JOURNAL OF BACTERIOLOGY, Oct. 1983, p. 409–413
0021-9193/83/100409-05$02.00/0

Vol. 156, No. 1

NOTES

Triple Fixation of *Bacillus subtilis* Dormant Spores

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Received 17 May 1983/Accepted 5 July 1983

A triple-fixation method with a sequential application of 5% glutaraldehyde, 1% osmium tetroxide, and 2% potassium permanganate gave superior preservation of the ultrastructure of *Bacillus subtilis* dormant spores with a thick spore coat.

A double-fixation method, using a combination of glutaraldehyde and the standard fixative (OsO_4) of Ryter et al. (7), has been widely employed to study the ultrastructure of many bacterial cells, including vegetative cells, sporulating cells, germinating spores, and some kinds of dormant spores; the method provides satisfactory preservation of these organisms. However, this fixation method was not adequate for the preservation of dormant spores with a thick spore coat, such as that found in *Bacillus subtilis* and *Bacillus coagulans*, because it resulted in a diminution in size of the cortex, a deformation of the spore protoplast, and a loss of structural detail of the cortex and spore protoplast (Fig. 1A). A triple-fixation method, using a sequential application of formaldehyde, glutaraldehyde, and OsO_4 (10), which is effective for fixing dormant spores of *Clostridium botulinum*, was also unsatisfactory for fixing dormant spores of *B. subtilis* because of a deformation of the spore coat and insufficient infiltration of resins (Fig. 1B).

In this study, many double- or triple-fixation procedures were examined to obtain satisfactory preservation of the ultrastructure of dormant spores of *B. subtilis*, and it was found that sequential application of glutaraldehyde, OsO_4, and $KMnO_4$ gave very acceptable images. Detailed information on this triple-fixation method is given below.

Dormant spores of *B. subtilis* PCI 219, prepared by growing the organism on nutrient agar at 37°C for 5 days, were harvested and washed five times with deionized water by centrifugation. Samples were prefixed in 5% glutaraldehyde buffered at pH 7.2 with 0.1 M sodium phosphate buffer for 5 to 8 h at 4°C and centrifuged at 1,500 × *g*. After being washed five times with 0.05 M sodium phosphate buffer (pH 7.2), the pellet was fixed in 1% OsO_4 buffered with 0.1 M sodium phosphate buffer (pH 7.2) for 3 days at 4°C and washed by centrifugation. The postfixation was performed with 2% $KMnO_4$ dissolved in deionized water for 3 h at ice-bath temperature, and the fixed sample was washed thoroughly with 0.05 M sodium phosphate buffer (pH 7.2) by centrifugation until the purple color of $KMnO_4$ in the supernatant was discharged. The sample was then suspended in molten 2% agar. After solidification, the agar was cut into 1-mm cubes. The agar cubes were dehydrated by passage through an ethanol series, substituted by *n*-butyl glycidyl ether, and embedded in Quetol 653 (2). Sections were cut on a Porter-Blum MT-2 ultramicrotome, stained with 6% uranyl acetate and Reynolds lead citrate (6), and examined in a JEOL JEM-200CX electron microscope at an accelerating voltage of 200 kV.

This triple-fixation method was effective for fixing dormant spores of *B. subtilis*, especially in regard to the following points: (i) providing a suitable contrast; (ii) presenting a large number of longitudinal sections of dormant spores with sufficient infiltration of resins; (iii) providing easy observation of a double density laminated surface layer (Fig. 2A), a laminar cortex, and a spore protoplast with its clear germ cell wall and granules (Fig. 2B), which were difficult to observe with other fixation methods. The postfixation with $KMnO_4$ is necessary for better preservation of the germ cell wall. The surface layer may correspond to the exosporium-like outer layer reported by Sousa et al. (9). A laminar cortex was observed more clearly with a double-fixation method of 4% paraformaldehyde and 1% OsO_4 than with the triple-fixation method (Fig. 3). With acid treatment of dormant spores of *Bacillus megaterium* and a variant *Bacillus cereus*, it has been reported that the swollen cortical remnant of spores is composed of five to seven concentric layers of dense filaments in the former (3) and four layers in the latter (11).

On the other hand, the weak points of this

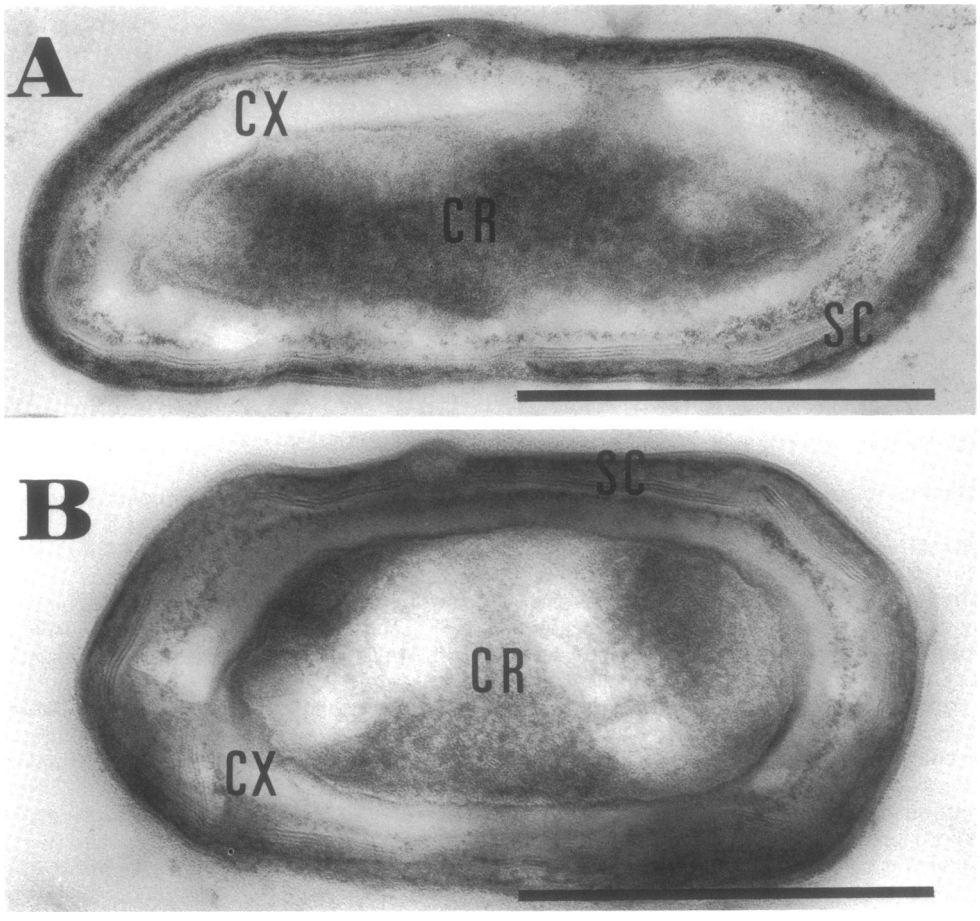

FIG. 1. (A) Dormant spores of *B. subtilis* fixed with a combination of glutaraldehyde and the standard fixative (OsO_4) of Ryter et al. (7), and (B) by the triple-fixation method which was reported by Stevenson et al. (10). With the former fixation, veronal acetate was employed as a buffer and dormant spores were fixed with 5% glutaraldehyde for 6 h at 4°C and subsequently with 1% OsO_4 for 3 days at 4°C. With the latter fixation, sodium phosphate buffer was used and dormant spores were fixed with 15% formaldehyde for 2 h at 4°C, 6% glutaraldehyde for 2 h at 4°C, and finally 1% OsO_4 for 3 days at 4°C. SC, Spore coat; CX, cortex; CR, core. Bar, 0.5 μm.

triple-fixation method were as follows: (i) there was some difficulty in making a cut with a glass knife because of two heavy-metal fixatives, i.e., OsO_4 and $KMnO_4$; (ii) there was a little swelling of the spore because of the small ratio of the major to minor axis compared with that of dormant spores treated by a double-fixation method shown in Fig. 1A; and (iii) unknown, granular, dense substances were present between the inner spore coat and cortex. Moreover, this fixation method was not applicable to either germinated spores of *B. subtilis* or dormant spores with a thin spore coat, such as *B. cereus*.

Dormant spores fixed successively with each fixative remained refractile and were dyed green by the Wirtz-Conklin spore stain method (4) (Table 1). The contents of protein, dipicolinic acid, and Ca^{2+} in dormant spores treated continuously with each fixative were almost similar to those for nontreated spores (Table 1). Moreover, amino acids, dipicolinic acid, and Ca^{2+} were not detected in any fixative after treatment of dormant spores (Table 1). These facts demonstrated that fewer biological components were leached from the dormant spores by the triple fixation method, and no signs of germination occurred during this process. The decrease of about two-thirds in the tyrosine content of dormant spores finally fixed with $KMnO_4$ (Fig. 4) may be due to a slight perturbation of the spore coat (Fig. 2A and B) because it has been reported that relatively tyrosine-rich polypeptides are present in *B. subtilis* spore coats (5).

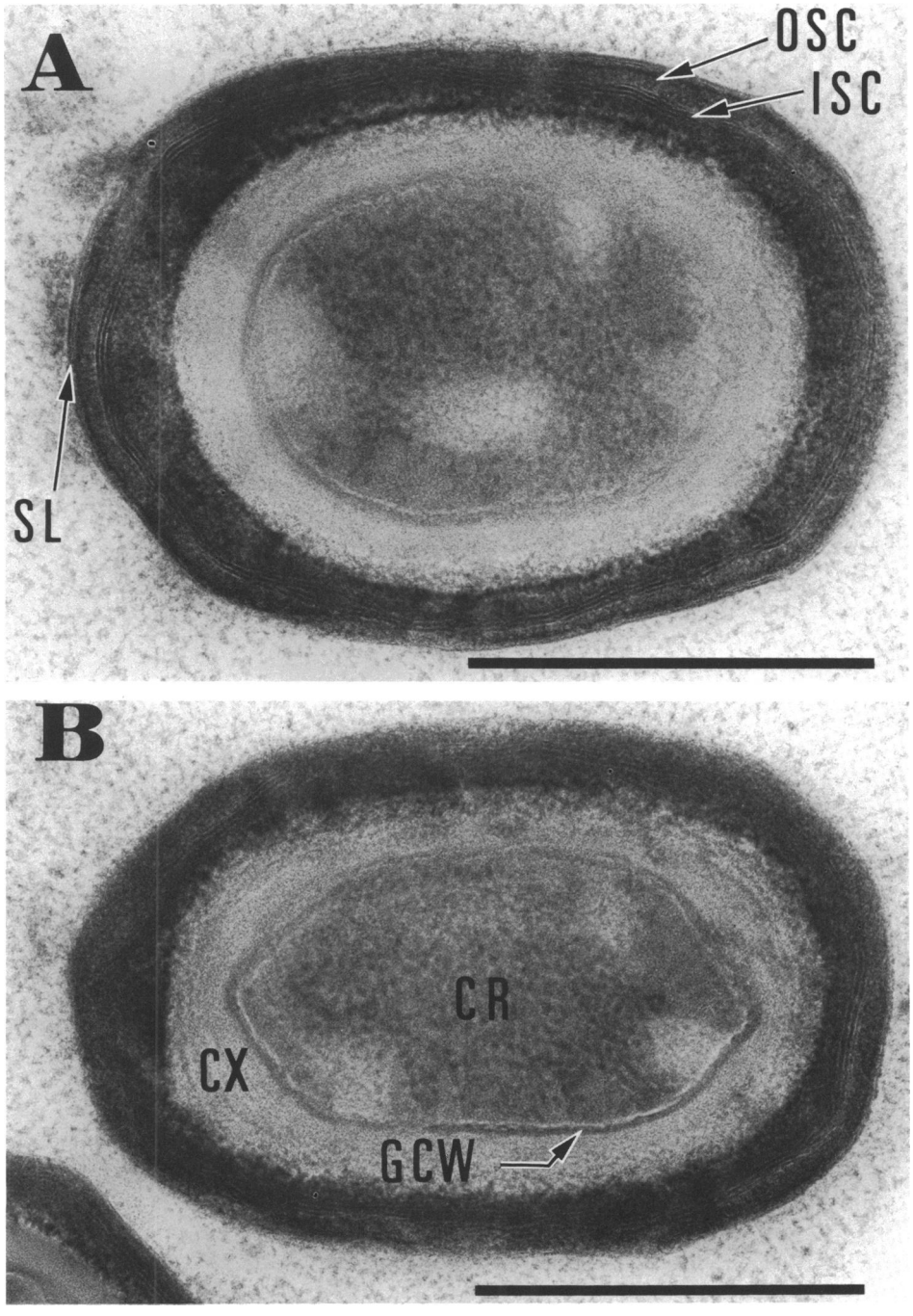

FIG. 2. Electron micrographs of thin sections of dormant spores of *B. subtilis* fixed by a triple-fixation method, using a sequential application of 5% glutaraldehyde, 1% OsO_4, and 2% $KMnO_4$. (A) Exterior ultrastructure. (B) Interior ultrastructure. SL, Surface layer; OSC, outer spore coat; ISC, inner spore coat; CX, cortex; GCW, germ cell wall; CR, core. Bar, 0.5 μm.

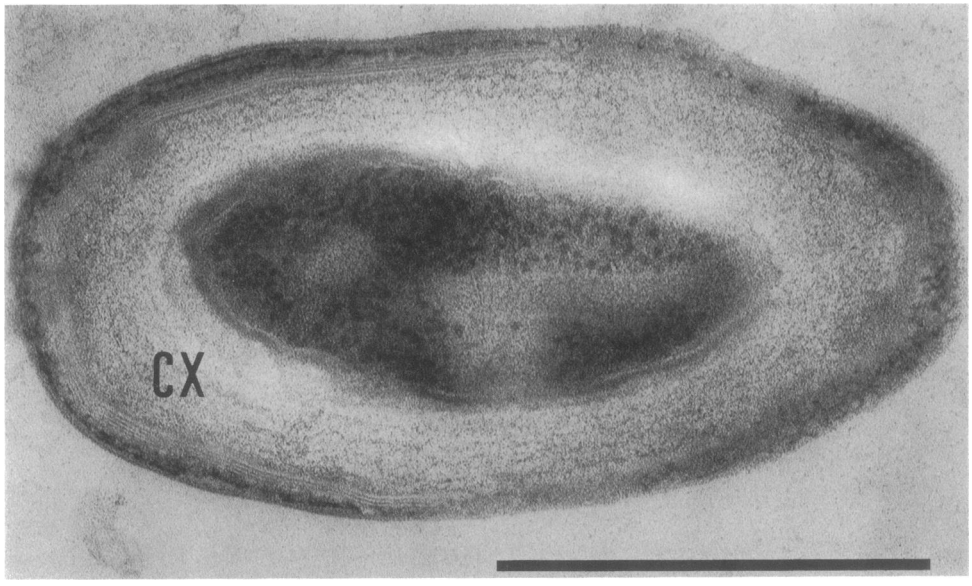

FIG. 3. Electron micrograph of a thin section of a dormant spore of *B. subtilis* prefixed with 4% paraformaldehyde for 15 h at 4°C and postfixed with 1% OsO_4 for 3 days at 4°C. CX, Cortex. Bar, 0.5 μm.

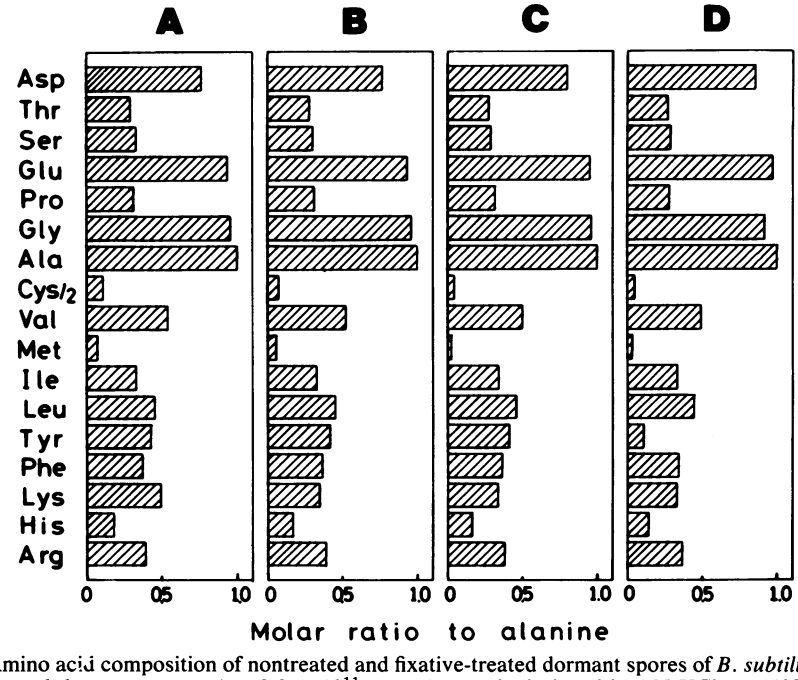

FIG. 4. Amino acid composition of nontreated and fixative-treated dormant spores of *B. subtilis*. Nontreated or fixative-treated dormant spores (ca. 2.9×10^{11} spores) were hydrolyzed in 6 N HCl at 110°C for 20 h. All hydrolyses were under vacuum and their amino acid compositions were routinely determined with a Hitachi 835 high-speed amino acid analyzer equipped with an autosampler, data processer, graphic printer, and recorder. Alanine, 1.0. (A) Nontreated dormant spores. (B) Glutaraldehyde-treated spores. (C) Glutaraldehyde and OsO_4-treated spores. (D) Glutaraldehyde, OsO_4, and $KMnO_4$-treated spores.

TABLE 1. Effect of treatment with each fixative on dormant spores of *B. subtilis*[a]

Treatment[b]	Spore content of:		
	Amino acid[c] (mg)	DPA[d] (mg)	Ca^{2+}[e] (mg)
None	49.2	12.2	4.1
Glutaraldehyde	47.9	12.2	3.6
Glutaraldehyde and OsO_4	47.0	12.7	3.5
Glutaraldehyde, OsO_4, and $KMnO_4$	47.4	12.2	3.3

[a] Amino acid, DPA, and Ca^{2+} contents in each fixative after treatment were not detected. Amino acid analysis was performed by using a Hitachi 835 high-speed amino acid analyzer, but the exact analytical detection of low levels of amino acid was difficult because of mixture with the fixative. DPA content was measured by the method described by Scott and Ellar (8). Ca^{2+} content was measured with a Hitachi atomic absorption spectrophotometer model 180-50. In all cases the fixed spores remained refractile when observed with phase-contrast microscopy. All fixed spores were stained green with the Wirtz-Conklin spore stain.

[b] About 2.9×10^{11} spores were treated.

[c] Calculated from the data shown in Fig. 4.

[d] DPA, Dipicolinic acid. Determined by the method described by Janssen et al. (1).

[e] Ca^{2+} content in a supernatant obtained by autoclaving dormant spores for 15 min at 15 lb/in^2, and centrifuging was measured by using a Hitachi atomic absorption spectrophotometer model 180-50.

It should be emphasized that the triple-fixation method described here is suitable for obtaining structural detail in the interior of dormant spores with a thick spore coat, including *B. subtilis*.

We are grateful to Roy H. Doi, Department of Biochemistry and Biophysics, University of California, Davis for his review and valuable criticism of the manuscript. We thank Taeko Hotta, Joint Laboratory, Nagoya City University Medical School, for performing amino acid analyses.

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