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Resistance of bacteria from cooling waters to bactericides

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SUMMARY

Bacteria from water cooling systems developed resistance to three different bactericides i.e. quarternary ammonium compound (QAC), isothiazolone and thiocarbamate. Resistance was induced by exposing isolates to increasing sublethal concentrations for a period of 10 weeks. *Bacillus subtilis* became resistant to 1000 mg l^{-1} QAC. Cross-resistance was also detected, e.g. isothiazolone induced resistance to QAC and thiocarbamate.

Bacteria in aqueous environments attach to surfaces where subsequent growth leads to formation of biofilms [13,18]. These biofilms promote corrosion of metal surfaces by a variety of mechanisms, e.g. by sulphate-reducing bacteria which grow in the anaerobic section of the biofilm [6]. This phenomenon is termed microbially induced corrosion (MIC). Industrial water systems (e.g. cooling water systems in power plants and mines) are treated with bactericides to eliminate or reduce corrosion. The various bacteria present differ in their susceptibility to bactericides [1].

The development of bacterial resistance to antibiotics is well established [7], and bacteria can acquire resistance to antiseptics such as quarternary ammonium compounds (QACs) [8,9,15,17] and biguanides [8,9] and to aldehyde-releasing bactericides such as hexahydro-1,3,5-triethyl-S-triazine [5]. Whether or not bacteria acquire resistance to water treatment bactericides is not known. Biofilm bacteria can be up to 100 times more resistant to chlorine dioxide than are free-floating ones [12]. Costerton and Lashen [4] reported inherent resistance of biofilm bacteria to several bactericides due to impermeability of bactericide into the extracellular polysaccharide layer surrounding cells.

Water cooling systems are often treated with isothiazolone or thiocarbamate-based bactericides [1]. Isothiazolones are non-oxidizing, do not release formaldehyde and are not membrane-active such as biguanides or QACs [19]. They react oxidatively with thiols to form disulphides [3]. The antimicrobial mechanism of thiocarbamates has not been reported to date. The objective of this study was to determine whether bacteria resident in water cooling systems become resistant to isothiazolone and thiocarbamate bactericides.

MATERIALS AND METHODS

Bacterial isolates

The strains studied were from our collection of isolates found dominant in cooling waters [2], and from later isolations. These were maintained on R2A nutrient agar slants [14] and subcultured monthly. Five strains were evaluated, i.e. *Pseudomonas cepacia*, *P. stutzeri*, *Bacillus cereus*, *B. subtilis*, and an *Aureobacterium* species.

Induction of resistance

The five strains were challenged with isothiazolone (a proprietary stabilized mixture of *N*-methyl isothiazolone and 5-chloro-*N*-methyl isothiazolone), with Na-diethyl dithiocarbamate and with benzalkonium chloride. As it has repeatedly been reported that bacteria develop resistance to benzalkonium chloride [9], cells were also challenged with tetradecyl-benzyl-dimethyl ammonium chloride to serve as a control. Minimum inhibitory concentrations (MIC) for the various isolates were initially determined as described previously [1]. Cultures were

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grown in 100-ml volumes of R2A broth [14] in a shaking waterbath at 28 °C. The pH was adjusted to 7.5 as water cooling systems are operated under slightly alkaline conditions. Bactericides were added at half the relevant MIC, and cultures were left to grow for 7 days before determining the new MIC as described below. Thereupon cultures were exposed to bactericide at half the new MIC.

Determination of minimum inhibitory concentration

MIC was determined weekly. Suspensions of challenged cells were inoculated onto three batteries of R2A agar, each containing one of the three bactericides in a range of concentrations. Cell suspensions were inoculated onto the surface of the agar media using a 19-point inoculator. All combinations of isolate and bactericide were inoculated onto every bactericide-containing agar in triplicate. Plates were incubated at 25 °C for 48 h. Growth on the surface of the bactericide-containing agar indicated resistance to the relevant bactericide at the specific concentration, and no growth indicated inhibition of growth. MIC was taken to be the lowest concentration of bactericide where no surface growth occurred.

RESULTS

Resistance

The results obtained are given in Table 1. Isolates acquired a three- to six-fold increase in resistance to isothiazolone. Resistance to the thiocarbamate increased four- to 20-fold, and to the QAC 12- to 100-fold. *Pseudomonas stutzeri* maintained its already high resistance to thiocarbamate. The 100-fold increase in resistance of *Bacillus* to the QAC is remarkable.

Cross resistance

Strains became more resistant to the isothiazolone when challenged with the thiocarbamate or the QAC, than when challenged with the isothiazolone. Resistance of *Bacillus* to the thiocarbamate was highest when strains were challenged with the QAC. *B. cereus* became 12 times

TABLE 1

Initial minimum inhibitory concentration (MIC) ($mg\,l^{-1}$ bactericide) of selected bacteria as isolated and MIC after 10 weeks of exposure to sublethal concentrations of three bactericides

Exposure to Isothiazolone[a]	Initial	Isothiazolone[a] (10 weeks)	Thiocarbamate[b] (10 weeks)	QAC[c] (10 weeks)
Bacillus cereus	10	40	40	200
B. subtilis	20	40	120	500
Aureobacterium species	20	60	300	100
Pseudomonas cepacia	10	60	500	200
P. stutzeri	10	60	500	1000
Exposure to Thiocarbamate[b]	**Initial**	**Isothiazolone[a] (10 weeks)**	**Thiocarbamate[b] (10 weeks)**	**QAC[c] (10 weeks)**
Bacillus cereus	10	70	40	100
B. subtilis	10	80	200	250
Aureobacterium species	50	80	500	800
Pseudomonas cepacia	30	80	500	250
P. stutzeri	500	80	500	1000
Exposure to QAC[c]	**Initial**	**Isothiazolone[a] (10 weeks)**	**Thiocarbamate[b] (10 weeks)**	**QAC[c] (10 weeks)**
Bacillus cereus	10	50	500	950
B. subtilis	10	70	500	1000
Aureobacterium species	20	80	500	600
Pseudomonas cepacia	40	80	500	1000
P. stutzeri	80	60	500	1000

[a] Proprietary stabilized mixture of *N*-methyl isothiazolone and 5-chloro-*N*-methyl isothiazolone.
[b] Na-diethyl dithiocarbamate.
[c] Tetradecyl-benzyl-dimethyl ammonium chloride.

more resistant to the thiocarbamate when challenged with the QAC. The isothiazolone induced resistance to the thiocarbamate in *Bacillus*, but to a lesser degree than thiocarbamate itself. Resistance of *Pseudomonas cepacia* to the thiocarbamate was induced equally strongly by all three bactericides. Similarly *P. stutzeri* became equally resistant to the QAC, independent of the challenging bactericide. Although the isothiazolone and the thiocarbamate induced resistance to QAC in *Bacillus*, *Aureobacterium* and in *P. cepacia*, cells exposed to the QAC were most resistant.

DISCUSSION

Gram-positive bacteria are more susceptible to QAC than are Gram-negative bacteria. This susceptibility is due to the nature of the Gram-positive cell wall which is mainly peptidoglycan [10]. QAC blocks oxygen uptake and alters permeability of membrane proteins [10]. No reports of QAC-resistant *Bacillus* species have appeared to date. However, the Gram-positive isolates studied became very resistant to the QAC. *Bacillus cereus* became resistant to 950 mg l^{-1} and *B. subtilis* to 1000 mg l^{-1}.

The development of cross-resistance encountered is interesting as bacterial resistance to antibiotics or antiseptics is usually specific [7,8]. Water treatment regimes are often alternated with the intention of avoiding development of a resistant population. Resistance of *P. cepacia* to thiocarbamate was the same for all three pre-exposures, and resistance of *P. stutzeri* to QAC was the same for all three pre-exposures. This suggests the development of a broad mechanism of resistance. QAC made *B. cereus* 12 times more resistant to thiocarbamate than did isothiazolone or thiocarbamate itself. Pretreatment of *B. subtilis* with QAC also made it more resistant to thiocarbamate than did thiocarbamate or isothiazolone. The selection pressure for *Bacillus* in QAC was higher than in thiocarbamate, resulting in a culture with a higher degree of protective properties towards QAC and thiocarbamate. However, *Bacillus* challenged with thiocarbamate was more resistant to isothiazolone than when challenged with QAC. Therefore the mechanism of resistance cannot be the same. However, it must be related in some way. The bactericides evaluated appear to promote development of a resistance factor. However, the nature of this resistance factor may differ somewhat from bactericide to bactericide. This could explain the differing cross-resistance found in many cases.

Bactericides are often alternated in water treatment programmes to prevent the development of resistant populations. The results presented here indicate that a bacterial population could become more resistant to a given bactericide after treatment with any other, than it would have been before. The mechanism of resistance, especially of Gram-positive bacteria will be studied in more detail.

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