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Immunoelectron Microscopic Localization of One of the Spore Germination Proteins, GerAB, in *Bacillus subtilis* Spores

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Ultrastructural localization of GerAB, one of the proteins of *Bacillus subtilis* spores related to L-alanine-initiated germination, was investigated by immunoelectron microscopy with antipeptide (residues 61 to 80 of GerAB) antiserum and a colloidal gold-immunoglobulin G complex. Immunogold particles were visualized in the boundary region between the cortex and coat of dormant spores, and they were broadly dispersed into the cortex region after germination.

Germination of *Bacillus subtilis* spores is triggered by L-alanine and many L-alanine analogs (8, 14). Some chemical characteristics, including the sizes and electrostatic properties of the recognition sites for the -CH_3, -COO-, and -NH-groups of the alanine molecule, of the receptor were determined in studies of the structure-activity relationships of alanine-related germinants and inhibitors (8, 14). Moreover, the alanine receptor was suggested to be located in a hydrophobic environment (15–17). However, the receptor itself has not been identified as protein molecules in the spore.

In genetic studies of germination mutants of *B. subtilis*, the *gerA* operon was demonstrated to be involved in L-alanine-initiated germination (11–13). The *gerA* operon encodes three membrane-associated proteins (GerAA, GerAB, and GerAC) which make up the alanine receptor complex (4, 12, 18). GerAB has a hydropathy profile characteristic of an integral membrane protein (18).

In this study, the localization of the GerAB protein in dormant and germinated spores was investigated by immunoelectron microscopy as described below. A peptide [amino acids 61 to 80, hereafter referred to as the GerAB(61–80) or 61–80 peptide] of the deduced GerAB protein, NTLIQKKHQTPSL PETLKEG (18), was synthesized with an Excell automated peptide synthesizer (MilliGen/Biosearch) and purified by reverse-phase liquid chromatography with a μBondasphere C_{18} column (Waters Chromatography), and its sequence was confirmed by analysis with a peptide sequencer (Applied Biosystems). The peptide was conjugated to bovine serum albumin by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma Chemical). Antibodies were raised against 0.4 mg of the conjugate emulsified in Freund's complete or incomplete adjuvant (Difco Laboratories) in a rabbit. Several booster injections were given subcutaneously at intervals of 1 week, and the animal was bled a week after the final booster. The titer of the specific antipeptide antiserum was 4,500 (the reciprocal of the highest dilution), as determined by a peptide enzyme-linked immunosorbent assay.

B. subtilis GSY225 and PCI219 were grown and sporulated on nutrient agar at 37°C for 5 days. Spores were harvested and washed repeatedly with deionized water at 4°C by centrifugation. Germinated spores were obtained by incubating dormant spores with 1 mM L-alanine–0.05 M phosphate buffer at pH 7.2 for 120 min. Both dormant and germinated spores were fixed with 4% freshly depolymerized paraformaldehyde–0.1 M phosphate buffer (pH 7.4) at 4°C for 4 h, washed three times in 10% sucrose–phosphate-buffered saline (PBS) (pH 7.2), and suspended in 2% agar. Small cubes were cut, dehydrated with a graded series of 30, 50, 70, 95 and 100% ethanol for 1 h at each ethanol concentration at −20°C and with 100% ethanol for 18 h at −20°C, and then suspended in LR-White resin (London Resin)–ethanol at 2:1, 1:1, 1:2, and 1:0 ratios for 2 h at each ratio at −20°C and finally in fresh LR-White for 18 h at −20°C. Samples were then placed in gelatin capsules filled with LR-White and 0.01% accelerator (London Resin) and polymerized by UV irradiation (model TUV-100 UV polymerizer; Dosaka EM) for 16 h at 4°C. Ultrathin sections were cut with a glass knife on a Reichert Ultracut-E ultramicrotome and mounted on Formvar-coated nickel grids (Veco Ni 200). The grids were placed on droplets of 0.1 M L-lysine–0.05 M Tris-HCl-buffered saline (TBS) (pH 7.2) for 30 min, on 1% gelatin–TBS for 30 min, and then onto a 1:100 dilution of rabbit anti-GerAB(61–80) peptide antiserum in 1% gelatin–TBS for 2 h at room temperature. The grids were washed two times by floating on droplets of TBS for 30 min, treated with a 1:30 dilution of colloidal gold (10-nm particle diameter)-conjugated goat anti-rabbit immunoglobulin G (IgG) (Zymed Laboratories) in 1% gelatin–TBS for 1 h, washed two times with TBS for 30 min each time, and finally washed with 0.01% Tween 20–PBS for 15 min. The sections were then contrasted by using 3% uranyl acetate for 15 min. Control sections were treated in a similar way by using preimmune serum from the same rabbit instead of anti-GerAB(61–80) peptide antiserum. The sections were observed with a JEM-200CX electron microscope operating at 80 kV.

In dormant spores (Fig. 1), immunogold staining was observed mainly within the spore integument, especially outside the cortex region. A significant number of the gold particles were distributed between the membranous thin structure surrounding the cortex and the inner coat region in cells of strain GSY225 (Fig. 1A) or from the membranous thin structure to the inner coat region in cells of strain PCI219 (Fig. 1B). Only a few gold particles were seen in the other regions, including the core, inner membrane, cortex, and outer coat (Fig. 1A and B). In germinated spores (Fig. 2), they were broadly dispersed in the cortex region but nearly absent from the cytoplasm and cytoplasmic membrane of the developing core (Fig. 2A and B). The distribution of gold particles seems to be compact in dormant spores and diffuse in germinated spores. In the latter, the GerAB proteins may spread in the interspace that arises by partial digestion of the cortex layer during germination. A

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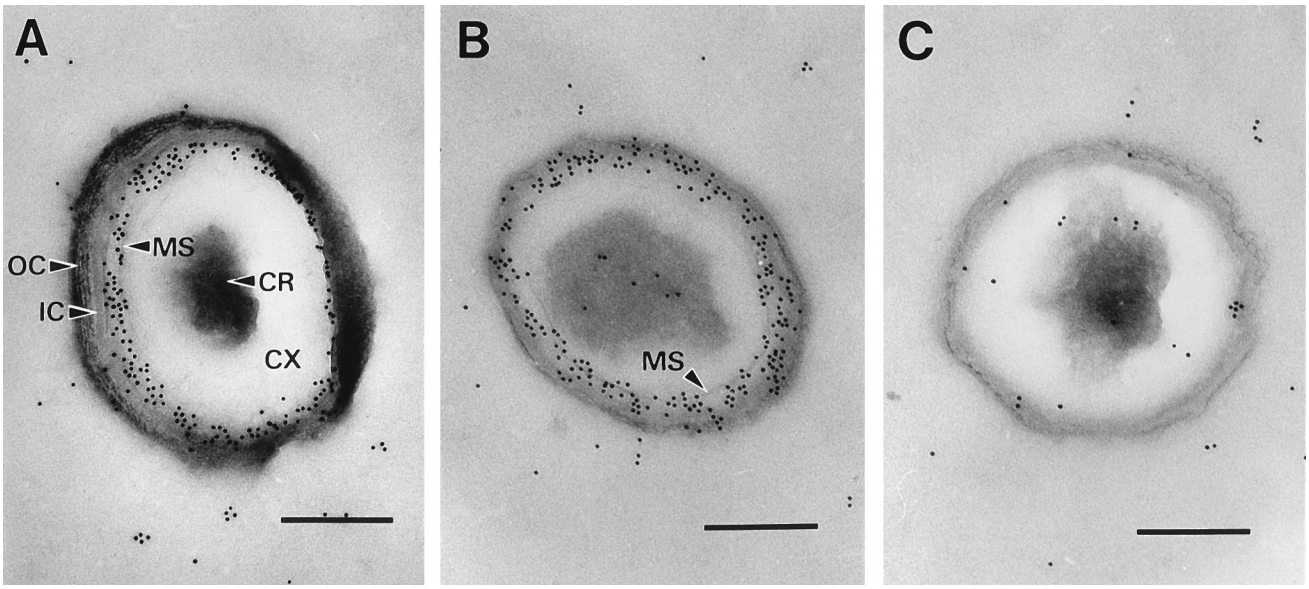

FIG. 1. Immunoelectron microscopic localization of the GerAB protein in dormant spores of *B. subtilis* GSY225 (A) and PCI219 (B and C). Thin sections of dormant spores fixed with 4% paraformaldehyde were stained with anti-GerAB(61–80) peptide antiserum and colloidal gold (10 nm)-IgG complex (A and B) or with preimmune serum and colloidal gold (10 nm)-IgG complex (C). OC, outer coat; IC, inner coat; CX, cortex; MS, membranous structure; CR, core. Bars, 200 nm.

greatly reduced level of staining was observed with the antiserum absorbed with the GerAB(61–80) peptide (data not shown), and only a few particles were seen with preimmune serum as a negative control (Fig. 1C and 2C).

These results demonstrated that the GerAB protein, or at least the GerAB(61–80) peptide, was expressed in significant amounts in dormant spores and that its localization site was the boundary region between the cortex and spore coat. GerAB is thought to be a membrane-integrated protein because of its highly hydrophobic nature (18). Spores contain two membranes: an inner membrane and an outer membrane. The inner membrane directly surrounds the spore core, whereas the outer membrane seems to lie more peripherally between the cortex and spore coat or among the layers of the spore coat (2). Although the outer membrane is barely seen as a bilayer structure in *B. subtilis* spores under electron microscopy (10), the presence of membrane proteins in the spore coat region was suggested by immunoelectron microscopy (5). Our earlier germination studies of *B. subtilis* spores from a chemical standpoint showed that the alanine receptor might be located in a hydrophobic environment (15–17). Thus, we propose here that the receptor for exogenous germinants or the spore germination apparatus, including the GerAB protein, is in the outer membrane or in the other hydrophobic environment just inside