Adaptation of bacterial cultures to non-oxidising water treatment bactericides

VS Brözel*, B Pietersen and TE Cloete
Environmental Biotechnology Laboratory, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0001, South Africa

Abstract

Bacterial communities in water-cooling systems treated with bactericides often become resistant to these bactericides. This has been ascribed to selection for resistant cells. Certain bacteria, having a high inherent susceptibility to water treatment bactericides, become dominant in systems after bactericide treatment. We investigated the idea that bacterial isolates adapt to growth in the presence of bactericides. Pure cultures of *Pseudomonas stutzeri* and *Bacillus cereus* were cultured repeatedly in the presence of sub-inhibitory concentrations of 2,2'-methylenebis(4-chlorophenol), sodium dimethyldithiocarbamate and isothiazolone. Both isolates adapted to growth in the presence of increasing concentrations of the bactericides. *P. stutzeri* adapted from 22 $\mu g \cdot m\ell^{-1}$ 2,2'-methylenebis(4-chlorophenol) to 80 $\mu g \cdot m\ell^{-1}$, from 12 $\mu g \cdot m\ell^{-1}$ Na dimethyldithiocarbamate to 310 $\mu g \cdot m\ell^{-1}$, and from 50 $\mu\ell \cdot \ell^{-1}$ isothiazolone to 250 $\mu\ell \cdot \ell^{-1}$. *B. cereus* adapted from 20 $\mu g \cdot m\ell^{-1}$ 2,2'-methylenebis(4-chlorophenol) to 75 $\mu g \cdot m\ell^{-1}$, from 6 $\mu g \cdot m\ell^{-1}$ Na dimethyldithiocarbamate to 132 $\mu g \cdot m\ell^{-1}$, and from 50 $\mu\ell \cdot \ell^{-1}$ isothiazolone to 300 $\mu\ell \cdot \ell^{-1}$. The phenomenon of resistance to water treatment bactericides can be ascribed not only to selection but also to adaptation.

Introduction

Surfaces in industrial water systems (e.g. cooling-water systems in power plants and mines) are prone to colonisation by bacteria. The resulting biofilms cause biofouling, leading to a decrease in system efficiency and life expectancy. The nature and mechanisms of biofouling have been reviewed extensively (Cloete et al., 1992; Ford and Mitchell, 1990). Many systems are treated with bactericides to eliminate or reduce biofouling. The various bacteria present differ in their susceptibility to the various bactericides available (Brözel and Cloete, 1991a). Some have a low degree of resistance under pure culture conditions, but play an important role in the microbial communities in cooling waters. Examples are *Pseudomonas stutzeri* and *Bacillus cereus* which often attain a dominant position in communities after bactericide treatment (Brözel and Cloete, 1992b). This indicates some form of adaptation to the bactericide over time (Jones et al., 1989). These bacteria appear to adapt to grow in the presence of otherwise inhibitory concentrations of certain bactericides. We have previously established that bacteria isolated from cooling-water systems do develop an increased level of resistance to bactericides, and grow in the presence of otherwise inhibitory concentrations (Brözel and Cloete, 1991b). Resistance can be defined in two ways, meaning either the ability of an organism and its progeny to multiply or to remain viable under conditions that would usually bring about the opposite (Gilbert and Wright, 1987). In this paper the first definition will hold as biofouling control aims at the prevention (i.e. inhibition) of bacterial growth and metabolic activity in water systems. The phenomenon of bacterial resistance to bactericides can be due to one of three reasons. Certain bacteria, notably gram-negative ones, have a high degree of inherent resistance to many bactericides due to the barrier nature of their cell envelope (Gilbert and Wright, 1987). Secondly, certain resistance mechanisms are genetically encoded and can be acquired by the contraction of an R plasmid (Franklin and Snow, 1981). Thirdly bacteria have, in certain cases, been reported to adapt to a more resistant physiological state (Jones et al., 1989). Decreased susceptibility of bacteria in cooling-water systems to non-oxidising bactericides after periods of treatment has been ascribed to the selection for less sensitive species (Characklis, 1990). Organisms less susceptible to the bactericide survive treatment and become dominant in the system, rendering the community more resistant to the following treatment.

Many authors have reported that bacteria acquire resistance to antiseptics such as quaternary ammonium compounds (QACs) (Heinzel, 1989; Jones et al., 1989; Sakagami et al., 1989) and biguanides (Heinzel, 1989; Jones et al., 1989). Development of resistance to aldehyde-releasing bactericides (e.g. hexahydro-1,3,5-triethyl-s-triazine) is also documented (Eagon and Barnes, 1986). Biofilm bacteria have been reported to be up to 100 times more resistant to ClO_2 than are free-floating ones (LeChevalier et al., 1988). Costerton and Lashen (1983) reported inherent resistance of biofilm bacteria to an isothiazolone-based bactericide due to the impermeability of the extracellular polysaccharide layer surrounding cells to the bactericide.

Water-cooling systems are often treated with isothiazolone, thiocarbamate or chlorinated phenol-based bactericides (Cloete et al., 1992). Isothiazolones are non-oxidising, do not release formaldehyde and are not membrane-active (Collier et al., 1990). They react oxidatively with thiols to form disulphides. Chlorinated phenols uncouple oxidative phosphorylation from respiration (Gilbert and Brown, 1978; Wallhäußer, 1988). The antimicrobial mechanism of thiocarbamates has not been reported to date.

The objective of this study was to investigate the rate at which pure cultures of bacteria, isolated from water-cooling systems, increase their tolerance to 3 selected bactericides, i.e. dichlorophen, thiocarbamate and isothiazolone during growth in the presence of sub-inhibitory concentrations of these bactericides. For this purpose we chose one gram-positive and one gram-negative isolate found to survive bactericide treatment in cooling-water systems, i.e. *Pseudomonas stutzeri* and *Bacillus cereus*.

*To whom all correspondence should be addressed.
Received 9 October 1992; accepted in revised form 27 January 1993.

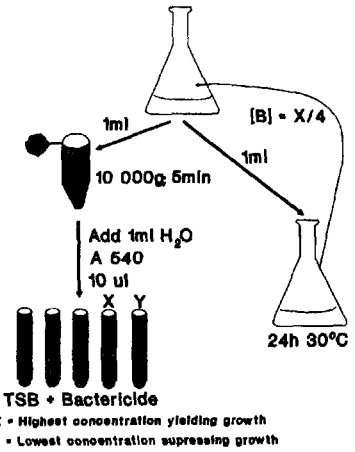

Figure 1
Diagrammatic representation of the protocol for the induction of resistance in bacterial isolates. (X/4 is one quarter of the previous MIC of bactericide; A_{540} is the absorbance at 540 nm)

Materials and methods

Cultures and media used

Two isolates found to attain a dominant position in cooling-water communities after various bactericide treatment regimes, i.e. *Pseudomonas stutzeri* and *Bacillus cereus* were used (Brözel and Cloete, 1992b). These were maintained on R2A agar slants (Brözel and Cloete, 1992a) containing 1% glycerol, and subcultured monthly. R2A medium was made up as follows (per litre): 0,5 g peptone (Biolab); 0,5 g yeast extract (Biolab); 0,5 g Casamino acids (Difco); 0,5 g glucose (BDH); 0,5 g starch (BDH); 0,3 g Na pyruvate (Merck); 0,3 g K_2HPO_4 (Merck) and 0,05 g $MgSO_4$ (Saarchem). For agar, 15 g.ℓ^{-1} agar (Biolab bacteriological grade) was added.

Bactericides evaluated

The following 3 bactericides were evaluated: Dichlorophen: 2,2'-methylenebis(4-chlorophenol) from Merck; thiocarbamate: sodium dimethyldithiocarbamate from Fluka. Isothiazolone (a stabilised mixture of N-methyl isothiazolone and 5-chloro-N-methyl isothiazolone) was supplied by Thor chemicals.

Determination of the minimum inhibitory concentration

The bacterial strains were cultured in 100 mℓ R2A broth (Brözel and Cloete, 1992a) under orbital shaking at 100 r·min^{-1} for 24 h at 30°C. The minimum inhibitory concentration (MIC) was determined as follows: One mℓ of culture was withdrawn, centrifuged at 10 000 x g for 5 min and resuspended in 1 mℓ sterile deionised water. This suspension was centrifuged at 10 000 x g for 5 min and resuspended in 1 mℓ sterile water. In the case of 2,2'-methylenebis(4-chlorophenol) and sodium dimethyldithiocarbamate, two loops full were inoculated into half-strength tryptic soy broth (TSB) (Biolab) containing various concentrations of the bactericides employed. In the case of isothiazolone, cell concentrations were determined spectrophotometrically at 540 nm and diluted to ca. 2 x 10^7 cfu.mℓ^{-1}. Aliquots of 10 $\mu\ell$ were inoculated into the bactericide-containing broths. Tubes were incubated at 30°C for 24 h. The lowest concentration of bactericide showing absence of growth, was taken to be the MIC.

Induction of resistance

One mℓ of the shake-culture was transferred to 100 mℓ fresh R2A broth containing bactericide at one quarter of the concentration of the MIC as determined. After 24 h growth the new MIC was determined as above and a new shake-culture was inoculated. This procedure was repeated 8 times in the case of dichlorophen and isothiazolone and 11 times in the case of thiocarbamate. In the case of isothiazolone, 3 parallel cultures of each isolate were exposed in order to determine whether the increase of resistance followed a fixed pattern, or whether it was a random process. A summary of the protocol is depicted in Fig. 1. Samples from broths were streaked out routinely onto R2A agar, and gram stains prepared to check for purity.

Results

2,2'-Methylenebis(4-chlorophenol)

Both initial strains did acquire a considerable degree of resistance over and above the initial value during growth in the presence of sub-inhibitory 2,2'-methylenebis(4-chlorophenol). The results are shown in Fig. 2 (A) and (B). *B. cereus* increased its resistance over the 15-d exposure period from 20 to 75 μg·mℓ^{-1}. The resistance of the *B. cereus* culture increased during the period of sub-inhibitory exposure. The rate of increase was, however, neither linear nor immediate. After 5 d the resistance dropped slightly, and started increasing again after 9 d. *P. stutzeri* increased its resistance from 22 to 80 μg·mℓ^{-1}. After 24 h of sub-inhibitory exposure, *P. stutzeri* became more susceptible, but after this its resistance increased. Again, the rate of increase was neither linear nor instantaneous.

Sodium dimethyldithiocarbamate

B. cereus and *P. stutzeri* became increasingly resistant to the thiocarbamate during sub-inhibitory exposure. The results are shown in Fig. 2 (E) and (F). *B. cereus* increased its resistance over the 15-d exposure period from 6 to 132 μg·mℓ^{-1}. The resistance of the *B. cereus* culture decreased slightly during the first 3 d of sub-inhibitory exposure. Hereafter it increased almost linearly. *P. stutzeri* increased in resistance from 12 to 310 μg·mℓ^{-1}. After 24 h of sub-inhibitory exposure, *P. stutzeri* doubled in resistance. Thereafter resistance increased slowly till 11 d of exposure, after which the rate of increase increased. After 19 d of exposure the resistance increased from 90 to 240 μg·mℓ^{-1} and then to 310 μg·mℓ^{-1}. As in the case of 2,2'-methylenebis(4-chlorophenol) the rate of increase was neither linear nor instantaneous.

Isothiazolone

The 3 different cultures increased in resistance to isothiazolone at similar rates, both in the case of *P. stutzeri* and *B. cereus* (Fig. 2 (C) and (D). Standard deviations between the 3 determinations was low. The development of resistance to isothiazolone was, therefore, not random, but followed a set pattern. The results indicate that the cultures studied would always develop resistance to a similar degree, and at a similar rate.

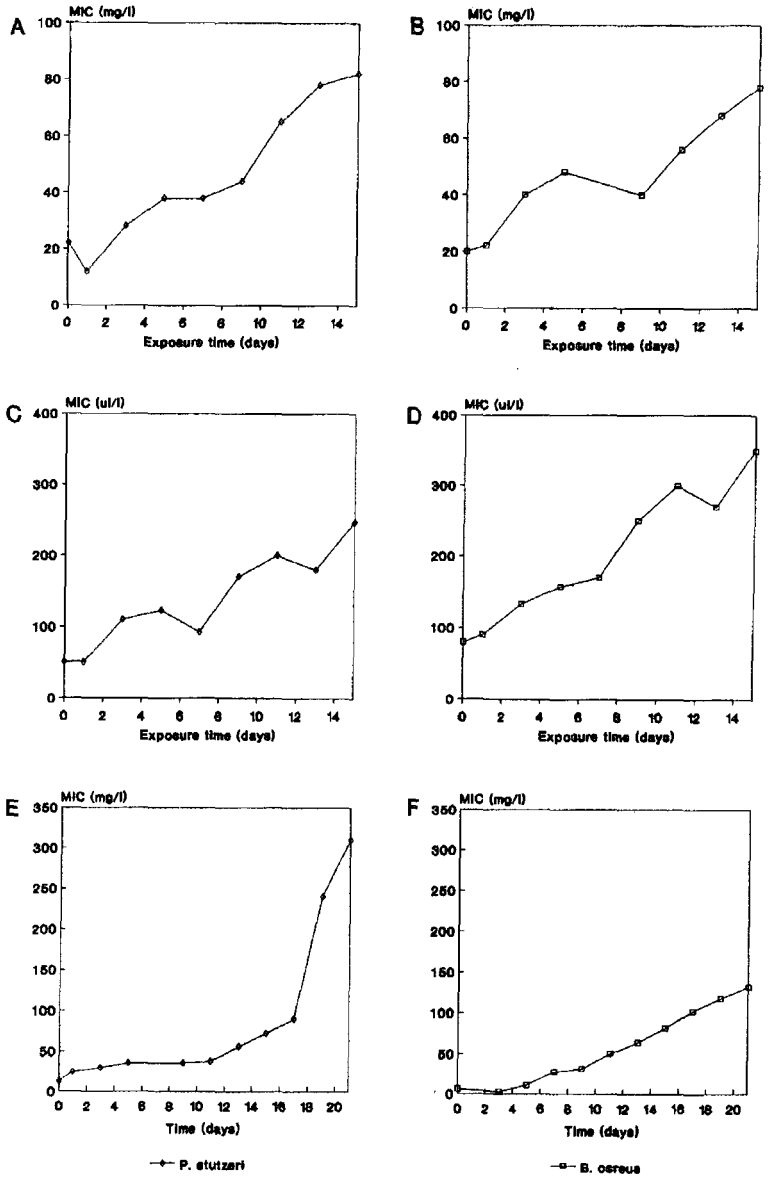

Figure 2

MIC of 3 water treatment bactericides to P. stutzeri *(A, C & E) and* B. cereus *(B, D & F) after growth in R2A broth containing one quarter the previous MIC of bactericide: A & B, 2,2'methylenebis (4-chlorophenol); C & D, isothiazolone (a mixture of N-methyl isothiazolone and 5-chloro-N-methyl isothiazolone); E & F, sodium dimethyldithiocarbamate*

B. cereus increased after every exposure from 50 $\mu\ell\cdot\ell^{-1}$ to 300 $\mu\ell\cdot\ell^{-1}$ after 11 d. It then dropped to 180 $\mu\ell\cdot\ell^{-1}$, and increased to 350 $\mu\ell\cdot\ell^{-1}$ after the 15-d exposure period. The increase in resistance over time was neither linear nor instantaneous. *P. stutzeri* resistance increased fivefold from 50 to 250 $\mu\ell\cdot\ell^{-1}$ over the 15-d exposure period. The resistance dropped from 123 $\mu\ell\cdot\ell^{-1}$ after 5 d to 93 $\mu\ell\cdot\ell^{-1}$ after 7 d. It then increased to 170 and to 200 $\mu\ell\cdot\ell^{-1}$, dropped to 180 and increased to 247 $\mu\ell\cdot\ell^{-1}$ after 15 d. Again the increase in resistance increased over time, but not in a linear fashion.

Discussion

The quantity of bactericide added to water in industrial cooling systems is calculated from minimum lethal concentrations determined under laboratory conditions. Due to the high cost of the volumes of antimicrobial agents required to treat the large volumes of water in industrial cooling systems, addition of bactericide is kept to a minimum. However, the active concentrations of bactericides in systems drop after addition due to a number of factors. Many bactericides react with constituents of the system, e.g. proteins, polysaccharides of the biofilm matrix, metal surfaces or with cells, resulting in depletion of the available pool (Characklis, 1990). System blow-down will result in loss of bactericide, and so will adsorption to surfaces. As a result the available concentration of bactericide in the cooling water is lower than required most of the time (Cloete et al., 1992). Therefore the bacterial communities are exposed to sub-

inhibitory concentrations of bactericide.

Bactericide treatment of water systems does not lead to sterilisation, and certain bacteria attain a dominant position over time (Brözel and Cloete, 1992b; Characklis, 1990). Characklis (1990) has ascribed the appearance of such more resistant bacteria to a process of selection. Hereby bacteria which are inherently more resistant to the bactericide, but which are suppressed by others under bactericide-free conditions, would attain the overhand and become dominant in the system. The data presented here demonstrate that certain bacteria are able to adapt to growth in the presence of previously inhibitory concentrations of 2,2'-methylenebis(4-chlorophenol), thiocarbamate and isothiazolone. The phenomenon of bacterial resistance to bactericides in systems can also be ascribed to a process of adaptation and not purely to selection. This would serve to explain why *P. stutzeri*, which is inherently susceptible to 2,2'-methylenebis (4-chlorophenol), was a dominant survivor 36 h after treatment of a system with 2,2'-methylenebis(4-chlorophenol) (Brözel and Cloete, 1992b).

2,2'-Methylenebis(4-chlorophenol)

The 2 strains adapted gradually to growth in the presence of inhibitory concentrations of 2,2'-methylenebis(4-chlorophenol). Resistance was not acquired directly, as is the case in antibiotic resistance or resistance to formaldehyde where formaldehyde dehydrogenase is constitutively encoded by a conjugable plasmid (Eagon and Barnes, 1986). As the bactericide has to enter through the outer cell membrane to reach its site of action in the cytoplasmic membrane, this adaptation would encompass an alteration in the permeability of the cell envelope to 2,2'-methylenebis (4-chlorophenol) (Gilbert and Wright, 1987). The observed pattern of increasing tolerance during continued exposure suggests adaptation to a resistant physiological state as in the case of biguanides (Jones et al., 1989).

Sodium dimethyldithiocarbamate

The resistance of *B. cereus* to Na dimethyldithiocarbamate can be ascribed to adaptation, as it follows near straight line or first order kinetics. The initial increase in resistance of *P. stutzeri* also appears to be due to adaptation. However, the increase in resistance of *P. stutzeri* to thiocarbamate after 19 d suggests some form of genomic change. No reports have, however, appeared to date on the mutagenicity of thiocarbamates.

Isothiazolone

Again resistance to inhibition increased during the period of exposure, indicating a mechanism of adaptation. The similarity in the kinetics of resistance development to isothiazolone between the 3 replicate cultures shows that the mechanism of adaptation is not random as the 3 cultures would otherwise yield differing data. A mutational change would also be a more random process, and a greater difference between the 3 data sets would be expected. 5-Chloro-N-methyl isothiazolone is, however, an established mutagen (Collier et al., 1990), and its effect on resistant cells will have to be established in more detail.

These results show that pure cultures of both *B. cereus* and *P. stutzeri* acquire increasing resistance to the non-oxidising bactericides evaluated during growth in the presence of sub-inhibitory concentrations of these bactericides. The decrease in susceptibility of bacterial communities to non-oxidising bactericides is, therefore, not necessarily due to selection for fitter species, but rather due to the adaptation of certain species to a more resistant physiological state.

Acknowledgements

We thank Edward P Wallace for technical assistance, Dr WF (Bill) McCoy for helpful discussions regarding the methodology and Thor chemicals for donating one of the bactericide samples. This research was supported by a grant for research by the Water Research Commission of South Africa.

References

BRÖZEL, VS and CLOETE, TE (1991a) Fingerprinting of commercially available water treatment bactericides in South Africa. *Water SA* **17**(1) 57 - 66.

BRÖZEL, VS and CLOETE, TE (1991b) Resistance of bacteria from cooling waters to bactericides. *J. Ind. Microbiol.* **8** 273 - 276.

BRÖZEL, VS and CLOETE, TE (1992a) Evaluation of nutrient agars for the enumeration of viable aerobic heterotrophs in cooling water. *Water Res.* **26** 1111 - 1117.

BRÖZEL, VS and CLOETE, TE (1992b) The effect of bactericide treatment on planktonic bacterial communities in water cooling systems. *Water SA* **18**(2) 87 - 92.

CHARACKLIS, WG (1990) Microbial biofouling control. In: Characklis WG and Marshall KC (eds.) *Biofilms.* John Wiley and Sons Inc., New York. 585 - 633.

CLOETE, TE, BRÖZEL, VS and VON HOLY, A (1992) Practical aspects of biofouling control in industrial water systems. *Int. Biodeterior. Biodegr.* **29** 299 - 341.

COLLIER, PJ, RAMSEY, A, WAIGH, RD, DOUGLAS, KT, AUSTIN, P and GILBERT, P (1990) Chemical reactivity of some isothiazolone biocides. *J. Appl. Bacteriol.* **69** 578 - 584.

COSTERTON, JW and LASHEN, ES (1983) The inherent biocide resistance of corrosion-causing biofilm bacteria. *Abstracts of the Natl. Ass. of Corrosion Eng. Conf.* 246/1 - 246/11.

EAGON, RG and BARNES, CP (1986) The mechanism of microbial resistance to hexahydro-1,3,5-triethyl-S-triazine. *J. Ind. Microbiol.* **1** 113 - 118.

FORD, T and MITCHELL, R (1990) The ecology of microbial corrosion. *Adv. Microbiol. Ecol.* **11** 231 - 262.

FRANKLIN, TJ and SNOW, GA (1981) *Biochemistry of Antimicrobial Action* (3rd edn.) Chapman and Hall, London.

GILBERT, P and BROWN, MRW (1978) Influence of growth rate and nutrient limitation on the gross cellular composition of *Pseudomonas aeruginosa* and its resistance to 3- and 4-chlorophenol. *J. Bacteriol.* **133** 1066 - 1072.

GILBERT, P and WRIGHT, N (1987) Non-plasmidic resistance towards preservatives of pharmaceutical products. In: Board, RG, Allwood, MC and Banks, JG (eds.) *Preservatives in the Food, Pharmaceutical and Environmental Industries.* Blackwell Scientific Publications, Oxford. 255 - 279.

HEINZEL, M (1989) The phenomena of resistance to disinfectants and preservatives. In: Payne, KR (ed.) *Industrial Biocides.* John Wiley and Sons, Chichester. 52 - 67.

JONES, MV, HERD, TM and CHRISTIE, HJ (1989) Resistance of *Pseudomonas aeruginosa* to amphoteric and quaternary ammonium biocides. *Microbios* **58** 49 - 61.

LECHEVALIER, MW, CAWTHON, CD and LEE, RG (1988) Inactivation of biofilm bacteria. *Appl. Environ. Microbiol.* **54** 2492 - 2499.

SAKAGAMI, Y, YOKOHAMA, H, NISHIMURA, H, OSE, Y and TASHIMA, T (1989) Mechanism of resistance to benzalkonium chloride by *Pseudomonas aeruginosa. Appl. Environ. Microbiol.* **55** 2036 - 2040.

WALLHÄUßER, KH (1988) *Praxis der Sterilisation: Desinfection - Konservierung Keimidentifizierung - Betriebshygiene.* Georg Thieme Verlag, Stuttgart.