean (fold) increase in MIC (fold range of MIC increases) |
|---|---|---|---|---|---|
| DDDMAC (μg/mL) | *E. coli* ATCC 10536 | 5.00[a] | DDDMAC *E. coli* 1 (dd) | 20.0 (10.0–50.0)[b] | 4 (2–10) |
| DDDMAC (μg/mL) | *E. coli* ATCC 10536 | 11.6 (5.2–18.0) | DDDMAC *E. coli* 2 (gp) | 108 (38.0–190)[c] | 9 (2–37) |
| DDDMAC (μg/mL) | *E. coli* ATCC 8739 | 76.4 (65.2–87.6) | DDDMAC *E. coli* 0 (gp) | 292 (223–368)[c] | 4 (3–6) |
| Eugenol (μg/mL) | *E. coli* ATCC 8739 | 657 (583–721) | Eugenol *E. coli* 0 (gp) | 996 (890–1060)[c] | <2 (<2–2) |
| Thymol (μg/mL) | *E. coli* ATCC 8739 | 151 (74.0–215) | Thymol *E. coli* 0 (gp) | 710 (543–1000)[c] | 5 (3–14) |
| DDDMAC (μg/mL) | *P. aeruginosa* ATCC 15442 | 1530 (950–2100) | DDDMAC *P. aeruginosa* (gp) | 3280 (2410–4200) | 2 (<2–4) |

dd, Disk diffusion; gp, gradient plate.
[a] MIC was always 5.00 μg/mL.
[b] *t*-Test not appropriate as agar MIC method was used here (provides discrete rather than continuous data).
[c] Significantly different from the parent strain [one-tailed, two-sample *t*-test assuming unequal variances (95% confidence interval)].

However, it remained sensitive to higher DDDMAC concentrations. The biocide gradient plate method produced five stable mutants with increased resistance to biocides, DDDMAC-*E. coli* 2 and 0 (from ATCC 10536 and 8739 respectively), DDDMAC-*P. aeruginosa*, eugenol-*E. coli* 0 (from ATCC 8739) and thymol-*E. coli* 0 (from ATCC 8739). Where appropriate, *t*-tests were carried out on the mutant stability test data (Table II). All of the *E. coli* assessed via the gradient plate MIC method had significantly higher MICs than their parent strain.

## Antibiotic susceptibility

Penicillin G, vancomycin and erythromycin did not produce zones of inhibition with the Gram-negative bacteria. Results for the other antibiotics tested are summarized in Table III. All five *E. coli* mutants were significantly (99% confidence interval) less susceptible to chloramphenicol (decreases were much larger with the gradient plate mutants than the disk diffusion mutant). The results for the other antibiotics were less marked, but some interesting trends were seen. All *E. coli* ATCC 8739 mutants (labelled 0; Table II) had inhibition zones significantly larger than those of the parent strain (ATCC 8739) when the aminoglycosides (gentamicin, tobramycin, amikacin, neomycin and kanamycin, except for DDDMAC *E. coli* 0 (gradient plate) and tobramycin), the quinolone ciprofloxacin, the third-generation cephalosporin, ceftazidime and polymyxin B were tested (Table III). The effect was greatest with ciprofloxacin, with mean zone sizes 6–7 mm larger than those of the standard strain. In contrast, DDDMAC *E. coli* 2 (gradient plate) had a significantly smaller mean zone size with ciprofloxacin than its parent strain. Tetracycline and the third-generation cephalosporin, cefuroxime, produced significantly smaller inhibition zones with the *E. coli* 0 mutants compared with the standard strain (ATCC 8739). Thymol *E. coli* 0 (gradient plate) also had a significantly reduced zone with carbenicillin. The DDDMAC *E. coli* 2 (gradient plate) mutant showed significantly smaller mean zone sizes than its parent with tetracycline, carbenicillin and piperacillin. Reductions in zone size were also recorded with DDDMAC *E. coli* ATCC 10536 (labelled 2; Table II), eugenol *E. coli* 0 and thymol *E. coli* 0 in the presence of ampicillin (a broad-spectrum penicillin, although without activity against *P. aeruginosa*).

## Gradient plate chloramphenicol MICs

The gradient plate MIC results (Figure 1) corresponded closely to those from the antibiotic disk

**Table III** Antibiotic susceptibility inhibition zones for parent and biocide tolerant strains

| Strain | Inhibition zone size (mm) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | G | NN | AN | N | K | CIP | CAZ | TE | CXM | AM | CAR | PB | PIP |
| *E. coli* ATCC 10536 | 28 | 23 | 22 | 22 | 22 | 21 | 34 | 31 | 22 | 24 | 22 | 29 | 18 | 31 |
| DDDMAC *E. coli* 1 (dd) | 24[a] | 22 | 22 | 23 | 22 | 22 | 33 | 32 | 22 | 26 | 22 | 29 | 17 | 31 |
| DDDMAC *E. coli* 2 (gp) | 12[a] | 22 | 22 | 23 | 23 | 22 | 31[a] | 31 | 20[a] | 20 | 17 | 25[a] | 18 | 28[a] |
| *E. coli* ATCC 8739 | 23 | 22 | 21 | 22 | 21 | 21 | 33 | 31 | 22 | 25 | 19 | 26 | 17 | 30 |
| Eugenol *E. coli* 0 (gp) | 12[a] | 27[b] | 26[b] | 28[b] | 26[b] | 26[b] | 40[b] | 35[b] | 17[a] | 20[a] | 15 | 26 | 21[b] | 32 |
| Thymol *E. coli* 0 (gp) | 12[a] | 28[b] | 26[b] | 26[b] | 26[b] | 24[b] | 39[b] | 35[b] | 16[a] | 16[a] | 12 | 24[a] | 23[b] | 31 |
| DDDMAC *E. coli* 0 (gp) | 7[a] | 27[b] | 23 | 26[b] | 25[b] | 24[b] | 40[b] | 38[b] | 16[a] | 17[a] | 18 | 28 | 22[b] | 34 |
| *P. aeruginosa* ATCC 15442 | 7 | 20 | 22 | 23 | 18 | 11 | 29 | 25 | 7 | 7 | 7 | 14 | 18 | 27 |
| DDDMAC *P. aeruginosa* (gp) | 9 | 19 | 23 | 22 | 18 | 10 | 31 | 30 | 9 | 7 | 7 | 20 | 19 | 29 |

Five repeat experiments performed except for DDDMAC *P. aeruginosa* (four repeats) and all ampicillin tests (two repeats). dd, Disk diffusion; gp, gradient plate; C, chloramphenicol; G, gentamicin; NN, tobramycin; AN, amikacin; N, neomycin; K, kanamycin; CIP, ciprofloxacin; CAZ, ceftazidime; TE, tetracycline; CXM, cefuroxime, AM, ampicillin, CAR, carbenicillin; PB, polymyxin B; PIP, piperacillin.

[a] Significantly smaller zone than the parent strain [one-tailed, two-sample *t*-test assuming unequal variances (99% confidence interval)].

[b] Significantly larger zone than the parent strain [one-tailed, two-sample *t*-test assuming unequal variances (99% confidence interval)].

diffusion tests and indicated the presence of significantly (99% confidence interval) elevated MICs to chloramphenicol in all of the *E. coli* mutants except DDDMAC *E. coli* from ATCC 10536 (labelled 1, disk diffusion; Table II).

## Cross-resistance to other biocides

An examination of the possible cross-resistance of the *E. coli* mutants to other biocides revealed that all three agents had significantly (one-tailed, *t*-test assuming unequal variance, 99% confidence interval) elevated MICs versus the DDDMAC, eugenol and thymol ATCC 8739 mutants. The ATCC 10536 DDDMAC gradient plate mutant had a significantly elevated MIC to thymol, but not to eugenol and the lack of cross-resistance with the disk diffusion isolate was confirmed (see Table IV).

## OMPs

Sample gels presented in Figure 2 were representative

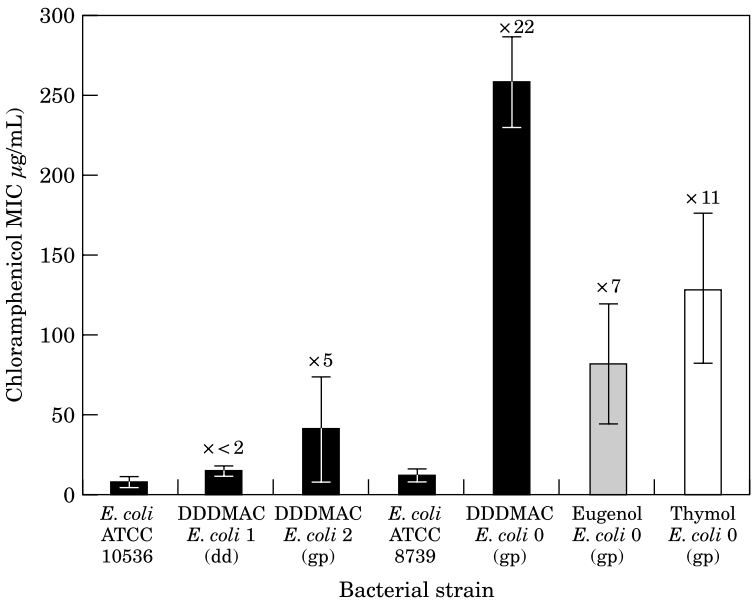


**Figure 1** Increases ( × fold) in chloramphenicol MIC of *E. coli* mutants produced in this study. All of the isolated *E. coli* strains had chloramphenicol MICs significantly higher than their parent strain [one tailed, two-sample *t*-test assuming unequal variances (99% confidence interval)]. dd, disk diffusion; gp, gradient plate.

**Table IV** Tolerance and cross-tolerance to DDDMAC, eugenol and thymol

| Organism | Mean MIC (standard deviation) and increase ( × fold) in MIC | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | DDDMAC (μg/mL) | | | Eugenol (μg/mL) | | | Thymol (μg/mL) | | |
| *E. coli* ATCC 10536 | 12.4 | (6.5) | - | 510 | (41) | - | 217 | (46) | - |
| DDDMAC *E. coli* 1 (dd) | 50.2[a] | (44) | × 4.0 | 405 | (226) | × 0.8 | 200 | (61) | × 0.9 |
| DDDMAC *E. coli* 2 (gp) | 114[a] | (71) | × 9.2 | 359 | (319) | × 0.7 | 351[a] | (59) | × 1.6 |
| *E. coli* ATCC 8739 | 122 | (51) | - | 613 | (26) | - | 266 | (50) | - |
| Eugenol *E. coli* 0 (gp) | 221[a] | (39) | × 2.2 | 960[a] | (124) | × 1.6 | 520[a] | (111) | × 2.0 |
| Thymol *E. coli* 0 (gp) | 251[a] | (34) | × 2.3 | 906[a] | (90) | × 1.5 | 546[a] | (115) | × 2.1 |
| DDDMAC *E. coli* 0 (gp) | 342[a] | (31) | × 0.9 | 769[a] | (89) | × 1.3 | 363[a] | (46) | × 1.4 |

[a] Significantly higher MIC than the parent strain [one-tailed, two-sample *t*-test assuming unequal variances (99% confidence interval)].

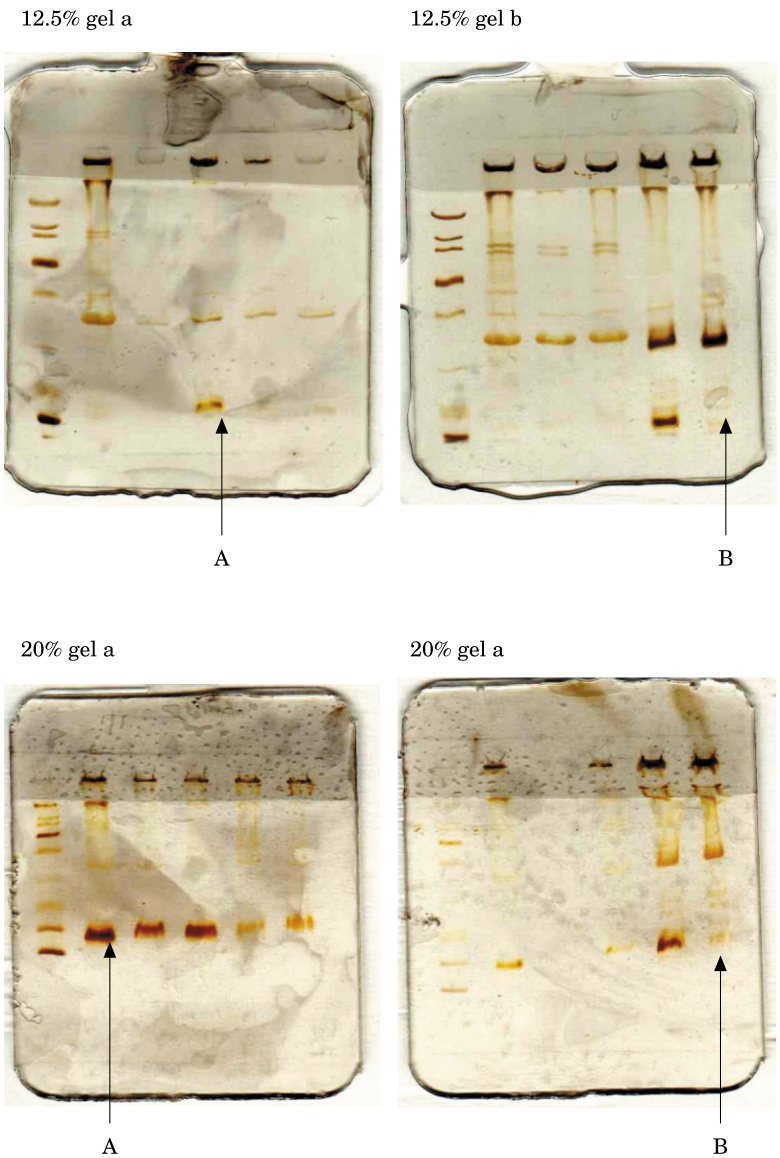


**Figure 2** SDS–PAGE gels showing Omps of standard strains and mutants. The samples loaded in the lanes of the 12.5 and 20% homogeneous gels were as follows (from left to right): Gel a: broad range standard, *E. coli* (ATCC 10536), *E. coli* 0 (ATCC 8739), eugenol *E. coli* 0, thymol *E. coli* 0, DDDMAC *E. coli* 0. Gel b: broad range standard, *E. coli* (ATCC 10536), DDDMAC *E. coli* 2, DDDMAC *E. coli* 1 (FD), *P. aeruginosa* (ATCC 15442), DDDMAC *P. aeruginosa*.

of the two extremes of results observed (i.e. presence and absence of bands).

### *E. coli*

Differences were seen between the samples and between the gels. The band indicated by arrow A (Figure 2) was present in some cases, but absent in others. This effect, although not consistent, did suggest that there may be some differences between the Omps of the standard strain and the mutant *E. coli* ATCC 8739 (*E. coli* 0), as the presence of this band was more common in the mutant strains than in the parent strain. It was usually absent (only visible on one in five gels) from the *E. coli* ATCC 10536 parent and mutant strains (Table V).

### *P. aeruginosa*

The results for *P. aeruginosa* were more pronounced (Table V). The band indicated by arrow B (Figure 2) was either absent or much fainter in all cases for the mutant compared with the parent strain. This suggests that the mutant strain has a decreased amount of this protein and that this may be contributing to its elevated MIC against DDDMAC.

## Discussion

This study demonstrated that it is possible to increase bacterial resistance to thymol, eugenol and DDDMAC. However, because of the limited extent to which the MICs were increased in the mutants, 'tolerance' to antimicrobials might be a more appropriate term in this case. Bacteria with a stable resistance were difficult to isolate and all mutants remained sensitive to higher concentrations of the agents. Bacteria with elevated MICs to TCC and ADMAO were not obtained. The *E. coli* mutants isolated via the gradient plate method showed low-level cross-resistance to DDDMAC, thymol and eugenol, [with the exception of DDDMAC *E. coli* 2 (gradient plate) and eugenol]. No cross-resistance was observed with the disk diffusion isolate. All *E. coli* mutants showed significantly (99% confidence interval) reduced susceptibility to at least one of the antibiotics tested, illustrating that the biocides might have the potential to select for antibiotic-resistant bacteria. However, three of the gradient plate mutants also demonstrated significantly greater susceptibility to some of the antibiotics. The comparison of inhibition zone size results shown in Table III with the values for MIC and zone breakpoints provided by Andrews et al.,[27] suggested that exposure to the biocides had, in some cases, changed their categorization from susceptible (implies that infection due to bacteria will probably respond to that antibiotic) to resistant (implies that infection due to bacteria tested will probably not respond to that antibiotic).[30] However, comparisons were only made for chloramphenicol, gentamicin, tobramycin, ciprofloxacin, tetracycline, ampicillin and piperacillin as Andrews et al.[27] do not provide breakpoints for Enterobacteriaceae and the other antibiotics used in this study. All of the gradient plate *E. coli* mutants could be classified as resistant to chloramphenicol, as they had a zone size of $\leq$20 mm indicating an MIC above the breakpoint of $\geq$16 mg/L. The *E. coli* gradient plate mutants had chloramphenicol MICs five to 22 times greater than those of the parent strains, with DDDMAC *E. coli* 0 (gradient plate) having the largest increase. Although the DDDMAC disk diffusion mutant did have a significantly smaller zone size to this antibiotic, this did not correspond to a change from sensitive to resistant. When the mutants showing increases in zone size were compared with antibiotic breakpoints (data were only available for gentamicin, tobramycin and ciprofloxacin), they represented an increase in the existing susceptibility rather than a change from resistant to sensitive. For example, all strains were sensitive to ciprofloxacin (zone sizes $\geq$18 mm), but the

**Table V** Molecular weight of bands A and B and their frequency of occurrence

| Strain | Band | Range of mol. wt (KDa) | Frequency (per five experiments) |
|---|---|---|---|
| *E. coli* (ATCC 10536) | A | 9.5 | 1 |
| *E. coli* 0 (ATCC 8739) | A | 11.4–13.0 | 2 |
| Eugenol *E. coli* 0 | A | 11.4–14.6 | 4 |
| Thymol *E. coli* 0 | A | 12.5–13.8 | 3 (one very faint) |
| DDDMAC *E. coli* 0 | A | 13.7–16.5 | 4 |
| DDDMAC *E. coli* 2 | A | Never present | 0 |
| DDDMAC *E. coli* 1 (FD) | A | 13.7 | 1 (one very faint) |
| *P. aeruginosa* (ATCC 15442) | B | 10.3–13.8 | 5 |
| DDDMAC *P. aeruginosa* | B | 11.1–14.7 | 2 (both less pronounced) |

*E. coli* 0 mutants had mean zone sizes 6–7 mm larger than those of the standard strain. The changes in inhibition zones for tetracycline indicated a slight decrease in susceptibility, as all strains including the parent strain were already resistant to this antibiotic. Thymol and DDDMAC *E. coli* 0 (gradient plate) were both resistant (zone $\leq$ 19 mm) to cefuroxime (parenteral) while other strains were sensitive (zone $\geq$ 20 mm). Eugenol and thymol *E. coli* 0 (gradient plate) and DDDMAC *E. coli* 2 (gradient plate) had inhibition zones of $\leq$17 mm indicating resistance to ampicillin not observed in the parent strains. However, due to the low number of repeat experiments for this antibiotic, statistical analysis was not carried out. DDDMAC *E. coli* 2 (gradient plate) was still sensitive to ciprofloxacin and piperacillin according to the criteria of Andrew et al.,[27] despite having significantly smaller zone sizes. Further studies are needed to examine whether these finding are of clinical relevance.

The lack of cross-resistance of the triclosan-resistant mutants to the biocides tested in this study, suggested that these biocides do not share their resistance mechanism(s) with the bisphenol. Preliminary indications have suggested that the resistance of the triclosan mutants is at least partly due to a combination of impermeability and efflux.[31] The resistance could be due to over-expression of *marA*, *soxS* or *acrAB*, which have been associated with triclosan resistance in *E. coli*. However, such over-expression has not previously been associated with such large increases in MIC,[23] and the efflux components of *E. coli* are known to use a large range of antibiotics and biocides as substrates.[32–34] This suggests that another mechanism is making these mutants more resistant to triclosan, whilst still permitting the biocides investigated to access their target site(s). This may involve the impermeability mentioned above,[31] perhaps coupled with mutations in the enoyl reductase gene (*fabI*) which have been shown to give rise to resistance to triclosan.[22] This lack of cross-resistance could be important in instances where an alternative biocide is needed to inactivate such triclosan-resistant strains.

SDS–PAGE of the Omps of Gram-negative bacteria reveals only a limited number of bands.[35] The mechanism of resistance for the *P. aeruginosa* and possibly the *E. coli* ATCC 8739 mutants may involve changes in some Omps, as differences were observed in the SDS–PAGE Omp extraction experiments. One of the important roles of the bacterial membrane transport systems is the provision of resistance to toxic compounds.[36] Efflux-mediated resistance has been shown to include a variety of structurally unrelated antimicrobial agents including antibiotics and biocides.[33,37] In *E. coli* Mar mutants, loss of the outer membrane porin OmpF results in low-level resistance to tetracycline, chloramphenicol and norfloxacin.[31] Mutations at the *mar* locus can also confer resistance via efflux mechanisms.[32] The reduced susceptibility of some of the mutants produced in this study to chloramphenicol and to some extent tetracycline, cefuroxime and ampicillin coupled with the increased Omp band frequency seen with some of the *E. coli* mutants suggested that these mechanisms might be involved. Efflux systems in *P. aeruginosa* regulated by *mexR* have been shown to use disinfectants as substrates[34] and are one possible cause of the elevated MICs reported here. However, the loss or fading of a band in the *P. aeruginosa* SDS–PAGE results could be accounted for by the loss or reduction in the amount of an Omp, such as a porin, which might be present in much lower amounts or absent in the mutant strain. Additional studies would be needed to substantiate these findings.

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## References

1. Blaszyk M, Holley RA. Interaction of monolaurin, eugenol and sodium citrate on growth of common meat spoilage and pathogenic organisms. *Int J Food Microbiol* 1998;**39**:175—183.
2. Vázquez BI, Fente C, Franco CM, Vázquez MJ, Cepeda A. Inhibitory effect of eugenol and thymol on *Penicillium citrinum* strains in culture media and cheese. *Int J Food Microbiol* 2001;**67**:157—163.
3. Chong BS, Owadally ID, Pitt Ford TR, Wilson RF. Antibacterial activity of potential retrograde root filling material. *Endod Dent Traumatol* 1994;**10**:66—70.
4. Torabinejad M, Hong CU, Pitt Ford TR, Kettering JD. Antibacterial effects of some root end filling materials. *J Endod* 1995;**21**:403—406.
5. Siqueira JF, Gonçalves RB. Antibacterial activities of root canal sealers against selected anaerobic bacteria. *Am Assoc Endod* 1996;**22**:79—80.
6. Kaplan AE, Gonzalez MI, Macchi RL, Molgatini SL. Antimicrobial effect of six endodontic sealers: an *in vitro* evaluation. *Endod Dent Traumatol* 1999;**15**:42—45.
7. Benecia F, Courrèges MC. *In vitro* and *in vivo* activity of eugenol on human herpesvirus. *Phytother Res* 2000;**14**:495—500.
8. Scheie AAA. Modes of action of currently known chemical

anti-plaque agents other than chlorhexidine. *J Dent Res* 1989;**68**:1609—1616.
9. Cosentino S, Tuberoso CIG, Pisano B, et al. *In-vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. *Lett Appl Microbiol* 1999;**29**: 130—135.
10. Sivropoulou A, Papanikolaou E, Nikolaou C, Kokkini S, Lanaras T, Arsenakis M. Antimicrobial and cytotoxic activities of *Origanum* essential oils. *J Agric Food Chem* 1996;**44**: 1202—1205.
11. Breneman DL, Hanifin JM, Berge CA, Keswick BH, Neumann PB. The effect of antibacterial soap with 1.5% triclocarban on *Staphylococcus aureus* in patients with atopic dermatitis. *Cutis* 2000;**66**:296—300.
12. Suller MTE, Russell AD. Antibiotic and biocide resistance in methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococcus. *J Hosp Infect* 1999;**43**:281—291.
13. Heinze JE, Yackovich F. Washing with contaminated bar soap is unlikely to transfer bacteria. *Epidemiol Infect* 1988;**101**: 135—142.
14. Nishihara T, Okamoto T, Nishiyama N. Biodegradation of dedecyldimethylammonium chloride by *Pseudomonas fluorescens* TN4 isolated from activated sludge. *J Appl Microbiol* 2000;**88**:641—647.
15. Farrell AP, Kennedy CJ, Wood A, Johnston BD, Bennett WR. Acute toxicity of a didecyldimethylammonium chloride-based wood preservative, Bardac 2280, to aquatic species. *Environ Toxicol Chem* 1998;**17**:1552—1557.
16. Morrel JJ, Smith SM. Chemically protecting cellulosic string from microbial attack. *For Prod J* 1995;**45**:84—89.
17. Šubík J, Takácsová G, PŠenák M, Devínsky F. Antimicrobial activity of amine oxides: mode of action and structure-activity correlation. *Antimicrob Agents Chemother* 1997;**12**: 139—164.
18. Glover RE, Smith RR, Jones MV, Jackson SK, Rowlands CC. An EPR investigation of surfactant action on bacterial membranes. *FEMS Microbiol Lett* 1999;**177**:57—62.
19. Birnie CR, Malamud D, Schnaare RL. Antimicrobial evaluation of *N*-alkyl betaines and *N*-alkyl-*N*, *N*-dimethylADMAOs with variations in chain length. *Antimicrob Agents Chemother* 2000;**44**:2514—2517.
20. Kopecká-Leitmanova A, Devinsky F, Mlynarcik D, Lacko I. Interaction of ADMAOs and quaternary ammonium salts with membrane and membrane-associated processes in *E. coli* cells: mode of action. *Drug Metabol Drug Interact* 1989;**7**: 29—51.
21. Schweizer HP. Triclosan: a widely used biocide and its link to antibiotics. *FEMS Microbiol Lett* 2001;**202**:1—7.
22. Slater-Radosti C, Aller GV, Greenwood R, et al. Biochemical and genetic characterization of the action of triclosan on *Staphylococcus aureus*. *J Antimicrob Chemother* 2001;**48**: 1—6.
23. McMurry LM, Oethinger M, Levy SB. Overexpression of *marA*, *SoxS*, or *acrAB* produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. *FEMS Microbiol Lett* 1998;**166**:305—309.
24. Russell AD, Suller MTE, Maillard J-Y. Do antiseptics and disinfectants select for antibiotic resistance? *J Med Microbiol* 1999;**48**:613—615.
25. McBain AJ, Gilbert P. Biocide tolerance and the harbingers of doom. *Int Biodeterior Biodegradation* 2001;**47**:55—61.
26. Moken MC, McMurry LM, Levy SB. Selection of multiple-antibiotic-resistant (mar) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the mar and *acrAB* loci. *Antimicrob Agents Chemother* 1997;**41**:2770—2772.
27. Andrews JM, The BSAC Working Party on Susceptibility Testing. BSAC standardized disc susceptibility testing method. *J Antimicrob Chemother* 2001;**48**:43—57.
28. Filip C, Fletcher J, Wulff L, Earhart GF. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. *J Bacteriol* 1973;**173**: 717—722.
29. Williams P, Gledhill P. Fractionation of bacterial cells and isolation of membranes and macromolecules. In: Denyer SP, Hugo WB, editors. *Mechanisms of Action of Chemical Biocides: Their Exploitation and Uses*. Oxford: Blackwell Science; 1991. p. 87—107.
30. MacGowan AP, Wise R. Establishing MIC breakpoints and the interpretation of in vitro susceptibility tests. *J Antimicrob Chemother* 2001;**48**(Suppl. 1):17—28.
31. Gomez-Escalada M, Maillard J-Y, Russell AD. Effects of triclosan-sensitive and resistant strains of Gram-negative bacteria. *Society for Applied Microbiology* 2001;30. 9—12 July, Swansea.
32. Cohen SP, McMurry LM, Hooper DC, Wolfson JS, Levy SB. Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in additions to OmpF reduction. *Antimicrob Agents Chemother* 1989;**33**:1318—1325.
33. Gambino L, Gracheck SJ, Miller PF. Overexpression of the MarA positive regulator is sufficient to confer multiple antibiotic resistance in *Escherichia coli*. *J Bacteriol* 1993; **175**:2888—2894.
34. Poole K. Efflux-mediated resistance to fluoroquinalones in Gram-negative bacteria. *Antimicrob Agents Chemother* 2000;**44**:2233—2241.
35. Benz R. Structure and function of porins from Gram-negative bacteria. *Annu Rev Microbiol* 1988;**42**:359—393.
36. Paulsen IT, Brown MH, Skurray RA. Proton-dependent multidrug efflux systems. *Microbiol Rev* 1996;**60**:575—608.
37. Poole K. Multidrug resistance in Gram-negative bacteria. *Curr Opin Microbiol* 2001;**4**:500—508.