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Assembly and Function of a Spore Coat-Associated Transglutaminase of *Bacillus subtilis*

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The assembly of a multiprotein coat around the *Bacillus subtilis* spore confers resistance to lytic enzymes and noxious chemicals and ensures normal germination. Part of the coat is cross-linked and resistant to solubilization. The coat contains ε-(γ-glutamyl)lysyl cross-links, and the expression of the gene (*tgl*) for a spore-associated transglutaminase was shown before to be required for the cross-linking of coat protein GerQ. Here, we have investigated the assembly and function of Tgl. We found that Tgl associates, albeit at somewhat reduced levels, with the coats of mutants that are unable to assemble the outer coat (*cotE*), that are missing the inner coat and with a greatly altered outer coat (*gerE*), or that are lacking discernible inner and outer coat structures (*cotE gerE* double mutant). This suggests that Tgl is present at various levels within the coat lattice. The assembly of Tgl occurs independently of its own activity, as a single amino acid substitution of a cysteine to an alanine (C116A) at the active site of Tgl does not affect its accumulation or assembly. However, like a *tgl* insertional mutation, the *tglC116A* allele causes increased extractability of polypeptides of about 40, 28, and 16 kDa in addition to GerQ (20 kDa) and affects the structural integrity of the coat. We show that most Tgl is assembled onto the spore surface soon after its synthesis in the mother cell under σ^K control but that the complete insolubilization of at least two of the Tgl-controlled polypeptides occurs several hours later. We also show that a multicopy allele of *tgl* causes increased assembly of Tgl and affects the assembly, structure, and functional properties of the coat.

The *Bacillus subtilis* spore is encased in a multilayered protein coat that confers resistance to peptidoglycan-breaking enzymes and noxious chemicals and also influences the germination response. Biogenesis of the spore coat relies on the ordered synthesis and assembly of over 40 polypeptides (5, 10, 11). The assembly process is controlled at various levels. First, the time and degree of expression of the genes encoding the various coat components are under complex regulation. Most, if not all, components of the coat are produced in the mother cell chamber of the sporulating cell (5, 10, 11, 34). Some coat proteins are synthesized prior to engulfment of the prespore by the mother cell, and those are under the control of σ^E, whose activity is influenced by the GerR and SpoIIID ancillary transcription factors (5, 6, 9, 10, 11). However, the synthesis of many other coat proteins and the deposition of most of the coat structural components are governed by σ^K, which replaces σ^E following engulfment completion (5, 9, 10, 11). The activity of σ^K is modulated by the GerE transcription factor, itself the product of a σ^K-controlled gene, which can act both as an activator or a repressor (5, 6, 10, 11, 41). Second, the formation of the spore coat is controlled by a class of unique morphogenetic proteins which are required for the assembly of several structural components at the surface of the developing spore (5, 10, 11). Third, the process is also controlled by posttranscriptional and posttranslational mechanisms which include proteolysis, glycosylation, and cross-linking. Together, these mechanisms are thought to contribute to the proper structural organization and properties of the coat structure (5, 10, 11).

The roles of several coat morphogenetic proteins have been examined in some detail (5, 10, 11, 22). However, our knowledge of the contribution of mechanisms such as protein cross-linking to the assembly process is still very limited. Nevertheless, about 30% of the total spore coat protein is refractory to extraction, forming an insoluble highly cross-linked fraction (5, 10, 30). Several coat proteins appear to be cross-linked, and the available evidence suggests that both reversible and irreversible cross-links occur within the coat (5, 10, 11, 12, 14, 20, 21, 42). In a classical study, Pandey and Aronson (30) have suggested the existence of *o*,*o*-dityrosine cross-links in purified coat material, an observation confirmed by more recent work (5). CotG and three other tyrosine-rich coat components, CotB, CotC, and CotU, have been recently shown to be present as multimers within the spore coat (12, 21, 14, 42). However, the nature of the cross-links in CotB, CotC, CotU, or CotG has not been reported, and even though other enzymes could, in principle, promote oxidative cross-linking of the coat

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structural components (see references 7 and 25 and references therein), a peroxidase activity associated with the *B. subtilis* spore has not yet been found.

In contrast, a transglutaminase activity is detected in sporulating cells and spores (16, 18, 37), and ε-(γ-glutamyl)lysyl cross-links have been detected in *B. subtilis* spores and in purified coat material (17). Moreover, the gene (*tgl*) for a transglutaminase was cloned following the purification of a spore-associated activity (16, 37). The enzyme could cross-link α-casein and bovine serum albumin in vitro, and a cysteine residue at position 116 was found to be essential for the reaction (16). Tgl-like polypeptides are present in other *Bacillus* and related spore-forming species but absent from the genomes of other bacteria, suggesting a specific role in sporulation (Fig. 1). Recently, it was shown that the deletion of *tgl* prevents cross-linking of GerQ, a coat protein that is also involved in spore germination (29, 31, 32). However, it is not known whether GerQ is directly cross-linked by Tgl (31). Other polypeptides have been suggested to be cross-linked by a transglutaminase in the coats, including the lysine- and glutamine-rich CotX protein, a component of the coat insoluble fraction and CotM, a member of the α-crystallin family of small heat shock proteins (12, 39).

Transglutaminases intervene in key biological processes, such as cytoskeletal scaffolding, the control of protein-protein interactions, blood clotting, wound healing, apoptosis, development and morphogenesis, or the keratinization of skin (8, 23). Because the ε-(γ-glutamyl)lysyl isopeptide is stable and proteolysis resistant, it contributes to the rigidity and resistance to chemical, enzymatic, physical, and mechanical decay of various tissues and cellular structures (8, 23). Here, we have investigated the assembly and function of Tgl. Our results suggest that Tgl may be present at various levels within the coats and that its assembly is independent of its activity. We also found that in addition to GerQ, three other polypeptides are more extractable from spores of a *tgl* deletion mutant or from those with an inactive Tgl. The complete insolubilization of at least two of the Tgl-controlled polypeptides occurs several hours later than when the assembly of Tgl is first detected. Tgl is required for the structural integrity of the outer coat layers, and increased production of Tgl interferes with the assembly and structure of the coat and alters its germination properties.

MATERIALS AND METHODS

Bacterial strains and general methods. All *B. subtilis* strains were congenic with the wild-type (wt) Spo$^+$ strain MB24 (*trpC2 metC3*) (Table 1). Luria-Bertani (LB) medium was used for the routine growth of *Escherichia coli* or *B. subtilis* strains, and sporulation was induced by nutrient exhaustion in Difco sporulation medium (DSM) (27). Formation of heat- and lysozyme-resistant spores and spore germination were assessed as previously described (12, 13). *Pfu* (Stratagene, La Jolla, CA) was used to generate all PCR products for cloning, and these were sequenced to verify the absence of mutations. The use of antibiotics, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), isopropyl-β-D-thiogalactopyranoside (IPTG), and all other general methods were described before (12, 13).

Deletion of the *tgl* gene. First, a 1,885-bp DNA fragment comprising the *tgl* promoter and coding regions was PCR amplified using primers *tgl*-3604D and *tgl*-5490R (Table 2) and cloned into pGem-T (Promega), creating pRZ80. Second, a fragment of 1,937 bp was released from pRZ80 with ApaI and SalI and inserted between the same sites of pLITMUS 39 (New England Biolabs) to form pRZ85. Lastly, a spectinomycin resistance (Sp^r) cassette obtained from pAH256 (13) with BglII and EcoRV was inserted between the BglII and BsgI sites of pRZ85 to give pRH83 (Fig. 2). Transformation of strain MB24 with ScaI-linearized pRH83 produced the Sp^r *tgl* null mutant AH2255 (Table 1), as the result of a double-crossover event (verified by PCR).

Insertion of *tgl* at the *amyE* locus. Plasmid pRZ85 (see above) was cleaved with SphI and SalI and cloned between the same sites of pDG364 (4) to give pAH2256 (Fig. 2). Following the transformation of *B. subtilis* AH2255 (Δ*tgl*::Sp) to chloramphenicol resistance (Cm^r) with PstI-linearized pAH2256, an AmyE$^-$ transformant was identified and named AH2257 (Table 1).

Point mutations in *tgl*. The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce point mutations in the *tgl* gene. Codon 116 of *tgl* (TGC) encoding a cysteine was changed to the alanine codon GCG using primers *tgl*-116D and *tgl*-116R (Table 2) and pRH80 as the template. Plasmid pAH2259 resulted from the mutagenesis. Similarly, the cysteine codon 169 (TGT) was changed to the alanine codon GCT using primers *tgl*-169aD and *tgl*-169aR (Table 2), and the resulting plasmid was named pAH2258. The DNA sequence of both plasmids was verified. Plasmids pAH2259 and pAH2258 were digested with SphI and SalI, and the *tgl*-containing fragment was inserted between the same sites of pDG364 (4) to form pAH2260 and pAH2262, respectively (Fig. 2). *B. subtilis* strain AH2255 was transformed with PstI-linearized pAH2260 or pAH2262 to obtain the Cm^r AmyE$^-$ strains AH2261 and AH2263, respectively (Table 1).

A multicopy allele of *tgl*. A fragment of 1,915 bp encompassing the *tgl* gene was released from pRZ85 with XhoI and PstI and cloned between the SalI and PstI sites of replicative plasmid pMK3 (36) to yield pAH2264. Transformation of MB24 (wt) and AH2255 (Δ*tgl*::Sp) with pAH2264 produced the neomycin-resistant (Nm^r) strains AH2265 and AH2266, respectively (Table 1).

Construction of a *tgl-lacZ* fusion. A 640-bp fragment comprising the *tgl* promoter region and part of its coding sequence (up to codon 22) was released from pRZ85 with EcoRI and BglII from pRZ85 and inserted into EcoRI- and BamHI-digested pJM783 (34) to yield pAH2273 (Fig. 2). The resulting *tgl-lacZ* fusion was then integrated at the *tgl* locus of strains MB24 (wt), AH74 (*sigK*::*erm*), and AH94 (*gerE36*) via a single reciprocal crossover (Campbell-type recombination), producing AH2205, AH2206, and AH2207, respectively (Table 1).

***cotX*, *cotY*, *cotZ*, and *gerQ* mutants.** Chromosomal DNA from strains JZ10 (Δ*cotX*::*neo*), JZ50 [Δ(*cotX cotYZ*)::*neo*], and JZ60 [Δ(*cotX cotYZ*)::*neo*] (39) was used to transform MB24 (wt) and AH2255 (Δ*tgl*::Sp) to Nm^r-yielding strains AH2193 and AH2194, and AH2195, AH2196, AH2197, and AH2198 (Table 1). To construct an insertion into the *gerQ* gene, a PCR fragment was first generated with primers *gerQ*-182D and *gerQ*-743R (Table 2) and cloned into pGem7-T (Promega) to produce pRZ87. Next, a Cm^r cassette was released from pMS38 (33) with SnaBI and EcoRV and inserted at the SmaI site of pRZ87 to yield pRZ90. Strains AH2203 and AH2204 (Table 1) resulted from the transformation of MB24 and AH2255, respectively, with ScaI-linearized pRZ90. In both, the *gerQ* locus was replaced with the Cm^r marker of pRZ90 (as confirmed by PCR).

Overproduction of Tgl for antibody production. First, the *tgl* coding sequence was amplified with primers *tgl*-192D and *tgl*-935R (Table 2). Next, the PCR product was cut with NdeI and SalI, and a fragment of 743 bp was inserted between the NdeI and XhoI sites of pET30a(+) (Novagen) to create pLOM4, in which the His_6 tag is fused at the 5′ end of *tgl*. The plasmid was introduced into *E. coli* strain Tuner (DE3) (pLacI) (Novagen), in which production of the His_6-Tgl fusion protein was induced by the addition of 1 mM IPTG at mid-log growth phase (optical density at 600 nm [OD_{600}] of about 0.2) in LB medium. The fusion protein was partially purified on His-Trap columns under denaturing conditions as recommended by the supplier (Amersham Biosciences) and used for antiserum production in rabbits (Eurogentec, Herstal, Belgium).

Analysis of Tgl synthesis and assembly onto the developing spore. Samples (10 ml) were collected from DSM cultures of various strains at the indicated times throughout sporulation, and the cells were lysed by passage through a French pressure cell at 19,000 lb/in^2 as described previously (42). Spore-containing and mother cell fractions were prepared essentially as described by Isticato et al. (14). First, the suspension was centrifuged at 12,000 × *g* for 20 min, and the pellet containing the developing spores was dissolved in sodium dodecyl sulfate loading dye (10). Fifty micrograms (mother cell) or 15 μg (spore fraction) of total proteins were resolved on 15% polyacrylamide gels containing sodium dodecyl sulfate (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) and transferred to nitrocellulose membranes as described previously (42). Immunoblot analysis was conducted as described previously (3, 42). Polyclonal anti-Tgl antiserum was used at a dilution of 1:10,000. A rabbit secondary antibody conjugated to horseradish peroxidase (Sigma) was used at a 1:10,000 dilution. The immunoblots were developed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Spore preparation and analysis of the coat fraction. Spores were harvested by centrifugation 24 h after the initiation of sporulation in DSM, washed in water, and purified on a step gradient of 20% to 50% of Gastrografin (Schering) as

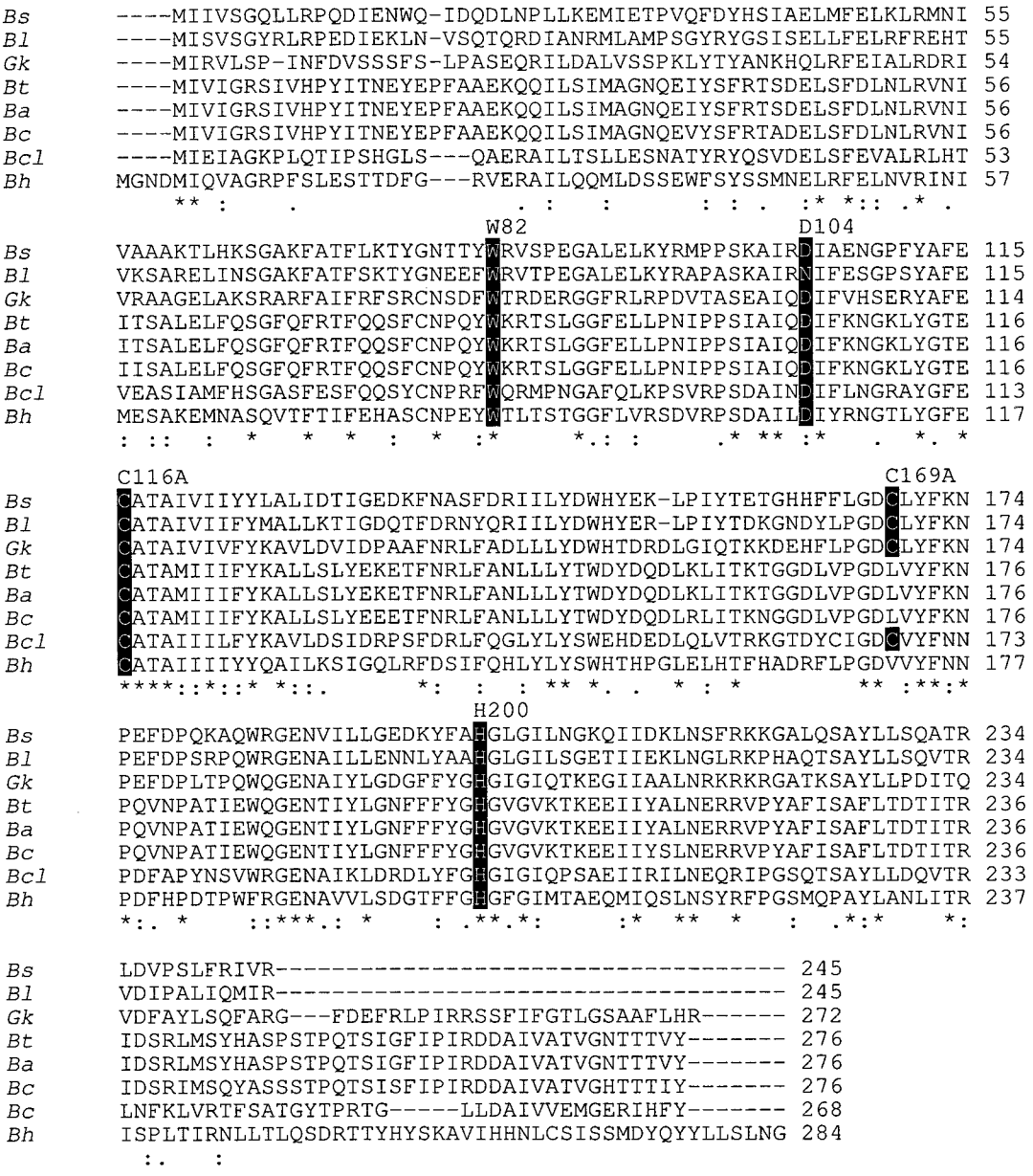

FIG. 1. Tgl is present in other spore formers. The figure represents a Clustal W alignment (www.ebi.ac.uk) of the Tgl proteins found in the following sporeformers: *Bs*, *B. subtilis* 168 (NP-391005); *Bl*, *Bacillus licheniformis* DSM 13 (YP-080406); *Gk*, *Geobacillus kaustophilus* HTA426 (BAD77199); *Bt*, *Bacillus thuringiensis* serovar konkukian strain 97-27 (YP-038027); *Ba*, *Bacillus anthracis anthracis* Sterne (accession number YP-030125); *Bc*, *Bacillus cereus* ATCC 14579 (EAL15447); *Bcl*, *Bacillus clausii* KSM-K16 (YP_175687); *Bh*, *Bacillus halodurans* C-125 (BAB07689). The sequences of all proteins were obtained through TIGR (www.tigr.org) and are shown with the amino acid residues in single-letter code. Only completed genomes were considered. The Tgl proteins were identical among the various sequenced strains of *B. anthracis* and those of *B. cereus* were highly similar, and only one protein from each species was included in the alignment. The asterisks below the alignment mark invariant residues, whereas conserved positions are indicated by dots. The two cysteines in the *B. subtilis* protein that were changed to alanine are shown against a black background, and the substitutions are indicated above the alignment. A highly conserved aspartate and invariant tryptophan and histidine residues (at the indicated positions in *B. subtilis* Tgl), which together with cysteine 116 may contribute to the enzyme's active site, are also shown against a black background.

described previously (12, 13). For the preparation of spores at hours 8 and 10 of sporulation in DSM, the cells in 80-ml cultures were sedimented by centrifugation, washed in 1 M KCl (1/4 of initial volume), and treated for 5 min on ice with 50 μg/ml lysozyme. The cells were collected by centrifugation, resuspended in 50 mM Tris HCl (pH 7.2), 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and lysed by passage through a French pressure cell at 19,000 lb/in^2. The lysed cells were then applied to a Gastrografin gradient as described above. Coat proteins were extracted from about two OD_{580} units of purified spores and electrophoretically resolved on 15% SDS-PAGE gels as described previously (10, 12). Proteins in the gels were either stained with Coomassie brilliant blue R250 or transferred to nitrocellulose for immunoblot analysis (as described above).

Electron microscopy analysis of spores. Spores were collected from 24- or 48-h DSM cultures, purified on Gastrografin gradients (see above), and processed for electron microscopy as described previously (12). The grids were observed and photographed on a JEOL transmission electron microscope at 60 kV.

Peptide mass fingerprinting. Protein-containing gel slices from Coomassie brilliant blue R250-stained SDS-PAGE gels were cut and washed with 200 μl of 20 mM NH_4HCO_3/50% (vol/vol) acetonitrile (ACN) for 30 min at 37°C. After

TABLE 1. Bacterial strains used in this study

Strain	Genotype and phenotype	Reference or source
MB24	*trpC2 metC3* wild type	Laboratory stock
JZ10	*trpC2 pheA1* Δ*cotX*::*neo* Nm^r	39
JZ50	*trpC2 pheA1* Δ(*cotX cotYZ*)::*neo* Nm^r	39
JZ60	*trpC2 pheA1* Δ(*cotYZ*)::*neo* Nm^r	39
AOB68	*trpC2 metC3* Δ*safA*::Sp Sp^r	28
AH77	*trpC2 metC3* Δ*sigK*::*erm* Em^r	Laboratory stock
AH94	*trpC2 gerE36*	Laboratory stock
AH2835	*trpC2 metC3* Δ*cotE*::Cm Cm^r	3
AH2884	*trpC2 metC3 gerE36*Δ*cotE*::Cm Cm^r	3
AH2203	*trpC2 metC3* Δ*gerQ*::Cm Cm^r	This work
AH2204	*trpC2 metC3* Δ*tgl*::Sp Δ*gerQ*::Cm Sp^r Cm^r	This work
AH2205	*trpC2 metC3 tgl*Ω*tgl-lacZ*[a] Cm^r	This work
AH2206	*trpC2 metC3* Δ*sigK*::*erm tgl*Ω*tgl-lacZ* Cm^r Em^r	This work
AH2207	*trpC2 metC3 gerE36 tgl*Ω*tgl-lacZ* Cm^r	This work
AH2255	*trpC2 metC3* Δ*tgl*::Sp Sp^r	This work
AH2257	*trpC2 metC3* Δ*tgl*::Sp Δ*amyE*::*tgl* Sp^r Cm^r	This work
AH2261	*trpC2 metC3* Δ*tgl*::Sp Δ*amyE*::*tgl* (C116A) Sp^r Cm^r	This work
AH2263	*trpC2 metC3* Δ*tgl*::Sp Δ*amyE*::*tgl* (C169A) Sp^r Cm^r	This work
AH2266	*trpC2 metC3* Δ*tgl*::Sp (pAH2264)[b] Sp^r Nm^r	This work
AH2265	*trpC2 metC3* (pAH2264) Nm^r	This work
AH2193	*trpC2 metC3* Δ*cotX*::*neo* Nm^r	This work
AH2194	*trpC2 metC3* Δ*cotYZ*::*neo* Nm^r	This work
AH2195	*trpC2 metC3* Δ(*cotX cotYZ*)::*neo* Nm^r	This work
AH2196	*trpC2 metC3* Δ*cotX*::*neo* Δ*tgl*::Sp Nm^r Sp^r	This work
AH2197	*trpC2 metC3* Δ*cotYZ*::*neo* Δ*tgl*::Sp Nm^r Sp^r	This work
AH2198	*trpC2 metC3* Δ(*cotX cotYZ*)::*neo* Δ*tgl*::Sp Nm^r Sp^r	This work

[a] The Ω symbol indicates the integration of plasmid pAH2273 at the *tgl* locus via a Campbell-type mechanism (single reciprocal crossover).
[b] Replicative plasmid pAH2264 is indicated in parentheses. The corresponding *B. subtilis* strains harbor a *tgl* multicopy allele (*tgl*^{MC}).

removal of the supernatant, 100 μl ACN was added, incubated for 15 min, and removed. This dehydration step was repeated. Trypsin solution containing 20 ng/μl trypsin (Promega, Madison, WI) in deionized water was added until the dried gel pieces stopped swelling, and digestion was performed for 16 to 18 h at 37°C. For peptide extraction, gel pieces were subsequently covered with 15 to 20 μl 5% (vol/vol) formic acid and incubated in an ultrasonic bath for 15 min. A second extraction was done with 15 to 20 μl 50% (vol/vol) ACN in an ultrasonic bath for 15 min. The peptide-containing supernatants were removed and subjected to mass spectrometric analysis. High pressure liquid chromatography separation was done on an Ultimate system (LC Packings, Amsterdam, The Netherlands). The system was coupled via a nanoLC inlet (New Objective, Woburn, MA) to a QSTAR Pulsar mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a nanoelectrospray source (Protana, Odense, Denmark). Peptides were desalted using a reversed-phase precolumn (PepMap microprecolumn, model no. C18, 300 μm [inside diameter] by 5 mm; LC Packings, Amsterdam, The Netherlands) with a flow rate of 30 μl/min using 100 μl of 0.1% (vol/vol) formic acid as solvent. The separation was performed via a reversed-phase nanocolumn (PepMap, C18, 75 μm [inside diameter] × 15 cm; LC Packings, Amsterdam, The Netherlands). As solvents, 0.05% (vol/vol) acetic acid (solvent A) and 90% (vol/vol) ACN-0.05% (vol/vol) acetic acid (solvent B) were used with a linear gradient from 5 to 50% of solvent B over 60 min. The eluted peptides were analyzed by tandem mass spectrometry (MS/MS). The instrument was running in automatic mode, allowing switching from MS to MS/MS mode. All peptides in a mass range from 300 to 2,000, with an intensity of at least 10 counts, were fragmented and an MS/MS spectrum was recorded in positive detection mode. The collision energy was adjusted automatically by the instrument. The resulting MS/MS data were analyzed with Bioanalyst software (Applied Biosystems, Foster City, CA) and the integrated Mascot script. For database searches, the Mascot search engine (Matrix Science, Ltd., London, United Kingdom) was used with a specific *Bacillus subtilis* sequence database.

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5′ to 3′)[a]
tgl-192D	GGGCTACATATGATTATTGTATCAGGAC
tgl-935R	CCGGTCGACGCGGACGATGCGGAAAAG AGAC
*tg*l-83D	GTTGTCTCGAGTCGTATCCTTCCGGC
tgl-3604R	TCTGCGAAGTCGACGGGCCTCGCACCCG
tgl-5490R	GTTGTCTCGAGTCGTATCCTTCCGGC
tgl-116D	CCCGTTTTATGCGTTTGAAGCGGCAACCGCA ATCGTTATC
tgl-116R	GATAACGATTGCGGTTGCCGCTTCAAACGC ATAAAACGGG
tgl-169D	GGACACCACTTTTTCCTTGGAGATGCTTTGT ATTTTAAGAATCCTG
tgl-169R	CAGGATTCTTAAAATACAAAGCATCTCCAAG GAAAAAGTGGTGTCC
gerQ-182D	CATAAAAAGGAGGAACCATATGAAACCC
gerQ-743R	CCCAAGCTTTCTTGGCGAATAGGACGCCAT GGAATATGG

[a] Engineered restriction sites or the sites of point mutations introduced into the *tgl* sequence are underlined.

β-Galactosidase assays. β-Galactosidase activity was measured with the substrate *o*-nitrophenol-β-D-galactopyranoside (ONPG) and expressed in Miller units as previously described (12, 13).

RESULTS

Expression of *tgl*. Transglutaminase activity was detected late during sporulation and in spores of *B. subtilis* as well as in sporulating cells of other *Bacillus* species (16, 18, 37). An inspection of the regulatory region of the *tgl* gene of *B. subtilis* has revealed sequences similar to those utilized by the σ^K form of RNA polymerase as well as possible GerE-binding sites and has led to the suggestion that *tgl* transcription could require both σ^K and GerE (16). Two studies in which DNA arrays were used have indeed assigned *tgl* to the σ^K regulon (6, 35). More-

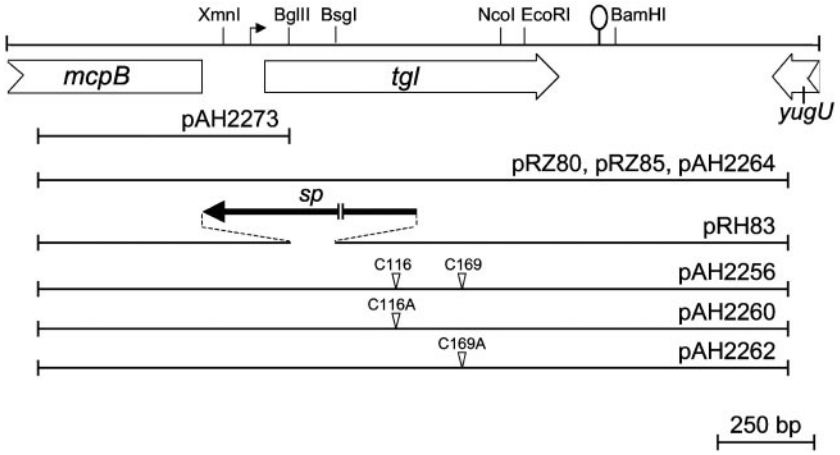

FIG. 2. The *tgl* locus. The figure depicts the genetic context of the *tgl* gene, which is flanked by *mcpB* and *yugG*, below a partial restriction map of the region (19). The stem-and-loop structure at the 3′ end of the *tgl* gene and the broken arrow preceding the *tgl* gene indicate a putative transcription terminator and the putative promoter, respectively. The figure also represents the DNA inserts in the indicated plasmids. Plasmid pRH83 was used to replace the wild-type sequence for a Sp^r cassette by a double-crossover event. pAH2273 was used to transfer a *tgl-lacZ* fusion to the *tgl* locus via a single reciprocal crossover; pAH2264 is a replicative plasmid carrying the entire *tgl* gene. Plasmids pAH2256, pAH2260, and pAH2262 are *amyE* integrational vectors carrying the wild-type *tgl* sequence or the C116A or C169A single amino acid substitution, respectively (positions indicated by the arrowheads). Other plasmids are described in the text.

over, one of these studies placed *tgl*, whose promoter was mapped, in the class of σ^K-dependent GerE-repressed genes (6) (Fig. 3B). Here, we have used a transcriptional fusion to *lacZ*, introduced via Campbell-type recombination at the *tgl* locus, to monitor expression of *tgl* in a wild-type strain and in congenic strains bearing mutations in the *sigK* or *gerE* gene (Table 1). We found that in a wild-type background (strain AH2205), the expression of *tgl-lacZ* commenced around hour 4 of sporulation, which is characteristic of many σ^K-controlled genes (see, for example, references 3 and 6), and was at background levels in a strain (AH2206) bearing a null allele of the *sigK* gene (encoding σ^K) (Fig. 3A). The expression of *tgl-lacZ* was slightly increased in a *gerE36* background (Fig. 3A, strain AH2207). We cannot exclude the possibility that sites located downstream of the fusion point (Fig. 3B) are needed for higher repression of *tgl-lacZ* by GerE. However, regardless of its effect on the transcription of *tgl-lacZ*, we found that the *gerE36* mutation did not affect the accumulation of Tgl relative to the wild-type in whole-cell extracts of sporulating cells (not shown).

Assembly of Tgl. Two lines of evidence suggest that the transglutaminase encoded by the *tgl* gene is associated with the *B. subtilis* spore. First, the Tgl protein was released from purified spores by incubation at a pH of 10.5 at 37°C, suggesting that the protein was attached to the external layers of the spore (36). Second, Tgl was found as a spore-associated protein of about 28 kDa in two proteomics-based studies (20, 21). Here, we have used a rabbit polyclonal antibody raised against a partially purified His_6-Tgl protein to investigate the association of Tgl with the spore coats. Sporulating cells of the wild-type strain MB24 were harvested at various times during sporulation and lysed, and the developing spores were separated from a mother cell fraction by centrifugation, as described in Materials and Methods (14). The presence of Tgl in the mother cell or spore fraction at the various time points was then investigated by immunoblot analysis with the Tgl-specific antibody. Tgl was first detected at hour 4 of sporulation as a band of about 28 kDa in the mother cell fraction of a wild-type strain (Fig. 4A), which agrees well with the predicted size (28.3 kDa) of the Tgl protein (16, 19). Tgl is detected in the spore fraction soon after (Fig. 4B, hour 5), and the amount of spore-associated Tgl increases significantly from hour 5 to hour 6. These results are consistent with the time of expression of *tgl-lacZ* (Fig. 3A). Moreover, even though a smaller amount of protein was analyzed (15 μg for the spore fraction compared to 50 μg for the mother cell fraction), the Tgl signal in the prespore fraction is comparable to that in the mother cell fraction (Fig. 4A and B). We conclude that Tgl is enriched in the spore fraction, and in confirmation of earlier work (20, 21, 37), we infer that Tgl is a bona fide coat component. Since a single Tgl band was always observed, we further infer that coat-associated Tgl is not cross-linked. We also note that some Tgl persists in the mother cell fraction at all time points examined.

Effects of mutations in *cotE*, *gerE*, and *safA* on the assembly of Tgl. To gain insight into the determinants for Tgl assembly, and into its possible location within the structure, we probed extracts of coat proteins solubilized from spores of mutants with lesions in the genes for important coat morphogenetic effectors. We examined the coats of *cotE* spores, whose most overt defect is the absence of assembly of the outer coat structure (40), *gerE*, whose spores do not show signs of assembly of the inner coat and have a greatly altered outer coat (5, 10, 26), and *safA*, whose spores assemble both an inner and an outer coat but show an incomplete and disorganized outer coat layer (28, 38). We also examined spores of a *cotE gerE* double mutant, whose spores failed to assemble both the inner and outer coat structures as judged by electron microscopy (5, 10). Spores from DSM cultures of various strains were collected 24 h after the onset of sporulation and purified by density gradient centrifugation to over 99% purity as judged by the inspection of the final suspensions by phase-contrast microscopy. Proteins were then extracted from the coats of purified

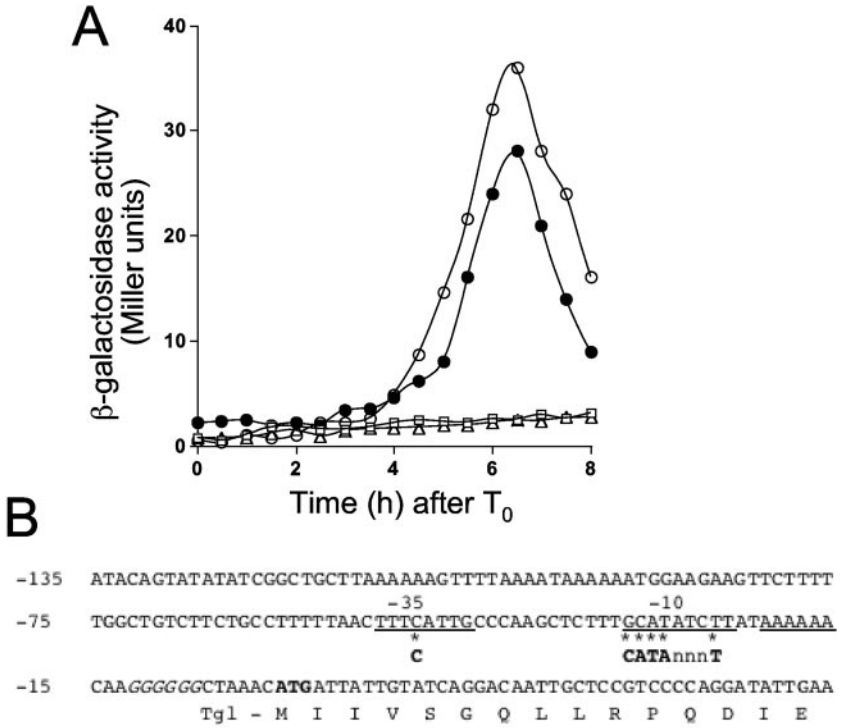

FIG. 3. Regulation of *tgl-lacZ* expression. (A) A *tgl-lacZ* fusion was inserted at the *tgl* locus of various strains, and samples were taken at the indicated times (in hours) after the initiation of sporulation in DSM to assay for β-galactosidase accumulation. Strains are as follows: AH2205 (*tgl-lacZ* in a wt background), closed circles; AH2206 (*sigK*::*erm tgl-lacZ*), open squares; AH2207 (*gerE36 tgl-lacZ*), open circles. The endogenous levels of β-galactosidase production were determined in the wild-type strain MB24 (triangles). Enzyme activity is expressed as Miller units. The graph shown is representative of three independent experiments. Panel B shows the sequence of the *tgl* promoter with the −10 and −35 regions (underlined) aligned to the consensus sequence for σ^K-dependent promoters (9). Identical bases are in bold and highlighted with an asterisk, whereas "n" represents any base. Transcription appears to initiate at the run of A residues (underlined type) just downstream of the −10 region (6). The ribosome binding site is indicated in italic letters and the start codon is shown in bold letters just downstream of the ribosome binding site.

spores, electrophoretically resolved, and subjected to immunoblot analysis with the anti-Tgl antibody. We found Tgl to be present at slightly reduced levels among the collection of proteins extracted from *cotE*, *gerE*, and *cotE gerE* mutant spores (Fig. 4E, lanes 1 to 5). In contrast, the *safA* mutation did not appear to affect the assembly of Tgl (Fig. 4E, lane 6). The slightly reduced levels of Tgl detected in the coats of *cotE*, *gerE*, and *cotE gerE* spores presumably reflect reduced association of Tgl with the coat and not reduced extractability of the protein, as it seems unlikely that the extractability of Tgl would be reduced in *cotE* or *gerE* mutants and increased in *safA* spores. In any case, the results suggest that Tgl resides at various levels within the coat lattice.

The activity of Tgl is not required for its own assembly within the spore coat. Our results suggest that Tgl is not cross-linked within the coats, but the activity of Tgl could be indirectly involved in its association with the surface of the developing spore. To test this, we analyzed the assembly of a Tgl active-site mutant. A cysteine at position 116 of Tgl was shown to be required for the Tgl-mediated cross-linking of bovine serum albumin in vitro, whereas a cysteine residue at position 169 did not affect enzyme activity (16). The cysteine residue at position 116 but not cysteine 169 is conserved among the Tgl proteins of other spore formers (Fig. 1). Moreover, results presented below suggest that C116 is also essential for the activity of Tgl in vivo. We tested whether either C116 or C169 had any role in the accumulation or the assembly of Tgl within the coat. We introduced single alanine substitutions at residue 116 (C116A) or 169 (C169A) within the *tgl* gene cloned in an *amyE* integrational plasmid (pAH2260 and pAH2262; Fig. 1) which, once inserted at the *amyE* locus, complements the effects of a *tgl* deletion allele for assembly (Fig. 4F, lane 3) and for function (see below). We have expressed the wild-type or mutated genes in *B. subtilis* at the *amyE* locus in the absence of the wild-type *tgl* gene. We found that neither mutation affected the accumulation of Tgl in sporulating cells (not shown). Moreover, neither the *tglC116A* (in strain AH2261) nor the *tglC169A* alleles (in strain AH2263) affected the level of Tgl associated with the coat (Fig. 4F, lanes 4 and 5, respectively). We infer that neither mutation severely affected the overall fold of the Tgl protein. We conclude that the activity of Tgl is not required for its assembly or stable association with the coat lattice.

Increased assembly of Tgl. Since we failed to identify a factor absolutely required for the assembly of Tgl, we wanted to test whether more Tgl could be assembled onto the developing spore. To test this, we made use of a strain (AH2266) harboring the entire *tgl* gene (tgl^{MC}) in a replicative plasmid. Samples were harvested from a sporulating culture of AH2266, fractionated into a mother cell and a spore fraction, and ana-

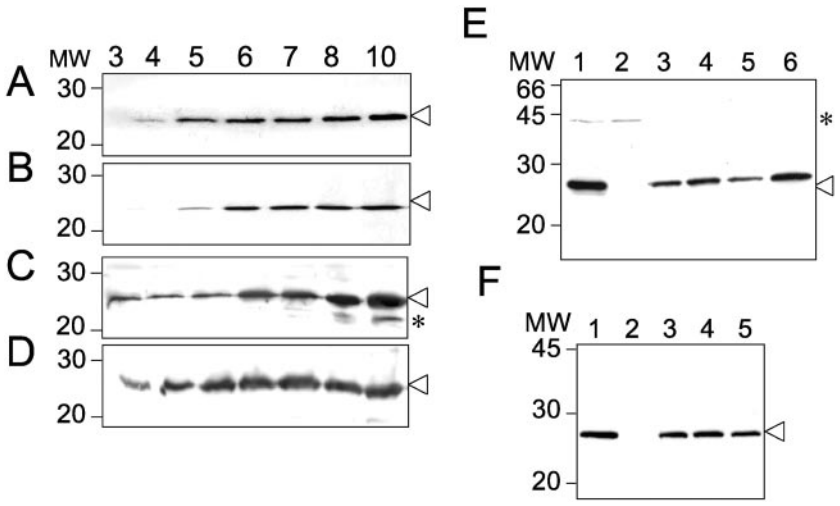

FIG. 4. Synthesis and assembly of Tgl into the spore coat. Samples were collected from sporulating cultures of a wild-type strain (MB24, panels A and B) and of a strain harboring a multicopy allele of *tgl* (AH2266, panels C and D). The cells were lysed and fractionated into mother cell (panels A and C) and prespore fractions (B and D) (see Materials and Methods). A total of 15 μg of the spore fraction or 50 μg of the mother cell fraction was then subjected to immunoblot analysis with an anti-Tgl polyclonal antibody. Note that the numbers above the lanes indicate time (in hours) after the onset of sporulation. To monitor the assembly of Tgl, the strains were grown in DSM and spores were purified by density gradient centrifugation as described in Materials and Methods. Proteins extracted from the coats of purified spores (2 OD_{580} units) were then subjected to immunoblot analysis with the anti-Tgl antibody (see Materials and Methods). Strains were as follows. Panel E: MB24 (wild type), lane 1; AH2255 (Δ*tgl*::Sp), lane 2; AH2835 (*cotE*), lane 3; AH94 (*gerE*), lane 4; AH2884 (*cotE gerE*), lane 5; AOB68 (*safA*), lane 6. Panel F: MB24 (wt), lane 1; AH2255 (Δ*tgl*::Sp), lane 2; AH2257 (Δ*tgl*::Sp *amyE*::*tgl*), lane 3; AH2261 (Δ*tgl*::Sp *amyE*::*tglC116A*), lane 4; AH2263 (Δ*tgl*::Sp *amyE*::*tglC169A*), lane 5. The open arrowhead indicates the position of Tgl. The asterisk in panel C marks the position of a band that may represent a Tgl degradation product, whereas in panel E the asterisk indicates a nonspecific band. MW, molecular weight in thousands.

lyzed for the presence of Tgl as described above for the wild-type strain. Tgl is detected in both the spore and mother cell fractions about 1 h earlier, from hour 3 of sporulation onwards, than in the wild-type strain (Fig. 4C and D). Note, however, that at this early time, the developing stage of the prespore may not allow its efficient separation from the mother cell fraction (27). From hour 4 onwards, when the prespore and mother cell fractions can be effectively separated (27), the level of Tgl in the spore fraction appears elevated relative to the wild-type strain (Fig. 4B and D). Moreover, it is higher in the spore fraction than in the mother cell fraction (Fig. 4C and D), indicating that more Tgl is being assembled in comparison to the wild-type strain. Thus, augmented expression of *tgl* results in increased assembly of Tgl but also increases the amount of Tgl that remains in the mother cell. Note that the signal in the mother cell fraction of the tgl^{MC} strain (AH2266) is also higher than for the mother cell fraction of a wild-type strain (Fig. 4A and C). Also, around hour 6, the signal in the mother cell fraction (Fig. 4C) increases abruptly, suggesting that at this time there might be some saturation of the prespore. Together with the observation that mutations in *cotE*, *gerE*, or *safA* (see above) did not preclude the recruitment of Tgl, it could be that the amount of Tgl assembled onto the spore is mainly controlled by its level of synthesis in the mother cell.

The activity of Tgl is required for proper assembly of the coat structure. Ragkousi and Setlow (31) reported on the effect of a *tgl* insertion and deletion allele on the assembly of the spore coat in *B. subtilis.* They found that the mutation resulted in increased extractability of a coat component called GerQ (31, 32). However, their analysis did not discriminate whether the effect was due to the absence of Tgl activity or to an indirect structural perturbation of the coat caused by the absence of the Tgl polypeptide. To investigate this, we compared the polypeptide composition of spores of a *tgl* null mutant with that of spores produced by strains with a point mutation in the active site of the enzyme. First, we purified spores of the wild-type strain and the *tgl* null mutant AH2255 (Table 1) and analyzed the profile of proteins that could be extracted from their coats and amenable to electrophoretic resolution. We found that in addition to the 20-kDa GerQ protein (see also below), the extractability of polypeptides of about 40, 28, and 16 kDa was also increased (Fig. 5A; the 40-, 28-, and 16-kDa polypeptides are labeled a, b, and c, respectively, in lane 2). We then examined the coats of the C169A mutant and found that the mutation, which does not affect the activity of Tgl in vitro (16), also did not affect, in any discernible manner, the polypeptide composition of the spore coat (Fig. 5A, lane 5). In contrast, spores of the C116A mutant showed increased extractability of polypeptides with the same electrophoretic mobility as those seen for the coats of Δ*tgl*::Sp null mutant spores (Fig. 5A, lanes 2 and 4). Therefore, the increased extractability of GerQ and the polypeptides of 40, 28, and 16 kDa is not caused by the absence of the Tgl protein from the coat structure, but rather by the absence of an active Tgl. As for GerQ (31), the presence of the 40-, 28-, and 16-kDa polypeptides in spores of the Δ*tgl*::Sp or *tglC116A* mutants additionally indicates that the recruitment of these species to the surface of the developing spore is *tgl* independent. We conclude that the activity of Tgl is responsible, directly or indirectly, for their insolubilization at the spore surface following their recruitment.

Sequences in the 40-, 28-, and 16-kDa bands. In an attempt to identify the *tgl*-controlled polypeptides, we isolated gel slices encompassing the 40-, 28-, 20-, and 16-kDa bands seen in the coat

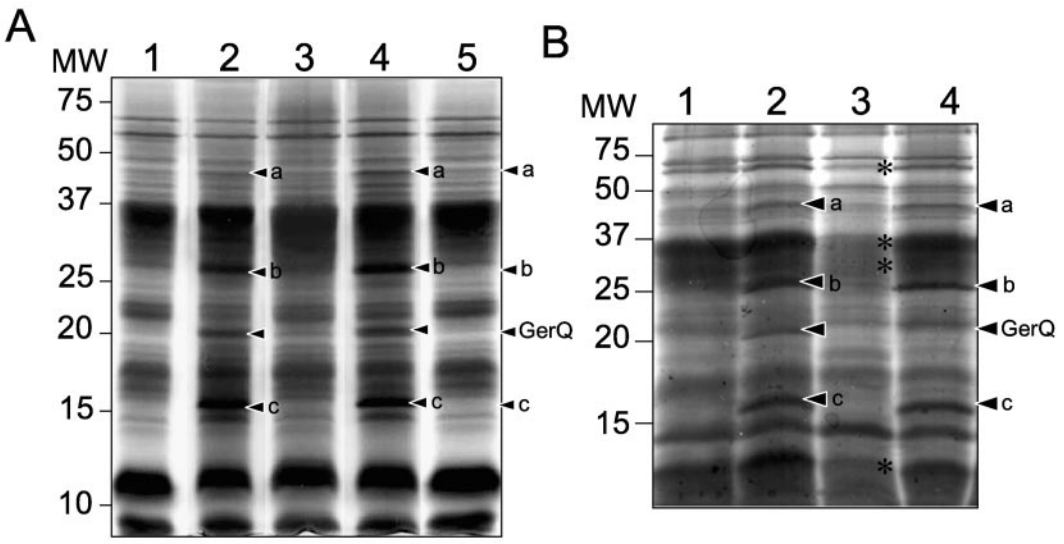

FIG. 5. Tgl activity is required for proper assembly of the coat structure. Spores were purified by density gradient centrifugation from DSM cultures of various strains 24 h after the onset of sporulation. The spore coat proteins were extracted from 2 OD_{580} units of a spore suspension and electrophoretically resolved, and the gels were stained with Coomassie brilliant blue (see Materials and Methods). For panel A, strains are as follows: MB24 (wt, lane 1); AH2255 (Δ*tgl*::Sp, lane 2); AH2257 (Δ*tgl*::Sp Δ*amyE*::*tgl*, lane 3); AH2261 (*tglC116A*, lane 4); AH2263 (*tglC169A*, lane 5). For panel B, strains are as follows: lane 1, MB24 (wt); lane 2, AH2255 (Δ*tgl*::Sp); lane 3, AH2203 (Δ*gerQ*::Cm); lane 4, AH2204 (Δ*tgl*::Sp Δ*gerQ*::Cm). Arrowheads in panels A and B show the position of GerQ (20 kDa) and of other polypeptides that are more extractable from the coats of the *tgl* deletion mutant (AH2255, lanes 2 in panels A and B) or the C116A (AH2261, lane 4 in panel A) point mutant and whose estimated molecular mass are as follows: a, 40 kDa; b, 28 kDa; c, 16 kDa. Asterisks in panel B, lane 3, mark the position of species of about 66 kDa (possibly CotB) and 8 kDa (presumably CotC) as well as two bands in the 36-kDa region (possibly two forms of CotG) (42) whose assembly or extractability is reduced in the spores of the *gerQ* mutant. MW, molecular weight in thousands.

extracts from Δ*tgl*::Sp spores, as well as slices from the same region of gels of wild-type coat extracts, and subjected duplicate samples to liquid chromatography (LC)–MS-MS analysis (see Materials and Methods). We have unequivocally identified peptides derived from the CotE (24-kDa), CotY (16-kDa), and GerQ (23-kDa) proteins in both the 40- and 28-kDa gel slices. The identification of GerQ-derived sequences by LC–MS/MS analysis in the 40- and 28-kDa regions in both the wild type and the *tgl* mutant supports the conclusion of Ragkousi and Setlow (31) that some of the GerQ multimers are formed in a Tgl-independent manner. Also, both CotE and CotY are found as cross-linked species within the coat (1, 20, 21, 39), explaining their presence in the 40- and 28-kDa regions of the gel. In confirmation of earlier work (31; see also above), in the 20-kDa region of both the wt and *tgl* coat protein extract, we found GerQ and in addition CotE, YfkD (29.4 kDa), YisY (30.4 kDa), and YckK (29.3 kDa). The latter three proteins have been identified previously and were proposed to be coat components (20, 21). However, the results of Ragkousi and Setlow (31) and our own (see below) clearly show that the 20-kDa band corresponds to GerQ, and hence, the presence of other sequences in this region is most likely Tgl independent. Lastly, in the 16-kDa slice, we found CotY as well as CotG-, CotE-, and YhxC (30.8 kDa)-derived sequences, the latter three probably due to proteolysis, as also detected by Kuwana et al. (20).

To independently test whether the 40-, 28-, or 16-kDa bands could contain GerQ or CotY, or otherwise GerQ- or CotY-controlled proteins, we made use of mutants. We first analyzed spores of a *gerQ* insertional mutant (AH2203) and found that the 20-kDa band was absent from the coats of *gerQ* mutant spores (Fig. 5A and B, lanes 1 and 3, respectively). Together with the mass spectrometry data, this suggests that a fraction of GerQ exists in part as a non-cross-linked form in the coats of wild-type spores. We also note that under our conditions, the *gerQ* mutation also seems to reduce the assembly or extractability of a 66-kDa polypeptide (possibly CotB) of two species in the 36-kDa region of the gel (presumably two forms of CotG) (42) and of a band of about 8 kDa (Fig. 5B, lane 3). We then analyzed the coats of a *gerQ tgl* double mutant (AH2204). We found that the 20-kDa species, but not those of 40, 28, and 16 kDa, was still missing from the coats of the *gerQ tgl* mutant, in confirmation of its assignment as GerQ (Fig. 5B, lane 4). In addition, the introduction of the *tgl* allele into the *gerQ* mutant restored the normal pattern of extractability of the 66-, 36- and 8-kDa species (Fig. 5B, compare lanes 3 and 4). Possibly, in the absence of GerQ the 66-, 36-, and 8-kDa species are cross-linked in a Tgl-dependent manner or their extractability is reduced because of abnormal cross-linking of other proteins. We also found that even though the *cotX*, *cotYZ*, and *cotXYZ* mutations affected the composition of the coat soluble fraction in several ways, they did not cause increased extractability of any of the *tgl*-controlled polypeptides (not shown). Moreover, the polypeptides in the 40-, 28-, 20-, and 16-kDa regions of a coat protein gel were still more extractable from *cotX tgl*, *cotYZ tgl*, or *cotXYZ tgl* spores (not shown).

Other proteins are known to be present as multimeric forms in the spore coat, including CotC (14), CotB (42), CotG (42), and CotE (1, 20; our unpublished results). We tested whether the *tgl* null allele affected the pattern of multimerization of any of these proteins as detected on one-dimensional gels by using

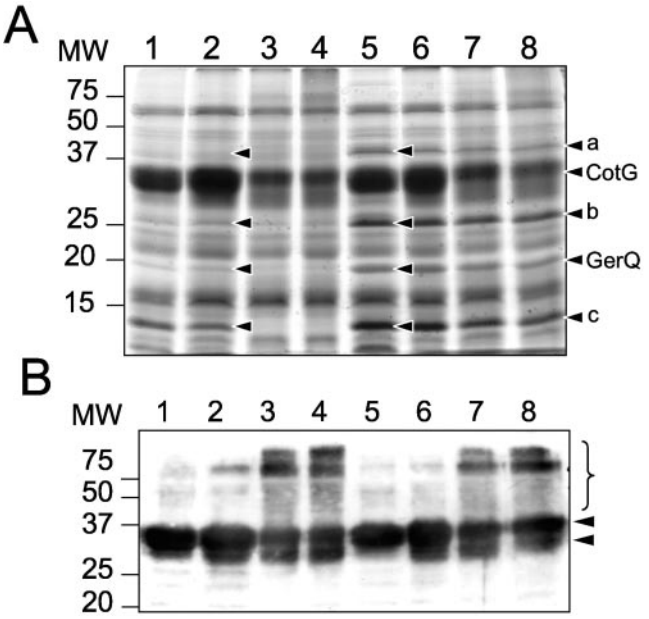

FIG. 6. Tgl-dependent insolubilization of coat proteins. Samples of sporulating cultures of the wild-type strain MB24 (lanes 1 through 4) or the Δ*tgl*::Sp mutant AH2255 (lanes 5 through 8) were collected at the following times after the onset of sporulation in DSM: hour 8, lanes 1 and 5; hour 10, lanes 2 and 6; hour 24, lanes 3 and 7; hour 36, lanes 4 and 8. The free spores present in the culture were then purified by density centrifugation as described in Materials and Methods. Panel A shows the profile of proteins extracted from spores purified at the indicated times and electrophoretically resolved on 15% acrylamide gels containing SDS. Panel B shows the immunoblot analysis of similar samples processed in parallel with an anti-CotG antibody of established specificity (42). In panel A, the positions of GerQ and other polypeptides whose extractability is *tgl* dependent (a through c) as well as the position of the abundant 36-kDa CotG protein are indicated. Note that band a is not readily apparent in lane 2, but its position is nevertheless indicated. The arrowheads in panel A indicate species which become less represented over time. The arrowheads in panel B indicate the two main forms of CotG found in the spore coat, whereas the brace indicates the position of multimeric forms of CotG with sizes larger than 36 kDa (42). MW, molecular weight in thousands.

specific antibodies. We found no discernible differences in the pattern of multimerization of CotC, CotB, CotE (not shown), or CotG (Fig. 6) between coat extracts from wild-type or *tgl* mutant spores, indicating that Tgl does not have a major role in multimerization of these proteins.

We conclude that Tgl controls the insolubilization of the 40-, 28-, and 16-kDa polypeptides independently of GerQ or of the proteins coded for by the *cotXYZ* cluster. We also conclude that Tgl is not decisively involved in the multimerization of the abundant spore coat components CotC, CotB, CotE, and CotG. The identities of the 40-, 28-, and 16-kDa polypeptides are presently unknown.

Tgl-dependent modification of the spore surface. Assembly of Tgl was detected soon after its synthesis (Fig. 4A to D), but the cross-linking of GerQ was reported to occur late in sporulation, presumably following lysis of the mother cell and the concomitant release of the spore into the culture medium (31). In an attempt to determine whether insolubilization of the 40-, 28-, and 16-kDa species herein found to be Tgl controlled (Fig. 5) coincided with the assembly of Tgl, we purified free spores from cultures of the wild-type strain or the Δ*tgl*::Sp mutant at various times after the onset of sporulation and after disruption of the mother cell with lysozyme (see Materials and Methods) and examined their coat protein profiles. The earliest time point included was hour 8, as the yield of spores before this time of sporulation was too low for this analysis. GerQ and the 28- and 16-kDa bands could be detected in the coats of wild-type spores prepared 8 or 10 h after the initiation of sporulation (Fig. 6A, lanes 1 and 2), but the extractability of these polypeptides (in particular that of the 16-kDa species) (Fig. 6A, band c) decreased in spores prepared 24 or 36 h after the onset of sporulation (Fig. 6A, lanes 3 and 4). In contrast, in the *tgl* mutant, GerQ and the 40-kDa (band a), 28-kDa (band b), and 16-kDa (band c) polypeptides appeared more extractable than in the wt at any time point tested (Fig. 6A, lanes 5 through 8). The decreases in the extractability of GerQ and of the 40-, 28-, and 16-kDa bands from wild-type spores are likely to be caused by their cross-linking. We base this assumption on the observation that the extractability of CotG, which clearly decreases in spores with 24 or 36 h, regardless of the presence of a *tgl* mutation (Fig. 6A and B), coincides with the detection of multimeric forms of CotG by immunoblot analysis (Fig. 6B) (42). Thus, most of CotG cross-linking occurs late, in a *tgl*-independent manner (see also above), and causes the reduction of the protein's representation in the coat soluble fraction. Since the *tgl* mutation increased the extractability of all four *tgl*-dependent polypeptides from hour 8 of sporulation onwards (Fig. 6A), Tgl appears to be active since at least this time of sporulation. Ragkousi and Setlow found similar levels of GerQ at hour 8 of sporulation in wild-type or *tgl* spores and little cross-linking of GerQ prior to hour 24 (31). However, these authors used a different method for the breakage of sporulating cells and coat protein extraction, which may have favored the extraction of GerQ monomers. In any case, our results suggest that although Tgl is active from hour 8 of sporulation onwards, it appears to become more active, at least towards GerQ and more markedly towards the 16-kDa band, at hour 24 of sporulation.

Properties of *tgl* mutant spores. Ragkousi and Setlow (31) have reported that spores of a *tgl* deletion mutant are not impaired in their resistance or germination properties. We have confirmed these results using spores prepared from 24- or 48-h cultures and made similar observations for the *tglC116A* (AH2261) mutant (not shown). In contrast to the deletion or point mutants, spores of AH2266 (tgl^{MC}) showed a dramatically altered profile of extractable coat proteins. For instance, the amount of the abundant CotG protein was reduced, and several other proteins found in the wild-type coats were greatly reduced or missing (Fig. 7A, lane 1). In contrast, a protein of about 28 kDa was overrepresented in the coats of AH2266 spores relative to the wild type (Fig. 7A, compares lanes 1 and 3). This protein appears to correspond to Tgl on the basis of immunoblot analysis (Fig. 7B). Moreover, AH2266 spores showed a faster germination rate than wild-type spores in the presence of L-alanine (Fig. 7C). In contrast, spores of the deletion or active-site mutants did not significantly differ from wild-type spores in their response to L-alanine (Fig. 7C).

Because we found that, in addition to GerQ, the assembly of at least three other polypeptides was altered in the *tgl* deletion or active-site C116A mutant and since the tgl^{MC} mutant had

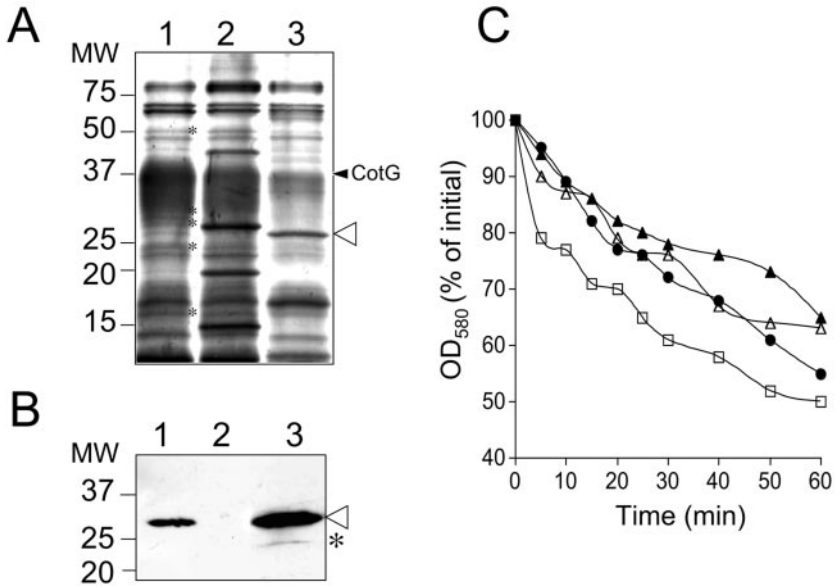

FIG. 7. A *tgl* multicopy allele interferes with coat assembly and function. Panel A shows a Coomassie blue-stained 15% SDS-PAGE gel of the proteins extracted from spores of the following strains, which were purified 48 h after the onset of sporulation: MB24 (wild type), lane 1; AH2255 (Δ*tgl*::Sp), lane 2; AH2266 (*tgl*^{MC}), lane 3. The asterisks indicate bands present in the wild-type coat extract (lane 1) that are reduced or missing from the coats of AH2264 (lane 3). Black or white arrowheads indicate the position of CotG or Tgl, respectively. Panel B shows the immunoblot analysis of similar samples resolved in parallel, using an anti-Tgl antibody. The white arrowhead indicates Tgl, and the asterisk indicates a cross-reactive species that may result from proteolysis. Note that the presence of the parental vector for pAH2264 (pMK3) does not change the profile of extractable coat proteins (3, 25). MW, molecular weight in thousands. Panel C shows the response of purified spores (48 h) of various strains to the germinant L-alanine. MB24 (wild type), closed circles; AH2255 (*tgl*::Sp), open triangles; AH2261 (*tglC116A*), closed triangles; AH2266 (*tgl*^{MC}), open squares.

greatly altered coats and altered germination in response to L-alanine, we examined mutant spores purified 24 or 48 h after the onset of sporulation in DSM by transmission electron microscopy. We found no differences between the 24- or 48-h spores for any of the strains examined, and the transmission electron micrographs documented in Fig. 8 refer to 48-h spores. In wild-type spores, the coat consists of a lightly staining lamellar inner layer closely apposed to a thicker electron-dense and striated outer coat (Fig. 8A) (5, 10, 11). Spores of both the *tgl*::Sp and *tglC116A* mutants did not show any discernible perturbation of the inner coat layers but showed a somewhat thinner outer coat of reduced electron density (Fig. 8B and C, respectively). Often, as in the specimens documented in Fig. 8 (panels B and C, but more apparent in the latter), the edge of the outer coat appeared less electron dense than the rest of the layer and showed several lamellae. This phenotype is reminiscent of that described for *cotM* mutant spores (13), but in our mass spectrometry analysis, we found no

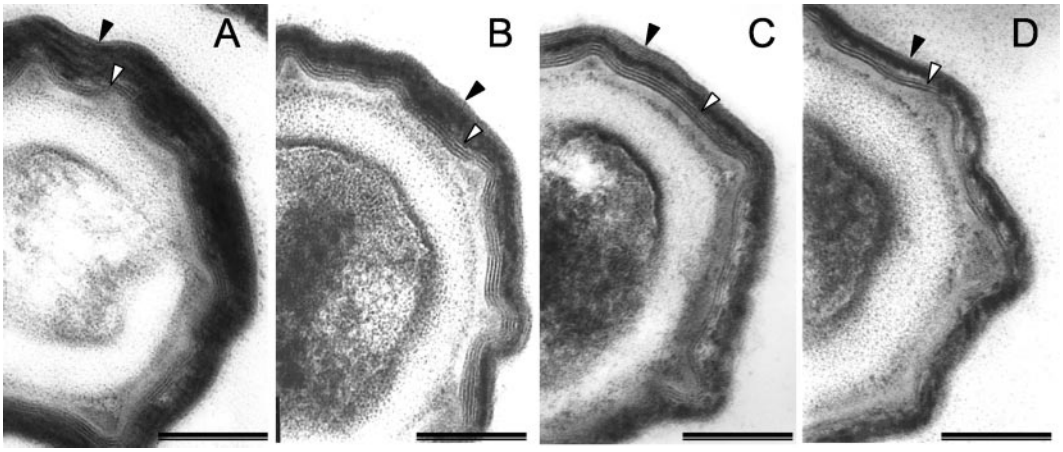

FIG. 8. Ultrastructural analysis of spores. The electron micrographs show sections of spores produced by the following strains: A, MB24 (wild type); B, AH2255 (Δ*tgl*::Sp); C, AH2261 (*tglC116A*); D, AH2264 (Δ*tgl*::Sp *tgl*^{MC}). The spores were collected from DSM cultures 48 h after the onset of sporulation, purified by density gradient centrifugation, and processed for electron microscopy as described in Materials and Methods. The black arrowheads point to the outer coat regions, whereas white arrowheads indicate the inner coat regions. Bars, 0.2 μm.

evidence for *tgl*-mediated cross-linking of CotM (see above). That spores of both the *tgl*::Sp and *tglC116A* mutants displayed similar features implies that these are caused by the absence of an active Tgl.

Compared to spores of the *tgl*::Sp or *tglC116A* mutants, spores of the tgl^{MC} mutant presented an even greater reduction of the outer coat layer, which also appeared to have lost most of its striation pattern (Fig. 8D). Spores of the tgl^{MC} mutant additionally presented a reduced inner coat, in which only one or two lamellae could be recognized, instead of the four to five normally observed, and the striated pattern of the outer coat was not as evident in wild-type spores (Fig. 8A and D). We conclude that Tgl contributes to the normal structure of the spore, in particular of its outer layers, and that although the coat can accommodate increased levels of Tgl, this occurs at the expense of proper assembly, structure, and normal germination properties.

DISCUSSION

The Tgl protein is a bona fide coat component whose production is governed by σ^K (6, 35; this work) and hence occurs at the time in development when most of the coat is deposited around the forming spore (5, 10, 11). Earlier work has shown that expression of the *tgl* gene is required for the insolubilization of a coat protein, GerQ, and has led to the suggestion that GerQ is a Tgl substrate (31, 32). However, the absence of Tgl from the coats could increase the extractability of GerQ independently of its enzymatic activity. We have found that the inactive C116A form of Tgl assembles at normal levels but is still required for the proper assembly of GerQ onto the coat, strengthening the view that assembly of GerQ requires the activity of Tgl and that GerQ is a Tgl substrate. The Tgl proteins are not closely related to other groups of transglutaminases, but the functional importance of the conserved C116 residue in the *B. subtilis* protein, as well as the presence of conserved Trp, His, and Asp residues (Fig. 1), suggests that they also use a papain-like active site to polymerize proteins via peptide-bound glutamine and lysine residues (15, 24). Inspection of the GerQ primary structure reveals a series of repeats in the form QQG encompassing the first 70 amino acids of the 181-residue-long protein (see Fig. 7 in reference 32). Possibly, the N-terminal region of GerQ could act as the acceptor for its Tgl-mediated cross-linking (8, 23).

GerQ is likely to be cross-linked to other coat proteins (31). GerQ recruits another coat protein, CwlJ, which is involved in spore germination (2, 29, 32), but the pattern of GerQ cross-linking is not affected by disruption of the *cwlJ* gene (31). We have found that in addition to GerQ, polypeptides of about 40, 28, and 16 kDa are more extractable from the coats of a *tgl* deletion or active-site mutant, and we suggest that like GerQ, they also serve as Tgl substrates. Their identification will have to wait for a more comprehensive analysis, including the use of two-dimensional gel separations in combination with mass spectrometry. However, the 40-, 28-, and 16-kDa polypeptides do not seem to be GerQ cross-linking partners because disruption of *gerQ* per se does not increase the extractability of proteins in these regions of a coat protein gel and because the extractability of these bands is still increased upon disruption of *tgl* in a *gerQ* background. Neither CotX (previously proposed to be subjected to transglutaminase-mediated cross-linking) nor CotY or the highly similar CotZ protein, all of which are part of the coat insoluble fraction (39), appears to be cross-linking partners for any of the Tgl-controlled polypeptides or otherwise contributes to their retention in the coat insoluble fraction. Also, in spite of certain ultrastructural similarities between *tgl* and *cotM* mutant spores (Fig. 8), we found no evidence for the *tgl*-mediated cross-linking of CotM, another protein previously proposed to be a substrate for a coat transglutaminase (13). More detailed studies will be needed to identify the putative GerQ cross-linking partners or other Tgl substrates.

Cross-linking of GerQ is not exclusively under Tgl control (31). GerQ does not contain cysteine residues but is rich in tyrosines and even though SodA, previously shown to be involved in the formation of *o*,*o*-dityrosine cross-links in the coat (12), has no role in the cross-linking of GerQ (31), it remains possible that GerQ is in part cross-linked by the action of a peroxidase. It could be that coat maturation involves a cascade of different types of cross-linking reactions (23). We have excluded the possibility that Tgl contributes significantly to the cross-linking of the tyrosine-rich CotB, CotC, or CotG protein or of the abundant CotE morphogenetic protein (14, 21, 22, 42). However, it is not yet known whether mutations in the genes for these proteins influence the pattern of Tgl-dependent cross-linking.

Ragkousi and Setlow (31) have found that cross-linking of GerQ occurs late, following lysis of the mother cell and spore release, and they have speculated that oxidative conditions could indirectly trigger Tgl-mediated cross-linking. We have found that Tgl influences the extractability of all four Tgl-dependent polypeptides at least from hour 8 of sporulation onwards. Presumably, there is little delay between the time of assembly of Tgl and the time at which its effects are first noticed. However, in agreement with the findings of Ragkousi and Setlow (31), we have also found that the complete insolubilization of at least GerQ and the 16-kDa polypeptide occurs only 24 h after the onset of sporulation. In keeping with their suggestion that oxidative conditions could in part control Tgl-mediated cross-linking, we have found that most of the cross-linking of CotG also takes place at hour 24 (42; see also below).

We found Tgl to be present, albeit at reduced levels, in the coats of *cotE*, *gerE*, and *cotE gerE* spores that fail to assemble the outer coat layer or with highly compromised inner and outer coat layers (5, 10, 26). In contrast, normal levels of Tgl were found in *safA* spores, in which the outer coat is incomplete and disorganized (28, 38). Together, these results suggest that Tgl is present at both the inner and outer coat levels. However, we note that the main perturbations of the coat structure in *tgl* mutants as detected by electron microscopy are at the level of the spore outer coat (Fig. 8). It could be that the inner coat/outer coat differentiation is in part determined by increased cross-linking of outer coat proteins and that Tgl could be more active in the outer coat. It will be interesting to determine the precise residence of Tgl or of the ε-(γ-glutamyl) lysine cross-link within mature spores. Following its synthesis, some Tgl persists in the mother cell. Other coat proteins that have been analyzed by this method, for example, CotC (14), CotB, and CotG (our unpublished results), are never detected in the mother cell fraction. This suggests that their assembly is prompt and complete. The persistence of Tgl in the mother cell cytoplasm may be related to the observation that the in-

creased recruitment of Tgl interferes with the assembly and structure of the coat and with the germination properties of the spore. We have previously noted that the expression of a multicopy allele of *cotA*, coding for an outer coat protein, does not interfere significantly with the assembly of the coat (25), whereas the expression of a multicopy allele of *oxdD*, encoding an inner coat component, strongly affects the assembly process (3). Since Tgl may also be located, at least in part, in the inner layers of the coat, it seems possible that its increased recruitment interferes with the assembly of other coat proteins.

No obvious resistance or germination phenotype could be associated with *tgl* mutants (31; this work). However, Tgl is important for the structural integrity of the outer coat structure in *B. subtilis* (this work), suggesting that the enzyme contributes to mechanical resistance (31) or to other spore properties that are not normally assessed in the laboratory.

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REFERENCES

1. **Aronson, A. I., L. Ekanayake, and P. C. Fitz-James.** 1992. Protein filaments may initiate the assembly of the *Bacillus subtilis* spore coat. Biochimie **74:** 661–667.
2. **Bagyan, I., and P. Setlow.** 2002. Localization of the cortex lytic enzyme CwlJ in spores of *Bacillus subtilis*. J. Bacteriol. **184:**1219–1224.
3. **Costa, T. V., L. O. Martins, U. Voelker, and A. O. Henriques.** 2004. Assembly of an oxalate decarboxylase produced under σ^K control into the *Bacillus subtilis* spore coat. J. Bacteriol. **186:**1462–1474.
4. **Cutting, S. M., and P. B. V. Horn.** 1990. Genetics analysis, p. 27–74. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons, Ltd., Chichester, England.
5. **Driks, A.** 1999. *Bacillus subtilis* spore coat. Microbiol. Mol. Biol. Rev. **63:**1–20.
6. **Eichenberger, P., M. Fujita, S. T. Jensen, E. M. Conion, D. Z. Rudner, S. T. Wang, C. Ferguson, K. Haga, T. Sato, J. S. Liu, and R. Losick.** 2004. The program of gene transcription for a single differentiating cell type during sporulation in *Bacillus subtilis*. PLoS Biol. **2:**e328. [Online.]
7. **Enguita, F. J., L. O. Martins, A. O. Henriques, and M. A. Carrondo.** 2003. Crystal structure of a bacterial endospore coat component: a laccase with enhanced thermostability properties. J. Biol. Chem. **278:**19416–19425.
8. **Griffin, M., R. Casadio, and C. M. Bergamini.** 2002. Transglutaminases: nature's biological glues. Biochem. J. **368:**377–396.
9. **Helmann, J. D., and C. P. Moran, Jr.** 2002. RNA polymerase and sigma factors, p. 289–312. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. American Society for Microbiology, Washington, D.C.
10. **Henriques, A. O., and C. P. Moran, Jr.** 2000. Structure and assembly of the bacterial endospore coat. Methods **20:**95–110.
11. **Henriques, A. O., T. Costa, L. O. Martins, and R. Zilhão.** 2004. The functional architecture of the spore coat, p. 34–52. *In* E. Ricca, A. O. Henriques, and S. M. Cutting (ed.), Bacterial spore formers: probiotics and emerging applications. Horizon Scientific Press, London, United Kingdom.
12. **Henriques, A. O., L. R. Melsen, and C. P. Moran, Jr.** 1998. Involvement of superoxide dismutase in spore coat assembly in *Bacillus subtilis*. J. Bacteriol. **180:**2285–2291.
13. **Henriques, A. O., B. W. Beall, and C. P. Moran, Jr.** 1997. CotM of *Bacillus subtilis*, a member of the α-crystallin family of stress proteins, is induced during development and participates in spore outer coat formation. J. Bacteriol. **179:**1887–1897.
14. **Isticato, R., G. Esposito, R. Zilhão, S. Nolasco, G. Cangiano, M. De Felice, A. O. Henriques, and E. Ricca.** 2004. Assembly of multiple CotC forms into the *Bacillus subtilis* spore coat. J. Bacteriol. **186:**1129–1135.
15. **Kashiwagi, T., K.-I. Yokoyama, K. Ishikawa, K. Ono, D. Ejima, H. Matsui, and E.-I. Suzuki.** 2000. Crystal structure of microbial transglutaminase from *Streptoverticillium mobarense*. J. Biol. Chem. **277:**44252–44260.
16. **Kobayashi, K., K.-I. Hashiguchi, K. Yokozeki, and S. Yamanaka.** 1998. Molecular cloning of the transglutaminase gene from *Bacillus subtilis* and its expression in *Escherichia coli*. Biosci. Biotechnol. Biochem. **62:**1109–1114.
17. **Kobayashi, K., Y. Kumazawa, K. Miwa, and S. Yamanaka.** 1996. ε-(γ-glutamyl)lysine crosslinks of spore coat proteins and transglutaminase activity in *Bacillus subtilis*. FEMS Microbiol. Lett. **144:**157–160.
18. **Kobayashi, K., S.-I. Suzuki, Y. Izawa, K. Yokozeki, K. Miwa, and S. Yamanaka.** 1998. Transglutaminase in sporulating cells of *Bacillus subtilis*. J. Gen. Appl. Microbiol. **44:**85–91.
19. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin, et al.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256.
20. **Kuwana, R., Y. Kasahara, M. Fujibayashi, H. Takamtsu, N. Ogasawara, and K. Watabe.** 2002. Proteomics characterization of novel spore proteins of *Bacillus subtilis*. Microbiology **148:**3971–3982.
21. **Lai, E.-M., N. D. Phadke, M. T. Kachman, R. Giorno, S. Vasquez, J. A. Vazquez, J. R. Maddock, and A. Driks.** 2003. Proteomic analysis of the spore coats of *Bacillus subtilis* and *Bacillus anthracis*. J. Bacteriol. **185:**1443–1454.
22. **Little, S., and A. Driks.** 2001. Functional analysis of the *Bacillus subtilis* morphogenetic spore coat protein CotE. Mol. Microbiol. **42:**1107–1120.
23. **Lorand, L., and R. M. Graham.** 2003. Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat. Rev. Mol. Cell Biol. **4:**140–156.
24. **Makarova, K. S., L. Aravind, and E. V. Koonin.** 1999. A superfamily of archaeal, bacterial, and eukaryotic proteins homologous to animal transglutaminases. Prot. Sci. **8:**1714–1719.
25. **Martins, L. O., C. M. Soares, M. M. Pereira, M. Teixeira, T. Costa, G. H. Jones, and A. O. Henriques.** 2002. Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. J. Biol. Chem. **277:**18849–18859.
26. **Moir, A.** 1981. Germination properties of a spore coat-defective mutant of *Bacillus subtilis*. J. Bacteriol. **146:**1106–1116.
27. **Nicholson, W. L., and P. Setlow.** 1990. Sporulation, germination and outgrowth, p. 391–450. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biology methods for *Bacillus*. John Wiley and Sons, Ltd., New York, N.Y.
28. **Ozin, A. J., A. O. Henriques, H. Yi, and C. P. Moran, Jr.** 2000. Morphogenetic proteins SpoVID and SafA form a complex during assembly of the *Bacillus subtilis* spore coat. J. Bacteriol. **182:**1828–1833.
29. **Paidhungat, M., K. Ragkousi, and P. Setlow.** 2001. Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca^{2+}-dipicolinate. J. Bacteriol. **183:**4886–4893.
30. **Pandey, N. K., and A. I. Aronson.** 1979. Properties of the *Bacillus subtilis* spore coat. J. Bacteriol. **137:**1208–1218.
31. **Ragkousi, K., and P. Setlow.** 2004. Transglutaminase-mediated cross-linking of GerQ in the coats of *Bacillus subtilis* spores. J. Bacteriol. **186:**5567–5575.
32. **Ragkousi, K., P. Eichenberger, C. van Ooij, and P. Setlow.** 2003. Identification of a new gene essential for spore germination of *Bacillus subtilis* spores with Ca^{2+}-dipicolinate. J. Bacteriol. **185:**2315–2319.
33. **Serrano, M., L. Côrte, J. Opdyke, C. P. Moran, Jr., and A. O. Henriques.** 2003. Expression of *spoIIIJ* in the prespore is sufficient for the activation of σ^G and for sporulation in *Bacillus subtilis*. J. Bacteriol. **185:**3905–3917.
34. **Serrano, M., R. Zilhão, A. J. Ozin, E. Ricca, C. P. Moran, Jr., and A. O. Henriques.** 1999. A *Bacillus subtilis* secreted protein with a role in endospore coat assembly and function. J. Bacteriol. **181:**3632–3643.
35. **Steil, L., M. Serrano, S. Hoevel, A. O. Henriques, and Uwe Völker.** 2005. Genome-wide analysis of temporally-regulated and compartment-specific gene expression during spore development in *Bacillus subtilis*. Microbiology **151:**399–420.
36. **Sullivan, M. A., R. E. Yasbin, and F. E. Young.** 1984. New shuttle vectors for *Bacillus subtilis* and *Escherichia coli* which allow rapid detection of inserted fragments. Gene **29:**21–26.
37. **Suzuki, S., Y. Izawa, K. Kobayashi, Y. Eto, S. Yamanaka, K. Kubota, and K. Yokozeki.** 2000. Purification and characterization of novel transglutaminase from *Bacillus subtilis* spores. Biosci. Biotechnol. Biochem. **64:**2344–2351.
38. **Takamatsu, H., T. Kodama, T. Nakayama, and K. Watabe.** 1999. Characterization of the *yrbA* gene of *Bacillus subtilis*, involved in resistance and germination of spores. J. Bacteriol. **181:**4986–4994.
39. **Zhang, J., P. Fitz-James, and A. I. Aronson.** 1993. Cloning and characterization of a cluster of genes encoding polypeptides present in the insoluble fraction of the spore coat of *Bacillus subtilis*. J. Bacteriol. **175:**3757–3766.
40. **Zheng, L., W. P. Donovan, P. Fitz-James, and R. Losick.** 1988. Gene encoding a morphogenetic protein required in the assembly of the outer coat of the *Bacillus subtilis* endospore. Genes Devel. **2:**1047–1054.
41. **Zheng, L. B., and R. Losick.** 1990. Cascade regulation of spore coat gene expression in Bacillus subtilis. J. Mol. Biol. **212:**645–660.
42. **Zilhão, R., M. Serrano, R. Isticato, E. Ricca, C. P. Moran, Jr., and A. O. Henriques.** 2004. Interactions among CotB, CotG, and CotH during assembly of the spore coat in *Bacillus subtilis*. J. Bacteriol. **186:**1110–1119.