The Effect of Hypochlorite on the Germination of Spores of *Clostridium bifermentans*

By LINDA R. WYATT AND W. M. WAITES
*Agricultural Research Council Food Research Institute,
Colney Lane, Norwich, NOR 70F*

(Received 6 June 1973)

Spores of *Clostridium bifermentans* are more sensitive to chlorine, added as sodium hypochlorite, than spores of other clostridia or of *Bacillus subtilis* (Dye & Mead, 1972). Spores of *C. bifermentans* or *B. subtilis*, damaged with chlorine, release dipicolinic acid (Dye & Mead, 1972; Alderton & Halbrook, 1971) and may therefore have increased permeability. We have isolated mutants of *C. bifermentans* which germinate more slowly than the parent strain, possibly because of reduced permeability to germinants (Wyatt & Waite, 1971). In view of these findings and the frequent use of chlorine as a disinfectant, we examined the effect of hypochlorite on the germination of mutant and parent spores.

METHODS

The parent strain of *Clostridium bifermentans*, the mutants derived from it and the preparation of spores were as described by Wyatt & Waite (1971). For treatment with hypochlorite, a suspension containing about 3.5 mg dry wt spores was centrifuged at 15000 g for 10 min and the spore pellet resuspended in 5 ml of a solution of 100 µg sodium hypochlorite/ml (B.D.H. Ltd, Poole) containing 50 µg free chlorine. The suspension was incubated for 10 min at 0 °C and centrifuged at 35000 g for 3 min, and the spores were washed once in 10 ml glass-distilled water and stored at 4 °C and then resuspended in 15 ml glass-distilled water and stored at 0 °C until required for measurement of germination rates.

Free chlorine was measured by the Palin method (Palin, 1957) using DPD tablets (B.D.H. Ltd), the extinction being read at 553 nm.

Germination rates were measured at 37 °C either spectrophotometrically or microscopically as described by Waite & Wyatt (1971). Results are expressed as the maximum rate of decrease in E_{600} calculated as a percentage of the initial extinction. The microscopic method was used when the germination rate was less than 0.4 and the rates calculated as described by Wyatt & Waite (1971). Germination was in NaCl (100 mM) and organic germinants as described. Germinant concentrations were (mM): L-alanine (Ala), 50; L-arginine (Arg), 5; L-phenylalanine (Phe), 5; L-lactate, 25; and glycine (Gly), 50. In addition all the germinant mixtures contained sodium phosphate (83 mM), pH 8.0 (with Ala), pH 6.25 (with Gly + Arg + Phe + L-lactate), or pH 7.5 (with all the other germinant mixtures).

RESULTS

Effect of hypochlorite on germination. The germination rate of spores of the wild-type was generally decreased slightly by pre-incubation at 0 °C with hypochlorite containing 10 µg free chlorine/ml, although the rate with L-alanine alone was almost doubled (Table 1).

Table 1. *Effect of hypochlorite on the germination of spores of wild-type and mutants of Clostridium bifermentans*

The germination rates of spores treated with sodium hypochlorite were compared with those of untreated spores as described in Methods.

Mutant	Treatment	Germination rate (%/min)					
		With Ala	With Ala	With Ala	With Ala	With Ala	With Gly
			+ Arg + Phe	+ Phe + L-lactate	+ Arg + L-lactate	+ Arg + Phe + L-lac tate	+ Arg + Phe + L-lactate
Wild-type	No hypochlorite	15	44	46	50	54	33
	With hypochlorite	29	39	35	41	40	28
1	No hypochlorite	0.01	1.8	6.7	2.1	11	6.9
	With hypochlorite	2.0	23	0.7	0.9	33	9.6
6	No hypochlorite	< 0.001	< 0.001	0.05	0.001	0.4	< 0.001
	With hypochlorite	0.002	0.9	4.6	1.8	4.8	< 0.001
3	No hypochlorite	< 0.001	< 0.001	0.04	0.001	0.5	< 0.001
	With hypochlorite	< 0.001	0.7	8.0	3.5	10	0.001

However, with four out of six of the germinant systems the rates of the mutants were increased by up to 3500 times and in some cases approached those of the wild-type. The greater increases generally occurred with germination systems containing Ala + Arg + Phe or Ala + Arg + L-lactate, and increases were small or absent when germination was with Gly + Arg + Phe + L-lactate. The presence of 100 or 250 mM-sodium phosphate (pH 7.0) during incubation with hypochlorite did not alter the effect on germination rates although the pH at the beginning of the incubation was dependent on the concentration of buffer. During the incubation the pH changed from 8.0 to 7.3 (unbuffered), 6.8 to 6.3 (100 mM) or remained at 6.8 (250 mM). The concentration of free chlorine decreased during the incubation from 10 µg/ml to less than 5 µg/ml.

DISCUSSION

Incubation with a solution of sodium hypochlorite increased the germination rate of spores of mutants of *Clostridium bifermentans* by up to 3500-fold and in some cases to about that of the wild-type. An iodophor increased colony formation by spores of *Bacillus subtilis* (Cousins & Allan, 1967) and the oxidizing agent, sodium perborate, increased the germination rate of spores of *B. cereus* (Cochran & Ordal, 1973). We have found that hypochlorite treatment results in about a 60% decrease in viable spores and Dye & Mead (1972) reported that similar concentrations of free chlorine rapidly killed spores of *C. bifermentans*. Previously we have produced more uniform and slightly greater increases in germination rate by treatment of spores of the mutants with 0.1 M-sodium hydroxide at 4 °C. Such treatment removes protein (Wyatt & Waites, 1971), possibly from the spore coat, and may increase the permeability of the spores to germinants (Waites, Wyatt & Arthur, 1972). Hypochlorite may act in a similar way, and this would explain the release of dipicolinic acid after chlorine treatment (Alderton & Halbrook, 1971; Dye & Mead, 1972).

The efficiency of chlorinating agents as disinfectants may be due, in part, to stimulation of spore germination followed by inactivation of the germinated spore.

The germination rates of the wild-type were slightly decreased by the hypochlorite in contrast to the effect on the mutants. We have found that the wild-type is more sensitive

than the mutants to lysozyme after incubation with sodium hydroxide (unpublished). This difference and the greater inhibition of the germination of the wild-type by hypochlorite may be due to differences between the spore coats of the wild-type and the mutants. The spore coat may act as a barrier to hypochlorite. Of the species examined by Dye & Mead (1972), spores of *Bacillus subtilis* were the most resistant to hypochlorite and loss of viability was preceded by a pronounced lag. If the spore coat is associated with hypochlorite resistance then such a result might be expected with spores with thick coats like those of *B. subtilis* (Wood, 1972), while spores of *Clostridium bifermentans*, which have much thinner coats (Waites *et al.* 1972), might be expected to be more sensitive to hypochlorite. Spores of *B. subtilis* deficient in cortex but with apparently normal spore coats are resistant to octanol (Fukuda & Gilvarg, 1968) and spore coats thus play a role in the resistance of spores to surfactants. Removal of spore-coat material might then be expected to make spores more sensitive to hypochlorite. Sodium hydroxide removes protein from spore coats of *B. subtilis* (Wood, 1972), and Cousins & Allan (1967) have shown that hypochlorite and sodium hydroxide act synergistically to kill spores of *B. subtilis*.

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**.
- COCHRAN, S. A. & ORDAL, Z. J. (1973). Oxidative activation of *Bacillus cereus* spores. *Applied Microbiology* **25**, 173-179.
- COUSINS, C. M. & ALLAN, C. D. (1967). Sporocidal properties of some halogens. *Journal of Applied Bacteriology* **30**, 168-174.
- DYE, M. & MEAD, G. C. (1972). The effect of chlorine on the viability of clostridial spores. *Journal of Food Technology* **7**, 173-181.
- FUKUDA, A. & GILVARG, C. (1968). The relationship of dipicolinate and lysine biosynthesis in *Bacillus megaterium*. *Journal of Biological Chemistry* **243**, 3871-3876.
- 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.
- 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. & ARTHUR, B. (1972). Effect of alkali treatment on the germination and morphology of spores of *Clostridium bifermentans*. In *Spores*, vol. 5, pp. 430-436. Edited by H. O. Halvorsen, R. Hanson and L. L. Campbell. Washington, D.C.: American Society for Microbiology.
- WOOD, D. A. (1972). Sporulation in *Bacillus subtilis*. Properties and time of synthesis of alkali-soluble protein of the spore coat. *Biochemical Journal* **130**, 505-514.
- WYATT, L. R. & WAITES, W. M. (1971). Studies with spores of *Clostridium bifermentans*; comparison of germination mutants. In *Spore Research 1971*, pp. 123-132. Edited by A. N. Barker, G. W. Gould and J. Wolf. London: Academic Press.