Short communication

The reaction of bacterial cultures to oxidising water treatment biocides

B Pietersen[1]*, VS Brözel[2]# and TE Cloete[1]

[1]*Environmental Microbiology Laboratory, Department of Microbiology and Plant Pathology, University of Pretoria 0002, Pretoria*

[2]*Department of Microbiology, University of the Western Cape,Private Bag X17, Bellville 7535, South Africa*

Abstract

The possible development of resistance of bacteria found dominant in industrial water systems to the oxidising biocides hypochlorous acid and 3-bromo-1-chloro-5,5-dimethylhydantoin was investigated. *Pseudomonas aeruginosa, P. stutzeri* and *Bacillus cereus* were cultured repeatedly in the presence of sub-inhibitory concentrations of hypochlorous acid and of 3-bromo-1-chloro-5,5-dimethylhydantoin. The minimum inhibitory concentrations (MIC) of hypochlorous acid and of 3-bromo-1-chloro-5,5-dimethylhydantoin decreased following initial exposure, but varied greatly during the period of the investigation. The MICs did stabilise towards the end of the study. Whereas the isolates used did not display classical resistance, they did respond variably to successive exposures, indicating that long-term treatment of water systems with hypochlorous acid or 3-bromo-1-chloro-5,5-dimethylhydantoin would yield variable degrees of control of microbial activity.

Introduction

Surfaces exposed to water are often colonised by bacteria which grow to form biofilms (Characklis, 1990). These bacterial biofilms cause biofouling of water systems, leading to a decrease in system efficiency, as well as to microbially influenced corrosion where metal surfaces are involved (Cloete et al., 1992). Many industrial water systems are treated with one, or a combination of biocides in order to eliminate or reduce biofouling. It has been established that bacteria resident in industrial water systems develop resistance to non-oxidising bactericides such as isothiazolone, dichlorophen, thiocarbamate and quaternary ammonium compounds (Brözel et al., 1993; Brözel and Cloete, 1993). Resistance develops over time and is not acquired but develops by adaptation during exposure to sub-inhibitory concentrations (Brözel and Cloete,1994). Resistant cells are able to grow in the presence of otherwise inhibitory concentrations of non-oxidising bactericides (Brözel et al., 1993).

The oxidising bactericides such as hypochlorous acid and 3-bromo-1-chloro-5,5-dimethylhydantoin are used in various applications to control or prevent bacterial biofouling in industrial water systems. It is not known whether bacteria in such water systems become more resistant to oxidising bactericides during prolonged periods of treatment (Brözel and Cloete, 1993). Certain cases have been reported (Cloete et al., 1992) where treatment regimes did not yield satisfactory results after a given period, posing the question of possible resistance. Certain bacteria are known to adapt rapidly to hydrogen peroxide by the so-called oxidising stress response, becoming more resistant following exposure to a low level of the oxidant. These include *Pseudomonas fluorescens* and *P. putida* (Katsuwon and Anderson, 1989), *Escherichia coli* (Storz et al., 1990) and *Bacillus subtilis* (Hartford and Dowds, 1992). If bacteria resident in industrial water systems possessed such an oxidising stress response, they too would become more resistant following exposure to initial low levels of oxidant. This would render treatment regimes ineffective because these bacteria would continue to grow.

The aim of this study was to investigate whether bacteria found dominant in the planktonic phase of industrial water systems (Brözel and Cloete, 1992) develop increased tolerance to the oxidising bactericides hypochlorous acid and 3-bromo-1-chloro-5,5-dimethylhydantoin under planktonic conditions.

Materials and methods

Cultures and media used

Three bacterial isolates found to attain a dominant position in cooling-water communities after various bactericide treatment regimes, *Pseudomonas aeruginosa, P. stutzeri* and *Bacillus cereus,* were used (Brözel and Cloete, 1992). These were maintained on R2A agar slants (Reasoner and Geldreich, 1985) 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 soluble starch (BDH); 0.3 g Na pyruvate (Merck); 0.3 g K_2HPO_4 (Merck); and 0.05 g $MgSO_4$ (Saarchem). For solid R2A medium, 12 g·ℓ$^{-1}$ agar (Biolab bacteriological grade) was added.

Bactericides evaluated

Hypochlorous acid was prepared fresh as an aqueous solution by dissolving $Ca(OCl)_2$ (Olin) in deionised water. 3-bromo-1-chloro-5,5-dimethylhydantoin (Aldrich) was also prepared fresh by dissolving 0.1 g in 1 mℓ ethanol (96 % m/v), and then adding sterile deionised water to 10 mℓ because it is difficult to dissolve in H_2O at this concentration.

Determination of the minimum inhibitory concentration

Bacterial strains were cultured in 100 mℓ R2A broth in 250 mℓ Erlenmeyer flasks under orbital shaking at 100 r·min^{-1} for 24 h at 30°C. R2A was chosen because it has a similar level of suspended organic content as many industrial waters, i.e. 2.8 g·ℓ$^{-1}$ as opposed

To whom all correspondence should be addressed.

☎ (021) 959-2140; F (021) 959-2769

* Present address: Institute for Pathology, University of Pretoria, PO Box 2034, Pretoria 0002, South Africa

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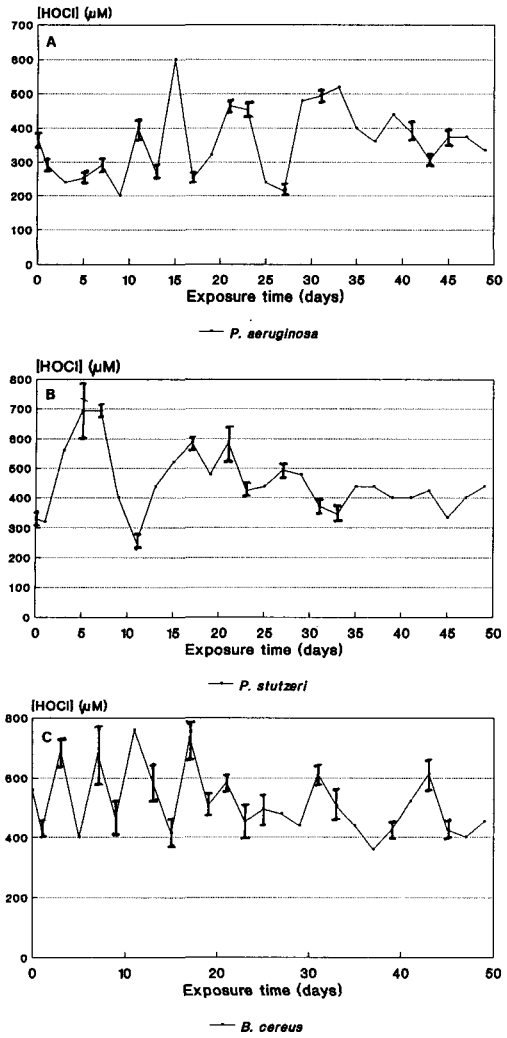

Figure 1
MIC of hypochlorous acid to P. aeruginosa *(A),* P. stutzeri *(B) and* B. cereus *(C) after growth in R2A broth containing one quarter the previous MIC of hypochlorous acid (see* ***Material and Methods*** *for details). The standard deviation is denoted by error bars, except in cases where this was too small to show.*

to 2.4 to 2.9 g·ℓ^{-1} (Howarth and McEwan, 1989). The MICs of the two bactericides were determined as previously described (Brözel et al., 1993). Briefly, 10 µl volumes of standardised suspensions of washed cells were inoculated into duplicate tubes of half-strength tryptic soy broth (Biolab) containing various concentrations (e.g. 100 to 800 µM·ℓ^{-1} in increments of 20 µM·ℓ^{-1}) of freshly added bactericide. Tubes were incubated at 30°C for 24 h. The lowest concentration of bactericide showing growth was taken to be the MIC, the actual value being the average of duplicate results.

Induction of resistance

One mℓ of the shake culture was transferred to each of three Erlenmeyer flasks containing 100 mℓ of fresh R2A broth and bactericide was added at one quarter of the MIC as determined. After 24 h growth with shaking (100 r·min^{-1}) at 30°C, the new MIC was determined as described above, and triplicate sets of flasks then similarly inoculated from these cultures. This procedure was repeated 26 times in the case of hypochlorous acid, and 23 times in the case of 3-bromo-1-chloro-5,5-dimethylhydantoin, each time using cells from the preceding cultures as inocula. Samples from the broths were streaked out routinely onto R2A agar, and Gram stains were prepared to check for purity.

Results

The MIC of hypochlorous acid varied considerably during the period of exposure (Fig. 1). While the interim values varied greatly, the values at the end of the 49 d period were similar to the MIC before exposure. In the case of *P. aeruginosa*, the MIC decreased following growth in the presence of hypochlorous acid, increased slightly again after 7 d, and only increased to above the initial value after 11 d. It then decreased and increased several times, stabilising to a degree 35 d after initiation of the experiment. At first, *P. stutzeri* adapted to growth in the presence of increasing concentrations of hypochlorous acid, but the MIC decreased and increased several times, stabilising to a degree 23 d after initial exposure at an MIC of 420 µMol·ℓ^{-1}, 80 µMol·ℓ^{-1} higher than the initial value. *B. cereus* reacted somewhat differently, with the MIC fluctuating around the initial value for 19 d, whereafter it stabilised for a short period, only to increase and decrease again.

Where cultures were grown in the presence of sub-inhibitory concentrations of 3-bromo-1-chloro-5,5-dimethylhydantoin, the MIC also varied greatly (Fig. 2). The concentration of ethanol added together with the 3-bromo-1-chloro-5,5-dimethylhydantoin never exceeded 0.05 % (v/v), so that the MIC was not influenced by ethanol. *P. aeruginosa* reacted in the same way as it did to hypochlorous acid, exhibiting a decrease in the MIC following exposure. The MIC recovered after 7 d, with the MIC increasing to above the initial value at 17 d following initial exposure. It then decreased, however, and the MIC value at the end of the experiment was slightly lower than at the start. Initially *P. stutzeri* was also more susceptible following exposure to 3-bromo-1-chloro-5,5-dimethylhydantoin, but became more resistant after 11 d of exposure. The MIC increased sharply at 17 d, only to decrease again. The

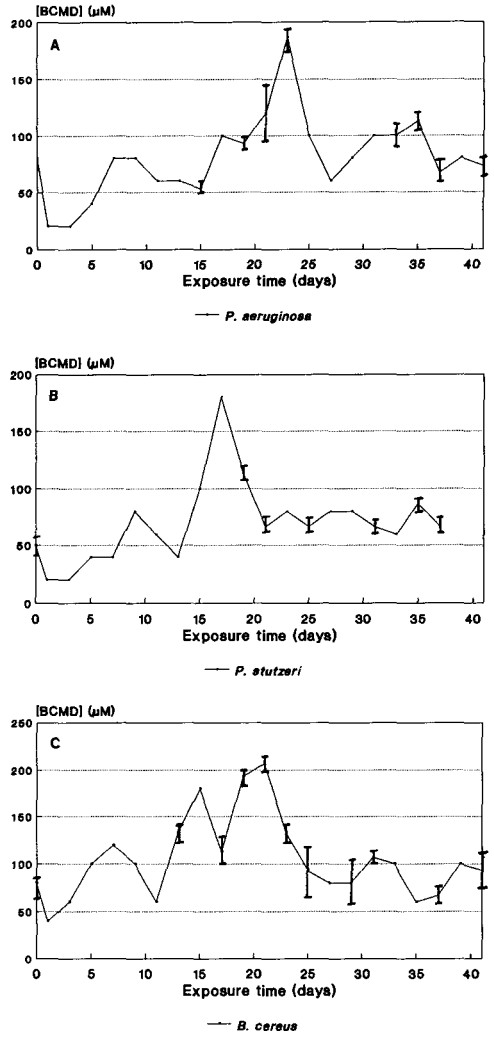

Figure 2
MIC of 1-bromo,3-chloro dimethylhydantoin to P. aeruginosa *(A),* P. stutzeri *(B) and* B. cereus *(C) after growth in R2A broth containing one quarter the previous MIC of 3-bromo-1-chloro-5,5-dimethylhydantoin (see* ***Materials and Methods*** *for details). The standard deviation is denoted by error bars, except in cases where this was too small to show*

MIC stabilised at about 74 µmol·ℓ^{-1}, 24 µmol·ℓ^{-1} higher than the initial value. The MIC for *B. cereus* also decreased, following initial exposure. It then increased, however, only decreasing to below the initial value at 11, 35 and 37 d following initiation of the experiment.

Discussion

The MIC values obtained were high, but this was due to reaction between some of the oxidant and components of the tryptic soy broth used in the assay. Although these values could not be taken as absolute values, increases and decreases would be real because the organic content of the broth was constant, meaning a fixed quantity of oxidant would be inactivated throughout.

Because all cultures became more susceptible following initial exposure to the oxidants, no stress response was demonstrated. In fact, all three cultures exhibited an inverse stress response. Two possible reasons are postulated: The first is that the response of these bacteria to hypochlorous acid and 3-bromo-1-chloro-5,5-dimethylhydantoin is different to their response to hydrogen peroxide. This is unlikely because the oxidising stress response mechanism is controlled by intracellular redox potential (Storz et al., 1990), which these oxidants and hydrogen peroxide affect similarly. Alternatively, the bacterial cultures under investigation react differently to general oxidative stress than did the classical stress response species such as *E. coli*, *P. fluorescens* and *P. putida*. *P. fluorescens* and *P. putida* (species displaying an oxidising stress response) are both root colonisers, whereas *P. aeruginosa* and *P. stutzeri* are not (Katsuwon and Anderson, 1989). These two groups also fall into different rRNA-homology groups within the fluorescent pseudomonads (Palleroni, 1984).

The increased level of tolerance of all cultures displayed at certain periods during exposure was not maintained, because cultures invariably became more susceptible again, the MIC dropping considerably. The relative stabilisation of the MIC after about one month compared with initial results indicated that cells had adapted in their mechanism of response to the oxidant. This indicated that there was no growing resistance in *P. aeruginosa*, *P. stutzeri* or *B. cereus* to either hypochlorous acid or 3-bromo-1-chloro-5,5-dimethylhydantoin. While bacteria dominant in industrial water systems do develop resistance to non-oxidising bactericides (Brözel and Cloete, 1993), they do not appear to develop resistance to the oxidising biocides. These results indicate that treatment regimes based on oxidising bactericides would, at first, yield excellent results especially since cultures are sensitised by initial exposure to the oxidant (Figs. 1 and 2). However, growth of the bacterial community would occur during later stages of treatment, due to sporadic increases in resistance. Where regimes are maintained beyond this, they would probably result in a decrease in bacterial numbers as reversion to initial degrees of susceptibility occurred, leading to good control. The inverse stress response of these bacteria to oxidising biocides, however, needs to be investigated in more detail.

Acknowledgements

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