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Chloroxylenol- and triclosan-tolerant bacteria from industrial sources

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Potential development of bacterial tolerance to biocides in the industrial environment is examined in this study. Bacteria tolerant to the phenolic-type agent *para*-chloro-*meta*-xylenol (PCMX) and the bis-phenol 2,4,4′-trichloro-2′-hydroxydiphenylether (triclosan) were isolated from industrial sources and identified. Minimum inhibitory concentrations (MICs) were determined and compared with those of culture collection (standard) strains. Of around 100 isolates originally obtained, most were naturally tolerant species such as *Pseudomonas* spp., or showed low tolerance levels. PCMX-tolerant isolates of *Pseudomonas stutzeri* and triclosan-tolerant isolates of *Citrobacter freundii* and *Acinetobacter johnsonii* were retained for further study. Of these, only *P. stutzeri* and *A. johnsonii* showed elevated tolerance compared with the standard strains. There was no evidence of tolerance to the other biocide except for *Pseudomonas aeruginosa* (an intrinsically tolerant microorganism), and tolerances were stable in the absence of selective pressure except for *A. johnsonii*. Attempts to select or generate increased tolerance in the standard strains were unsuccessful. High tolerances in terms of MIC were not reflected in terms of lethal effects. This study did not produce any evidence suggesting that the presence of residual biocide concentrations in the industrial environment promotes the emergence of bacterial tolerance for them.
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Introduction

Bacterial resistance to antibiotics continues to pose a worldwide problem [1] and the issue of biocide resistance is becoming increasingly important [19]. Furthermore, there is the possibility of linkage between antibiotic and biocide resistance, implying that improper use of biocides could select for antibiotic-resistant bacteria [20]. Clearly, further studies of these issues are required [19].

The definition of ''resistance'' as applied to biocides varies greatly throughout the literature [9]. When applied to antibiotics, the term describes a level of susceptibility that renders an agent ineffective at attainable *in vivo* concentrations, but cited examples of biocide resistance do not usually correlate with failure of the product at in-use concentrations [8]. Here we use the term ''tolerance'' to describe bacteria that demonstrate reduced susceptibility to biocides in terms of high or elevated biocide minimum inhibitory concentrations (MICs), and possibly a lowered killing effect.

Several previous investigations showed that bacterial tolerance can result from single or repeated exposure to sublethal concentrations of biocide [11,13,23]. In industrial environments such as biocide manufacturing facilities and laboratories, this type of exposure is likely to take place *in situ* and this raises the possibility of the development of bacterial tolerance in such situations.

Para-chloro-*meta*-xylenol (PCMX) [4,17] is a halogenated phenolic, and is considered one of the oldest antimicrobials in use today [4]. It is a constituent of some disinfectant formulations, and is also used as a topical and urinary antiseptic, and as a preservative in pharmaceutical and cosmetic products [25]. PCMX disrupts microbial cell membranes, causing leakage of cell constituents [17].

2,4,4′-Trichloro-2′-hydroxydiphenylether (triclosan) [6] is a broad-spectrum antimicrobial, widely used in skincare products, oral care products and deodorant formulations [13]. More recently, it has been incorporated into plastic kitchenware products [2]. Originally shown to act on the microbial cell membrane [6], recent research has shown that low concentrations of triclosan specifically inhibit the enoyl-acyl carrier protein reductase FabI [7,16]. This has had serious implications in the use of this phenolic, as it has been claimed that triclosan might induce the emergence of bacterial resistance to chemotherapeutic drugs that have the same target [21]. This aspect has not been studied here.

The aim of this study was to investigate potential bacterial tolerance to PCMX and triclosan in the industrial environment, and to determine the nature of any such tolerance, should it occur.

Materials and methods

Collection of isolates

Duplicate swab samples were taken from a variety of sites in a factory and laboratories of two biocidal-product manufacturing companies, one in the UK and one in Germany. Sites were chosen according to likely regular exposure to PCMX and/or triclosan (Table 1). Swabs were incubated in Brain Heart Infusion broth (Oxoid, Basingstoke, UK) at 37°C for 24 h or 22°C for 48 h. Following incubation, cultures were streaked on Tryptone Soya Agar (TSA, Oxoid) plates containing either PCMX (Reckitt

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Table 1 Sampling sites at the two facilities

Site	No. of samples	Likely biocide exposure
UK Company		
Wet area	4	PCMX
Dispensary	4	PCMX
Mobile cleaning unit	1	PCMX
Manufacturing area	8	PCMX
Formulation laboratory	8	PCMX and triclosan
Microbiology laboratory	4	PCMX and triclosan
German Company		
Microbiology laboratory	15	Triclosan
Production area	6	Triclosan

Benckiser Healthcare, Hull, UK) at concentrations of 100–500 μg ml^{-1} or triclosan (Ciba Specialty Chemicals, Manchester, UK) at 0.1–100 μg ml^{-1}, according to likely exposure at the sampling site. Dimethylsulfoxide (DMSO, Sigma Ltd., Poole, UK) or ethanol (Sigma) were used as biocide co-solvents, at concentrations previously shown to be non-inhibitory. Tolerant isolates were retained on TSA containing PCMX at 100 μg ml^{-1} or triclosan at 1 μg ml^{-1} to avoid loss of any unstable tolerance, that is, tolerance that could be lost in the absence of biocide exposure.

Identification of isolates

Colony morphology indicated that *Pseudomonas aeruginosa* was common among the tolerant isolates. This was first determined by selection on Pseudomonas Agar F (Difco, Detroit, MI, USA). ''Orientation tests'' were then conducted on the remaining isolates: Gram stain, KOH test [5] to confirm the Gram result, Oxidase test using ''Identification Sticks Oxidase'' (Oxoid) and Catalase test using ''API Colour Catalase'' [3] (Biomérieux, Basingstoke, UK). This enabled most of the isolates to be identified with API commercial kits (Biomérieux) [3], API 20E, API 20 NE and API Staph. Most remaining unidentified species were not examined further as they demonstrated low biocide tolerance, but one triclosan-tolerant isolate was identified professionally by NCIMB Ltd., Aberdeen, UK, with biochemical testing followed by 16s rDNA analysis.

Bacterial tolerances were compared with published MIC data [4,6,10,17,25] and industrial data where possible. Isolates showing unexpectedly high levels of tolerance were retained for further study, along with one highly intrinsically tolerant species for comparison.

Determination of MIC values

MIC values for PCMX or triclosan were determined by spotting 1 μl volumes of overnight TSB culture (*ca* 10^9 cfu ml^{-1}) on TSA plates containing increasing concentrations of PCMX or triclosan, with a Denley Multipoint Inoculator (Denley, Billingshurst, UK). Plates were incubated for 24 h at 37°C or 48 h at 22°C according to the original isolation temperature and then examined for growth of culture spots. The lowest biocide concentration inhibiting growth was taken as the MIC. Tolerance to the other biocide was tested in the same manner. Experiments were conducted in triplicate.

Stability of tolerance

MICs were tested following 2, 4, 8, 12 and 16 subcultures in TSB without biocide, to determine whether tolerances were stable in the absence of selective pressure.

Culture collection strains

Standard strains corresponding to the retained industrial isolates were purchased from NCIMB. Comparisons of tolerance were initially by MIC values determined as described above.

Selection of increased tolerance — standard strains

Where MIC levels demonstrated by industrial isolates were significantly higher than those of the corresponding standard strains, the following methods were employed to attempt to select or generate similarly high tolerance in the standard strains:

(i) ''Heavy inoculum'' method: One hundred microliters of overnight TSB culture (*ca* 10^9 cfu ml^{-1}) was spread on TSA plates containing PCMX at 100–500 μg ml^{-1} or triclosan at 1.0–100 μg ml^{-1}. Plates were incubated at 37°C for 3 days or 22°C for 6 days according to the incubation temperature for the strain. Colonies demonstrating tolerance were subcultured and MICs tested as described above.

(ii) ''Disk diffusion'' method: Sterile paper disks (Whatman, Maidstone, UK) were soaked in PCMX or triclosan, concentrations as above, and placed on TSA plates swabbed with cells from

Table 2 Identification of isolates from the two industrial facilities

Organism	API	Frequency	Tolerance level (μg ml^{-1})[1]
UK Company PCMX-tolerant isolates			
Alcaligenes xylosoxidans	20 NE	1	>500
Pseudomonas aeruginosa[2]	–	24	>500
Pseudomonas fluorescens	20 NE	1	>500
Unidentified (not in database)	20 NE	1	>500
Pseudomonas stutzeri	**20 NE**	**5**	**300–500**
Pseudomonas, low discrimination	20 NE	3	100–500
UK Company triclosan-tolerant isolates			
Citrobacter freundii	**20 E**	**1**	**>100**
Pseudomonas aeruginosa[3]	**–**	**12**	**>100**
Unidentified (not in database)	20 NE	1	>100
Unidentified (no kit suitable)	–	**5**	**1–100**
Pantoea/Erwinia spp.	20 E	1	1–10
Pantoea spp.	20 E	1	1–10
Unidentified (no result with kit)	Staph	1	1–10
German Company triclosan-tolerant isolates			
Pseudomonas putida	20 NE	1	>100
Unidentified (no result with kit)	20 E	1	1–10
Stenotrophomonas maltophilia	20 NE	1	1–10
Brevibacterium vesicularis	20 NE	2	0.1–1.0
Enterobacterium, low discrimination	20 E	2	0.1–1.0
Klebsiella pneumoniae	20 E	1	0.1–1.0
Pseudomonas, low discrimination	20 NE	1	0.1–1.0
Staphylococcus cohnii cohnii	Staph	2	0.1–1.0
Staphylococcus cohnii urealyticum	Staph	1	0.1–1.0
Staphylococcus epidermidis	Staph	1	0.1–1.0
Staphylococcus hominis	Staph	1	0.1–1.0
Staphylococcus, low discrimination	Staph	4	0.1–1.0
Unidentified, no result with kit	Staph	2	0.1–1.0
Unidentified (no kit suitable)	–	12	0.1–1.0

Bold print designates isolates retained for further study: (i) a PCMX-tolerant *P. aeruginosa* from the ''wet area,'' (ii) a PCMX-tolerant *P. stutzeri* from the manufacturing area, (iii) triclosan-tolerant *C. freundii* from the microbiology laboratory and (iv) an unidentified triclosan-tolerant species from the formulation laboratory.
[1]Highest concentration at which the microorganism was isolated.
[2]Twenty strains identified as *P. aeruginosa* with Pseudomonas Agar F.
[3]Strains identified as *P. aeruginosa* with Pseudomonas Agar F.

Table 3 Biocide tolerances — MICs of PCMX and triclosan against industrial isolates and culture collection strains

Species	MIC PCMX (μg ml^{-1})	MIC Triclosan (μg ml^{-1})
PCMX-tolerant isolates and standard strains		
P. aeruginosa IS	>1000	>100
P. aeruginosa NCIMB 10421	>1000	>100
P. stutzeri IS	**465–470**	<5
P. stutzeri NCIMB 11358	**200–220**	<5
Triclosan-tolerant isolates and standard strains		
C. freundii IS	180–185	>100
C. freundii NCIMB 11490	165–170	>100
A. johnsonii IS	80–85	**>100**
A. johnsonii NCIMB 12460	80–85	**<5**

IS designates industrial strain. Bold print highlights large MIC differences between industrial and standard strains.

overnight TSB culture. Following a 1-h diffusion period, plates were incubated as above and then examined for tolerant colonies within inhibition zones surrounding the disks. Any such colonies were subcultured and MICs tested.

(iii) "Repeated exposure" method: Following estimation of broth MIC values, overnight TSB cultures were used to inoculate TSB containing either PCMX or triclosan at sub-MIC concentrations. Following overnight incubation, these cultures were used to inoculate further TSB+biocide mixtures. This process was repeated to give five overnight biocide exposures. MICs were tested following the first and fifth exposure, to check for elevated tolerance.

Lethal effects of biocides

Where a large difference in MIC had been shown between the industrial and standard strains, suspension tests were carried out to compare lethal effects on strains. Cell suspensions were not washed, since it was found that washing and centrifuging in distilled water, phosphate buffer or saline caused high variability in results (with *Pseudomonas stutzeri*) or loss in viability in controls (with *Acinetobacter johnsonii*) (data not shown). One milliliter of overnight TSB culture was added to 9 ml biocide solution, to give the required final biocide concentrations of 150–250 μg ml^{-1} (PCMX) or 10–50 μg ml^{-1} (triclosan). After a contact time of 5 min at 22°C, 1 ml of this mixture was added to 9 ml of neutraliser [5% Tween 80, 1.5% lecithin (Sigma)] to quench biocide activity. Serial dilution in distilled water was then carried out and survivors counted by the drop-counting method. Control experiments were carried out where biocide was excluded from the reaction vessel.

DMSO was used as a co-solvent where required, at concentrations of 1–5%, which were previously shown to have no killing effect (results not shown). In addition, independent experiments were carried out to confirm non-toxicity of the neutraliser and its efficacy to quench biocidal activity (results not shown). Five replicates were conducted for all experiments.

Antibacterial activity was expressed as reduction factors, that is, logarithmic reductions in viable organisms:

$$\text{Reduction factor} = \log_{10}\ \text{CFU ml}^{-1}_{(\text{control})} - \log_{10}\ \text{CFU ml}^{-1}_{(\text{treated})}$$

Log reductions were compared with either two-sample *t*-tests, Mann–Whitney *U* test, one-way ANOVA or Kruskal–Wallis test using Minitab Release 13 (Minitab Inc., PA, USA).

Results

Identification of isolates

A total of around 100 isolates was obtained and identified (Table 2). High tolerances to either PCMX or triclosan were shown by a number of isolates from the UK company. For PCMX, around 67% of these were identified as intrinsically insusceptible Gram-negative rods such as *Pseudomonas* species, especially *P. aeruginosa*. There was much greater variety amongst tolerant isolates from the German company with numerous staphylococci, but tolerance levels for these strains were low.

The unidentified triclosan-resistant Gram-negative isolate (tolerance level >100 μg ml^{-1} triclosan) was identified by biochemical testing and 16s rDNA analysis as *A. johnsonii*.

Isolates retained for further study are shown in Table 2 (bold print). One strain of the highly intrinsically tolerant *P. aeruginosa* was kept for comparison. With the exception of *P. stutzeri*, all strains were isolated at 22°C.

Determination of MIC values

MIC values for the two biocides are shown in Table 3. It can be seen that there was no evidence of tolerance to the other biocide except with *P. aeruginosa*. This was expected since standard strains of this species are intrinsically tolerant to a number of biocides and resistant to antibiotics [18].

Table 4 Stability of tolerance

No. of sub-cultures	MIC (μg ml^{-1}) following subcultures			
	PCMX-tolerant isolates		Triclosan-tolerant isolates	
	P. aeruginosa IS	*P. stutzeri* IS	*C. freundii* IS	*A. johnsonii* IS
Original	>1000	450–500	>100	>100
2	950–1000	450–500	>100	>100
4	>1000	450–500	>100	<5*
6	>1000	450–500	>100	<5*
12	>1000	450–500	>100	<5
16	>1000	450–500	>100	<5

IS designates industrial strain.
*Some organisms retained the original tolerance, others did not (see text).

Stability of tolerance

MIC values following repeated subculture without biocide are shown in Table 4. Tolerance to biocides was stable in the absence of selective pressure except for *A. johnsonii*, for which tolerance to triclosan was lost after 4–12 subcultures without biocide. Furthermore, some individuals within each population lost triclosan tolerance before others. Several colonies were cultured for MIC testing at each step, and after four and six subcultures, some cultures retained the original tolerance of >100 μg ml^{-1}, whereas others did not.

Stability testing therefore produced "revertant" strains of *A. johnsonii*, having lost tolerance to triclosan. One such revertant strain was retained for further study.

Culture collection strains

The standard strain of *P. aeruginosa* purchased from NCIMB was that recommended for use in disinfectant testing. The other strains purchased were the corresponding type strains of the isolate studied. Table 3 shows these strains along with determined MIC values, compared with the industrial strains.

For the most part, MICs of these strains were very similar to those of the industrial isolates (Table 3), suggesting natural intrinsic tolerance levels. However, the MIC of PCMX for the standard strain of *P. stutzeri* was around half that of the industrial isolate (bold print, Table 3). Furthermore, the triclosan MIC of the standard strain of *A. johnsonii* was far lower than that of the industrial strain (bold print, Table 3), similar to that of the revertant sensitive strain produced by stability testing experiments.

Selection of increased tolerance

The "heavy inoculum" method produced only colonies at near-MIC values, 200 μg ml^{-1} PCMX for *P. stutzeri* and 1 μg ml^{-1} triclosan for standard and revertant strains of *A. johnsonii*. These colonies showed poor growth, and when subcultured, the MIC values were similar to those of the parent strains.

Disk diffusion was only carried out for triclosan with *A. johnsonii* strains, since PCMX diffused poorly in agar. No tolerant organisms were present in inhibition zones from disks soaked in triclosan at any of the concentrations.

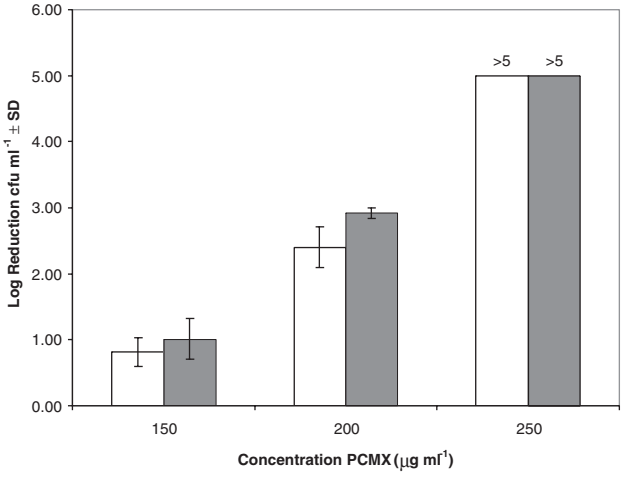

Figure 1 Lethal effects of PCMX on *P. stutzeri* SS and IS. □ *P. stutzeri* standard strain (SS); ■ *P. stutzeri* industrial strain (IS).

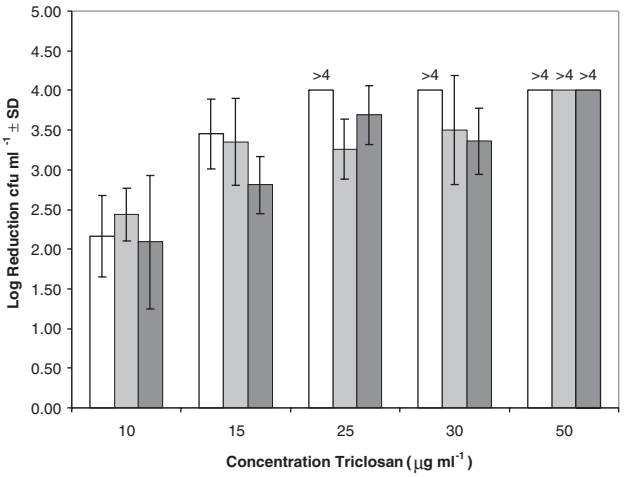

Figure 2 Lethal effects of triclosan on *A. johnsonii* SS, RS and IS. □ *A. johnsonii* standard strain (SS); ▒ *A. johnsonii* revertant strain (RS); ■ *A. johnsonii* industrial strain (IS).

Broth MIC values for the standard strain of *P. stutzeri* were 120–130 μg ml^{-1}; repeated biocide exposures were therefore carried out at 50 and 100 μg ml^{-1}. For *A. johnsonii*, broth MICs were 0.04–0.06 and 0.06–0.08 μg ml^{-1} for the standard and revertant strains, respectively, so exposures were carried out at 0.02 and 0.04 μg ml^{-1}. In all cases, the resulting organisms had similar agar MIC values to the parent strains following five overnight exposures.

Lethal effects of biocides

Figure 1 shows the lethal effects of PCMX against the two strains of *P. stutzeri*. The industrial strain (IS) was in fact subject to a greater log reduction than the standard strain (SS), and at a concentration of PCMX 200 μg ml^{-1}, although small, this difference was significant (Mann–Whitney *U* test, $p=0.0122$). PCMX 250 μg ml^{-1} resulted in >5-log reduction for both strains.

Figure 2 shows a similar situation for standard (SS), revertant (RS) and industrial strains (IS) of *A. johnsonii*. At 10 and 15 μg ml^{-1} triclosan, there was no significant difference in log reductions for the standard, revertant and industrial strains (one-way ANOVA, $p=0.622$ and Kruskal–Wallis test, $p=0.063$, respectively). Triclosan at 25 and 30 μg ml^{-1} produced greater than a 4-log reduction in the standard strain only, although values for the revertant and industrial strains were only slightly lower, and for these, there was no significant difference in log reduction (two-sample *t*-test, $p=0.127$). Triclosan 50 μg ml^{-1} produced greater than a 4-log reduction in all three strains.

Discussion

Most of the resistant species identified in this study showed intrinsic tolerance to the biocides tested, such as *Pseudomonas* spp., especially *P. aeruginosa*, and *C. freundii*. High and stable MIC values can be expected in these species [4,6].

Of all strains isolated, only two isolates showed increased tolerance, *P. stutzeri* to PCMX and *A. johnsonii* to triclosan. However, these tolerances were only demonstrated in terms of MIC since neither species appeared significantly more difficult to kill in a suspension test than the corresponding standard strain.

Furthermore, lethal concentrations of biocides were low, especially for *A. johnsonii* at 25–50 μg ml^{-1}.

The reliability of MICs as indicators of biocidal efficacy is a matter of some debate as elevated MIC values do not necessarily correlate with low rates of bacterial kill [22]. MICs alone are not considered a reliable indicator of antibacterial efficacy [15] and our results support this contention. In addition, the tolerance to PCMX shown by *P. stutzeri* was relatively low and the triclosan tolerance of *A. johnsonii*, although high, was unstable in the absence of selective pressure. Hence, the industrial significance of these tolerances is unlikely to be great. It was surprising that ostensibly sub-inhibitory concentrations (as determined by agar MIC methods) had a marked lethal effect when used in liquid suspension tests. This was observed repeatedly but the reason is unknown. There might be binding of biocides to agar or, alternatively, totally soluble actives might not be achieved in agar media.

The impact of industrial biocide exposure on the elevated MICs of *P. stutzeri* and *A. johnsonii* is unclear at present. None of the laboratory methods employed to select for increased tolerance from standard or revertant strains was successful. Nonetheless, *Acinetobacter* spp. are known to develop antibiotic resistance quickly in response to antimicrobial challenge [24].

The mechanisms by which these strains developed biocide tolerance are currently being investigated, together with their susceptibility to chemotherapeutic agents.

Brief details of this work were presented recently [14]; to the best of our knowledge, this is the first published study to investigate potential biocide tolerance in the industrial environment. Despite growing concerns regarding biocide resistance, and the fact that bacteria isolated in this study were most likely exposed to PCMX and/or triclosan on a regular basis, no evidence was found that suggested that the presence of continuous selective pressure leads to development of bacterial tolerance to biocides. On the basis of studies with clinical and industrial isolates, Lambert *et al* [12] have concluded that multiple antibiotic resistance was related to the use of antibiotics rather than to the use of biocides.

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