Involvement of Superoxide Dismutase in Spore Coat Assembly in Bacillus subtilis

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Endospores of Bacillus subtilis are enclosed in a proteinaceous coat which can be differentiated into a thick, striated outer layer and a thinner, lamellar inner layer. We found that the N-terminal sequence of a 25-kDa protein present in a preparation of spore coat proteins matched that of the Mn-dependent superoxide dismutase (SOD) encoded by the sodA locus. sodA is transcribed throughout the growth and sporulation of a wild-type strain and is responsible for the SOD activity detected in total cell extracts prepared from B. subtilis. Disruption of the sodA locus produced a mutant that lacked any detectable SOD activity during vegetative growth and sporulation. The sodA mutant was not impaired in the ability to form heat- or lysozyme-resistant spores. However, examination of the coat layers of sodA mutant spores revealed increased extractability of the tyrosine-rich outer coat protein CotG. We showed that this condition was not accompanied by augmented transcription of the cotG gene in sporulating cells of the sodA mutant. We conclude that SodA is required for the assembly of CotG into the insoluble matrix of the spore and suggest that CotG is covalently cross-linked into the insoluble matrix by an oxidative reaction dependent on SodA. Ultrastructural analysis revealed that the inner coat formed by a sodA mutant was incomplete. Moreover, the outer coat lacked the characteristic striated appearance of wild-type spores, a pattern that was accentuated in a cotG mutant. These observations suggest that the SodA-dependent formation of the insoluble matrix containing CotG is largely responsible for the striated appearance of this coat layer.

An important determinant of the resistance, longevity, and germination properties of Bacillus subtilis endospores is a proteinaceous structure known as the coat. The coat is assembled from a heterogeneous (in both size and amino acid composition) group of over 2 dozen polypeptides and in its final state is differentiated into a lamella-like inner layer and a striated, electron-dense outer layer (1, 11, 18, 50). Biogenesis of the spore coat is the result of a complex process of macromolecular assembly that is controlled at different levels. It involves intricate genetic regulation, with the sequential participation of at least four mother cell-specific transcription factors in the order σE, SpoIID, σK, and GerE (31, 46). The transcriptional control guarantees that the production of coat structural components, as well as the morphogenetic proteins that guide their assembly, occurs in the mother cell chamber of the spore and is differentially regulated in the inner versus outer coat layers. However, assembly of the inner or outer coat layers does not closely reflect the order of transcription of coat structural genes (cot) but rather is largely dependent on a topological plan that is laid down early in the process and requires the proteins of three morphogenetic genes, spoIVA, spoVID, and cotE (3, 40, 45, 50). Mutations in these genes affect the assembly of many coat proteins around the forespore but, at least in some extent, do not interfere with the interactions among specific components. Coat structural proteins of spoIVA and spoVID mutants can still associate to form long swells of coat material in the mother cell cytoplasm (3, 40, 45). Evidently, assembly of the spore coat involves interactions among individual coat polypeptides and mechanisms that promote interactions of higher-order building blocks.

The mechanisms enforcing these interactions are poorly understood, but the available evidence points to proteolysis and reversible or irreversible protein cross-linking (2, 8, 24, 25, 48). Inter- or intramolecular cross-linking is likely to be an important determinant of the spore coat functional architecture, since about 30% of the total coat protein is confined in a fraction that is refractory to extraction under reducing conditions and to electrophoretic analysis (37, 48). Recently, (ε-γ)-glutamyl-lysyl isopeptide bonds were detected in spores and purified coat material (25). A transglutaminase activity was subsequently purified, and the corresponding gene was cloned and characterized (24). (ε-γ)-Glutamyl-lysyl isopeptide bonds are known to be present in other biological structures such as the eye lens crystallin and keratins (16, 18, 47). In other systems, dityrosine cross-links are generated by the activity of peroxidase with H2O2. Formation of O,O-dityrosine bonds is known to take place in the hardening of the nematode cuticle, the insect egg chorion, and the sea urchin fertilization membrane (13, 27, 43). Dityrosine bonds are also important in elicitor- and wound-induced oxidative cross-linking of plant cell wall proteins (6, 26). In all cases, cross-linking of structural proteins results in the insolubilization of specific components and confers a high degree of chemical and mechanical resistance on the final structure (16, 43, 47). Because purified coat material has a high tyrosine content, Pandey and Aronson (37) proposed that O,O-dityrosine formation could be an important mechanism in coat assembly. However, the demonstration of dityrosine cross-links in coat material has been difficult (15). In addition, the putative peroxidase(s) has not been found, nor has a system for the generation of H2O2.

In this report, we provide evidence reinforcing the view that oxidative cross-linking is an important mechanism in spore coat assembly. We found that a sodA mutant lacking an Mn-
type superoxide dismutase (SOD) produces spores with altered coat layers. We propose that SodA is required to fix at least one major coat structural protein, CotG, into a structure from which it is not easily extracted. This process has dramatic consequences for the architecture of the coat.

MATERIALS AND METHODS

Bacterial strains and general methods. With the exception of ZB307 (51), all of the B. subtilis strains utilized in this study are congenic derivatives of Spore strain MB24 (Table 1). Escherichia coli DH5α (Bethesda Research Laboratories) was used for routine molecular cloning procedures. Luria-Bertani medium was used for the routine growth of E. coli or B. subtilis. Difco sporulation medium was used for sporulation of B. subtilis (35). The extent of sporulation was measured by the titer of heat, chloroform, or lysozyme CFE per milliliter at 18 h after the onset of sporulation (19). All of the other general techniques used were described previously (18, 19).

Extraction and analysis of spore coat proteins. Coat proteins were extracted from Renografin-purified spores as described before (18, 19). Usually, about 105 spores were fixed in 3 ml of 4% paraformaldehyde, and aqueous 4% uranyl acetate at room temperature. Following de- inactivation of the spore coat, the protein was digested with proteinase K (24) and subjected to two-dimensional gel electrophoresis (PAGE). Protein bands were stained with Coomassie blue. Titration experiments were conducted to determine the optimal concentration of proteinase K.

Electron microscopy. Spores were purified on Renografin step gradients as previously described (18, 19). Under native conditions, about 104 spores were fixed in 3 ml of 4% paraformaldehyde, and aqueous 4% uranyl acetate at room temperature. The cells were further processed for electron microscopy. Staining of internal structures was enhanced with additional 1-h steps in 0.1% tannic acid, 1% osmium tetroxide, and aqueous 4% uranyl acetate at room temperature. Following dehydration in a graded ethyl alcohol series, the cells were rehydrated in ultraviolet-visibility embedding medium (25). Postsection staining was completed with 2% alcoholic uranyl acetate and calcined lead citrate (17) for 5 min each. Observation and photography of the samples were performed with a Philips CM-10 transmission electron microscope operated at 80 kV.

RESULTS

Mn-dependent SOD is associated with the inner coat layers. Coat proteins were extracted from purified spores produced by a cotE deletion mutant, by treatment with SDS and mercaptoethanol (DTT) as previously described (18, 19). This procedure extracts a heterogeneous protein sample from wild-type spores and a less complex one from cobalt mutant spores. After transfer of the electrophoretically resolved proteins to a polyvinylidene difluoride membrane, we obtained the N-terminal amino acid

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or phenotype</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>MB24</td>
<td>trpC2 metC3 Spo</td>
<td>Laboratory stock;</td>
</tr>
<tr>
<td>ZB307</td>
<td>SPlc2de2:ZaTn917:pSK2Δa10</td>
<td>Laboratory stock;</td>
</tr>
<tr>
<td>AH394</td>
<td>trpC2 metC3 SPlacZ</td>
<td>E. Ricca; 41</td>
</tr>
<tr>
<td>AH763</td>
<td>trpC2 metC3 SPcoclEucz</td>
<td>This work</td>
</tr>
<tr>
<td>AH1490</td>
<td>trpC2 metC3 sodA::pAH402</td>
<td>This work</td>
</tr>
<tr>
<td>AH1494</td>
<td>ZB307 SPlcoglacZ</td>
<td>This work</td>
</tr>
<tr>
<td>AH1495</td>
<td>trpC2 metC3 SPcoglglacZ</td>
<td>This work</td>
</tr>
<tr>
<td>AH1496</td>
<td>trpC2 metC3 sodA::pAH402</td>
<td>This work</td>
</tr>
<tr>
<td>AH1497</td>
<td>trpC2 metC3 cotG::cat</td>
<td>This work</td>
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<tr>
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<td>This work</td>
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<td>AH1517</td>
<td>ZB307 SPlcoglHlacZ</td>
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<td>trpC2 metC3 sodA::pAH402</td>
<td>This work</td>
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<tr>
<td>AH64</td>
<td>trpC2 metC3 ΔcotE::cat</td>
<td>Laboratory stock;</td>
</tr>
<tr>
<td>AH94</td>
<td>trpC2 metC3 gerE::M</td>
<td>Laboratory stock;</td>
</tr>
</tbody>
</table>

TABLE 1. B. subtilis strains used in this study

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The sequence of a 25-kDa polypeptide and found that it matched that of several Mn-dependent SODs. We cloned a 550-bp DNA fragment internal to the sodA gene into an integrational plasmid, creating pAH402 (Fig. 1). Sequence analysis of the cloned insert confirmed that a fragment of a SOD-encoding gene had been cloned. The complete sequence of the gene encoding the Mn-dependent SOD from B. subtilis (yQD or sodA) was later deposited in the GenBank database (accession no. D84432) (29). The sodA gene is predicted to encode a 25.3-kDa polypeptide, in good agreement with our initial estimation. Alignment of the complete amino acid sequence with those of other bacterial enzymes confirmed its assignment as an Mn-dependent SOD (29, 38, 39). Four conserved residues are ligands to the metal cofactor: the histidines at positions 27, 82, and 168 and the aspartate at position 164. Moreover, among the residues involved in discriminating between iron or manganese as the cofactor, the two glycines at positions 77 and 78, the histidine at position 79, the phenylalanine at position 85, the glutamine at position 149, and aspartate 150 are nearly invariant in the Mn-dependent SODs (38, 39, and data not shown). In the structurally related Fe-dependent enzymes, the corresponding positions are preferentially occupied by an alanine, a glutamine, a tyrosine, an alanine and a glycine (38). sodA is expressed throughout growth and sporulation. Because the enzyme was found to be associated with the coat layers, we wanted to know if the enzyme activity could be detected in sporulating cells at the time of coat formation or in purified spores. We prepared whole-cell lysates during the growth and sporulation of wild-type strain MB24 (Fig. 2). The replicase gel was then stained for SOD activity by the NBT method (see Materials and Methods). A single activity band was detected during the logarithmic phase of growth (log) and at various times (in hours) after the initiation of sporulation (T<sub>0</sub>). Crude extracts were prepared, and samples of protein (30 µg) were resolved on nondenaturing 12.5% polyacrylamide gels Lanes: 1: log; 2: T<sub>0</sub>; 3: T<sub>3</sub>; 4: T<sub>6</sub>; 5: T<sub>9</sub>. The gels were stained for SOD activity by the NBT method. The arrow indicates the position of the acharomatic bands produced by SOD activity.

The yggE locus is not required for viability, SOD activity, or sporulation. Examination of the sodA region of the chromosome identified an associated downstream open reading frame designated yggE in the sequence given GenBank accession no. D84432 (29). The start codon of yggE is separated from the sodA stop codon by only 33 bp, and no possible transcription terminators or obvious promoters could be identified in the intergenic region (Fig. 1). The 429 residue YggE protein (about 48 kDa) is predicted to have 10 to 12 hydrophobic segments, indicating a possible association with the membrane. Interestingly, genes encoding highly similar products are located downstream of SOD genes in B. stearothermophilus (accession no. P28754) and B. caldotenax (accession no. X62682). Because these observations suggested a functional linkage between sodA and yggE, we wanted to know whether the absence of the SOD activity band in strain AH1490 was due to a polar effect on the expression of yggE. For this purpose, strain AH1499 was analyzed for the presence of a SOD activity band on native gels (the strain carries a disruption of the yggE locus caused by the integration of pAH406). Strain AH1499 retained SOD activity and produced wild-type levels of heat- and lysozyme-resistant spores (data not shown). We concluded that the band observed in SOD activity gels is sodA dependent and that expression of this activity does not require yggE function. We further concluded that yggE is not essential for growth of B. subtilis.

sodA mutants form spores with altered coat layers. We hypothesized that SodA could have a role in spore coat assembly, since the enzyme was found in preparations of coat proteins and because the SOD activity was detected in whole-cell extracts at times that included the period during sporulation.
when the coat is assembled. To examine whether SodA participates in spore coat assembly, we analyzed by gel electrophoresis the profile of SDS–DTT-extractable proteins from the coat of sodA mutant spores. In parallel, coat proteins were extracted from equal numbers of wild-type (strain MB24) and gerE (AH94) or cotE (AH64) mutant spores, which lack the inner or outer coat layer, respectively (30, 50). The samples of purified coat material were analyzed by SDS–15% PAGE, and examples are shown in Fig. 3. Approximately the same amount of spore suspension was used in all cases, as confirmed by the relative intensity of a band of about 15 kDa (asterisk in lane 1) whose extractability remained essentially unaffected in cotE and gerE mutants (Fig. 3, lanes 3 and 4, respectively) compared to that in wild-type spores (lane 2).

The main difference revealed in the pattern of electrophoretically resolved proteins extracted from the coats of sodA mutant spores is that a protein of about 36 kDa was much more abundant in the extract from the mutant spores than in that from wild-type spores (Fig. 3). Several lines of evidence indicate that the 36-kDa protein is CotG. Its apparent size corresponds to that of CotG (41), and it is missing from the coats of both a cotE (Fig. 3, lane 3) and a gerE (lane 4) mutant. CotG is an outer coat protein (and therefore dependent on CotE for assembly), and its production is controlled at the transcriptional level by the GerE protein (41). Finally, the N-terminal sequence analysis of the 36-kDa protein produced a single sequence of eight residues that matched that of CotG.

Disruption of the yggE locus did not affect the extractability of CotG (data not shown). Thus, the effect seen in the AH1490 mutant cannot be attributed to a polar effect of the sodA insertion mutation. A band corresponding to a protein of the size of CotB (9) was also more abundant in the extract from the sodA mutant (Fig. 3, lane 1), although this effect was not always as pronounced as in this particular gel. This band exhibited the same dependency on cotE reported previously (50) for the assembly of CotB into the coat (Fig. 3, lane 3).

SodA controls the assembly of CotG into coat layers. Transcription of the cotG gene by the σ83 form of RNA polymerase is dependent on DNA-binding protein GerE (41). GerE is a regulator that affects the transcription of several cot genes (49).

We examined the transcription of a cotG-lacZ fusion integrated in single copy in the prophage SPβ in wild-type and sodA mutant cells. We found that the pattern of cotG-lacZ expression in the sodA mutant did not differ from that observed in wild-type cells (Fig. 4). Moreover, cotG-lacZ expression in the sodA mutant was still dependent on GerE (data not shown). We conclude that the sodA::pAH402 allele did not affect the transcription of cotG. Assembly of CotG into the outer coat layers is known to depend on CotE and CotH (33, 41). Expression of cotE-lacZ and cotH-lacZ in the sodA mutant did not differ from the profile obtained with wild-type cells (data not shown). Furthermore, the extractability of proteins of the size of CotE (24 kDa) or CotH (43 kDa) was not altered in coat material purified from sodA mutant spores (Fig. 3), suggesting that their production or assembly was unaffected. These results indicate that the increased representation of CotG in the extracts of coat proteins from spores of the sodA mutant is not caused by increased transcription of the cotG gene or by increased levels of the proteins that normally recruit CotG for assembly. It seems more likely that loss of SodA results in a change in CotG that makes it more easily extractable from the spore coat.

Ultrastructural analysis reveals that SodA and CotG are important determinants of outer coat organization. Because the sodA null allele affected the extraction properties of an abundant coat component, we thought the mutation would have an impact on the ultrastructural characteristics of the coat structure. We examined purified wild-type and AH1490 spores by electron microscopy. Figure 5A is an electron micrograph of a thin section of a wild-type spore. The coat, delimited by the two arrows, consisted of a lightly staining lamellar inner sublayer closely apposed to a thick, electron-dense, and multilayered outer coat (1, 18, 50). The inner coat structure usually displayed two to five lamellae, whereas the outer coat showed a characteristic pattern of striations, also two to five, depending on the section considered. In contrast, SodA mutant spores had a reduced inner coat and a highly diffuse outer coat which had lost its striated appearance (Fig. 5B). In addition, both...
coat structures did not seem to associate tightly. We reasoned that if increased extractability of CotG correlated with a decrease in outer coat structural organization, then complete loss of CotG should have a much greater impact on coat morphology. As predicted, the cotG mutation caused a dramatic alteration in the morphology of the coat layers: spores of a cotG insertion mutant had an expanded outer coat that had completely lost its electron density and multilayered type of organization (Fig. 5C). Note that the outer coat appeared to be sandwiched between the inner coat lamellae and a thin layer of material at its outer edge that is slightly more electron dense than the rest of the structure. The significance of this layer is unknown. sodA-cotG double-mutant cells formed spores whose coat layers did not differ greatly from those of a cotG single mutant, suggesting that sodA acts mainly through cotG (Fig. 5D). These characteristics are consistent with a model in which CotG plays a major role in the organization of the outer coat, possibly by forming an insoluble matrix that is the basis for its characteristic multilayered appearance. We propose that SodA is used to cement CotG into this insoluble matrix.

**DISCUSSION**

An Mn-dependent SOD encoded by the sodA locus of *B. subtilis* was found to be associated with spore coat proteins. SODs catalyze the disproportionation of superoxide radicals to hydrogen peroxide and oxygen and are thought to have an important role in defending the organism against the toxic effects of oxygen (12). In agreement with the results of Casillas-Martinez and Setlow (7), we detected a single SOD activity throughout the growth and sporulation of *B. subtilis*. This activity is dependent on the sodA locus, since its inactivation results in cells that lack detectable SOD activity (7 and this work) and sodA is transcribed during growth and sporulation (this work). In confirmation of the results of Casillas-Martinez and Setlow (7), we found that sodA is not essential for viability in rich medium or sporulation and that inactivation of the sodA locus is not compensated for by the expression of other forms of the enzyme. In the bacterium *E. coli*, the sodB gene (encoding an Fe-dependent SOD) is expressed constitutively, whereas sodA encodes an inducible enzyme (12). It is unclear why in *B. subtilis* and at least two other members of the gram-positive group (14, 34) the constitutive SOD activity appears to be Mn, as opposed to Fe, dependent. The gene downstream from sodA, yqgE (29), which appears to be associated with genes encoding Mn-containing SODs in at least two other spore-forming *Bacillus* species, is also dispensable for growth or sporulation. In addition, yqgE is not required for the expression of sodA activity in crude extracts. However, the fact that it probably encodes a membrane protein and the cross-species conservation of the sodA-yqgE unit prompt us to speculate that the activity of SodA might be somehow coupled to a membrane-associated function.

Casillas-Martinez and Setlow (7) showed that sodA plays no role in the resistance of *B. subtilis* spores to oxidizing agents. Our observations suggest an alternative role for SodA during sporulation. Our results suggest that SodA participates in the assembly of the spore coat, possibly by activating the oxidative cross-linking of a specific coat structural component. Although we initially found SodA in a preparation of coat proteins, it is not known whether SodA is enriched in this fraction or whether most of the cell’s SodA is located elsewhere. Since *H$_2$O$_2$*, produced by SodA would be diffusible, the involvement of SodA in spore coat formation does not require that SodA be a component of the coat. SodA mutant spores exhibit increased extractability of a previously characterized coat protein, CotG, an abundant spore coat component. We found that the sodA mutation does not affect cotG promoter activity. We also found that the sodA mutation does not affect the expression of the cotE and cotH loci (the only known requirements for CotG assembly [33, 41]) or the abundance of the corresponding products in the SDS-DTT-extractable fraction of coat proteins. The CotG protein is tyrosine rich and is organized in nine repeats of a 13-amino-acid sequence whose con-
sens is $nN'KKS\gamma R/C\gamma S/T nN'KKSRS$ (the residues in the smaller font indicate the least-conserved positions) (41). The relatively high level of tyrosines suggests that CotG is a potential substrate for a peroxidase that catalyzes the polymerization of CotG via dityrosine cross-links. We propose that CotG can exist in two forms, a monomeric, soluble form that can be detected by analyzing a sample of purified coat material by SDS-PAGE and a polymeric, cross-linked form that is insoluble and not amenable to electrophoretic resolution. In our model, SodA is involved in the production of $H_2O_2$, which could be used by a peroxidase for the cross-linking of certain coat proteins (e.g., CotG). Therefore, CotG could partition between a soluble, easily extractable fraction and an insoluble (cross-linked or polymeric) fraction. The polymerization of CotG is represented schematically, since the exact stoichiometry of the reaction is unknown. The model does not require SodA to be a coat structural component because $H_2O_2$ is diffusible.

\[ 2O_2^- + 2H^+ \xrightarrow{SodA} H_2O_2 + O_2 \]

\[ \text{CotG} \xrightarrow{\text{Peroxidase}} (\text{CotG})_n + H_2O \]

FIG. 6. Model for the role of SOD in spore coat assembly. According to the model, SodA is involved in the production of $H_2O_2$, which could be used by a peroxidase for the cross-linking of certain coat proteins (e.g., CotG). Therefore, CotG could partition between a soluble, easily extractable fraction and an insoluble (cross-linked or polymeric) fraction. The polymerization of CotG is represented schematically, since the exact stoichiometry of the reaction is unknown. The model does not require SodA to be a coat structural component because $H_2O_2$ is diffusible.

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