Utility of Sodium Hypochlorite for Ultrastructure Study of Bacterial Spore Integuments

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ABSTRACT

RODE, L. J. (The University of Texas, Austin), AND M. GLENN WILLIAMS. Utility of sodium hypochlorite for ultrastructure study of bacterial spore integuments. *J. Bacteriol.* 92:1772-1778. 1966.—Spores of *Bacillus megaterium* are partially dissolved by sodium hypochlorite. Spore integuments become visible during the dissolution, and ultrastructural features may be detected. Three distinct integument types are described for *B. megaterium* QM B1551 with the use of this technique. Since a variety of microbial cells are affected by sodium hypochlorite, its use may be applicable to ultrastructure study of cells other than bacterial spores.

The effect of sodium hypochlorite on microbial cells is drastic. Meyer (4) observed that *Bacillus* cells were dissolved almost completely by alkaline sodium hypochlorite, but that the lipid cellular inclusions remained intact. The latter observation subsequently served as the basis of a procedure for estimating the poly- β -hydroxybutyrate granule content of *Bacillus* spp. (11). According to Salton (9), cell walls and glycosaminopeptide of gram-positive organisms are solubilized by strong (10%) sodium hypochlorite in the cold. However, the use of sodium hypochlorite as an aid in studying cellular structure has apparently not been described. This report demonstrates its utility for examining ultrastructure of bacterial spore integuments. The general term "integuments" is used to denote all spore envelopes or covering structures observed.

MATERIALS AND METHODS

Spores. Spores of *B. megaterium* QM B1551 and *B. megaterium* ATCC 19213 ["Texas" strain (5)] were produced and handled according to methods described previously (5).

Preparation of spore integuments and cell walls. Spore integuments and cell walls were prepared with the Nossal cell disintegrator according to a procedure described previously (7).

Sodium hypochlorite. Solutions were prepared in demineralized water from household liquid "bleach" (W-P Brand, Waples-Platter Co., Ft. Worth, Tex.) containing 5.25% sodium hypochlorite as the active ingredient.

Optical density (OD) measurements. The capacity of sodium hypochlorite to reduce the OD of cellular suspensions was followed by use of a Klett-Summer-

son photoelectric colorimeter equipped with filter no. 54. Initial readings were approximately 100 Klett-Summerson units; data are presented as percentage of initial Klett-Summerson readings (OD, per cent) plotted against time.

Treatment of spores with sodium hypochlorite. Thoroughly washed spores were added to dilute solutions of sodium hypochlorite and incubated at 37 C. For certain electron microscope studies, spores were added to the sodium hypochlorite solution at intervals during incubation to provide, within a single preparation, integuments in varying stages of digestion. After the desired incubation, the tubes were chilled rapidly and centrifuged for 10 min at $10,000 \times g$, and the integument sediment was washed three times with cold, demineralized water.

Electron microscopy. Specimens were viewed both directly and as replicas by use of an RCA EMU-3G electron microscope with double condenser, a 35-m μ objective aperture, and an accelerating voltage of 100 kv. Micrographs were taken at initial magnifications of 5,000 to 18,000 $\times$ on Dupont Cronar Ortho S Litho film. For preparation of replicas, specimens were placed on mica squares, permitted to dry in the air, and shadowed at an angle of approximately 35° with platinum. After this, the specimens were coated with carbon, the carbon films were removed from the mica by flotation onto water, and the cellular material was dissolved from the carbon replicas with 0.5% sodium hypochlorite. After overnight treatment with sodium hypochlorite, the carbon replica films were washed thoroughly with water and lifted onto copper grids for examination.

RESULTS

Effect of sodium hypochlorite on OD of cell suspensions; concentration effect. When spores of *B. megaterium* QM B1551 were suspended in

solutions of sodium hypochlorite, a precipitous decline in turbidity occurred (Fig. 1; see 11). The lag period and rate of clearing were observed to be a function of the concentration of sodium hypochlorite. During this turbidity decrease, the spores, viewed with the phase microscope, progressively lost refractility, darkened, and a blanching of the central portion of the cell ensued. Eventually, only integument structures remained discernible; these became progressively more faint until they were almost invisible. Intermediate cellular stages persisted for greater periods of time in the lower concentrations of sodium hypochlorite. These phase microscope and turbidimetric observations are in agreement with those of Williamson and Wilkinson (11).

Effect on spores, spore integuments, vegetative cells, and vegetative cell walls. Vegetative cells and spores, and likewise their respective integuments, were affected by sodium hypochlorite (Fig. 2). The rate and extent of OD reduction was greater with spores and spore integuments than with vegetative cells and cell walls. The higher residual turbidity noted for vegetative cells was probably due to the resistance to sodium hypochlorite of the poly- β -hydroxybutyrate granules present in

FIG. 1. Optical density reduction of suspensions of spores of *Bacillus megaterium* QM B1551 by sodium hypochlorite; the effect of concentration. Incubation was at 37 C.

FIG. 2. Optical density reduction of suspensions of spores, spore integuments, vegetative cells, and vegetative cell walls of *Bacillus megaterium* ATCC 19213, by 0.5% sodium hypochlorite. Incubation was at 37 C.

such cells. Although general solubilization of cell structures, with the exception of poly- β -hydroxybutyrate granules, may be deduced from these data, different rates of solubilization are indicated.

Effect of pH. The effect of aqueous sodium hypochlorite on spores of *B. megaterium* QM B1551 was influenced by pH; an alkaline solution effected a more rapid reduction of OD and the ultimate clearing was greater than with solutions acidified with HCl (Fig. 3).

Electron Microscopy.

B. megaterium QM B1551. Untreated spores of this strain, viewed directly, appear opaque and featureless but possess characteristic polar knobs (Fig. 4; 3). This appearance is a consequence of the prominent equatorial ridge or fold possessed by this and some other strains of *B. megaterium* (Fig. 5; 1, 3). The spore surface appears textured and pitted, but neither ultrastructural repeating patterns nor ordered structure is apparent (Fig. 5). Upon treatment with sodium hypochlorite,

FIG. 3. Optical density reduction of suspensions of spores of *Bacillus megaterium* QM B1551 by 0.1% sodium hypochlorite; the effect of pH. Incubation was at 37 C.

the spores lose opacity owing to dissolution and extrusion of the cellular contents, and the integument structures become visible (Fig. 6). With prolonged treatment, the integuments become progressively thinner and ultrastructural details may be discerned.

Three distinctive integument types have been observed in spore suspensions of *B. megaterium* QM B1551 treated with sodium hypochlorite. These include a membranous integument, a fibrillar integument, and a prominent, often characteristically cleaved, outermost integument (Fig. 7).

The membranous integument is thin and featureless; it is perhaps identical with the internal membranous element described by Sacks and

Thomas (8) for *B. macerans* spores. An isolated membranous integument (Fig. 8) exhibits folds and a torn margin through which the cell contents may have been expelled.

A thick outer integument, seemingly cleaved in a fashion analogous to that described by Levinson and Wrigley (3) for the germination process, is shown in Fig. 9. Underlying this integument, and protruding beyond its margin, is a portion of a fibrillar integument (Fig. 9 and 10). The parallel fibrils, the beaded appearance, and the 100-A diameter of the fibrils of such integuments (Fig. 11) suggest an identity with the structure described by Thomas (10). Employing a microincineration technique, Thomas described such an integument structure, which was characterized as a 100-A, fibrillar, textured, middle-coat layer of *B. megaterium* spores. It may correspond also to the "matrix of beaded threads" described by Knaysi and Hillier (2). The fibrillar arrangement and beaded appearance are visible also when such integuments are viewed directly (Fig. 15 and 16).

Free fibrils were encountered frequently in suspensions of *B. megaterium* QM B1551 spores treated with sodium hypochlorite (Fig. 13). The appearance suggests that these were "shed" by integument structures while drying on the mica surface during specimen preparation. Such free fibrils (Fig. 14) have the 100-A diameter.

B. megaterium ATCC 19213. Morphologically, spores of this strain resemble the single-coat strains of *B. megaterium* (e.g., strain KM) described by Fitz-James and Young (1), and lack the polar knobs and the equatorial ridge of *B. megaterium* QM B1551.

Neither the distinctive fibrillar integuments nor free fibrils which characterize strain QM B1551 spores were encountered with this strain. Extremely thin integuments with a meshwork appearance were observed (Fig. 17), however, as well as integuments with marked striations (Fig. 18) which resemble somewhat the fibrillar integuments of strain QM B1551. The appearance of replicas suggests, however, that these striations

FIG. 4-10. *Bacillus megaterium* QM B1551. Fig. 4 and 6, direct observation; Fig. 5, 7, 8, 9, and 10, replicas; Fig. 4 and 5, untreated spores; Fig. 6-10, integuments from combined suspension of spores treated with 0.2% sodium hypochlorite at 37 C for periods ranging from 15 to 60 min.

FIG. 4. Opaque, featureless spores, one exhibiting a prominent polar knob (arrow). $\times 15,000$.

FIG. 5. Single spore with textured surface and equatorial ridge (arrow). The double polar knob appearance is evident from the shadow. $\times 17,500$.

FIG. 6. Integuments from spore suspension treated with sodium hypochlorite. $\times 15,000$.

FIG. 7. Three integument types: a cleaved prominent outer integument (O), a smooth membranous integument (M), and a cluster of fibrillar integuments (F). $\times 25,000$.

FIG. 8. Membranous integument with folds and a ruptured margin (arrow). $\times 25,000$.

FIG. 9. Cleaved outer integument with a portion of the underlying fibrillar integument protruding beyond its margin (arrow). $\times 30,000$.

FIG. 10. Enlargement of the area indicated by arrow in Fig. 9. $\times 72,000$.

FIG. 11–14. *Bacillus megaterium* QM B1551. Replicas of integuments from combined suspension of spores treated with 0.2% sodium hypochlorite at 37 C for periods ranging from 15 to 60 min.

FIG. 11. Folded fragment of fibrillar integument exhibiting parallel fibrils with 100-A diameter and a nonfibrillar appearance on the reverse side (arrows). $\times 50,000$.

FIG. 12. Contrasting detail of outer integument (O) and fibrillar integument (F). The repeating pattern of the latter is superimposed on the former in the region of overlay (margin indicated by arrows). $\times 36,000$.

FIG. 13. Outer integuments and isolated fibrils (arrow) presumably derived from fibrillar integuments such as are shown in Fig. 11 and 12. $\times 25,000$.

FIG. 14. Enlargement of isolated fibrils indicated by arrow in Fig. 13. These have the 100-A diameter and are variable in length. $\times 150,000$.

FIG. 15–19. Integuments from spore suspensions treated with 0.05% sodium hypochlorite. Action of the sodium hypochlorite was stopped after 40 min at 37 C with 0.3% sodium thiosulfate.

FIG. 15 and 16. *Bacillus megaterium* QM B1551, direct observation.

FIG. 15. Fibrillar integument exhibiting tear resulting from cleavage of adjacent fibrils (arrow). $\times 48,000$.

FIG. 16. Fibrillar integument with isolated fibrils projecting from margin (arrows). $\times 48,000$.

FIG. 17–19. *Bacillus megaterium* ATCC 19213; Fig. 17 and 18, direct observation; Fig. 19, replica.

FIG. 17. Three integuments which emphasize the thinness which results from the action of sodium hypochlorite. $\times 27,500$.

FIG. 18. Integument exhibiting parallel fibrils, folds, and a tear (arrow) which emphasizes the fibrillar arrangement. $\times 44,800$.

FIG. 19. Striations characterize the outermost integument surface of this spore type, apparently a consequence of the fibrillar structure shown in Fig. 18. The inner surface (arrow) seems to lack this pattern. $\times 52,800$.

TABLE 1. Effect of sodium hypochlorite on the optical density of cell suspensions of a variety of microbial types

Organism	Reduction of OD ^a
Bacterial spores ^b	
<i>Bacillus megaterium</i>	89
<i>B. cereus</i>	90
<i>B. subtilis</i>	77
Gram-positive bacteria ^c	
<i>B. megaterium</i>	65
<i>B. cereus</i>	75
<i>Micrococcus luteus</i>	84
Gram-negative bacteria ^d	
<i>Escherichia coli</i>	67
<i>Rhizobium lupini</i>	70
<i>Saccharomyces cerevisiae</i>	60
Soil fungus (spores) ^e	66
Soil actinomycete (spores) ^f	80

^a Exposure to sodium hypochlorite, 0.5%, varied from 10 to 30 min at 37 C. Values depict maximal reduction of OD observed.

^b Similarly affected were spores of five other strains of *B. megaterium*, and one strain each of *B. cereus*, *B. subtilis*, *B. larvae*, and *B. licheniformis*.

^c Also *B. cereus* var. *mycoides*, *B. thermoacidurans*, *Staphylococcus aureus*, and *Lactobacillus bulgaricus*.

^d Also *Aerobacter aerogenes*, *Azotobacter agilis*, *Azotobacter vinelandii*, *Serratia marcescens*, and *Spirillum serpens*.

^e Also spores of two other unidentified soil fungi.

^f Also spores of two other unidentified soil actinomycetes.

may be present on the outer surface of the primary integument (coat) of this strain (Fig. 19). This conclusion is supported by the observation that such striations may be seen also on the surface of resting and germinated spores of this spore type (*unpublished data*).

DISCUSSION

The solubilizing effect of sodium hypochlorite on bacterial spores has been exploited and found to be a useful means of obtaining spore integuments for ultrastructure study. Conventional procedures such as mechanical rupture or sonic treatment for obtaining integuments are not required with this treatment. Spore integuments persist long after cellular integrity has been destroyed. They are digested and become progressively thinner during prolonged exposure to the

chemical, and structural details and resistant features may be detected. Manipulation of concentration, pH, temperature, and time of exposure to sodium hypochlorite permits one to observe cellular elements in varying stages of digestion.

Although this report has been limited to an electron microscope study of *B. megaterium* spores, the action of sodium hypochlorite is not limited to this organism. A variety of microbial cells is affected by the chemical (Table 1), and the technique may prove generally applicable to ultrastructure studies.

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