Location of Bacterial Polysaccharide During Various Phases of Growth

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Received for publication 4 February 1969

Polysaccharide-containing material was located by histochemical staining on ultrathin sections of *Bacillus cereus* and several clostridial species during various phases of growth. These components were located along the cell wall in young vegetative cells and along the developing cortex in sporulating cells. In *B. cereus* any carbohydrate-containing material present along the forespore membrane was too small to be detected by direct staining. However, in three clostridial species such material was detected at all stages of forespore development.

The use of the silver-methenamine technique to stain polysaccharide-containing material on ultrathin sections of bacteria was described by Walker and Short (11). This paper describes the application of this technique to stain sections of *Bacillus cereus* and several clostridial species at various stages of sporulation and germination to obtain more precise information on the times of synthesis of these components.

**MATERIALS AND METHODS**

**Organisms.** The following organisms were used in the present study: *B. cereus* var. terminalis, *Clostridium botulinum* type C (Wellcome Research Laboratories Culture Collection) CN4946, *C. bifermentans* CN1617, and *C. sporogenes* (Leeds University Bacteriology Collection) L. 206.

**Preparation of organisms and staining technique.** Ultrathin sections of the appropriate organism were prepared as described previously (9-11) and stained by the following methods.

(i) The sections were floated onto 0.05% periodic acid and allowed to oxidize for 20 min. After rinsing with two changes of distilled water, they were transferred by a wire loop onto the staining solution which consisted of 10 ml of 0.25% silver nitrate, 0.3 mg of methenamine, 8 ml of 5% borax, and water to 30 ml (5). Staining was carried out at 50°C for 2 hr. After two rinses in distilled water, the sections were transferred to 0.05% sodium thiosulfate for 1 min, and after a final rinse with water they were picked up on Formvar-coated grids. Stained sections were examined with a Philips EM 200 electron microscope at 60 kv.

(ii) The second method was the same as method i, except that the staining solution used was a mixture consisting of 10 ml of 0.25% silver nitrate, 0.3 mg of methenamine, 10 ml of ethyl alcohol, and 10 ml of distilled water. Sections were stained at room temperature for 2 to 6 hr.

With both of these methods the degree and specificity of staining were similar. However, the need to maintain the temperature of the staining solution at 50°C in the first method often resulted in distortion of the specimen. For this reason the second method was developed. The alcoholic staining solution described could be used at room temperature to give excellent staining without distortion of the specimens. The staining time varied from 2 to 6 hr, depending on the specimen and different batches of staining solution. This could be monitored by removing sections at different times and examining them with the microscope.

**RESULTS**

In general, sections were free from extraneous deposits of silver and other electron-dense materials, although the manipulation involved during the staining involves the possibility of such contamination. In some instances (Fig. 9 and 13) such deposits were obtained, but these did not interfere with a reasonable interpretation of the pictures.

**Staining of unoxidized sections.** Sections which were treated with the silver stain without having undergone prior oxidation with periodic acid are shown in Fig. 1 and 2. Deposition of silver occurred on the wall of the young vegetative cell but not on the spore membranes. The electron-dense granules appeared to be larger and less numerous than those observed in oxidized sections (Fig. 3).

**Staining of oxidized sections.** (i) In young vegetative cells of *B. cereus*, silver grains were located only along the cell wall and developing cross walls (Fig. 3). At subsequent stages leading to sporulation, deposits of silver were again seen along the vegetative cell wall, though not along the developing forespore membrane (Fig. 4 and 5). No staining of the forespore septum was observed until after completion of this membrane (Fig. 6 to 11). Only the vegetative cell wall
FIG. 1 and 2. Ultrathin sections of *B. cereus* stained with silver without prior oxidation. (Fig. 1) Young vegetative cell; (Fig. 2) sporulating cell. Coarse deposits of silver are seen along the cell wall (CW) but not along the spore cortex (CO). Marker represents 0.2 μm.
Fig. 3–5. Ultrathin sections of *B. cereus* oxidized with periodic acid and stained with silver. (Fig. 3) Young vegetative cell; (Fig. 4) poly-β-hydroxybutyric acid granule formation; (Fig. 5) forespore development. Fine deposits of silver are seen along the cell wall and developing cross wall (CW) but not along the forespore membrane (FM). Poly-β-hydroxybutyric granules (PHB) are seen developing in the early stages of sporulation. Marker represents 0.2 µm.
FIG. 6-9. Ultrathin sections of sporulating cells of B. cereus showing forespore formation. (Fig. 6 and 7) Untreated; (Fig. 8 and 9) oxidized with periodic acid and stained with silver. Deposits of silver are evident around the cell wall (CW) but not along the forespore membrane (FM) even after the latter is fully completed. Marker represents 0.2 μm.
and the developing cortex were stained during subsequent development of the spore coat and exosporium (Fig. 12 to 15). The cortical membrane appears to stain very intensely. In freshly germinating spores, silver was located along the region of the disintegrating cortex and developing cell wall (Fig. 16) but not along the spore coat and exosporium.

(ii) Similar results were found with C. botulinum type C, C. sporogenes, and C. bifermentans. In young vegetative cells, silver was again located only along the cell wall and developing cross walls (Fig. 17, 18, and 19), though in some instances there was a suggestion of layering within the cell wall (Fig. 18). In contrast to the staining in B. cereus, the developing forespore membrane was stained with silver at all stages of formation (Fig. 20 to 23). During development of the spore coat and exosporium, and during germination, a distribution of deposited silver was observed similar to that in the corresponding stages in B. cereus (Fig. 24 to 27).

DISCUSSION
The distribution of polysaccharide-containing material at various stages of sporulation and germination of B. cereus, C. botulinum type C, C. sporogenes, and C. bifermentans described in this paper extends results previously published (11). In general, silver nitrate is reduced in two ways: (i) nonspecifically by reduced osmium present in the section and (ii) specifically by aldehydes released from the polysaccharide-containing material in the bacterial section by oxidation. Reduced osmium bound by the cell wall is responsible for the silver deposited along this structure in unoxidized sections. On the other hand, the cortex which binds little osmium is unstained. The pattern produced after oxidation under the conditions used is quite different and reflects reduction due to specific release of alde-
Fig. 12 and 13. Ultrathin sections of sporulating cells of B. cereus showing developing spore. (Fig. 12) Untreated; (Fig. 13) similar section oxidized with periodic acid and stained with silver. Deposits of silver are seen along the cell wall (CW) and developing cortex (CO); the cortical membrane (CM) is stained very intensely. No deposits of silver are seen along the developing spore coat (SC) or exosporium (E). Marker represents 0.2 μm.
Fig. 14 and 15. Ultrathin sections of sporulating cells of B. cereus showing mature spores. (Fig. 14) Untreated; (Fig. 15) similar section oxidized with periodic acid and stained with silver. Deposits of silver are seen along the cell wall (CW) and cortex (CO), particularly the cortical membrane (CM), but not along the spore coat (SC) or exosporium (E). Marker represents 0.2 μm.
Fig. 16. Ultrathin section of germinating spore of B. cereus oxidized with periodic acid and stained with silver. Deposits of silver are seen along the region of the disintegrating cortex (CO) but not along the spore coat (SC) or exosporium (E). Marker represents 0.2 μm.

Hydes from polysaccharide-containing material after removal of reduced osmium by reoxidation. In this study, an attempt was made to determine more accurately the times of synthesis of these components by staining sections obtained during various stages of the life cycle. In the case of B. cereus, the results obtained with this technique appear to indicate that any polysaccharides present along the forespore septum are too small to be detected by direct staining, and it is only after completion of the forespore membrane that any significant amount of polysaccharide-containing material is synthesized. On the other hand, in three species of clostridia, it was possible to demonstrate polysaccharide-containing material along the forespore septum at all stages of development. It is difficult to be certain, at this stage, whether these results reflect fundamental qualitative or merely quantitative differences in the sporulation patterns of Bacillus and Clostridium. The suggestion that the space between the invaginating membranes of the forespore includes small amounts of cell wall material, which may serve as a primer in the assembly of second-stage spore cell wall primordium and cortex, has already been made (3). Furthermore, Fitz James (2) showed that, in sporulating mutants of B. cereus, cell wall material was laid down between the forespore membrane at an early stage of forespore formation, although, in these circumstances, cell wall material accumulates more rapidly owing to blocking of subsequent stages. Staining of the cortex is explainable both by reference to its mode of development (1, 8) and to chemical and antigenic studies (9, 12). The cortical membrane appears to be very intensely stained, presumably owing to a more dense accumulation of cortical material in this structure.

In a previous paper (11), staining with silver was attributed to carbohydrates present in mucopolysaccharide material. This terminology was based on an analogy of the use of this technique to locate similar compounds in animal tissues. However, this may not be entirely justified when applied to bacteria. It is pertinent,
therefore, to consider in more detail the types of compounds present in bacteria which may be stained by using this technique. Compounds which are susceptible to such oxidation under the conditions used are those containing 1:2 glycol groups; in bacteria such compounds are present in a number of structural polysaccharides. For example, L-rhamnose, present in both poly- and lipopolysaccharide, N-acetylglucosamine in mucopentite, and glucuronic acid in teichuronic acid are certainly susceptible to this type of oxidation. Various sugars present in
Ultrathin sections showing forespore development in clostridia oxidized with periodic acid and stained with silver. (Fig. 20 and 22) C. sporogenes; (Fig. 21 and 23) C. bifermentans. Deposits of silver are seen along the cell wall (CW) and also along the developing forespore membrane (FM). Marker represents 0.2 μm.
Fig. 24–25. Ultrathin sections of sporulating cells of developing spores of C. botulinum type C. (Fig. 24) Untreated. The cortex (CO) is seen developing between the outer (OM) and inner forespore membranes (IM). (Fig. 25) Similar section oxidized with periodic acid and stained with silver. Deposits of silver are seen along the cell wall (CW) and developing cortex (CO) but not along the spore coat (SC). Marker represents 0.2 μm.
FIG 26. Ultrathin section of sporulating cell of C. bifermentans oxidized with periodic acid and stained with silver. Deposits of silver are seen along the cell wall (CW) and cortex (CO) but not along the spore coat (SC). Marker represents 0.2 μm.

FIG. 27. Ultrathin section of germinating spore of C. bifermentans showing outgrowth; oxidized with periodic acid and stained with silver. Deposits of silver are seen along the cell wall (CW) of the developing vegetative cell but not along the spore coat (SC) or exosporium (E). Marker represents 0.2 μm.
lipopolysaccharide, such as adequose, colitose, paratose, and tyvelose, may also be oxidized. For a more detailed account of these structures see Mandelstam and McQuillen (4).

Recent electron-microscopic studies on cell wall anatomy showed that in _B. megaterium_ the cell wall is built up of two layers: a rigid one made of mucopolymer and a plastic one containing teichoic acid (6). In _B. polymyxa_ the cell wall was shown to contain three layers: an outer protein layer, an intermediate polysaccharide layer, and an inner mucoprotein layer (7). The suggestion of layering observed in the preparations of young vegetative cells of _C. botulinum_ type C may well be due to staining of carbohydrates in different structural layers. In other photographs it appears that it is the mucoprotein layer which is staining most intensely. The resolution obtainable with the present method is inadequate for examining structural details of this type. It is hoped that with subsequent refinements, perhaps combined with enzymatic digestion, the technique might be made more specific for individual components.

ACKNOWLEDGMENTS

We thank R. O. Thomson for his help and advice in preparing the manuscript and throughout this work.

LITERATURE CITED