NOTES

Fixation of Mature Spores of Clostridium botulinum

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A triple fixation method using a sequential application of 15 or 30% formaldehyde, 6% glutaraldehyde, and 1% osmium tetroxide resulted in excellent fixation of mature spores of Clostridium botulinum.

The Ryter-Kellenberger fixation method (7, 12) and fixation schedules utilizing a combination of glutaraldehyde and osmium tetroxide (OsO_4) have been widely used to study spore ultrastructure (13). These methods give excellent preservation of vegetative cells and sporulating cells; however, with the onset of spore maturation, the preservation of the spore interior is greatly diminished (2, 3). Difficulty in obtaining structural detail in the interior of mature spores has been discussed by other investigators (1–3, 9, 10). Inadequate fixative penetration through thick spore coats is a major problem in the fixation of mature spores (5, 14) and results in a loss of structural detail of the inner spore coats and spore protoplast (Fig. 1).

In this study, excellent preservation of the ultrastructure of mature spores of Clostridium botulinum was obtained by sequential application of formaldehyde (HCHO), glutaraldehyde, and OsO_4 fixatives. A similar triple fixation procedure has been used by one of us (E.V.C.) for fungal spores. We are unaware of any study employing HCHO as a fixative for bacterial spores.

Spores of C. botulinum type A (National Canners Association strain 78A) were produced in a diphase medium consisting of a solid phase (10% egg-meat medium (Difco), 2% yeast extract (Difco), 1% (NH_4)_2SO_4, 0.5% glucose, 0.15% cysteine-HCl, 2% agar) and a liquid phase [1% (NH_4)_2SO_4, and 0.5% glucose]. After 300 ml of the solid phase had solidified in a 1-liter flask, 500 ml of hot liquid phase was added along with 25 ml of spore inoculum; the medium was incubated in a nitrogen atmosphere at 30 ˚C. The spores were harvested by centrifugation at 3,000 × g for 10 min, washed twice with distilled water, and resuspended in molten 2% agar; after solidification, the agar was cut into 1-mm cubes which were subjected to triple fixation procedures with various concentrations of formaldehyde.

Fixative solutions were prepared from stock solutions of 40% HCHO derived from paraformaldehyde powder (11), 50% glutaraldehyde, and crystalline OsO_4. Final concentrations of HCHO used were 4, 8, 15, and 30% for 2 hr. Glutaraldehyde and OsO_4 were used at concentrations of 6 and 1% for 2 and 18 hr, respectively. All fixatives were buffered at pH 7 with 0.1 M sodium phosphate buffer; 15 and 30% HCHO were also used without buffering at pH 10 and 11, respectively. The HCHO and glutaraldehyde fixatives were used at 21 C; OsO_4 was used at 4 C.

After fixation, the agar cubes were dehydrated in an acetone series and embedded in Epon 812 epoxy resin (8). Thin sections were cut with a diamond knife, stained with uranyl magnesium acetate and Millonig’s lead hydroxide (4), and examined in an RCA EMU-3 model E electron microscope at an accelerating voltage of 50 kv.

Sequential application of HCHO, glutaraldehyde, and OsO_4 gave better fixation of mature spores than the standard fixation methods (Fig. 2). Varying the HCHO concentration used in the initial fixation period had a slight effect on the adequacy of fixation. Spores fixed with lower concentrations (4 and 8%) of

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FIG. 1. Mature spore of Clostridium sporogenes, enclosed within its sporangium, fixed with the Rytter-Kellenberger method (7, 12). Although preservation of the vegetative cell is excellent, detail is lacking in the spore interior (photo courtesy of L. M. Santo, Univ. of California, Davis). C, spore coat; CX, cortex; CY, vegetative cell cytoplasm. Bar equals 0.2 μm.

FIG. 2. Mature spore of Clostridium botulinum exhibits a well-defined, multilayered exosporium, electron-dense outer coat layer, and a thick inner coat after triple fixation. The darkly stained ribosomes and nucleoid areas are also clearly differentiated in the spore interior. C, spore coat; CX, cortex; N, nucleoid area; R, ribosomes; E, exosporium. Bar equals 0.2 μm.
HCHO were incompletely fixed and insufficiently infiltrated with embedding resin. Sections of these spores were prone to electron beam damage, resulting in a loss of the spore protoplast during examination in the electron microscope. At higher concentrations (15 and 30%), spore ultrastructure was well-preserved and exhibited minimum beam damage. A significant decrease in the quality of fixation was noted when either the HCHO or glutaraldehyde fixation steps were eliminated. Varying the pH of the HCHO fixative from pH 7 to 11 had no noticeable effect on the adequacy of fixation.

The use of HCHO to fix relatively impermeable bacterial spores and their contents is logical since the penetration velocity of HCHO is greater than that of other aldehyde fixatives or OsO4 (6). The triple fixation method presented here provides an effective method of fixing mature bacterial spores, such as those of *C. botulinum*.

LITERATURE CITED


