CotG controls spore surface formation in response to the temperature of growth in Bacillus subtilis

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Summary

Bacterial spores of the Bacillus genus are ubiquitous in nature and are commonly isolated from a variety of diverse environments. Such wide distribution mainly reflects the spore resistance properties but some Bacillus species can grow/sporulate in at least some of the environments where they have been originally isolated. Growing and sporulating at different conditions is known to affect the structure and the resistance properties of the produced spore. In B. subtilis the temperature of growth and sporulation has been shown to influence the structure of the spore surface throughout the action of a sporulation-specific and heat-labile kinase CotH. Here we report that CotG, an abundant component of the B. subtilis spore surface and a substrate of the CotH kinase, assembles around the forming spore but also accumulates in the mother cell cytoplasm where it forms aggregates with at least two other coat components. Our data suggest that the thermo-regulator CotH contributes to the switch between the coat of 25°C and that of 42°C spores by controlling the phosphorylation levels of CotG that, in turn, regulates the assembly of at least two other coat components.

Introduction

Spore formers are mostly Gram-positive bacteria of the Bacillus and Clostridiales genera sharing the ability to differentiate into a quiescent and resistant cell, the spore, in response to environmental conditions no longer allowing cell growth. The spore can survive almost indefinitely without water and nutrients and at conditions of extreme temperature and pH, and in the presence of toxic chemicals and lytic enzymes (McKenney et al., 2013). Although quiescent, the spore senses the environment and responds to the presence of nutrients by germinating and forming a new cell able to grow and eventually sporulate (Christie and Setlow, 2020). Spore resistance is in part due to the spore structure, characterized by a dehydrated cytoplasm surrounded by protective layers: a peptidoglycan-like cortex and a proteinaceous and multilayered coat (McKenney et al., 2013). In some species, such as Bacillus anthracis or Clostridiodes difficile, the outer coat is surrounded by the exosporium, an additional layer formed by proteins and glycoproteins (McKenney et al., 2013). In B. subtilis, the model system for spore formers, the coat is surrounded by the crust, an additional layer formed by proteins and glycoproteins, in some respect resembling the exosporium of other species (McKenney et al., 2013).

Spores are ubiquitous in nature and are commonly isolated from almost all environments where they have been searched for, from desert sands and deep sediments, to the gut of animals and insects (Istitico et al., 2020). In some of these diverse environments, such as the animal gut (Cutting, 2011), the rhizosphere of plants (Timmusk et al., 2011) and the deep marine sediments (Cupit et al., 2019), spores of some Bacillus species germinate, proliferate and sporulate, indicating a physiological role of aerobic spore formers in those specific environments. The conditions of growth and sporulation influence the structure of the spore (Garcia et al., 2010; Abhyankar et al., 2016) and recent reports have shown that the temperature of sporulation modifies the structure of the spore coat and of the exosporium in B. cereus (Bressurrelsoard et al., 2016) and induces a switch between two alternative types of spore surfaces in B. subtilis (Istitico et al., 2020). The latter study identified CotH, an atypical Ser/Tyr kinase, as a major thermo-regulator of the spore surface, more abundant and active at low (25°C) than at high (42°C) temperatures and able to induce a switch from the 25°C to the 42°C spore surface (Istitico et al., 2020).

In B. subtilis the gene coding for the kinase CotH is clustered with the genes coding for two coat proteins that
are the only two CotH substrates, CotB and CotG (Nguyen et al., 2016; Freitas et al., 2020). While the cotB gene follows the 3’ end of cotH and is independently transcribed (Donovan et al., 1987), the cotG gene is at the 5’ end of cotH but divergently transcribed and entirely contained between the promoter and the coding region of cotH (Giglio et al., 2011). Both cotG and cotH genes are transcribed by SigK-controlled RNA polymerase but are differently regulated by the transcription factor GerE, which acts as an activator of cotG (Sacco et al., 1995) and a repressor of cotH (Naclerio et al., 1996). Although cotG mRNA is complementary to the 5’ un-translated part of the cotH mRNA, it does not have a role in cotH expression (Giglio et al., 2011).

CotG is an abundant coat protein of 195 amino acids with a strong positive charge (Sacco et al., 1995) and is listed in the top-20 supercharged proteins present in data banks (Ma et al., 2020). Such positive charge is, at least partially, balanced by its extensive and CotH-dependent phosphorylation (Saggese et al., 2014; Nguyen et al., 2016; Freitas et al., 2020). In a B. subtilis mutant lacking CotH, unphosphorylated CotG affects the coat assembly of other proteins, such as CotC and CotU (Saggese et al., 2014).

It has been previously reported that cotG is transcribed about twofold more efficiently in cells sporulating at 25°C or 37°C than at 42°C, while cotC, cotU and cotH have similar transcription levels at the three temperatures (Nicolas et al., 2012). In addition, the temperature also controls the stability of the kinase CotH, which is more stable at low than high temperatures (Isticato et al., 2020). As a consequence, at a low temperature (25°C), when the kinase CotH is abundantly present and active, its substrates CotB and CotG are efficiently phosphorylated and assembled on the coat surface contributing to the lamellar-like and electron-dense appearance of 25°C spores (Isticato et al., 2020). At a high temperature (42°C) CotH is less active (or totally inactive), CotB and CotG are less efficiently phosphorylated and assembled on the coat where are replaced by other proteins, including CotC and CotU, which contribute to the granular and thick appearance of the coat of 42°C spores (Isticato et al., 2020). The focus of this study is CotG and its negative effect on the assembly of CotC and CotU. We report that such negative effect is due to the unphosphorylated form of CotG that accumulates in the mother cell cytoplasm and forms aggregates with CotC and CotU. It is proposed that the negative effect observed in a mutant lacking CotH is the exacerbation of a regulatory role of CotG, that in a wild type cell controls the amounts of CotC and CotU assembled around the forming spore in response to the temperature of growth and sporulation.

Result and discussion

CotG accumulates in the mother cell cytoplasm

The cotG and cotH genes are both controlled by the sporulation-specific sigma factor of the RNA polymerase SigK in conjunction with the transcriptional factor GerE that activates cotG (Sacco et al., 1995) and represses cotH (Naclerio et al., 1996), presumably regulating the timing of cotG and cotH expression during sporulation. We monitored the expression of both genes simultaneously in sporulating cells of a strain carrying the cotH and cotG genes translationally fused to the lacZ and gusA genes of E. coli respectively. As expected, the cotH::lacZ fusion was expressed earlier than the cotG::gusA and its highest levels of expression were observed 5 h after the beginning of sporulation (T5) when cotG expression had not yet started (Fig. 1A). At late sporulation times, cotH expression

Fig. 1. Timing of production of CotG and CotH during sporulation. A. Temporal expression of the cotH (closed symbols) and cotG (open symbols) genes in B. subtilis strain GC224, carrying the cotG::gusA and cotH::lacZ fusions. B. Detection of CotH and CotG proteins by Western blotting analysis of proteins extracted from wild-type sporulating cells and mature spores reacted against anti-CotH or anti-CotG primary antibodies. Peroxidase-conjugated anti-rabbit secondary antibodies were used to visualize the recognized proteins.
rapidly declined while cotG expression increased (Fig. 1A). Consistently, in a wild type strain [PY79 (Youngman et al., 1984)] the kinase CotH was detected in extracts from sporulating cells 5 h after the beginning of sporulation (T5), reached its maximal abundance 1 h later (T6) and then rapidly decreased while CotG appeared at T6 and became abundant at later times (Fig. 1B). As previously reported (Freitas et al., 2020), CotG was extracted from prespores and mature spores in two forms, a slower migrating form of about 36 kDa (CotG36) and a faster migrating form of about 32 kDa (CotG32) (Fig. 1B). CotG36 appeared first at T6, and CotG32 1 h later, although was less abundant than CotG36 (Fig. 1B). Then, at T8 and in mature spores the two CotG forms were similarly represented (Fig. 1B).

The observation that CotG is produced and accumulates at late time of sporulation, when the amount of the specific kinase CotH is decreasing (Fig. 1), raised the possibility that some CotG molecules could be present in sporangia in an unphosphorylated form. Repeated attempts to detect CotG by Western blotting in the mother cell cytoplasm were unsuccessful, therefore a fluorescence microscopy approach was followed using strain RH296 of *B. subtilis* carrying the cotG gene fused to the gene coding for the red fluorescent protein (RFP) of the coral *Discosoma* sp. (Donadio et al., 2016; Istitcato et al., 2020). In an otherwise wild type strain most CotG-RFP molecules were localized around the forming spore (Fig. 2A); however, some CotG-RFP molecules were also observed in the mother cells, concentrated in well-defined small spots (Fig. 2A). In an isogenic strain lacking the kinase CotH (RH298), CotG is not phosphorylated and not assembled around the forming spore (Freitas et al., 2020); as a consequence a strong fluorescence signal (CotG-RFP) almost completely filled the mother cell cytoplasm (Fig. 2A).

The dependency of CotG-RFP accumulation in the mother cell cytoplasm on CotH (Fig. 2A) makes it unlikely that the fluorescence observed in the mother cell of a wild type strain (Fig. 2A) was due to RFP alone (i.e. due to RFP cleaved off from CotG). However, to confirm this point, an immunofluorescence microscopy analysis was performed with anti-CotG antibody. As shown in Fig. 2B, CotG-specific fluorescence signals were observed around the forming spore and in the mother cell cytoplasm of a wild type strain, where they formed well-defined spots similar to those observed with CotG-RFP (Fig. 2A). In a mutant lacking CotH all CotG-dependent fluorescence was found in the mother cell cytoplasm (Fig. 2B).

Results of Figs 1 and 2 indicate that in a wild type strain some CotG molecules do not assemble around the spore and accumulate in the mother cell cytoplasm in well-defined spots. In a mutant lacking CotH such accumulation is massive and obviously formed by unphosphorylated CotG.

*CotG is mostly unphosphorylated in the mother cell cytoplasm of wild type sporangia*

To verify whether the CotG molecules present in the mother cell of a wild type strain were phosphorylated,
immuno-fluorescence experiments were performed with anti-PKC antibody (Cell Signalling Technology), recognizing phosphorylated serine residues surrounded by a lysine or an arginine. In a wild type strain (PY79) a strong fluorescence signal was observed associated to the forming spore while only a modest and diffuse signal was visible in the mother cell cytoplasm (Fig. 3). No fluorescence was observed in a mutant lacking CotH (AZ607), indicating that the fluorescence in wild type sporangia was only due to the kinase activity of CotH (Fig. 3). In a mutant lacking CotB (AZ668), the other substrate of the kinase CotH, fluorescence signals were only due to the CotH-dependent phosphorylation of CotG and appeared mainly present around the forming spore (Fig. 3). In sporangia of strain AZ668 only a minimal part of the fluorescence (due to phosphorylated CotG molecules) was found in the cytoplasm, never forming the well-defined fluorescence spot observed in Fig. 2.

Based on these results, we infer that in a wild type strain most CotG molecules are phosphorylated and assembled around the spore, the remaining CotG molecules are mostly unphosphorylated and form well-defined spots in the mother cell cytoplasm.

**Unphosphorylated CotG forms aggregates with CotC and CotU**

Accumulation of massive amounts of unphosphorylated, supercharged CotG in the mother cell cytoplasm of a mutant strain lacking CotH (Fig. 2A) could be the cause of the mis-assemblage of the coat proteins CotC and CotU previously observed in mature spores of a cotH mutant strain (Saggese et al., 2014). To verify such hypothesis, immunofluorescence microscopy experiments were performed with anti-CotC antibody, known to recognize both CotC and CotU proteins (Isticato et al., 2004; Istitico et al., 2008). In a wild type strain (PY79), fluorescence signals were mostly observed around the forming spore and only a weak fluorescence was localized in the mother cell (Fig. 4). Fluorescence signals similar to those observed in wild type sporangia but of reduced intensity were also observed in mutants lacking CotC (RH101) or CotU (RH202) (Fig. 4), indicating that the fluorescence observed in wild type sporangia was due to both CotC and CotU.

In a mutant lacking CotH (AZ607) the CotC/CotU-dependent fluorescence signals appeared were differently distributed between the forming spore and mother cell with respect to the wild type strain, with a stronger signal in the mother cell cytoplasm and weaker signal around the spore than in the isogenic wild type strain (Fig. 4). The weak assemblage of CotC/CotU around the forming spore is however transient, since no CotC/CotU are then observed around mature spores of a mutant lacking CotH (Saggese et al., 2014). Therefore, in a mutant lacking CotH both unphosphorylated CotG (Fig. 2) and CotC/CotU (Fig. 4) accumulated in the mother cell cytoplasm in well-defined spots. Such effect is CotG-dependent since it was not observed in a mutant lacking CotG (AZ604) (Fig. 4).

A fluorescence microscopy analysis of the B. subtilis strain RH406, carrying the RFP fused to CotG and the green fluorescent protein (GFP) fused to CotC (Istitico et al., 2020) confirmed that both CotC and CotG behave similarly and showed that the two proteins co-localize in the mother cell cytoplasm (Fig. 5). In an otherwise wild type strain both CotG-RFP and CotC-GFP were mostly localized around the forming spores but were also present in the mother cell cytoplasm (Fig. 5). When CotH was not present, only few CotG-RFP and CotC-GPF molecules were around the forming spore while most of them failed to assemble around the spore and were co-localized in aggregates in the mother cell cytoplasm (Fig. 5).

**Fig. 3.** Fluorescence microscopy detection of phosphorylated CotG. Immunofluorescence analysis of sporangia of wild type (PY79) and isogenic mutants lacking cotH (AZ607) and cotB (AZ668) with anti-PKC antibody. For all panels, the percentage of cells showing the indicated fluorescence is reported in Supplementary Material Table S3. Representative examples are reported by phase-contrast (PC) and fluorescence microscopy and by merging the images (Merge). In PC panels dotted yellow ovals indicate the position of the forming spore. The exposure time was 200 ms in all cases. Scale bar 1 μm.
The behaviour observed with CotC/CotU was not observed with other coat proteins, independently from their dependence on CotG in a mutant lacking CotH (Saggese et al., 2014). Indeed, CotS (affected by CotG), CotA and CotZ (not affected by CotG) (Saggese et al., 2014) were all found assembled around the forming spore with no fluorescence signals in the mother cell cytoplasm, both in a wild type and in a strain bearing a cotH null mutation (Fig. 6). Results of Fig. 6 then suggest that the formation of CotG-dependent aggregates is not common to all coat proteins but rather a specific property of CotC and CotU.

CotG controls the assembly of CotC and CotU in wild type sporangia in response to the temperature

A fluorescence microscopy analysis of B. subtilis strains carrying the cotC::gfp or the cotG::rfp fusion was performed on sporangia grown at 25°C and 42°C. Since the temperature affects the growth rate, the entry into the sporulation cycle and the time of spore formation, sporangia of similar age were produced at the different temperatures as previously described (Isticato et al., 2020). As shown in Fig. 7A, CotC and CotG molecules showed an opposite behaviour in sporangia grown at 25°C or 42°C. At 25°C CotG-RFP was abundant around the spore and formed well-defined spots in the cytoplasm while CotC-GFP was almost absent around the spore and localized in well-defined spots in the cytoplasm (Fig. 7A). At 42°C CotG-RFP was almost absent.

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around the spore and formed well-defined spots in the cytoplasm while CotC-GFP was abundant around the spore and in minimal amounts in the cytoplasm (Fig. 7A). At 42°C CotG-RFP appeared less abundant than at 25°C, suggesting a reduced production of the protein. To clarify this point an isogenic strain carrying a translational cotG::lacZ fusion (Sacco et al., 1995) was grown at 25°C, 37°C and 42°C. As shown in Fig. 7B, the expression of the cotG genes was highest at 25°C, about twofold lower at 37°C and about threefold lower at 42°C, in agreement with a previous transcriptomic study (Nicolas et al., 2012).

To evaluate the phosphorylation status of CotG at different temperatures, an immunofluorescence analysis with anti-PKC on sporangia growing at 25°C and 42°C was performed. At low temperature CotG around the spore appeared mostly phosphorylated while in the mother cell cytoplasm was mostly unphosphorylated (Fig. 7C), similarly to what was observed at 37°C (Fig. 3). As expected by the low activity of the kinase CotH at 42°C (Isticato et al., 2020), at high temperature only a minimal amount of CotG was phosphorylated and exclusively found around the forming spore (Fig. 7C).
Altogether, results of Fig. 7 suggest that at 25°C unphosphorylated CotG molecules accumulate in the cytoplasm sequestering most CotC (and presumably CotU) molecules in well-defined cytoplasmic spots and thus impairing their assembly around the spore. At 42°C less CotG molecules are produced, a minimal amount is phosphorylated and assembled around the spore while the remaining part is unphosphorylated and forms...

Fig. 7. Effect of the sporulation temperature on CotG and CotC assembly.
A. Fluorescence microscopy analysis of strains carrying the cotC::gfp (RH238) or cotG::rfp (RH296) fusion in sporulating cells produced at 25°C or 42°C. The percentage of cells showing the indicated fluorescence is reported in Supplementary Material Table S3. Representative examples are reported for each experiment and strain. The exposure time was 500 ms in all cases. Scale bar 1 μm.
B. cotG-directed β-galactosidase synthesis during sporulation at 25°C, 37°C and 42°C. Due to the reduced growth rate at 25°C, cells were collected at late times according to Istitato et al. (2020).
C. Immunofluorescence analysis of wild type (PY79) sporangia growing at 25°C or 42°C with anti-PKC antibody. The percentage of cells showing the indicated fluorescence is reported in Supplementary Material Table S3. Representative examples are reported by phase-contrast (PC) and fluorescence microscopy and by merging the images (Merge). In PC panels dotted yellow ovals indicate the position of the forming spore. The exposure time was 200 ms in all cases. Scale bar 1 μm.

Fig. 8. Working model of CotG, CotC and CotU assembly at 37°C (A), in a mutant lacking CotH (B) and in a wild type strain at 25°C (C) or 42°C (D). Phosphorylated and unphosphorylated CotG molecules are boxed in red and yellow respectively.
aggregates with CotC (and presumably CotU), slightly reducing their assembly around the spore.

A model to explain the assembly of CotG and its regulatory role on the assembly of CotC/CotU is proposed in Fig. 8. In a wild type strain growing at 37°C, CotG is readily phosphorylated by CotH originating CotG\textsubscript{36} and CotG\textsubscript{32} (boxed in red in Fig. 8A). Some CotG molecules produced later in sporulation, when the kinase is no longer synthesized, are not phosphorylated and accumulate in the mother cell cytoplasm (boxed in yellow in Fig. 8A), where they form aggregates with CotC/CotU. As a result, both CotG and CotC/CotU are found around the spore and in the cytoplasm with CotG localization depending on its phosphorylation level (and therefore on CotH) and CotC/CotU localization depending on the level of unphosphorylated CotG (Fig. 8A). Under unphysiological conditions (in a mutant lacking CotH), all CotG molecules are unphosphorylated, massively accumulate in the cytoplasm sequestering all CotC/CotU molecules (Fig. 8B), and causing the previously reported negative effect of CotG on CotC/CotU assembly (Saggese et al., 2014). At 25°C CotG is abundant both around the spore and in the cytoplasm, where it sequesters a large part of the CotC/CotU molecules limiting their assembly around the spore (Fig. 8C). At 42°C less CotG is produced but being almost completely unphosphorylated, does not assemble and accumulates in the cytoplasm sequestering some CotC/CotU molecules and thus regulating their assembly around the spore (Fig. 8D).

Conclusions

The main results in this manuscript are that \emph{B. subtilis} controls by CotH-mediated phosphorylation the assembly of CotG and that unphosphorylated CotG accumulates in the mother cell cytoplasm forming aggregates with CotC and CotU. These results provide a possible mechanism to explain how \emph{B. subtilis} builds different spores at different temperatures with those produced at 25°C being lamellar and heavily electron dense and those produced at 42°C appearing granular and thick (Isticato et al., 2020).

It has been previously proposed that CotH, a thermo-labile kinase, phosphorylates CotG and CotB that are then readily assembled on the forming spores and contribute to the structure/function of the 25°C spore (Isticato et al., 2020). At 42°C CotH is almost totally absent/inactive, its phosphorylation targets, CotG and CotB, are almost undetectable on the spore surface where are replaced by other coat components, including CotC and CotU (Isticato et al., 2020). Results reported here suggest that unphosphorylated CotG forms aggregates with CotC and CotU, sequesters them in the mother cell cytoplasm and renders them unavailable for coat assembly. Since the level of phosphorylated/assembled or unphosphorylated/unassembled CotG depends on the action of the thermo-sensor CotH, a hierarchical model is proposed to explain the effects of the temperature on the formation of the spore surface, with the temperature acting on cotG transcription (by still unexplored mechanism) and on the thermo-labile kinase CotH that controls its substrate CotG that, in turn, controls the assembly of CotC/CotU.

The unphosphorylated CotG accumulated in the cytoplasm has a strong positive charge [Net Charge Density (NCD) = 0.30 as defined by Ma et al., 2020] that may be responsible for the formation of the cytoplasmic aggregates. This hypothesis is supported by the previous observation that a mutant form of CotG, deleted of the central region where most of the positively charged amino acids are concentrated, and showing an NCD = 0.15, does not have any negative effect on the assembly of CotC/CotU (Saggese et al., 2016).

CotG of \emph{B. subtilis} is not highly conserved but a CotG-like protein, with a positive charge and a modular structure similar to those of CotG, is common in almost all \emph{Bacillus} species containing a CotH orthologue (Saggese et al., 2016). CotG and CotH are functionally linked to each other and their coding genes have a conserved organization with cotG entirely contained between the promoter and the coding part of cotH (Giglio et al., 2011), suggesting that the two proteins may have a similar role in the structure and function of the \emph{Bacillus} spore. Understanding whether the CotH-CotG system responds to the temperature and influences the spore surface structure also in other \emph{Bacillus} species, confirming the role of the positive charge of CotG in the formation of aggregates and clarifying the mechanisms by which cotG transcription is controlled by the temperature will all be challenging tasks of future research projects.

Experimental procedures

\textbf{Bacterial strains and molecular procedures}

\textit{Bacillus subtilis} strains are listed in Table S1 and are all isogenic derivatives of strain PY79 (Youngman et al., 1984). Plasmid amplification for nucleotide sequencing, subcloning experiments, and transformation of \emph{Escherichia coli} competent cells were performed with \emph{E. coli} strain DH5\textalpha. Bacterial strains were transformed by previously described procedures: CaCl\textsubscript{2}-mediated transformation of \emph{E. coli} cells (Sambrook et al., 1989), competent cells and two-step transformation of \emph{B. subtilis} (Cutting and Vander Horn, 1990). DNA ligation, isolation of plasmids and restriction digestion were performed by using standard methods. Chromosomal DNA extraction
B. subtilis was carried out as described previously (Cutting and Vander Horn, 1990).

B. subtilis strain construction

- strain expressing cotG::gusA and cotH::lacZ fusions

A 406 bp DNA fragment containing the cotG promoter region and the region coding for 40 amino acids (from the N-terminal end) was PCR amplified using chromosomal DNA of the PY79 strain as a template and priming the reaction with oligonucleotides H2 and G2 (Table S2). The PCR product was cloned in the pTOPO vector (Invitrogen) yielding plasmid pFV200. The gusA gene (1812 bp) was excised from the pMLK83 plasmid (Karow and Piggot, 1995) by EcoRI digestion and cloned in pFV200 linearized by EcoRI digestion in frame with cotG codons, yielding plasmid pFV201. The gene fusion was then moved into plasmid pER82 (Ricca et al., 1992) by subcloning into the BamHI restriction site. The resulting plasmid, pFV202, carried the gene fusion and a kanamycin-resistance gene between two parts of the amyE gene of B. subtilis. This plasmid was analysed by restriction analysis, linearized by digestion with PstI and used to transform competent cells of the B. subtilis strain PY79. Kanamycin-resistant clones were the result of a double-crossover recombination event, resulting in the chromosome. All Kanamycin-resistant clones were tested by PCR using chromosomal DNA as a template and oligonucleotides AmyS and AmyA (Table S2) to prime DNA amplification. One positive clone (GC223) was used to extract the chromosomal DNA that was used to transform competent cells of strain GC222 carrying the cotH::lacZ fusion integrated at the cotH locus (Baccigalupi et al., 2004) and yielding strain GC224 (cotH::lacZ cotG::gusA).

- strain expressing the cotG::rfp fusion in a mutant lacking CotG and CotH

Chromosomal DNA-mediated transformation of competent cells of the B. subtilis strain AZ603 (ΔcotG ΔcotH::neo) (Saggese et al., 2014) with chromosomal DNA of B. subtilis strain RH296 (amyE::cotG::rfp) (Pettrillo et al., 2020) was used to yield strain RH298 (ΔcotG ΔcotH::neo amyE::cotG::rfp).

- strain expressing the cotG::rfp and cotC::gfp fusions in a mutant lacking CotG and CotH

Chromosomal DNA-mediated transformation of competent cells of the B. subtilis strain RH406 (amyE::cotG::rfp cotC::gfp) (Pettrillo et al., 2020) with chromosomal DNA of B. subtilis strain AZ603 (ΔcotG ΔcotH::neo) (Saggese et al., 2014) was used to yield strain AZ720 (ΔcotG ΔcotH::neo amyE::cotG::rfp cotC::gfp).

- strain expressing the cotA::gfp in a mutant lacking CotH

Chromosomal DNA-mediated transformation of competent cells of the B. subtilis strain AZ565 (cotA::gfp) (Isticato et al., 2013) with chromosomal DNA of B. subtilis strain AZ607 (ΔcotG ΔcotH::neo amyE::cotG) (Saggese et al., 2014) was used to yield strain RH240 (ΔcotG ΔcotH::neo amyE::cotG cotA::gfp).

β-Galactosidase and β-glucuronidase assay

The specific β-galactosidase activity was determined using o-Nitrophenyl-β-D-Glucuronide as substrate. One millilitre of cotH::lacZ-bearing cells was collected, during sporulation, at the times indicated, and assayed as previously described (Ricca et al., 1992). The specific β-glucuronidase activity was determined using p-Nitrophenyl-β-o-Glucuronide as substrate, as previously reported (Melville et al., 1994). One millilitre of cotG::gusA-bearing cells was collected, during sporulation, at the times indicated, and assayed as previously described (Melville et al., 1994).

Spore coat proteins extraction and Western blot analysis

Sporulation of wild-type and recombinant strains was induced in Difco sporulation medium at 25°C, 37°C or 42°C, with vigorous shaking. During sporulation, at different times, forespores or mature spores were collected as described in Istiticato et al. (2004) and washed four times with distilled water and purified as described in Nicholson and Setlow (1990). Spore coat proteins were extracted from a suspension of 1 × 10⁸ mature spores or from a suspension of forespores fractions, by treatment at 65°C in SDS-DTT. The extracted proteins were quantified through a Bio-Rad DC protein assay kit (Bio-Rad), then 20 μg of total spore coat proteins were fractionated on 15% polyacrylamide gels and electro-transferred on a nitrocellulose filter (Bio-Rad) for Western blot analysis. Monoclonal antibodies were used at working solution of 1:7,000 for CotG or 1:150 for CotH specific detection. Then, a horseradish peroxidase-conjugated anti-rabbit secondary antibody was used (Santa Cruz). Western blot filters were visualized by the SuperSignal West Pico chemiluminescence (Pierce) method as specified by the manufacturer.

Fluorescence and immunofluorescence microscopy

Fluorescence microscopy analyses were carried out using an Olympus BX51 fluorescence microscope fitted with a 100× objective UPlanF1. FITC (U-MIB2) and TRITC (U-MWIG2) filters were used to detect the green or red fluorescence signals respectively. Images were captured using an Olympus DP70 digital camera equipped with Olympus U-CA Magnification Changer and
processed with Image Analysis Software (Olympus) for minor adjustments of brightness, contrast and colour balance and for creation of merged images. SmillaEnlarger 0.9.0 software was used to increase images quality. Immunofluorescence assay was performed as described in Manzo et al. (2013), with some modifications. One millilitre of sporulating cells was fixed for 1 h at room temperature in 80% methanol, then washed and incubated overnight at 4°C in 100% methanol. Fixed sporangia were permeabilized by incubating samples with a GTE (5% glucose, 0.01 M EDTA pH 8.0, 20 mM Tris–HCl pH 7.5) – 0.2 mg ml⁻¹ lysozyme solution and rapidly placed on a coverslip previously treated for 5 min with poly-L-Lysine. The coverslips were air dried, and cells were pretreated with 1% (wt./vol.) dried milk in phosphate-buffered saline (PBS, pH 7.4), prior to 2 h incubation at 4°C with the monoclonal anti-CotC (1:200), anti-CotG (1:500) or anti-PKC (1:500) primary antibodies. After four washes with PBS, the samples were incubated with a 64-fold diluted anti-rabbit secondary antibody, conjugated with Fluorescein isothiocyanate, FITC (BETHYL) for 2 h at room temperature in the dark. After four washes, the coverslips were mounted onto microscope slides, adding one drop of PBS, and analysed by fluorescence microscopy, as described below. Sporangia with only addition of the secondary-FITC conjugated antibody were used as a control of the specificity of this technique.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Appendix S1:** Supplementary Information.