J. Biochem. **139**, 887–901 (2006)
doi:10.1093/jb/mvj096

Modification of GerQ Reveals a Functional Relationship between Tgl and YabG in the Coat of *Bacillus subtilis* Spores

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Received January 16, 2006; accepted March 19, 2006

Here we describe the functional relationship between YabG and transglutaminase (Tgl), enzymes that modify the spore coat proteins of *Bacillus subtilis*. In wild-type spores at 37°C, Tgl mediates the crosslinking of GerQ into higher molecular mass forms; however, some GerQ multimers are found in *tgl* mutant spores, indicating that Tgl is not essential. Immunoblotting showed that spores isolated from a *yabG* mutant after sporulation at 37°C contain only very low levels of GerQ multimers. Heat treatment for 20 min at 60°C, which maximally activates the enzymatic activity of Tgl, caused crosslinking of GerQ in isolated *yabG* spores but not in *tgl/yabG* double-mutant spores. In addition, the germination frequency of the *tgl/yabG* spores in the presence of L-alanine with or without heat activation at 60°C was lower than that of wild-type spores. These findings suggest that Tgl cooperates with YabG to mediate the temperature-dependent modification of the coat proteins, a process associated with spore germination in *B. subtilis*.

Key words: crosslinking, processing, protein modification, spore germination, transglutaminase.

Bacteria such as *Bacillus subtilis* change from vegetative cells to spores in response to specific environmental conditions such as nutritional insufficiency. Spores possess extremely high dormancy, show unique morphologies, and are remarkably resistant to heat, lysozyme, and harsh chemicals (*1*). Spore formation is the result of a complex, highly controlled process of macromolecular assembly (*2*). Endospore formation involves a series of temporally and spatially ordered changes in cell morphology and gene expression. Genes involved in sporulation are mostly transcribed by RNA polymerase, which contains sporulation-specific sigma factors during sporulation (*3*). Two sigma factors, SigF and SigG, are active specifically in the prespore, whereas SigE and SigK control transcription in the mother cell (*3*).

The spores of *B. subtilis* consist of a spore coat, cortex, and core. The spore coat acts as a barrier, protecting the spore from damage and providing resistance to chemicals and lytic enzymes. Over 40 polypeptides must be properly assembled on the developing forespores to generate the spore coat (*4*). These proteins are synthesized in the mother cell and then assembled into a thick layer by post-translational modification (*4*). Several types of post-translational modification are found in coat proteins, including glycosylation, proteolytic processing, and crosslinking (*5*). SodA, OxdD (YoaN), YabG, and Tgl are particularly important in this regard (*4*). SodA (superoxide dismutase) is required for the assembly of coat protein CotG into the insoluble spore matrix (*6*), and OxdD (YoaN) is an oxalate decarboxylase that associates with the spore coat structure (*7*).

Little change in the spore profile is seen in *sodA* and *oxdD* mutants (*6*, *7*), but significant changes are observed in *yabG* and *tgl* mutants (*8*, *9*). The *yabG* gene is transcribed by SigK-containing RNA polymerase and encodes a protease required for the processing of CotF, CotT, SafA (YrbA), SpoIVA, YeeK, and YxeE during sporulation (*8*, *10*). The *tgl* gene is also transcribed by the SigK-containing RNA polymerase, and its expression is controlled by GerE, a transcriptional regulator (*9*, *11*). The *tgl* gene encodes a transglutaminase [EC 2.3.2.13] that catalyzes the formation of epsilon-(gamma-glutamyl) lysine isopeptide bonds between peptides, and can, therefore, crosslink and polymerize various proteins (*12*). Tgl mediates the crosslinking of GerQ, a spore coat protein involved in spore germination (*13*, *14*). GerQ is also required for the inclusion of CwlJ, an *N*-acetylmuramoyl-L-alanine amidase, in the spore coat (*13*, *15*). Tgl appears to be involved in the insolubilization of a set of 40, 28, and 16 kDa spore coat polypeptides independently of GerQ (*9*).

Although bacterial spores are metabolically dormant, they can return to active metabolism through the process of outgrowth and germination (*16*). When the spores are activated prior to the addition of a germinant, they initiate germination more rapidly and completely. The response of the spores to germination agents is affected by environmental conditions, including chemicals, pH, temperature, and pressure. Heating is the most widely used method of spore activation (*16*). Generally, heat activation is required for the measurement of germination (*17*). For some species, the heat activation process is reversible (*18*), but the precise changes induced by heat activation are not clear. Here, we describe a possible relationship between YabG and Tgl in the crosslinking of the coat proteins and the heat activation of spore germination.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and General Techniques—The *B. subtilis* and *Escherichia coli* strains

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Table 1. **Bacterial strains and plasmids used in this study.**

Strains	Genotype/description	Source, refrence, or construction
B. subtilis		
168	*trpC2*	1A1 (*Bacillus* Genetic Stock Center)
SGF602C	*trpC2 sigF*::*cat*	(*21*)
SGE603C	*trpC2 sigE*::*cat*	(*21*)
SGG604C	*trpC2 sigG*::*cat*	(*21*)
SGK605C	*trpC2 spoIVCB (sigK)*::*cat*	(*21*)
MTB862 (GERE5E)	*trpC2 gerE*::pMutin3	(*29*)
MTB905 (COTE5E)	*trpC2 cotE*::pMutin3	(*32*)
YRBA5E	*trpC2 safA (yrbA)*::pMutin3	This work (pYRBA5E, 168)
S4A5E	*trpC2 spoIVA*::pMutin3	This work (pS4A5E, 168)
S6D5E	*trpC2 spoVID*::pMutin3	This work (pS6D5E, 168)
COTF5E	*trpC2 cotF*::pMutin3	This work (pCOTF5E, 168)
COTT5E	*trpC2 cotT*::pMutin3	This work (pCOTT5E, 168)
YWDL5E	*trpC2 ywdL*::pMutin3	This work (pYWDL5E, 168)
YABG5E	*trpC2 yabG*::pMutin3	This work (pYABG5E, 168)
TGL5C	*trpC2 tgl*::*cat*	This work (pTGL5C, 168)
YABG5ETGL	*trpC2 yabG*::pMutin3 *tgl* ::*cat*	This work (TGL5C, YABG5E)
TGL8G	*trpC2 tgl-gfp cat*	This work (pTGL8G, 168)
TGL8GCOTE	*trpC2 cotE*::pMutin3 *tgl-gfp cat*	This work (pTGL8G, COTE5E)
TGL8GYRBA	*trpC2 safA(yrbA)*::pMutin3 *tgl-gfp cat*	This work (pTGL8G, YRBA5E)
TGL8GS6D	*trpC2 spoVID*::pMutin3 *tgl-gfp cat*	This work (pTGL8G, S6D5E)
TGL8GGERE	*trpC2 gerE*::pMutin3 *tgl-gfp cat*	This work (pTGL8G, GERE5E)
TGL8GYABG	*trpC2 yabG*::pMutin3 *tgl-gfp cat*	This work (pTGL8G, YABG5E)
E. coli		
JM109	*relA supE44 endA1 hsdR17 gyrA96 mcrA mcrB + thiΔ(lac-proAB) / F′(traD36 proAB + lacIq lacZΔM15)*	(*26*)
Plasmids		
pMutin3	*bla erm lacZ lacI Pspac*	(*20*)
pYABG5E	*bla erm yabG′-lacZ lacI Pspac-′yabG*	This work
pS4A5E	*bla erm spoIVA′-lacZ lacI Pspac-′spoIVA*	This work
pYRBA5E	*bla erm safA (yrbA)′-lacZ lacI Pspac-′safA (yrbA)*	This work
pS6D5E	*bla erm spoVID′-lacZ lacI Pspac-′spoVID*	This work
pCOTF5E	*bla erm cotF′-lacZ lacI Pspac-′cotF*	This work
pCOTT5E	*bla erm cotT′-lacZ lacI Pspac-′cotT*	This work
pYWDL5E	*bla erm ywdL′-lacZ lacI Pspac-′ywdL*	This work
pCAT5	*cat*	(*21*)
pTGL5C	*cat tgl′*	This work
pTUE1122	*bla lacI tac* promoter His6	(*23*)
pYWDL1A	*bla lacI tac* promoter *ywdL* His6	This work
pMALEH6	*bla lacI tac promoter malE His6*	(*32*)
pMCOTF1A	*bla lacI tac promoter malE cotF His6*	This work
pMCOTT1A	*bla lacI tac promoter malE cotT His6*	This work
pGFP7C	*gfp cat*	This work
pTGL8G	*tgl-gfp cat*	This work

and plasmids used in this study are listed in Table 1. The *B. subtilis* strains are all derivatives of strain 168; those constructed in this work were prepared by transformation with plasmid DNA and confirmed by PCR. *E. coli* strain JM109 was used for the production of plasmids. The oligonucleotides used for PCR amplifications are listed in Table 2.

We used a Campbell-type single-crossover recombination method to construct a series of insertion mutants of *B. subtilis* in this study (*19–21*). Segments of the *cotF*, *cotT*, *safA (yrbA)*, *spoIVA*, *spoVID*, *yabG*, and *ywdL* genes were PCR-amplified (primers listed in Table 2), cut with *Hin*dIII and *Bam*HI at primer-based sites, and inserted into the *Hin*dIII/*Bam*HI-digested pMutin3 vector to obtain plasmids pCOTF5E, pCOTT5E, pYRBA5E, pS4A5E, pS6D5E, pYABG5E, and pYWDL5E, respectively (Table 1) (*20*). Segments of the *tgl* gene were PCR-amplified (primers listed in Table 2), cut with *Hin*dIII and *Bam*HI at primer-based sites, and inserted into the *Hin*dIII/*Bam*HI-digested pCAT5 vector to obtain plasmid pTGL5C (*21*). These plasmids were used to transform *B. subtilis* 168 by a Campbell-type single-crossover recombination with selection for erythromycin resistance (0.5 μg/ml erythromycin) or chloramphenicol resistance (5 μg/ml chloramphenicol)

Table 2. **Oligonucleotide primers used in this study.**

Name	Sequence	Added site	Resulting construct
COTF159R	5′-AAAGGATCCTCAACACCCTGAATAGAC-3′	*Bam*HI	pCOTF5E
COTF18	5′-CAGAAGCTTGGCTTGGCATGAAACATT-3′	*Hin*dIII	pCOTF5E
COTT11	5′-ATTAAGCTTTGAATGAACAGTCATTTG-3′	*Hin*dIII	pCOTT5E
COTT129R	5′-GGGGGATCCGGATAATACGGTCTTGGA-3′	*Bam*HI	pCOTT5E
GFP4	5′-CAGCTCGAGACGTAAAGGAGAAGAACTTTTC-3′	*Xho*I	pGFP7C
GFP714R	5′-ATGGTGATGAGATCTTTTGTATAGTTCATCC-3′	*Bgl*II	pGFP7C
COTF3	5′-TTTGGATCCGGATGAACGCAGAACATTG-3′	*Bam*HI	pMCOTF1A
COTF479R	5′-TTGCTCGAGTTCATTGAAATGGCTCTGTTG-3′	*Xho*I	pMCOTF1A
COTT3	5′-GTTGGATCCTAGGATTACCCTTTGAATGAAC-3′	*Bam*HI	pMCOTT1A
COTT246R	5′-CCTCGAGTAACCGTAACCTCCCCCAT-3′	*Xho*I	pMCOTT1A
S4A350R	5′-AGGATCCGCGGCCCGTTTTCATC-3′	*Bam*HI	pS4A5E
S4A57	5′-TAAGCTTAGGAGTCGTAGGTGCTG-3′	*Hin*dIII	pS4A5E
S6D57	5′-AAGAAGCTTGGACAGGAAGTTTCTGAACTGC-3′	*Hin*dIII	pS6D5E
S6D178R	5′-GGAGGATCCGATCTATGTTGTACTCACC-3′	*Bam*HI	pS6D5E
TGL22	5′-AGGAAGCTTGCTCCGTCCCCAGGA-3′	*Hin*dIII	pTGL5C
TGL305R	5′-GGAGGATCCATCGCTTTTGAAGGCGGC-3′	*Bam*HI	pTGL5C
TGL71	5′-TCTGGATCCGCTCTTAAAAGAGATG-3′	*Bam*HI	pTGL8G
TGL737R	5′-GGGCTCGAGTAGCGGACGATGCGGAAA-3′	*Xho*I	pTGL8G
YABG220R	5′-GAGGGATCCATTCATTCTGCTCTCATCT-3′	*Bam*HI	pYABG5E
YABG69	5′-CGAAAGCTTGGAATAGAGCAAACAAGCAA-3′	*Hin*dIII	pYABG5E
YRBA299R	5′-AAAGGATCCATCGGCGGGACATACGGC-3′	*Bam*HI	pYRBA5E
YRBA30	5′-AAGAAGCTTCGCTCTGGAAAATAGCTG-3′	*Hin*dIII	pYRBA5E
YWDL544R	5′-TTCAGATCTTGGCGAATAGGACGC-3′	*Bgl*II	pYWDL1A
YWDLM396	5′-GTTCTCGAGAGTATGCTAGAAATGGA-3′	*Xho*I	pYWDL1A
YWDL190R	5′-CGGGGATCCAAATCCCTGCTGTCC-3′	*Bam*HI	pYWDL5E
YWDL40	5′-GCAAAGCTTTGATAATATGCAGGGGTAT-3′	*Hin*dIII	pYWDL5E
TGL3	5′-GCTGGATCCGATTATTGTATCAGGACAATTG-3′	*Bam*HI	*tgl* RNA probe
TGL700RT7	5′-TAATACGACTCACTATAGGGCGAGTGGTCGCCT GAGACAGAAG-3′	T7 promoter	*tgl* RNA probe

Oligonucleotides used for PCR amplifications are listed. The T7 promoter sequence is underlined. PCR products were digested with restriction enzymes at the primer-derived sequences, and inserted into restriction enzyme–digested plasmids to generate the plasmids listed in Table 1.

to yield strains COTF5E, COTT5E, YRBA5E, S4A5E, S6D5E, YABG5E, YWDL5E, and TGL5C, respectively (Table 1) (*19–21*). The chromosomal DNA YABG5E was introduced into TGL5C to construct the double-mutant YABG5ETGL. The recombination of DNA was confirmed by PCR.

The gene encoding green fluorescent (GFP) protein mutant 3 (*22*) was amplified using oligonucleotide primers GFP4 and GFP714R (Table 2). The PCR product was digested at the *Xho*I and *Bgl*II sites introduced by the primers, and inserted into *Xho*I/*Bgl*II-digested pCAT5 to create plasmid pGFP7C (Table 1). The *gfp* gene makes an in-frame fusion at the 3′ end in this plasmid with the sequence encoding a 6x His tag. Oligonucleotide primers TGL71 and TGL737R were used to amplify the *tgl* gene fragment from the *B. subtilis* 168 chromosome (Table 2). The PCR product was digested at the *Bam*HI and *Xho*I sites introduced by the primers, and then inserted into *Bam*HI/*Xho*I-digested pGFP7C to create plasmid pTGL8G (Table 1). Strains 168, MTB862 (GERE5E), MTB905 (COTE5E), YRBA5E, S6D5E, and YABG5E were transformed with this plasmid by a single crossover recombination with selection for chloramphenicol resistance (5 μg/ml) yielding strains TGL8G, TGL8GGERE, TGL8GCOTE, TGL8GYRBA, TGL8GS6D, and TGL8GYABG, respectively (Table 1). The authentic *tgl* gene was replaced by the *tgl-gfp* fusion in these strains. The recombination of DNA was confirmed by PCR.

To construct a series of plasmids, DNA fragments encoding *cotF*, *cotT*, and *ywdL* were PCR-amplified (see Table 2 for primers), digested with restriction enzymes, and inserted into *Bam*HI/*Xho*I-digested pMALEH6 or *Xho*I/*Bgl*II-digested pTUE1122 (which contains a 6x His tag) to give recombinant plasmids pMCOTF1A, pMCOTT1A, and pYWDL1A, respectively (Table 1) (*23*). The plasmids were integrated into the chromosomal DNA by homologous recombination, and the resulting transformants were verified by PCR.

B. subtilis strains were grown in Difco Sporulation (DS) medium (Difco) (*24*). The conditions for sporulation of *B. subtilis* were as previously described (*25*). Recombinant DNA techniques were carried out according to standard protocols (*26*). Preparation of competent cells, transformation, and preparation of chromosomal *B. subtilis* DNA were carried out as previously described (*27*).

RNA Preparation and Northern Analysis—Total RNA was prepared from *B. subtilis* cells as described previously (*28*). Northern analysis, hybridization, and detection were performed using the DIG Northern Starter Kit (Roche) according to our previous report (*29*). RNA probes for Northern hybridization were synthesized using T7 RNA polymerase with PCR products as templates. The 0.8-kb

probe for *tgl*, corresponding to nt 3 to 700 downstream of the translation initiation codon of *tgl*, was prepared by PCR using primers TGL3 and TGL700RT7 (Table 2). RNA probes specific for *tgl* were labeled with the Roche digoxigenin labeling system as previously described (*29*).

Phase Contrast and Fluorescence Microscopy—Aliquots of the cultures of strains harboring the *tgl-gfp* fusion on the chromosome sporulated in DS medium were transferred to a microscope slide. Fluorescence due to the GFP fusion protein was observed under a BX51 fluorescent microscope with a GFP mirror cube unit (Olympus). The images were captured with a CoolSNAP ES/OL cooled charge-coupled device camera (Roper Scientific), and processed with RS Image Express ver. 4.5 (Roper Scientific).

Preparation of Spores—The *B. subtilis* strains were grown in DS medium at 37°C as described previously (*21*). Mature spores were harvested 18 h after the cessation of exponential growth (T18) and washed once with 10 mM Tris-HCl (pH 7.2). The spore samples were then prepared by a modification of the procedure described by Kuwana *et al*. (*21*). To remove cell debris and vegetative cells, the pellets were suspended in 0.1 ml lysozyme buffer (10 mM Tris-HCl, pH 7.2 with 1% [w/v] lysozyme) and incubated at room temperature for 10 min. In some experiments, complete protease inhibitor cocktail (Roche) was added to the lysozyme buffer. The pellets were then washed repeatedly with buffer (10 mM Tris-HCl, pH 7.2 and 0.5 M NaCl) at room temperature. After this treatment, more than 99% of the spores were refractive, and almost no dark or gray spores were visible by phase contrast microscopy. Furthermore, the samples contained neither vegetative cells nor cell debris (data not shown).

Heat Treatment—For heat activation, equilibrated spore cells were left unheated or heated at the appropriate temperature (37, 45, or 60°C) for 20 min and then cooled on ice immediately before solubilization of the spore proteins. For inhibitory assays, the cells were treated for 5 min on ice with 20 mM ammonium sulfate (pH 7.2) or control buffer (10 mM phosphate buffer, pH 7.2) prior to heat activation.

Solubilization of Proteins from Mature Spores—Spore proteins were solubilized in 0.1 ml of loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% [w/v] SDS, 10% [v/v] 2-mercaptoethanol, 10% [v/v] glycerol, and 0.05% [w/v] bromophenol blue) and boiled for 5 min (*21*). A Bio-Rad RC DC protein assay kit was used to measure the amount of protein in the sample as described previously (*30*). The proteins were separated by 14% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250 (CBB) (*8*).

Purification of Recombinant, CotF, CotT, and GerQ (YwdL) Proteins from E. coli—*E. coli* cells were transformed with recombinant plasmids pMCOTF1A, pMCOTT1A, and pYWDL1A. The resulting transformants were grown at 37°C for 3 h in 200 ml of Luria broth supplemented with ampicillin (50 μg/ml). The culture was then supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside and incubated for an additional 3 h at 37°C. The His-tagged recombinant proteins were purified by affinity chromatography on Ni-NTA agarose beads (Qiagen), and were further purified by electroelution from the subsequent SDS-PAGE as previously described (*31*).

Preparation of Antisera against MalE-CotF, MalE-CotT, and GerQ (YwdL)—Purified MalE-CotF, MalE-CotT, or GerQ (YwdL) (1 ml of 0.2 mg/ml) and 16 mg of killed *Mycobacterium tuberculosis* cells (Difco) were mixed with 2 ml of complete Freund's adjuvant (Difco), and 3 ml of each emulsion was injected into healthy rabbits. After 2 weeks, the rabbits were injected with solutions of MalE-CotF, MalE-CotT, or GerQ (YwdL) in incomplete Freund's adjuvant (Difco). Two weeks after the second immunization, antisera for MalE-CotF, MalE-CotT, and GerQ (YwdL) were isolated as previously described (*31*). The rabbit antisera against SpoIVA and SafA (YrbA) were collected as described previously (*8*).

SDS-PAGE and Immunoblotting—Protein samples were analyzed by 14% SDS-PAGE as described previously (*21*). A Bio-Rad RC DC protein assay kit was used to measure the quantity of protein in the sample as described previously (*30*). Immunoblotting was performed using rabbit immunoglobulin G (IgG) against SpoIVA, SafA (YrbA), MalE-CotF, MalE-CotT, YaaH, and GerQ (YwdL) as described previously (*10*, *31*, *32*). In agreement with a previous report (*32*), we found that the anti–*E. coli* MalE antiserum did not react with any spore proteins of *B. subtilis* (data not shown).

Spore Germination—For studies on the effect of the germination temperature, purified spores were heat-activated at 37, 45, or 60°C for 20 min, cooled, and suspended in 10 mM Tris-HCl (pH 7.2) buffer to an optical density of 0.5 Abs at 600 nm. Spores were then mixed with either L-alanine (10 mM) or AGFK (10 mM L-asparagine, 10 mM D-glucose, 10 mM D-fructose, and 10 mM potassium chloride). Germination was monitored for up to 120 min by measuring the decrease in optical density at 660 nm of the spore suspension at 37°C (*33*).

RESULTS

Expression Pattern and Transcription Unit of the tgl Gene—Previous studies using a *tgl-lacZ* transcriptional fusion have indicated that transcription of the *tgl* gene requires SigK-containing RNA polymerase, and that it is repressed by the regulatory protein GerE (*9*). We confirmed the transcription unit of the *tgl* gene by Northern hybridization (Fig. 1). A probe specific for *tgl* first detected a 0.8-kb transcript 4 h after the onset of sporulation. This indicates that the *tgl* gene, which is composed of 738 nucleotides, is monocistronically transcribed. We then examined the dependency of *tgl* expression on sigma factors SigF, SigE, SigG, and SigK and the transcriptional regulator GerE. We performed Northern hybridization with RNA prepared from sigma factor mutant cells and from *gerE* mutant cells 6 h after the onset of sporulation. We were unable to detect *tgl* mRNA in the *sigF*, *sigE*, *sigG*, and *sigK* mutant strains, but the level of the *tgl* transcript was apparently increased in the *gerE* mutant. In the region upstream of the *tgl* gene, we found the consensus sequence for SigK-containing RNA polymerase and putative GerE-binding sites as previously reported (*11*). This suggests that the expression of *tgl* is dependent on SigK-containing RNA polymerase and negatively controlled by GerE in the mother cell compartment.

Localization of Tgl-GFP during Sporulation—We constructed a gene encoding a Tgl-GFP fusion to examine the localization of Tgl in the spores. We introduced the *tgl-gfp* fusion into the chromosome of the wild-type and *cotE*, *gerE*, *safA*, *spoVID*, and *yabG* mutant strains.

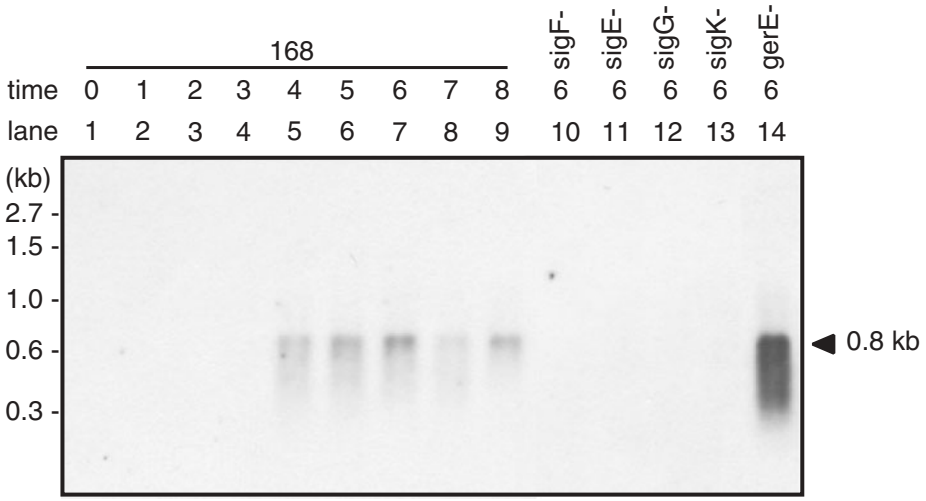

Fig. 1. **Northern blot analysis of *tgl* mRNA.** Total RNA was prepared from sporulating cells, and mRNAs were detected by Northern hybridization using probes specific for *tgl*. The arrowheads indicate the position of *tgl* mRNA hybridized with the digoxigenin-labeled RNA probe. Transcription of *tgl* in strain 168 (lanes 1–9), *sigF* mutant (lane 10), *sigE* mutant (lane 11), *sigG* mutant (lane 12), *sigK* mutant (lane 13), and *gerE* mutant (lane 14) cells was analyzed by Northern hybridization. The time (h) after the onset of sporulation is shown at the top.

CotE, SafA, and SpoVID are required for the proper assembly of many coat proteins (*25*, *34*, *35*). The resulting transformants were grown in DS medium and analyzed 8 h after the onset of sporulation (Fig. 2). In the wild-type transformants, Tgl-GFP was detected around the outsides of the forespores, but not in the mother cell compartment (Fig. 2B). The fluorescence of Tgl-GFP was concentrated at both polar ends of developing forespores (Fig. 2, B and C). In the *cotE*, *gerE*, *safA*, and *spoVID* mutant transformants, Tgl-GFP was abnormally detected in the mother cell compartment and/or around the outsides of forespores (Fig. 2, E, H, K, and N). The fluorescence of Tgl-GFP was enhanced in the *gerE* mutant, and was slightly decreased in the *cotE* and *safA* mutant strains. These results suggest that Tgl is synthesized in the mother cell compartment and assembles in the spore coat under the guidance of CotE, SafA, and SpoVID. In the *yabG* mutant, Tgl-GFP located around the outside of the forespores, but did not concentrate at their polar ends (Fig. 2Q).

Protein Composition of Spores—To clarify the functional relationship between YabG and Tgl, we analyzed the spore proteins prepared from wild-type and mutant strains of *tgl*, *yabG*, and *tgl*/*yabG* by SDS-PAGE followed by CBB staining (Fig. 3). Proteins were solubilized from the spores that had been collected and purified 18 h after the cessation of sporulation. The protein profile of the wild-type and *yabG* mutant spores was almost the same as previously reported (Fig. 3, lanes 1 and 2; 8, 9). The P55, P45, P29, P21, P20, P18, and P15 bands detected in the sample from the *yabG* spores correspond to SpoIVA, SafA (large form), SafA (small form), CotF, YxeE, YeeK, and CotT (*8*). The positions of SafA (small form), CotF, and YxeE are slightly different than in the previous report because we changed the concentration of acrylamide in the gel as well as the method for preparing the spore proteins (see "MATERIALS AND METHODS"). We analyzed the protein profile of the insertion mutants of *cotF*, *yxeE*, *yeeK*, and *cotT* in the background of the *yabG* mutant, and confirmed that the P21, P20, P18, and P15 bands in the *yabG* mutant spores correspond to CotF, YxeE, YeeK, and CotT, respectively (data not shown). Two bands denoted P31 and P23, with molecular masses of 31 and 23 kDa, were newly detected in this study (Fig. 3, lane 2). We assume that these two bands separated from other bands and became distinguishable because of the high percentage (14%) acrylamide gel. The protein bands with molecular masses of 42, 28, 23, and 17 kDa in the sample from the *tgl* spores were named P42, P28, P23, and P17, respectively (Fig. 3, lane 3). Zilhao *et al.* also reported 40, 28, 20, and 16 kDa proteins specific to the *tgl* mutant (*9*). These are identical to the P42, P28, P23, and P17 of the *tgl* spores in this study. The P23 band in the extract from the *yabG* spores probably corresponds to the monomer form of GerQ, and its level was clearly decreased in the extract from the *gerQ*/*yabG* double-mutant spores (data not shown).

The protein composition of the *tgl*/*yabG* spores was more similar to that of the *yabG* spores than the *tgl* spores (Fig. 3, lane 4). The sample from the *tgl*/*yabG* spores also contained an additional P34 band that was unclear in other samples. The *tgl*/*yabG* double-mutant lost two bands with molecular masses of 65 and 35 kDa corresponding to coat proteins CotB and CotG (Fig. 3, lane 2; 21, 36, 37). Other minor differences were found but not reproducible (data not shown). These findings suggest that the *yabG* mutant has a dominant effect on the *tgl* mutant, and prevents the production of P42, P28, and P17 in *tgl*/*yabG* spores.

Identification of a Common Substrate for YabG and Tgl—LC-MS/MS analysis of the proteins in the *tgl* spores by Zilhao *et al.* revealed candidate substrates for Tgl (*9*). In the current study, we used immunoblotting to try to identify the coat protein that is modified by both YabG and Tgl. We analyzed the proteins extracted from the *tgl* and the *tgl*/*yabG* spores with the anti-SpoIVA, -SafA, -CotF, -CotT, and -GerQ antisera. YxeE and YeeK are also modified by YabG, but anti-YxeE and anti-YeeK antisera were not available for this study. We used purified spores obtained from culture in DS medium at 37°C for 24 h. Mature spores were prepared in the presence of a protease inhibitor cocktail to prevent protein degradation (see "MATERIALS AND METHODS"). We then extracted the spore proteins and performed immunoblotting for SpoIVA, SafA, CotF, CotT, and GerQ (Fig. 4). The specificities of the antisera were confirmed using strains that were null for the expression of each gene (data not shown). Arrowheads in Fig. 4 indicate the position of each protein.

The molecular masses of SpoIVA, SafA, CotF, CotT, and GerQ estimated from immunoblotting agreed well with the predicted molecular masses as well as with previous

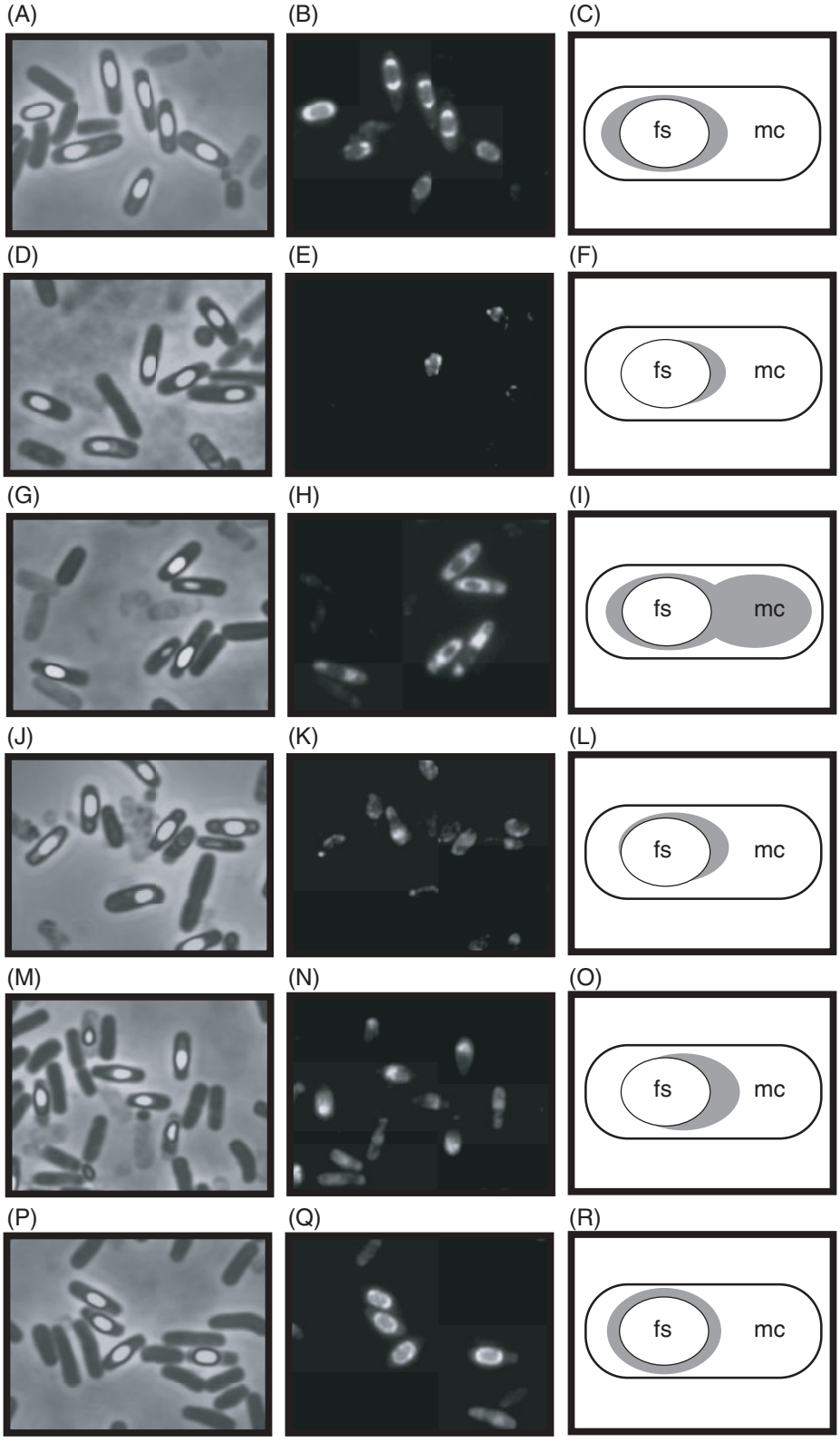

Fig. 2. **Detection of Tgl-GFP fusion in sporulating cells.** TGL8G (A and B), TGL8GCOTE (D and E), TGL8GGERE (G and H), TGL8GYRBA (J and K), TGL8GS6D (M and N), and TGL8GYABG (P and Q) strains carrying *tgl-gfp* fusion were grown in DS medium at 37°C, and the cells were collected 8 h after the onset of sporulation. The cells were analyzed by phase-contrast microscopy (A, D, G, J, M, and P) and fluorescence microscopy (B, E, H, K, N, and Q). (C, F, I, L, O, and R) Models of the localization of Tgl-GFP during sporulation. Grey shading, Tgl-GFP; fs, forespore; mc, mother cell.

results (*10*, *14*, *30*, *32*). A 55 kDa band, corresponding to intact SpoIVA, was detected in the *yabG* and the *tgl*/*yabG* mutant spores (Fig. 4, A and D). No proteins cross-reacted with the anti-SpoIVA antiserum in the samples from wild-type or *tgl* spores (Fig. 4, A and C). The low-molecular mass bands were probably the products of degradation or processing in the *yabG* mutant (Fig. 4A, lane 2). The P55 bands from the *yabG* and the *tgl*/*yabG* mutant spores

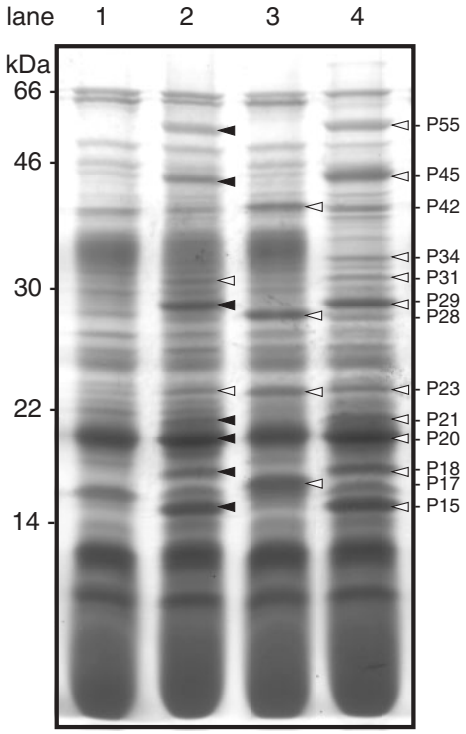

Fig. 3. **SDS-PAGE analysis of proteins solubilized from spores.** Cells were cultured for 24 h in DS medium at 37°C. Purified spores were prepared using lysozyme buffer containing a complete protease inhibitor cocktail (Roche). Protein samples were solubilized from the spores by boiling in the presence of SDS and 2-mercaptoethanol. The solubilized proteins were separated by SDS-PAGE (14% gel) and stained with CBB. Lane 1, wild-type spores; lane 2, *yabG* mutant spores; lane 3, *tgl* mutant spores; lane 4, *tgl*/*yabG* mutant spores. Filled arrowheads indicate the significantly increased or decreased bands that were identified in our previous study (*8*): SpoIVA (55 kDa), SafA (YrbA; 45 kDa), SafA (31 kDa), CotF (23 kDa), YxeE (21 kDa), YeeK (18 kDa), and CotT (15 kDa). Open arrowheads indicate bands showing increases or decreases in mutant spores as compared to wild-type spores.

shown in Fig. 3 probably are SpoIVA. Previous studies have indicated that SafA is synthesized as 45 and 31 kDa polypeptides that are proteolytically processed into smaller molecules (*10*, *25*, *38*). Using anti-SafA antiserum, a band with a molecular mass of 30 kDa was detected in the extracts from wild-type spores (Fig. 4B, lane 1). Bands with molecular masses of 45 and 31 kDa were detected in *yabG* and *tgl*/*yabG* spores (Fig. 4B, lanes 2 and 4).

We detected 42 and 30 kDa forms of SafA in *tgl* spores (Fig. 4B, lane 3). A 45 kDa form of SafA was also detected in the sample from *tgl* spores. We speculate that the 42 and 30 kDa forms of SafA are the processed products of the 45 and 31 kDa forms of SafA, respectively. The density of the 30 kDa SafA band was greatly increased in *tgl* spores as compared to wild-type spores. Bands in each lane smaller than 30 kDa are likely the products of degradation or processing, and the high-molecular mass bands detected in each lane are presumably parts of crosslinked complexes. We speculate that the P45 and P29 bands in extracts from *yabG* and *tgl*/*yabG* mutant spores shown in Fig. 3 correspond to the 45 and 31 kDa forms of SafA that reacted with the anti-SafA antiserum, respectively. The 42 and 30 kDa forms of SafA in *tgl* spores were probably detected as the P42 and P28 bands in Fig. 3, respectively.

Both CotF and CotT are synthesized as precursor forms and proteolytically processed in the wild-type strain (*39*, *40*). A 21 kDa form of CotF was detected in the *yabG* and the *tgl*/*yabG* mutant spores (Fig. 4C). This size is slightly different than in our previous results (23 kDa; *8*). A 15 kDa form of CotT was also detected in the *yabG* and the *tgl*/*yabG* mutant spores (Fig. 4D). Neither CotF nor CotT was detected in wild-type or *tgl* mutant spores. These 21 and 15 kDa proteins detected in the *yabG* and *tgl*/*yabG* mutant spores are probably the precursors of CotF and CotT, respectively (*8*, *39*, *40*). We speculate that the processed forms of CotF and CotT in the wild-type and *tgl* spores are insoluble under our experimental conditions.

The size of GerQ (YwdL) is estimated to be 20,132 Da based on its primary amino acid sequence (Japan Functional Analysis Network of *B. subitlis* database; http://bacillus.genome.jp). GerQ is reported to exist as an 18 or 20 kDa monomer and as a 40 kDa or larger multimer (*9*, *14*). In the wild-type spores, we found both monomeric (23 kDa) and multimeric forms (40 kDa and higher molecular masses) of GerQ due to crosslinking by Tgl (Fig. 4E, lane 1). The high-molecular mass species of GerQ are produced 48 h after the initiation of sporulation and are efficiently extracted from spores by treatment at 70°C for 30 min in the presence of 0.1 M NaOH and 1% SDS (*14*). We purified spores 18 h after the initiation of sporulation and extracted proteins in the absence of NaOH to avoid the deactivation of spore enzymes. Using the anti-GerQ antiserum, we detected bands with molecular masses of 40 and 23 kDa in the sample of wild-type spores (Fig. 4E, lane 1). In this study, the size of the GerQ monomer was estimated to be 23 kDa because we used different conditions for preparing the spore proteins and for SDS-PAGE than in previous studies (*9*, *14*). The 23 kDa form of GerQ was present in all extracts (Fig. 4E), and the 40 kDa multimer form was present in the extract from wild-type but not the *yabG* or *tgl*/*yabG* mutant spores (Fig. 4E, lane 1, 2, and 4). Less GerQ multimer was detected in the *tgl* spores than in the wild-type spores as described previously (Fig. 4E, lane 3) (*14*). YaaH is involved in germination, and is expressed by SigE-containing RNA polymerase during sporulation (*33*). YaaH has been reported to exist as a 50 kDa monomer in wild-type cells (*30*, *32*). The density of the 50 kDa YaaH band in each lane was visually equal (Fig. 4F), so we used YaaH as a loading control in the following experiments. We also investigated the specificity of anti-GerQ antiserum by immunoblotting analysis (Fig. 5A). We examined GerQ in extracts from the wild-type spores and *gerQ* spores. We detected bands with molecular masses of 40 and 23 kDa and bands smaller than 20 kDa in the wild-type spores (Fig. 5A, lane 1). There were no bands in extracts from *gerQ* spores (Fig. 5A, lane 2). Bands smaller than 20 kDa are probably fragments of GerQ generated by proteolysis. These results suggest that YabG is also involved in the crosslinking of GerQ (Figs. 4E and 5A). YaaH was analyzed as a control by using anti-YaaH antiserum, and was detectable in extracts from the wild-type spores and *gerQ* spores (Fig. 5B).

Temperature-Controlled Modification of GerQ—We identified GerQ as the common substrate of Tgl and YabG. *In vitro*, the enzymatic activity of Tgl is optimal at 60°C

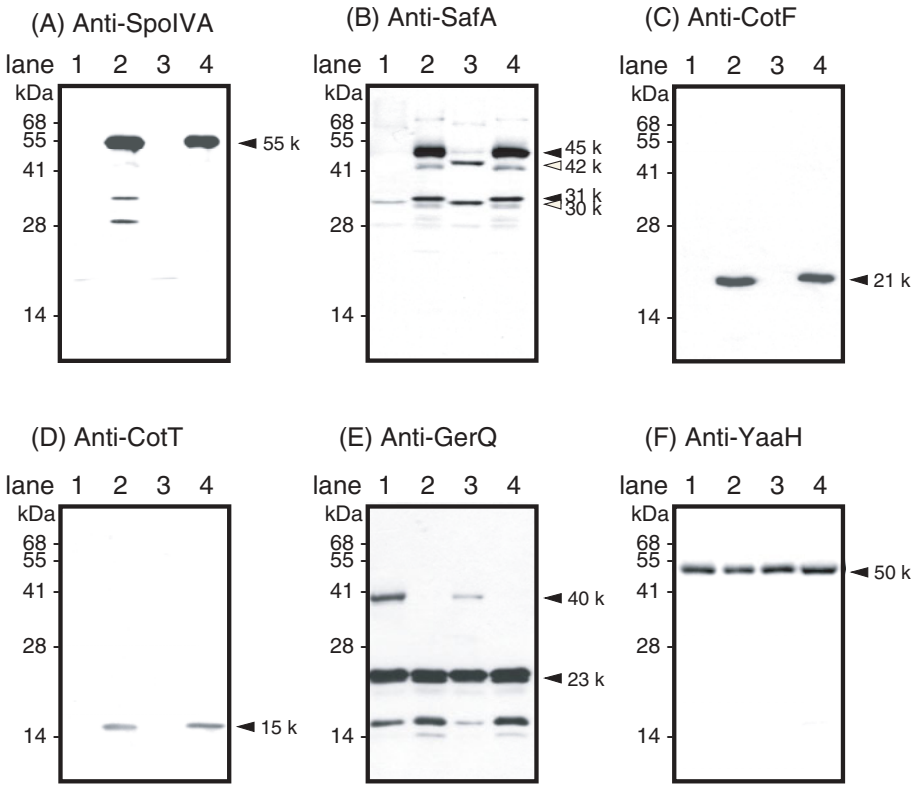

Fig. 4. **Immunoblotting identification of the proteins affected by YabG and Tgl.** Cells were cultured for 24 h in DS medium at 3°C. Purified spores were prepared in lysozyme buffer containing a complete protease inhibitor cocktail (Roche). Protein samples (10 μg) were solubilized from wild-type cells (lane 1), *yabG* mutant spores (lane 2), *tgl* mutant spores (lane 3), and *yabG*/*tgl* double-mutant spores (lane 4). The samples were resolved by 14% SDS-PAGE, and immunoblotting was performed with anti-SpoIVA (A), anti-SafA (B), anti-CotF (C), anti-CotT (D), anti-GerQ (E), and anti-YaaH (F) antisera. The arrowheads show the position of each protein. Filled arrowheads indicate estimated molecular masses for the previously reported bands (*8*), and open arrowheads indicate new bands detected in the mutants in this study.

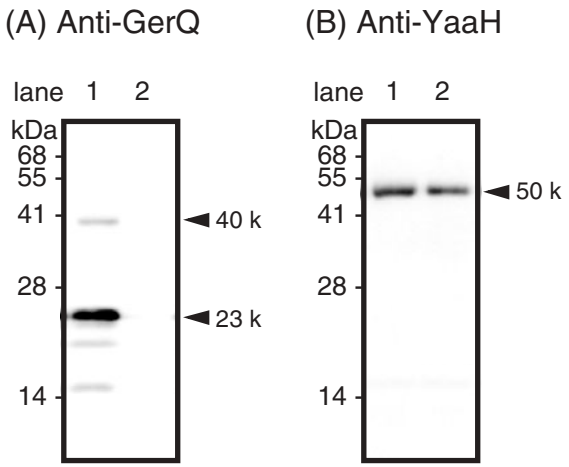

Fig. 5. **Specificity of the anti-GerQ antiserum in spores.** Cells were cultured for 24 h in DS medium at 37°C. Purified spores were prepared in lysozyme buffer containing a complete protease inhibitor cocktail (Roche). Protein samples (10 μg) were solubilized from wild-type cells (lane 1) and *gerQ* mutant spores (lane 2). The samples were resolved by 14% SDS-PAGE, and immunoblotting was performed with anti-GerQ (A) and anti-YaaH (B) antisera. The arrowheads show the position of each protein.

(*12*). We, therefore, speculated that, at 37°C, Tgl is not fully active and that the crosslinking of GerQ is dependent on YabG. We, therefore, investigated the modification of GerQ at several temperatures. We used spores purified from the wild-type and *yabG*, *tgl*, and *tgl*/*yabG* mutant strains that had been cultured in DS medium at 37°C for 24 h. We incubated the purified spores for 20 min at 37, 45, 60, or 80°C in the absence of protease inhibitor cocktail (see "MATERIALS AND METHODS"). We then extracted total proteins and performed immunoblotting of the samples with the anti-GerQ antiserum (Fig. 6). Both the monomer (23 kDa) and multimer (40 kDa) forms of GerQ were detected in the samples from the wild-type spores (Fig. 6A). The density of the GerQ multimer band decreased after the wild-type spores were heated at 60 or 80°C (Fig. 6A, lanes 4 and 5). The amount of the 40 kDa form of GerQ in the extract from the *yabG* mutant spores was significantly increased by heat treatment at 60°C (Fig. 6B, lane 4). The amount of GerQ multimer in the extracts from the *tgl* spores was not substantially changed by heat treatment, and was lower than in the extract from the wild-type spores (Fig. 6C). The 40 kDa form of GerQ was not detected in the extract from the *tgl*/*yabG* mutant spores regardless of heat treatment (Fig. 6D). The density of YaaH in each lane was visually equal (Fig. 6, lower panel). These results show that, at 60°C, Tgl mediates the crosslinking of GerQ even in the absence of YabG.

Time Required for Crosslinking of GerQ—We examined the time required for the crosslinking of GerQ in heat-treated spores. We incubated the purified spores at 60°C for 0, 0.5, 1, 5, 10, 15, 20, or 30 min, extracted the total protein, and analyzed the samples by immunoblotting with the antiserum against GerQ. The 23 and 40 kDa GerQ bands in the wild-type spores decreased gradually and slightly by long-term incubation at 60°C (Fig. 7A). The density of YaaH in each lane was visually equal (Fig. 7, lower panel). In the *yabG* mutant spores, the 23 kDa GerQ protein was detected in all lanes, but the intensity of the 40 kDa band gradually increased after 5 or more min of heat treatment (Fig. 7B). In the *tgl* mutant and the

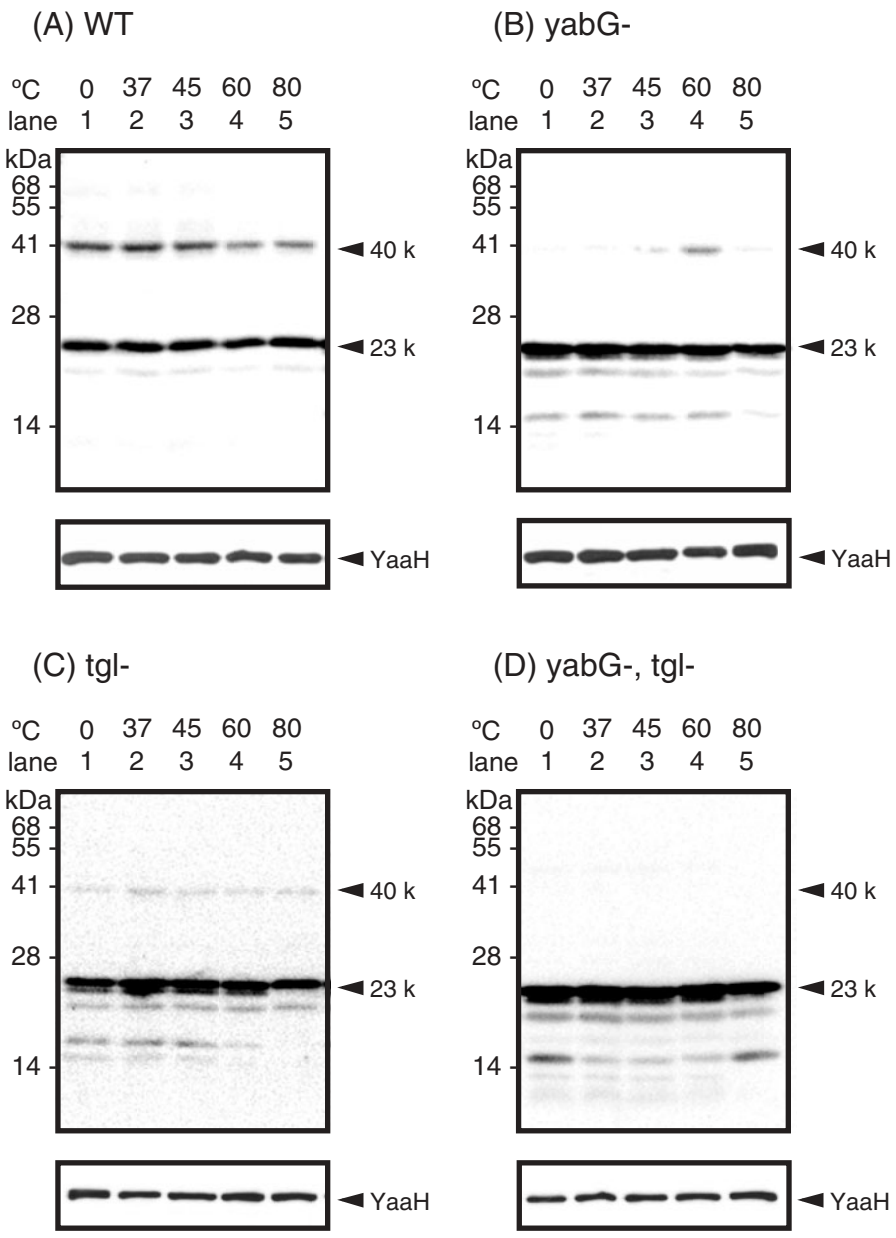

Fig. 6. **Immunoblot analysis of GerQ in heat-treated spores.** Cells were cultured for 24 h in DS medium at 37°C. Purified spores were prepared without complete protease inhibitor cocktail (Roche) in lysozyme buffer. After purification, the spores were incubated for 20 min at 0, 37, 45, 60, or 80°C. The temperature (°C) of heat treatment is shown at the top. The protein samples (10 μg) were solubilized from wild-type cells (A), *yabG* mutant spores (B), *tgl* mutant spores (C), and *tgl*/*yabG* mutant spores (D). The samples were resolved by 14% SDS-PAGE, and immunoblotting was performed with anti-GerQ antiserum. Arrowheads show the positions of GerQ (upper panel). YaaH was analyzed as a loading control by using anti-YaaH antiserum (lower panel).

tgl/*yabG* mutant, the 23 kDa form of GerQ was detected in all lanes (Fig. 7, C and D). The 40 kDa form of GerQ did not increase in the samples from the *tgl* or the *tgl*/*yabG* mutant spores even after incubation at 60°C for 30 min (Fig. 7, C and D). These results show that 20 min is sufficient for GerQ crosslinking.

Inhibition of the Enzymatic Activity of Tgl by Ammonium Sulfate—The enzymatic activity of Tgl is reported to be inhibited by ammonium sulfate (*12*). We incubated the purified spores at 60°C for 20 min in the presence or absence of 20 mM ammonium sulfate, and then extracted the total proteins and analyzed them by immunoblotting with the anti-GerQ antiserum. A 23 kDa band corresponding to the monomer form of GerQ was detected in all samples regardless of heat treatment or the presence of ammonium sulfate (Fig. 8, lanes 1–16). The 40 kDa form of GerQ was detected in all samples from wild-type spores (Fig. 8, lanes 1–4). After heat treatment of the wild-type spores, the level of the GerQ multimer decreased slightly in both the presence and absence of ammonium sulfate (Fig. 8, lanes 3–4). The production of the 40 kDa form of GerQ by incubation at 60°C was inhibited by ammonium sulfate in the *yabG* spores (Fig. 8; lanes 5–8). In the *tgl* spores, a low level of the 40 kDa form of GerQ was detected regardless of heating or the addition of ammonium sulfate (Fig. 8, lanes 9–12). Little of the 40 kDa form of GerQ was detected in the samples of the *tgl*/*yabG* double mutant (Fig. 8, lanes 13–16). The density of YaaH in each lane was visually equal (Fig. 8, lower panel). These results show that GerQ crosslinking in the *yabG* spores at 60°C is specifically mediated by Tgl.

Effect of Heat Activation on Spore Germination—We examined the effects of heat activation on the germination

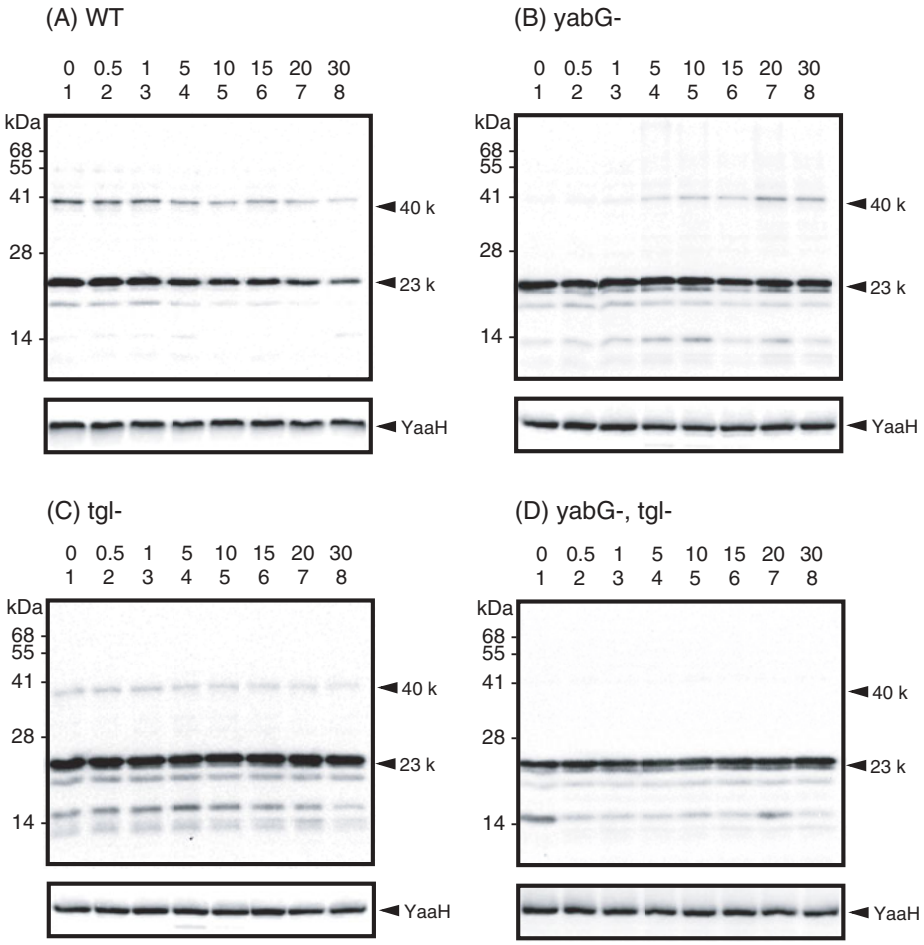

Fig. 7. **Immunoblot analysis of GerQ in spores incubated at 60°C.** Cells were cultured for 24 h in DS medium at 37°C. Purified spores were prepared without complete protease inhibitor cocktail (Roche) in lysozyme buffer. After purification, the spores were incubated at 60°C for the indicated time (min; top of lanes 1–8). The protein samples (10 μg) were solubilized from wild-type spores (A), *yabG* mutant spores (B), *tgl* mutant spores (C), and *tgl*/*yabG* mutant spores (D). The samples were resolved by 14% SDS-PAGE, and immunoblotting was performed with anti-GerQ antiserum. Arrowheads show the positions of GerQ (upper panel). YaaH was analyzed as a loading control by using anti-YaaH antiserum (lower panel).

of the wild-type, *yabG* mutant, *tgl* mutant, and *tgl*/*yabG* mutant spores. Germination was measured by monitoring the decrease in the optical density at Abs 600 nm of the spore suspension upon the addition of L-alanine or AGFK (a mixture of L-asparagine, D-glucose, D-fructose, and potassium chloride) at 37°C with or without heat activation at 60°C (Fig. 9). Without heat activation, the frequency of L-alanine–stimulated germination of the *yabG* and *tgl* mutant spores was slightly lower that that of wild-type spores. Spores of the *tgl*/*yabG* double mutant were less responsive to L-alanine without heating. After heating the spores at 60°C, L-alanine stimulated germination of the *yabG* spores was accelerated to a similar extent as the wild-type spores (Fig. 9B). In the presence of L-alanine, the germination of the *tgl* and the *tgl*/*yabG* mutant spores was also activated by heating, although the frequency of germination was lower than that of the wild-type or the *yabG* mutant spores (Fig. 9B). Heat activation at 60°C had little effect on the response of the various spores to AGFK (Fig. 9, C and D).

DISCUSSION

Assembly of Coat Modification Enzymes—At least three sporulation-specific enzymes, including OxdD, Tgl, and YabG, are involved in the modification of *B. subtilis* coat proteins; however, these proteins have little or no effect on the morphology of the spores or on their resistance to heat, lysozyme, or organic solvents (*4*, *7*, *8*, *14*). These enzymes are synthesized in the mother cell compartment under the control of SigK (*7*–*9*, and this study). OxdD and YabG are spore coat proteins whose localization is dependent on CotE and SafA (*7*, *8*).

We confirmed that a Tgl-GFP fusion protein also localizes around developing forespores (Fig. 2, A and B). Using immunoblotting with an anti-Tgl antiserum, Zilhao *et al.* found that Tgl is associated with the purified spores of wild-type and *cotE*, *gerE*, and *safA* mutant strains of *B. subtilis* (*9*). Tgl-GFP is abnormally distributed on the forespores of *cotE*, *safA*, and *spoVID* mutants. We conclude that Tgl is a coat protein that requires CotE, SafA, and SpoVID for proper assembly on developing forespores. In the *gerE* mutant, a part of Tgl-GFP assembles around forespores, and the remainder is found in the mother cell compartment. We assume that excessive Tgl is produced in the absence of the negative regulator, GerE, so that it has no place to assemble on the coat layers, or that some assembly factors are not functional under such conditions. Tgl-GFP condenses at the polar cap regions of developing wild-type forespores but not *yabG* mutant forespores. We assume that this difference is not important for the function of Tgl because a portion of Tgl-GFP locates at the polar cap regions of the developing forespores, and because Tgl is active at 60°C in the *yabG* mutant.

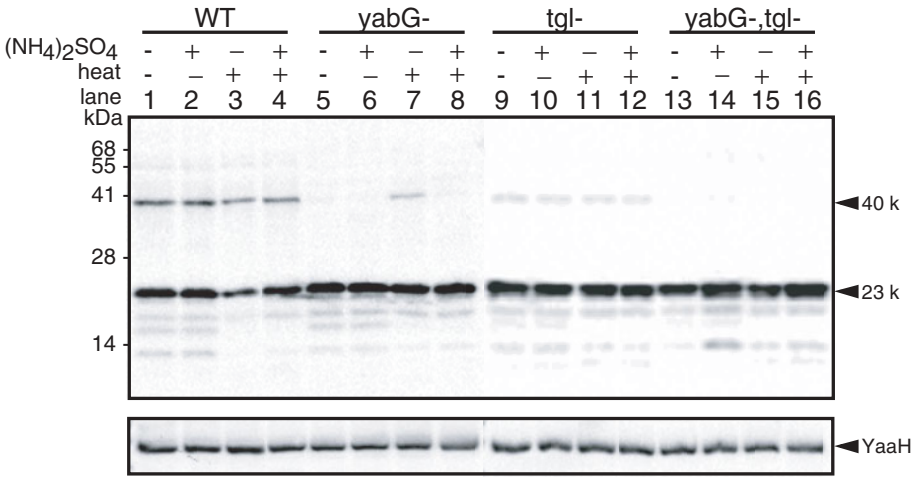

Fig. 8. **Inhibition of GerQ crosslinking by ammonium sulfate.** Cells were cultured for 24 h in DS medium at 37°C. Spores were prepared without complete protease inhibitor cocktail (Roche) in lysozyme buffer. Equilibrated spore samples were incubated for 5 min on ice with 20 mM ammonium sulfate (pH 7.2) (even-numbered lanes) or control buffer (10 mM phosphate buffer, pH 7.6; odd-numbered lanes). The spores were then analyzed directly (lanes 1, 2, 5, 6, 9, 10, 13, and 14) or incubated at 60°C for 20 min (lanes 3, 4, 7, 8, 11, 12, 15, and 16). Protein (10 μg) was solubilized from wild-type spores (lanes 1–4), *yabG* mutant spores (lane 5–8), *tgl* mutant spores (lanes 9–12), or *tgl*/*yabG* mutant spores (lanes 13–16). The samples were resolved by 14% SDS-PAGE, and immunoblotting was performed with anti-GerQ antiserum. Arrowheads show the positions of GerQ (upper panel). YaaH was analyzed as a loading control by using anti-YaaH antiserum (lower panel).

Protein Modification Mediated by YabG and Tgl—YabG is a protease involved in the modification of SpoIVA, SafA, CotF, YxeE, YeeK and CotT (*8*), and Tgl mediates the crosslinking of GerQ and other unidentified proteins (*9*, *14*). Using SDS-PAGE followed by CBB staining, we found nine bands (P55, P45, P29, P23, P21, P20, P18, and P15) that were specific to or increased in the extracts of *yabG* spores. The P55, P45, P29, P21, P20, P18, and P15 bands correspond to SpoIVA, SafA (large form), SafA (small form), CotF, YxeE, YeeK, and CotT, respectively (*8*). The remaining band, P23, may correspond to the monomer form of GerQ because it was not detected in the *gerQ*/*yabG* mutant spores (data not shown). Using LC-MS/MS to analyze the 40, 28, 20, and 16 kDa bands specific to the *tgl* spores, Zilhao *et al.* found that the 40 and 28 kDa bands contained CotE, CotY, and GerQ; the 20 kDa band contained CotE, GerQ, YfkD, YisY, and YckK; and the 16 kDa band contained CotG, CotE, CotY, and YhxC (*9*). We also found that GerQ is a substrate for Tgl but we did not identify the other proteins in this study.

The size of GerQ multimer in the extract of wild-type spores was found to be approximately 40 kDa by immunoblotting. This analysis also clearly showed that the amount of GerQ multimer is decreased in *yabG*, *tgl*, and *tgl*/*yabG* mutant spores; however, the multimer form of GerQ is a minor component of the 40 kDa band and was not identified by CBB staining. The 42 and 30 kDa forms of SafA that accumulated in the *tgl* spores were possibly intermediates because they were not detected in the *yabG* or *tgl*/*yabG* mutant spores (Fig. 4B). The P17 band in the *tgl* spores was not detected in the *tgl*/*yeeK* spores, suggesting that YeeK is also a common substrate for YabG and Tgl (Takamatsu et al., unpublished observations). The protein profile of the *tgl*/*yabG* spores is similar to that of the *yabG* spores except for the 65, 36, and 34 kDa bands. The 65 and 36 kDa bands missing in the *tgl*/*yabG* spores probably correspond to CotB and CotG, which are remarkable proteins that are dependent on CotH (*41*, *42*). These results indicate that the protein modification system comprising of YabG and Tgl may be related to the interaction among the CotB, CotG, and CotH proteins. Finally, we could not identify any of the proteins in the 34 kDa band found in the spore extracts.

Crosslinking of GerQ at 37°C Is Dependent on YabG—High-molecular mass forms of GerQ were not present in the extract from the *yabG* or *tgl*/*yabG* mutant spores under our experimental conditions, indicating that YabG is also involved in the crosslinking of GerQ. YabG is a sporulation-specific protease involved in the processing and degradation of some coat proteins (*8*). There are three possible explanations for why GerQ crosslinking at 37°C is dependent on YabG. First, in addition to Tgl, YabG may mediate the crosslinking of GerQ. So YabG is conserved in *Bacilli* and *Clostridia*, but it is not related to other transglutaminases, including *B. subtilis* Tgl. A second possibility is that YabG is required for the activity of Tgl. The transglutaminase from *Streptomyces mobaraensis* is secreted as an inactive precursor protein that is processed and activated by an endoprotease (*43*, *44*). Similarly, in *Streptoverticilliu mobaraense*, the transglutaminase is produced as an inactive proenzyme and is C-terminally cleaved to the mature, active form (*45*). Despite this, previous immunoblotting experiments with anti-Tgl antiserum have not detected processed products of *B. subtilis* Tgl (*9*). A third possibility for the YabG-dependent crosslinking of GerQ at 37°C is that YabG modifies GerQ so that it can be crosslinked by Tgl and/or other enzymes.

Here, we suggest a model for the crosslinking of GerQ and other coat proteins (Fig. 10A). Our results indicate that Tgl, YabG, and other enzymes are involved in a dual modification system of the spore coat proteins. The coat proteins are synthesized as precursor forms in the mother cell compartment of developing forespores. First, YabG changes the precursors into intermediates. These

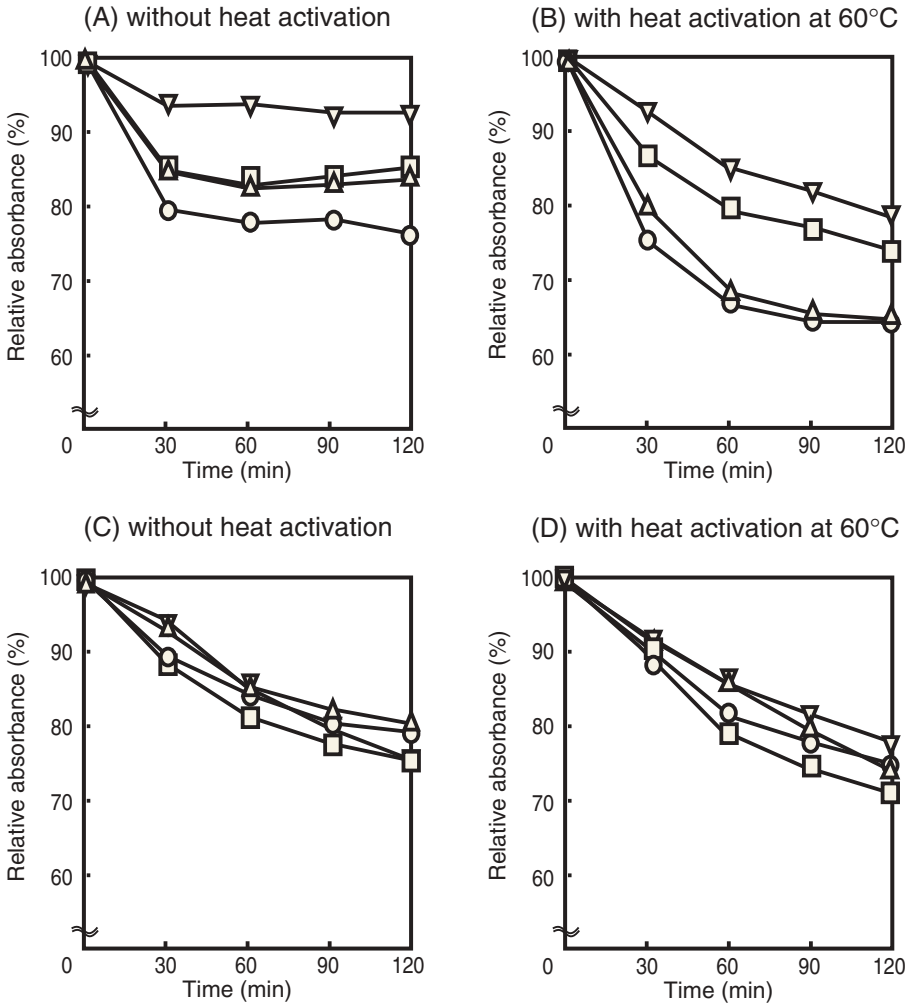

Fig. 9. **Germination assay.** Spores of the wild-type (open circles), *yabG* mutant (open upward triangles), *tgl* mutant (open squares), and *tgl*/*yabG* mutant (open downward triangles) were analyzed directly (A, C) or were heat-activated for 20 min at 60°C (B, D). After heat activation, the cells were incubated at 37 °C with L-alanine (10 mM) or AGFK (10 mM L-asparagine, 10 mM D-glucose, 10 mM D-fructose, and 10 mM potassium chloride). The germination of each spore type was monitored by measuring the optical density at Abs 600 nm at the indicated times after the addition of L-alanine (A, B) or AGFK (C, D). The relative absorbance corresponds to the efficiency of germination.

intermediates are then capable of being crosslinked by Tgl and/or other enzymes. Low levels of the high-molecular mass forms of GerQ were detected in the *tgl* spores (Fig. 4) (*14*). Other enzyme(s) besides Tgl are probably present and mediate the crosslinking of the monomer form of GerQ. We cannot determine which proteins are candidates for the Tgl-independent crosslinking of GerQ at 37°C, but there is some evidence that YabG functions prior to Tgl at this temperature. The protein profile of the *tgl*/*yabG* mutant spores is similar to that of the *yabG* mutant spores but not the *tgl* mutant spores. For example, the protein extract from the *tgl* spores included the processed forms of SafA, but those of the *yabG* and the *tgl*/*yabG* mutant spores included intact SafA. We speculate that SafA is synthesized as large (45 kDa) and small (31 kDa) forms, processed by YabG to 42 and 30 kDa intermediate forms, and finally incorporated into some insoluble materials by Tgl-dependent crosslinking. On the other hand, we could not detect any intermediate forms of GerQ in the *tgl* spores by SDS-PAGE or immunoblotting. Some amino acid residues in the coat proteins are cleaved by proteases including YabG, and this cleavage causes a change in their structure. As a result, Tgl recognizes the functional group of the intermediates. YabG likely cleaves amino acid residues near the N- or C-termini of GerQ and/or some other protein that forms a complex with GerQ. It is also possible that YabG modifies some amino acid residues of GerQ without requiring its cleavage. Finally, very low levels of SpoIVA, CotF, and CotT were found in the samples of the wild-type and the *tgl* mutant spores. These proteins may be crosslinked by enzymes other than Tgl so that they are incorporated into insoluble materials, or they may completely digested by YabG at 37°C.

Crosslinking of GerQ at 60°C Is Independent of YabG— Our results and previous reports indicate that Tgl and GerQ are present in the spore coat of *B. subtilis*, which is a hard proteinaceous shell that protects the spores from lytic enzymes and physical disruption (*9*, *14*). It is conceivable that small molecules such as amino acids but not lytic enzymes can pass through the spore coat (*2*). Thus, Tgl cannot come into contact with GerQ if they are assembled separately and tightly attached to the coat. Tgl mediates the crosslinking of GerQ after lysis of the mother cell, and the majority of the high-molecular-mass forms of GerQ were previously reported to appear 48 h after the initiation of sporulation (*14*). This indicates that Tgl mediates the crosslinking of GerQ after its assembly into the spore coat. We, therefore, speculate that Tgl is not tightly fixed, but rather can move within the proteinaceous shell.

Our results indicate that the amounts of both the monomer and multimer forms of GerQ decrease in the sample of

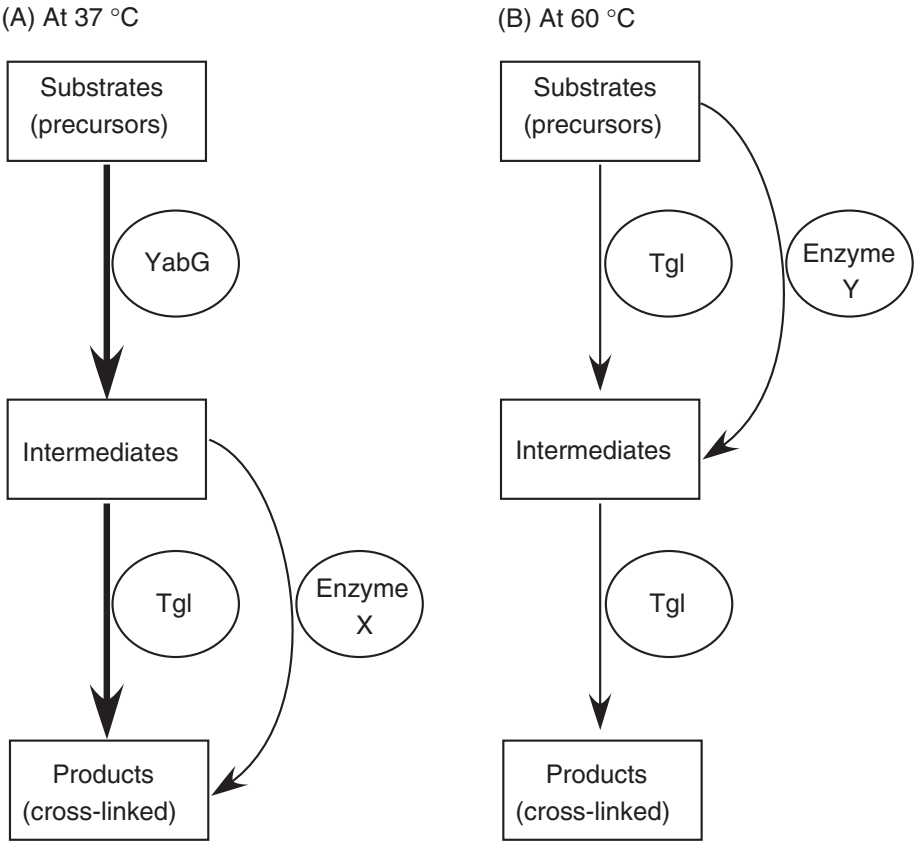

Fig. 10. **Schema for protein modification by YabG and Tgl.** Shown is a possible model for the crosslinking of coat proteins. The coat proteins are synthesized in precursor forms in the mother cell compartment during sporulation. Some coat proteins are processed or cleaved into intermediates by a protease such as YabG, after which they are further modified by other enzymes. (A) At 37°C, precursor proteins (substrates) are first processed into intermediates by YabG. The proteins are then probably crosslinked by Tgl and/or Enzyme X. (B) At 60°C, precursor proteins (substrates) are first modified into intermediates by Enzyme Y but are not modified by YabG. The proteins are then probably crosslinked by Tgl. Because this temperature is optimal for the enzymatic activity of Tgl, the two-step modification of precursors into crosslinked products is presumably mediated by Tgl alone. Bold lines show major pathways, and thin lines show minor or other pathways.

the wild-type spores after long-term incubation at 60°C. GerQ was probably crosslinked and became insoluble under our experimental conditions. On the other hand, in the *yabG* mutant spores, the the amount of the monomer form of GerQ did not decrease, and the high-molecular-mass form of GerQ was produced after incubation at 60°C. This reaction is probably dependent on Tgl because no GerQ multimer was visible in the extract of the *tgl*/*yabG* spores. Moreover, the crosslinking of GerQ in the *yabG* spores at 60°C was reduced in the presence of ammonium sulfate, an inhibitor of Tgl. Small amounts of the high-molecular mass forms of GerQ were detected in the *tgl* mutant spores with or without heat treatment at 60°C. They were also probably produced at 37°C in the absence of Tgl. Our results further show that the enzymatic activity of Tgl is retained in the purified spores.

We suggest the following explanations for the observations at 60°C (Fig. 10B). (i) The structure of GerQ is changed by heat treatment at 60°C in the absence of YabG, and the crosslinking of GerQ is mediated by Tgl. Some thermophilic and/or thermostable protease(s) likely modify the monomer form of GerQ (precursor form) into an intermediate form capable of being crosslinked by Tgl. Indeed, several proteases, including AprX, Epr, FtsH, and Mpr, have been detected in the purified spores by LC-MS/MS (*21*). Of these, Mpr is a moderately thermophilic protease with an optimal temperature at 50°C, and it retains 30% of its enzymatic activity even at 85°C (*46*). (ii) Tgl is independently capable of crosslinking GerQ at 60°C, a temperature at which Tgl is most active. Tgl may directly crosslink the monomer form of GerQ without requiring the formation of intermediates. Alternatively, Tgl mediates both the processing and crosslinking of GerQ; however, a BLAST sear*ch showed t*hat Tgl of *B. subtilis* is not similar to any known proteases.

Multiple Functions of Transglutaminase—A search of the complete genome sequences of *Bacilli* and closely related species, including *B. anthracis*, *B. cereus*, *B. clausii*, *B. halodurans*, *B. licheniformis*, *B. thuringensis*, and *Geobacillus kaustophilus* (DNA Data Bank of Japan), identified only one protein similar to *B. subtilis* Tgl in each microorganism. In addition, a BLAST search showed that *B. subtilis* Tgl does not show significant similarity to other transglutaminases. Transglutaminases are multifunctional enzymes that are widely distributed in eukaryotes and prokaryotes (*47*). Human transglutaminase factor XIII has a cysteine protease-like active site that mediates a crosslinking reaction (*48*). Also, the protein disulfide isomerase PDI-3 from *Caenorhabditis elegans* has transglutaminase activity that is involved in the control of body shape (*49*). Conversely, a tissue-type transglutaminase has been shown to have protein disulfide isomerase activity (*50*). We assume that other unknown enzyme(s) also have activities similar to these transglutaminases in *B. subtilis*. Indeed, YebA of *B. subtilis* belongs to a transglutaminase-like super family that has conserved cysteine protease domains found in several archaeal, bacterial, and eukaryotic genomes, although its function has not yet been determined (*47*, BLAST search).

Spore Germination with Heat Activation—In *B. subtilis*, *B. megaterium*, *B. cereus*, and *Clostridium perfringenes*, spore germination is activated by heat treatment

(*51–54*), but the detailed mechanisms for this phenomenon have not yet been identified. The germination frequency of the *yabG* mutant and wild-type spores in the presence of L-alanine was enhanced to the same extent by heat activation at 60°C (Fig. 9). We further showed that GerQ is crosslinked by Tgl in wild-type and *yabG* mutant spores following heat activation at 60°C. On the other hand, almost no GerQ multimers were produced in the *tgl* and the *tgl*/*yabG* mutant spores upon heating at 60°C. The germination of the *tgl* spores and the *tgl*/*yabG* mutant spores in the presence of L-alanine was also stimulated by heating, but was slower than the germination of wild-type and *yabG* mutant spores.

GerQ is essential for germination in the presence of Ca-DPA chelate (*13*). We attempted to examine the effect of heat treatment on the germination of *yabG* spores in the presence of Ca-DPA, but did not obtain a sufficient level of germination (data not shown). GerQ is also essential for the assembly of CwlJ in the spore coat (*13*), but mutation of *cwlJ* has no effect on spore germination (*15*). We, therefore, conclude that YabG and Tgl influence the germination properties of the spores by altering the crosslinking of GerQ and other proteins. Electron microscopy has revealed that *tgl* mutants have altered coat structures (*9*). We, therefore, suspect that the crosslinking of GerQ and/or other coat proteins participates in the breakdown of the spore structure during germination.

Spores of *B. cereus* are activated by heat treatment at 65°C for 45 min or 34°C for 48 h (*52*). We assume that the mechanism of spore germination in *B. subtilis* is very similar to that in *B. cereus* because the germination genes are highly conserved (*55–57*). A BLAST search revealed that YabG and Tgl are also conserved in *Bacilli*. Thus, the protein modifications mediated by YabG and Tgl may be involved in the germination of spores of other *Bacillus* species. We suggest that there is a comprehensive interaction among YabG, Tgl, and their substrates in the mature spores.

This work was supported by Grants-in-Aid for Young Scientists (B) (KAKENHI 15710151) and the Sumitomo Foundation (No. 050407).

REFERENCES

1. Setlow, P. (1993) Spore structure proteins in *Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics* (Sonenshein, A.L., Hoch, J.A., and Losick, R., eds.) pp. 801–809, American Society for Microbiology, Washington, DC
2. Driks, A. (1999) *Bacillus subtilis* spore coat. *Microbiol Mol Biol Rev.* **63**, 1–20
3. Errington, J. (1993) *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis [Review]. *Microbiol. Rev.* **57**, 1–33
4. Henriques, A.O., Costa, T.V., Martins, L.O., and Zilhao, R. (2004) The functional architecture and assembly of the spore coat in *Bacterial Spore Formers, Probiotics and Emerging Appoications* (Ricca, E., Henriques, A.O., and Cutting, S.M., eds.) pp. 65–85, Horizon Bioscience, Norfolk, UK
5. Henriques, A.O. and Moran, C.P. Jr. (2000) Structure and assembly of the bacterial endospore coat [Review]. *Methods* **20**, 95–110
6. Henriques, A.O., Melsen, L.R., and Moran, C.P. Jr. (1998) Involvement of superoxide dismutase in spore coat assembly in *Bacillus subtilis*. *J. Bacteriol.* **180**, 2285–2291
7. Costa, T., Steil, L., Martins, L.O., Volker, U., and Henriques, A.O. (2004) Assembly of an oxalate decarboxylase produced under sigmaK control into the *Bacillus subtilis* spore coat. *J. Bacteriol.* **186**, 1462–1474
8. Takamatsu, H., Kodama, T., Imamura, A., Asai, K., Kobayashi, K., Nakayama, T., Ogasawara, N., and Watabe, K. (2000) The *Bacillus subtilis yabG* gene is transcribed by SigK RNA polymerase during sporulation, and *yabG* mutant spores have altered coat protein composition. *J. Bacteriol.* **182**, 1883–1888
9. Zilhao, R., Isticato, R., Martins, L.O., Steil, L., Volker, U., Ricca, E., Moran, C.P. Jr., and Henriques, A.O. (2005) Assembly and function of a spore coat-associated transglutaminase of *Bacillus subtilis*. *J. Bacteriol.* **187**, 7753–7764
10. Takamatsu, H., Imamura, A., Kodama, T., Asai, K., Ogasawara, N., and Watabe, K. (2000) The *yabG* gene of *Bacillus subtilis* encodes a sporulation specific protease which is involved in the processing of several spore coat proteins. *FEMS Microbiol. Lett.* **192**, 33–38
11. Kobayashi, K., Hashiguchi, K., Yokozeki, K., and Yamanaka, S. (1998) Molecular cloning of the transglutaminase gene from *Bacillus subtilis* and its expression in *Escherichia coli*. *Biosci. Biotechnol. Biochem.* **62**, 1109–1114
12. Suzuki, S., Izawa, Y., Kobayashi, K., Eto, Y., Yamanaka, S., Kubota, K., and Yokozeki, K. (2000) Purification and characterization of novel transglutaminase from *Bacillus subtilis* spores. *Biosci. Biotechnol. Biochem.* **64**, 2344–2351
13. Ragkousi, K., Eichenberger, P., van Ooij, C., and Setlow, P. (2003) Identification of a new gene essential for germination of *Bacillus subtilis* spores with Ca^{2+}-dipicolinate. *J. Bacteriol.* **185**, 2315–2329
14. Ragkousi, K. and Setlow, P. (2004) Transglutaminase-mediated cross-linking of GerQ in the coats of *Bacillus subtilis* spores. *J. Bacteriol.* **186**, 5567–5575
15. Ishikawa, S., Yamane, K., and Sekiguchi, J. (1998) Regulation and characterization of a newly deduced cell wall hydrolase gene (*cwlJ*) which affects germination of *Bacillus subtilis* spores. *J. Bacteriol.* **180**, 1375–1380
16. Setlow, P. and Johnson, E.A. (1997) Spores and their significance in *Food Microbiology: Fundamentals and Frontiers* (Doyle, M.P., Beuchat, L.R., and Montville, T.J., eds.) pp. 30–65, American Society for Microbiology, Washington, DC
17. Nicholson, W.L. and Setlow, P. (1990) Sporulation, germination, and outgrowth in *Molecular Biological Methods for Bacillus* (Harwood, C.R. and Cutting, S.M., eds.) pp. 391–450, John Wiley and Sons Ltd, West Sussex, England
18. Lee, W.H. and Ordal, Z.J. (1963) Reversible activation for germination and subsequent changes in bacterial spores. *J. Bacteriol.* **85**, 207–217
19. Errington, J. (1990) Gene Cloning Techniques in *Molecular Biological Methods for Bacillus* (Harwood, C.R. and Cutting, S.M., eds.) pp. 175–220, John Wiley and Sons Ltd, West Sussex, England
20. Vagner, V., Dervyn, E., and Ehrlich, S.D. (1998) A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* **144**, 3097–3104
21. Kuwana, R., Kasahara, Y., Fujibayashi, M., Takamatsu, H., Ogasawara, N., and Watabe, K. (2002) Proteomics characterization of novel spore proteins of *Bacillus subtilis*. *Microbiology* **148**, 3971–3982
22. Cormack, B.P., Valdivia, R.H., and Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**, 33–38
23. Nakane, A., Takamatsu, H., Oguro, A., Sadaie,Y., Nakamura, K., and Yamane, K. (1995) Acquisition of azide-resistance by elevated SecA ATPase activity confers

azide-resistance upon cell growth and protein translocation in *Bacillus subtilis*. *Microbiology* **141**, 113–121
24. Schaefer, P., Millet, J., and Aubert, J.P. (1965) Catabolic repression of bacterial sporulation. *Proc. Natl. Acad. Sci. USA* **54**, 704–711
25. Takamatsu, H., Kodama, T., Nakayama,T., and Watabe, K. (1999) Characterization of the *yrbA* gene of *Bacillus subtilis*, involved in resistance and germination of spores. *J. Bacteriol.* **181**, 4986–4994
26. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory manual*, 2nd eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Cutting, S.M. and Vander Horn, P.B. (1990) Genetic analysis in *Molecular Biological Methods for Bacillus* (Harwood, C.R. and Cutting, S.M., eds.) pp. 22–74, John Wiley and Sons Ltd, West Sussex, England
28. Igo, M.M. and Losick, R. (1986) Regulation of a promoter that is utilized by minor forms of RNA polymerase holoenzyme in *Bacillus subtilis*. *J. Mol. Biol.* **191**, 615–624
29. Kuwana, R., Yamamura, S., Ikejiri, H., Kobayashi, K., Ogasawara, N., Asai, K., Sadaie, Y., Takamatsu, H., and Watabe, K. (2003) *Bacillus subtilis spoVIF (yjcC)* gene, involved in coat assembly and spore resistance. *Microbiology* **149**, 3011–3021
30. Kuwana, R., Okumura, T., Takamatsu, H., and Watabe, K. (2005) The *ylbO* gene product of *Bacillus subtilis* is involved in the coat development and lysozyme resistance of spore. *FEMS Microbiol Lett.* **242**, 51–57
31. Takamatsu, H., Chikahiro, Y., Kodama, T., Koide, H., Kozuka, S., Tochikubo, K., and Watabe, K. (1998) A spore coat protein, CotS, of *Bacillus subtilis* is synthesized under the regulation of sigmaK and GerE during development and is located in the inner coat layer of spores. *J. Bacteriol.* **180**, 2968–2974
32. Kuwana, R., Ikejiri, H., Yamamura, S., Takamatsu, H., and Watabe, K. (2004) Functional relationship between SpoVIF and GerE in gene regulation during sporulation of *Bacillus subtilis*. *Microbiology* **150**, 163–170
33. Kodama, T., Takamatsu, H., Asai, K., Kobayashi, K., Ogasawara, N., and Watabe, K. (1999) The *Bacillus subtilis yaaH* gene is transcribed by SigE RNA polymerase during sporulation, and its product is involved in germination of spores. *J. Bacteriol.* **181**, 4584–4591
34. Zheng, L.B., Donovan, W.P., Fitz-James, P.C., and Losick, R. (1988) Gene encoding a morphogenic protein required in the assembly of the outer coat of the *Bacillus subtilis* endospore. *Genes Dev.* **2**, 1047–1054
35. Beall, B., Driks, A., Losick, R., and Moran, C.P. Jr. (1993) Cloning and characterization of a gene required for assembly of the *Bacillus subtilis* spore coat. *J. Bacteriol.* **175**, 1705–1716
36. Donovan, W., Zheng, L.B., Sandman, K., and Losick, R. (1987) Genes encoding spore coat polypeptides from *Bacillus subtilis*. *J. Mol. Biol.* **196**, 1–10
37. Sacco, M., Ricca, E., Losick, R., and Cutting, S. (1995) An additional GerE-controlled gene encoding an abundant spore coat protein from *Bacillus subtilis*. *J. Bacteriol.* **177**, 372–377
38. Ozin, A.J., Costa, T., Henriques, A.O., and Moran, C.P. Jr. (2001) Alternative translation initiation produces a short form of a spore coat protein in *Bacillus subtilis*. *J. Bacteriol.* **183**, 2032–2040
39. Bourne, N., Fitz-James, P.C., and Aronson, A.I. (1991) Structural and germination defects of *Bacillus subtilis* spores with altered contents of a spore coat protein. *J. Bacteriol.* **173**, 6618–6625
40. Cutting, S., Zheng, L.B., and Losick, R. (1991) Gene encoding two alkali-soluble components of the spore coat from *Bacillus subtilis*. *J. Bacteriol.* **173**, 2915–2919
41. Naclerio, G., Baccigalupi, L., Zilhao, R., De Felice, M., and Ricca, E. (1996) *Bacillus subtilis* spore coat assembly requires *cotH* gene expression. *J. Bacteriol.* **178**, 4375–4380
42. Zilhao, R., Serrano, M., Isticato, R., Ricca, E., Moran, C.P. Jr., and Henriques, A.O. (2004) Interactions among CotB, CotG, and CotH during assembly of the *Bacillus subtilis* spore coat. *J. Bacteriol.* **186**, 1110–1119
43. Zotzel, J., Keller, P., and Fuchsbauer, H.L. (2003) Transglutaminase from *Streptomyces mobaraensis* is activated by an endogenous metalloprotease. *Eur. J. Biochem.* **270**, 3214–3222
44. Zotzel, J., Pasternack, R., Pelzer, C., Ziegert, D., Mainusch, M., and Fuchsbauer, H.L. (2003) Activated transglutaminase from *Streptomyces mobaraensis* is processed by a tripeptidyl aminopeptidase in the final step. *Eur. J. Biochem.* **270**, 4149–4155
45. Pasternack, R., Dorsch, S., Otterbach, J.T., Robenek, I.R., Wolf, S., and Fuchsbauer, H.L. (1998) Bacterial pro-transglutaminase from *Streptoverticillium mobaraense*-purification, characterisation and sequence of the zymogen. *Eur. J. Biochem.* **257**, 570–576
46. Rufo, G.A. Jr., Sullivan, B.J., Sloma, A., and Pero, J. (1990) Isolation and characterization of a novel extracellular metalloprotease from *Bacillus subtilis*. *J. Bacteriol.* **172**, 1019–1023
47. Makarova, K.S., Aravind, L., and Koonin, E.V. (1999) A superfamily of archaeal, bacterial, and eukaryotic proteins homologous to animal transglutaminases. *Protein Sci.* **8**, 1714–1719
48. Pedersen, L.C., Yee, V.C., Bishop, P.D., Le Trong, I., Teller, D.C., and Stenkamp, R.E. (1994) Transglutaminase factor XIII uses proteinase-like catalytic triad to crosslink macromolecules. *Protein Sci.* **3**, 1131–1135
49. Eschenlauer, S.C. and Page, A.P. (2003) The *Caenorhabditis elegans* ERp60 homolog protein disulfide isomerase-3 has disulfide isomerase and transglutaminase-like cross-linking activity and is involved in the maintenance of body morphology. *J. Biol. Chem.* **278**, 4227–4237
50. Hasegawa, G., Suwa, M., Ichikawa, Y., Ohtsuka, T., Kumagai, S., Kikuchi, M., Sato, Y., and Saito, Y. (2003) A novel function of tissue-type transglutaminase: protein disulphide isomerase. *Biochem. J.* **373**, 793–803
51. Adams, D.M. and Busta, F.F. (1972) Ultrahigh-temperature activation of a low-temperature *Bacillus subtilis* spore germination system. *Appl. Microbiol.* **24**, 418–423
52. Keynan, A., Evanchik, Z., Halvorson, H.O., and Hastings, J.W. (1964) Activation of Bacterial endospores. *J. Bacteriol.* **88**, 313–318
53. Levinson, H.S. and Hyatt, M.T. (1970) Effects of temperature on activation, germination, and outgrowth of *Bacillus megaterium* spores. *J. Bacteriol.* **101**, 58–64
54. Craven, S.E. and Blankenship, L.C. (1987) Changes in the hydrophobic characteristics of *Clostridium perfringens* spores and spore coats by heat. *Can. J. Microbiol.* **33**, 773–776
55. Clements, M.O. and Moir, A. (1998) Role of the *gerI* operon of *Bacillus cereus* 569 in the response of spores to germinants. *J. Bacteriol.* **180**, 6729–6735
56. Barlass PJ, Houston CW, Clements MO, Moir A. (2002) Germination of *Bacillus cereus* spores in response to L-alanine and to inosine: the roles of *gerL* and *gerQ* operons. *Microbiology* **148**, 2089–2095
57. Hornstra, L.M., de Vries, Y.P., de Vos, W.M., Abee, T., and Wells-Bennik, M.H. (2005) *gerR*, a novel *ger* operon involved in L-alanine- and inosine-initiated germination of *Bacillus cereus ATCC 14579*. *Appl. Environ. Microbiol.* **71**, 774–781