The Effect of Transition Metal Ions on the Resistance of Bacterial Spores to Hydrogen Peroxide and to Heat

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The presence of 10 μM - Cu^{2+} increased the lethal effect of hydrogen peroxide on spores of *Clostridium bifermentans* but not on those of *Clostridium sporogenes* PA 3679, *Clostridium perfringens*, *Bacillus cereus* or *Bacillus subtilis* var. *niger*. Cu^{2+} at 100 μM also increased the lethal effect of heat on spores of *C. bifermentans* but not on those of *B. subtilis* var. *niger*. The rate and extent of Cu^{2+} uptake by spores of *C. bifermentans* and *B. subtilis* var. *niger* were similar, but examination of unstained sections of spores by electron microscopy suggested that Cu^{2+} is bound by the protoplasts of spores of *C. bifermentans* but not of *B. subtilis* var. *niger*.

INTRODUCTION

Transition metal ions inhibit the germination of bacterial spores (Levinson & Sevag, 1953; Krishna Murty & Halvorson, 1957; Murakami, 1971). We have shown previously that addition of 10 μM - Cu^{2+} caused a fivefold increase in the lethal effect of H_2O_2 on spores of *Clostridium bifermentans* and that addition of other divalent metal ions also slightly increased the kill (Bayliss & Waites, 1976). Preliminary results suggested that such ions do not markedly increase the effect of H_2O_2 on spores of other species. This paper reports a more detailed study of the influence of metal ions on the resistance of spores of several species to H_2O_2 and to heat.

METHODS

Organisms, spore preparation and maintenance of cultures. The strains of *Clostridium bifermentans*, *Bacillus cereus* and *Bacillus subtilis* var. *niger* used and the methods of spore preparation were those described previously (Bayliss & Waites, 1976; Wyatt & Waites, 1975). *Clostridium sporogenes* PA 3679 and *Clostridium perfringens* NCTC 8238 were obtained from Dr G. C. Mead of this laboratory. Spores of *C. sporogenes* PA 3679 were prepared by growth at 33 °C under H_2/CO_2 (9:1, v/v) for 3 d on a medium containing (g l⁻¹): skim milk powder (Oxoid), 50; yeast extract (Difco), 3; peptone (Oxoid), 5; trypticase (BBL), 5; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.072; cysteine hydrochloride (BDH), 0.5; agar (Davis), 15; adjusted to a final pH of 7.0 to 7.2 with 1 M-NaOH. Spores of *C. perfringens* were prepared by growth at 33 °C for 48 h in the medium of Kim *et al.* (1967). Spores were washed five times with glass-distilled water at 4 °C before storing in glass-distilled water; those of *B. cereus* were stored at -20 °C and the remainder at 1 °C. The anaerobes were maintained in Robertson's cooked meat medium (double quantity meat, Southern Group Laboratories) and the aerobes on heart infusion agar (Difco).

Treatment of spores with metal ions, hydrogen peroxide, heat and urea/mercaptoethanol. Spores were treated at about 0.7 mg dry wt ml⁻¹. Unless otherwise stated, metal ions were pre-incubated with spores for 30 min at 37 °C. In some experiments the spores were then washed twice by centrifugation with glass-distilled water at 4 °C, but essentially the same results were achieved with or without washing.

Hydrogen peroxide (Analar grade, BDH) at the required concentration was added to spores in 100 mM-sodium phosphate buffer pH 7.0 which had been pre-incubated at the required temperature in polypropylene

Fig. 1. Effect of heat and Cu^{2+} on the colony-forming ability of spores of *C. bifermentans*. Spores were incubated with (●) or without (○) $100 \mu\text{M-Cu}^{2+}$ for 30 min at 37°C before centrifugation and washing as described in Methods. After resuspension in glass-distilled water, the spores were heated at 85°C and samples were removed at intervals for dilution and plating.

centrifuge tubes. Spores were also heated in glass-distilled water alone. After incubation, the tubes were cooled rapidly to 1°C and the contents were diluted immediately with glass-distilled water.

Spores were treated for 90 min at 37°C with 4 M-urea in 10% (v/v) mercaptoethanol, adjusted to pH 10.3 with 6 M-NaOH. After treatment, the spores were cooled rapidly to 1°C , centrifuged for 10 min at 15000 g and 4°C , and washed twice by further centrifugation with glass-distilled water.

Colony formation, germination and outgrowth. Colony formation of *B. cereus*, *B. subtilis* and *C. bifermentans* was examined as described previously (Wyatt & Waites, 1975). *Clostridium sporogenes* PA 3679 was plated on the reinforced clostridial medium of Hirsch & Grinsted (1954) solidified with 1.6% (w/v) agar (Davis), and *C. perfringens* on the trypticase/yeast extract (TCYS) medium of Adams (1973). Germination of *C. bifermentans* was measured spectrophotometrically with the 'Ala+' germination system (Bayliss & Waites, 1976) as described by Waites & Wyatt (1971) or, after treatment with urea/mercaptoethanol, with lysozyme (Wyatt & Waites, 1974) by counting 100 spores using a phase-contrast microscope and scoring dark (germinated) and bright (ungerminated) spores. Outgrowth of *C. bifermentans* was studied at 37°C in medium containing casein hydrolysate flushed with H_2/CO_2 (9:1, v/v) as described by Waites & Wyatt (1974).

Turbidity and spore dry weight. Samples were removed during outgrowth and their turbidity was measured at 600 nm (A_{600}). Dry weights were estimated by converting absorbance to spore dry weight using a standard calibration curve.

Measurement of Cu^{2+} in spores by atomic absorption spectroscopy. Spore suspensions were digested in 6 M-HCl and diluted for analysis. The copper content was determined using a Varian AA6 spectrophotometer with an oxidizing air/acetylene flame. Conditions used were a wavelength of 324.7 nm, a slit width of 0.5 nm, and a lamp current of 5.0 mA. Concentrations were determined from a calibration curve obtained with standard solutions of CuCl_2 (BDH, for atomic absorption spectroscopy).

Preparation of samples for electron microscopy. Spore suspensions were fixed with buffered 3% (v/v) glutaraldehyde for 3 h (without any subsequent fixation with osmium tetroxide) before washing overnight in buffer (Gordon *et al.*, 1963). Samples were then dehydrated and embedded, and sections were examined with or without staining with uranyl acetate and lead acetate as described previously (Waites *et al.*, 1972).

RESULTS

Effect of metal ions on the resistance of spores to hydrogen peroxide and to heat

The presence of $10 \mu\text{M-Cu}^{2+}$ during treatment of spores of *C. sporogenes* PA 3679, *C. perfringens*, *B. subtilis* or *B. cereus* with H_2O_2 failed to increase the lethal effect and even at $100 \mu\text{M}$, Cu^{2+} , Co^{2+} , Zn^{2+} , Fe^{2+} , Ni^{2+} , Mn^{2+} , Mg^{2+} or Ca^{2+} did not increase the kill of spores of *B. subtilis*.

Divalent metal ions such as Cu^{2+} increase the hydrolysis of peptides at 75°C (Hill, 1965) suggesting that they might also decrease the heat resistance of spores. The colony-forming ability of spores of *C. bifermentans* was reduced substantially during heating at 85°C after

Fig. 2. Effect of heat or H_2O_2 on the colony-forming ability of spores of *C. bifermentans* grown in the presence or absence of $100 \mu M$ - $CuSO_4$. Spores grown in the presence (●) or absence (○) of $100 \mu M$ - $CuSO_4$ were (a) heated at $85^\circ C$ or (b) incubated at $25^\circ C$ with H_2O_2 , before dilution and plating as described in Methods.

Fig. 3. Effect of heat and Cu^{2+} on the germination rate of spores of *C. bifermentans*. Spores were incubated with (●) or without (○) $10 \mu M$ - Cu^{2+} for 30 min at $37^\circ C$ before washing and heating at $70^\circ C$. Samples were removed at intervals and their germination rate with the 'Ala+' system was determined spectrophotometrically.

pre-incubation in $100 \mu M$ - Cu^{2+} (for example, about sixfold after heating for 75 min; Fig. 1). Addition of $100 \mu M$ - Cu^{2+} to the medium on which spores of *C. bifermentans* were produced reduced their resistance to heat by up to 15-fold and to H_2O_2 by up to 200-fold (Fig. 2). Heating spores of *B. subtilis* at $85^\circ C$ for 60 min in the presence or absence of $100 \mu M$ - Cu^{2+} produced the same number of survivors (48%).

To produce colonies, spores must germinate and outgrow to form vegetative cells which are able to divide. We therefore examined the effect of heat with and without added Cu^{2+} on the ability of spores of *C. bifermentans* to germinate and outgrow. Spores heated at $70^\circ C$ after pre-incubation with $10 \mu M$ - Cu^{2+} (Fig. 3) or with various divalent metal ions at $100 \mu M$ (Table 1) germinated more slowly with the 'Ala+' germination system than did spores heated in the absence of these ions. Spores incubated with $100 \mu M$ - Cu^{2+} at $1^\circ C$ outgrew at the same rate as spores incubated in the absence of Cu^{2+} , but spores heated at $75^\circ C$ with

Table 1. *Effect of divalent metal ions and heat on the germination rate of spores of C. bifermentans*

Spores of *C. bifermentans* were incubated for 5 min at 37 °C with the metal ion (100 μ M) indicated before centrifuging and washing. The spores were then resuspended in glass-distilled water and heated at 70 °C for 0, 30 or 60 min before germination with the 'Ala+' germination system.

Metal ion	Germination rate (% min ⁻¹)		
	After heating at 70 °C for:		
	Unheated	30 min	60 min
None	91	94	73
Cu ²⁺	101	16	3
Fe ²⁺	104	42	17
Zn ²⁺	97	59	34
Ni ²⁺	80	86	40
Co ²⁺	84	65	47
Mn ²⁺	87	67	52

Fig. 4. Effect of heat and Cu²⁺ on the germination and outgrowth of spores of *C. bifermentans*. Spores were heated at 75 °C for 10 min either with (■) or without (□) 100 μ M-Cu²⁺, and the subsequent changes in turbidity during incubation in outgrowth medium were compared with those for unheated spores pre-incubated with (●) or without (○) 100 μ M-Cu²⁺ at 1 °C for 60 min.

100 μ M-Cu²⁺ germinated and outgrew more slowly than spores heated in the absence of added Cu²⁺ (Fig. 4).

Spores treated with urea/mercaptoethanol germinated in the presence of lysozyme, but this germination was completely inhibited by pre-incubation with 100 μ M-Cu²⁺ and, to a lesser extent, by similar concentrations of other metal ions (Table 2). Pre-incubation with 100 μ M-Cu²⁺ before treatment with urea/mercaptoethanol did not affect germination initiated by lysozyme.

Uptake of Cu²⁺ by spores of C. bifermentans and B. subtilis

Incubation of spores of *C. bifermentans* and *B. subtilis* with 100 μ M-Cu²⁺ at 37 °C resulted in rapid uptake of Cu²⁺, about 30% of which was lost during germination (Table 3). Since about 30% of the spore dry weight is lost during germination (Gould, 1969), this result might suggest binding of Cu²⁺ equally throughout the spore. Washing ungerminated spores with 0.03 M-HCl removed about 90% of the Cu²⁺, while treating them with urea/mercaptoethanol removed more than 97 and 99% of Cu²⁺ from ungerminated spores of *C. bifermentans* and *B. subtilis*, respectively. Washing spores with HCl may remove metal ions from the spore coat (Murrell & Warth, 1965) and treatment with urea/mercaptoethanol ruptures

Table 2. *Effect of metal ions on germination with lysozyme of spores of C. bifermentans after treatment with urea/mercaptoethanol*

Spores of *C. bifermentans* were treated with urea/mercaptoethanol before washing as described in Methods, incubating with the metal ion indicated, washing and incubating at 37 °C with 100 µg lysozyme ml⁻¹ in the presence of 100 mM-sodium phosphate buffer pH 7.0. The percentage of dark (germinated) spores was determined after 120 min by counting 100 spores using a phase-contrast microscope.

Metal ion (µM)	Phase-dark spores after 120 min (%)
None	82
Cu ²⁺ (10)	62
Cu ²⁺ (20)	31
Cu ²⁺ (50)	27
Cu ²⁺ (100)	4
Zn ²⁺ (100)	29
Ni ²⁺ (100)	40
Fe ²⁺ (100)	65
Mn ²⁺ (100)	75
Co ²⁺ (100)	82

Table 3. *Uptake and subsequent loss of Cu²⁺ by spores of C. bifermentans and B. subtilis var. niger*

Spores of *C. bifermentans* and *B. subtilis* var. *niger* were incubated at 37 °C with 100 µM-Cu²⁺ and the Cu²⁺ present in the spores was measured at intervals as described in Methods. Spores pre-incubated at 37 °C with 100 µM-Cu²⁺ for 20 min were also (a) germinated with the 'Ala+' system, (b) washed by centrifugation with 0.03 M-HCl or (c) treated with urea/mercaptoethanol, before the Cu²⁺ present was determined.

Treatment	Cu ²⁺ present in spores (µg g ⁻¹)	
	<i>C. bifermentans</i>	<i>B. subtilis</i> var. <i>niger</i>
Incubated with 100 µM-Cu ²⁺ for (min):		
0	185	265
1	7200	6900
5	7500	7500
10	8300	8100
90	12700	9040
Preincubated with 100 µM-Cu ²⁺ then		
(a) germinated	9300	6050
or (b) washed with 0.03 M-HCl	1000	834
or (c) treated with urea/mercaptoethanol	300	35

disulphide bonds in the spore coat (Gould & Hitchins, 1963). It was probable, therefore, that Cu²⁺ was bound to the spore coat. However, examination of unstained electron micrographs of thin sections of spores showed that incubation with 100 µM-Cu²⁺ for 30 min at 37 °C, before washing with glass-distilled water, increased the electron density of the protoplasts of spores of *C. bifermentans* (Fig. 5a, c) but not those of spores of *B. subtilis* (Fig. 5b, d). The protoplasts of spores of *C. bifermentans* also became more electron dense when sections were incubated with 100 µM-Cu²⁺ but those of *B. subtilis* did not, suggesting differences in the availability of binding sites for Cu²⁺.

Staining with uranyl acetate and lead acetate after sectioning increased the electron density of spores of both species. With spores of *C. bifermentans* the protoplasts increased in electron density, but those not pre-incubated with Cu²⁺ remained much less electron dense (compare Fig. 6a and 6b), suggesting that the sites binding Cu²⁺ were different from those binding UO₂²⁺ and Pb²⁺. With spores of *B. subtilis* the coats and cortices increased in electron density but the protoplasts remained unstained.

Spores of *C. bifermentans* pre-incubated with 100 µM-Fe²⁺ showed electron-dense areas

Fig. 5. Effect of Cu^{2+} on the ultrastructure of spores. Spores of *C. bifermentans* (a, c) and *B. subtilis* (b, d) were incubated for 30 min at 37 °C with (a, b) or without (c, d) 100 μM - Cu^{2+} before washing with glass-distilled water, fixing with glutaraldehyde, embedding and sectioning as described in Methods. Bar markers represent 0.25 μm .

around the exosporia (Fig. 7a) or, where these were damaged or absent, around the coats (Fig. 7b). We have shown that the resistance of spores of *C. bifermentans* to H_2O_2 was only slightly decreased by the presence of Fe^{2+} (Bayliss & Waites, 1976). It is probable, therefore, that only those metal ions like Cu^{2+} which bind to protoplasts are able to decrease spore resistance.

DISCUSSION

We have shown that Cu^{2+} markedly increased the lethal effect of H_2O_2 (Bayliss & Waites, 1976) and of heat on spores of *C. bifermentans* but not on those of the other species examined, suggesting that certain sites in spores of *C. bifermentans* must be especially sensitive and/or accessible to Cu^{2+} . Spores of *B. subtilis* and *C. bifermentans* took up Cu^{2+} at about the same rate but examination of unstained sections of spores by electron microscopy suggested that

Fig. 6. Effect of Cu^{2+} on the ultrastructure of spores of *C. bifermentans* post-stained with uranyl acetate and lead acetate. Spores were incubated with (a) or without (b) $100 \mu\text{M-Cu}^{2+}$, as described for Fig. 5, before fixing, embedding and staining as described in Methods. Bar markers represent $0.25 \mu\text{m}$.

Fig. 7. Effect of Fe^{2+} on the ultrastructure of spores of *C. bifermentans*. Spores were incubated with $100 \mu\text{M-Fe}^{2+}$ before treating as described for Fig. 5. Bar markers represent $0.25 \mu\text{m}$.

Cu^{2+} was bound by the protoplasts of spores of *C. bifermentans* but not those of spores of *B. subtilis*. Since most of the metabolism of an outgrowing spore will be in the protoplast, such differences are consistent with the greater lethal effect of Cu^{2+} on *C. bifermentans*. The greater resistance to Cu^{2+} and H_2O_2 of *B. cereus*, *C. sporogenes* PA 3679 and *C. perfringens* would suggest that, as with *B. subtilis*, Cu^{2+} was not bound to the protoplasts. The inability

of Cu^{2+} to bind to protoplasts of spores of *B. subtilis* in section suggests that such differences depend more on the availability of metal-binding sites than on spore permeability. However, previous studies which have shown that metal ions can decrease the germination or viability of spores of other strains (Levinson & Sevag, 1953; Krishna Murty & Halvorson, 1957; Murakami, 1971; Wallen, 1976) suggest that metal ions may be able to bind to sensitive sites within spores of some species.

Examination of electron micrographs of thin sections of spores of *C. bifermentans* suggested that Cu^{2+} did not bind to the cortices. Beveridge & Murray (1976) found that the ability of Fe^{2+} and Cu^{2+} to stain isolated walls of vegetative cells of *B. subtilis* was diminished after digestion by lysozyme, suggesting that peptidoglycan in the wall bound both Fe^{2+} and Cu^{2+} . The peptidoglycan of the spore cortex is structurally similar, although not identical, to that of walls of vegetative cells (Tipper & Gauthier, 1972) so that the cortex might be expected to bind Cu^{2+} . If the cortex was highly expanded compared with the vegetative cell wall, as suggested by Gould & Dring (1975), fewer Cu^{2+} binding sites/unit area would result in an apparently unstained cortex. Lysozyme initiates germination-like changes by degrading the spore cortex (Gould & Hitchins, 1963). We have shown that Cu^{2+} prevented germination initiated by lysozyme, suggesting that metal ions can bind to the cortex but at too few sites to be detected by electron microscopy.

With spores of *C. bifermentans*, ions other than Cu^{2+} were less effective in potentiating the action of heat and H_2O_2 and inhibiting germination with lysozyme. Similarly, Fe^{2+} did not bind to spore protoplasts. Riemann (1961) and Albert (1950) showed that the stability constants of Cu-dipicolinic acid, Cu-EDTA and Cu-glycine were higher than for other metal ions. In our work the order of effectiveness of metal ions varied; for inhibition of germination by lysozyme ($\text{Cu}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Fe}^{2+} > \text{Mn}^{2+} > \text{Co}^{2+}$) the order was similar to the stability constants for metal-dipicolinic acid and metal-amino acid complexes ($\text{Cu}^{2+} > \text{Ni}^{2+} > \text{Zn}^{2+} > \text{Fe}^{2+} > \text{Mn}^{2+}$; Riemann, 1961; Albert, 1950) but those for potentiating the effect of H_2O_2 ($\text{Cu}^{2+} > \text{Fe}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+} > \text{Zn}^{2+}$; Bayliss & Waite, 1976) and heat ($\text{Cu}^{2+} > \text{Fe}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+} > \text{Mn}^{2+}$) were different. Since the action of metal ions on H_2O_2 is probably to catalyse free radical production (King & Gould, 1969), there is no reason to suggest that they bind to particular sites in the spore protoplast but it would be of interest to determine the sites at which Cu^{2+} acts to decrease heat resistance and inhibit germination by lysozyme.

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