Influence of Transition Metals Added during Sporulation on Heat Resistance of Clostridium botulinum 113B Spores

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Sporulation of Clostridium botulinum 113B in a complex medium supplemented with certain transition metals (Fe, Mn, Cu, or Zn) at 0.01 to 1.0 mM gave spores that were increased two to sevenfold in their contents of the added metals. The contents of calcium, magnesium, and other metals in the purified spores were relatively unchanged. Inclusion of sodium citrate (3 g/liter) in the medium enhanced metal accumulation and gave consistency in the transition metal contents of independent sporum crops. In citrate-supplemented media, C. botulinum formed spores with very high contents of Zn (~1% of the dry weight). Spores containing an increased content of Fe (0.1 to 0.2%) were more susceptible to thermal killing than were native spores or spores containing increased Zn or Mn. The spores formed with added Fe or Cu also appeared less able to repair heat-induced injuries than the spores with added Mn or Zn. Fe-Increased spores appeared to germinate and outgrow at a higher frequency than did native and Mn-increased spores. This study shows that C. botulinum spores can be sensitized to increased thermal destruction by incorporation of Fe in the spores.

The safety of low-acid canned foods depends on a thermal treatment sufficient to give a 12 decimal reduction of spores of group 1 Clostridium botulinum (11, 27). Few practical methods are available that sensitize clostridial spores to heat. Alderton and co-workers (1–4) found that spores can be heat sensitized by removal of metals by acid treatment. Heat resistance was regained by replacement of metals at increased pH. Heat sensitization of spores by acid stripping has been confirmed and expanded by other researchers (6, 9, 21, 23).

Sporulation in C. botulinum is influenced by transition metals, especially Zn and Cu (15). In the anaerobic growth environments of clostridia, transition metals would be expected to have important roles in the sporulation and resistance properties of spores. Metals that undergo redox changes (including Fe, Mn, and Cu) tend to precipitate as the oxides or hydroxides in aerobic environments, but they are more soluble and biologically available at low redox potentials. During metabolism, clostridia also produce organic acids that assist in solubilizing metals and making them available for growth and incorporation into spores. There is little published information describing quantitative analyses and physiological variations of the metal contents of purified C. botulinum spores. In this study, we show that C. botulinum accumulates high concentrations of certain transition metals during sporulation, particularly Zn, and also that incorporation of certain metals results in spores having widely different thermal resistances.

MATERIALS AND METHODS

Organism and sporulation. C. botulinum 113B (group 1, proteolytic) was used throughout this study. Its nutritional requirements and methods of culture were previously described (29). Spores were produced and purified to ≥98% purity as previously reported (15).

Spore analyses and heat resistance determinations. Spores were enumerated and analyzed for metals as described earlier (15). Heat resistance profiles were determined by two independent methods.

(i) Method 1 (performed at the Food Research Institute, University of Wisconsin). A 1-ml sample of purified, cleaned, and counted spores in distilled water was pipetted at 1.0 × 10^6 to 3.0 × 10^6 spores per ml into thin-walled, glass 20-ml ampoules, which were sealed with a bunsen burner. More thermal kills were done with cleaned and purified spores. However, to determine whether the cleaning process influenced the rate of kill, uncleaned preparations were also used. Thermal resistances were determined by heating ampoules of spores at 110°C in an oil bath. Duplicate ampoules were removed at 2.5, 5, 7.5, and 10 min and immediately cooled on ice. Heated spores were diluted in 100 mM sodium phosphate buffer (pH 7.1) containing 0.85% sodium chloride (phosphate-buffered saline) and plated on C. botulinum isolation agar consisting of the following (per liter): Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 40 g; Na_2HPO_4, 5.0 g; NaCl, 2.0 g; MgSO_4, 0.2 ml of a 5% solution; glucose, 2.0 g; yeast extract, 5.0 g; distilled water, 900 ml; egg yolk suspension, 100.0 ml (from three egg yolks mixed in 50 ml of sterile phosphate-buffered saline); agar, 20 g. Since the egg yolk agar is difficult to prepare, modified PA3679 agar (MPA) (see below) was used in most experiments. The plates were incubated for 2 to 14 days (see Results) in an anaerobic chamber (80% nitrogen, 10% carbon dioxide, 10% hydrogen), and the colonies were counted.

(ii) Method 2 (performed at Campbell Soup Co., Camden, N.J.). An independent method was used to accurately determine D and Z values (see below) for native spores or for spores produced in media containing 1 mM Mn or 1 mM Fe. To determine the heat resistances, 0.5 ml of heat-shocked (80°C for 15 min) spore suspensions (~1.5 × 10^7 spores per ml) were thermally processed at 92.2°C (198°F), 95°C (203°F), and 100°C (212°F) by fully immersing the tubes in a water bath. Heat resistances were also determined at 104.4°C (220°F), 107.2°C (225°F), and 110°C (230°F) under pressurized steam in a thermal death time retort. The heated spore suspensions were cooled on ice and serially diluted in 0.5% peptone–water, and 1 or 0.1 ml of the dilutions was

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dispensed in petri dishes. The spores were overlaid with MPA medium (13) at 55°C, allowed to solidify, and incubated for 6 days at 35°C in a Coy Anaerobic chamber in an atmosphere of 10% CO₂, 5% H₂, and 85% N₂. The viable counts per millilitre were converted to log₁₀ units and plotted as a function of time at a specified temperature. The slope in the linear portion of the plot was used to determine the D values (time at a specified temperature to reduce the spore population by 90% or 1 log), and Z values (number of Fahrenheit degrees for thermal destruction curve to change 1 log unit) were calculated from a plot of D values as a function of temperature.

RESULTS

Production of spores in media containing transition metals. C. botulinum sporulated well in media containing 0.01 to 1.0 mM Fe, Cu, Mn, or Zn. Final concentrations of free endospores ranged from 1.9 × 10⁷ to 6.9 × 10⁹ (Table 1). On preparation of independent batches of spores, we found that considerable variation occurred from batch to batch in the contents of transition metals accumulated from the media. The addition of sodium citrate (3 g/liter) to the medium, however, resulted in consistent metal accumulation and rates of thermal destruction of the spores (see below).

Analyses of purified spore crops by inductively coupled plasma emission spectrometry (15) indicated that there were substantial changes in the metal contents of spores grown with elevated levels of metals in the medium. The C. botulinum spores under all conditions accumulated high concentrations of Ca and Zn. Supplementation of the medium with 0.01 or 1.0 mM Zn resulted in spores containing even higher contents of Zn (Table 1). These data indicate that C. botulinum has a high avidity for Zn and that spores, especially those produced in media containing sodium citrate, have very high contents of Zn. Supplementation with Fe or Cu gave spores that had increased contents of these metals, but their contents were still much less than Zn. Mn supplementation did not give a significantly higher Mn content in the spores.

Influence of metals on spore germination and outgrowth. Throughout this study the percentage of heat-shocked spores that germinated and formed colonies was determined and compared with microscopic counts. Considerable variability in percent spore germination and outgrowth was observed among spore batches. However, Fe-increased spores appeared to germinate and outgrow more frequently than did Mn-increased spores. Fe spores were also more susceptible to thermal destruction as described below.

Heat resistance of spores prepared in the metal-supplemented media. The spores produced in the metal-supplemented media were tested for heat resistance. To determine whether the cleaning and washing procedure affected heat sensitivity, spores were heat killed at various times during the purification procedure. No differences in rates of thermal destruction were observed for unwashed, washed, and Renografin-purified spores (data not shown). To determine the variation between individual thermal kills, independent kills were done in triplicate. There was only slight variation among triplicate samples, and the difference between metal types was significant. The reproducibility of heat resistance for spores grown on Zn and Fe are shown in Fig. 1.

The spores produced in the metal-supplemented media differed considerably in their heat sensitivities.

![FIG. 1. Rates of thermal killing at 110°C for triplicate samples of C. botulinum 113B spores produced in medium containing 1 mM ZnSO₄ (●) or FeSO₄ (○).](http://aem.asm.org/)

**TABLE 1.** Spore formation and metal contents of spores produced in media supplemented with transition metals

<table>
<thead>
<tr>
<th>Metal added in sporation medium (mM)</th>
<th>Final no. of spores/ml</th>
<th>Conc of metal in spores (μg/g of dry wt)</th>
<th>Conc of metal in medium (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ca</td>
<td>Mg</td>
</tr>
<tr>
<td>None (native)</td>
<td>2.4 × 10⁷</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn (0.01)</td>
<td>2.4 × 10⁷</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn (1.0)</td>
<td>8.3 × 10⁷</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe (0.01)</td>
<td>3.9 × 10⁷</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe (1.0)</td>
<td>2.8 × 10⁷</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn (0.01)</td>
<td>3.1 × 10⁷</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn (1.0)</td>
<td>6.9 × 10⁷</td>
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<tr>
<td>Cu (0.01)</td>
<td>7.6 × 10⁷</td>
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</tr>
<tr>
<td>Cu (1.0)</td>
<td>1.9 × 10⁷</td>
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</table>

Spore formation and metal contents of spores produced in media supplemented with transition metals.

**Notes:**

- Metals were dissolved in 0.1 N HCl and filter sterilized, and 5 ml of the metal solution was added to 4.3 liters of medium. This addition did not alter the initial or final pH of the culture. Sodium citrate (3 g/liter) was added to all media.

- Native spores.

- NA, Not available.
grown in Fe and Cu were consistently more susceptible to thermal inactivation than were spores grown in Mn or Zn (Fig. 2). Native spores (sporulated in medium without added metals) and heat resistances very similar to those of spores grown in Mn and Zn. Quantitative determination of the heat resistance of native spores and Fe- and Mn-supplemented spores at several different temperatures indicated that native and Mn-supplemented spores had similar heat resistances at each temperature tested, but the Fe-supplemented spores were considerably more sensitive (Fig. 3). At 110°C (230°F), the native and Mn-supplemented spores had D values of 0.9 and 0.8 min, respectively, whereas the D value for Fe-supplemented spores could not be accurately determined owing to their rapid rate of destruction (the $D_{110^\circ C}$ was estimated to be 0.4 min). At lower temperatures the same pattern was observed (Fig. 3), but the difference in heat resistance between native and Mn- and Fe-supplemented spores was increased. For example, the $D_{107.2^\circ C}$ (225°F) values were 1.4, 1.0, and 0.4 min for native, Mn-supplemented, and Fe-supplemented spores; the $D_{100^\circ C}$ (212°F) values were 7.0, 7.5, and 1.1 min; and the $D_{90^\circ C}$ (203°F) values were 36.3, 47.8, and 3.8 min. A $D_{91.1^\circ C}$ value of 16.7 min was found for the Fe-supplemented spores (data not shown); the $D_{91.1^\circ C}$ values for native and Mn-supplemented spores were not determined but were estimated to be approximately 100 min. These quantitative determinations clearly showed that the Fe-increased spores were sensitized to thermal inactiva-
tion and that there was little difference in rates of kill between the native and Mn-supplemented spores.

The D values were plotted logarithmically as a function of temperature to determine the Z values (Fig. 4). The Z values determined from the linear portion of the plots for the native and Mn- and Fe-supplemented spores were 12.5, 11.5, and 12.5°F, respectively.

Recovery of colonies on extended incubation. After heat treatments, spores were plated on MPA and incubated for 2 to 14 days in an anaerobic chamber, and colonies were counted after various durations of incubation. Colony counts increased fivefold on extended incubation after 5 and 10 min of heating for Zn-supplemented spores but not for Fe- and Cu-supplemented spores (data not shown). These results suggested that Mn- and Zn-supplemented spores but not the Fe-supplemented spores were able to repair thermal injuries.

DISCUSSION

Development of methods to accelerate the destruction of C. botulinum spores during thermal processing could potentially reduce the thermal processing requirements and improve the organoleptic and nutritional properties of foods. We found that certain transition metal ions, particularly Fe and Cu, when incorporated during sporulation, increased the rates of thermal destruction.

Several investigators have shown that there is considerable variation in ions incorporated into spores depending on the mineral composition of the medium (5, 7, 10, 16, 17, 19, 26; see references 20 and 22 for reviews). Amaha and Ordal (5) found that Ca and Mn in the medium increased the heat resistance of spores of Bacillus coagulans subsp. thermoacidurans. Slepecky and Foster (26) and Levinson and Hyatt (17) demonstrated that sporulating cultures of Bacillus megaterium have a strong tendency to accumulate metals, including Ca, Zn, Mn, Ni, and Cu, and that the ion contents of the spores affected heat resistance. Aoki and Slepecky (7) showed that the inclusion of Mn ions in the medium of Bacillus fastidiosus increased heat resistance and dormancy. Nearly all studies on the influence of mineral nutrition have been done with Bacillus spp., and there is little information available for the clostridia. In this study we found that C. botulinum spore mineral composition and heat resistance depended critically on the minerals available in the growth medium. In particular, inclusion of Fe and Cu sensitized C. botulinum spores to heat.

The mechanism by which Fe and Cu accelerate heat inactivation of the spores is not known. The inability of heated Fe or Cu spores to grow on extended incubation suggests that destruction of a lytic enzyme with consequent delayed germination (1, 7) is not the cause of the greater thermal susceptibility found in this study. Moreover, MPA and egg yolk agar used for spore recovery contain lysozyme to facilitate germination.

Determination of the location of the added metals in the spores would help us to understand the mechanism by which the metals decrease thermal resistance. If Fe and Cu are mostly located on the surface, then killing may result from destruction of the cortex peptidoglycan polymer (12). Little information is available concerning the location of transition metals in clostridial spores. Johnstone et al. (14) reported that Mn (99%) is located in the core of B. megaterium, whereas Zn (98%) is located in the coats. The location of iron and copper was not determined. Rosson and Nealon (24) reported that spores of a marine Bacillus sp. bound and oxidized manganese on the spore surface. If the Fe and Cu are internalized in C. botulinum spores, then they may react with essential macromolecules. Fe and Cu are redox active transition metals and may catalyze hydrolytic reactions (30) and also spontaneously autoxidize, generating free radicals (18). Free radicals damage DNA (18) and other important cellular components. Similarly, Zn and Mn can catalyze hydrolytic reactions (30), but incorporation of these ions did not accelerate thermal inactivation in this study. Mn and Zn are well known to associate with nucleic acids and can provide protection against alterations. Mn was demonstrated to protect DNA in vitro against heat denaturation (28). In certain biological systems Mn is also an effective scavenger of free radicals (8). Understanding the mechanism of Fe- and Cu-enhanced inactivation will require determination of their location and identification and characterization of damaged cellular components.

During examination of the metal contents of spores, we unexpectedly found that C. botulinum spores from cultures grown in medium containing sodium citrate contain very high contents of Zn, comparable to the content of Ca present. High contents of Zn may be a characteristic peculiar to C. botulinum spores. Vegetative cells of C. botulinum 113B effectively scavenge Zn from the medium. Uptake is enhanced by inclusion of sodium citrate in the growth medium. High concentrations of Zn in spores is apparently not a property of Bacillus spores. In Bacillus cereus, Ca comprises 2.5% of the dry weight, whereas Zn comprises only 0.08% (25). Similarly, spores of Bacillus steatorrhophilus contain 0.02 to 0.2% Zn, and Ca comprises 4.2 to 15.9% of the dry weight. Analyses of the spores of other Bacillus species have confirmed that Zn is a relatively minor mineral component (14, 16, 19, 20, 22). Determination of whether the presence of high levels of Zn is a property of other Clostridium species will require further studies.

ACKNOWLEDGMENTS

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