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Biocide susceptibility and intracellular glutathione in *Escherichia coli*

John S. Chapman, Megan A. Diehl and Richard C. Lyman

Rohm and Haas Co. Inc. Research Center, 727 Norristown Road, Spring House, PA 19477, USA

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SUMMARY

Inhibition of growth and speed of kill by biocides with different mechanisms of action was examined with respect to intracellular glutathione levels. A strain deficient in intracellular glutathione was hypersusceptible to electrophilic biocides, with the exception of an isothiazolone biocide. Growth inhibition by quaternary ammonium compounds and radical-generating biocides was unaffected by intracellular glutathione levels. Speed of kill experiments demonstrated a faster rate of killing by formaldehyde in both log and stationary phase cultures of the glutathione-deficient strain as compared to its wild-type parent. Glutathione levels had no effect on the speed of kill by hydrogen peroxide in log phase cultures, but resulted in an increased rate of killing in stationary phase cultures. Stationary phase cultures of the glutathione-deficient strain were killed by a quaternary ammonium biocide at a slower rate than the glutathione-replete strain. These studies provide information about both the mechanism of action of biocides as well as the role of glutathione in determining microbicide susceptibility.

INTRODUCTION

Biocides are synthetic antimicrobial compounds which are used to protect industrial systems such as water treatment or metal working fluid systems and to preserve products ranging from cosmetics to wood [2]. The mechanisms by which biocides and preservatives work have been classified generally as either membrane active or electrophilic [14]. Membrane active agents such as quaternary ammonium compounds alter the permeability or integrity of cellular membranes, while electrophilic agents nonspecifically attack cellular nucleophiles such as protein sulfhydryls. A third mechanistic class is represented by the radical-generating compounds, particularly peroxides.

The influence of physiological variation and environmental factors on biocide susceptibility has received relatively little attention, especially when compared to antibiotic susceptibility. Knowledge of the effect of such factors on biocide susceptibility may prove useful in both biocide selection and use patterns.

The intracellular tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) is the predominant intracellular nucleophile within bacteria, reaching concentrations as high as 10 mM [13]. Glutathione protects protein sulfhydryls in the cell from electrophilic substances by presenting a competing, nonlethal target [10]. Regulation of glutathione levels and the role of glutathione in 'normal' metabolism in bacteria are not well studied. The growth of cells treated with the glutathione synthesis inhibitor L-buthionine-D,L-sulfoximine is not inhibited [8] (personal observations), and in cells in which glutathione biosynthesis has been genetically inactivated there is little or no penalty in terms of growth rate [7]. Thus, under non-stressed conditions glutathione is apparently dispensable. However, glutathione-deficient bacteria are hypersusceptible to electrophilic agents such as formaldehyde, glyoxal, diamide, and n-ethyl maleimide [5,7]. In eukaryotes, glutathione is also capable of participating in the detoxification of radicals via a glutathione peroxidase [12]; *E. coli* lacks this enzyme [16] and thus glutathione provides no protection against attack by hydrogen peroxide or redox cycling agents [7] in log phase bacteria.

In this study, we examined the role of intracellular glutathione in determining growth inhibition and rate of killing by biocides having different mechanisms of action.

MATERIALS AND METHODS

Organisms

The *E. coli* strain used in this work are AB1157 (wild-type for glutathione biosynthesis) and JTG10 (gshA20:TN10 derivative of AB1157), which were a gift of Dr Bruce Demple, Harvard University. The glutathione synthetase gene has been inactivated in strain JTG10 by transposon insertion [7].

Chemicals

The following chemicals were purchased from Sigma Chemical Co., St Louis, MO: cefazolin, formaldehyde, hydrogen peroxide, T-butyl hydroperoxide, and methyl viologen. The remainder of the antimicrobial compounds used are the active ingredients of commercially available biocides which were obtained from the Rohm and Haas Co. Biocides Archive, Spring House, PA.

Correspondence to: J.S. Chapman, Rohm and Haas Co. Inc. Research Center, 727 Norristown Road, Spring House, PA 19477, USA.

Susceptibility determinations

Relative susceptibilities were examined by determination of the zones of inhibition of growth of a lawn of cells around a paper disc spotted with antimicrobial compound. The amounts of antimicrobial applied per disc (μg $disc^{-1}$) in a 10-μl volume were: cefazolin, 30; formaldehyde, 37; hexahydro-1,3,5,-triazine, 78; 5-hydroxymethyl-1-aza,3,7-dioxabicyclo-(3,3,0)-octane, 50; dimethylhydroxyl-5,5′-dimethylhydantoin, 55; 1-bromo-1-nitrostyrene, 20; *N*-(hydroxymethyl)-*N*-(1,3-dihydroxymethyl-2,5-dioxo-4-imidazolidinyl)-*N*-(hydroxymethyl)-urea, 50; 2,2-dibromonitrilopropionamide, 20; 2-bromo-2-nitropropan-1,3-diol, 20; 5-bromo-5-nitro,1,3-dioxane, 20; 5-chloro-2-methylisothiazol-3-one + 2-methylisothiazol-3-one, 10; hydrogen peroxide, 300; tert-butyl hydroperoxide, 300; methyl viologen, 100; dimethylbenzalkonium chloride, 50; poly[ethoxy(dimethyliminio)-ethylene-(dimethyliminio)]-di-chloride, 60. The organisms were cultured in tryptic soy broth (TSB) at 37 °C for 18–24 h. Fresh cultures were started in TSB and the cultures grown to an A_{660} value of 0.4 (mid-log phase). Approximately 1×10^7 cells were spread on each TSB plate, and the plates allowed to dry at room temperature before placement of the disk and spotting it with biocide. After 24 h incubation at 37 °C, the diameter of each zone of inhibition was measured. At least two measurements of diameter were determined for each zone. The experiment was repeated three times.

Speed of kill activity of 25 μg ml^{-1} benzalkonium chloride (BAC), 750 μg ml^{-1} formaldehyde (FA), and 500 μg ml^{-1} hydrogen peroxide (HP) was determined using a most probable number (MPN) method. The MPN method was adapted to 96-well microtiter plates; serial dilutions were performed with a Biomek 1000 Workstation (Beckman Instruments, Somerset, NJ) and were performed as 1 : 5 dilutions rather than 1 : 10 dilutions to increase the resolution of the method. Cultures were grown overnight in TSB at 37 °C and used directly (stationary phase cultures), or fresh cultures were started and grown to A_{660} of 0.4 (log phase cultures). Dilutions were made such that the exposure media contained approximately 2×10^8 CFU ml^{-1}. Biocide (or water for the control) was added to a flask containing 10 ml of culture, and aliquots removed at intervals. Aliquots were serially diluted 1 : 5 in TSB with 200 μg ml^{-1} sodium thioglycollate, and the plates incubated for 48 h at 37 °C. Four replicates of each sample were serially plated, and the number of CFU ml^{-1} surviving estimated by determining the last well to show growth. The limit of detection of this method for BAC and HP was 125 CFU ml^{-1} because of incomplete inactivation of biocide resulting in growth inhibition in the first two wells of the MPN plate. These experiments were repeated 3–4 times. Student's *t*-test was applied to the data to determine if differences observed were statistically significant. Differences were deemed significant if α was equal to or less than 0.005.

RESULTS AND DISCUSSION

Table 1 presents the relative susceptibilities of *E. coli* strains AB1157 and JTG10 to a number of antimicrobial

TABLE 1

Susceptiblities of *E. coli* strains AB1157 and JTG10 to various biocides. Susceptibility is expressed as the diameter of the zone of inhibition in mm. Each value is the mean plus and minus the standard error from at least three experiments

Biocide	AB1157	JTG10
Controls		
Cefazolin	17.3 ± 1.2	17.9 ± 0.8
Formaldehyde	8.4 ± 1.1	15.4 ± 1.1
Electrophiles		
Hexahydro-1,3,5,-triethyl triazine	16.8 ± 1.4	26.4 ± 0.7
5-hydroxymethoxy-methyl-1-aza, 3,7-dioxabicyclo-(3,3,0)octane	17.9 ± 3.3	28.7 ± 2.1
Dimethylhydroxyl-5,5′-dimethyl-hydantoin	11.3 ± 0.3	17.4 ± 1.1
1-bromo-1-nitrostyrene	16.9 ± 0.5	24.8 ± 1.3
N-(hydroxymethyl)-*N*-(1,3-dihydroxy-methyl-2,5-dioxo-4-imidazolidinyl)-*N*-(hydroxymethyl)-urea	11.2 ± 0.4	18.1 ± 0.6
2,2,-dibromonitrilopropionamide	10.9 ± 0.5	13.6 ± 0.9
2-bromo-2-nitropropane-1,3-diol	13.9 ± 0.7	18.6 ± 2.5
5-bromo-5-nitro-1,3,-dioxane	11.3 ± 0.3	19.9 ± 0.7
5-chloro-2-methylisothiazol-3-one + 2-methylisothiazol-3-one	13.2 ± 1.1	12.4 ± 1.5
Radical-generating compounds		
Hydrogen peroxide	24.2 ± 1.7	25.4 ± 2.4
T-butyl hydroperoxide	11.4 ± 0.9	11.6 ± 1.2
Methyl viologen	16.5 ± 0.7	17.0 ± 1.1
Membrane-active compounds		
Dimethylbenzylalkonium chloride	24.5 ± 2.1	25.1 ± 2.0
Poly[ethoxy(dimethyliminio)-ethylene-(dimethyliminio)]-dichloride	11.2 ± 0.4	10.9 ± 0.6

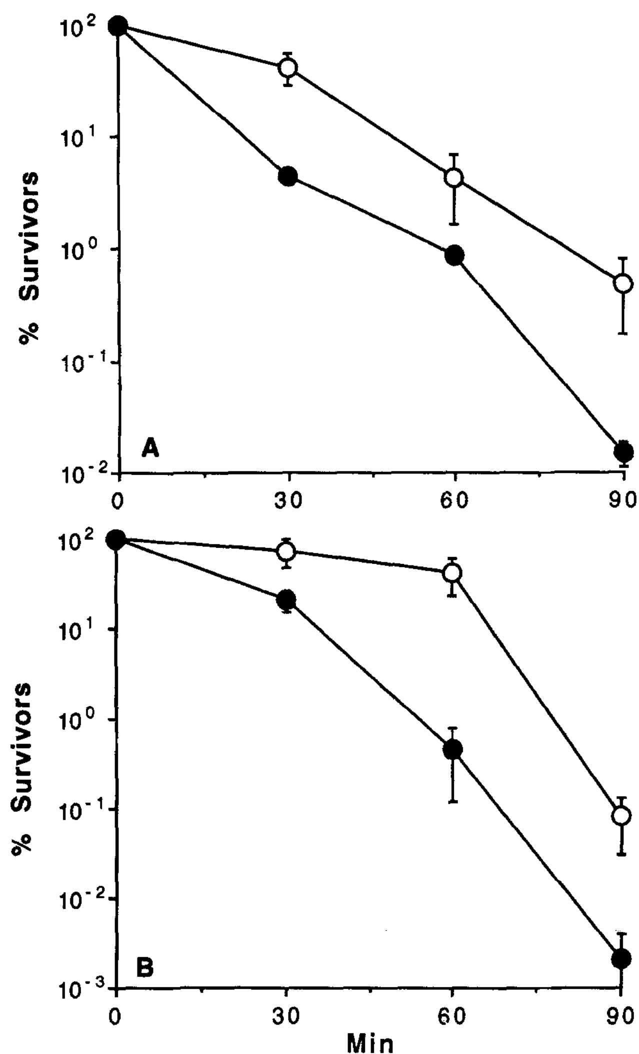

Fig. 1. Lethal challenge of AB1157 (○) and JTG10 (●) by 750 ppm formaldehyde. **A**, log phase cultures; **B**, stationary phase cultures. Initial culture density was approximately 2×10^8 cells ml^{-1}. Each point represents the mean of 3–4 determinations with 4 replicates.

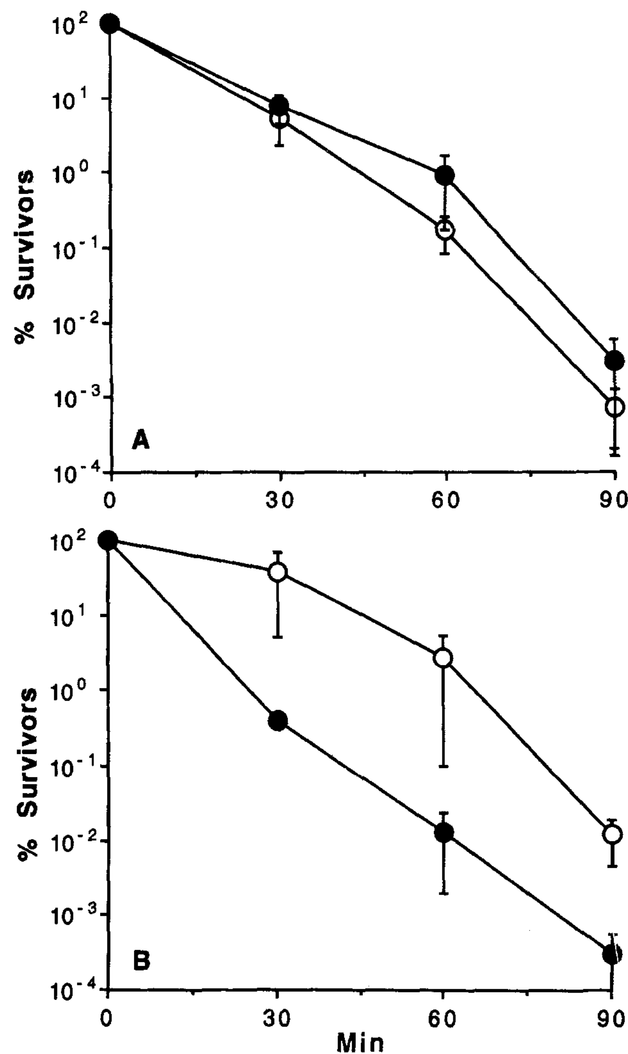

Fig. 2. Lethal challenge of AB1157 (○) and JTG10 (●) by 500 ppm HP. **A**, log phase cultures; **B**, stationary phase cultures. Initial culture density was approximately 2×10^8 cells ml^{-1}. Each point represents the mean of 3–4 determinations with 4 replicates.

agents representing each of the mechanisms associated with biocides. FA is included as the positive control; it is the classic electrophilic microbicide and previous work has shown strain JTG10 to be hypersusceptible [7]. Cefazolin served as a negative control; it is a broad spectrum cephalosporin and would not be expected to be affected by intracellular glutathione levels.

The first group of biocides have all been postulated to have significant electrophilic components in their antimicrobial mechanism [14]. As Table 1 shows, all the biocides in this group but one demonstrated greater activity against the glutathione-deficient strain JTG10 than against AB1157. The notable exception is the 3 : 1 mixture of 5-chloro-2-methylisothiazol-3-one and 2-methylisothiazol-3-one [11]. Isothiazolones have been postulated to exert their antimicrobial activity via electrophilic attack on protein sulfhydryls [4,14]. Thus, the failure of glutathione-deficiency to result in increased susceptibility to isothiazolones indicates the in vivo mechanism of isothiazolones may not be limited to electrophilic attack. Results generated in our lab (Chapman, J.S., unpublished results) indicate the intracellular generation of radicals may have a significant role in the antimicrobial mechanism of isothiazolones.

The demonstration of increased susceptibility by JTG10 to 2-bromo-2-nitropropane-1,3-diol (BNPD) is also noteworthy. Stretton and Manson [17] suggested that BNPD is capable of generating radicals within cells and these radicals are responsible for the antibacterial activity observed. Subsequent work [15] provided support for this theory by demonstrating that the addition of BNPD to a solution of cysteine results in oxygen consumption, implying the formation of superoxide radicals. In addition, Ames et al. [1] showed that the reduction potential of BNPD is within the range where biological reduction is possible. However, increased susceptibility in JTG10 implies a significant role for electrophilic attack in the mechanism of BNPD. It is

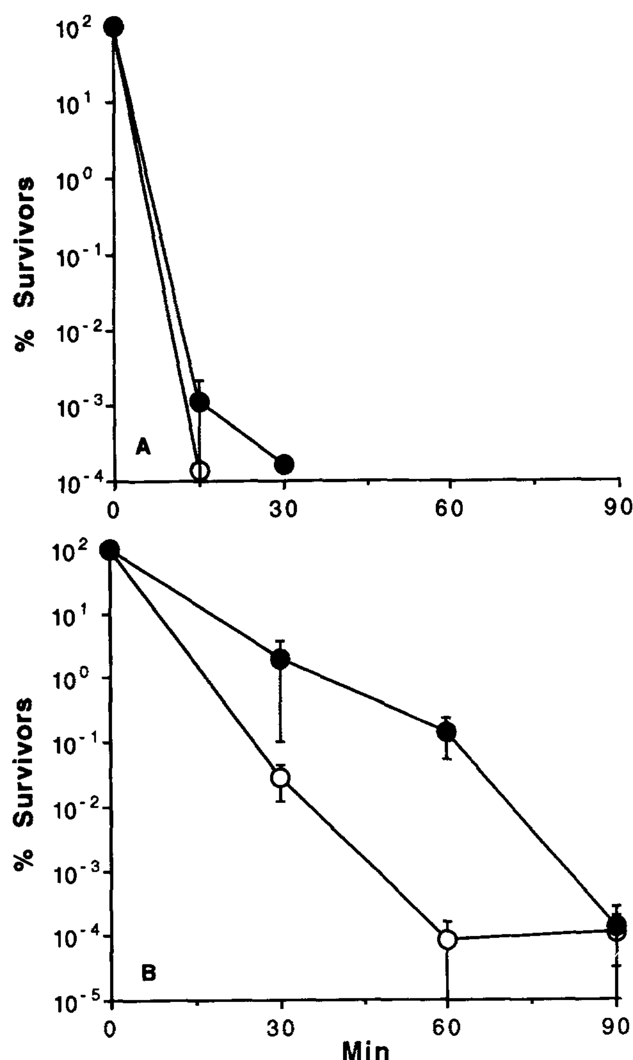

Fig. 3. Lethal challenge of AB1157 (○) and JTG10 (●) by 25 ppm BAC. **A**, log phase cultures; **B**, stationary phase cultures. Initial culture density was approximately 2×10^8 cells ml^{-1}. Each point represents the mean of 4 determinations in 4 replicate experiments.

possible that both mechanisms, electrophilic attack and radical generation, contribute to the antimicrobial activity of BNPD.

The effect of glutathione on the speed of kill activity of formaldehyde was examined (Fig. 1). In both log and stationary phase cultures (Fig. 1(A,B)) strain JTG10 was killed at a faster rate than was strain AB1157. The increased susceptibility of log phase JTG10 to FA killing has been demonstrated previously [7]. Stationary phase cultures of both strains were killed by FA at a slower rate than their respective log phase counterparts. The decreased susceptibility of stationary phase cultures to a number of antimicrobial stresses, although largely unexplained, is nonetheless well documented [3,6].

Tert-butyl hydroperoxide and hydrogen peroxide (HP) generate radicals by decomposition while methyl viologen generates radicals by participating in a redox cycle with cellular constituents [9]. Table 1 shows that glutathione levels do not affect the size of the zone of inhibition produced by these radical-generating compounds. There was also no effect on the rate at which HP killed log phase cultures of both strains (Fig. 2(A)) [7]. However, in stationary phase cultures glutathione deficiency resulted in relatively greater speed of kill by HP (Fig. 2(B)). The stationary phase cultures of strain AB1157 were more resistant to HP killing, while strain JTG10 was more sensitive.

Zones of inhibition produced by the membrane-active quaternary ammonium compounds were unaffected by intracellular glutathione levels (Table 1). Since these compounds exert their antibacterial activity by disrupting membrane integrity, this is not unexpected. Log phase cultures of both strains were rapidly killed by BAC (Fig. 3(A)). However, stationary phase strain AB1157 was killed at a faster rate than stationary phase strain JTG10 (Fig. 3(B)). This difference in the rate at which the two strains were killed was unexpected, and the basis for the difference remains unexplained. Log phase cultures of both strains were more susceptible to BAC killing than were stationary phase cultures.

Thus, the level of intracellular glutathione is a major determinant of susceptibility to electrophilic biocides. In log phase cells glutathione has no role in determining susceptibility to radical-generating biocides or membrane-active agents, although it appears glutathione may be involved in determining susceptibility to these biocides in stationary phase cultures. The role of glutathione in stationary phase physiology is currently unknown. The relationship between variations in physiology of the bacterial cell and biocide susceptibility is important to understand in order to apply biocides efficiently in a world seeking to reduce the introduction of toxic chemicals into the environment.

The relative susceptibilities of strains JTG10 and AB1157 to biocides is a useful tool in investigations of in vivo antimicrobial mechanisms. The observed discrepancies in the predicted behavior of the paired strains to the isothiazolone biocide and BNPD suggests a re-evaluation of the models describing their antimicrobial mechanisms may be necessary.

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