APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1983, p. 1374–1379
0099-2240/83/041374-06$02.00/0

Vol. 45, No. 4

Proposed Mechanism for Sensitization by Hypochlorite Treatment of *Clostridium botulinum* Spores†

PEGGY M. FOEGEDING‡ AND F. F. BUSTA*
Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota 55108

Received 23 August 1982/Accepted 19 January 1983

Hypochlorite-treated *Clostridium botulinum* 12885A spores, but not buffer-treated spores, could be germinated with lysozyme, indicating that their coats are made permeable to lysozyme by hypochlorite treatment so that the cortex is accessible. Hypochlorite-treated spores and spores extracted with 8 M urea–2-mercaptoethanol (pH 3.0) were sensitive to certain components of recovery media, but spores sensitized to lysozyme by other treatments were not. These data indicate that hypochlorite does more than increase coat permeability to lysozyme. Scanning electron microscopy revealed a more open-appearing surface of hypochlorite-treated spores, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that a greater amount of protein was removed from hypochlorite-treated and other lysozyme-sensitized spores than from buffer-treated spores. The data suggest that spore coat proteins may be removed by hypochlorite treatment, and this may be responsible for the sensitivity of spores and for their observed ability to germinate in lysozyme.

Wyatt and Waites (18) have shown that hypochlorite treatment of *Clostridium bifermentans* spores sensitizes the spores to lysozyme germination. Spores typically do not germinate in lysozyme unless procedures are used which alter the coats, making the spores permeable to lysozyme (2, 3, 6, 10, 15, 18). If permeability barriers are altered, spores may demonstrate not only lysozyme sensitivity but increased sensitivity to recovery media components as well. Other damaging treatments are manifested by sensitivity to components of recovery media. For example, *Clostridium botulinum* spores damaged by heat are sensitive to the presence of sodium chloride in the recovery medium (13). A previous study (5) in our laboratory has suggested that hypochlorite treatment of *C. botulinum* spores may injure the spores so that they are not enumerated by a most probable number procedure with modified peptone colloid medium (MPCM) but they are enumerated by a colony count procedure with modified peptone yeast extract glucose (PYEG) agar. Buffer-treated spores are enumerated essentially equally by both procedures. Germination responses of spores of *Bacillus* or *Clostridium* species also may be altered by hypochlorite treatment (5, 17, 18). The injury and altered germination requirements of the spores may have been the result of changes in the spore surface.

The objectives of this research were to detect induced sensitivity in hypochlorite-treated spores to provide information on altered structure and resistance barriers; to visualize, by scanning electron microscopy, any structural alterations in spores after hypochlorite treatment; and to determine whether protein is removed from the spores by hypochlorite treatment.

MATERIALS AND METHODS

Spores. *C. botulinum* 12885A was used in these experiments. The culture origin and 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 (5). Heat activation was always within 6 h of germination.

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 (5).

Coat extraction treatments. Several treatments which have been used successfully to remove coat layers of *Bacillus* and *Clostridium* spores were used in these experiments. These reagents, conditions, and references to the sources of the treatments are detailed in Table 1. In each case, heat-shocked spores (ca. 2×10^7 spores per ml) were pelleted, the supernatant fluid was discarded, and the spore pellet was mixed and incubated with the reagent at the indicated time and temperature. The volume of reagent used was equal to the initial volume of spores. The treated spores were washed and used for germination, enumeration, or

† Paper no. 13,042, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul, Minn.

‡ Present address: Department of Food Science, North Carolina State University, Raleigh, NC 27650.

TABLE 1. Treatments for extracting spore coats

Reagent composition[a]	Incubation		Reference
	Temp (°C)	Time (h)	
0.1 N NaOH	0	0.5	15
500 μM DTT (pH 10)[b]	35	1.5	2
50 mM DTT, 4 M urea (pH ca. 8.9)[c]	35	2.5	
10% (wt/vol) MCE, 4 M urea (pH ca. 6.5)[c]	35	2.5	19
10% (wt/vol) MCE, 8 M urea (pH 3)[d]	35	2.5	15
Distilled water (pH 3)[d]	0	0.5	

[a] All reagents were filter sterilized and held at 4°C for no more than 3 weeks.
[b] pH adjusted with NaOH.
[c] pH not adjusted.
[d] pH adjusted with HCl.

electron microscopy as described below. In some cases, the supernatant fluids from the treatments were dialyzed and concentrated for electrophoresis as detailed below. The reagent suppliers were as follows: dithiothreitol (DTT), Sigma Chemical Co., St. Louis, Mo.; urea, Fisher Scientific Co., Pittsburgh, Pa., or Spectrum Chemical Manufacturing Corp., Redondo Beach, Calif.; 2-mercaptoethanol (MCE), Matheson, Coleman and Bell, Norwood, Ohio.

Washing spores. After treatments of the spores, and before any germination experiments and electron microscopy, the spores were washed with cold sterile distilled water as previously detailed (5). However, before enumeration of the treated spores, washes were performed with cold sterile 0.3 M sucrose (pH 9.0) to minimize aggregation of the spores (4). If enumeration, microscopy, and germination data were desired, spores were washed with sucrose, and a sample was removed for enumeration; the remaining spores were washed with water and used for germination or microscopy experiments.

Germination procedures. The germination procedures were described previously (5). Two germination media were used in these experiments. Medium AB^+LX contained 4.5 mM L-alanine (Sigma), 55 mM $NaHCO_3$ (Matheson, Coleman and Bell), 9 mM DL-calcium lactate (Fisher), 100 mM Tris-hydrochloride buffer (pH 7.0), and 0.8 mM sodium thiosulfate; it was prepared as described previously (5). The pH of medium AB^+LX was 7.36. A lysozyme solution was used as the germination medium for some experiments (5).

Colony counts. Sensitivities to media components were determined by the recovery of spores in various media. Lee tubes (12) with variations of modified PYEG agar (8) without vitamin K_1 and with 14 mM $NaHCO_3$ or with MPCM (7) were used for these experiments.

Preparation and use of PYEG Lee tubes was as detailed by Foegeding and Busta (5). Salt solution components and suppliers were detailed previously (5). The PYEG variations included the addition of 0.005% lysozyme or 2.5% NaCl. Lysozyme (U.S. Biochemical Corp., Cleveland, Ohio) was incorporated at 0.005% by adding 0.2 ml of a freshly prepared filter-sterilized 0.35% solution in 0.05 M potassium phosphate buffer (pH 8.1) per tube of autoclaved tempered (46 to 48°C) medium. Sodium chloride (Spectrum) was incorporated by adding the desired amount before adjusting the pH.

MPCM was prepared and used as follows. A mixture of 20 g of tryptose (Difco Laboratories, Detroit, Mich.), 5 g of NaCl, 1 g of dextrose, 15 g of agar (Difco), and 1 liter of distilled water was made and steamed for 20 min at 100°C. Next, 0.2 g of iron sulfate and 0.3 g of sodium thiosulfate (Fisher) per liter were mixed into the medium. MPCM Lee tubes were dispensed, autoclaved, inoculated, mixed, incubated, and counted in the same way as PYEG Lee tubes. The MPCM variations included the addition of either $NaHCO_3$ or yeast extract (BBL Microbiology Systems, Cockeysville, Md.). Sodium bicarbonate was added after autoclaving as detailed for PYEG Lee tubes (5) so that the concentration was 14 mM. Yeast extract (1%) was added before autoclaving.

For comparisons of sensitivities, the recovery of the spores in the media variations was reported as a percentage of the total population. The total population was considered to be that population from each treatment which was enumerated in PYEG medium.

Electron microscopy. Treated, water-washed spores were pelleted in a centrifuge, and the supernatant fluid was discarded. The spore pellets were brought to approximately five times the initial volume with distilled water. One drop of each spore suspension was placed onto a prepared specimen mount, and the mounts were dried at 37°C or at room temperature. Aluminum specimen mounts (Structure Probe, Inc., West Chester, Pa.) were prepared by painting the surface of clean mounts with conductive carbon paint and firmly placing a clean, dust-free, round glass cover slip (diameter, 1.27 cm; thinness, 2; Ernest F. Fullam, Inc., Schenectady, N.Y.) onto the moist paint surface. The edges of the glass cover slip were coated with carbon paint to provide a conductive bridge from the top of the cover slip to the aluminum stub. The dried samples were mounted on a rotary stage and coated in a vacuum evaporator with a thin layer (ca. 2 nm) of 60% gold–90% palladium alloy.

Coated samples were held in a desiccator jar until microscopic observation. A Philips 500X electron microscope operated at 12 kV acceleration voltage was used.

Dialysis and concentration of spore extracts. The supernatant fluids obtained from 2 ml of spores treated with each treatment (500 μM DTT, 8 M urea plus MCE [pH 3], 4 M urea plus MCE, 28 μg of available chlorine [AC] per ml) and from extracts from 6 ml of buffer-treated spores were dialyzed against repeated changes of distilled water at 4°C overnight. Dialysis tubing with a cutoff of 3,500 molecular weight was used (Arthur H. Thomas Co., Philadelphia, Pa.; no. 3787-H47). Dialyzed samples were frozen, lyophilized, and stored at 4°C for subsequent electrophoresis.

Electrophoresis. The lyophilized samples were rehydrated with 100 μl of deionized water. A 50-μl portion of a 2:1 (vol/vol) mixture of extract and tracking dye was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (11), followed by Coomassie brilliant blue staining. Molecular weight standards (Pharmacia Fine Chemicals, Piscataway, N.J.) with molecular weights given parenthetically included: phosphorylase *b* (94,000), albumin

(67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and alpha-lactalbumin (14,400).

RESULTS

Spore sensitivities. Comparisons were made between buffer-treated and hypochlorite-treated spores on the one hand and spores which had been treated with reagents known to alter coats of various *Bacillus* or *Clostridium* spores on the other. These comparisons of their abilities to germinate in lysozyme and their sensitivities to components of recovery media are presented in Table 2. Each of the spores from hypochlorite or various coat extraction treatments germinated 50 to 62% in medium AB^+LX. All of the spores from hypochlorite or various coat extraction treatments germinated in the presence of lysozyme, except for buffer-treated spores, which did not germinate in lysozyme in 2 h. When lysozyme germination occurred, the extent of germination was small, from 8 to 29%. Spores treated with 12 and 28 μg of AC per ml germinated 22 and 19%, respectively, in lysozyme. The data indicate that the spore coats were altered by all of the treatments except buffer treatment; consequently, the cortex was accessible to lysozyme, and germination occurred in the presence of lysozyme.

The recovery of the spores from hypochlorite or various coat extraction treatments in several media is also detailed in Table 2. When the recovery of buffer-treated spores served as a control, the spores from the various treatments did not show any marked sensitivities except for the spores treated with 28 μg of AC per ml and those treated with urea plus MCE (pH 3). The spores treated with 28 μg of AC per ml were especially sensitive to sodium chloride in the medium. Spores treated with 28 μg of AC per ml and spores treated with urea plus MCE (pH 3) were recovered at low levels by MPCM Lee tube enumeration, despite the addition of sodium bicarbonate or yeast extract, both of which (when compared with MPCM Lee tube data) improved the recovery of spores receiving other treatments. The addition of lysozyme to PYEG Lee tubes had only a minimal effect on the recovery of any of the spores from the various treatments. The presence of 0.5% NaCl in

TABLE 2. Germination of treated spores in lysozyme and their sensitivity to medium components

Treatment[a]	% Germination at 2h		Population (% of CFU/ml in PYEG Lee tubes)					
			PYEG Lee tubes containing:			MPCM + 1.5% agar Lee tubes containing:		
	AB^+LX (2)[b]	Lysozyme[c] (2)	Lysozyme (1)	0.5% NaCl (2)	2.5% NaCl (4)	No addition (4)	$NaHCO_3$[d] (2)	Yeast extract[e] (2)
Hypochlorite								
0 μg/ml	60	0	64	88	71	28	70	23
12 μg/ml	62	22	75	104	43	28	80	48
28 μg/ml	60	19	68	43	19	6	18	16
0.1 N NaOH (30 min, 0°C)	62	22	56	120	68	30	73	36
500 μM DTT (90 min, 35°C)	50	12	75	89	58	15	73	23
50 μM DTT + 4 M urea (2.5 h, 35°C)	58	29	61	100	74	23	87	50
MCE + 4 M urea (2.5 h, 35°C)	56	25	68	88	57	31	56	10
MCE + 4 M urea (pH 3, 2.5 h, 35°C)	56	28	75	87	62	8	14	5
Water (pH 3, 30 min, 0°C)	54	8	72	ND[f]	78	29	ND	ND

[a] See text and Table 1 for details of treatments.
[b] Numbers in parentheses show numbers of replicate trials averaged.
[c] Lysozyme (0.1 mg/ml) in 0.05 M potassium phosphate buffer (pH 8.1) at 35°C.
[d] $NaHCO_3$ (14 mM) added after autoclaving.
[e] Yeast extract (1%) added before autoclaving.
[f] ND, Not done.

PYEG had essentially no negative effect on recovery except in the case of spores treated with 28 μg of AC per ml.

Structure. Scanning electron microscopy was used to visualize any surface effects of the various treatments on the spores. Figure 1 shows representative micrographs of the spores from various treatments. The spores were approximately 1 μm in diameter. Figures 1A and B are micrographs of buffer-treated spores. The spore surfaces were generally irregular in shape (Fig. 1A), although smooth spores (Fig. 1B) were occasionally observed. Spores treated with hypochlorite or by the other treatments were generally more smoothly shaped but had a more open appearance (Fig. 1C and D).

Proteins were removed from spores treated with 500 μM DTT, 8 M urea plus MCE (pH 3), 4 M urea plus MCE, 28 μg of AC per ml, and buffer. The electrophoretogram obtained from the spore extracts and molecular weight standards is presented in Fig. 2. Much less protein was removed from buffer-treated spores than from the spores receiving other treatments. The extract from three times more spores was loaded onto the gels, and yet the bands were much more faint. In addition, only two visible protein bands resulted from the extract from buffer-treated spores, whereas three or more bands were visible from the other extracts.

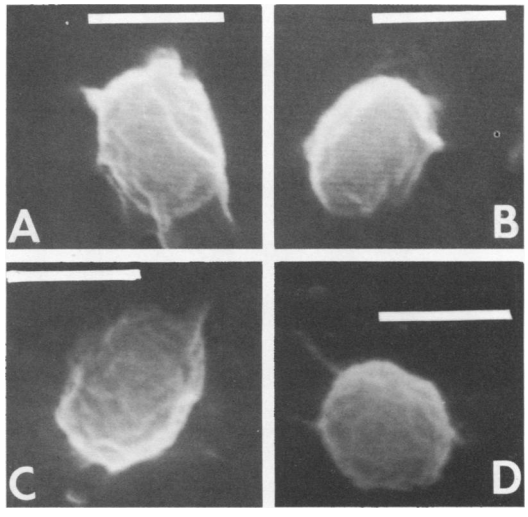

FIG. 1. Scanning electron micrographs of spores treated with 0 μg of AC per ml (A, B), 500 μM DTT (C), and 28 μg of AC per ml (D). Bar, 1 μm. Details of the treatments are given in Table 1.

DISCUSSION

Wyatt and Waites (19) have shown that *C. bifermentans* spores are sensitized to lysozyme germination by treatment with hypochlorite, and others (9, 16, 17) have indicated that hypochlorite may alter the permeability of spores by removing part of the spore coat. Usually, germination with lysozyme occurs only after treatment with agents which are known to remove or alter the coats of spores. Spores of *Clostridium perfringens* 8-6 lack a spore coat and germinate only in the presence of lysozyme (14). Hypochlorite treatment and treatments which have been shown to remove or alter the coats of other *Bacillus* or *Clostridium* spores sensitize *C. botulinum* spores to lysozyme germination. The data indicate that hypochlorite treatment alters the spore coat so that it is permeable and the cortex is accessible to lysozyme. None of the treatments generates dependence on lysozyme. Dependence of germination on lysozyme was observed by Labbe et al. (10) after treating *C. perfringens* spores with sodium hydroxide and was indicated by Vary (15) after treating *Bacillus megaterium* with 8 M urea–10% MCE at pH 3.0. Alderton et al. (1) found that adding lysozyme to media improved the recovery of heat-damaged *C. botulinum* spores, but we did not observe this benefit.

The treatments which sensitized the spores to lysozyme germination did not necessarily cause sensitivity to components of recovery media. Only the hypochlorite and urea plus MCE (pH 3) treatments resulted in spores with increased sensitivities to recovery medium components. These data suggest that hypochlorite had an effect on the spores which was more complex than simply making the coats permeable to lysozyme and was similar to the effect of treatment with urea plus MCE (pH 3). Differences in the sensitivities resulting from the treatments were not unexpected. Various spore extraction procedures differentially remove coat layers (9, 10, 15). For example, using transmission electron microscopy, Labbe et al. (10) found that 0.1 N NaOH and 50 mM DTT extractions of *C. perfringens* removed different portions of the coat and that 7 M urea–10% MCE (pH 2.8) caused the spore subcoat to have a granular appearance not detected after other extraction procedures.

Scanning electron microscopy studies indicated that the surfaces of the *C. botulinum* spores were more open in appearance as a result of each of the treatments which sensitized the spores to lysozyme. The surface changes were subtle and appeared similar for all of the treatments. A greater amount of protein was removed from *C. botulinum* spores which were sensitized to lysozyme by hypochlorite or other treatments than was removed from buffer-treated spores. The increased amount of protein or other components which was removed from the

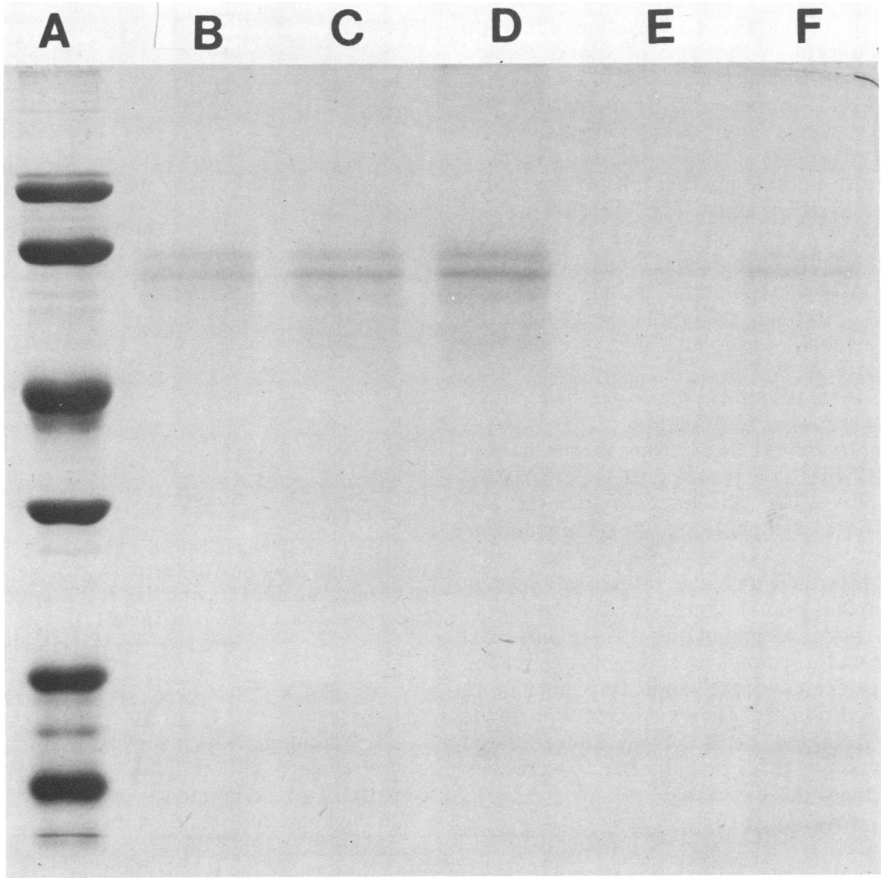

FIG. 2. Electrophoretogram of extracts from treated spores. (A) Molecular weight standards. Treatments: (B) 4 M urea plus MCE; (C) 500 μM DTT; (D) 8 M urea plus MCE (pH 3); (E) 0 μg of AC per ml; (F) 28 μg of AC per ml. The extract from three times as many spores was used for the treatment with 0 μg of AC per ml compared with the extracts for the other treatments.

spores may have been responsible for lysozyme sensitivity and the open spore appearances. These data support previous indications (9, 16, 17, 19) that hypochlorite may act on spores by removing part of the spore coat, thereby altering the permeability of the spores.

ACKNOWLEDGMENTS

This research was supported in part by University of Minnesota Agricultural Experiment Station project 18-59. P.M.F. was supported by General Foods-Institute of Food Technologists and Ralston Purina Food Science Fellowships.

We thank Cindy L. Nelson and Lorraine B. Smith for assistance in the laboratory and with figure preparation. We are sincerely grateful to Gilbert G. Ahlstrand for invaluable assistance with the electron microscopy.

LITERATURE CITED

1. **Alderton, G., J. K. Chen, and K. A. Ito.** 1974. Effect of lysozyme on the recovery of heated *Clostridium botulinum* spores. Appl. Microbiol. **27**:613–615.
2. **Aronson, A. I., and P. C. Fitz-James.** 1971. Reconstitution of bacterial spore coat layers in vitro. J. Bacteriol. **108**:571–578.
3. **Cassier, M., and A. Ryter.** 1971. Sur un mutant de *Clostridium perfringens* donnant des spores sans tuniques à germination lysozyme-dépendante. Ann. Inst. Pasteur (Paris) **121**:717–732.
4. **Fitz-James, P. C.** 1971. Formation of protoplasts from resting spores. J. Bacteriol. **105**:1119–1136.
5. **Foegeding, P. M., and F. F. Busta.** 1983. Hypochlorite injury of *Clostridium botulinum* spores alters germination responses. Appl. Environ. Microbiol. **45**:1360–1368.
6. **Gould, G. W., and A. D. Hitchens.** 1963. Sensitization of bacterial spores to lysozyme and to hydrogen peroxide with agents which rupture disulfide bonds. J. Gen. Microbiol. **33**:413–423.
7. **Greenberg, R. A., B. O. Bladel, and W. J. Zingelmann.** 1966. Use of the anaerobic pouch in isolating *Clostridium botulinum* spores from fresh meats. Appl. Microbiol. **14**:223–228.
8. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
9. **Kulikovsky, A., H. S. Pankratz, and H. L. Sadoff.** 1975. Ultrastructural and chemical changes in spores of *Bacillus cereus* after action of disinfectants. J. Appl. Bacteriol. **38**:39–46.

10. **Labbe, R. G., R. R. Reich, and C. L. Duncan.** 1978. Alteration in ultrastructure and germination of *Clostridium perfringens* types A spores following extraction of spore coats. Can. J. Microbiol. **24**:1526–1536.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
12. **Ogg, J. E., S. Y. Lee, and B. J. Ogg.** 1979. A modified method for the cultivation and enumeration of anaerobic bacteria. Can. J. Microbiol. **25**:987–990.
13. **Pivnick, H., and C. Thacker.** 1970. Effect of sodium chloride and pH on initiation of growth by heat-damaged spores of *Clostridium botulinum*. Can. Inst. Food Technol. J. **3**:70–75.
14. **Sacks, L. E.** 1981. Influence of cations on lysozyme-induced germination of coatless spores of *Clostridium perfringens* 8–6. Biochim. Biophys. Acta **674**:118–127.
15. **Vary, J. C.** 1973. Germination of *Bacillus megaterium* spores after various extraction procedures. J. Bacteriol. **116**:797–802.
16. **Wyatt, L. M., and W. M. Waites.** 1971. Studies with spores of *Clostridium bifermentans*: comparison of germination mutants, p. 123–132. *In* A. N. Barker, G. W. Gould, and J. Wolf (ed.), Spore research 1971. Academic Press, Inc., (London) Ltd., London.
17. **Wyatt, L. R., and W. M. Waites.** 1973. The effect of hypochlorite on the germination of spores of *Clostridium bifermentans*. J. Gen. Microbiol. **78**:383–385.
18. **Wyatt, L. R., and W. M. Waites.** 1974. The effect of sodium hydroxide or dithiotheritol-urea on spores of germination mutants of *Clostridium bifermentans*. J. Gen. Microbiol. **84**:391–394.
19. **Wyatt, L. R., and W. M. Waites.** 1975. The effect of chlorine on spores of *Clostridium bifermentans*, *Bacillus subtilis* and *Bacillus cereus*. J. Gen. Microbiol. **89**:337–344.