The Effect of Chlorine on Spores of *Clostridium bifermentans*, *Bacillus subtilis* and *Bacillus cereus*

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(Received 24 February 1975)

SUMMARY

The effect of chlorine on the germination, outgrowth, colony formation and structure of spores of *Clostridium bifermentans*, *Bacillus subtilis* var. *niger* and *Bacillus cereus* was examined. Chlorine decreased heat resistance and slowed or prevented germination and swelling, but spores that did swell were usually able to elongate to form vegetative cells. Chlorine removed protein from spores, apparently from the coat, and allowed lysozyme to initiate germination. Treatment with other agents that remove spore-coat protein increased the lethal effect of chlorine by as much as 4000-fold, suggesting that coat protein protects spores against chlorine.

INTRODUCTION

Chlorine, often as hypochlorite, is widely used to kill bacteria and will rapidly oxidize proteins (for a review, see Trueman, 1971). Knox *et al.* (1948) first suggested that enzymes which contain SH-groups are especially sensitive to chlorine. Bacterial spores are more resistant to chlorine than vegetative cells (Phillips, 1952) but the reasons for this extra resistance are not understood. Most previous studies on spores have been confined to the effect of chlorine on colony formation. Recently, spores damaged by chlorine have been shown to lose dipicolinic acid during heating (Alderton & Halbrook, 1971; Dye & Mead, 1972). In this study, the strains used were: *Clostridium bifermentans*; a mutant of *C. bifermentans* which germinates more slowly than the parent strain (Wyatt & Waite, 1971); *Bacillus subtilis* var. *niger*; and *B. cereus*. We have investigated the stage at which the formation of vegetative cells from spores is blocked by chlorine treatment, and the reasons for the extra resistance of spores to chlorine.

METHODS

Organisms, spore preparation and maintenance of cultures. The strains of *Clostridium bifermentans* used (parent and mutant 1) and the preparation and storage of spores have been described previously (Waite & Wyatt, 1971; Wyatt & Waite, 1971). These organisms were maintained in the reinforced clostridial medium of Hirsch & Grinstead (1954). *Bacillus cereus* T, obtained from W. L. King, Unilever Research, Sharnbrook, Bedford, and *Bacillus subtilis* var. *niger*, from Dr J. Payne of this laboratory, were maintained on nutrient agar slopes and spores were produced by growth on potato agar plates as described by Gould, Stubbs & King (1970). After two to three days' incubation at 30 °C, the spores were collected and washed five (*B. cereus*) or ten (*B. subtilis*) times at 4 °C with glass-distilled water before resuspending in glass-distilled water and storing at 0 °C.

Treatment of spores with chlorine. Spores (about 3.5 mg dry wt) were centrifuged, resuspended in 5 ml of a solution of sodium hypochlorite (BDH) containing 250 mM-sodium

phosphate buffer pH 7.0, incubated for 10 min at 0 °C, and then washed with glass-distilled water as described previously (Wyatt & Waites, 1973). The strength of the hypochlorite solution was estimated as free chlorine; free chlorine was measured by the Palin method (Palin, 1957) with diethyl-*p*-phenylene diamine (DPD) tablets (BDH) and using the extinction coefficient at 553 nm.

Treatment of spores with dithiothreitol, urea-dithiothreitol or urea-mercaptoethanol. Spores (about 700 µg dry wt/ml) were treated with 200 µM-dithiothreitol, with 50 µM-dithiothreitol in 4 M-urea (Aronson & Fitz-James, 1971) or with 4 M-urea with 10 % (v/v) mercaptoethanol at pH 10.3 (Gould & Hitchins, 1963) and then washed four times with glass-distilled water by centrifugation at 4 °C.

Outgrowth, colony formation and germination. Outgrowth of *C. bifermentans* was tested at 37 °C in medium containing casein hydrolysate and flushed with H₂ + CO₂ (90:10, v/v), while colony formation was tested on reinforced clostridial medium solidified with 1.5 % (w/v) agar as described previously (Waites & Wyatt, 1974). Spores of *B. cereus* and *B. subtilis* were outgrown in heart infusion broth (Difco) and colony-forming ability determined by incubating for 2 days at 30 °C on nutrient agar (Oxoid) or reinforced clostridial medium (solidified with 1.5 % agar). Essentially the same results were achieved on both solid media. Colony counts were made in triplicate. During outgrowth, samples of 100 organisms were counted under a phase-contrast microscope and scored as phase-bright (refractile), phase-dark (non-refractile), swollen, or elongated spores. In some experiments spores were heated for 10 min, either at 80 °C for *B. cereus* and *B. subtilis*, or at 70 °C for *C. bifermentans* as described by Waites & Wyatt (1974), before examination of outgrowth, colony formation or germination. Germination was measured spectrophotometrically as described by Waites & Wyatt (1971). The concentrations of germinants which were the optimal for the species were (mM): *B. subtilis* – L-alanine (5.0), potassium chloride (100) and sodium phosphate buffer pH 7.5 (83); *B. cereus* – L-alanine (1.0), inosine (1.0), sodium chloride (100) and sodium phosphate buffer pH 7.5 (83); *C. bifermentans* – L-alanine (50), L-arginine (5), L-phenylalanine (5), L-lactate (25), sodium chloride (100) and sodium phosphate buffer pH 7.5 (83).

Polyacrylamide gel electrophoresis of spore extracts. Extracts were made from 14 mg dry wt of spores by treatment with 1 ml hypochlorite containing 10 mg free chlorine, with 1 ml 50 µM-dithiothreitol in 4 M-urea, 200 µM-dithiothreitol (Aronson & Fitz-James, 1971), or with 4 M-urea plus 10 % (v/v) mercaptoethanol (Gould & Hitchins, 1963), and added in a maximum volume of 0.2 ml containing 100 µg protein in 10 % (w/v) sucrose to polyacrylamide gels. Electrophoresis was carried out as described by Davis & Ornstein (1961) but without the large-pore gel, in vertical columns for about 60 min at 5 mA/running tube and 4 °C. Bromophenol blue (0.001 %, w/v) was used as a marker, and amido black (1.0 %, w/v) in acetic acid (7 %, v/v) as a stain. Destaining was with several changes of acetic acid (7 %, v/v) during 36 h at room temperature.

Spore dry weight. Dry weights were estimated spectrophotometrically. Extinction coefficients were measured at 600 nm and converted to spore dry weight using a standard calibration curve relating E_{600} to spore dry weight.

Protein estimations. These were made by the method of Lowry *et al.* (1951) with bovine plasma albumin fraction V (Armour Pharmaceuticals Ltd, Eastbourne) as a standard.

Fig. 1. Effect of chlorine and heat on the germination and outgrowth of spores of *B. subtilis*. Spores of *B. subtilis* were incubated in heart infusion broth (a) without chlorine treatment, (b) after treatment with 50 µg chlorine/ml as described in Methods, or (c) after treatment with 50 µg chlorine/ml and heating at 80 °C for 10 min. Samples were removed at intervals and the percentage of refractile (●), non-refractile (○), swollen (■) or elongated (□) spores determined by counting 100 organisms by phase contrast microscopy.

RESULTS

Effect of chlorine on germination, outgrowth and colony formation

The development of a vegetative bacterium from a bacterial spore involves loss of refractility (germination) followed by swelling and elongation of the germinated spore (outgrowth). With *B. subtilis* most untreated spores had lost refractility by 30 min, had swollen by 90 min, and had elongated to form vegetative organisms by 5 h (Fig. 1a). After treatment with 25 µg free chlorine/ml these processes were only slightly delayed but, while treatment with 50 µg free chlorine/ml markedly retarded outgrowth (Fig. 1b), 100 µg free chlorine/ml prevented germination within 6 h. A similar effect was obtained with spores of *B. cereus*, except that treatment with 50 µg free chlorine/ml prevented germination. With spores of *C. bifermentans*, treatment with 20 µg free chlorine/ml markedly delayed outgrowth (Fig. 2), while 50 µg free chlorine/ml allowed about 60% to germinate within 6 h but completely prevented swelling. After treatment with chlorine, the percentage of viable spores, measured by colony-forming ability, was quantitatively similar to the percentage of spores able to form vegetative bacteria except that in some experiments the appearance of colonies was delayed. A slow-germinating mutant of *C. bifermentans* whose germination was markedly increased by chlorine treatment (Wyatt & Waites, 1973) was also examined. Colony formation from spores of the mutant was less sensitive to chlorine than it was with the parent strain; for example, when treated with 10 µg free chlorine/ml for 10, 30 or 60 min,

Fig. 2. Effect of chlorine on the germination and outgrowth of spores of *C. bifermentans*. Spores of *C. bifermentans* were incubated in a medium containing casein hydrolysate, (a) without chlorine treatment or (b) after treatment with 20 µg chlorine/ml as described in Methods. Symbols as for Fig. 1.

Table 1. *Effect of heat on germination of spores after treatment with chlorine*

Spores of *C. bifermentans*, *B. cereus* and *B. subtilis* were treated with chlorine at the concentrations described and then germinated either with or without heating as described in Methods.

Chlorine concn (µg free chlorine/ml)	Germination rate (%/min)					
	<i>C. bifermentans</i>		<i>B. cereus</i>		<i>B. subtilis</i>	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
0	56	57	22	38	7.9	7.1
10	24	19	25	48	—	—
20	0.4	0.3	19	5.6	—	—
30	—	—	2.5	2.5	5.8	3.0
40	0.02	*	—	—	—	—
50	*	*	1.0	<0.2	3.3	1.5
100	*	*	—	—	0.002	0.001
150	—	—	—	—	<0.001	*

* Some spores had lost refractility before addition of germinants.

the number of colonies formed by each strain (expressed as a percentage of the colonies formed by untreated spores) was 35, 13 or 1.0% for the parent and 47, 43 or 35% for the mutant.

Effect of heat on spores treated with chlorine

Dye & Mead (1972) showed that colony formation by spores of *C. bifermentans* and *C. perfringens* which survived treatment with chlorine, was more sensitive to heat than that of untreated spores. In the present study with *B. subtilis*, *B. cereus* and *C. bifermentans*, after chlorine treatment had reduced the colonies formed to 1%, a heat treatment reduced the colonies formed to 0.2%. After less severe chlorine treatments heating did not reduce the percentage of survivors.

Chlorine-treated spores of *C. bifermentans* and *B. subtilis* germinated more slowly after heating, but those of *B. cereus* which require heat activation (Keynan *et al.* 1964) germinated more rapidly after heating following treatment with 10 µg free chlorine/ml (Table 1). Heating also slowed the elongation of spores of *B. subtilis* pretreated with 50 µg free chlorine/ml (Fig. 1c) but did not significantly delay outgrowth of spores treated with 25 µg or less of free chlorine/ml.

Fig. 3. Effect of chlorine on the colony-forming ability of spores pretreated with dithiothreitol or dithiothreitol-urea. Spores pretreated (closed symbols) or not (open symbols) with dithiothreitol (*C. bifermentans*, ■, □) or dithiothreitol-urea (*B. cereus*, ▲, △ and *B. subtilis* ●, ○) were treated with chlorine at the concentrations described and the percentage of spores able to form colonies determined as described in Methods.

Table 2. Effect of lysozyme on germination of spores of *C. bifermentans* after treatment with chlorine

Spores were treated with the concentrations of chlorine indicated, and then incubated either at 4 °C in glass-distilled water for 7 days, or at 37 °C for 20 h in sodium phosphate buffer (83 mM, pH 7.0) with or without lysozyme at 100 µg/ml.

Chlorine concn (µg free chlorine/ml)	Percentage of non-refractile spores after treatment with:		
	Glass-distilled water	Sodium phosphate buffer	Sodium phosphate plus lysozyme
0	2	1	6
10	3	7	39
20	2	8	69
40	14	98	78
50	51	—	—

Effect of chlorine on spore constituents

Dithiothreitol, dithiothreitol with urea, or urea with mercaptoethanol, have been shown to remove protein from spore coats without affecting colony formation (Aronson & Fitz-James, 1971). Pretreatment with such agents markedly reduced the ability of spores of *B. subtilis* and *B. cereus* to form colonies after subsequent treatment with chlorine; results for dithiothreitol with urea are shown in Fig. 3. With *C. bifermentans*, dithiothreitol had a similar effect (Fig. 3). The other pretreatments were not tested with *C. bifermentans* because they markedly reduced colony formation even in the absence of chlorine treatment. Polyacrylamide gel electrophoresis of protein extracted from spores of *B. subtilis*, *B. cereus* or *C. bifermentans*, by dithiothreitol-urea or a solution containing 10 mg free chlorine/ml,

Table 3. *Effect of lysozyme on colony formation after treatment of spores of C. bifermentans with chlorine*

Spores of *C. bifermentans* were treated with chlorine at the concentration described, spread on re-inforced clostridial medium solidified with 1.5 % agar containing 0, 1.0, 2.5 or 5.0 μg lysozyme/ml, and incubated as described in Methods.

Chlorine concn (μg free chlorine/ml)	$10^{-7} \times$ Colonies formed/ml, at lysozyme concn of (μg /ml):			
	0	1.0	2.5	5.0
0	92	100	93	80
10	24	24	29	24
50	3.5	4.8	4.9	4.1

produced an identical band to that extracted from spore coats of *C. bifermentans* by sodium hydroxide (Wyatt & Waite, 1971; Waite, Wyatt & Arthur, 1972), although other less dense bands similar to those demonstrated by Vary (1973) were also found in the dithiothreitol-urea extracts. Since agents which remove spore coat are known to allow germination by lysozyme (Gould, Stubbs & King, 1970; Aronson & Fitz-James, 1971; Wyatt & Waite, 1974), we examined the ability of lysozyme to promote germination of spores of *C. bifermentans* after treatment with chlorine (Table 2). Spores treated with 20 μg free chlorine/ml lost refractility with lysozyme but from the periphery only, the centre remaining refractile, while spores treated with 40 μg free chlorine/ml lost peripheral refractility on incubation in the absence of lysozyme, as did spores of *B. subtilis* treated with 100 μg free chlorine/ml. Heating increased twofold the number of spores of *C. bifermentans* which lost refractility with lysozyme after treatment with 10 μg free chlorine/ml.

Lysozyme (5 μg /ml) in the medium did not increase the colony formation of spores of *C. bifermentans* treated with 10 or 50 μg free chlorine/ml by more than 5 % (Table 3), and similar results were obtained with *B. subtilis* after treatment with 25 or 50 μg free chlorine/ml. It is apparent, therefore, that when chlorine damages the spore coat sufficiently to allow lysozyme to initiate germination, it usually also damages the spore sufficiently to prevent outgrowth of the germinated spore.

DISCUSSION

We have shown that chlorine prevents colony formation by spores of *B. subtilis*, *B. cereus* and *C. bifermentans* by preventing germination or swelling. After treatment with chlorine, germination, outgrowth and colony formation were all more sensitive to heat. Chlorine removed a protein from spores of *B. subtilis*, *B. cereus* and *C. bifermentans* which had the same electrophoretic mobility on polyacrylamide gel as the protein removed from spore coats of *C. bifermentans* by sodium hydroxide (Wyatt & Waite, 1971; Waite *et al.* 1972). Mild treatments with either sodium hydroxide or chlorine increase the germination rate of slow-germinating mutants of *C. bifermentans* (Wyatt & Waite, 1971, 1973), perhaps by removing protein and increasing the permeability of the spore to germinants, while the coats of spores treated with higher concentrations of chlorine were sufficiently permeable to allow lysozyme to promote germination. Such results, together with the loss of dipicolinic acid from spores after treatment with chlorine (Alderton & Halbrook, 1971; Dye & Mead, 1972) and the germination-like changes which occurred during incubation in water of spores treated with high concentrations of chlorine, suggest that chlorine disrupts first the spore coat and secondly layers nearer to the spore centre; at any rate

where chlorine inactivated the germination mechanism, even when lysozyme was able to initiate germination, most spores were damaged to the extent that further development was prevented.

Vary (1973) has shown that sodium hydroxide and other treatments which remove protein from spores of *Bacillus megaterium* also increase the germination rate and in some cases allow germination to occur with a wider variety of compounds. We have found similar effects with *C. bifermentans* (Waites *et al.* 1972). Neither we nor Vary have found that such procedures decrease the heat resistance of spores, in contrast to the effect of chlorine.

Germination and colony formation of the slowly germinating mutant 1 (Wyatt & Waites, 1973) were more resistant to chlorine than was the case with the parent strain. Germination of spores of mutant 1 was previously found to be more resistant to successive treatments with sodium hydroxide and lysozyme (Wyatt & Waites, 1974). Since sodium hydroxide and chlorine allow lysozyme to degrade the spore cortex by increasing the permeability of the spore coat, differences in the spore coat between the wild type and mutant 1 may account for differences in resistance to both chlorine and sodium hydroxide. We have found that treatments which remove spore-coat protein increase the lethal effect of chlorine on spores of *C. bifermentans*, *B. subtilis* and *B. cereus* by as much as 4000-fold (Fig. 3). These results suggest that the coat protects spores against chlorine. The coat may also protect spores against other chemicals, since Freese *et al.* (1970) showed that spores of *B. subtilis* which were deficient in cortex but had apparently normal coats were resistant to octanol. However, since spores treated with urea-mercaptoethanol are about 100 times more resistant than vegetative cells to chlorine (cf. Phillips, 1952), then either some remaining coat material protects the spores or the inner layers of the spore have properties which confer extra resistance.

We are grateful to Dr G. C. Mead and Dr J. L. Peel for useful discussions.

REFERENCES

- ALDERTON, G. & HALBROOK, W. U. (1971). Action of chlorine on bacterial spores. *Bacteriological Proceedings*, 12.
- ARONSON, A. I. & FITZ-JAMES, P. C. (1971). Reconstitution of bacterial spore coat layers *in vitro*. *Journal of Bacteriology* 108, 571-578.
- DAVIS, B. J. & ORNSTEIN, L. (1961). *Disc Electrophoresis*. Rochester, U.S.A.: Distillation Products Industries.
- DYE, M. & MEAD, G. C. (1972). The effect of chlorine on the viability of clostridial spores. *Journal of Food Technology* 7, 173-181.
- FREESE, E. B., COLE, R. M., KLOFAT, W. & FREESE, E. (1970). Growth, sporulation and enzyme defects of glucosamine mutants of *Bacillus subtilis*. *Journal of Bacteriology* 101, 1046-1062.
- GOULD, G. W. & HITCHINS, A. D. (1963). Sensitization of bacterial spores to lysozyme and to hydrogen peroxide with agents which rupture disulphide bonds. *Journal of General Microbiology* 33, 413-423.
- GOULD, G. W., STUBBS, J. M. & KING, W. L. (1970). Structure and composition of resistance layers in bacterial spore coats. *Journal of General Microbiology* 60, 347-355.
- HIRSCH, A. & GRINSTED, E. (1954). Methods for the growth and enumeration of anaerobic spore-formers from cheese with observations on the effect of nisin. *Journal of Dairy Research* 21, 101-110.
- KEYNAN, A., EVENCHIK, Z., HALVORSON, H. O. & HASTINGS, J. W. (1964). Activation of bacterial endospores. *Journal of Bacteriology* 88, 313-318.
- KNOX, W. F., STUMPF, P. K., GREEN, D. E. & AUERBACH, V. H. (1948). The inhibition of sulfhydryl enzymes as the basis of the bactericidal action of chlorine. *Journal of Bacteriology* 55, 451-458.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193, 265-275.
- PALIN, A. T. (1957). The determination of free and combined chlorine in water by the use of diethyl-p-phenylene diamine. *Journal of the American Water Works Association* 49, 873-880.

- PHILLIPS, C. R. (1952). Relative resistance of bacterial spores and vegetative bacteria to disinfectants. *Bacteriological Reviews* **16**, 135-138.
- TRUEMAN, J. R. (1971). The halogens. In *Inhibition and Destruction of the Microbial Cell*, pp. 137-183. Edited by W. B. Hugo. London: Academic Press.
- VARY, J. C. (1973). Germination of *Bacillus megaterium* spores after various extraction procedures. *Journal of Bacteriology* **116**, 797-802.
- WAITES, W. M. & WYATT, L. R. (1971). Germination of spores of *Clostridium bifermentans* by certain amino acids, lactate and pyruvate in the presence of sodium or potassium ions. *Journal of General Microbiology* **67**, 215-222.
- WAITES, W. M. & WYATT, L. R. (1974). The outgrowth of spores of *Clostridium bifermentans*. *Journal of General Microbiology* **84**, 235-244.
- WAITES, W. M., WYATT, L. R. & ARTHUR, B. (1972). Effect of alkali treatment on the germination and morphology of spores of *Clostridium bifermentans*. In *Spores V*, pp. 430-436. Edited by H. O. Halvorson, R. Hanson and L. L. Campbell. Washington, D.C.: American Society for Microbiology.
- WYATT, L. R. & WAITES, W. M. (1971). Studies with spores of *Clostridium bifermentans*: comparison of germination mutants. In *Spore Research-1971*, pp. 123-131. Edited by A. N. Barker, G. W. Gould and J. Wolf. London: Academic Press.
- WYATT, L. R. & WAITES, W. M. (1973). The effect of hypochlorite on the germination of spores of *Clostridium bifermentans*. *Journal of General Microbiology* **78**, 383-385.
- WYATT, L. R. & WAITES, W. M. (1974). The effect of sodium hydroxide or dithiothreitol-urea on spores of germination mutants of *Clostridium bifermentans*. *Journal of General Microbiology* **84**, 391-394.