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

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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 & Waites, 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 & Waites (1971). For treatment with hypochlorite, a suspension containing about 3.5 mg dry wt spores was centrifuged at 15,000 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 35,000 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 Waites & 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 & Waites (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 I. 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.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Treatment</th>
<th>With Ala + Arg</th>
<th>With Ala + Phe</th>
<th>With Ala + L-lactate</th>
<th>With Ala + Arg + Phe + L-lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>No hypochlorite</td>
<td>15</td>
<td>44</td>
<td>46</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>With hypochlorite</td>
<td>29</td>
<td>39</td>
<td>35</td>
<td>41</td>
</tr>
<tr>
<td>1</td>
<td>No hypochlorite</td>
<td>0-01</td>
<td>1-8</td>
<td>6-7</td>
<td>2-1</td>
</tr>
<tr>
<td></td>
<td>With hypochlorite</td>
<td>2-0</td>
<td>2-3</td>
<td>0-7</td>
<td>0-9</td>
</tr>
<tr>
<td>6</td>
<td>No hypochlorite</td>
<td>&lt; 0-001</td>
<td>&lt; 0-001</td>
<td>0-05</td>
<td>0-001</td>
</tr>
<tr>
<td></td>
<td>With hypochlorite</td>
<td>0-002</td>
<td>0-9</td>
<td>4-6</td>
<td>1-8</td>
</tr>
<tr>
<td>3</td>
<td>No hypochlorite</td>
<td>&lt; 0-001</td>
<td>&lt; 0-001</td>
<td>0-04</td>
<td>0-001</td>
</tr>
<tr>
<td></td>
<td>With hypochlorite</td>
<td>&lt; 0-001</td>
<td>0-7</td>
<td>8-0</td>
<td>3-5</td>
</tr>
</tbody>
</table>

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 approach 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.

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*.

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REFERENCES


