

## Effect of triclosan or a phenolic farm disinfectant on the selection of antibiotic-resistant *Salmonella enterica*

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**Objective:** To determine the effect of growth of five strains of *Salmonella enterica* and their isogenic multiply antibiotic-resistant (MAR) derivatives with a phenolic farm disinfectant or triclosan (biocides) upon the frequency of mutation to resistance to antibiotics or cyclohexane.

**Methods:** Strains were grown in broth with or without the biocides and then spread on to agar containing ampicillin, ciprofloxacin or tetracycline each at 4× MIC or agar overlaid with cyclohexane. Incubation was for 24 and 48 h and the frequency of mutation to resistance was calculated for strains with and without prior growth with the biocides. MICs were determined and the presence of mutations in the *acrR* and *marR* regions was determined by sequencing and the presence of mutations in *gyrA* by light-cycler analysis, for a selection of the mutants that arose.

**Results:** The mean frequency of mutation to antibiotic or cyclohexane resistance was increased ~10- to 100-fold by prior growth with the phenolic disinfectant or triclosan. The increases were statistically significant for all antibiotics and cyclohexane following exposure to the phenolic disinfectant ( $P \leq 0.013$ ), and for ampicillin and cyclohexane following exposure to triclosan ( $P \leq 0.009$ ). Mutants inhibited by >1 mg/L ciprofloxacin arose only from strains that were MAR. Reduced susceptibility to ciprofloxacin (at 4× MIC for parent strains) alone was associated with mutations in *gyrA*. MAR mutants did not contain mutations in the *acrR* or *marR* region.

**Conclusions:** These data renew fears that the use of biocides may lead to an increased selective pressure towards antibiotic resistance.

Keywords: ciprofloxacin, efflux, cyclohexane, MAR, *gyrA*

### Introduction

Antiseptics and disinfectants (biocides) are freely available without prescription, unlike antibiotics, and as such are used on a daily basis in homes, schools, hospitals, restaurants, farms, abattoirs, other work places and in health care products.<sup>1–4</sup> Reduced susceptibility of bacteria to certain antimicrobial agents and some biocides due to efflux has been demonstrated.<sup>5</sup> Therefore, use of some biocides may select reduced susceptibility not only to the biocides used but possibly to antibiotics also.<sup>4,6</sup>

Antiseptics and disinfectants usually have multiple, non-specific modes of action to kill bacteria and, therefore, any resistance is unlikely to arise by a single mutational step as typically occurs with antibiotic resistance.<sup>2,7</sup> One exception to this generalization is mutation in *Escherichia coli fabI*, a gene that encodes

enoyl-ACP reductase or FabI, a highly conserved enzyme involved in fatty acid biosynthesis.<sup>8–10</sup> Resistance to triclosan in *E. coli* can be acquired through a missense mutation in *fabI*.<sup>9</sup> With the exceptions of resistance to some metals and organomercurials, plasmids are not normally associated with elevated levels of antiseptic or disinfectant resistance in Gram-negative bacteria.<sup>1</sup> However, reduced susceptibility in bacteria to biocides first described in the 1950s and 1960s is apparently increasing.<sup>3</sup> In particular, cationic agents (quaternary ammonium compounds, chlorhexidine, diamidines and acridines) and triclosan have been implicated as a possible cause for the selection and persistence of bacterial strains with reduced susceptibility (typically MIC values 4- to 8-fold higher than for wild-type strains) to a range of agents, including antibiotics.<sup>3</sup> Additionally, chemicals containing phenolic rings, which have been used as disinfectants in various

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forms from as far back as 1867,<sup>3,7,11</sup> have been shown to depress the multiple antibiotic resistance locus *marRAB* causing increased efflux,<sup>12</sup> which is increasingly recognized as a common resistance mechanism to both biocides and antibiotics.<sup>5</sup> In *E. coli* and *Salmonella enterica*, reduced susceptibility to biocides and antibiotics giving rise to multiple antibiotic resistance (MAR) is generally attributed to up-regulation of the AcrAB–TolC efflux system.<sup>13–15</sup> In *E. coli* and *S. enterica* up-regulation of both the *marRAB* and *soxRS* loci can cause up-regulation of *acrAB* giving rise to MAR.<sup>12,16–18</sup> In *E. coli* and *S. enterica*, overexpression of *acrAB*, *marRAB* and *soxRS* genes can all lead to low level resistance to antibiotics such as  $\beta$ -lactams, chloramphenicol, fluoroquinolones and tetracyclines,<sup>12,14,16–19</sup> to increased organic solvent tolerance<sup>18,20</sup> and to decreased susceptibility to disinfectants such as pine oil<sup>21</sup> and triclosan.<sup>2</sup> Whilst efflux can be involved in fluoroquinolone resistance,<sup>13,14</sup> clinical resistance specifically to quinolone antibiotics is generally attributed to alterations in the target enzymes, DNA gyrase and topoisomerase IV, which are essential for DNA replication.<sup>22</sup> DNA gyrase is composed of two GyrA and two GyrB subunits, encoded by the *gyrA* and *gyrB* genes, respectively.<sup>22</sup> Topoisomerase IV is composed of two subunits: ParC is homologous to GyrA and is encoded by *parC* whilst ParE is homologous to GyrB and is encoded by *parE*.<sup>22</sup> Mutations in the *gyrA* gene are reported as the most common cause of fluoroquinolone resistance in Gram-negative bacteria.<sup>22</sup>

Chromosomal mutation leading to antibiotic resistance has been recognized for decades but few studies have been carried out to determine whether mutation confers resistance to antiseptics and disinfectants.<sup>1</sup> It is known that hospital isolates of Gram-negative bacteria invariably show reduced susceptibility to biocides compared with laboratory strains and this suggests that mutation and selection may play a role in the reduced susceptibility of these isolates.<sup>1</sup> Triclosan, a synthetic bisphenol compound, was first used in the early 1970s<sup>3,23</sup> and was apparently used for 30 years before reduced susceptibility was reported.<sup>24</sup> Triclosan is now used widely in hand soaps, surgical soaps, shower gels, deodorant soaps, hand lotions and creams, toothpaste, mouthwashes and deodorants.<sup>7</sup>

The hypothesis explored in this present study was that growth in the presence of triclosan and/or phenolic farm disinfectant enriches for *S. enterica* with reduced susceptibility/resistance to antibiotics and this can be detected when strains are subsequently exposed to antibiotics or cyclohexane. To explore this hypothesis, the development of antibiotic, cyclohexane and biocide resistance in *S. enterica* (Dublin, Enteritidis and Typhimurium) wild-type and isogenic MAR strains was investigated. Representative mutants were analysed for mutations in the *gyrA*, *acrR* and *marRO* regions.

## Materials and methods

### Bacterial strains

Ten strains were investigated comprising five wild-type strains and a single isogenic MAR derivative of each. The wild-type strains were derived from the collection at the Veterinary Laboratories Agency, Weybridge, UK, and were *S. enterica* Dublin (993/96), *S. enterica* Enteritidis (LA5 and 2363/97) and *S. enterica* Typhimurium (3530/96 and 3992/96), which were previously determined to be cyclohexane susceptible<sup>20</sup> and are hereafter referred to as

non-MAR parent strains. The MAR derivatives were derived by serial passage on agar plates supplemented with tetracycline or chloramphenicol according to the method of George & Levy<sup>25</sup> with the exception that strains were grown on Luria–Bertani (LB) agar and the incubation temperature was 30°C. These MAR derivatives were all cyclohexane resistant<sup>20</sup> and are hereafter referred to as MAR parent strains.

### Growth media and antimicrobials

Antibiotics and chemicals were obtained from Sigma–Aldrich (Poole, Dorset, UK) except ciprofloxacin, which was kindly donated by Bayer (Newbury, Berkshire, UK) and triclosan (Irgasan DP300), which was kindly donated by Ciba Consumer care (Macclesfield, Cheshire, UK). The phenolic farm disinfectant (PFD) was a blend of high boiling point tar acids and organic acids. For all procedures, strains were grown overnight in LB broth or agar except for agar dilution MICs, for which Iso-Sensitest agar was used. All incubation temperatures were 37°C except for incubation of plates overlaid with cyclohexane, which were incubated at 30°C.<sup>26</sup> Antibiotics and biocides were dissolved on the day of use and diluted in sterile distilled water before adding to agar or broth. The PFD was diluted to give a percentage value rather than mg/L for MIC determination.

### MICs and cyclohexane resistance

Agar doubling dilution MICs of the PFD, triclosan and the antibiotics ampicillin, chloramphenicol, ciprofloxacin and tetracycline, and cyclohexane resistance were determined as described previously.<sup>26</sup> Broth MICs of the two biocides were determined by serially diluting biocides in LB broth inoculated with 10<sup>5</sup> cfu/mL of an overnight broth culture with incubation overnight at 37°C. The MIC was recorded as the lowest concentration of biocide that inhibited growth.

### Determination of the frequency of mutation to resistance

The frequency of mutation to antibiotic, biocide and cyclohexane resistance was determined for strains grown with and without the biocides. To do this, strains were grown overnight at 37°C in one of the three media: LB broth alone, LB broth with triclosan (1/4 to 1/2 broth MIC) or LB broth with PFD (1/4 to 1/2 broth MIC). Viable counts were determined<sup>27</sup> and strains were sub-cultured (100 mL per plate, ~10<sup>9</sup> cfu/mL) from each medium on to either LB agar containing 4× MIC of either ampicillin, ciprofloxacin or tetracycline, or agar overlaid with cyclohexane (for the five cyclohexane-susceptible non-MAR parent strains only). Strains grown in LB broth alone were sub-cultured on LB agar supplemented with triclosan or PFD at 4× MIC for each strain. Plates were incubated at 37°C for 48 h except for the determination of cyclohexane resistance, for which plates were incubated at 30°C for 24 h. The number of colonies that grew were recorded at 24 and 48 h as appropriate.

The frequency of mutation to resistance was calculated as the number of colonies growing in the presence of antibiotic or biocide per mL of inoculum divided by the viable count (cfu/mL) of the inoculum. Where mutants occurred, up to five colonies were picked at random from each plate per condition and stored on Dorset egg slopes for subsequent analyses.

### Light-cycler *gyrA* mutation assay (GAMA)

The 10 test strains and up to five mutants from these selected from each test condition (i.e. selected from ampicillin, ciprofloxacin, tetracycline or cyclohexane plates after growth with and without

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biocides) were examined for mutations in the quinolone resistance determining region (QRDR) of *gyrA* as described previously.<sup>28</sup>

### Mutations in *acrR* and *marRO*

To attempt to elucidate whether there were specific mutations in *acrR* and *marR* and their relevant promoter regions which may be responsible for the MAR phenotype, the *acrR* gene and its promoter region (*S. Typhimurium* EMBL accession number AE008717, nucleotide numbers 19332–20313) and the *marR* gene and its promoter region (*S. Typhimurium* EMBL accession number U54468, nucleotide numbers 450–1645) of all parents strains (non-MAR and MAR) and representative mutants were determined and compared with the published *S. Typhimurium* sequence. To do this, the regions were amplified by PCR, with the primers shown in Table 1, using the Expand High Fidelity PCR System (Roche) according to the manufacturer's instructions. Sequencing reactions were determined using a BigDye DNA sequencing kit (Applied Biosystems) according to the manufacturer's instructions using the sequencing primers listed in Table 1 with the PCR amplicon DNA as template. Sequencing reactions were run on an ABI PRISM 3770 automated DNA sequencer.

### Statistical analysis

The mutation frequencies were transformed to their logarithm to base 10 for the analysis because of the large ranges, and the results are presented as geometric means calculated by back transformation. The data were analysed by three-way analysis of variance (ANOVA)<sup>29</sup> to determine whether there was a significant effect of the biocide treatment on the numbers of mutants occurring when strains were subsequently exposed to antibiotics or cyclohexane. A separate analysis was carried out for each antibiotic. All the statistical models included factors for strain and biocide (control, triclosan, PFD). The strain factor was subdivided to enable testing of the non-MAR parent/MAR parent difference, except in the analysis of the cyclohexane results where only non-MAR strains were used. When the three-way ANOVA showed that growth with either triclosan or PFD had a significant effect at  $P < 0.05$  on the isolation of resistant mutants when

strains were subsequently exposed to antibiotics or cyclohexane, Student's *t*-tests based on the residual mean squares from the analyses of variance were used to compare individual means.

## Results

### Frequency of mutation to resistance

None of the 10 parent strains (non-MAR and MAR) tested grew on  $4 \times$  MIC of PFD. The mean frequency of mutation to resistance when plated on to ampicillin, ciprofloxacin, tetracycline or cyclohexane, without prior growth with the biocides, was between  $<5 \times 10^{-9}$  and  $1 \times 10^{-8}$  (Table 2), but following growth for 24 h with either of the biocides at 1/4 to 1/2 their broth MIC in broth culture at 37°C, the mean frequency of mutation to resistance when plated on to ampicillin, ciprofloxacin, tetracycline or cyclohexane was  $\geq 10$ -fold greater (Table 2). Specifically, growth with PFD led to statistically significant increases in the mean frequency of mutation to resistance to ampicillin, ciprofloxacin, tetracycline and cyclohexane (Table 3). Growth with triclosan led to increases in the mean frequency of mutation to resistance to ampicillin, ciprofloxacin, tetracycline and cyclohexane of  $\geq 10$ -fold, but only the increased frequency of mutation to resistance to ampicillin and cyclohexane resistance was statistically significant (Table 3).

The mean frequency of mutation to resistance was  $\sim 10$ -fold higher for MAR parent strains compared with non-MAR parent strains for strains grown in antibiotic-free broth and plated on to ciprofloxacin or tetracycline and for strains grown with triclosan and plated on to ciprofloxacin (Table 2). Overall, the mean frequency of mutation to resistance was statistically significantly higher for MAR parent strains compared with non-MAR parent strains when grown with PFD and when plated on to ciprofloxacin (Table 3).

However, not all strains gave rise to mutants (Table 2). The incidence of strains giving rise to mutants was higher after plating on to ciprofloxacin than after plating on to either

**Table 1.** PCR and sequencing primers

PCR and sequencing primers <sup>a</sup>	F or R <sup>b</sup>	5' to 3' DNA sequence of primers	EMBL accession no., nucleotide position
<i>acrR</i> -P	F	TGTGACAACCCCAACTTCTGG	AE008717, 19332–19352
<i>acrR</i> -P	R	TGTAACAAACAGAATAGCGACAC	AE008717, 20291–20313
<i>acrR</i> -S	F1	GCATCAGAACGACCGCCA	AE008717, 19418–19435
<i>acrR</i> -S	F2	GCGTTTGTCTCGCAGCA	AE008717, 19667–19684
<i>acrR</i> -S	F3	CGTAGCCTTTGTCTGGAAA	AE008717, 19995–20013
<i>acrR</i> -S	R1	CGTAGGCGCGAGCTTCTTTTT	AE008717, 20170–20187
<i>acrR</i> -S	R2	CTCAATCTCAAGCTCACCAAT	AE008717, 19812–19832
<i>acrR</i> -S	R3	ACGCAGCAATGGGTTTA	AE008717, 19514–19530
<i>marRO</i> -P	F	AAAGCATTCTGAAGCCGATAA	U54468, 450–470
<i>marRO</i> -P	R	TTTTTGCGGATTTCCGTCATTTT	U54468, 1622–1645
<i>marRO</i> -S	F1	GCCACCATCATAATAGCGAAGAC	U54468, 554–576
<i>marRO</i> -S	F2	TAGTTACAAGTTATCACAGCACAA	U54468, 850–873
<i>marRO</i> -S	F3	TCGATCTCGGCGCATTGACG	U54468, 1181–1200
<i>marRO</i> -S	R1	CGATCCAGTCCAAAATGCTATGAA	U54468, 1470–1493
<i>marRO</i> -S	R2	CGCAGCGTATCGAGCAAAGCACT	U54468, 1114–1138
<i>marRO</i> -S	R3	CAGGTTATAGTTGTAGAGG	U54468, 819–838

<sup>a</sup>P, PCR primer; S, sequencing primer.

<sup>b</sup>F, forward; R, reverse.

**Table 2.** Frequency of mutation to reduced susceptibility to antibiotics<sup>a</sup> and cyclohexane resistance for *S. enterica* strains grown in media with and without biocides

Treatment; frequency or incidence of reduced susceptibility <sup>a</sup>	Frequency of mutants (geometric mean) and incidence (i.e. number of strains and repeats/total tested) at which mutants arose for non-MAR and MAR parent strains									
	ampicillin			ciprofloxacin			tetracycline			cyclohexane non-MAR
	non-MAR	MAR	all	non-MAR	MAR	all	non-MAR	MAR	all	
Control frequency	$<5 \times 10^{-9}$	$<7 \times 10^{-9}$	$<6 \times 10^{-9}$	$4 \times 10^{-9}$	$1 \times 10^{-8}$	$7 \times 10^{-9}$	$2 \times 10^{-9}$	$1 \times 10^{-8}$	$8 \times 10^{-9}$	$1 \times 10^{-9}$
Control incidence	0/10	0/10	0/20	3/10	4/10	7/20	0/10	2/10	2/20	1/5
Triclosan frequency	$4 \times 10^{-8}$	$1 \times 10^{-8}$	$2 \times 10^{-8}$	$8 \times 10^{-9}$	$2 \times 10^{-8}$	$1 \times 10^{-8}$	$2 \times 10^{-8}$	$1 \times 10^{-8}$	$2 \times 10^{-8}$	$6 \times 10^{-6}$
Triclosan incidence	4/10	1/10	5/20	6/10	5/10	11/20	2/10	1/10	3/20	5/5
PFD frequency	$3 \times 10^{-8}$	$4 \times 10^{-8}$	$3 \times 10^{-8}$	$1 \times 10^{-8}$	$5 \times 10^{-8}$	$3 \times 10^{-8}$	$2 \times 10^{-8}$	$6 \times 10^{-8}$	$3 \times 10^{-8}$	$5 \times 10^{-8}$
PFD incidence	1/10	1/10	2/20	2/10	5/10	7/20	0/10	2/10	2/20	4/5

PFD, phenolic farm disinfectant.

<sup>a</sup>Reduced susceptibility to antibiotics, i.e. growth at 4 × MIC for the parent strain.

**Table 3.** Statistical comparisons for mean frequency to reduced susceptibility under different conditions and for non-MAR versus MAR parent strains

Comparison	Conditions under which mutants were selected and associated <i>P</i> values						
	control	triclosan	PFD	AMP	CIP	TET	Cyc
Non-MAR versus MAR	0.067	0.224	<b>0.048</b> (MAR)	0.667	<b>&lt;0.001</b> (MAR)	0.126	–
Control versus triclosan	–	–	–	<b>0.009</b> (Tric)	0.089	0.091	<b>&lt;0.001</b> (Tric)
Control versus PFD	–	–	–	<b>&lt;0.001</b> (PFD)	<b>&lt;0.001</b> (PFD)	<b>&lt;0.001</b> (PFD)	<b>0.013</b> (PFD)

PFD, phenolic farm disinfectant; Cyc, cyclohexane; Tric, triclosan.

Bold type indicates where a significantly higher ( $P < 0.05$ ) mean frequency of mutation occurred for a given strain type (non-MAR or MAR) or growth condition (control, PFD, Tric) as shown in brackets.

ampicillin or tetracycline, irrespective of whether strains were grown with or without the two biocides before being plated on to media with antibiotics.

#### Resistance phenotypes of mutants

Mutants were obtained from *S. Dublin* 993/96 non-MAR parent after plating on to ciprofloxacin with and without prior growth with PFD, but not after plating on to ampicillin or tetracycline alone (Table 4). The mutants following plating on to ciprofloxacin were 4-fold less susceptible to this agent (Table 4). Growth of *S. Dublin* 993/96 non-MAR parent with triclosan, irrespective of the antibiotic on to which it was plated, gave rise to mutants that had decreased susceptibility to ampicillin, chloramphenicol, tetracycline (at least 4-fold) and triclosan (at least 2-fold) and were resistant to cyclohexane (results not shown), i.e. were MAR, although only a 2-fold decrease in susceptibility to ciprofloxacin was observed for these mutants (Table 4).

No mutants were selected from the *S. Enteritidis* LA-5 non-MAR parent when plated on to antibiotic-containing agar. However, following growth with triclosan, plating on to ampicillin and ciprofloxacin gave rise to mutants and following

growth with PFD, plating on to ampicillin alone gave rise to mutants. All of these mutants tested showed the typical MAR phenotype (Table 4). *S. Enteritidis* LA-5 MAR parent (i.e. MAR prior to experimentation), gave rise to mutants with reduced susceptibility to ciprofloxacin whether plated on to ciprofloxacin alone or after prior growth with PFD or triclosan (Table 4). When *S. Enteritidis* 2363/97 non-MAR parent was pre-grown with triclosan and then plated on to ampicillin, the mutants were all MAR (Table 4).

Mutants derived from *S. Typhimurium* 3530/96 non-MAR parent after plating on to ciprofloxacin alone were 8-fold less susceptible to this agent (Table 4). Growth of *S. Typhimurium* 3530/96 non-MAR parent with triclosan gave rise to mutants when plated on to ampicillin, ciprofloxacin or tetracycline, or agar overlaid with cyclohexane and these were all MAR (Table 4). Growth of *S. Typhimurium* 3530/96 non-MAR parent with PFD gave rise to mutants when plated on to cyclohexane only; these mutants were all MAR (Table 4). *S. Typhimurium* 3992/96 MAR parent (i.e. MAR prior to experimentation) gave rise to mutants with reduced susceptibility to ciprofloxacin when plated on to ciprofloxacin with or without prior growth with PFD or triclosan (Table 4).

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**Table 4.** MICs for *S. enterica* Dublin, Enteritidis and Typhimurium strains following growth with or without biocide and subsequent plating on medium with antibiotics

Serotype, ref. no., phenotype for parent strains	Biocide passage <sup>a</sup>	Plated on to <sup>b</sup>	MIC (mg/L or % v/v for PFD) of					
			AMP	CHL	CIP	TET	PFD	Tric
Dublin 993/96 non-MAR	–	–	1	4	0.03	1	0.06	0.25
	–	CIP (0.06)	2 <sup>c</sup>	4 <sup>c</sup>	<b>0.13<sup>c</sup></b>	1 <sup>c</sup>	0.06 <sup>c</sup>	0.25 <sup>c</sup>
	Tric (0.06)	AMP (4)	<b>16<sup>d</sup></b>	<b>32<sup>d</sup></b>	0.06 <sup>d</sup>	<b>8<sup>d</sup></b>	0.13 <sup>d</sup>	<b>1<sup>d</sup></b>
	Tric (0.06)	CIP (0.06)	<b>8<sup>d</sup></b>	<b>32<sup>d</sup></b>	0.06 <sup>d</sup>	<b>8<sup>d</sup></b>	0.13 <sup>d</sup>	<b>2<sup>d</sup></b>
	Tric (0.06)	TET (4)	<b>8<sup>d</sup></b>	<b>32<sup>d</sup></b>	0.06 <sup>d</sup>	<b>8<sup>d</sup></b>	0.06 <sup>d</sup>	0.5 <sup>d</sup>
	PFD (0.06)	CIP (0.06)	1 <sup>c</sup>	4 <sup>c</sup>	<b>0.13<sup>c</sup></b>	1 <sup>c</sup>	0.06 <sup>c</sup>	0.25 <sup>c</sup>
Enteritidis LA5 non-MAR	–	–	1	4	0.03	1	0.03	0.5
	Tric (0.06)	AMP (4)	<b>16<sup>d</sup></b>	<b>16<sup>d</sup></b>	<b>0.13<sup>d</sup></b>	<b>4<sup>d</sup></b>	0.06 <sup>d</sup>	1 <sup>d</sup>
	Tric (0.06)	CIP (0.13)	<b>8<sup>d</sup></b>	<b>16<sup>d</sup></b>	<b>0.25<sup>d</sup></b>	<b>4<sup>d</sup></b>	0.06 <sup>d</sup>	0.05 <sup>d</sup>
	PFD (0.06)	AMP (4)	<b>8<sup>d</sup></b>	<b>32<sup>d</sup></b>	<b>0.13<sup>d</sup></b>	<b>8<sup>d</sup></b>	0.06 <sup>d</sup>	1 <sup>d</sup>
Enteritidis LA5 MAR	–	–	8	32	0.06	8	0.06	1
	–	CIP (0.25)	8 <sup>c</sup>	16 <sup>c</sup>	<b>2<sup>c</sup></b>	8 <sup>c</sup>	0.06 <sup>c</sup>	1 <sup>c</sup>
	Tric (0.13)	CIP (0.25)	4 <sup>c</sup>	16 <sup>c</sup>	<b>2<sup>c</sup></b>	8 <sup>c</sup>	0.06 <sup>c</sup>	1 <sup>c</sup>
	PFD (0.06)	CIP (0.25)	8 <sup>c</sup>	32 <sup>c</sup>	<b>4<sup>c</sup></b>	8 <sup>c</sup>	0.06 <sup>c</sup>	1 <sup>c</sup>
Enteritidis 2363/97 non-MAR	–	–	1	4	0.03	1	0.06	0.25
	Tric (0.06)	AMP (4)	<b>8</b>	<b>32</b>	0.06	<b>4</b>	0.06	<b>0.5</b>
Typhimurium 3530/96 non-MAR	–	–	1	4	0.015	1	0.06	0.25
	–	CIP (0.06)	1 <sup>c</sup>	4 <sup>c</sup>	<b>0.13<sup>c</sup></b>	1 <sup>c</sup>	0.06 <sup>c</sup>	0.25 <sup>c</sup>
	Tric (0.06)	AMP (4)	<b>16<sup>d</sup></b>	<b>32<sup>d</sup></b>	<b>0.06<sup>d</sup></b>	<b>4<sup>d</sup></b>	0.13 <sup>d</sup>	<b>1<sup>d</sup></b>
	Tric (0.06)	CIP (0.06)	<b>4<sup>d</sup></b>	<b>32<sup>d</sup></b>	<b>0.13<sup>d</sup></b>	<b>4<sup>d</sup></b>	0.13 <sup>d</sup>	<b>1<sup>d</sup></b>
	Tric (0.06)	TET (4)	<b>8<sup>d</sup></b>	<b>32<sup>d</sup></b>	<b>0.13<sup>d</sup></b>	<b>4<sup>d</sup></b>	0.13 <sup>d</sup>	0.5 <sup>d</sup>
	Tric (0.06)	Cyc	2 <sup>d</sup>	<b>32<sup>d</sup></b>	<b>0.06<sup>d</sup></b>	<b>4<sup>d</sup></b>	0.06 <sup>d</sup>	0.5 <sup>d</sup>
	PFD (0.06)	Cyc	2 <sup>d</sup>	<b>16<sup>d</sup></b>	<b>0.06<sup>d</sup></b>	<b>4<sup>d</sup></b>	0.06 <sup>d</sup>	0.5 <sup>d</sup>
Typhimurium 3992/96 MAR	–	–	8	16	0.03	4	0.06	1
	–	CIP (0.5)	8 <sup>c</sup>	16 <sup>c</sup>	<b>2<sup>c</sup></b>	4 <sup>c</sup>	0.06 <sup>c</sup>	1 <sup>c</sup>
	Tric (0.5)	CIP (0.5)	8 <sup>c</sup>	16 <sup>c</sup>	<b>2<sup>c</sup></b>	4 <sup>c</sup>	0.13 <sup>c</sup>	<b>8<sup>c</sup></b>
	PFD (0.03)	CIP (0.5)	8 <sup>c</sup>	16 <sup>c</sup>	<b>2<sup>c</sup></b>	4 <sup>c</sup>	0.13 <sup>c</sup>	1 <sup>c</sup>

Tric, triclosan; PFD, phenolic farm disinfectant; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; Cyc, cyclohexane; TET, tetracycline.

Bold type indicates mutants that showed a decrease in susceptibility of  $\geq 4$ -fold compared with the parent strain.

<sup>a</sup>mg/L triclosan or % v/v PFD that strains are grown in broth with prior to antibiotic exposure.

<sup>b</sup>mg/L antibiotic in plates.

<sup>c</sup>Strains that acquired a mutation in *gyrA*.

<sup>d</sup>Strains that became cyclohexane resistant.

### Triclosan resistance

Mutants of 3530/96 MAR parent (i.e. MAR prior to experimentation) and 2363/97 non-MAR parent were obtained after plating on to agar containing triclosan alone (data not shown). The MICs of triclosan were increased by  $\leq 32$ -fold but were unaltered for ampicillin, ciprofloxacin, chloramphenicol or tetracycline (data not shown). Strain 3992/96 MAR parent (i.e. MAR prior to experimentation) pre-grown with triclosan and plated on to ciprofloxacin-containing agar gave rise to a mutant that was less susceptible to ciprofloxacin (and had a mutation in *gyrA*). This mutant showed reduced susceptibility to triclosan also (MIC increased 8-fold), but the MICs of ampicillin, chloramphenicol and tetracycline were unchanged (Table 4).

### Ciprofloxacin resistance was not always associated with *gyrA* mutation or cyclohexane resistance

All 10 parent test strains (non-MAR and MAR) had wild-type *gyrA* prior to experimentation. Only when plated on to ciproflox-

acin (with or without prior growth with PFD or triclosan) did the MAR parent strains become additionally resistant to ciprofloxacin (Table 4). The MICs of ciprofloxacin for these strains increased up to 64-fold (ciprofloxacin MICs 1–4 mg/L). A total of 54 mutants from plating on to ciprofloxacin were analysed for mutations in *gyrA*. Twenty of these 54 mutants (19/20 from non-MAR parents) showed reduced susceptibility to ciprofloxacin but without mutations in the *gyrA* QRDR and 11/19 of these were cyclohexane resistant and MAR. The remaining 34/54 mutants had mutations in the *gyrA* QRDR; 26/34 of these mutants arose from MAR parent strains. The most common substitution was Ser-83 to Phe. Higher MICs of ciprofloxacin ( $>0.5$  mg/L) were observed for those mutants that had mutations in *gyrA* and were cyclohexane resistant.

### Mutations in *acrR* and *marR*

The *acrR*, *marR* and upstream promoter sequences of *S. Typhimurium* 3992/96 non-MAR and MAR parent strains were

identical to the published sequences. *S. Typhimurium* 3530/96 non-MAR and MAR parent strains had a single base pair substitution in the *marR* coding sequence, which was silent. *S. Dublin* 993/96 and *S. Enteritidis* LA5 and 2363/97 parent strains (both non-MAR and MAR) showed serotype-associated differences from the *S. Typhimurium* sequence in *marR* and its promoter region (accession nos. AJ011765 and AJ011766, respectively) and in *acrR* and its promoter region (accession nos. AY340595 and AY340596, respectively). Additionally, *S. Dublin* 993/96 MAR parent harboured a single base pair substitution in the *acrR* region compared with *S. Dublin* 993/96 non-MAR parent but this mutation was silent.

None of the four mutants from passage of *S. Typhimurium* 3530/96 non-MAR parent with triclosan and subsequent plating on to ampicillin, ciprofloxacin, tetracycline or cyclohexane had mutations in *acrR*, *marR* or the relevant promoter regions compared with the sequence of the parent strain.

## Discussion

In the present studies, the effect of growth with PFD or triclosan upon the selection of antibiotic resistance (at 4× MIC for the parent strain) in three serovars of *S. enterica* was investigated. Plating on to agar with triclosan, but not with PFD, gave rise to mutants with reduced susceptibility to triclosan. Growth with sub-inhibitory concentrations of PFD or triclosan followed by plating on to agar with antibiotics or cyclohexane led to significantly higher numbers of mutants with reduced susceptibility to antibiotics (4-fold increase in MIC) or which were cyclohexane tolerant. The mutants from plating on to antibiotics fell into two main phenotypes, those that were MAR and showed cross-resistance to cyclohexane, and those plated on to ciprofloxacin and showed reduced susceptibility to this agent only. The mutants plated on to ampicillin, tetracycline or cyclohexane were all MAR although a few mutants showed reduced susceptibility to antibiotics in the absence of cyclohexane resistance. The MAR mutants generally showed a 4- to 8-fold decrease in susceptibility to ampicillin, chloramphenicol, ciprofloxacin and tetracycline although there were instances where only a 2-fold decrease in susceptibility to ciprofloxacin was seen. This possibly reflects experimental variation or reduced efflux for ciprofloxacin compared with the other antibiotics, although this was not investigated. Eight of the mutants from plating on to ciprofloxacin showed reduced susceptibility to ciprofloxacin in the absence of mutations in *gyrA* or an obvious MAR phenotype; it is possible that such strains may have had mutations in target genes other than *gyrA*, such as *gyrB*.<sup>22</sup> There were also some mutants which only showed an increase in resistance to triclosan (i.e. were not MAR) and this may possibly be attributed to a specific mutation such as a mutation in *fabI*,<sup>8–10</sup> but this was not determined.

It has been argued that laboratory selection of resistant mutants by low concentrations of triclosan is irrelevant to current usage of this agent, since triclosan concentrations in commercial preparations range from 600 to 20 000 mg/L,<sup>7</sup> which far exceed the MIC values of triclosan even for resistant strains of *E. coli* and *Salmonella*.<sup>4</sup> However, triclosan in toothpaste and soaps will be diluted with water usually, and soap has been shown to reduce the efficacy of triclosan ~20-fold.<sup>50</sup> For PFD, recommended usage is 0.25–0.87% v/v and in a farm environment PFD will come into contact with materials that will dilute

it, especially organic materials, which are known to reduce the efficacy of disinfectant.<sup>31</sup> Some of the mutants selected after growth with triclosan required 8 mg/L for inhibition. It may be that such strains could survive brief exposure to triclosan, when the original preparation is diluted, or when its activity is reduced by the presence of soap or other antagonistic agents. The PFD MICs were 0.06–0.13% for all strains; these concentrations are only just below the lower usage level. It can also be argued that in a real life situation, biocides are unlikely to come into contact with bacteria in pure culture. As such, with respect to triclosan, it is possible that if the environment is dominated by triclosan-insusceptible species, these would become clonally expanded at the expense of any mutant clones but further work would be needed to verify if this is the case.

Other workers have shown that overexpression of *marA*, *soxS* or *acrAB* produces reduced susceptibility to triclosan in *E. coli*.<sup>2</sup> In the present study, growth with triclosan or PFD led to the increased isolation of MAR mutants of *S. enterica* when followed by plating on media with antibiotics or cyclohexane. We tested whether mutations were present in the MAR mutants by sequence analysis of the *acrR* and *marRO* regions but no amino acid substitutions were found, suggesting the involvement of other loci. Further work is in progress.

Reduced susceptibility to ciprofloxacin (up to 128-fold) associated with mutation in *gyrA* occurred only after plating on to ciprofloxacin and greater numbers of mutants arose from the MAR parent strains. This would suggest that MAR strains might more readily acquire mutations in *gyrA* than non-MAR strains. Alternatively, the presence of pre-existing MAR affords a level of protection such that the bacteria survive and are thereby exposed to the selective pressure longer. Therefore, the difference in the frequencies of mutation may reflect relative survival rather than actual mutation frequency *per se*. The MICs of ciprofloxacin for *gyrA* mutants that were cyclohexane resistant were as high as 4 mg/L. Previous studies have shown that *Salmonella* which have both *gyrA* mutations and are cyclohexane resistant, tend to be more resistant than strains which just have *gyrA* mutations, or are cyclohexane resistant without mutations in *gyrA*.<sup>28,32</sup>

In conclusion, the present study has shown that growth of *Salmonella* with sub-inhibitory concentrations of biocides followed by plating on to media with antibiotics leads to increased selection of strains with reduced susceptibility to antibiotics and in some instances the level of resistance seen was clinically relevant. This was particularly apparent for the MAR strains exposed to ciprofloxacin. These findings suggest that the use of biocides alone or combined with antibiotic treatment may exert increased selective pressure on bacteria to acquire antibiotic and biocide resistance.

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