Characterization and Deposition of the Proteins in the Outermost Layer of Bacillus megaterium Spore

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Abstract It was proved that three spore coat proteins of 48, 36, and 22 kDa (P48, P36, and P22) were the components of the outermost layer (OL) of Bacillus megaterium ATCC 12872 spore by analysis of the isolated OL. And it was indicated that these proteins were deposited not by disulfide bond, but by ionic and/or hydrophobic bonds on the spore. Among them, P36 and P22 were expected to be located on the very surface of the spore by immunological analysis. In the OL deficient mutant of B. megaterium ATCC 12872, MAE05, whose spore was lacking in these OL proteins and galactosamine-6-phosphate polymer, both P36 and P22 were present in the mother cell cytoplasm and deposited on the forespores, but they disappeared with the lysis of mother cells. An OL protein-releasing factor having proteolytic activity was detected in the culture supernatant at the late sporulating stage of both the wild-type and the mutant strains. But the factor could not act on the proteins of the mature spores and the forespores at t10 (tn indicates n hr after the end of exponential growth) of the wild-type strain. Moreover, P36 and P22 were found in the spores of a revertant of MAE05 which could form galactosamine-6-phosphate polymer, suggesting that this sugar polymer played the role in protecting the OL proteins against the protease-like substance after the deposition.

Bacillus megaterium ATCC 12872 (QM B1551) consists of two layers, the inner coat (IC) and the outermost layer (OL). Ogaki et al (18) reported that the main components of OL were galactosamine-6-phosphate polymer and polypeptides. But nothing has been known about polypeptides in OL, because they did not analyze the intact OL but the resistant fraction of the spore coat, a resistant residue against sonication, alkali-treatment, and pronase digestion prepared by Kondo-Foster's method (14). All the OL deficient mutant spores of ATCC 12872 lacked three major spore coat proteins (SCP) of 48, 36, and 22 kDa (P48, P36, and P22) as previously reported (22). This result suggested that these proteins were the components of OL.

Many studies have been carried out about the ultrastructure, composition, synthesis and processing of polypeptides/proteins of the spore coat (1, 2, 6, 7, 9,
11, 20, 21). Nevertheless many problems still remain unresolved about deposition, namely, processing, assembly of SCP and their regulation. Some spore coat mutants have been used to resolve these subjects. Lysozyme-sensitive coatless mutant of Bacillus cereus could synthesize SCP precursors and process them but the processed SCP were recovered from cytoplasm, not from the spore surface, and the alteration in the outer forespore membrane was suggested from the morphological analysis (19). Protease deficiency and its association with defects in spore coat structure were proposed in B. cereus and B. subtilis germination mutants with alteration in spore coat structure (4, 12). Recently Donovan et al (5) cloned genes encoding some spore coat polypeptides of B. subtilis, but any one of the proteins examined was found not to have a significant effect on the incorporation of other polypeptides into the spore coat by insertional inactivation of spore coat genes. In B. megaterium, we found no report concerning the study of deposition mechanism of SCP using spore coat defective mutant spores.

This study was conducted to identify and characterize the proteins in OL and to better understand the reason why P48, P36, and P22 were missing in these OL deficient mutant spores.

**MATERIALS AND METHODS**

*Bacterial strains and growth conditions.* B. megaterium ATCC 12872 was obtained from the American Type Culture Collection, and the OL deficient mutants, MAE02, MAE04, and MAE05, were isolated in our laboratory (22). Cells of these strains were grown and sporulated in SNB medium at 30 C as previously described (22). Phase-bright spores reach maximum at t8 (tₙ indicates n hr after the end of exponential growth) and this stage was used as an internal marker for the timing of developmental events.

*Isolation of the intact OL.* The suspension (20 mg dry weight/ml deionized water) of spore coat preparation (18) was sonicated at 30-sec intervals for a total sonication time of 3 min with the small probe of a Heat Systems Ultrasonics sonicator, model W-220F, at 4 C. After sonication, the suspension placed on the tube containing the ten stepwise Urografin density gradients (density range, 1.28-1.37 g/cm³) was centrifuged as previously described (22). Two bands of density, 1.28 and 1.35 g/cm³, respectively, were collected by a syringe, washed with deionized water several times after centrifugation (10,000×g for 20 min), and lyophilized after dialysis against deionized water for three days.

*Electron microscopy.* Specimens were prepared, stained, sectioned, and observed under an electron microscope (Hitachi, HU-120, 80 kV) as described previously (16).

*SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis.* SDS-PAGE was performed on 1 mm thick vertical slab gels of 12.5% polyacrylamide at a constant current of 30 mA at room temperature. For visualization, gels were stained with Coomassie brilliant blue R-250 or immunologically. Immunoblot analysis was carried out by the method of Imagawa et al (9).
**PROTEINS IN THE SPORE OUTERMOST LAYER**

*Extraction of SCP.* Spores, a spore coat preparation, or an OL preparation were suspended (10 mg dry weight/ml) in an alkaline solution (pH 9.8) of 1% sodium dodecyl sulfate, 50 mM dithiothreitol, and 0.1 M NaCl (SDS/DTT) or other solubilizing solutions and SCP were extracted at 37°C for 2 hr. After centrifugation at 10,000 × g for 10 min at room temperature, extracts were stored at −20°C.

*Preparation of anti-surface-protein IgG.* Spores were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight and dehydrated with 10 and 15% sucrose for 4 hr, respectively, and finally with 20% sucrose overnight. All procedures were performed at 4°C. Fixed spores, after washing with 0.1 M sodium phosphate containing 0.2 M NaCl, were suspended in the same buffer containing anti-SCP IgG. After incubation at 25°C for 30 min for adsorption and washing with the same buffer for removal of unadsorbed IgG, 0.005 M glycine-HCl buffer (pH 2.5) was added for elution of anti-surface-protein IgG, and then the supernatant after centrifugation was neutralized with 2 M Tris-HCl (pH 8.0).

*Preparation of mother cell cytoplasm and forespore fractions.* Sporulating cells were harvested from 100 ml of the culture, washed with 0.85% NaCl, and suspended in 5 ml of 50 mM Tris-HCl buffer (pH 8.0). After disruption of the cells by sonication (model NS-250, Nihonseiki) at 30-sec intervals for a total time of 3–5 min, the suspension was centrifuged at 15,000 × g for 30 min at 4°C. The supernatant was used as the mother cell cytoplasm fraction and the precipitate was used as the forespore fraction after washing with deionized water.

*Detection of SCP-releasing factor (SRF).* The forespore fraction (10 ml) of the culture at t10 was incubated with 1 ml of the culture supernatant or the mother cell cytoplasm fraction at 30°C for 1 hr. After incubation, the forespore fraction was washed with deionized water and SCP was extracted with SDS/DTT solution at 37°C for 2 hr. Immunoblot analysis was carried out on the extracted SCP.

*Isolation of the revertants of MAE05.* A spontaneous streptomycin-resistant mutant of MAE05, MAE05ST, was isolated at first by plating the culture on nutrient agar containing 100 μg streptomycin per ml. According to the method previously described (22), revertants were isolated. Approximately 10⁹ spores of MAE05ST were centrifuged on Urografin density gradient (density range, 1.26–1.34 g/cm³) and the diluents of the fraction of low density (less than 1.32 g/cm³) were spread and sporulated on glutamate agar at 30°C for two days. After sporulation, the spores with low hydrophobic surface like the wild-type were selected by salt aggregation test.

*Chemicals.* Urografin (Schering AG, West Germany), Nonidet P-40 (Sigma), streptomycin (Wako) were used. All reagents employed in this study were of analytical grade, unless otherwise stated.

**RESULTS**

*Isolation of OL from the Spore Coat*

Electron microscopic observation of a large gap between OL and IC in *B. megaterium* spores as previously described (3), prompted us to try to separate OL
from IC mechanically. Two sharp bands appeared at the density of 1.28 g/cm³ and 1.35 g/cm³ when Urografin density gradient centrifugation was carried out on the spore coat preparation after sonication for 3 min in deionized water, but no clear separation was achieved without sonication. Coat-like fragments were observed in the higher density band but not in the lower density band under the phase-contrast microscope. In the OL deficient mutant MAE05, only one band was found at the density of 1.28 g/cm³. Moreover, the main component of OL, hexosamine, was detected only in the higher density band (data not shown). Figure 1 is the electron micrograph of the thin sections of the higher density band and the intact spore showing that the higher density band is OL free from fibrous IC.

Analysis of the Proteins in OL

Because the intact OL could be isolated as a higher density band, the OL proteins were analyzed. In SDS/DTT-soluble fraction of the OL preparation, a trace amount of cysteine was detected, whereas in SDS/DTT-insoluble fraction,
lysine was rich, more than 25% of the total residues (data not shown). Figure 2 shows SDS-PAGE pattern of the SDS/DTT-soluble proteins from the OL preparation together with those of the spore coat preparation and spores of both the wild-type and the OL deficient mutant MAE05. Three proteins, P48, P36, and P22, which are lacking in the OL deficient mutant spores, are found in the OL preparation.

**Solubilization of P48, P36, and P22 with Various Chemical Reagents**

To know how these OL proteins are deposited on the spore, SCP were extracted with various chemical reagents from the wild-type spores and the proteins solubilized were analyzed by SDS-PAGE (Fig. 3). Almost all proteins including P48, P36, and P22 were solubilized with 1% SDS and 0.1 M NaCl, and P48 with 1 M NaCl, but no proteins were solubilized with 4 M urea, 50 mM ethylenediaminetetraacetate (EDTA), and 2% Nonidet P-40.

**Surface Protein of the Mature Spore**

The location of these OL proteins was examined by the immunological method. Figure 4 shows the result of the immunoblot analysis of SCP with anti-surface-protein IgG. P36 and P22 reacted with anti-surface-protein IgG, but P48 did not.

**Detection of P48, P36, and P22 in Mother Cell Cytoplasm**

All of the OL deficient mutant spores including MAE05 spore lack these OL proteins.
proteins (22). Absence of these proteins in the mature spore does not necessarily mean no synthesis of them, because the mutation of the gene responsible for processing or assembly may be possible. To know the reason for no deposition, we first analyzed the mother cell cytoplasmic fraction by immunoblot method using anti-SCP IgG (Fig. 5). In the MAE05 strain, P36 and P22 were detected in this

Fig. 3. SDS-PAGE of SCP obtained by extraction with various solutions. Lane 1, 1% SDS, 0.1 M NaCl, 50 mM DTT (pH 9.8); lane 2, 1% SDS, 0.1 M NaCl (pH 9.8); lane 3, 2% Nonidet P-40; lane 4, 1.0 M NaCl; lane 5, 0.3 M NaCl; lane 6, 0.3 M NaSCN; lane 7, 50 mM EDTA; lane 8, 4.0 M urea.

Fig. 4. Immunoblot analysis for surface-protein of spores. SCP, extracted with SDS/DTT solution, was transferred to nitrocellulose paper after SDS-PAGE and the paper was treated with anti-SCP IgG (lane 1), and with anti-surface-protein IgG (lane 2).
fraction at $t_6$ and $t_8$ respectively, as well as in the wild-type strain, but P48 was not. The same result was obtained on the other OL deficient strains, MAE02 and MAE04. As the slightly stained band near P48, which is also found in Fig. 2, lane 4, did not react with anti-P48 IgG (data not shown), this is not the band of the degradation product of P48.

**Deposition of P36 and P22 on the Forespores**

P36 and P22 are not detected in mature spores of MAE05 although they exist in mother cell cytoplasm. Therefore, SCP on the forespores were analyzed by the immunoblot analysis (Fig. 6). Both P36 and P22 were deposited on the forespores at $t_8$, but began to disappear from $t_{12}$. Observation under the phase-contrast microscope revealed that the lysis of mother cells of MAE05 began at $t_{12}$ and free spores were released from most sporulating cells at $t_{17}$.

**SCP-Releasing Factor (SRF) in the Culture Supernatant**

Disappearance of P36 and P22 deposited on the forespores with the lysis of the mother cells suggested the existence of SRF. When SRF was screened by using forespores at $t_{10}$ of MAE05, SRF was found only in the culture supernatant after $t_{12}$ and P22 was more susceptible than P36. Figure 7, lane 2, indicates that SRF is found in the culture supernatant of the wild-type strain as well as the mutant.
strain and lane 3 indicates that its activity is inhibited by 10 mM EDTA and 2 mM phenylmethylsulfonyl fluoride (PMSF), and SRF is expected to be a protease-like substance. But the factor did not act on the forespores at t19 and the mature spores of the wild-type strain, suggesting that some protective mechanism exists in the wild-type strain.

Deposition of P36 and P22 on the Mature Spore of the Revertant

The streptomycin-resistant (100 µg/ml) mutant of MAE05, MAE05ST, reverted spontaneously at frequencies of more than $1 \times 10^{-9}$. The revertant had the translucent OL by electron microscopy without staining. The hexosamine content in a resistant fraction and the result of a salt aggregation test were consistent with that of the wild-type strain (data not shown). Figure 8 shows SDS-PAGE of SCP of the spores of MAE05ST, the revertant, and the wild-type strain. In the revertant, P36 and P22 were deposited and moreover P48 was synthesized and deposited.

DISCUSSION

Sonication allowed the coat preparation to separate into two bands by Urografin density gradient centrifugation and the higher density band was OL, because
Fig. 7. Digestion of P36 and P22 on the forespore with culture supernatant. The forespore fraction of MAE05 at t10 was incubated with the culture supernatant at t12 during sporulation, and immunoblot analysis was carried out on the extracted SCP. Lanes 1 and 2, incubated with culture supernatant of MAE05 and the wild-type strain; lane 3, incubated with culture supernatant of MAE05 containing 10 mM EDTA and 2 mM PMSF; lane 4, not treated.

Fig. 8. SDS-PAGE of SCP from the revertant spore. Lane 1, spore of the wild-type strain (ATCC 12872); lane 2, spore of the streptomycin-resistant OL deficient mutant (MAE05ST); lane 3, spore of the revertant.
this band was the thick and dense layer with no fibrous IC structure and hexosamine was detected only in this band. The missing higher density band in the OL deficient mutant spores provided another supportive evidence for this. Isolation of OL made it possible to analyze the proteins in the intact OL. It was concluded that three proteins (P48, P36, and P22) absent in the mature spores of the OL deficient mutants are the components of OL, since they were found in SDS/DTT-soluble proteins of the higher density band. Recently, Imagawa et al (10) reported that P48 antigen was found only in the OL region from immunoelectron microscopic studies.

SDS/DTT-soluble proteins of the OL fraction contained only trace cysteine and P48, P36, and P22 were solubilized without the reducing agent, DTT, and hence we think these OL proteins are not deposited by disulfide bond like in B. cereus (1), but by ionic and/or hydrophobic bonds. By immunochemical technique, P36 and P22 were found to be on the very surface of the spore, but P48 was not. Since Imagawa et al (9) reported that P48, P36, and P22 were deposited successively on the forespore after synthesis, P48 may be on the basal layer of OL.

In the spore of OL deficient mutant, MAE05, which could not synthesize P48 and galactosamine-6-phosphate polymer, P36 and P22 were detected in the mother cell cytoplasmic fraction but not deposited on the mature spore. This had suggested that P48 and/or galactosamine-6-phosphate polymer were necessary for the deposition of these proteins. But this assumption was proved to be incorrect by immunoblot analysis of the forespore fraction; P36 and P22 were deposited temporarily on the forespore and they disappeared by the action of SCP-releasing substance in the culture supernatant. Because the releasing activity was inhibited by EDTA and PMSF and some extracellular proteases have been reported in B. megaterium sporulating cells (13, 15), the factor in the culture supernatant must be a protease-like substance from sporulating cells. P22 was more susceptible than P36 to SRF. Since both P36 and P22 in the cytoplasm fraction were degraded equally with SRF (data not shown), the assembly of these proteins in the forespore of MAE05 may participate in the susceptibility to SRF, and further work is required.

We reported that galactosamine-6-phosphate polymer begins to be deposited on the forespore from t9 (17) and may be responsible for the surface hydrophobicity of the spore (22). In this study, it was indicated that this sugar polymer does not participate in the deposition of P36 and P22, but may play the role in protecting these OL proteins against the releasing factor after deposition, because loss of these proteins was not observed in the mature spore and the forespore at t10 of the wild-type strain where the sugar polymer had been deposited. SDS-PAGE of SCP of the revertant also supported this hypothesis. At present it remains unknown how P36 and P22, also P48, are assembled specifically on the forespores, but MAE05 may be used to elucidate this problem. MAE05, as well as MAE02 and MAE04, was a phenotypically pleiotropic mutant, but the revertant could synthesize both P48 and galactosamine polymer. Since the revertants were isolated at the frequency of more than $1 \times 10^{-9}$, MAE05 might be a single mutant and the synthesis of P48 and galactosamine polymer was dependently regulated during morphogenesis.
of OL. Recently we isolated Tn917 insertion OL deficient mutants and their phenotypes supported the above assumption (unpublished data). Holland et al (8) suggested that ger E, which appears to be concerned in the regulation of spore coat formation, encodes the polypeptides that may have DNA-binding property. Further experiments are in progress to clarify this mutated gene product and its function in the regulation of morphogenesis of OL.

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