A protein phosphorylation module patterns the *Bacillus subtilis* spore outer coat

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Abstract

Assembly of the *Bacillus subtilis* spore coat involves over 80 proteins which self-organize into a basal layer, a lamellar inner coat, a striated electrondense outer coat and a more external crust. CotB is an abundant component of the outer coat. The C-terminal moiety of CotB, SKR, formed by serine-rich repeats, is polyphosphorylated by the Ser/Thr kinase CotH. We show that another coat protein, CotG, with a central serine-repeat region, SKR, interacts with the C-terminal moiety of CotB and promotes its phosphorylation by CotH in vivo and in a heterologous system. CotG itself is phosphorylated by CotH but phosphorylation is enhanced in the absence of CotB. Spores of a strain producing an inactive form of CotH, like those formed by a cotG deletion mutant, lack the pattern of electrondense outer coat striations, but retain the crust. In contrast, deletion of the SKR region, has no major impact on outer coat structure. Thus, phosphorylation of CotG by CotH is a key factor establishing the structure of the outer coat. The presence of the cotB/cotH/cotG cluster in several species closely related to *B. subtilis* hints at the importance of this protein phosphorylation module in the morphogenesis of the spore surface layers.

KEYWORDS
CotB, CotG, CotH, protein kinase, protein phosphorylation, spore coat
1 | INTRODUCTION

Bacterial endospores (spores for simplicity) are a dormant cell type formed by a diverse group of bacteria within the Firmicutes phylum. Sporulation occurs within a sporangium formed by a larger mother cell, and a smaller forespore, or future spore. At the end of the differentiation process, and upon lysis of the mother cell, the spore is released into the environment. In Bacillus subtilis, the outermost spore layer is the coat, a protein-bound organelle that protects mature spores and mediates their interaction with abiotic and biotic surfaces and also with germinants (Henriques and Moran, 2007; McKenney et al., 2013; Driks and Eichenberger, 2016). The coat comprises a basal layer, a lamellar inner coat, a striated electrodense outer coat and a more external crust (reviewed by Henriques and Moran, 2007; McKenney et al., 2013; Driks and Eichenberger, 2016). In species of the B. cereus/B. anthracis/B. thuringiensis group, the more external spore layer is an exosporium, formed by a basal layer and a hair-like nap that projects from it; this layer is separated from the coat by an interspace of variable length (Stewart, 2015).

Synthesis of the coat and crust proteins relies on a mother cell type-specific transcriptional cascade involving two RNA polymerase sigma factors and three ancillary transcription factors, in the order $\sigma^E$, SpoIIID and GerR, $\sigma^K$ and GerE (Henriques and Moran, 2007; McKenney et al., 2013; Driks and Eichenberger, 2016). The morplogenetic proteins that govern basal layer (SpoVA and SpoVM), inner coat (SaFA), outer coat (CoTE) and crust assembly (CoTZ), are produced early in development, when the larger mother cell begins engulfment of the smaller forespore, and are recruited to the mother cell proximal forespore pole (MCP) to form an organizational center responsible for the assembly of the various coat sub-layers (McKenney et al., 2013;2010; Shuster et al., 2019). In the second step of coat assembly, termed enencasement, the coat proteins start surrounding the forespore, some tracking the engulfing membranes, others in successive waves during and following engulfment completion; the waves in enccasement are determined by the deployment of the mother cell transcriptional cascade (McKenney et al., 2013).

Self-assembly mechanisms and post-translational modifications of the coat proteins such as proteolytical processing, glycosylation and cross-linking that also play important roles in coat assembly and maturation (McKenney et al., 2013; Driks and Eichenberger, 2016). SpoIVA, for example, is an ATPase that self-assembles into cables, in an ATP-dependent manner, that covers the surface of the forespore to form the coat basal layer (Ramamurthi and Losick, 2008). An important question is how the structural features of the spore surface layers arise from individual components and recent work has shed light onto the assembly of the crust in B. subtilis and the exosporium in the B. cereus/B. anthracis/B. thuringiensis group. Crust and exosporium proteins self-assemble forming two-dimensional sheets with a hexagonal lattice and three-dimensional stacks which reproduce structures seen in mature spores (Ball et al., 2008; Kallas et al., 2011; Jiang et al., 2015). However, how the structural features of the coat layers emerge is unknown.

Here, we are concerned with the formation of the electrodense striated outer coat layer of B. subtilis spores. CotB and CotG are two abundant outer coat components (Donovan et al., 1987; Sacco et al., 1995; Zilhao et al., 2004) and an earlier study has suggested that CotG is an important structural determinant of this layer (Henriques et al., 1998). The cotB and cotG genes are clustered together with a third gene, cotH, which is also an important determinant of outer coat assembly (Sacco et al., 1995; Naclerio et al., 1996; Zilhao et al., 1999; Kim et al., 2006; Saggese et al., 2014) (Figure 1a). CotH has an atypical protein kinase-like (PKL) fold and is distantly related to the Fam20C-family of secretory pathway kinases (Galperin et al., 2012; Nguyen et al., 2016). Both CotB and CotG possess a series of continuous Ser/Lys-rich tandem repeats, CotB in its C-terminal moiety (the SKR$^B$ region) and CotG in its central part (the SKR$^G$ region) (Sacco et al., 1995; Saggese et al., 2014) (Figure 1b,c). Although CotB is synthesized as a 46 kDa species (CotB-46) the main form of the protein detected in mature spores has an apparent mass of 66 kDa (CotB-66) (Sacco et al., 1995; Naclerio et al., 1996; Zilhao et al., 2004). Accumulation of CotB-66 in spores requires both CotH and CotG (Zilhao et al., 2004). CotB is phosphorylated in the SKR$^B$ region (Saggese et al., 2014; Rosenberg et al., 2015; Nguyen et al., 2016; Ravikumar et al., 2018). It is likely that CotB-66 is a polyphosphorylated form of the protein; not only is CotB-46 phosphorylated by CotH in vitro to produce a species with an apparent mass of about 66 kDa, but a phosphorylated protein at about 66 kDa is detected in extracts from WT spores but not in those obtained from a cotH mutant (Nguyen et al., 2016). CotG is also phosphorylated in the SKR$^G$ region during spore coat assembly and a peptide derived from SKR$^G$ is phosphorylated by CotH in vitro (Saggese et al., 2014; Nguyen et al., 2016). Thus, the kinase activity of CotH is required for the phosphorylation of CotG and CotB, and possibly of additional spore coat proteins (Nguyen et al., 2016).

The cotB/cotH/cotG cluster is found in several species closely related to B. subtilis and also in Geobacillus (Sacco et al., 1995; Naclerio et al., 1996; Todd et al., 2003; McPherson et al., 2010; Saggese et al., 2016). While CotB homologues are found in most spore-forming Bacilli, CotG-like proteins are found in nearly all spore-forming species that also code for a CotH homologue (Galperin et al., 2012; Saggese et al., 2016). In the B. cereus group, for instance, ExsB, a CotG-like protein required for attachment of the exosporium to the coat, is phosphorylated at multiple Thr residues within a central repeat region; this phosphorylation most likely relies on a CotH homologue (McPherson et al., 2010; Nguyen et al., 2016). Importantly, CotG was also proposed to be a crust component in B. subtilis (McPherson et al., 2010). Two CotB paralouges are also found in the coat/exosporia of B. cereus/B. anthracis/B. thuringiensis spores (Abhyankar et al., 2013; Abhyankar et al., 2017), suggesting that the cotB/cotG/cotH cluster may participate in determining the structural pattern seen in spores of these organisms: a thin outer coat/interspace/exosporium versus the thick outer coat/crust architecture of B. subtilis spores.

Previous studies on the characterization of the cotB/cotH/cotG cluster in B. subtilis made use of polar insertional mutations, and it is unclear how the absence of active CotH impacts the overall structure of the coat and what is the contribution of phosphorylated CotG and CotB. Also, unclear is how CotG influences the phosphorylation
We now show that CotG is required for the efficient phosphorylation of CotB-46 by CotH, both during coat assembly and also in a heterologous system. Conversely, phosphorylation of CotG in a CotH-dependent manner is favored in the absence of CotB-46. We show that CotG interacts with the C-terminal region of CotB and we propose that this interaction promotes phosphorylation of CotB-46, while inhibiting the phosphorylation of CotG in the presence of CotH. We show that phosphorylation of CotB-46 and CotG occurs at the surface of the developing spore. While deletion of the SKRB region has no impact on the structure of the coat, we show that mutants lacking active CotH or CotG form an amorphous outer coat which is still delimited at its outer edge by the crust. Thus, CotH and CotG establish the normal striated pattern of the outer coat. Strikingly, the outer coat region of cotH or cotG mutants resembles the interspace and exosporium of B. cereus/B. anthracis/ B. thuringiensis spores, suggesting that CotH and CotG may have a role in determining the structural and functional features of the spore surface layers in a wide range of organisms.

2 | RESULTS

2.1 | Phosphorylation sites in CotB and CotG

A striking feature of CotB and CotG is the presence in the C-terminal half of CotB and in the central part of CotG, of direct repeats of a...
sequence rich in Ser, Lys and Arg residues (Sacco et al., 1995; Giglio et al., 2011; Saggese et al., 2016) (Figures 1 and S1). The SKR^G region, in CotB, is formed by four direct repeats whereas SKR^G, in CotG, is formed by 19 direct repeats of five to seven amino acids (Sacco et al., 1995; Giglio et al., 2011; Saggese et al., 2016; Uversky, 2017). Both SKR^G and SKR^G are likely to be disordered (Figure S1) (Romero et al., 1997; Liu and Huang, 2014). Moreover, the Ser residues, but only once per molecule. However, the dramatic shift of CotB-46 to CotB-66 in the presence of CotG and CotH suggests that CotB is polyphosphorylated. Here, we were able to show multiple serine phosphosites in a single peptide, demonstrating the occurrence of multiple phosphosites within one CotB molecule (Figure 1d; see also Table S4).

2.2 The kinase activity of CotH explains its morphogenetic role

Using a phosphospecific antibody raised against the consensus phosphorylation site recognized by protein kinase C (PKC), Nguyen and co-authors showed the presence of phosphorylated proteins in spore coat extracts (Nguyen et al., 2016). Bands of about 30 kDa, inferred to be CotG and of 66 kDa inferred to correspond to CotB-66, were detected in extracts from WT spores, and in spores formed by a cotH mutant expressing WT cotH in trans; these bands were not detected in spores producing catalytically inactive forms of CotH from the same ectopic site in the chromosome, or in spores of a cotH null mutant (Nguyen et al., 2016). Additional phosphorylated species were detected, suggesting that CotH could phosphorylate other coat proteins (Nguyen et al., 2016), presumably those known to be dependent on CotH (see below). However, in the study of Nguyen and co-workers, a Coomassie-stained gel of coat extracts was not shown, precluding an evaluation of the impact of the catalytic mutants on the overall assembly of the coat. To more directly test the impact of CotH activity on coat assembly, we first constructed a strain producing a catalytically inactive form of CotH. Previous work has shown that the substitution of Asp228 in CotH by a Gln, renders the protein inactive (Nguyen et al., 2016; see also below). Here, the WT or a cotH^D228Q alleles were inserted at the non-essential amyE locus in a cotH insertion mutant; both alleles were placed under the control of the e^K-dependent promoter known to drive expression of cotH and which is located downstream of the 3’-end of cotG (Giglio et al., 2011) (Figures 1a and 2a). To avoid insertion of a second copy of cotG at amyE, the region corresponding to the long 5’-untranslated region of the cotH mRNA was not included in our construct (Figure 2a). Deletion of this region, however, does not affect the accumulation of CotH or spore coat formation (Giglio et al., 2011).

The coat protein profile of a cotH insertion mutant was originally established after SDS/DTT extraction of the spore coat proteins (Naclerio et al., 1996). At least nine proteins are absent from spores of a cotH insertion mutant, including CotG-36 and CotB-66, CotS (41 kDa), CotSA (42.9 kDa), CotQ (50 kDa), CotU (11.6 kDa) and CotC (14.8 kDa), YusA (30.4 kDa) and CotZ (16.5 kDa; but see below) (Naclerio et al., 1996; Zilhao et al., 1999; 2004; Baccigalupi et al., 2004; Kim et al., 2006; Giorno et al., 2007; see also below), or of CotH itself was not detected, at any time point, in our analysis (Table S4). At hour 5 of sporulation, pSer was identified for at least eight positions in the SKR^G region of CotG, some of which within the phosphorylated peptides detected by Saggese and co-authors, who were unable to identify the specific phosphosites (Saggese et al., 2014) (Figure 1c and Table S4). Four of the pSer sites and additionally Thr147, were also found in other studies (Saggese et al., 2014; Nguyen et al., 2016; Ravikumar et al., 2018) (Figure 1c). In addition, four pSer and three pTyr were detected outside the SKR^G region (Saggese et al., 2014; Ravikumar et al., 2018). Thus, at least 14 residues of CotG can be phosphorylated.

For CotB, also at hour 5, we identified 30 pSer, one pTyr and one pArg residue, all of which in the SKR^B region (Figure 1c). Of those, 12 pSer were also detected in other studies, along with a different pTyr residue (Rosenberg et al., 2015; Ravikumar et al., 2018). CotB was also found to be phosphorylated at two other Ser residues upstream of the SKR^B region (Ravikumar et al., 2018). Thus, CotB can be phosphorylated at a minimum of 35 residues, including 30 Ser residues within the SKR^G region (Figure 1c). To date, site-specific phosphorylation of CotB was only shown for a single phosphosite per molecule (Saggese et al., 2014; Nguyen et al., 2016; Ravikumar et al., 2018), leaving the possibility that CotB could be phosphorylated at several serine residues, but only once per molecule. However, the dramatic shift of CotB-46 to CotB-66 in the presence of CotG and CotH suggests that CotB is polyphosphorylated. Here, we were able to show multiple serine phosphosites in a single peptide, demonstrating the occurrence of multiple phosphosites within one CotB molecule (Figure 1d; see also Table S4).
in SDS/DTT coat extracts; presumably not sufficient protein is extracted by this method to allow detection by our antibody. For reference, however, an SDS-PAGE analysis of coat extracts obtained from cotH and cotH<sup>D228Q</sup> spores by SDS/DTT extraction is shown in Figure S2a (see also the Supporting Information). The collection of coat proteins obtained from cotH spores following NaOH extraction revealed the absence of CotB-66, CotG-36 and CotC and at least four other proteins (Figure 2b, red, grey and blue arrows in the top panel). In addition, at least three proteins were more extractable from cotH or cotH<sup>D228Q</sup> spores (Figure 2b, brown arrows). Importantly, the pattern of extractable coat proteins from cotH or cotH<sup>D228Q</sup> spores was nearly identical, and insertion of the WT cotH
allele at amyE restored the WT pattern to either mutant, as assessed by Coomassie staining (Figure 2b, top). Spores of a cotH insertion mutant as well as those of the catalytically inactive cotHD228Q are impaired in L-alanine-triggered spore germination (Naclerio et al., 1996; Nguyen et al., 2016). A mixture of L-Asn, glucose, fructose and KCl (AGFK) activates a second pathway of nutrient-induced spore germination (Setlow, 2014). We show that cotH or cotHD228Q spores are impaired in AGFK-triggered spore germination and that complementation with the WT allele restores normal germination (Figure S2B). Thus, the kinase activity of CotH influences the two known pathways of nutrient-induced spore germination (Setlow, 2014; Setlow et al., 2017).

Consistent with previous results (Giglio et al., 2011), immunoblot analysis of coat extracts prepared from purified spores shows that the level of coat-associated CotHWT produced from the amyE locus under the control of the native cotH promoter was similar to that found for a WT strain (Figure 2b, bottom panel). We note, however, that in the complementation strain the level of CotB-66 and CotG-36, is increased (Figure 2b, blue and grey arrows in the upper panel). It seems plausible that a slight increase in the level of CotH produced from amyE, not perceptible by immunoblot analysis, accounts for the increase in the representation of CotB-66 and CotG-36. Importantly, the level of coat-associated CotH WT was only slightly lower that of the WT protein, indicating that the activity of CotH is not required for its own assembly (Figure 2b). In contrast, the catalytic inactive CotHD228Q was not incorporated into the coat, unless overproduced from stronger promoters (Nguyen et al., 2016). Therefore, the cotHD228Q mutant may more accurately reflect the impact of the absence of active CotH on the overall assembly and properties of the spore coat. While it seems plausible that the assembly of the CotH-controlled proteins requires their phosphorylation by CotH, the possibility that CotH acts as a priming kinase for another, as yet unknown kinase has been raised and cannot presently be excluded (Nguyen et al., 2016).

### 2.3 | CotG is required for the phosphorylation of CotB-46 in spores

CotH catalyzed incorporation of 32P from [γ-32P]-ATP into purified CotB-46, shifting its SDS-PAGE mobility to the 66 kDa region of the gel (Nguyen et al., 2016). Thus, in the spore coat, CotB-66 is also likely to arise through extensive phosphorylation of CotB-46, an inference in line with the detection of a phosphorylated species of about 66 kDa in spores of strains producing CotHWT but not CotH Δ cotH228Q (Nguyen et al., 2016). A phosphorylated species inferred to be CotG was also detected (Nguyen et al., 2016). In this study, we wanted to examine extracts of a cotG mutant, since CotG is known to be required for the formation of CotB-66 in spores (Naclerio et al., 1996; Zilhao et al., 2004), whereas in the study of Nguyen and co-authors CotB-66 could be formed through direct phosphorylation of purified CotB-46 by CotH, that is, in the absence of CotG (Nguyen et al., 2016). Coat protein extracts were prepared from purified spores of several strains by NaOH treatment, analyzed by SDS-PAGE and Coomassie staining and additionally by immunoblotting with anti-phosphoserine (pSer), anti-CotB and anti-CotG antibodies before and after incubation with alkaline phosphatase. CotB-66 was detected in Coomassie-stained gels of WT extracts but was absent from cotG, cotH (in these two mutants only CotB-46 is detected) and cotB spore coat extracts (Naclerio et al., 1996; Zilhao et al., 2004) (Figure 2c, top panel). CotB-66 reacted with the anti-pSer and anti-CotB antibodies (Figure 2c, two middle panels). A form of CotB, CotB-34, detected by immunoblotting in all the extracts except in those from cotB and cotH mutants, did not react with anti-pSer (Figure 2c); it may correspond to a proteolytic fragment encompassing the N-terminal moiety of the protein (expected size of about 28.7 kDa; see also below). Treatment of the WT spore coat extracts with calf intestinal phosphatase (AP) caused disappearance of CotB-66 from the Coomassie-stained gels and from the immunoblots in which anti-CotB or anti-pSer antibodies were used (Figure 2c). Concurrently, AP treatment resulted in the appearance of CotB-46, which reacted with the anti-CotB antibody but not with anti-pSer (Figure 2c). The presence of CotB-66 was restored in the cotH complementation strain and treatment with AP strongly reduced its level; because the level of CotB-66 is higher in this strain than in the WT, however, the AP treatment did not completely convert CotB-66 into CotB-46. Rather, it resulted in a smear corresponding to several bands below CotB-66, which reacted both with anti-CotB and anti-pSer antibodies (Figure 2c). No form of CotB reacted with the anti-pSer or the anti-CotB antibodies in the coat extract prepared from cotH or cotHD228Q spores (Figure 2c). Furthermore, in the cotG mutant only CotB-46 form is detected with the anti-CotB antibody and no form of CotB reacted with the anti-pSer antibody (Figure 2c). Together, these results indicate that CotB-46 is not phosphorylated (or its phosphorylation is below our detection level), and that CotB-66 is formed from CotB-46 through CotH-mediated phosphorylation, which is promoted by CotG.

In the WT, CotG is detected in coat extracts as a diffuse band around 36 kDa (CotG-36) when extracts are prepared by SDS/DTT treatment (Naclerio et al., 1996, Zilhao et al., 2004; see also Figure S2a). Following NaOH treatment CotG-36 is only weakly detected by Coomassie staining (Figure 2b,c, top) but immunoblotting with an anti-CotG antibody reveals its presence in the extracts (Figure 2c, bottom panel). CotG-36 was also not detected by the anti-pSer antibody (Figure 2c). Nevertheless, CotG-36 may correspond to a phosphorylated form of CotG because it is no longer detected by immunoblotting following AP treatment of the extracts prepared from WT spores (Figure 2c, lane 2). Several bands are detected below CotG-36 in the ΔcotH complemented by the cotHWT at amyE after AP treatment (Figure 2C, lane 8); this again suggests that CotG-36 is phosphorylated but that the AP treatment was not complete. For this strain, however, the signal for CotG-36 is also stronger than for the WT (Figure 2c, compare lanes 1 and 6). Also, in cotB spores and also in the cotH inser- tional mutant complemented with WT cotH at amyE, at least one additional anti-CotG-reactive band is detected above CotG-36.
In an attempt to determine whether full-length CotG could be phosphorylated by CotH, at least in the absence of CotB, we first overproduced CotHWT or CotHD228Q with a C-terminal Strep-tag in E. coli, and the two proteins were affinity purified (see Material and Methods). The presence of the Strep-tag did not impair kinase activity, as shown by the formation of CotB-66 when cotH was induced together with cotB and cotG (Figure S4). Purified CotHWT but not CotHD228Q showed auto-phosphorylation activity in the presence of [γ-32P]ATP (Nguyen et al., 2016) (Figure S5a). Moreover, CotHWT but not CotHD228Q showed trans-phosphorylation activity when incubated with purified CotB-46 in the presence of [γ-32P]ATP (Figure S5b). Although labeling of CotB-46 was detected, formation of CotB-66 was not, presumably because under our experimental conditions the degree of phosphorylation of CotB-46 in the absence of CotG is insufficient to alter its electrophoretic mobility (Nguyen et al., 2016; Figure S5b). Labeled CotHWT was only detected in the absence of CotB-46, suggesting rapid transfer of phosphoryl groups to CotB. Both the auto- and trans-phosphorylation activities of CotH were insensitive to Staurosporine (Ruegg and Burgess, 1989; Xiao et al., 2013; see also the Supporting Information).

To test whether CotH could directly phosphorylate CotG, we used immunoblotting to monitor the accumulation of CotG over time following IPTG induction of cotG alone or upon co-induction of cotH (Figure 4). Under these conditions, CotG was initially detected as a species of about 34 kDa (Figure 4, red arrow in the two panels) that may correspond to the un-phosphorylated form of the protein. Over time, in the presence of CotHWT, but not in the presence of CotHD228Q, CotG-36 accumulated (Figure 4, blue arrow). Additional species, with an apparent molecular weight greater than 36 kDa, were also detected upon prolonged incubation with CotHWT (Figure 4b, black arrows). Importantly, formation of all the
forms of CotG above CotG-34 decreased when cotB was co-induced (Figure 4b). We conclude that full-length CotG is directly phosphorylated by CotH. Moreover, phosphorylation of CotG appears less efficient in the presence of CotB.

### 2.6 CotG interacts with the C-terminal region of CotB

We have shown before that CotB and CotG self-interact and that CotB interacts with CotG (Zilhao et al., 2004). That the SKR region of CotB is the site of CotB-46 phosphorylation, together with the requirement for CotG for the formation of CotB-66, suggested to us that CotG could specifically interact with the C-terminal moiety of CotB. To test this possibility, we used a GAL4-based yeast two-hybrid interaction assay (Zilhao et al., 2004). We fused the entire coding sequence of CotB (CotBFL) or CotG to either the activation (AD) or the DNA-binding domain (BD) of GAL4 yeast transcriptional activator GAL4 (Table 1). In addition, the sequences coding for the N- (CotBN, residues 1-213) or C-terminal (CotB C, residues 195-380) regions of CotB FL were also fused to both the AD and BD of GAL4. Interactions between the various fusion proteins were assessed in a

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**FIGURE 3** CotG is necessary and sufficient for the efficient phosphorylation of CotB-46 by CotH. CotHWT (a) or CotHD228Q (b) were overproduced from a T7lac promoter in E. coli strains alone or together with the indicated proteins (+ signs). The strains were grown in autoinduction medium, whole cell extracts prepared and the proteins resolved by SDS–PAGE. Coomassie stained gels (top) and the immunoblot analysis with anti-CotB, anti-pSer and anti-CotG antibodies, as indicated, are show for both (a) and (b). The position of relevant species is indicated on the right side of the panel. The parentheses in (a) and (b) (middle panels) indicates possible degradation forms of CotB. See also Figure S3, which shows the same gels stained with Sypro Ruby or Pro-Q Diamond. In panels (a) and (b): blue arrow, CotB-66; red arrow, CotB-46; brown arrow, a form of CotB with a mobility between that of CotB-46 and CotB-66; black arrows, species with an apparent molecular weight higher than that of CotB-66; green arrow, CotHWT (or CotHD228Q); light grey, CotG-34; dark grey, CotG-36. The position of molecular weight markers is shown on the left side of all panels [Colour figure can be viewed at wileyonlinelibrary.com]
yeast reporter strain using a colony lift assay that monitors expression of the \textit{lacZ} gene (Zilhao et al., 2004). Under our experimental conditions, background levels of \(\beta\)-galactosidase activity, determined by co-transforming the same cells with the two empty vectors, were negligible (Table 1). Also, no \(\beta\)-galactosidase activity was detected when individual fusion proteins were expressed with the corresponding empty vector control. We found an interaction between \(CotB^{FL}\) and itself and a stronger interaction of \(CotB^{N}\) with itself (Table 1). This suggests that the SKR\(B\) region of \(CotB\) somehow reduces the ability of the N-terminal moiety to self-interact. In contrast, we found no evidence for an interaction of \(CotB^{C}\) with itself. Both \(CotB^{FL}\) and \(CotB^{C}\) interacted with \(CotG\), but \(CotB^{N}\) did not (Table 1). Thus, it is the C-terminal moiety of \(CotB\) that interacts with \(CotG\). Possibly, \(CotB\) interacts with \(CotG\) via the SKR\(B\) region, and this interaction may be part of the mechanism by which \(CotG\) promotes phosphorylation of \(CotB^{46}\). It also seems possible that this interaction somehow reduces the ability of \(CotH\) to phosphorylate \(CotG\).

### 2.7 The kinase activity of \(CotH\) patterns the spore outer coat

Deletion of \(cotH\) brings about drastic alterations on the ultrastructure of the spore outer coat as viewed by transmission electron microscopy (TEM) (Zilhao et al., 1999). We examined spores of the \(\Delta cotH\) and \(cotHD228Q\) mutants along with spores of the congenic WT strains, by TEM. In both the WT and in the \(\Delta cotH\) mutant complemented by the WT \(cotH\) allele at \(amyE\), the surface layers show the expected structural features: a lamellar inner coat, an outer coat formed by electrodense striations and a well-defined crust region at the edge of the spore (Henriques et al., 1998; Figure 5, first two top panels). In both \(cotG\) and \(cotHD228Q\) mutants, and although the region normally occupied by the outer coat had approximately the same width as in WT spores, the electrodense outer coat striations were absent and replaced by partially structured material (Figure 5). The outer edge of the coat, however, showed a defined contour. In addition, the inner coat lamellae appeared reduced in both mutants (Figure 5).

### TABLE 1 Detection of \(lacZ\) transcription by colony lift assays in yeast diploid strains Y187/Y190 containing fusions of products encoded by \(cotG\), \(cotH\) and full-length, N- or C-terminal regions of \(cotB\) to GAL4 activation and binding domains

| Activation domain fusion\(^a\) | DNA-binding domain fusion\(^b\) |
|-------------------------------|-------------------------------|
| pACT-2 (AD)                   | pAS2-1 (BD) (CotB-FL-BD)     |
| pACT-B/(CotB-FL-AD)           | pAS-B (CotB-N-BD)            |
| pAC-BN/(CotB-N-AD)            | pAS-BN (CotB-C-BD)           |
| pAC-BC/(CotB-C-AD)            | pAS-G (CotG-BD)              |
| pAC-G/ (CotG-AD)              | pAS-H (CotH-BD)              |

\(^a\)Description of plasmid constructs in pACT-2 (contains GAL4 activation domain (AD)) that were transformed into yeast strain Y190 (Clontech).

\(^b\)Description of plasmid constructs in pAS2-1 (binding domain (BD) that were transformed into yeast strain Y187 (Clontech).

\(^c\)\(=\) represent the time for detection of blue colonies on a colony lift assay for \(lacZ\) expression; +++, + and − indicates development of color in 30 min, 1 hr, and greater than 1 hr, respectively, as determined by testing three independent colonies for each pairwise combination.
The structure of the coat in the 
cotH or 
cotHD228Q mutants is reminiscent of that reported for spores of a 
cotG insertional mut - 
tant, in which the electrodense outer coat striations are replaced 
by partially structured material, but the outer edge of the coat re -
 mains delimited by a thin well-defined layer (Henriques et al., 1998). 
Thus, 
cotG was proposed to be an important structural organizer of 
the spore outer coat. As already mentioned, however, the 
cotH pro -
moter is now known to be located downstream of the 3′-end of 
cotG and 
cotG insertional mutations predictively exert a polar effect on 
cotH expression (Giglio et al., 2011) (Figure 1a). We, therefore, con-
structed a strain bearing an in-frame 
cotG deletion and characterized 
the spores formed by this new mutant by TEM. We found that spores 
of the new 
cotG deletion mutant to be essentially indistinguishable 
from those of the insertional mutant used before (Henriques et al., 1998) 
(Figure 5). Together, these results not only support the origi-
nal conclusion that CotG is a key organizer of the spore outer coat 
(Henriques et al., 1998) but further suggests that the kinase activity 
of CotH is required for proper formation of the spore outer coat, via 
cotG. Possibly, the phosphorylation of CotG stabilizes the protein, 
while allowing it to promote formation of the structural pattern nor-
mally seen for the outer coat. Both the phosphorylation of CotG and 
CotB-46 are likely to occur mostly at the spore surface (Figure S6; 
see also the Supporting Information).

2.8 | The SKR^B region is dispensable for proper coat 
morphogenesis

Because CotB-66 is not formed in cotG mutants, we reasoned that 
the CotH-dependent phosphorylation of CotB-46, promoted by 
CotG, could also be a key determinant for the structural organiza-
tion of the outer coat. If so, then spores of a cotB mutant could share 
some of the structural features observed for cotH or cotG spores. 
To test this, we first constructed a cotB in-frame deletion mutant 
and examined the spores produced by this strain by TEM. We found 
that spores of a ΔcotB mutant had a thinner outer coat but that the 
pattern of electrodense striations was retained (Figure 5, red arrow). 
This suggests that phosphorylation of CotB-46 is not a main deter-
minant of the structural organization of the outer coat.

To further test this inference, we constructed strains bearing 
an in-frame deletion of the cotB gene and either a WT cotB allele 
or an allele with an in-frame deletion of the SKR^B region at amyE

**FIGURE 5** CotH patterns the spore outer coat. Transmission electron microscopy of spores produced by the following strains, as indicated: wild type (wt), ΔcotH/cotH^wt, ΔcotH, ΔcotH/cotH^D228Q, ΔcotG, ΔcotB and ΔcotB complemented in trans with either WT cotB or cotB^SKR, ΔcotXYZ and ΔcotG/ΔcotXYZ. Spores were collected from DSM cultures 24 hr after the initiation of sporulation. The two bottom panels show a field of ΔcotG/ΔcotXYZ spores (left) and higher magnification images (two last panels on the right) of the region encircled. The red arrow points to the outer coat or electrondense outer coat material, the yellow arrow to the inner coat, and the blue arrow to the crust region. The green arrows in the ΔcotXYZ panel point to discontinuities in the outer coat layer [Colour figure can be viewed at wileyonlinelibrary.com]
(Figure S7a,b). Analysis of the proteins extractable from the coat of ΔcotB/cotBWT and ΔcotB/cotBΔSKR spores show a similar collection of proteins except for the absence of CotB-66 from spores of the latter strain, an observation confirmed by Immunoblot analysis (Figure S7c). Thus, as previously reported for a cotB insertional allele (Naclerio et al., 1996; Zilhao et al., 2004), neither the ΔcotB nor the cotBΔSKR alleles cause gross alterations in the composition of the coat. Spores of the ΔcotB mutant with either WT cotB or cotBΔSKR in trans were then analyzed by TEM (Figure 5). Both showed an electro dense striated outer coat and appeared indistinguishable from WT spores (Figure 5). Thus, the SKRΔ region and hence phosphorylation of CotB-46, is not a pre-requisite for the structural organization of the outer coat. By comparison with the thinner outer coat of ΔcotB spores, we infer that the CotB NTD contributes to the normal thickness of the spore outer coat.

2.9 | CotH activity is not essential for crust assembly

A thin, well-defined layer forms the outer edge of the coat in ΔcotH, ΔcotB or ΔcotG mutants (Henriques et al., 1998; Zilhao et al., 1999; Figure 5). This structure could correspond to the crust, or a crust basal-layer. Assembly of the crust is dependent on the outer coat morphogenetic proteins CotO and CotE (McKenney et al., 2010; Plomp et al., 2014; Krajcikova et al., 2017; Bartels et al., 2019; Shuster et al., 2019); CotE also controls CotH assembly (Naclerio et al., 1996; Zilhao et al., 1999; Isticato et al., 2015). Yet, at least the crust protein CotW is largely independent of CotH for assembly (Kim et al., 2006; Shuster et al., 2019). To test whether CotH was required for assembly of the crust, and since CotZ is at the top of the hierarchy for crust assembly (Imamura et al., 2010; McKenney et al., 2010; Bartels et al., 2019; Shuster et al., 2019), we examined the localization of a CotZ-GFP fusion in spores of the WT and in spores of the ΔcotH, ΔcotG and ΔcotB mutants. We found that CotZ-GFP formed a complete ring of fluorescence around 62% of the WT spores scored, and a polar cap in 38% of the spores (Figure 6). These numbers did not differ much for the cotHD228Q, ΔcotB, ΔcotG or ΔcotH spores, suggesting that in these mutants the crust is still assembled. We note, however, that in spores of the strain bearing the wt cotH allele at amyE, the cap pattern of CotZ-GFP fluorescence was reduced to 13%, while the complete circle pattern increased to 87% (Figure 6). The wt cotH allele at amyE complements a cotH deletion for spore morphogenesis (Giglio et al., 2011; see also above and Figure 5, three first panels). Moreover, expression of cotH from amyE did not affect the accumulation of CotH as assessed by immunoblot analysis (Giglio et al., 2011; see also Figure 2B). However, and as mentioned above, an increase in the level of CotB-66 and CotG-36 was noticed in extracts from ΔcotH/cotHWT spores (Figure 2B). It seems possible that a slight increase in cotH expression at amyE, not detected by immunoblot analysis, alters crust assembly (promoting formation of the full circle pattern of CotZ-GFP), as assessed using a GFP fusion. In any event, while largely dispensable for crust assembly, CotH may somehow influence formation of this structure.

We introduced a deletion of the cotX, cotY and cotZ genes (ΔcotXYZ) (Zhang et al., 1993) into the ΔcotG mutant, as an independent test of the idea that the thin structure seen at the edge of ΔcotG spores corresponds to the crust. As a control, we also examined spores of the ΔcotXYZ mutant. As previously reported (Zhang et al., 1993), the outer coat of ΔcotXYZ spores is less organized and

**FIGURE 6** Localization of CotZ-GFP in mature spores. Spores of the indicated mutants expressing a cotZ-GFP translational fusion were purified and analyzed for the localization of the fusion protein. The percentage of the two main patterns of fluorescence observed for CotZ-GFP, full circle and one or two polar caps (a single cap is represented for simplicity), relative to the total number of spores scored (at least 75 spores were scored for each strain) is indicated for each strain. The red arrows show spores with the complete circle pattern of CotZ-GFP fluorescence, the blue arrows the two caps pattern, and the yellow arrows the single cap pattern. Scale bar, 0.2 μm [Colour figure can be viewed at wileyonlinelibrary.com]
shows discontinuities around the periphery of the spores (Figure 5). Consistent with the view that the crust forms the edge of the outer coat region in ΔcotG spores, this layer is absent from spores of the ΔcotG/ΔcotXYZ mutant. Strikingly, the spores are often surrounded by detached electrondense material that often projects into the surrounding medium forming long twirls (Figure 5). We posit that this material likely to correspond to the patches of electrondense amorphous material seen in the outer coat region of ΔcotG spores.

The phenotype of ΔcotXYZ and ΔcotG/ΔcotXYZ spores suggests that the crust has a role in maintaining the integrity and localization of the outer coat or outer coat material. The data are also consistent with a model in which cotG and cotH are essential determinants of the normal patterning of the outer coat, that the kinase activity of CotH is required mainly for outer coat assembly, and that the normal structural organization of the outer coat is not an essential pre-requisite for crust assembly.

3 | DISCUSSION

CotH is a eukaryotic-type Ser/Thr kinase responsible for the phosphorylation of at least 39 serine residues within the SKR8 region of CotB-46 and at least 13 serine residues in the SKRG region of CotG (in addition to a threonine residue; Figure 1c). Moreover, we show for the first time that CotB-46 is phosphorylated multiple times per molecule (Figure 1d). It is the extensive phosphorylation of CotB-46 that slows its mobility under SDS–PAGE conditions causing the protein to migrate with an apparent mass of 66 kDa. Evidence also suggests that the phosphorylation of CotG results in the formation of CotG-36, the main form of the protein found in the coat by Coomassie staining, as well as other forms of CotG, of higher apparent mass, that are also detected in the coat by immunoblotting (Zilhao et al., 2005; this work). We have used the catalytically inactive CotH(D228Q) protein, to examine the overall role of CotH in coat assembly and structure. Another catalytically inactive form of CotH, CotH(D251A), has been analyzed for the presence of phosphorylated proteins in the coat by immunoblotting, but gels documenting the collection of proteins extracted from spores of the mutants have not been reported (Nguyen et al., 2016). CotH(D228Q) results in spores which lack the same proteins absent from a cotH insertional mutant. Importantly, while CotH(D228Q) is assembled into the spore coat when produced under the control of its normal promoter, CotH(D251A), was only assembled when overproduced from the PcotA or PgerE promoters (both σE dependent and GerE-repressed) or the PcotE P2 promoter (σK-controlled) (Nguyen et al., 2016). Altering the time and level of cot gene expression, however, may impact drastically on assembly of the coat (see for example, (Costa et al., 2007)). Moreover, overexpression of cotH bypasses the need for CotE for the assembly of several CotE-dependent proteins (Isticato et al., 2013). Thus, CotH(D228Q) reveals that the absence of kinase activity and most likely no other perturbation in the assembly pathway independently of kinase activity, has a global impact on coat assembly. The proteins missing in cotH(D228Q) spores include CotB-66, CotG and CotC, as well as other CotH-controlled proteins that may also be phosphorylated by CotH. Other than CotB and CotG, the phosphorylation of other CotH-dependent proteins was not detected in our study. However, several coat proteins phosphorylated at Ser residues, including the CotH-controlled CotO, and YhcQ, YdfQ, GerW and YtxO, have been detected during spore germination (Rosenberg et al., 2015). Spores of a cotHD251A mutant are also impaired in L-Ala-triggered germination (Nguyen et al., 2016), although part of the effect could result from secondary effects of the overexpressed allele on coat assembly. In any event, and since cotHD228Q spores are impaired in AGFK-triggered germination, the kinase activity of CotH directly affects the two pathways known to control the germination of spores in response to nutrients (Setlow, 2014).

Co-production of CotB and CotH in E. coli is not sufficient to shift migration of the protein to the 66 kDa region of the gel; in the presence of CotG, however, CotB-66 is promptly and efficiently formed (Figure 3). Conversely, the CotH-dependent phosphorylation of CotG is enhanced in E. coli, in the absence of CotB-46. These reactions mimic the situation during coat assembly (Figure 7a). In the absence of CotG, CotB-46 may be phosphorylated, but not sufficiently to alter its SDS–PAGE migration noticeably (Figure 7a). In contrast, in the absence of CotB, phosphorylation of CotG seems more extensive (Figure 7a). How CotG stimulates the phosphorylation of CotB-46 is unknown. Phosphorylation of CotG, however, is not a pre-requisite for the interaction with and the phosphorylation of CotB-46 by CotH, because the interaction between CotG and CotB is detected in yeast cells in the absence of CotH (Table 1) and because CotG(SKRG) still promotes formation of CotB-66 in vivo, in a CotH-dependent manner (Saggese et al., 2016). This observation does not support a scenario in which CotG transfers phosphoryl groups to CotB. CotG binds to the C-terminal moiety of CotB (Table 1) and it seems plausible that this interaction involves the N- and C-terminal regions of CotG flanking the SKRG region (Saggese et al., 2016) (Figure 7b). Possibly, binding of CotG to CotB alters the conformation of and/or exposes the SKR8 region, predicted to be disordered, facilitating phosphorylation by CotH (Figure 7b). In contrast, the interaction of CotG with CotB may impair phosphorylation of CotG by CotH.

The SKR8 region is also predicted to be disordered and may be susceptible to proteolysis (Giglio et al., 2011; Saggese et al., 2016). The repeat region of B. anthracis ExsB, a CotG homologue, was also found to be extremely sensitive to proteolysis (McPherson et al., 2010). Phosphorylation may thus stabilize the protein, explaining why CotG does not accumulate in spores unable to produce active CotH or in E. coli (Zilhao et al., 2004; Isticato et al., 2015; Nguyen et al., 2016; this work). Previous work has shown that CotH stabilizes two other outer coat proteins, CotC and CotU, in the mother cell when their assembly is prevented by deletion of cotE (Isticato et al., 2004; 2008; 2013). Thus, CotH-mediated phosphorylation may serve a general role in the stabilization of coat proteins following their synthesis in the mother cell and in promoting the formation of protein complexes competent for assembly, although at least CotB-46 is assembled in the absence of CotH (Isticato et al., 2013; 2015; this work). It is presently unknown whether formation of CotB-66
can occur in the mother cell if, as in mutants such as spoIVA, assembly of the coat/crust around the spore is blocked. It is clear, however, that CotH is active at the spore surface where, over time, CotB-46 is converted into CotB-66 and CotG is converted into forms of apparent mass ≥ 36 kDa (Figure S6). Moreover, we have shown, in previous work, that forms of CotG of apparent mass of 50, 75 and 150 kDa, continue to accumulate following spore release from the mother cell (Zilhao et al., 2005). These species may correspond to phosphorylated forms of CotG, because the co-production of CotG with CotH in E. coli (in the absence of CotB) results in the accumulation of forms of the protein with about the same apparent masses (Figure 4b).

cotH is transcribed mainly under the control of σK and is repressed by GerE; thus, it occurs in a pulse prior to the main period of cotB and cotG production. It seems possible that CotB is phosphorylated first, and only after spore release from the mother cell, is the full phosphorylation of CotG attained (Zilhao et al., 2005). If phosphorylation of CotG continues following spore release, then it may depend on environmental conditions that control the activity of CotH (Isticato et al., 2020). Whether phosphorylation of CotB-46, CotG and other coat proteins occurs before or after the spore is released from the mother cell and the source of ATP used in the reaction are unanswered questions. The phosphorylation of CotB and CotG may be irreversible, since no phosphatase is known to be expressed late in the mother cell and because phosphorylated CotB and CotG is detected in germinating spores (Rosenberg et al., 2015).

Formation of a normal striated outer coat requires CotG and CotH as in spores unable to produce either protein, the outer coat region is expanded, lacks striations and contains disorganized, amorphous material (Figure 5); thus, the cotB, cotG, cotH cluster carries
a functional module that patterns the spore outer coat (Figure 7c). Since the SKR region is dispensable for normal coat morphogenesis, it seems likely that CotG is the main structural organizer of the outer coat (Henriques et al., 1998). However, and although we have not detected phosphorylation of these proteins in our study (Table S4), the contribution of other proteins phosphorylated by CotH (Rosenberg et al., 2015) to the normal structural organization of the outer coat cannot be ruled out. The expanded outer coat region seen in cotG and cotH spores bears resemblance to the interspace in spores of the B. cereus group, which may also contain coat and exosporium proteins (reviewed by Stewert, 2015). It is tempting to suggest that the phosphorylation of ExsB by CotH at Thr residues, versus the phosphorylation of CotG at Ser residues may be part of the reason why the two structural patterns (coat/crust or coat/interspace/exosporium) emerge. We note that ExsB is required for attachment of the exosporium to the coat (McPherson et al., 2010), although under certain conditions a reduction or the absence of ExsB may also result in a less robust exosporium located closer to the coat (Aronson et al., 2014).

The resemblance between cotH and cotG spores is consistent with the lack of accumulation of CotG in the absence of active CotH (Henriques et al., 1998; Saggese et al., 2014). Moreover, it suggests that the main determinant of the striated pattern of the outer coat is CotG and no other CotH-dependent protein (Figure 7c). Only CotB-46 and CotC seem absent from extracts of cotG spores (Sacco et al., 1995; Zilhao et al., 2004); all other outer coat proteins are recruited to the spore surface but may lack a scaffold formed by CotG to become organized into a striated pattern (Henriques et al., 1998; this work). Remarkably, since deletion of the SKR region has no major impact on the structure of the outer coat, the rationale for polyphosphorylation of CotB-46 remains mysterious. One possibility is that it controls the timing and/or phosphorylation level of CotG (see above).

The role of CotH may be largely confined to patterning of the outer coat, since in cotB, cotG or cotH mutants the crust layer is still present (Figure 5). This conclusion is in line with previous work showing that in cotH and cotB mutants the localization of only one crust protein, CotW, was perturbed, and only slightly (Bartels et al., 2019) and that in cotG and cotH mutants, the localization of CotX and importantly of CotZ, was not affected (Shuster et al., 2019). Moreover, atomic force microscopy revealed that the assembly of an amorphous and a rodlet layer that appear to be part of the crust is not affected by deletion of CotB or CotH (Plomp et al., 2014). Although formation of the outer coat is required for assembly of the crust (McKenney et al., 2010; Plomp et al., 2014; Bartels et al., 2019; Shuster et al., 2019), it follows that the normal structure of the outer coat is not a pre-requisite for crust formation (Figure 7c). In the absence of the crust proteins CotX, CotY and CotZ, however, the outer coat is incomplete. Moreover, the electrodense patches of amorphous material seen in the outer coat region of cotG spores form long twirls that project from the spore surface (Figure 5). Thus, formation of the crust is required for normal assembly of the outer coat, and/or for maintaining the structural integrity and localization of this layer.

The observation that CotZ-GFP formed a complete circle around only about 62% of the WT spores scored, while in the remaining the fusion protein decorated about 2/3 of the circumference of the spore (Figure 6b), suggests that the crust is a discontinuous, non-uniform structure. CotZ-GFP was found to be enriched at the MCP spore pole when sporulation was induced by nutrient exhaustion, as we have done here (Imamura et al., 2010), but not when sporulation was induced by resuspension (McKenney et al., 2010; McKenney and Eichenberger, 2012), conditions under which CotZ is required to anchor the crust to the coat at the middle of the spore (Bartels et al., 2019). A non-uniform crust may have functional significance. It calls to mind the assembly of the ‘bottle cap’ in the B. cereus/B. anthracis group, a specialized spore structure required for germination (Steichen et al., 2007). The cap forms at the MCP pole during the early stages in exosporium assembly before the rest of the exosporium and covers about 1/3 of the spore circumference (Steichen et al., 2007). Strikingly, the B. subtilis CotZ and CotY crust proteins are highly similar, and are paralogues of B. cereus/B. anthracis CotY and ExsY (Boydston et al., 2006; Johnson et al., 2006; Redmond et al., 2004, Zhang et al., 1993; reviewed by Stewart, 2015). CotY is a cap-specific protein dispensable for assembly of a complete exosporium, whereas ExsY is required for assembly of the non-cap part of the exosporium (Thompson et al., 2007; 2012; Thompson and Stewart, 2008). Possibly, CotZ and CotY in B. subtilis also have specific roles in the assembly of a non-uniform crust.

The formation of membraneless organelles in eukaryotic cells often occurs by intracellular phase separation (Brangwynne, 2013; Aguzzi and Altmeyer, 2016; Mitrea and Kriwacki, 2016). Although the assembly of nuclear and cytoplasmic ribonucleoprotein particles such as P bodies, Cajal bodies, germ and stress granules has been the most intensely studied examples, phase separation may also explain the formation of RNA-independent multiprotein assemblies such as centrosomes (Brangwynne, 2013; Aguzzi and Altmeyer, 2016; Mitrea and Kriwacki, 2016). Phase separation often involves intrinsically disordered proteins (IDP’s), which contain low complexity regions rich in a subset of amino acids including Arg and Ser, thought to promote self-aggregation (Aguzzi and Altmeyer, 2016; Mitrea and Kriwacki, 2016; Uversky, 2017). Importantly, phase transitions may be regulated by phosphorylation. One example is the phosphorylation of Ser-rich IDP’s, which induces RNA granule disassembly in Caenorhabditis elegans, while assembly is promoted by their dephosphorylation (Wang et al., 2014). The presence of intrinsically disordered regions in both CotB and CotG, rich in Ser, Lys and Arg, and their likely multisite phosphorylation during coat assembly suggests that some of the principles involved in the assembly of membraneless organelles by phase separation may apply to the process of coat assembly. If so, the widespread occurrence of CotB and CotH orthologues, as well as CotG-like proteins among spore-forming Bacilli (Giglio et al., 2011; Galperin et al., 2012; Saggese et al., 2016) suggests that these principles could apply to the organization of the spore surface layers in many species. In any event, the differential phosphorylation of homologous proteins may help explain how a common kit of structural components gives rise to the diverse structural features found at the surface of spores of different spore-forming Firmicutes.
4 | MATERIAL AND METHODS

4.1 | Bacterial strains, media and general techniques

The bacterial strains used in this study are listed in Table S1. Luria-Bertani (LB) medium was routinely used for growth of *E. coli* and *B. subtilis* strains, and sporulation was induced by nutrient exhaustion in liquid Difco sporulation medium (DSM) (Nicholson and Setlow, 1990b). The high fidelity *Phusion* DNA polymerase (Finnzymes) was used in all PCR reactions and the products sequenced to ensure that no unwanted mutations were introduced. Antibiotics were used as described before (Zilhão et al., 2005). All other general methods were as described before (Cutting, 1990; Henriques et al., 1995).

4.2 | Plasmids

Details of the construction of all plasmids used in this study can be found in the Supporting Information section. The sequence of all primers used is given in Table S2, and all plasmids are listed in Table S3.

4.3 | Spore production, purification and spore coat extraction

Mature spores were harvested 24 hr after the onset of sporulation and purified by centrifugation through density gradients of metrizoic acid (Henriques et al., 1995). Proteins were extracted from purified spores using either NaOH or SDS/DTT and fractionated on 12.5% or 15% SDS–PAGE gels as indicated in the figure legends (Henriques et al., 1995). The gels were stained with Coomassie blue R-250 or transferred to nitrocellulose membranes for immunoblotting (described below).

4.4 | *B. subtilis* whole cell extracts and immunoblot analysis

Whole cell lysates were prepared from sporulating cultures of *B. subtilis* and resolved by SDS–PAGE (Seyler et al., 1997). α5, CotB, CotG and CotH were immunodetected in spore coat extracts or whole cell lysates using rabbit polyclonal antibodies of established specificity and as previously described (Zilhão et al., 1999; 2004; Fujita, 2000). An anti-Phosphoserine antibody was obtained from Milipore and used according to the manufacturer’s guidelines.

4.5 | Phosphatase treatment

Proteins were extracted from wild-type purified spores with 0.1M NaOH for 15 min at 4°C. After centrifugation at 12,000 × g, for 10 min at 4°C the supernatant containing the coat proteins was neutralized with 0.1M HCl. The lysate was then subject to treatment with alkaline phosphatase (FastAP, from Fermentas); two enzyme units were added to 50 μl of neutralized extract and the mixture incubated at 37°C for 20 min, prior to addition of SDS–PAGE loading dye to stop the reaction.

4.6 | Overproduction of CotB-His6, CotG-His6 and CotH

The various recombinant proteins were overexpressed in *E. coli* BL21(DE3) by a modified autoinduction method (Fernandes et al., 2015). After the induction period, cells were collected by centrifugation (at 12,000 × g, for 10 min at 4°C) and resuspended in one tenth of the culture volume of buffer W (100 mM Tris-HCl, pH 8.0 and 150 mM NaCl). The cells were then lysed by passage through a French Press cell at 19,000 lb/in² as described previously (Henriques et al., 1995). Samples of the whole cell lysates were electrophoretically resolved on 12% SDS–PAGE gels. Staining of SDS–PAGE gels with Pro-Q® Diamond and SYPRO® Ruby staining was as described by the manufacturer (Invitrogen). The PeppermintStick phosphoprotein molecular weight standard was also used as described by the manufacturer (Invitrogen); in this marker, ovalbumin (45 kDa) and β-casein (23.6 kDa) are phosphorylated. A FLA-5100 fluorescence scanner (from Fuji) was used for imaging of the stained gels.

4.7 | Purification of CotB, CotH and CotH<sub>D228Q</sub>

CotB-His<sub>6</sub> and CotH-Strep-tag proteins were overexpressed in *E. coli* BL21(DE3) as described above (Fernandes et al., 2015). After the induction period, cells were collected by centrifugation (at 12,000 × g, for 10 min at 4°C). Cells with CotB-His<sub>6</sub> were resuspended in one tenth of Start buffer (10 mM imidazole, 20 mM phosphate, 0.5 M NaCl), containing 1 M phenylmethanesulfonyl fluoride (PMSF). Lysates were prepared by a passage through a French press (19,000 psi) and centrifuged (at 12,000 × g, for 10 min at 4°C). Since most of CotB-His<sub>6</sub> is present in the insoluble fraction, Start buffer containing urea (8 M) was added to the debris to increase the solubility of CotB-His<sub>6</sub>. The sample was stirred for 45 min and centrifuged (at 12,000 × g, for 10 min). CotB-His<sub>6</sub> was purified by Ni<sup>2+</sup>-NTA affinity chromatography (Qiagen). The Ni<sup>2+</sup>-NTA affinity purified protein was analyzed by 12.5% SDS–PAGE. Fractions containing CotB-His<sub>6</sub> were pooled and dialyzed against Start buffer without urea. Cells with CotH-Strep-tag or CotH<sub>D228Q</sub>-Strep-tag were resuspended in one tenth of buffer W (see above), containing 1 M PMSF. Lysates were prepared by a passage through a French press (19,000 psi) and centrifuged (at 12,000 × g, for 10 min at 4°C). CotH-Strep-tag or CotH<sub>D228Q</sub>-Strep-tag were purified using Strep-Tactin Sepharose (IBA). The affinity purified protein was analyzed by 12.5% SDS–PAGE.

4.8 | Kinase activity assays

Purified CotH (0.5-1μM) and/or CotB (2 μM) were incubated with 0.1 mM of unlabeled ATP and 1 μCi of [γ<sup>32</sup>P]ATP in kinase buffer...
(50 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM MgCl₂, 10 mM MnCl₂ and 0.5 mM TCEP) at 37°C for 5, 10 and 30 min. For kinase inhibition, staurosporine (Sigma) was added at concentrations of 7, 70 or 700 μM. The reactions were stopped by addition of SDS-PAGE loading buffer and the proteins resolved by SDS-PAGE. The gels were dried, exposed to a Phospho Screen and imaged using a Storm phosphorimager (GE Healthcare).

4.9 | Germination efficiency

Purified spores were heat activated as previously described (Cutting, 1990) and diluted in 10 mM Tris-HCl pH 8.0 buffer containing 1 mM glucose, 1 mM fructose and 10 mM KCl. After 15 min at 37°C, L-asparagine was added to a final concentration of 10 mM, and the optical density of the suspension at 580 nm was measured at 10 min intervals until a constant reading was reached.

4.10 | Fluorescence microscopy

Purified spores were resuspended in 0.1 mL of PBS. Fluorescence microscopy was performed as previously described (Serrano et al., 2011), and images were analyzed with Metamorph v7.7 (Molecular Devices). For quantitation of the subcellular localization of CotZ-GFP, at least 100 spores were randomly examined and scored.

4.11 | Transmission electron microscopy

For thin sectioning transmission electron microscopy (TEM) analysis, B. subtilis spores were purified by density gradient centrifugation as described above. Samples were processed for TEM essentially as described previously (Henriques et al., 1998) and imaged on a Hitachi H-7650 Microscope equipped with an AMT digital camera operated at 120 keV.

4.12 | Mass spectrometry

Cultures of B. subtilis cells were induced to sporulate by resuspension in Sterlini–Mandelstam (SM) medium (Nicholson and Setlow, 1990a) and samples were taken every hour for 5 hr. Then, the cells were sedimented at 4°C and 8,000 × g for 10 min. The cell pellets were resuspended in 20 mL of lysis/binding buffer (8M Urea, 20mM Tris-HCl pH 8.0, 150mM NaCl, 1mM PMSF, 1mM β-glycerol phosphate, 1mM sodium orthovanadate, 2.5mM sodium pyrophosphate and 10mM sodium fluoride) and disrupted using a cell-disruptor (Constant Systems Limited). The lysate was centrifuged for 30 min at 15,000 × g to precipitate the cell debris. Then, free cysteines were alkylated with 5mM lodoacetamide and incubated for 30 min in the dark. Then, the urea concentration was decreased to below 1M by the addition of 50 mM Tris-HCl pH 8 and 150 mM NaCl and proteins were subsequently digested with trypsin (1:20, Promega) for 12 hr. Then, phosphopeptide enrichment was performed using TitanSphere TiO2 beads (GL Science) according to a previously described protocol (Elsholz et al., 2012). Briefly, the peptide mix was acidified using 1% TFA and solution was cleared by centrifugation for 5 min at 15,000 × g at room temperature. The supernatant was purified using a C18 Sep-Pak columns (Waters) and eluted with 50% acetonitrile and 0.1% TFA. Samples were lyophilized and resuspended in 73% Acetonitrile, 25% lactic acid and 2% TFA, mixed with 30 μg beads (GLC Science) and incubated at room temperature for 30 min. Beads were loaded onto a C18 spin column (Nest group) and washed three times with 80% acetonitrile and 2% TFA. Peptides were eluted with 50 μl 5% NH₄OH followed by 50 μl 50% acetonitrile. Enriched peptides were separated and analyzed by LC-MS/MS using an Easy-nLCII HPLC system (Thermo Fisher Scientific) coupled directly with an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) at the Harvard Mass Spectrometry and Proteomics Resource Laboratory, FAS Center for Systems Biology. Raw data were processed using the Max Quant software, as described (Elsholz et al., 2012). The probabilities for phosphorylation at each possible phosphorylation site on a given peptide were calculated and only phospho-site with a probability higher than 0.75 were accepted (Elsholz et al., 2012; see Table S4).

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.