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Organization and Evolution of the *cotG* and *cotH* Genes of *Bacillus subtilis*▽†

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The *cotG* and *cotH* genes of *Bacillus subtilis* encode two previously characterized spore coat proteins. The two genes are adjacent on the chromosome and divergently transcribed by σ^K, a sporulation-specific σ factor of the RNA polymerase. We report evidence that the *cotH* promoter maps 812 bp upstream of the beginning of its coding region and that the divergent *cotG* gene is entirely contained between the promoter and the coding part of *cotH*. A bioinformatic analysis of all entirely sequenced prokaryotic genomes showed that such chromosomal organization is not common in spore-forming bacilli. Indeed, CotG is present only in *B. subtilis*, *B. amyloliquefaciens*, and *B. atrophaeus* and in two *Geobacillus* strains. When present, *cotG* always encodes a modular protein composed of tandem repeats and is always close to but divergently transcribed with respect to *cotH*. Bioinformatic and phylogenic data suggest that such genomic organizations have a common evolutionary origin and that the modular structure of the extant *cotG* genes is the outcome of multiple rounds of gene elongation events of an ancestral minigene.

Endospore-forming bacteria are Gram-positive microorganisms belonging to different genera and including more than 200 species (13). The common feature of these organisms is the ability to form a quiescent cellular type called an endospore (spore) in response to harsh environments. The spore can survive in this dormant state for long periods, resisting a vast range of stresses, such as high temperature, dehydration, absence of nutrients, and presence of toxic chemicals. When the environmental conditions ameliorate, the spore germinates, originating a vegetative cell able to grow and to sporulate again. Spore resistance is made possible by the presence of the spore coat, a multilayered structure composed by more than 70 proteins synthesized in the mother cell compartment of the sporangium and assembled around the forming spore (16). Coat formation is finely controlled through various processes acting at the transcriptional or posttranslational level. The synthesis of coat proteins is regulated by a cascade of at least five transcription factors: σ^E and σ^K (two mother cell-specific σ factors of the RNA polymerase), SpoIIID and GerE (two transcriptional regulators acting in conjunction with σ^E and σ^K, respectively) (18), and GerR (initially found to control at least 14 σ^E genes [6] and more recently identified as affecting directly or indirectly also some σ^K-dependent genes [3]). The assembly of coat components on the surface of the forming spore is governed by a subset of morphogenetic proteins that guide the correct packaging process (16). The main morphogenetic factors are SpoIVA, CotE, and SafA (25). SpoIVA (5, 33) is assembled into the basement layer of the coat and is anchored to the outer membrane of the forespore through its C terminus that contacts SpoVM, a small, amphipathic peptide embedded in the forespore membrane (24, 30, 31). SpoIVA controls the assembly of most coat components either directly or through SafA and CotE, proposed as key regulators of the inner coat and the outer coat, respectively (25). CotE self-interacts (23) and assembles into a ring that surrounds the SpoIVA basement structure (40). The inner layer of the coat is then formed between the SpoIVA layer and the CotE ring, while the outer coat is formed outside the CotE ring (25, 40). SafA and CotE have been proposed to interact with most coat components based on the results of a fluorescence microscopy analysis of a collection of strains carrying *cot-gfp* fusions (21, 25). Biochemical experiments have confirmed the direct interaction of CotE with two outer coat components and have revealed the essential role of CotE in mediating their interaction (20).

Other morphogenetic proteins include CotH and CotG (16). CotH plays a role in the assembly of at least 9 other coat components, including CotG (21), and in the development of lysozyme resistance of the mature spore (28, 41). CotG is needed for the conversion of CotB from an immature 44-kDa form into a mature 66-kDa form (42). The structural genes coding for CotH and CotG are clustered together on the *Bacillus subtilis* chromosome but are divergently transcribed (28). While CotH is a 42.8-kDa protein found in several *Bacillus* species and also in some *Clostridium* species (16), CotG is a 24-kDa protein containing nine tandem repeats of a 13-amino-acid stretch in its central part (34) and so far has been found only in *B. subtilis* (16).

Here we report that *cotH* expression depends on a newly identified promoter located 812 nucleotides upstream of the coding region. The long sequence at 5′ end of *cotH* is most likely not translated and completely overlaps the divergent *cotG* gene (see Fig. 1A). The apparent lack of function of the long 5′ untranslated region along with the evidence that the

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cotG-*cotH* genome organization is not conserved in *Bacillus* species prompted us to investigate the evolutionary origin of the *cotG* gene and of the *cotH*-*cotG* gene organization.

MATERIALS AND METHODS

Bacterial strains and transformation. *B. subtilis* PY79 (39) was used as recipient strain in transformation procedures. Plasmid amplification for DNA sequencing, subcloning experiments, and transformation of *Escherichia coli* competent cells were performed with *E. coli* strain DH5α (36). Bacterial strains were transformed by previously described procedures, i.e., $CaCl_2$-mediated transformation of *E. coli* competent cells (36) and two-step transformation of *B. subtilis* (4).

Genetic and molecular procedures. Isolation of plasmids, restriction digestion, and ligation of DNA, were carried out by standard methods (36). Chromosomal DNA from *B. subtilis* was isolated as described elsewhere (4).

Deletion analysis, transcriptional gene fusions, and β-galactosidase assays. Deletion mutants of DNA upstream of the *cotH* coding part were constructed starting from *B. subtilis* strain GC237, containing a *cotH*::*lacZ* translational fusion inserted at the *cotH* locus (2). Deletion mutants were obtained by PCR using chromosomal DNA of strain GC237 as a template. PCRs were carried out with oligonucleotide lacZ2 (5′-<u>GAATTC</u>TATTTTTGACACCAGACC-3′ [underlined is the EcoRI site]), annealing at the 3′ end of the *lacZ* gene, and one of the following oligonucleotides: Del4, Del1, Del2, Del5, and Del3 (see Table S1 in the supplemental material). Amplified fragments were subcloned in pGEM-T Easy vector, sequenced to ensure there were no mutations (BMR Genomics), digested with BamHI/EcoRI, and cloned in the integrative vector pDG364 (4), previously restricted with the same enzymes. Gene fusions were then inserted at the *amyE* locus of a wild-type (PY79) strain of *B. subtilis* by a double reciprocal crossing-over event.

The integrative vector pSN32 (26) was used to obtain a transcriptional fusion of the *cotH* promoter to the *lacZ* gene of *E. coli*. A genomic fragment containing the *cotH* promoter was PCR amplified by using chromosomal DNA of strain PY79 as a template and oligonucleotides Del3 and G27 as primers (see Table S1 in the supplemental material). Purified fragments were cloned into pGEM-T Easy vector (Promega), excised by EcoRI/BamHI digestion and cloned upstream of the *lacZ* gene into the pSN32 vector (26) restricted with the same enzymes. The resulting plasmid was linearized and used to transform competent cells of *B. subtilis* strain PY79. The obtained strain, AZ530, contained the *cotH*::*lacZ* transcriptional fusion at the *amyE* locus of the chromosome. The fusion was then moved by chromosome-mediated transformation into an isogenic strain carrying null mutations in either *spoIVB* (*spoIVB*::*erm*) or *gerE* (*gerE36*). Specific β-galactosidase activity was determined using *o*-nitrophenol-β-D-galactoside (ONPG) as the substrate (4). Samples of cells (1 ml each) bearing the fusion were collected, during sporulation, at the indicated times and assayed as previously described (4).

Construction of a *cotH* internal deletion mutant. DNA coding for the 5′ part of *cotH* mRNA extending from nucleotide −843 to nucleotide −34 (with respect to the first base of *cotH* coding sequence) was deleted using the gene splicing by overlap extension (SOEing) technique (17). Briefly, two PCR products were obtained with oligonucleotide pairs Del3/H33s (to amplify the *cotH* promoter region) and H32s/H13 (extending 34 bases upstream of the ATG to the unique EcoRI site internal to the *cotH* coding sequence) (see Table S1 in the supplemental material). The obtained products were used as templates to prime a third PCR with the external primers Del3 and H13 (Table S1). The modified version of *cotH* was cloned into the vector pER19 (32), and the correct gene fusion was verified by sequencing reactions. The resulting plasmid, pRG25, was introduced by single reciprocal (Campbell-like) recombination at the *cotH* locus of the *B. subtilis* chromosome. Several chloramphenicol-resistant clones were analyzed by PCR to select the clone containing the modified *cotH* promoter sequence upstream the entire *cotH* gene.

Primer extension analysis. Total RNA was extracted from the wild-type strain PY79 and the isogenic mutant strains carrying null mutations in *spoIVB* (*spoIVB*::*erm*) or *gerE* (*gerE36*), 5 h after the onset of sporulation using the Qiagen minikit (Qiagen, Milan, Italy) according to the manufacturer's instructions. Total RNAs were dissolved in 50 μl of RNase-free water and stored at −80°C. The final concentration and quality of the RNA samples were estimated spectrophotometrically and by agarose gel electrophoresis with ethidium bromide staining. Total RNAs were treated with RNase-free DNase (1 U/μg of total RNA; Fermentas) for 30 min at 37°C, and the reaction was stopped with DNase inactivation reagent. For primer extension experiments, 10 μg of total RNA was used with [γ-^{32}P]dATP (GE Healthcare)-labeled oligonucleotide G25 (see Table S1 in the supplemental material), deoxynucleoside triphosphates (dNTPs), and reverse transcriptase (Stratagene) to prime cDNA synthesis as previously described (28) The reaction products were fractionated on 6 M urea–6% polyacrylamide gels, along with DNA sequencing reactions using pNC12 (carrying the *cotH*-*cotG* chromosomal fragment) as a template primed with the same oligonucleotide.

For reverse transcription-PCR (RT-PCR) analysis a sample containing 2 μg of DNase-treated RNA was incubated with oligonucleotide H at 65°C for 5 min and slowly cooled to room temperature to allow the primer annealing. The mixture was incubated at 50°C for 1 h in the presence of 1 μl AffinityScript multiple-temperature reverse transcriptase (Stratagene), 4 mM dNTPs, reaction buffer (Stratagene), and 10 mM dithiothreitol (DTT). The enzyme was inactivated at 70°C for 15 min. One-tenth of the reaction mix was used as a template in PCRs using oligonucleotide H coupled with oligonucleotides Del4, G22, G24, and Del5 (see Table S1 in the supplemental material). For a control, PCRs were carried out with RNA alone to exclude the possibility that the amplification products could derive from contaminating genomic DNA.

Spore purification, extraction of spore coat proteins, and Western blot analysis. Sporulation was induced in Difco sporulation medium (DSM; Difco) by the exhaustion method as described elsewhere (4). After a 30-h incubation at 37°C, the spores were collected, washed four times, and purified as described by Nicholson and Setlow (29) by using overnight incubation in H_2O at 4°C to lyse residual sporangial cells. Spore coat proteins from *B. subtilis* PY79 and of the Δ*cotH* mutant (AZ535) were extracted either from a suspension of purified spores by using an SDS-DTT extraction buffer as previously described (29) or from sporulating cells harvested at various times after the onset of sporulation. In the latter case, sporulating cells were washed three times, suspended in 100 μl of lysozyme solution (25 mM Tris-HCl [pH 7.5], 50 mM glucose, 10 mM EDTA, 1% lysozyme) and incubated for 5 min on ice. The suspension was then boiled in 2% (vol/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, 62.5 mM Tris-HCl (pH 6.8), and 0.05% (wt/vol) bromophenol blue for 5 min. The concentration of extracted proteins was determined by using Bio-Rad DC protein assay kit (Bio-Rad), and 20-μg samples of total spore coat proteins were fractionated on 12.5% SDS-polyacrylamide gels and electrotransferred to nitrocellulose filters (Bio-Rad) for Western blot analysis following standard procedures. CotH-specific antibody was used at a working dilution of 1:5,000, and a horseradish peroxidase (HRP)-conjugated anti-rabbit antibody was utilized as secondary antibody (Santa Cruz). Western blot filters were visualized by the SuperSignal West Pico chemiluminescence (Pierce) method as specified by the manufacturer.

Homolog retrieval and phylogenetic analysis. BLAST probing of the DNA and protein databases was performed with the BLASTn and BLASTp options of the BLAST program (1), using default parameters with no filters. Nucleotide sequences were retrieved from the GenBank and EMBL databases. The ClustalW program (38) was used to align the gene sequences obtained with the most similar ones retrieved from the databases. Each alignment was checked manually, corrected, and then analyzed using the neighbor-joining method (37) and the model of Kimura 2-parameter distances (22). Phylogenetic trees were constructed with the aligned sequences using Molecular Evolutionary Genetics Analysis 5 software (35). The robustness of the inferred trees was evaluated by 1,000 bootstrap resamplings.

RESULTS

A long upstream region is required for *cotH* expression. It has been previously reported that a strain carrying a translational *cotH*::*lacZ* fusion placed at the *cotH* locus of the *B. subtilis* chromosome showed a sporulation-specific and GerE-dependent β-galactosidase activity starting from 4 h after the onset of sporulation (2). When the same gene fusion containing about 250 bp upstream of the first *cotH* codon was moved at the *amyE* locus of the *B. subtilis* chromosome, no β-galactosidase activity was observed (data not shown). To define the DNA region upstream of the first codon required for the ectopic expression of *cotH*, we performed a deletion analysis: DNA fragments containing the entire *lacZ* gene fused in frame at the beginning of the *cotH* coding region (2) and extending for 1145 (Del3), 891 (Del5), 654 (Del2), 399 (Del1), and 262 (Del4) bp upstream of the first *cotH* codon (Fig. 1A) were PCR

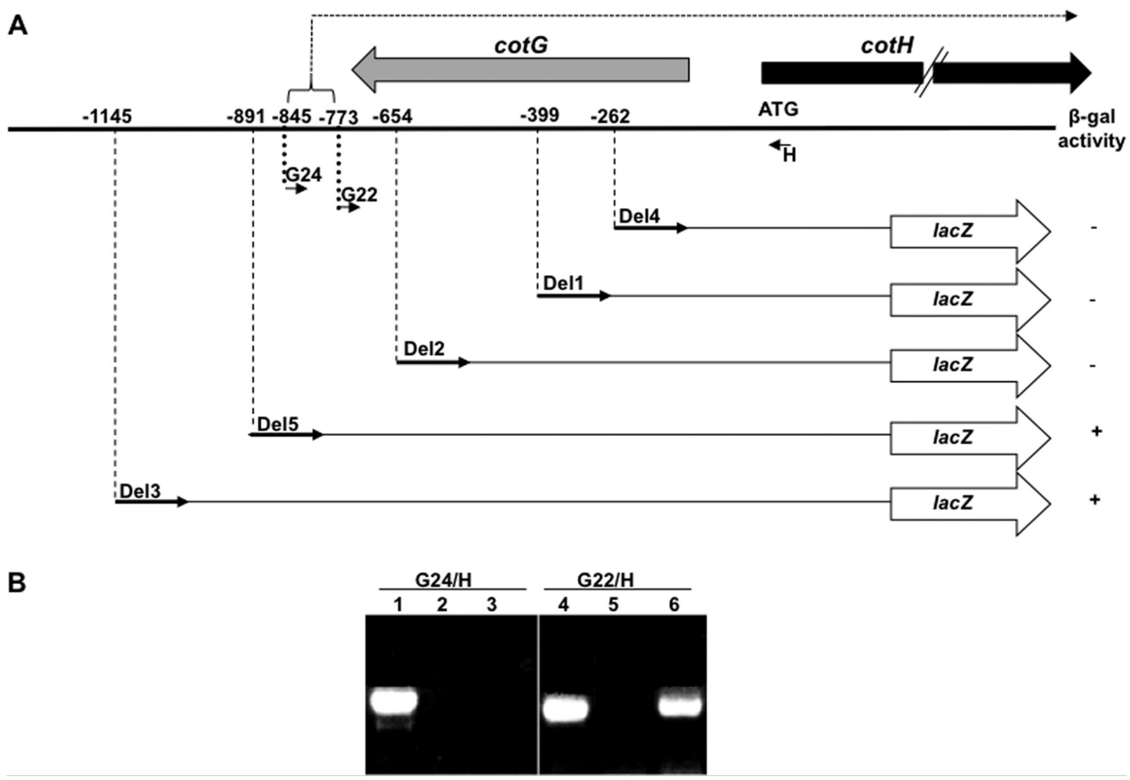


FIG. 1. (A) Deletion analysis of the DNA region upstream of the *cotH* coding part. Numbers indicate positions on the DNA sequence, considering the first base of the translation start site as +1. The *lacZ* gene of *E. coli* is fused in frame to the *cotH* coding part as described by Baccigalupi et al. (2). β-gal, β-galactosidase. Thin, short arrows indicate the positions of the oligonucleotides used in the RT-PCR experiment corresponding to panel B, while thick gray and black arrows indicate the coding parts of *cotG* and *cotH*, respectively. The dashed arrow indicates the mRNA produced from the *cotH* promoter. (B) Agarose gel electrophoresis used to analyze the extension products of an RT-PCR experiment. Total RNA was extracted from sporulating cells 5 h after the onset of sporulation from a wild-type (PY79) strain. cDNA synthesis was primed with oligonucleotide H (panel A; Table 1), while the amplification reactions were primed with oligonucleotide pairs H/G22 and H/G24 (panel A; Table 1). Control experiments were performed using chromosomal DNA as a template (lanes 1 and 4) or mRNA without the addition of the RT enzyme (lanes 2 and 5). cDNA was used as a template in the reactions of lanes 3 and 6.

amplified and cloned into plasmid pBK2, a pDG364 derivative (4). Plasmid DNA was then used to integrate all fusions at the *amyE* locus of the *B. subtilis* chromosome. As shown in Fig. 1A, only strains carrying the two longest fusions (Del3 and Del5) showed β-galactosidase activity. Those activities were indistinguishable from each other and from that observed with a strain carrying the translational fusion integrated at the *cotH* locus (2; not shown). This observation, together with the total absence of β-galactosidase activity of strains carrying fusions Del1, Del2, and Del4 strongly suggested that the DNA region spanning from position −654 to −891 is essential for *cotH* gene expression.

The region upstream of the *B. subtilis cotH* gene is transcribed. The long region required for *cotH* expression could be either the binding site for transcriptional activators or part of the transcription unit. We used an RT-PCR approach to discriminate between the two possibilities. For this purpose, total RNA was extracted from sporulating cells of a wild-type *B. subtilis* strain (PY79) 5 h after the initiation of sporulation and used as a template to produce cDNA with the synthetic oligonucleotide H (Fig. 1A; see Table S1 in the supplemental material) to prime the reaction. cDNA was then PCR amplified with oligonucleotide H and a collection of oligonucleotides annealing in the upstream region. We observed an amplification product of the expected size with oligonucleotide G22 and all oligonucleotides annealing downstream from it, while no PCR product was obtained with oligonucleotide G24 or all oligonucleotides annealing upstream of it. The amplifications performed with oligonucleotide pairs G22/H and G24/H are shown in Fig. 1B.

Therefore, these data indicate that the DNA region upstream of the *cotH* coding part is transcribed up to, at least, position −773 and that the *cotH* transcriptional start site is likely located downstream of position −845.

The analysis of the transcribed DNA region upstream of the *cotH* coding region revealed the presence of a long open reading frame (ORF), extending for 570 bp, and of several other short ORFs in the same orientation as *cotH*. However, all those ORFs do not have typical ribosome binding sites upstream of potential start codons. In addition, a translational fusion of the *lacZ* gene of *E. coli* and the longest ORF failed to produce β-galactosidase in both rich and sporulation-inducing media (not shown). Based on those results, we suggest that the long DNA region upstream of the *cotH* coding part is transcribed but not translated in the direction of *cotH*.

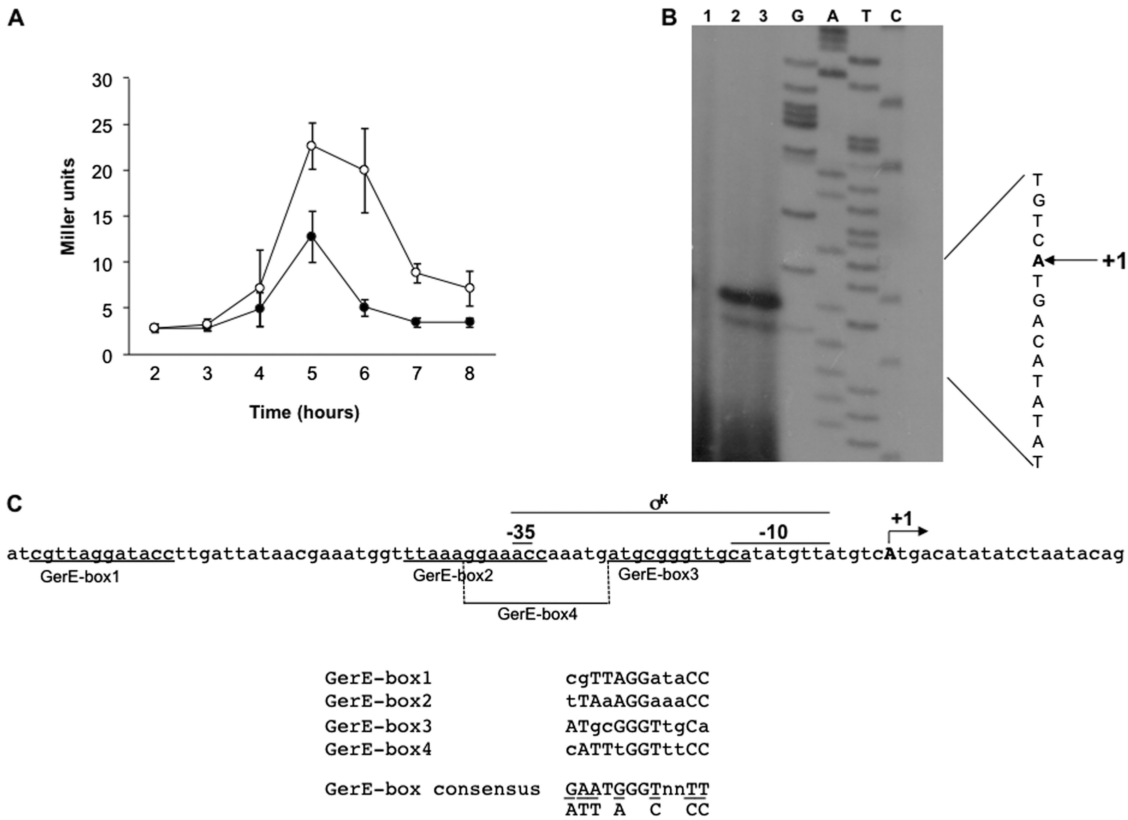


FIG. 2. (A) Expression of a *cotH*::*lacZ* transcriptional fusion during sporulation in an otherwise wild-type (closed symbols) or *gerE* null mutant (open symbols) strain. Samples were collected at various times after the onset of sporulation. Enzyme activity is expressed in Miller units. Data are the means of three independent experiments, and error bars indicate the standard deviations. (B) Primer extension analysis of the *cotH* promoter region performed with total RNA extracted from sporulating cells 5 h after the onset of sporulation from a mutant that fails to make SigK (*spoIVB*::*erm*; lane 1), a *gerE* null mutant (*gerE36*; lane 3), and a wild-type strain (PY79; lane 2). Primer extension and sequencing reactions were primed with the synthetic oligonucleotide G25 (Table 1). (C) *cotH* promoter region. The transcription start site ("A") is in bold and indicated as +1. The putative promoter sequences are indicated and the putative GerE boxes underlined. A comparison of the four GerE boxes with the GerE consensus is also reported.

Identification and analysis of the *cotH* promoter. To characterize the *cotH* promoter, we constructed a transcriptional gene fusion by using a 372-bp DNA region comprised of oligonucleotides Del3 and G27 (complementary to G22; Fig. 1A), containing the *cotH* promoter and the coding sequence of the *Escherichia coli lacZ* gene. The gene fusion was integrated at the *amyE* locus of the *B. subtilis* chromosome and the β-galactosidase activity measured at various times after the onset of sporulation in an otherwise wild-type strain and a collection of congenic sporulation mutants. As reported in Fig. 2A, we observed that β-galactosidase activity commenced 4 h after the onset of sporulation (T4), reached its maximum 1 h later (T5), and then decreased (Fig. 2A). Expression of *cotH*::*lacZ* was not detected in a mutant that fails to make σ^K (*spoIVB*::*erm*; data not shown), while the expression in a strain not producing GerE (*gerE36*; open symbols) was higher than that in an isogenic wild type (Fig. 2A, filled symbols). These results are in agreement with previous data obtained with a translational *cotH*::*lacZ* fusion (2) and confirm that *cotH* is transcribed by σ^K-containing RNA polymerase and is negatively regulated by GerE.

A primer extension experiment was then performed to map the *cotH* transcriptional start site. As shown in Fig. 2B, an extension product was obtained and appeared to be specific, since it was not observed when total RNA from a mutant that fails to make SigK (*spoIVB*::*erm*) was used for the extension reaction (Fig. 2B, lane 1) and was more intense with RNA from a strain not producing GerE (*gerE36*) than from a wild type (Fig. 2B, lanes 3 and 2, respectively). The 5′ terminus (indicated as +1) was mapped 812 bp upstream of the *cotH* first codon. The analysis of the nucleotide sequence upstream of the *cotG* 5′ terminus revealed the presence of a sequence perfectly matching all positions of the −10 and −35 consensus for a σ^K promoter (Fig. 2C) (15). As indicated in Fig. 2C, sequences resembling the proposed binding site for the GerE protein (18) are also present upstream of the promoter (GerE box 1), overlapping the −35 region (GerE box 2), between the −35 and −10 regions (GerE box 3), and on the opposite strand overlapping the −35 region (GerE box 4), consistent with a negative role of GerE on *cotH* transcription.

The 5′ part of *cotH* mRNA does not control CotH synthesis. The 812-bp RNA sequence extending from the transcriptional start site (+1) to the first codon was analyzed using the RNAfold Web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) and showed the potentials for extensive RNA secondary structures (see Fig. S2 in the supplemental mate-

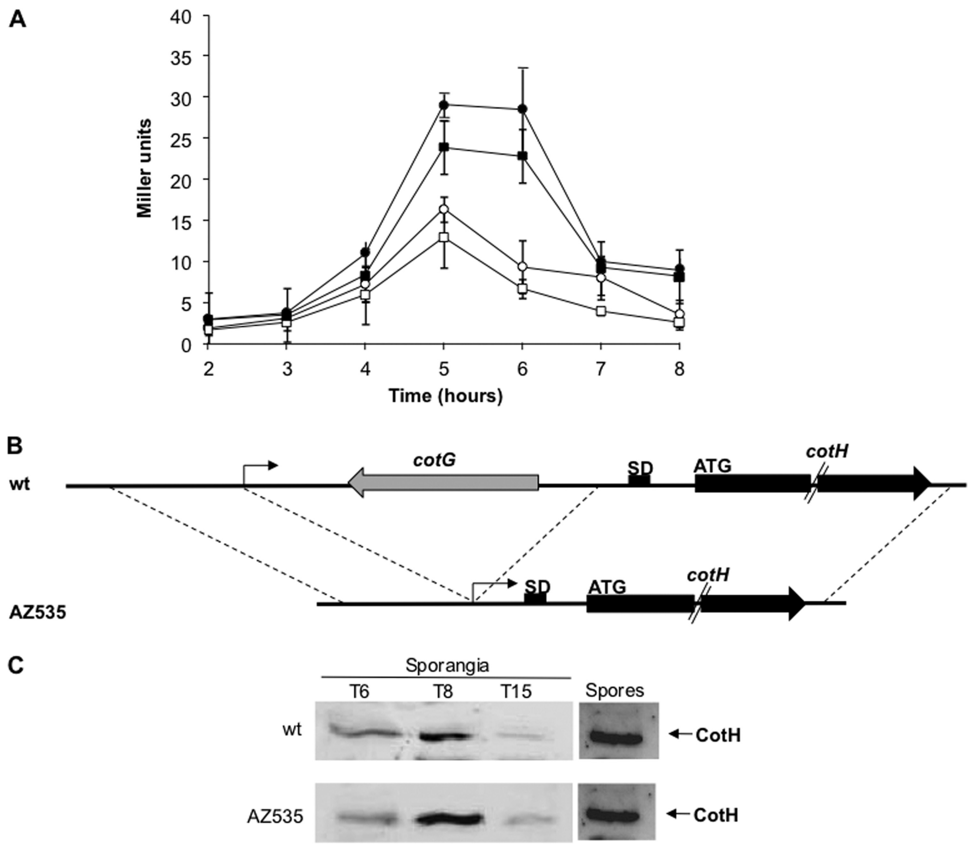


FIG. 3. (A) β-Galactosidase activity of strains carrying the *cotH* promoter and either 53 (squares) or 812 (circles) bp downstream of the transcription start site fused to the *lacZ* gene of *E. coli.* Gene fusions were inserted in an otherwise wild-type (open symbols) or *gerE* null mutant (closed symbols) strain. Samples were collected at various times after the onset of sporulation, and enzyme activity is expressed in Miller units. Data are the means of three independent experiments, and error bars indicate the standard deviations. (B) Construction of the deletion mutant. (C) Western blot of proteins extracted from sporulating cells or from purified spores of a wild-type strain and of an isogenic mutant carrying the deletion indicated in panel B. Proteins were fractionated on 15% polyacrylamide gels, electrotransferred to membranes, and reacted with anti-CotH antibody.

rial). Although *cis*-acting regulatory RNA elements (riboswitches) in *B. subtilis* have an average length of 360 bp (19), we decided to analyze whether the long 5′ part of *cotH* mRNA had a regulatory role on the downstream coding region. With this aim, we first constructed two transcriptional gene fusions by using the coding sequence of the *E. coli lacZ* gene as a reporter gene and DNA regions containing the *cotH* promoter and either 53 or 812 bp downstream of the transcriptional start site. Therefore, the two fusions contained only the initial 53 bp or the entire 5′ untranslated region of *cotH*. Both gene fusions were checked by nucleotide sequence analysis and integrated at the *amyE* locus of the *B. subtilis* chromosome. The β-galactosidase activity was then measured at various times after the onset of sporulation in an otherwise wild-type strain and in an isogenic strain not producing GerE (*gerE36*). As reported in Fig. 3A, similar levels of β-galactosidase activity were observed with the short (open and closed squares in wild-type and *gerE* backgrounds, respectively) or the long (open and closed circles in wild-type and *gerE* backgrounds, respectively) fusion, suggesting that the 5′ region did not affect the transcription of the *cotH* coding part.

In addition, we constructed a deletion mutant lacking 777 bp (from the transcriptional start site to 34 bp upstream of first codon) at the 5′ part of *cotH* (Fig. 3B). In this deletion mutant (AZ535), the *cotH* promoter was then positioned just upstream of the *cotH* ribosome binding site and the coding part. Sporulating cells of the wild type and the isogenic deletion mutant were harvested at various times during sporulation, lysed, and analyzed by Western blotting with anti-CotH antibody, as indicated in Materials and Methods. Similar amounts of CotH were found in the sporangia and on purified spores of both strains, indicating that, at least in these experimental conditions, the 5′ part of *cotH* mRNA does not affect production and assembly of CotH within the coat (Fig. 3C).

Purified spores of the wild type and the isogenic deletion mutant (AZ535) did not show any difference when analyzed for their heat resistance (10 and 30 min at 80°C), lysozyme (300 μg/ml) resistance, and Asn-induced germination (not shown), supporting the idea that the 5′ part of *cotH* mRNA does not affect CotH synthesis and spore coat formation.

The *cotG-cotH* chromosomal region. The unusual size and the lack of a regulatory function of the 5′ portion of *cotH* mRNA, together with the presence of the divergent *cotG* gene between the promoter and the beginning of the coding region of *cotH* (Fig. 1A), prompted us to investigate in more detail the *cotG-cotH* chromosomal region. We used the amino acid se-

TABLE 1. List of *cotG* genes retrieved from completely sequenced genomes

Organism	Accession no.	E value	Protein length (aa)
B. subtilis subsp. *subtilis* strain 168	NP_391488.1	4e−92	195
B. subtilis subsp. *spizizenii* strain W23	YP_003867887.1	1e−61	198
B. amyloliquefaciens FZB42	YP_001422884.1	6e−31	176
B. atrophaeus 1942	YP_003975035.1	2e−28	174
Geobacillus sp. strain WCH70	YP_002950034.1	6e−05	77
Geobacillus sp. strain Y4.1MC1	YP_003988838.1	5e−04	183

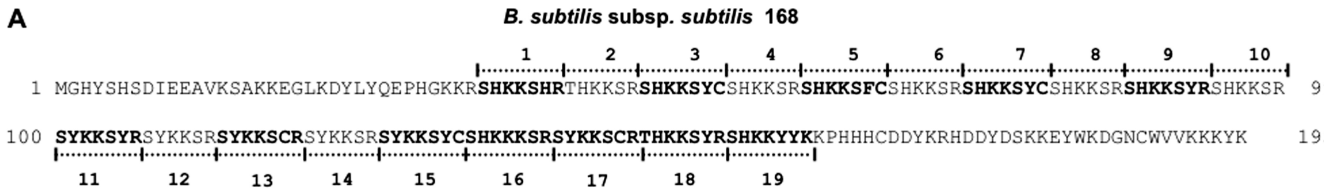
quence of the *B. subtilis* subsp. *subtilis* strain 168 CotG protein (accession number NP_391488.1) as a query in a BLAST (1) analysis to probe all translated ORFs of all completely sequenced prokaryotic genomes. The scanning of the 1,006 genomes (917 from bacteria and 89 from archaea) using the default parameters with no filters retrieved only six sequences with an E value below the default parameters used, four from bacilli and two from geobacilli (Table 1). The six CotG sequences exhibited different lengths, ranging from 77 (*Geobacillus* sp. WCH70) to 198 (*B. subtilis* subsp. *spizizeni* strain W23) amino acid residues (Table 1). Since our BLAST analysis included 76 entire genomes of *Bacillales*, results of Table 1 confirmed the very narrow phylogenetic distribution of *cotG* and indicated, as a consequence, that the *cotG-cotH* gene organization found in *B. subtilis* is not prevalent among spore-forming bacilli. Next, we analyzed the *cotG-cotH* chromosome organization in the six *cotG*-containing organisms. The *cotG* ortholog is adjacent to a divergently transcribed *cotH* ortholog in all four *Bacillus* sequences and in one of the two *Geobacillus* sequences, *Geobacillus* sp. Y4.1MC1 (see Fig. S3 in the supplemental material). In *Geobacillus* sp. strain WCH70, *cotG* and *cotH* orthologs are divergently transcribed but are separated by an ORF coding for a putative transposase of the IS*116*/IS*110*/IS*902* protein family (Fig. S3). RT-PCR experiments indicate that, as in *B. subtilis*, also in *B. amyloliquefaciens* FZB42 and *B. atrophaeus* 1942, *cotG* lies within the *cotH* transcript (see Fig. S4 in the supplemental material). Additional experiments will be needed to clarify if a similar situation also occurs in geobacilli. Those results indicate that while the *cotG-cotH* gene organization is not prevalent among spore-forming bacilli, it is conserved in all organisms that contain a *cotG* ortholog, suggesting its common evolutionary origin. This hypothesis is also supported by the analysis of a phylogenetic tree based on CotH amino acid sequence, showing that the six CotG-containing strains joined the same cluster (see Fig. S5 in the supplemental material).

Structure, origin and evolution of the *cotG* gene. A Clustal W analysis (38) performed on the six CotG sequences of Table 1 revealed that conserved amino acids were more abundant at the N-terminal region (amino acids 1 to 45) than at the C terminus and not apparent in the internal part of the proteins (see Fig. S6 in the supplemental material). When the same analysis was limited to CotG of bacilli, conserved amino acids were found also in the central parts of the proteins and were more apparent in the C-terminal regions (see Fig. S7 in the supplemental material).

It has been previously reported that the central part of CotG of *B. subtilis* 168 is characterized by the presence of 9 repeats of 13 amino acids (34). We now suggest for that part of CotG a more complex organization with 7 tandem repeats of 7 and 6 amino acids followed by 5 repeats of 7 amino acids (Fig. 4A). At the DNA level, this part of *cotG* consists of 19 paralogous regions of two different sizes: 12 21-bp-length copies and 7 18-bp-length copies (Fig. 4B). The intriguing modular structure of the internal part of this gene suggests that it is the outcome of several rounds of gene elongation events of an ancestral module, as postulated for other bacterial genes (9, 10, 11, 12). A plethora of different molecular pathways might have occurred to originate the extant 19 modules, starting from a single ancestral one. Useful hints on the most probable pathway might be inferred by the analysis of the number of mismatches existing between the different modules (Tables 2, 3, and 4). As shown in these tables, most of the mutations fell in the 21-bp modules (modules 1, 3, 5, 7, 9, 11, 13, 15, 16, 17, 18, and 19), whereas the seven 18-bp modules (modules 2, 4, 6, 8, 10, 12, and 14) remained almost identical. In addition, comparing the tandem organized module pairs (1-2 versus 3-4 and 3-4 versus 5-6, etc.), the sequence divergence between each pair of 39-bp modules is very low.

Assuming that the evolution of this gene has followed the most parsimonious pathway, data from Tables 2 to 4 suggest two alternative possibilities: either (i) the ancestral module was 21 bp long (module 1 in Fig. 4A) and elongated, giving rise to another module which underwent an evolutionary divergence consisting of the deletion of the last triplet and additional base pairs substitutions, resulting in a 18-bp module (module 2 of Fig. 4A), or (ii) the ancestral module was 18 bp long (module 2 in Fig. 4A) and elongated, giving rise to a new copy that diverged by acquiring a triplet (module 1 in Fig. 4A). Either way, modules 1 and 2 would have formed the first tandem module (1-2) of 39 bp, which in turn elongated, originating module 3-4, and the two copies diverged, differing by only 6 bp (5 base substitutions between modules 1 and 3 and 1 between modules 2 and 4). This evolutionary pathway predicts that the newly generated 39-bp module elongated until the construction of the first 14 modules. Then, the last part of the repeated region (consisting of five 21-bp repeats) would have originated via duplication of one or more 21-bp modules. Figure 5 shows a model of such an evolutionary process, considering the 21-bp module as the ancestral one. The last five modules (15, 16, 17, 18, and 19) are the most divergent ones; however, the traces of their origin from the other paralogous 21-bp long modules are evident.

In all CotG-containing bacilli, CotG has a modular structure, although the numbers and the lengths of the repeats differ in the four microorganisms (Fig. 6A). Indeed, 20 repeats were found in the CotG of *B. subtilis* subsp. *spizizeni* W23, and there were 15 in *B. amyloliquefaciens* FZB42 and *B. atrophaeus* 1942. This finding suggests that, starting from the same ancestral gene, a different number of elongation events in the four different bacteria originated the extant *cotG* genes. A similar scenario can be depicted for CotG of the two CotG-containing *Geobacillus* strains, where the central part of CotG has a modular structure but the various modules do not share a significant degree of sequence identity/similarity with those of *B. subtilis*. In *Geobacillus* sp. strain WCH70, CotG is only 77 amino acids long (Table 1) and contains three repeats (Fig. 6B), while in *Geobacillus* sp. strain Y4.1MC1, CotG is 183



B *B. subtilis* subsp. *subtilis* 168

Nucleotide sequence	Module number	Encoded sequence
AGTCATAAAAAGTCGCACCGC	1	SHKKSHR
ACTCACAAAAAATCTCGC	2	THKKSR
AGCCATAAAAAATCATACTGC	3	SHKKSYC
TCTCACAAAAAATCTCGC	4	SHKKSR
AGTCACAAAAAATCATTCTGT	5	SHKKSFC
TCTCACAAAAAATCTCGC	6	SHKKSR
AGCCACAAAAAATCATACTGC	7	SHKKSYC
TCTCACAAGAAATCTCGC	8	SHKKSR
AGCCACAAAAAATCGTACCGT	9	SHKKSYR
TCTCACAAAAAATCTCGC	10	SHKKSR
AGCTATAAAAAATCTTACCGT	11	SYKKSYR
TCTTACAAAAAATCTCGT	12	SYKKSR
AGCTATAAAAAATCTTGCCGT	13	SYKKSCR
TCTTACAAAAAATCTCGC	14	SYKKSR
AGCTACAAAAAGTCTTACTGT	15	SYKKSYC
TCTCACAAGAAAAAATCTCGC	16	SHKKKSR
AGCTATAAGAAGTCATGCCGC	17	SYKKSCR
ACACACAAAAAATCTTATCGT	18	THKKSYR
TCCCATAAGAAATACTACAAA	19	SHKKYYK

FIG. 4. (A) Amino acid sequence of CotG of *B. subtilis* subsp. *subtilis* 168. The 19 modules present in the central part of the protein are indicated. The 7-amino-acid modules are in bold. (B) The 19 paralogous regions, 12 of 21 bp and 7 of 18 bp, encoding the protein modules.

amino acids long and contains two almost identical repeats of 49 amino acids (Fig. 6C). Although those modules have a primary structure different from that present in CotG-containing bacilli, it is likely that also in this case, gene elongation events occurred during evolution to originate the extant *cotG* orthologs.

DISCUSSION

A first result of this work is the identification of the *cotH* transcriptional start site 812 bp upstream of the first codon. A transcriptional start site for the *cotH* gene had been previously mapped 99 bp upstream of the first codon (28). However, we believe that the −99 site is not an internal promoter and that its erroneous identification was due either to RNA processing events or to the inhibition of cDNA synthesis during primer extension experiments (27). This hypothesis is supported by the following evidence: (i) the *in silico* analysis of the untranslated region showed the potentials for extensive secondary RNA structures (see Fig. S2 in the supplemental material), (ii) DNA fragments containing the upstream promoter, but not the putative internal promoter, were able to drive transcription (Fig. 1A and B, 2A, and 3B), and (iii) RT-PCR experiments failed to detect mRNA synthesis in fragments containing only the putative internal promoter (not shown).

While the correct position and sequence of the *cotH* promoter have been identified in this study, our results confirm previous data based on the analysis of a translational gene

TABLE 2. Numbers of mutations between the different 21-bp modules of the *B. subtilis* 168 *cotG* gene

Module	No. of mutations in module[a]:											
	1	3	5	7	9	11	13	15	16	17	18	19
1	—	5	7	7	5	6	7	5	11	6	8	11
3		—	4	2	2	3	2	4	10	5	7	8
5			—	2	2	3	2	4	9	8	6	11
7				—	1	1	2	3	10	6	7	9
9					—	3	2	4	12	7	5	9
11						—	0	2	12	5	5	9
13							—	2	13	4	8	10
15								—	13	6	6	11
16									—	10	8	9
17										—	10	10
18											—	10
19												—

[a] Dashes are shown to indicate the same module in the row and column.

TABLE 3. Numbers of mutations between the different 18-bp modules of the *B. subtilis* 168 *cotG* gene

Module	No. of mutations in module[a]:						
	2	4	6	8	10	12	14
2	—	1	0	0	1	0	0
4		—	0	0	1	0	0
6			—	0	1	0	0
8				—	1	0	0
10					—	1	1
12						—	0
14							—

[a] Dashes are shown to indicate the same module in the row and column.

TABLE 4. Numbers of mutations between the different tandem modules of the *B. subtilis* 168 *cotG* gene

Module pair	No. of mutations in module pair[a]:					
	3–4	5–6	7–8	9–10	11–12	13–14
1–2	6	7	7	6	6	7
3–4	—	4	2	3	3	2
5–6		—	2	3	3	2
7–8			—	2	1	2
9–10				—	4	3
11–12					—	0
13–14						—

[a] Dashes are shown to indicate the same module in the row and column.

fusion (2) indicating that *cotH* transcription is driven by the sporulation-specific σ factor σ^K of the RNA polymerase and that the transcriptional regulator GerE acts as a repressor of *cotH* expression.

The long 5′ part of *cotH* mRNA (812 bp from +1 to the first codon) is most likely not translated in the direction of *cotH*. It contains several open reading frames (ORFs), but none of them has typical ribosome binding sites upstream of potential start codons. Additional experiments would be needed to definitely conclude that none of those ORFs is translated.

Long 5′ untranslated regions are not common in bacteria unless they are *cis*-acting regulatory RNA elements (riboswitches) that control the expression of the downstream coding regions. An average length of 360 bp has been determined for such regulatory elements in *B. subtilis* (19). The 5′ untranslated part of *cotH* mRNA is unusually long (812 bp) and can form secondary structures potentially involving the *cotH* ribosome binding site (see Fig. S2 in the supplemental material) but does not seem to have a regulatory role on the downstream coding region. Although we cannot rule out the possibility that the 5′ untranslated region of *cotH* mRNA may have a regulatory role in particular environmental conditions, data reported in Fig. 3 do not show any effect on CotH synthesis in standard laboratory conditions (Materials and Methods).

The unusual size of the 5′ end of *cotH* and the lack of a regulatory role in the analyzed conditions were puzzling and induced us to analyze in more detail the presence of the divergently transcribed gene *cotG*, entirely contained between the *cotH* transcriptional and translational start sites (Fig. 1A). The analysis of the structure, organization, and phylogenetic distribution of *cotG* revealed that it is most likely the outcome of gene elongation events involving an ancestral module. Gene elongation increases the size of genes by duplication of internal motifs and represents one of the most important mechanisms in the evolution of complex genes from simple ones (8, 11). A gene elongation event can be the outcome of an in-tandem duplication of a DNA sequence. When the elongation event involves an entire gene, a deletion of the intervening sequence between the two copies occurs, and this is followed by a mutation converting the stop codon of the first copy into a sense codon and resulting in the fusion of the two gene copies (7). When the elongation event involves only an internal region of

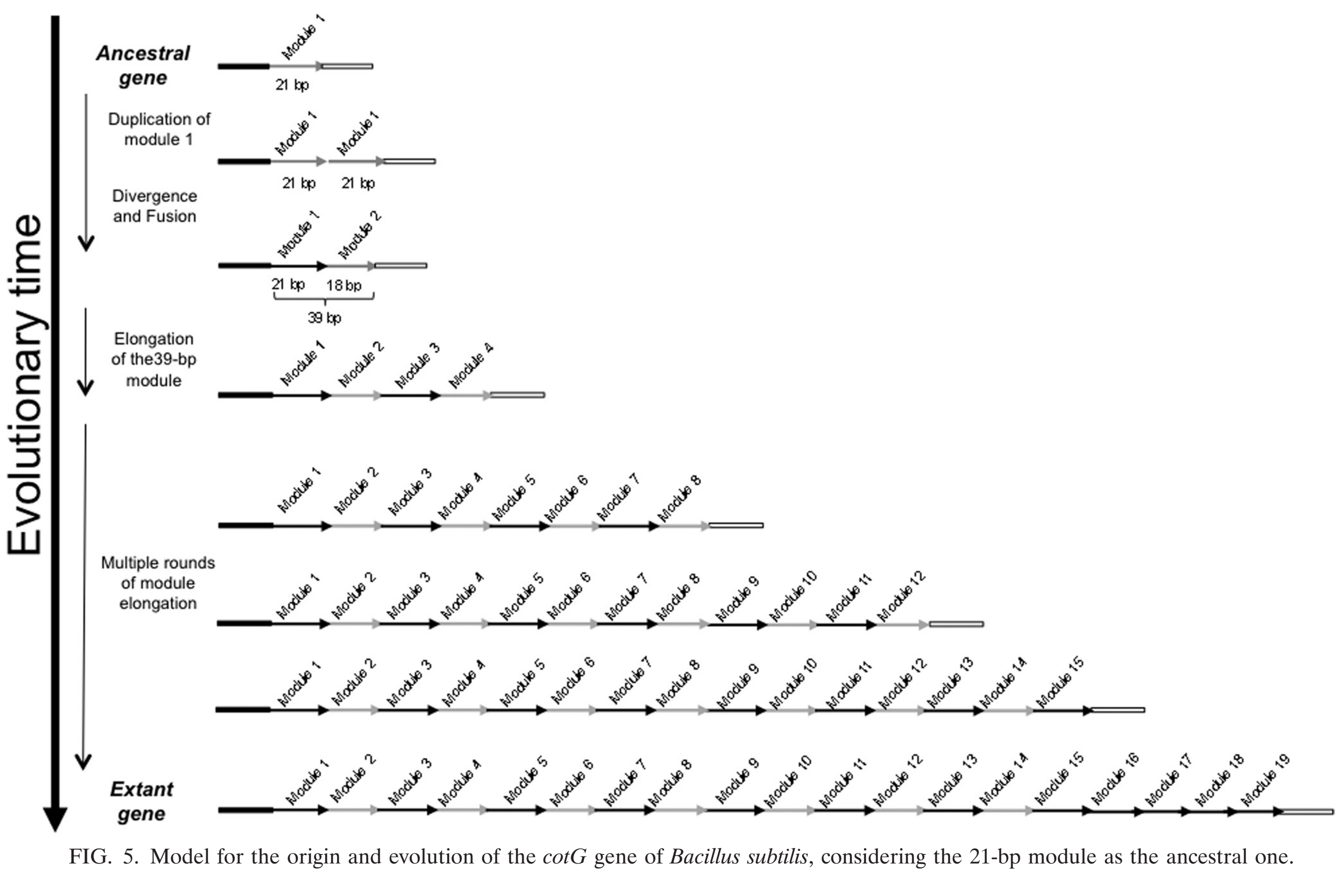


FIG. 5. Model for the origin and evolution of the *cotG* gene of *Bacillus subtilis*, considering the 21-bp module as the ancestral one.

A

B. subtilis subsp. *spizizenii* W23	*B. amyloliquefaciens* FZB42	*B. atrophaeus* 1942
SHKKSHR	KHKKSR	SHKKSYC
SHKKSR	SHKKSR	SHKKSR
SHKKSYC	SHKKSCR	SHKKSNC
SHKKSR	SHKKSR	SYKKSR
SHKKSYC	SHKKSCR	SHKKSYC
SHKKSR	SHKKSR	SHKKSR
SYKKSYC	SHKKSR	SHKKSFC
SHKKSR	SHKKSR	SHKKKSR
SYKKSYC	SHKKSR	SYKKSYR
SHKKSR	SHKKSR	SHKKKSR
SYKKSYC	SHKKSR	SYKKSFC
SHKKSR	SHKKSR	SHKKKSR
SYKKSYC	SRKKSCR	SYKKSFC
SHKKSR	SHKKSR	SHKKKSR
SYKKSYR	SHKKSCR	SHKKSYR
SYKKYR		
SYKKSCR		
THKKSYC		
SHKKK		
SHKKYYK		

B

***Geobacillus* WCH70**

1 MSVYTQDDIQKAVKELETEGLEDFLYQEPVGRKHSSGRKHSSGRKHSSHRTSCFRPRRKRRYWRDSNMHTLWLELDD 77

C

***Geobacillus* Y4.1MC1**

1 MRTYTHDDIQNAVIEMESGGLSGFLYQEPTGNKSKKHRSHHKRRTGYTSHSRSTNRSRQTQRTSRTSGASRSGRTDRTGRTSRSSGTSRSHRTHHT 96

97 TSHTSRSRSTNRSRQTQRTGRTSGTSRSGRTDRTGRTSRSSGTSRSHRTHHTNHASHSHRTHRSRRTKYFKRKKRYWDNGNMIMLWLG 183

FIG. 6. (A) Amino acid modules of CotG of *B. subtilis* subsp. *spizizenii* W23, *B. amyloliquefaciens* FZB42, and *B. atrophaeus* 1942. (B) Amino acid sequence of CotG of *Geobacillus* WCH70. The three identical modules of 6 amino acids present in the central part of the protein are boxed. (C) Amino acid sequence of CotG of *Geobacillus* Y4.1MC1. The two 49 repeats present in the central part of the protein are boxed.

a gene, as in the case of *cotG*, a gene rearrangement occurs to position the duplicated sequences in the same frame. Examples of tandem arrays of multiple short repeats within a gene in pathogenic bacteria have been described (27). In those cases, low-complexity regions have been hypothesized to originate by mutational processes, such as slipped-strand mispairing and unequal crossing-over taking place during DNA replication (27). Two or more paralogous moieties (modules) that constitute or are contained in the new gene may diverge and undergo further duplication events, leading to a gene constituted by or containing more repetitions. The biological significance of gene elongation might rely on (i) the improvement of the function of a protein by increasing the number of active sites and/or (ii) the acquisition of an additional function by modifying a redundant segment. Examples of genes sharing internal sequence repetitions have been described in both prokaryotes and eukaryotes (see reference 14 and references therein). The most extensively documented example in prokaryotes is represented by the pair of genes *hisA* and *hisF*, showing an evident split into two modules half the size of the entire gene (8, 9, 12).

All six CotG-containing strains have significantly different GC contents in their *cotG* genes with respect to their entire genomes (Table 5). In particular, in the four bacilli, the GC content of *cotG* is lower than that in their entire genomes, while in the two geobacilli, the GC content of the gene is higher than that in the genome (Table 5). However, this difference is mainly due to the repeated part of the *cotG* genes of geobacilli that have GC contents significantly higher than that of their entire *cotG* genes (Table 5).

At the protein level, homologies are found in the N- and C-terminal regions of all analyzed CotG proteins (see Fig. S6 in the supplemental material), while the central part shows

TABLE 5. GC contents of *cotG* genes compared to those in the corresponding entire genomes

Organism	% GC		
	Entire *cotG*	Repeated region of *cotG*	Entire genome
Bacillus subtilis subsp. *subtilis* strain 168	38.3	36.7	43.5
Bacillus subtilis subsp. *spizizenii* strain W23	36.3	36.4	44.0
Bacillus amyloliquefaciens FZB42	41.4	42.3	46.5
Bacillus atrophaeus 1942	36.3	37.0	43.0
Geobacillus sp. strain WCH70	47.9	59.2	42.8
Geobacillus sp. strain Y4.1MC1	51.9	60.4	44.0

homologies only excluding the *Geobacillus* strains from the analysis (see Fig. S7 in the supplemental material). Based on this, on the narrow phylogenetic distribution of *cotG*, and on the different GC contents of *cotG* genes with respect to their entire genomes, we hypothesize that an ancestral *cotG* gene was constituted by the 5′ and 3′ regions and by an internal low-complexity region acting as an ancestral module. This ancestral minigene was either acquired by bacilli and geobacilli through independent horizontal gene transfer events from a still-unknown microorganism or acquired by a common ancestor of all CotG-containing bacteria. The latter hypothesis is supported by the chromosomal organization of *cotG-cotH* locus, similar in all six CotG-containing microorganisms. The internal low-complexity region of *cotG* genes would have then evolved independently through elongation events.

In *B. subtilis*, *cotG* is entirely contained between the promoter and coding part of *cotH*, and a similar situation has been observed also in *B. amyloliquefaciens* and *B. atrophaeus.* It is not clear whether this chromosomal organization has given a selective advantage to the cell; however, this event has been fixed by evolution. It is instead clear that, soon after the insertion of *cotG*, the cell has adjusted the regulation of the *cotG-cotH* expression in order to avoid the collision of RNA polymerase molecules during transcription of *cotG* and *cotH*. Although the genome organization of the *cotH-cotG* cluster is conserved and is likely to have occurred only once during evolution, we still do not know whether in the geobacilli the presence of *cotG* has caused the detachment of the promoter from the coding part of *cotH*, and further studies will be needed to address this point.

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