The Location of Mucopolysaccharides on Ultrathin Sections of Bacteria by the Silver Methanamine Staining Technique

By P. D. WALKER AND J. SHORT

The Wellcome Research Laboratories, Beckenham, Kent, England

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SUMMARY

The silver methanamine technique was used to locate mucopolysaccharides in ultrathin sections of bacteria. Polysaccharides were located along the cell wall and developing cross-walls in young vegetative forms and along the developing cortex in sporulating bacilli. The results are discussed in relation to morphological and chemical studies.

INTRODUCTION

The details of sporulation and germination in bacteria have been extensively studied by the technique of ultrathin sectioning. Species studied include both those in the genus *Bacillus* (Young & Fitz-James, 1959*a, b*; 1962; Fitz-James, 1960; Ohye & Murrell, 1962; Kawata, Inoue & Takagi, 1963) and in the genus *Clostridium* (Hashimoto & Naylor, 1958; Takagi, Kawata & Yamamoto, 1960; Fitz-James, 1962). More recently electron-microscope studies have been combined with immunological and cytochemical studies to demonstrate the location of antigens (Thomson, Walker & Hardy, 1966; Walker, Baillie, Thomson & Batty, 1966; Walker, Thomson & Baillie, 1967*a, b*) and enzymes (van Iterson & Leene, 1964*a, b*; Sedar & Burde, 1965*a, b*; Baillie, Thomson, Batty & Walker, 1967) during various phases of growth.

Proteins and nucleic acids have been located on ultrathin sections of bacteria by observing the changes in structure produced following extraction of these components by digestion with specific enzymes (Granboulan & Leduc, 1967). Although proteins could be digested by proteinases in ultrathin sections of osmium-treated cells (Monneron & Bernhard, 1966), satisfactory digestion of nucleic acids with nucleases was only obtained after aldehyde fixation and the use of a water-soluble resin (Granboulan & Leduc, 1967). Nermut (1967) attempted to stain ultrathin sections of *Bacillus megaterium* for polysaccharides using Ruthenium red, although the results were not very satisfactory. Various methods have been devised for staining ultrathin sections of animal tissues to demonstrate carbohydrates such as glycogen, mucopolysaccharides and glycoproteins. One of these, the silver methanamine technique, was first devised for conventional light microscopy (Gomori, 1946; Jones, 1957) but was later extended to the demonstration of polysaccharide-rich structures in tissues using the electron microscope (Churg, Mautner & Grisham, 1958; De Martino & Zamboni, 1967). In the present paper modifications of this technique are described for the staining of bacterial polysaccharides during phases of sporulation and germination.

METHODS

Organisms. The following organisms were used: *Clostridium bifermentans* (Wellcome Research Laboratories collection) CN1617; *C. botulinum* type C CN4946; *C. sporogenes* (Leeds University Bacteriology Department collection) L206; *Bacillus cereus* var. *terminalis*.

Sporulation. In the case of *Clostridium bifermentans* and *C. sporogenes* the deposit from 25 ml. of a 6 hr sporulating culture in heart infusion broth (Difco) was fixed for 18 hr in the fixative of Kellenberger, Ryter & Séchaud (1958). Similar deposits of sporulating bacteria of *C. botulinum* type C and *Bacillus cereus* were prepared from 18 hr cultures in Robertson meat broth and the sporulating medium of Young (1958), respectively.

Germination. Spore suspensions of *Clostridium bifermentans* and *Bacillus cereus* were heat-shocked at 65° for 10 min. and incubated in nutrient broth containing 5 mM-L-alanine. Samples were removed after 5 min. and 1 hr for centrifugation and fixation.

Preparation of ultrathin sections. After fixation all specimens were dehydrated in ethanol and embedded in Maraglas (Freeman & Spurlock, 1962). Sections were cut with an L.K.B. 'Ultratome' on to distilled water and transferred by means of a wire loop on to the various staining solutions. After the final rinse the sections were collected on 200-mesh formvar-coated grids and examined in a Philips EM 200 electron microscope at 60 kV.

Staining solutions and method of staining. The method used was essentially that of Marinozzi (1961) as modified by Short (1968). To 10 ml. 0.25 % (w/v) silver nitrate add 10 ml. absolute ethanol, 0.3 g. methanamine and 10 ml. distilled water.

Method. Float the sections on to 0.5 % periodic acid, to oxidize them, for 10–20 min.; wash in two changes of distilled water; transfer to the silver solution in a covered receptacle and leave for 2 hr at room temperature (about 22°); wash thoroughly four or five times in distilled water; transfer to 0.5 % sodium thiosulphate for 1–2 min.; wash in distilled water; mount on grids.

RESULTS

The results of staining ultrathin sections of *Clostridium bifermentans* with silver are shown in Pls. 1, 2 and 3; in each case similar sections are compared before and after treatment. It can be seen in Pl. 1, fig. 1 that silver deposits were arranged along the cell wall of the young vegetative form and along the developing cross-wall, with little staining of the cytoplasm; there was considerable difference in contrast between the oxidized and unoxidized sections. Staining of the developing spore is shown in Pl. 2, fig. 2. Both the vegetative cell wall and the developing cortex are stained with silver grains. In the section of germinating spores, shown in Pl. 3, fig. 3, only the cell wall of the emerging vegetative form is stained.

A similar picture is shown for sporulating forms of *Clostridium botulinum* type C (Pl. 4, figs. 4, 5) and *C. sporogenes* (Pl. 4, fig. 6). In both cases the cell wall and developing cortex, but not the spore coat, were stained by silver deposits. Very similar results were found both in sporulating forms and in germinating spores of *Bacillus cereus*. In freshly germinated spores of *B. cereus* only the region of the disintegrating cortex and developing vegetative cell wall was stained (Pl. 4, fig. 7).

DISCUSSION

Staining of carbohydrates results from the reduction of the silver solution by free aldehyde groups released from the mucopolysaccharides by oxidation; electron-dense silver deposits are thus formed at the site. Oxidation also serves to re-oxidize reduced osmic acid which would, if not removed, also reduce silver. During fixation of biological specimens with osmic acid, lower oxides of osmium are formed which blacken the cells and are responsible for much of the electron-density of the sections. Treatment of sections with periodic acid re-oxidizes the reduced osmium by converting it to the soluble tetroxides. This results in a decrease in electron-density in the specimen, particularly in the membranes (Silva, 1967).

The development of silver grains along the cell wall is in agreement with the demonstration of polysaccharide components in isolated walls of both Gram-positive and Gram-negative bacteria by Salton (1953). Staining of the cortex is also explicable by reference to its mode of development and to chemical studies on disintegrated spores. It is known that the cortex is secreted between the two membranes of the fore-spore, which develops as an invagination of the cytoplasmic membrane (Fitz-James, 1960; Ohye & Murrell, 1962). The cortex is in close contact with the 'outer' side of the cytoplasmic membrane, which would normally be in contact with the bacterial cell wall and can thus be regarded, in part, as an endogenous cell wall (Warth, Ohye & Murrell, 1963*a, b*). From their chemical studies on disintegrated spores these workers have also suggested that the spore cortex and bacterial cell wall have a similar chemical composition. Their observation that the spore coat and exosporium consist mainly of protein is supported by our observation that membranes of neither were stained with silver grains.

In some previously reported studies the chemical analyses of various fractions of bacteria have been complicated by the fact that these have not always been homogeneous. For example, it was pointed out by Warth *et al.* (1963*b*) that while the analysis of spore-coat preparations by several workers had indicated the presence of small amounts of hexosamine or DPA material, this could be explained by the presence of small amounts of adherent cortical material in their preparations. The advantage of direct staining is that the chemical components can be identified *in situ* without any undue distortion of the structure. As far as is known this is the first successful application of this method to the demonstration of bacterial carbohydrate *in situ*.

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EXPLANATION OF PLATES

Abbreviations used: *CW* = cell wall; *CO* = cortex; *SC* = spore coat; *E* = exosporium;
N = nuclear material.

PLATE 1

Fig. 1. Section of young vegetative cells of *Clostridium bifermentans* CN1617 unoxidized (L) and oxidized with periodic acid (R); stained with silver. Silver grains can be seen along the cell wall and developing cross-wall. $\times 65,000$.

PLATE 2

Fig. 2. Section of sporulating cells of *C. bifermentans* CN1617 unoxidized (L) and oxidized with periodic acid (R); stained with silver. Deposits of silver are located along the cell wall and cortex, not the spore coat. L, $\times 60,000$; R, $\times 76,250$.

PLATE 3

Fig. 3. Section of germinating spore of *C. bifermentans* CN1617, showing outgrowth after 1 hr incubation unoxidized, L; and oxidized with periodic acid, R; stained with silver. Deposits of silver grains are seen along the cell wall of developing vegetative cell, but not along the spore coat or exosporium. L, $\times 54,400$; R, $\times 46,000$.

PLATE 4

Figs. 4, 5. Section of sporulating cells of *C. botulinum* type C CN4946 after oxidation and staining with silver. Deposits of silver are arranged along the vegetative cell wall and along the cortex. Fig. 4, $\times 45,750$; fig. 5, $\times 61,000$.

Fig. 6. Section of sporulating cells of *C. sporogenes* L206 after oxidation and staining with silver. Deposits of silver are evident along the vegetative cell wall and cortex, but not the spore coat. $\times 45,750$.

Fig. 7. Section of germinating spore of *Bacillus cereus* var. *terminalis* (5 min. incubation), after oxidation and staining with silver. Deposits of silver are evident along cortex and developing vegetative cell wall. $\times 93,500$.