Journal of Industrial Microbiology & Biotechnology (1997) 18, 223–225

Reversible sporicidal action of cuprous ions

GC Rutherford and RE Marquis

Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642-8672, USA

At 10 mM, Cu^{+} was highly protective against killing of spores of *Bacillus megaterium* ATCC 19213 by H_2O_2, while at higher concentrations, from 15–100 mM, killing was augmented. In contrast, Cu^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+} or Co^{3+} ions acted only protectively. Cu^{+} itself was sporicidal in the absence of H_2O_2 or ascorbate, and its sporicidal action did not depend on generation of highly reactive oxygen species. It appeared that killing involved either inhibition of germination or copper toxicity to germinated cells in that Cu^{+}-inactivated spores did not germinate readily but chemical decoating of the cells prior to plating on a solid medium resulted in reversal of the sporicidal effect.

Keywords: sporicides; bacterial spores; copper toxicity; oxidative damage

Introduction

Copper is well known to be toxic to living cells, and many microorganisms can protect themselves against copper toxicity either by means of efflux systems to move the mineral out of the cytoplasm or by production of copper-binding proteins. The general topic of resistance to heavy metals of environmental concern was reviewed recently by Ji and Silver [2]. It is generally considered that the toxicity of copper derives in part from its capacity to catalyze oxidative damage through so-called Fenton reactions in which cuprous ion reacts with hydrogen peroxide to yield cupric ion, hydroxyl anion and the very damaging hydroxyl radical. Copper bound to the exterior of cells, as well as that in the cytoplasm, may be toxic. For example, in *Escherichia coli* copper associated with the outer membranes can be involved in redox cycling that mediates hydroperoxide toxicity to the organism [4]. Presumably, in this situation, radicals produced outside of the cell or at the cell surface can cause damage to cell membranes or possibly penetrate into the cytoplasm. Of course, copper is needed in small amounts by most cells, and the need is often for the functioning of enzymes protective against oxidative damage such as superoxide dismutases or cytochrome oxidases.

Bacterial endospores have inherent resistance to heavy metals by virtue of their protective coat structures and their dormancy. Basically, the cells do not produce hydroperoxides, and so Fenton reactions cannot occur, except of course if hydroperoxides are purposely added to the cells. Currently, hydroperoxides are very widely used sporicides, especially for aseptic packaging and processing. The history of use of copper as a sporicide was reviewed by Russell [5], and copper salts have recently been shown to be virucidal and sporicidal, primarily admixed with ascorbate and hydroperoxides [6–8]. These cidal actions are thought to involve oxidative damage following production of radicals from hydrogen peroxide. However, contrary findings that copper ions can actually be protective against hydroperoxide damage have been reported [3,10,11]. The work described here was undertaken to define more clearly the nature of the sporicidal action of copper salts.

Correspondence: RE Marquis, Box 672, University of Rochester Medical Center, Rochester, NY 14642–8672, USA
Received 12 July 1996; accepted 3 November 1996

Materials and methods

Spores

Bacillus megaterium ATCC 19213 was grown in the medium of Slepecky and Foster [12] at 32°C with shaking. The spores produced were purified by differential centrifugation and stored as a pellet under 95% ethanol until used.

Assays of sporicidal activity

Suspensions of spores containing approximately 10^8 colony-forming units per ml in water were used. They generally were diluted 1 : 5 to prepare the final mix, which contained 1% Difco peptone and other ingredients for the specific sporicidal assay. For nearly all assays reported, the pH of the final mix was adjusted to 7, and the temperature was maintained before and after mixing at 50°C by use of a heat block. Samples were taken at intervals and diluted in 1% Difco peptone broth at room temperature. Addition of neutralizers such as sulfhydryl compounds for hydroperoxides or chelators of mineral ions did not enhance colony counts when peptone solution was used as diluent. Samples (0.1 ml) of appropriately diluted suspensions were spread plated on tryptic-soy agar and incubated at 37°C until colony formation was complete. In general, assays were carried out at least twice to assess repeatability of results.

Results

Sporicidal potency of Cu^{+} ions

At 15 to 50 mM Cu^{+} is cidal for spores of *B. megaterium* ATCC 19213 at 50°C and pH 7 (Figure 1a). The D value (time for killing 90% of the spore population) was only about 20 min during the first hour of exposure to Cu^{+} at a concentration of 50 mM. Killing of spores by Cu^{+} was not highly temperature sensitive, and the rate of killing at 4° or 25°C was basically the same as that at 50°C (data not shown). Cu^{+} was not sporicidal at a concentration of

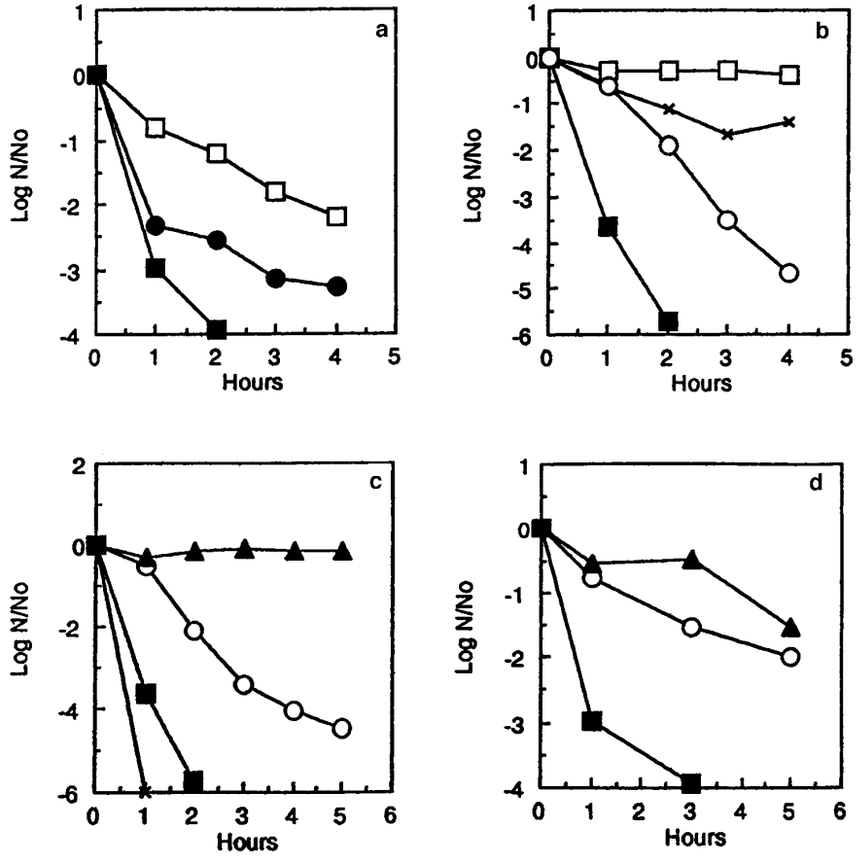

Figure 1 Sporicidal action of Cu^+ for spores of *B. megaterium* ATCC 19213 and effects of copper ions on killing by hydroperoxide. Data are shown for (a) spores treated at 50°C and pH 7 with 15 mM (□), 25 mM (●) or 50 mM (■) CuCl; (b) for spores treated with 0.1% H_2O_2 alone (○) or 0.1% H_2O_2 plus 50 mM $FeSO_4$ (×) or 50 mM $CuCl_2 \cdot 2H_2O$ (□) or 50 mM CuCl (■); (c) for spores treated with 0.1% H_2O_2 alone (○) or 0.1% H_2O_2 plus 10 mM (▲), 50 mM (■) or 100 mM (×) CuCl; and (d) for spore killing at 50°C and pH 7 by 5 mM ascorbate (▲), 0.03% H_2O_2 (○) or 50 mM Cu^+ (■).

10 mM but, as shown in Figure 1, D values of, respectively, 75 and 25 min were achieved with 15 and 25 mM Cu^+.

Augmentation of killing by hydroperoxide

Addition of 50 mM Cu^+ to 0.1% (32.6 mM) H_2O_2 resulted in enhanced sporicidal activity (Figure 1b). The average D value for the H_2O_2 alone was some 30 min, while the mixture had an average D value over the first hour of killing of about 16 min, or only slightly less than the value obtained when 50 mM Cu^+ was used alone. 50 mM Fe^{2+} or Cu^{2+} both acted to protect the spores against the lethal action of H_2O_2 (Figure 1b). Similar protection occurred with Fe^{3+}, Co^{2+}, or Co^{3+} at concentrations ranging from 10 to 100 mM.

Results of further titration of the capacities of Cu^+ to augment H_2O_2 killing of *B. megaterium* spores are presented in Figure 1c. Again, the D value for killing by 0.1% H_2O_2 alone was about 30 min during exponential killing. Addition of 50 mM Cu^+ resulted in more rapid killing, with a D value of some 16 min, while addition of 100 mM Cu^+ reduced the D value by some 10 min indicated by a six-log reduction in log N/N_o after 1 h of exposure; the D value in this experiment for 100 mM Cu^+ was some 11 min. In contrast, addition of 10 mM Cu^+ effectively protected the spores against killing by H_2O_2, as found previously [11].

Ascorbate is commonly added to enhance lethality of mixtures of H_2O_2 and a reduced transition metal ion. Ascorbate alone at a concentration of 0.1% (5 mM) had a slight sporicidal effect, possibly due to a low level of oxidative cycling with radical production (Figure 1d). H_2O_2 (0.03%) also had an expected mild sporicidal activity at the experimental temperature of 50°C. When the ascorbate and hydroperoxide were combined, the killing was essentially the same as that of the H_2O_2 alone. As shown, 50 mM Cu^+ was again toxic, and addition of 0.1% ascorbate, 0.03% H_2O_2 or a combination of the two did not enhance the toxicity of Cu^+ (data not shown because the killing curves were all nearly identical).

Effects of decoating

Spores of *B. megaterium* inactivated with Cu^+ did not undergo germination after heat activation and exposure to 1 mM L-alanine and 1 mM inosine, as indicated by no change in light absorbance and no loss of refractility visible in the phase microscope. However, they could be decoated by the procedure of Aronson and Horn [1] involving exposure to 50 mM dithiothreitol, and 0.8% sodium dodecyl sulfate at pH 10.3 and 50°C for 90 min. The decoated spores were largely recovered on plates of tryptic-soy agar (Table 1). Plate counts of spores exposed to Cu^+ and then

Table 1 Recovery of spores inactivated by cuprous ion is enhanced by decoating

Cu^+ (mM)	Decoating before plating	Average count[a] (CFU ml^{-1})
15	No	$5.0\ (\pm 2.4) \times 10^3$
15	Yes	$2.6\ (\pm 1.2) \times 10^5$
25	No	$6.3\ (\pm 4.7) \times 10^2$
25	Yes	$3.1\ (\pm 1.7) \times 10^5$

[a]The average initial count was $1.5\ (\pm 0.8) \times 10^6$ CFU ml^{-1}. The numbers in parentheses are standard errors of the mean; in all cases the number of separate determinations, run on separate days, was four, and spores were treated with Cu^+ for 3 h at pH 7 and 50°C.

decoated were always somewhat lower than those of untreated spores, possibly because of damage during decoating or losses during manipulation, but it was clear that Cu^+ killing of spores could be reversed by decoating the cells. Each of the components of the decoating mix was effective for increasing the spore count. Sodium dodecyl sulfate (0.8%) was the most effective. In a typical experiment, the count in a spore suspension exposed to 0.5% Cu^+ was reduced from 7.2×10^8 to 6.0×10^4 CFU ml^{-1}. If the suspension of Cu^+-treated spores was incubated for 90 min at pH 10.3 or in 50 mM dithiothreitol solution or in 0.8% sodium dodecyl sulfate solution, plate counts were increased, respectively, to 1.4×10^6, 1.5×10^6 and 7.0×10^7. Repeated exposure of the reversibly inactivated spores to 0.8% sodium dodecyl sulfate solution resulted in essentially total recovery of the original population (data not shown). Addition of lysozyme to decoated spores over a range of concentrations did not further enhance recoveries of Cu^+-inactivated spores subsequently decoated. Moreover, washing Cu^+-inactivated spores with water or solutions containing 50 mM ethylene-diamine-tetraacetate or *o*-phenanthroline did not result in higher recoveries, and these findings suggest that the copper was tightly bound to the spores.

Discussion

Cu^+ at concentrations above about 15 mM is sporicidal but addition of hydroperoxides or ascorbate is not required to achieve spore killing. Thus, there is question about whether or not Cu^+ enhances oxidative damage as an important part of its sporicidal action. Instead, it seems that Cu^+ at higher concentrations is acting mainly as a toxic, heavy-metal ion. At lower concentrations, here 10 mM, it clearly was protective against hydroperoxide damage, as were other reduced transition metal ions, such as Fe^{2}+ and Co^{2+}. Oxidized transition metal ions, including Cu^{2+}, can also be protective, although they are generally less effective than the reduced forms [3,10,11]. In fact, spore killing by the reduced Cu^+ but not the oxidized Cu^{2+} may be related to the unusual, oxidized state of the spore [9]. Thus, Cu^+ may be retained because it reacts with the spore by way of oxidation-reduction reactions, while Cu^{2+} does not. The protective effects of transition metal ions against hydroperoxide damage have not been interpreted mechanistically, although they could depend on the ions being excluded by the spore and then reacting externally with hydroperoxides before they can penetrate through the coat-outer-membrane complex into the spore.

The finding that decoating can reverse the lethality of Cu^+ suggests that the ion is bound to the coats. Removing the coats then removes the copper. Thus, the spores are made superdormant by Cu^+. Moreover, if the spores do germinate, the copper in the vicinity of the germinated cell may prevent outgrowth. Clearly, Cu^+-induced lethality is reversible, and this reversibility, along with concerns about environmental problems, may limit wide use of Cu^+ as a sporicide.

Acknowledgements

This work was supported by the Center for Aseptic Processing and Packaging Studies, which is a US National Science Foundation Industry/University Cooperative Research Center based at North Carolina State University and University of California at Davis.

References

1 Aronson AI and D Horn. 1972. Characterization of the spore coat protein of *Bacillus cereus*. In: Spores V (Halvorson HO, R Hanson and LL Campbell, eds), pp 19–27, American Society for Microbiology, Washington DC.
2 Ji G and S Silver. 1995. Bacterial resistance mechanisms for heavy metals of environmental concern. J Ind Microbiol 14: 61–75.
3 Marquis RE, GC Rutherford, MM Faraci and SY Shin. 1995. Sporicidal action of peracetic acid and protective effects of transition metal ions. J Ind Microbiol 15: 486–492.
4 Rodriguez-Montelongo L, LC de la Cruz-Rodriguez, RN Farias and EM Massa. 1993. Membrane-associated redox cycling of copper mediates hydroperoxide toxicity in *Escherichia coli*. Biochim Biophys Acta 1144: 77–84.
5 Russell AD. 1990. Bacterial spores and chemical sporicidal agents. Clin Microbiol Rev 3: 99–119.
6 Sagripanti J-L. 1992. Metal-based formulations with high microbicidal activity. Appl Environ Microbiol 58: 3157–3162.
7 Sagripanti J-L, LB Routson and CD Lytle. 1993. Virus inactivation by copper or iron ions alone and in the presence of peroxide. Appl Environ Microbiol 59: 4374–4376.
8 Sagripanti J-L and A Bonifacino. 1996. Comparative sporicidal effects of liquid chemical agents. Appl Environ Microbiol 62: 545–551.
9 Setlow B and P Setlow. 1993. Binding of small, acid-soluble spore proteins to DNA plays a significant role in the resistance of *Bacillus subtilis* spores to hydrogen peroxide. Appl Environ Microbiol 59: 3418–3423.
10 Shin SY and RE Marquis. 1994. Sporicidal activity of tertiary butyl hydroperoxide. Arch Microbiol 161: 184–190.
11 Shin SY, EG Calvisi, TC Beaman, HS Pankratz, P Gerhardt and RE Marquis. 1994. Microscopic and thermal characterization of hydrogen peroxide killing and lysis of spores and protection by transitional metal ions, chelators, and antioxidants. Appl Environ Microbiol 60: 3192–3197.
12 Slepecky RA and JW Foster. 1959. Alteration in metal content of spores of *Bacillus megaterium* and the effects on some spore properties. J Bacteriol 78: 117–123.