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Proposed Role of Lactate in Germination of Hypochlorite-Treated *Clostridium botulinum* Spores†

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Clostridium botulinum 12885A spores treated with hypochlorite required added DL-calcium lactate for L-alanine germination. Lactate was the active component of calcium lactate. Equimolar concentrations of L-malate, but not of DL-propionate, could replace lactate, suggesting that the alpha-hydroxy acid structure is important. Neither lactate nor malate was an effective germinant for buffer-treated or hypochlorite-treated spores. If the L-alanine concentration was increased 100-fold (to 450 mM), the lactate germination requirement was overcome. The data suggest that the L-alanine germination sites were modified by hypochlorite so that a higher concentration of alanine was required for activity. Lactate appeared to be an activator of modified or non-hypochlorite-modified L-alanine germination sites.

A previous study (2) in our laboratory suggested that hypochlorite treatment of *Clostridium botulinum* spores may injure the spores so that they are not enumerated by a most probable number procedure with modified peptone colloid medium but are enumerated by a colony count procedure with modified peptone yeast extract glucose agar. Buffer-treated spores were enumerated essentially equally by both procedures. Other investigators have suggested that the germination responses of spores of *Bacillus* spp. or *Clostridium* spp. may be altered by hypochlorite treatment as well (2, 9, 10). *C. botulinum* spores which have been injured by hypochlorite require DL-calcium lactate for L-alanine germination, whereas buffer-treated spores do not (2). Waites and Wyatt (6) have reported that the germination of *C. bifermentans* spores in suboptimal L-alanine concentrations is stimulated by lactate. The objective of this study was to provide information on the role of lactate in the germination of hypochlorite-treated spores so that knowledge of the effect of hypochlorite on *C. botulinum* spores would be enhanced.

MATERIALS AND METHODS

Spores. *C. botulinum* 12885A was used in these experiments. The culture origin, spore crop preparation, storage (4°C, distilled water), trypsin plus lysozyme cleaning, and heat activation (80°C, 15 min) have been detailed previously in this journal (2).

Hypochlorite treatment. Samples of heat-activated spores were treated with hypochlorite in 100 mM potassium phosphate buffer, pH 7.0, for 2 min at 25°C as detailed previously (2).

Germination procedures. Germination medium components, their concentrations, and the codification system are detailed in Table 1. All media ranged from pH 7.27 to 7.40. The preparation of germination media and the experimental method have been described previously (2).

RESULTS

Lactate. The influence of DL-calcium lactate on the L-alanine germination of buffer-treated and hypochlorite-treated spores is evident in Fig. 1. There was a stepwise decrease in the percentage of germination in media AB^+X, TB^+X, and TB^+LX (see Table 1) owing to treatment of the spores with increasing concentrations of hypochlorite. However, despite hypochlorite treatment, spores germinated to essentially the same extent in 2 h in medium AB^+LX. The data illustrate the importance of a combination of calcium lactate plus L-alanine for the germination of hypochlorite-treated spores. Spores treated with up to 28 μg of available chlorine (AC) per ml showed germination to an extent essentially equivalent to that of buffer-treated spores only in the medium with 4.5 mM L-alanine plus 9 mM calcium lactate in addition to 55 mM sodium bicarbonate, 0.8 mM sodium thiosulfate, and Tris-hydrochloride buffer. Tryptose could not fulfill the alanine requirement of hypochlorite-treated spores.

To determine whether the beneficial effect of calcium lactate observed in medium AB^+LX

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TABLE 1. Germination media

Code	Component	Concn[a]
A	L-Alanine (Sigma Chemical Co., St. Louis, Mo.)	4.5 mM
B^+	Sodium bicarbonate (Matheson, Coleman and Bell, Norwood, Ohio)	55 mM
$CaCl_2$	Calcium chloride (Fisher Scientific Co., Pittsburgh, Pa.)	9 mM
L	DL-Calcium lactate (Fisher)	9 mM
M	L-Sodium malate (NaOH-neutralized malic acid, Sigma)	18 mM
NaL	DL-Sodium lactate (Fisher)	9 mM
P	DL-Sodium propionate (NaOH-neutralized propionic acid, Fisher)	18 mM
T	Tryptose (Difco Laboratories, Detroit, Mich.)	1.8%
X	Tris-hydrochloride buffer (THAM, Fisher), pH 7.0 at 35°C	100 mM
	Sodium thiosulfate (Fisher)[b]	0.8 mM

[a] Concentrations listed have taken into account dilution owing to inoculation.

[b] Sodium thiosulfate was present in each medium and was not designated by a code.

was due to the Ca^{2+} or the lactate anion, a comparison was made of the respective influences of added calcium lactate, sodium lactate, and calcium chloride to the basic medium AB^+X (Fig. 2). Sodium lactate was incorporated in medium AB^+X at amounts equimolar to the calcium lactate (9 mM) and also at twice the molarity so that the molarity of the lactate anion would be equivalent to that in calcium lactate. The extent of germination at 2 h of *C. botulinum* 12885A spores treated with 28 μg of AC per ml was essentially equal to that of buffer-treated spores of the same strain in media containing calcium or sodium lactate. In addition, the extents of germination were essentially the same in each of the three lactate-containing media for any given spore treatment (0 or 28 μg of AC per ml). However, the extent of germination of spores treated with 28 μg of AC per ml was decreased by 27% compared with that of buffer-treated spores in medium AB^+X with 9 mM $CaCl_2$ added. The germination of buffer-treated spores in the medium with added $CaCl_2$ was decreased by 6 to 18% compared with that of buffer-treated spores in media with added calcium or sodium lactate. The extents of germination in AB^+X controls were 57 and 5% for spores treated with 0 and 28 μg of AC per ml, respectively (data not shown). These data indicate that Ca^{2+} may have contributed to some extent to the germination of hypochlorite-treated spores, but lactate in the absence of Ca^{2+} contributed the full beneficial germination effect. Two questions arise from observing a lactate requirement for the L-alanine germination of hypochlorite-treated spores. First, what role was lactate playing in initiating germination? Second, what was significant about the structure of lactate which allowed it to have the observed role?

A comparison of the effects of L-sodium malate and DL-calcium lactate on the germination of buffer-treated and hypochlorite-treated spores is presented in Fig. 3. The hypochlorite-treated spores germinated in 2 h to essentially the same extent as buffer-treated spores in media with either lactate or malate. This was not observed in the medium with neither lactate nor malate. The extents of germination of buffer-treated spores in the three media were similar, although slightly less if lactate or malate was not present. Germination of hypochlorite-treated spores in medium with propionate was poor. In both AB^+X and AB^+PX media (see Table 1), the germination of spores treated with 28 μg of AC per ml was 18 to 22%, whereas buffer-treated spores germinated 60 to 64% in these media. (Germination in paired AB^+LX comparisons for the AB^+PX germination trials was 69 to 72% for spores treated with both 0 and 28 μg of AC per ml).

The germination responses of hypochlorite-treated and buffer-treated spores owing to calcium lactate and sodium malate are presented in Table 2. There was negligible germination of both buffer-treated and hypochlorite-treated spores in media B^+X, B^+LX, and B^+MX (see Table 1). Results with hypochlorite-treated spores were similar to results with buffer-treated spores. The data suggest that neither lactate nor malate was an effective germinant of buffer-treated or hypochlorite-treated spores, contributing only ca. 6 to 9% to the 2-h germination

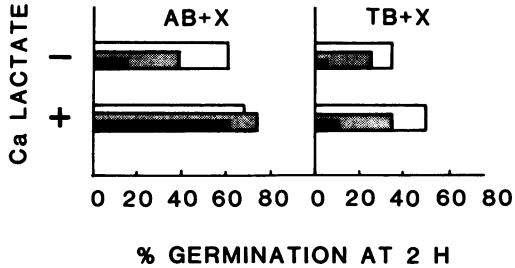

FIG. 1. Influence of DL-calcium lactate on the L-alanine germination of hypochlorite-treated *C. botulinum* spores in AB^+X and TB^+X media. Data represent averages of duplicate trials. AC concentrations, 28 (■), 12 (▦), and 0 (□) μg/ml. Calcium lactate (9 mM) was either present (+) or absent (−) from each medium.

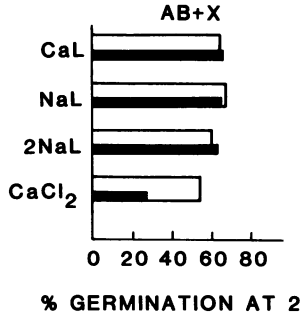

FIG. 2. Influence of calcium and lactate on the germination of buffer-treated and hypochlorite-treated *C. botulinum* 12885A spores. Additions to the AB^+X medium base included 9 mM DL-calcium lactate (CaL), 9 mM DL-sodium lactate (NaL), 18 mM DL-sodium lactate (2NaL), and 9 mM calcium chloride ($CaCl_2$). Data represent averages of duplicate trials. Symbols as in legend to Fig. 1; data were not taken for spores treated with 12 μg of AC per ml.

responses observed in media AB^+LX and AB^+MX.

L-Alanine concentration. The effect of increasing the L-alanine concentration 10- and 100-fold, from 4.5 to 45 and 450 mM, on the germination of buffer-treated and hypochlorite-treated spores in four basic media is presented in Fig. 4. The data from the AB^+X media (see Table 1) made with various L-alanine concentrations demonstrate that increasing the L-alanine concentration to about 450 mM permitted hypochlorite-injured spores to germinate to nearly the full extent of buffer-treated spores. In the ATB^+X media, similar results owing to increasing the L-alanine concentration were seen. However, in the ATB^+X media, the inhibition of L-alanine germination owing to tryptose was also a factor (2). As the L-alanine concentration was increased, the inhibition owing to tryptose was overcome, and the extent of germination of hypochlorite-treated spores approached that of buffer-treated spores. In the media with both alanine and lactate (AB^+LX media) but without tryptose, the L-alanine concentration had little influence on the extent of germination. Hypochlorite-treated and buffer-treated spores germinated to essentially equal extents in 2 h in medium AB^+LX, and increasing the L-alanine concentration above 4.5 mM was inconsequential. In the ATB^+LX media, the inhibitory effect of tryptose was again apparent since hypochlorite-treated spores germinated to a lesser extent than buffer-treated spores in each of the media. Yet the inhibition was nearly overcome by 450 mM L-alanine in combination with lactate. Germination in 2 h of spores treated with 28 μg of AC per ml was decreased by 11% compared with the germination of buffer-treated spores.

A comparison of the effect of lactate plus L-alanine to a 100-fold higher L-alanine concentration on the germination of buffer-treated and hypochlorite-treated spores is presented in Fig. 5. Neither the presence of lactate nor a 100-fold increase in L-alanine affected the extent of germination of buffer-treated spores. Germination of hypochlorite-treated spores was decreased by 36% compared with that of buffer-treated spores in medium AB^+X. However, if lactate was present or if the L-alanine concentration was increased to 450 mM, the extents of germination of hypochlorite-treated spores were the same or nearly the same as those of buffer-treated spores. The greatest difference was in medium $(100A)B^+X$, in which the germination in 2 h of spores treated with 28 μg of AC per ml was decreased by 11% compared with the germination of buffer-treated spores. The difference may have been less in media with > 450 mM L-alanine.

DISCUSSION

Previously, we reported that hypochlorite treatment of *C. botulinum* spores alters the rate and extent of germination in some media compared with the germination of buffer-treated spores (2). Hypochlorite-treated spores required DL-calcium lactate plus L-alanine for complete germination (germination equal to that of buffer-treated spores), but buffer-treated spores germinated to essentially the same extent whether calcium lactate was present or not. Lactate was the active component of calcium lactate. Malate but not propionate could fulfill the lactate germination requirement of hypochlorite-treated spores. The pK values of the acids indicate that the charges on lactate, propionate, and malate in these media are −1, −1, and −2, respectively. The data suggest that a hydroxyl group alpha to a carboxyl group is a structural feature of lactate and malate which is important in permitting the

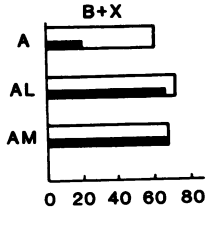

FIG. 3. Comparison of the influence of L-sodium malate with that of DL-calcium lactate on the L-alanine germination of hypochlorite-treated *C. botulinum* spores in B^+X medium. Data represent averages of duplicate trials. Symbols as in legend to Fig. 1.

TABLE 2. Germination owing to lactate and malate of AC-treated *C. botulinum* spores

Medium	% Germination at 2 h with AC concn (μg/ml)[a]:	
	0	28
B^+X	2	1
B^+LX	11	7
B^+MX	10	9

[a] Averages of duplicate trials are presented.

full germination response of hypochlorite-treated spores in media AB^+LX and AB^+MX (Fig. 3; see Table 1). Ando (1) has also reported that anions of alpha-hydroxy acids, including lactate, are beneficial for the germination of *C. botulinum* spores, and Waites and Wyatt (6) have reported that lactate stimulates the germination of *C. bifermentans* spores.

Further support for the activity of an alpha-hydroxy acid comes from comparing the extents of germination in $(10A)B^+X$ medium to the extents in AB^+LX medium (Fig. 4). There was a 34% decrease in the germination of spores treated with 28 μg of AC per ml compared with the germination of buffer-treated spores in medium $(10A)B^+X$, yet the extents of germination were essentially equal in AB^+LX medium, despite hypochlorite treatment. The concentration of L-alanine in $(10A)B^+X$ medium was 45 mM. Thus, 40.5 mM excess alanine was present compared with that in AB^+LX medium. These data indicate that L-alanine, although structurally similar to lactate, could not fulfill the observed lactate germination requirement of hypochlorite-treated spores.

Two possible roles for lactate and malate in the germination process are as germinants and as stimulants of germination. Lactate and malate were poor germinants for spores treated with both 0 and 28 μg of AC per ml. These data do not support the proposal that the functioning of lactate and malate as germinants explains the observed requirement of hypochlorite-treated spores for them. The greater extent of germination of buffer-treated spores in AB^+LX or AB^+MX medium compared with AB^+X medium, however, may have been the result of lactate or malate functioning as germinants. Ando (1) indicated that lactate is not an effective germinant of *C. botulinum* spores and probably acts in a synergistic manner, with L-alanine as the main germinant.

The data indicate that L-alanine was the germinant but that 4.5 mM L-alanine was fully active as a germinant of hypochlorite-treated spores only in the presence of lactate or malate. However, in media without lactate or malate, hypochlorite-treated spores germinated to an extent nearly equal to that of buffer-treated spores if the alanine concentration was increased 100-fold to 450 mM. These data suggest that one effect of hypochlorite may be to alter the configuration of the alanine germination site so that a higher alanine concentration is required by hypochlorite-treated spores to get a response equivalent to the site in its native state. Furthermore, the data suggest that lactate or malate may have functioned as an activator of the altered alanine germination site. One model could be that lactate or malate functions by allosteric-type activity, interacting at or near the altered L-alanine germination site and causing the configuration of the altered germination site to be changed back to the native or an active state. Thus, the low L-alanine concentration (4.5 mM) was active. This model is supported by the fact that lactate and malate are not germinants but act synergistically with L-alanine in the germination of hypochlorite-treated spores. Also, the need for lactate or malate is overcome by increasing the L-alanine concentration. The necessity for further work to confirm this model is obvious. However, others have suggested that allosteric activity of germination sites is involved in germination (1, 4, 5, 7). Another hypothesis is that lactate may activate non-hypochlorite-altered germination sites of both hypochlorite-treated and buffer-treated spores.

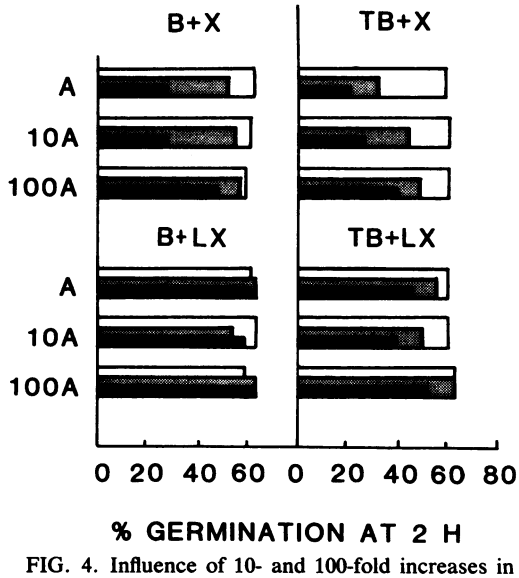

FIG. 4. Influence of 10- and 100-fold increases in the L-alanine concentration on the germination of hypochlorite-treated *C. botulinum* spores in four different media. Data represent averages of duplicate trials. Symbols as in legend to Fig. 1. Media were formulated with 4.5 mM (A), 45 mM (10A), and 450 mM (100A) L-alanine.

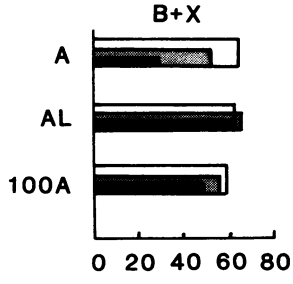

FIG. 5. Comparison of the influences of DL-calcium lactate and a 100-fold increase in the alanine concentration on the L-alanine germination of hypochlorite-treated *C. botulinum* spores in B^+X medium. Data represent averages of duplicate trials. Symbols as in legend to Fig. 1. Media were formulated with 4.5 mM L-alanine (A), 4.5 mM L-alanine plus 9 mM DL-calcium lactate (AL), and 450 mM L-alanine (100A).

This might increase the activity of the sites, explaining the observed increases in the extents of germination. Lactate appeared to be important primarily for the germination of hypochlorite-treated spores. There could be several reasons for this. The lactate site may have been more accessible because of the hypochlorite treatment (8, 9) or it may have been formed because of the hypochlorite treatment. We have discussed modifications of *C. botulinum* spores by hypochlorite elsewhere (3). If the alanine concentration was lower and suboptimal, a synergistic effect of lactate on germination may have been expressed by buffer-treated spores. In fact, Waites and Wyatt (6) have reported synergism owing to lactate if *C. bifermentans* spores were germinated in suboptimal concentrations of L-alanine. Other explanations exist.

Previously, we presented evidence indicating that tryptose or its components interferes with the L-alanine germination of buffer-treated and hypochlorite-treated spores (2). Furthermore, the data suggest that the nature of the interference is competitive inhibition of L-alanine germination by a tryptose component. These data support the assumption of competitive inhibition. In the ATB^+X media, increasing the alanine concentration resulted in an increase in the extent of germination. Two simultaneous occurrences which may explain the observed increases in the extents of germination in the ATB^+X media are that (i) competition owing to a tryptose component(s) was partially overcome by increasing the alanine, and (ii) the proposed altered germination site reacted more readily with higher alanine concentrations.

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