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"Protein-protein interactions during assembly of the *Bacillus subtilis* spore coat"

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Chapter 1

Introduction and Outline of this thesis

Bacterial spore

The bacterial spore is a dormant, highly resistant cell type, which allows spore-forming organisms to survive adverse conditions, such as starvation, high temperatures, ionizing radiations, mechanical abrasion, chemical solvents, detergents, hydrolytic enzymes, desiccation, pH extremes and antibiotics (Nicholson, el al., 2000).

Spore formers mainly belong to the *Bacillus* and *Clostridium* genera. The most studied species of these two genera is *Bacillus subtilis*, a model system for gram-positive bacteria.

Spore formation involves the cooperation of two sister cells in a series of developmental changes culminating in the packaging of one cell into a tough resistant coat. The other cell contributes most of its resources to the process and then lyses. Unlike most adaptive responses in bacteria, sporulation takes many hours and includes major changes in cellular morphology as well as in biochemistry and physiology. Because it also involves the differentiation of two cells that start out with identical genomes, it incorporates two of the main characteristics of developmental processes in all organisms, i.e., temporal change and cellular differentiation (Errington, J., 1993). Morphological and metabolic diversity between two cells genetically identical is possible because they follow two different gene expression programmes of the gene expression. In spore-forming bacteria the

mechanisms responsible for the establishment of cell-specific gene expression and the temporal control of gene expression is due, in large part, to sequential appearance of four transcription factors, called sigma factors, alternate to σ^A factor active during vegetative life (σ^F, σ^E, σ^G, σ^K), which bind to core of RNA polymerase and direct it to transcribe only from promoters of sporulation genes, called *spo* (Losick and Stragier, 1992). The process of spore formation is presented in the fig. 1.

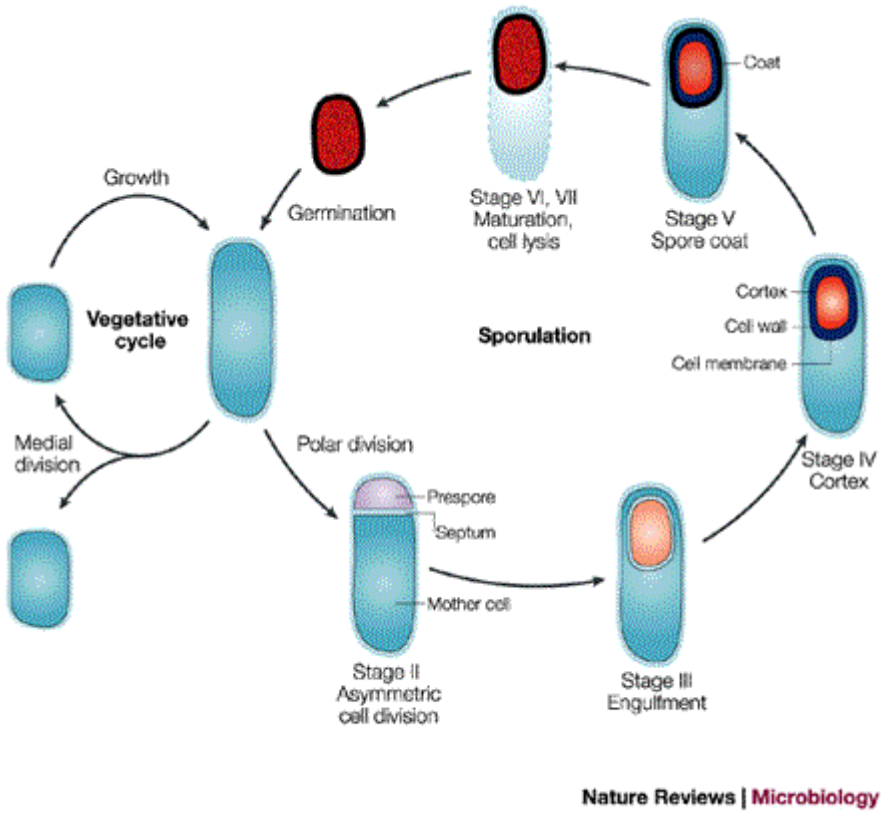

Fig. 1: schematic representation of sporulation and germination in *Bacillus subtilis*

The spore can stay in the environment as quiescent cell for a long time, but it must be able to return to active growth when nutrient is again available, through a process called germination (Fig.1) . This is possible because, although dormant, spores are in alert: they continuously monitor the environment for the renewed presence of nutrient.

Sporulation and germination in *Bacillus subtilis*

Morphological stages

The morphological process of sporulation is driven by a temporally and spatially controlled program of gene expression. Commencement of this program requires that the cell has reached a certain stage in the cell cycle (Grossman, 1995; Errington et al, 1996), that the tricarboxylic acid cycle is intact (Sonenshein, 1995), that at least one extracellular pherormone is present in the appropriate amount, and that an unknown environmental stimulus has activated a complex phosphorylation cascade (Hoch, 1993).

When these conditions are met (during starvation), sporulation ensues and the pattern of vegetative gene expression is largely replaced with the specialized program of sporulation gene expression.

An early morphological event is the formation of an asymmetrically placed division septum, which divides the cell into the forespore and

mother cell compartments (Fig. 1). The smaller compartment (the forespore) will go on to become the spore. The larger compartment (the mother cell) will serve to nurture the spore until its development is complete. After the sporulation septum is laid down, the sporulation gene expression program splits and two distinct programmes became active, one in each of the resulting cellular compartments. As a result of these two divergent programmes of gene expression, the spore will be built from the outside (as a result of protein synthesis in the mother cell) and from the inside (as a consequence of the proteins produced in the forespore). After that, the edge of the septum migrates in the direction of the forespore pole of the cell, pinching the forespore compartment off to become a protoplast which sits free in the mother cell cytoplasm and is surrounded by a double layer of membranes. Following the completion of the engulfment, two types of peptidoglycans are layered between the inner and outer membranes surrounding the forespore. The surface of the inner spore membrane is the site of assembly of a thin layer of peptidoglycan called the primordial germ cell wall, similar in composition to the vegetative cell wall that serves as the primordial wall of the newly formed vegetative cell following spore germination. The outer forespore membrane is the site of assembly of a second, thicker, and chemically distinct layer of peptidoglycan called the spore cortex which is essential for the

attainment and maintenance of the dehydrated state of the spore core, for spore mineralization and for dormancy (Henriques and Moran, 2007).

Concomitantly with cortex formation, the protein coat is deposited around the outer surface of the outer membrane. The coat is evident by about the fifth hour of sporulation. Two major coat layers can be discerned by electron microscope analysis: a darkly staining outer coat and a more lightly staining lamellar inner coat. The final step is the lysis of the mother cell and the release of the fully formed spore. When enviromental conditions are suitable, the spore can germinate and thereby convert back into a growing cell. When this occurs, first the spore core rehydrates and swells and then cortex and coat crack, releasing the nascent cell (Fig. 1).

Genetic control of sporulation

A cascade of sigma factors directs sporulation gene expression to occur at the right time and place. The first sporulation-specific sigma factor to direct sporulation-specific gene expression is σ^H, that in combination with the major housekeeping factor σ^A, directs the expression of a large set of genes, some of which play early roles in sporulation and in the appearance of the sporulation septum. σ^A activates the expression of sporulation genes in conjunction with the

transcription factor Spo0A, which becomes active immediately after sporulation is initiated. Once the sporulation septum is formed, σ^F becomes active in the forespore compartment, although is present in both cell compartments. This restriction of activity to only once cell type is a consequence of an anti-sigma factor called SpoIIAB and an additional set of regulatory proteins. The σ^F regulon includes genes involved in engulfment, and also the gene *spoIIIG* that encodes the next sigma factor to be active in the forespore (Sun et al., 1989). Once σ^F is active, σ^E becomes active in the mother cell. σ^E is synthesized as a proprotein, requiring the removal of its amino terminus before becoming active. This processing, which is restricted to the mother cell (Ju et al., 1997), converts the membrane-associated proprotein into a cytoplasmic factor capable of directing transcription (Hofmeister, 1998). An additional forespore-specific proteolytic event removes pro-σ^E from the forespore (Pogliano et al., 1997). Result of σ^E activity is the engulfment of the forespore compartment, with the formation of a free protoplast encircled by a double membrane layer. It also directs the appearance of an important transcription factor, SpoIIID (Kroos et al., 1989; Kunkel et al., 1989), as well as an inactive proform of σ^K. SpoIIID works with σ^E to activate a second phase of mother cell gene expression. After engulfment, the next sigma factor, σ^G, becomes active in the forespore, probably thanks the

presence of a recently identified channel, that connect the two compartments of a sporangium. This channel is formed by the protein products (AA-AH) of a eight-cistron *spoIIIA* operon, which is transcribed in the mother cell under the σ^E control, and by a forespore protein Q (Camp and Losick, 2009). The gene encoding σ^G is under the control of σ^F and is expressed just after the appearance of the sporulation septum. However, the gene product remains inactive until forespore engulfment is complete (Stragier, 1992).

The cytoplasm of the forespore dehydrates soon after σ^G becomes active. σ^K is activated by a proteolysis event that appears to occur on the outer forespore membrane, which is in contact with the mother cell cytoplasm. σ^K is required for a variety of events that occur late in sporulation, including the synthesis of the coat. The last known phase of mother cell gene expression is directed by σ^K along with the small DNA-binding protein GerE (Zheng et al., 1990). GerE controls coat protein genes and genes that may be involved in the glycosylation of the coat. The mechanism of regulation of gene expression in the two cell types, that I have briefly summarized, was proposed in 1992 (Stragier and Losick), and called criss-cross regulation. Since then, it has benn confirmed by various different experimental evidences and is schematically reported in the fig. 3.

Germination and outgrowth

When the environmental conditions became favorable for the vegetative life, the spore that continuously monitor the environment, returns to active growth, through a process called germination. It is an irreversible process, like sporulation (Parker et al., 1996), that involves a series of rapid degradative reactions, leading to dismantlement of the unique spore structure and loss of spore dormancy and resistance. The subsequent steps that lead to cell-enlargment and cell-division are termed *outgrowth*, and are considered a separate process, distinct from germination. Outgrowth takes more than one hour to occur while germination takes just few minutes. The amino-acids necessary in this step, for the protein during synthesis, during outgrowth are provided by the degradation of the coat components and of the SASP (Small Acid Soluble Proteins, DNA binding proteins responsible of UV resistance) degradations. The dormant spore is equipped with sensors to choose the right moment for germination. These sensors are specific proteins called nutrient receptors, and are located at the inner membrane (Hudson et al., 2001; Paidhungat et al., 2001).

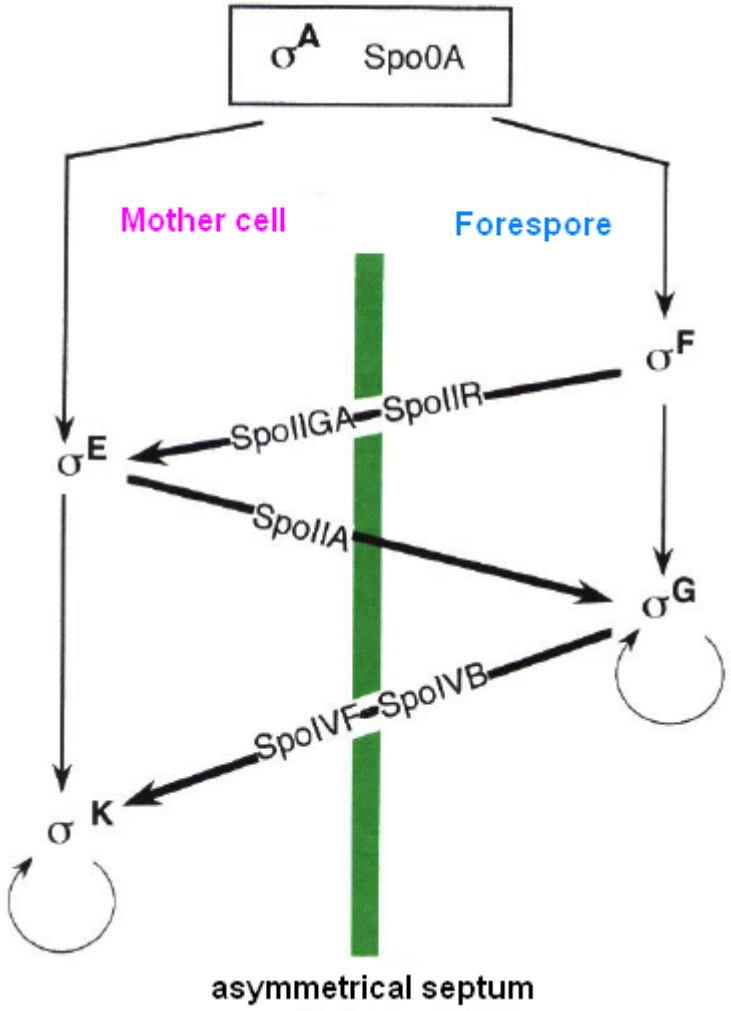

Fig. 2: C*riss-cross regulation*. The thin arrows indicate the transcriptional control in the two different cells, while the thick arrows indicate the three checkpoints that coordinate the gene expression of one cell with the morphological changes of the other.

They recognize specific molecules as a signal of nutrient-rich conditions. Effective germinants are amino-acids, sugars and robosides (Hornstra et al., 2005). In laboratory conditions, germination is efficiently induced by L-alanine and a mixture of molecules known as AGFK (asparagines, glucose, fructose, KCl). The

correct assembly of the coat is also important for a proper germination, in fact null mutant for *cotT* and *cotD*, show defect in germination, also because the coat is responsible to suppress spontaneous, unwanted germination. Moreover, different classes of mutants are identified, that are not able or less able to germinate in presence of L-alanine and AGFK. These differences are probably a consequence of presence of different receptors nutrient-specific (Moir and Smith, 1990).

Spore structure and spore coat

The spore consists of various structural features: a core, surrounded by the inner membrane, the cortex, the outer membrane and the coat (Fig. 2). The core is the innermost part of the spore. It contains the spore cytoplasm with all cellular components, such as cytoplasmatic proteins, ribosomes and DNA. The physical state of the core cytoplasm, however, is far from regular in comparison to vegetative cell cytoplasm, having a water content of only 30-50%, instead of the 70-88% of the vegetative cytoplasm (Potts, 1994; Setlow, 1994; 2000).

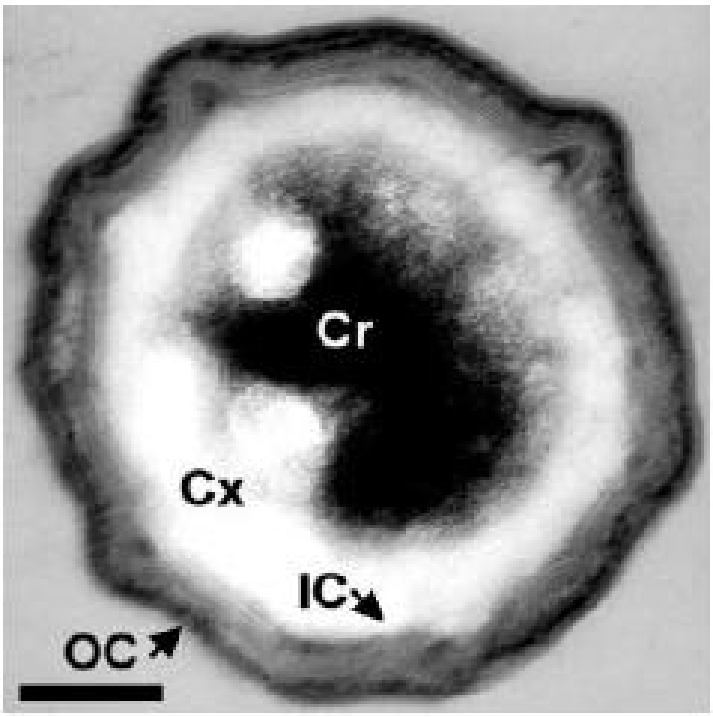

Fig. 3: *Bacillu subtilis* spore ultrastructure. The figure shows the spore core (Cr), the cortex peptidoglycan layer (Cx), the inner coat (IC) and the outer coat (OC) (Silvaggi et al, 2004).

This dehydratated state plays an important role in spore longevity, dormancy and resistance. The core harbors large quantities of SASP (Setlow, 1988), that form a complex with the spore DNA, thereby forcing the DNA into a special compressed format (Douki et al., 2004), which protects the DNA against many types of damage. The spore core is surrounded by the inner membrane. It is in a special, compressed way and it becomes the cytoplasmic membrane of the nascent vegetative cell upon germination. The inner membrane is also the site where spore germination receptors are located, thus it a structure of prime importance for spore resistance and germination.

Around the inner membrane is built the cortex. It is a thick cell wall composed of specifically modified peptidoglycan (Warth and Strominger, 1972). The cortex is of a crucial importance for the maintenance of spore core dehydration and thus resistance and dormancy. Its specific structure is conserved among species, and may play a role in spore heat resistance, although a clear correlation between cortex structure and heat resistance has not been found. During germination, the cortex peptidoglycan is rapidly degraded by lytic enzymes already present in the dormant spore. The inner part of the cortex lacks the specific modifications that are characteristic of cortex peptidoglycan and is called the germ cell wall or primordial cell wall. The germ cell wall is not degraded upon germination and forms the initial cell wall of the freshly germinated spore.

Around the cortex lies the relatively poorly studied outer membrane. The outer membrane, deriving from engulfment, has opposite polarity with respect to the inner membrane.

The most external structure of the spore is the coat, a complex multilayered structure, that plays roles in spore resistance, germination, and apparently possesses enzymatic functions that may possibly permit interactions with other organisms in the environment. It consist of two main layers (Fig. 3): the inner layer (thick 20-40 nm) is formed by the juxtaposition of three to six lamellae aligned along

the periphery of the spore, and the outer layer (thick 40-90 nm) consisting of four to five electron-dense striations that also align parallel to the spore surface (Henriques and Moran, 2007). Both coat layers are packed closely together and appear thicker at the spore poles and thinner along its sides (Driks, 1999). It is not yet known precisely what factors determine the lamellar or striated pattern of the inner and outer coat layers. It may reflect in part the successive overlay of the coat proteins during the assembly process, as well as the physical properties of the coat components, but is demonstrated that both layers are important to confer to the spore resistance and for germination (Ricca, et al., 1997). There is another layer, called undercoat, between the cortex and inner coat layers. The total number of coat proteins is estimated to be more than 70. At least 20 coat proteins have demonstrated enzymatic function. Some govern the assembly process by posttranslationally modifying proteins at the spore surface, whereas others affect spore protection or germination.

The interactions between various coat components, inferred only on the base of by genetic dependence, form a complex network and are schematically reported in Fig. 4 (Kim, et al., 2006).

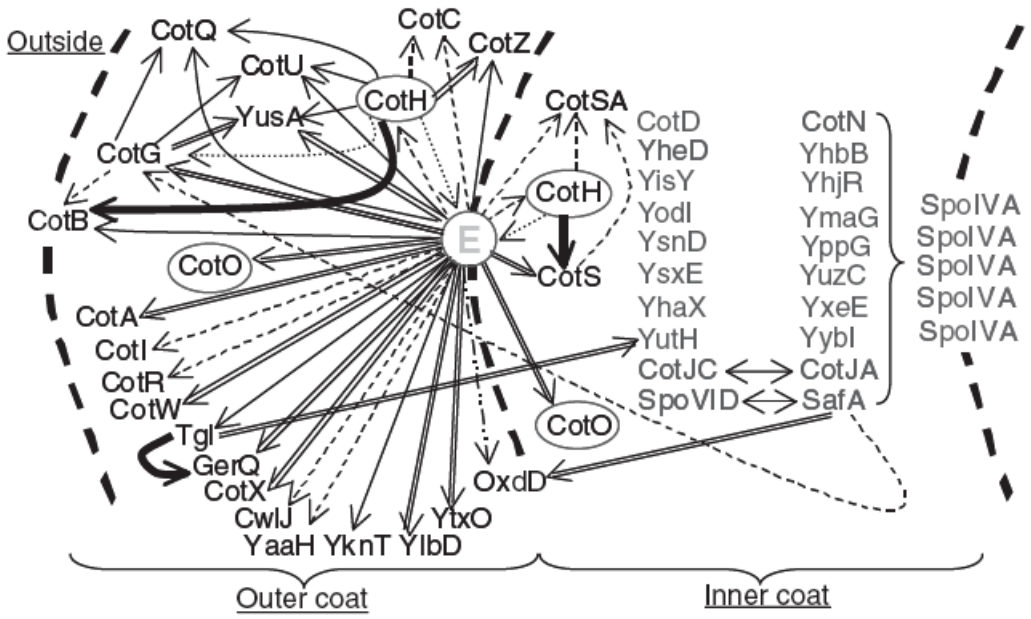

Fig. 4: Model of the coat protein interaction network (Kim, et al., 2006).

Analysis of localization of various coat protein and their timing of appearance, suggest that the assembly of the two layers does not occur from inner to outer, but that it is under a complex control mechanism acting at two levels: the transcriptional level, controlling the temporal synthesis of the various protein; and at the post-translational level, with various kinds of modifications and with the assembly of coat components (Driks et al., 1994; Ricca et al., 1997).

Role of gene expression in coat assembly

Coat assembly is mainly a function of the mother cell and covers a period of about 6 h, beginning with asymmetric division of the sporangial cell. Thus, expression of genes coding for coat components

(*cot* genes) is under control of the two transcription factors that regulate mother cell gene expression: σ^E and σ^K; and three DNA-binding proteins called: SpoIIID, GerR and GerE. Due to the action of those transcriptional factors it is possible to identify four classes of *cot* genes (Fig. 5).

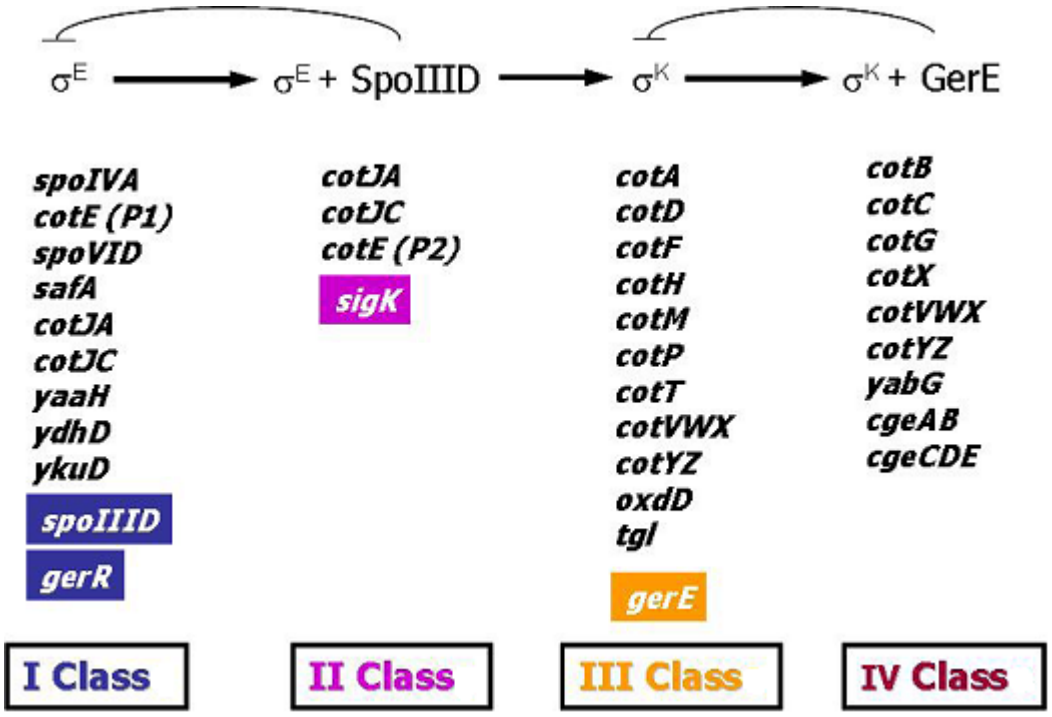

Fig. 5: Program of mother cell gene expression.

The gene expression programme in the mother cell compartment involves the activation of 383 genes, that represent about 9% of *Bacillus subitlis* genome, and it is started with activation of σ^E . It directs the expression of at least two coat genes, *cotE* and *cotJ*. Expression of *cotE* relies on two promoters, designed P1 and P2.

Transcription from P1 is initiated soon after asymmetric division and is shut off by the repressive action of SpoIIID. Transcription from P2 appears to be under the joint control of σ^E and SpoIIID, but remains on after the activation of σ^K, to be repressed in the final stages of sporulation by the GerE) (Costa, et al., 2006). σ^E is also responsible for the expression of the genes coding for the coat morphogenetic proteins SpoIVA and SpoVID, from pro-SigK and for genes coding for the transcriptional factorsSpoIIID and GerR. SpoIIID can act as a repressor or as an activator while GerR, seems towork only as a repressor of genes activate by σ^E (Eichenberger et al., 2004). After engulfment, σ^K directs the expression of a large group of coat protein genes. The first σ^K –controlled regulon is composed of *cotA*, *cotD*, *cotF*, *cotH*, *cotM*, *cotT*, *cotV*, *cotW*, *cotY*, and *cotZ*. The transcription factor gene *gerE* is also part of this regulon. GerE works in conjunction with σ^K to activate a final regulon, encompassing *cotB*, *cotC*, *cotG*, *cotS*, *cotV*, *cotW*, *cotX*, *cotY*, and *cotZ*. GerE can also modulate the expression of genes in the σ^K regulon. It down regulates *cotA* and *cotM* and activates *cotD*, *cotV*, *cotW*, *cotX*, *cotY*, and *cotZ*. A further level of complexity in the control of mother cell gene expression comes from a feedback-like regulation in which late regulatory events modulate ones that were initiated earlier. For example, σ^K down regulates transcription of the gene encoding σ^E,

thereby helping to terminate expression of σ^E–directed genes. GerE is also able to down regulate the activity of σ^K. The production of the spore coat proteins in the correct cellular compartment and at the proper time is critical to the formation of the coat. Mutants in which the time of σ^K activation is altered, and the coat components of class III and IV, are synthesized one hour earlier than in wild type cells, produced spores impaired in their germination efficiency (Cutting et al., 1991; Ricca et al., 1992).

Post-translational regulation during coat formation

In coat formation, regulation of gene expression regulation is associated to another regulation level, involving various coat proteins with morphogenetic activity, i.e. proteins that do not affect the sunthesis of other coat proteins, but affect their assembly within the coat layers. The most important morphogenetic proteins are: SpoIVA, SpoVID, CotE, CotH and are all produced in the mother cell compartment (Driks et al., 1994; Ricca et al., 1997). SpoIVA is produced in the mother cell early in sporulation, under the control of σ^E and is essential for the assembly of both the spore cortex and the coat layers.

spoIVA localizes at or near the mother cell surface of the septum and at later stages, following engulfment of the forespore by the mother

cell, the protein forms a shell that surrounds the forespore. Recent studies (Ramamurthi and Losick, 2008) have demonstrated that SpoIVA is an ATPase and that it allows its self assembly. It has been proposed that two interaction sites are involved in assembling of SpoIVA: one is used for ATP-dependent polymerization, and the second for lateral interactions between the polymers. Localization of SpoIVA marks the forespore outer membrane for its use both in the synthesis of the cortex and as the site of coat attachment. Mutants carrying a *spoIVA* null allele, show a coat correctly formed with inner and outer part but dispersed in the mother cell cytoplasm and not assembled around the forespore (Roels et al., 1992; Stevens et al., 1992). So its role is probably to attach the undercoat to the forespore surface from the mother cell side (Driks et al., 1994).

SpoIVA is also required for the localization of second key coat morphogenetic protein, SpoVID (Beall et al., 1993), to the surface of the developing spore. SpoVID governs a morphogenetic transition, called spore encasement, using a N-terminal, that seems to be essential to form multimeric structures (Wang et al., 2009). *spoVID* mutants like *spoIVA* mutants, have a spore coat dispersed in the cytoplasm.

The localization of SpoIVA to the forespore outer membrane is a prerequisite also for the localization of a third morphogenetic protein, CotE (Zheng et al., 1988). It localizes about 75 nm from the forespore

outer membrane and later encircles the engulfed forespore. Electron microscope analysis have shown that *cotE* mutants totally lack of the outer coat layer (Zheng et al., 1988). The region delimited by the SpoIVA and the CotE ring is referred to as matrix or precoat. The precoat is of unknown composition, but it most likely contains proteins that are synthesized early and recruited under SpoIVA control. Probably the inner coat proteins assemble in the space between CotE and the forespore surface, forming the inner lamellar layer (Driks, et al., 1994). Instead, the outer coat proteins assemble simultaneously around the CotE layer to form the electrondense outer layer (fig 6). Assembly of the outer coat involves the cooperation of CotE with the action of the additional morphogenetic protein CotH (Nacleiro et al., 1996). CotH may function in part in the mother cell cytoplasm, perhaps as a protease inhibitor or *chaperone* (Baccigalupi et al., 2004; Isticato et al., 2004), as it is required for the stabilization of CotG and CotC. Since assembly of CotB depends on expression of *cotG*, a hierarchical control CotH-CotG-CotB has been proposed (Zilhao et al., 2004).

Some additional morphogenetic proteins also contribute to coat formation. An exsample is CotM that is implicated in the outer layer formation; *cotM* mutants show a reduced amount of various outer coat proteins.

Post- translational modifications

In addition to structural and morphogenetic coat proteins, there are coat proteins involved in the post-translational modification of various coat components. One important type of modifications is the cross-linking that contributes to the strength of the coat. Several types of cross-linked species have been implicated in coat assembly, including disulfide cross-links and dityrosine cross-links (Henriques and Moran, 2007).

The potential importance of dityrosine species on the integrity of the coat is reinforced by the preponderance of residues of tyrosine in the sequences of coat proteins. A trasglutaminase has been identified in the coat layers and the its structural gene, *tgl,* identified (kobayashi et al., 1996). This enzyme generate a ε-(γ-glutamyl)lysine cross-link among the R groups of different amino-acids, belonging at the same chain or also at different chains.

It has been also demonstrated that the assembly of GerQ within the is dependent on Tgl activity (Zilhao et al., 2005).

In addition to modification by cross-linking, there is evidence for glycosylation of coat proteins late in development. A cluster of genes, *cge*, activated under the control of GerE, was identified (Roels et al., 1992). One of these, *cgeD*, is similar to glycosyl transferases that participate in polysaccharide biosynthesis. Mutations that deleted

genes in this cluster resulted in spores that appeared normal by the standard assays and by electron microscopy. However, these spores tended to aggregate and had abnormal adsorption properties, suggesting a surface alteration. It is possible that the *cge* locus encodes one or more coat proteins, but it is perhaps more likely that it is responsible for a terminal step in coat formation that involves the glycosylation of the coat.

Another cluster of 10 genes which also show homology to polysaccharide biosynthetic genes, and whose expression is repressed by GerE, was identified (Hullo et al.). One of these, *spsA*, is very similar to *cgeD*. The deletion of the *sps* locus does not result in a gross morphological defect in spore structure, but the spores are very hydrophobic. So, the glycosilation of the spore surface could determine the hydrophobicity of the spore and therefore its adherence properties (Wiencek, et al., 1990).

An additional post-translational modification that has been shown to occur during coat formation is the proteolytic processing of certain coat proteins, such as CotF and CotT. The enzymes responsible for these cleavage events have not been identified, but a GerE-dependent coat associated protease activity has been found.

From the results discussed above, it is possible to propose a tentative model to explain how the layered structure of the coat is established

and how coat formation is directed to occur at the location of the spore surface (Fig. 6). In this view, there are four major steps in coat assembly: binding of SpoIVA to the forespore surface, formation of the precoat (both of these steps occur under the control of σ^E), a σ^K-dependent phase of inner and outer coat layer assembly, and a σ^K-plus-GerE-dependent phase of inner and outer coat layer assembly that includes post-assembly modification of the coat.

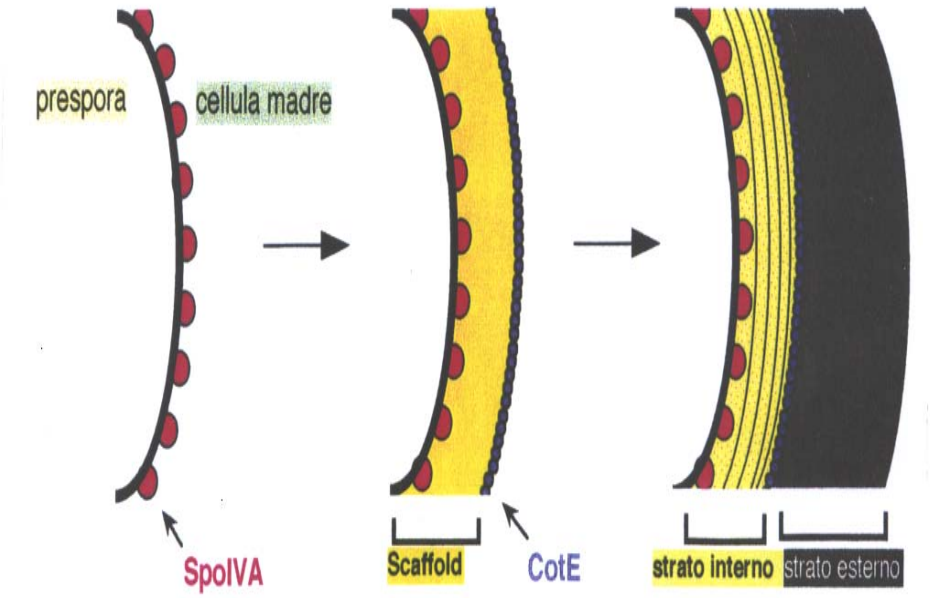

Fig. 6: Spore coat assembly model

Recombinant spores as vaccine vehicular.

Considerable efforts have recently been devoted to the development of oral vaccines, that are able to provide better levels of local immunity against pathogens which enter the body primarily through the mucosal

surface (Fischetti et al., 1996). Since mucosal immunisation using soluble antigens has long been known to generate poor immune responses due to antigen degradation in the stomach, limited absorption and tolerance, different approaches have been undertaken to develop carrier systems displaying heterologous antigens on the surface of microbial cells and viruses. Because of the thicker cell wall, gram-positive bacteria are viewed as preferential candidates over gram-negative bacteria for the development of bacterial biocatalysts and whole-cell adsorbents. Among them, *B. subtilis* offers additional advantages due to the detailed knowledge of its spore structure and the availability and ease of advanced genetic tools and genomic data that facilitate the construction of recombinant spore. In fact more recent is a strategy to engineer the *Bacillus subtilis* endospore to display heterologous proteins on its surface. There are many potential advantages with the utilization of spores: (i) a high stability of the expression system is ensured by the well-documented resistance and stability of the bacterial spore (Driks et al., 1999; Henriques et al., 2000); (ii) a good safety record is ensured by the use of spores of species including *Bacillus subtilis*, *B. clausii*, *B. coagulans*, *B. cereus*, and *B. natto* as food additives in human and animal food preparations and as prescription or non prescription products for the treatment of gastrointestinal disorders ; (iii) simple and economic production of

large amounts of spores is ensured by already available and commonly used procedures for industrial-scale production and commercialization of several spore-based products (Green et al., 1999; Hoa et al., 2000). The spore-display system, thus far used to express antigens (Isticato et al. 2001; Mauriello et al. 2004; Duc et al. 2003b) and enzymes, is based on the construction of gene fusions between heterologous DNA (codifying for a *passenger protein*) and a *B. subtilis* gene coding for a component of the coat (*carrier protein*), the proteinaceous structure surrounding the spore (Henriques et al., 2000) (Fig. 7).

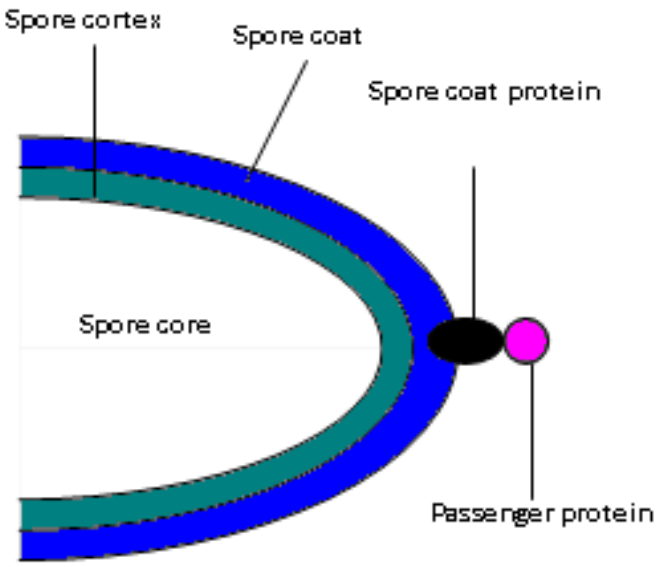

Fig. 7: Dsiplay system on spore surface using the coat protein as carrier

Expression on spore surface

Two proteins of *Bacillus subtilis* spore coat, CotC and CotB, have been successfully used to display heterologous antigens on the spore surface. Several antigens have been exposed on the spore surface using either CotB or CotC as a carrier. However, display details have been characterized only for two antigens: the C fragment of the tetanus toxin (TTFC) of *Clostridium tetani* and the B subunit of the heat labile toxin (LTB) of *Escherichia coli* (Isticato et al., 2001; Mauriello et al., 2004).

Surface display of CotB has been demonstrated by cytofluorimetry data (Isticato et al., 2001) and, then, by an atomic force microscopy analysis (Chada et al., 2003) (Fig. 8). CotB is a protein of 380 amino-acids characterized by an hydrophilic at the C-terminus part, consisting of a motive of 27 amino-acids rich in serine, lysine and glutamine residues, repeated three times. The *cotB* gene codes for a 46-kDa polypeptide (CotB-46) which is post-translationally converted into a form of about 66kDa (Zilhao et al., 2004). This form is the only one found in a coat protein extract, and it is demonstrated that its formation depends from presence of other coat components, such as CotH and CotG. Probably it is the result of a cross-linked homodimer with irregular mobility, or a heterodimer perhaps containing CotG (Zilhão et al., 2004).

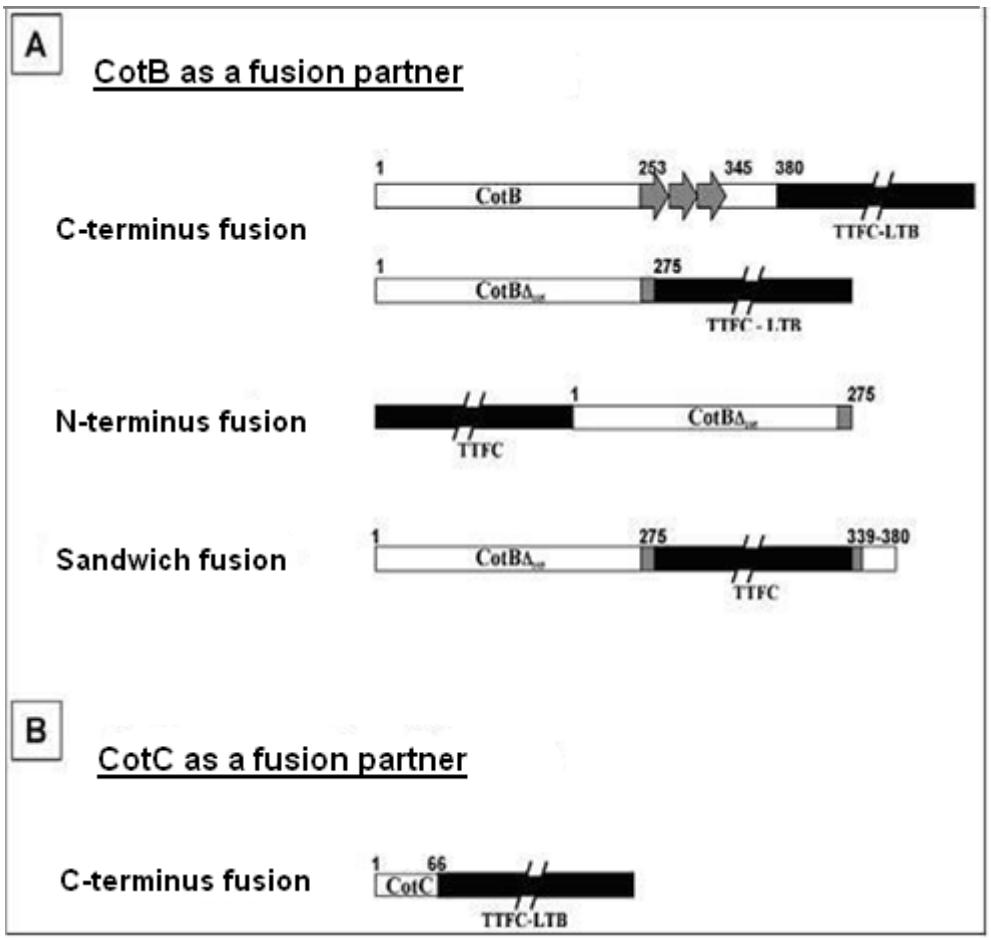

Fig. 8: Surface display in *Bacillus subtilis*. The black part correspond to the heterologous proteins.

To avoid stability problems the DNA coding for the 27 amino-acid repeats was not included in the gene fusion between *cotB* and DNA coding for TTFC. The expression of chimeric protein was put under the transcriptional and translational control of *cotB* gene and integrated on the *B. Subtilis* chromosome.

CotC was initially selected as a carrier for spore surface display for its abundance in the outer coat layer and used to express TTFC and LTB (Mauriello et al., 2004).

CotC is a protein rich in tyrosine (30.3%), lysine (28.8%) and aspartic acid (18.2%) and together with CotB and CotG represents the 50% of the soluble coat proteins. CotC is assembled in four forms (12kDa, 12.5kDa, 21kDa, 30kDa) CotE- and CotH-dependent (Isticato et al., 2004).

CotB-TTFC, CotC-TTFC and CotC-LTB fusions do not effect the resistance of recombinant spores to toxic agents and do not alter the sporulation and germination processes (Isticato et al., 2001; Mauriello et al., 2004).

A recent study has shown that both wild type and recombinant spores expressing LTB fused to CotC are phagocitated by human THP-1 macrophage cells that mimic blood monocyte-derived macrophages (Ceragioli et al., 2009). In the *in vitro* system spore uptake was very inefficient with a percentage of phagocytosis equivalent to about 2.5% and the phagocytated spores germinated and were then killed before cell growth commenced (Ceragioli et al., 2009). The analysis of infected macrophages did not reveal cytotoxic effects exerted by *B. subtilis* spores, supporting the safety of wild type and recombinant spores of *B. subtilis*.

Outline of this thesis

Bacillus subtilis is a model organism to study a variety of biological phenomena, fram gene expression to microbial differentiation, protein-protein interactions and formation of subcellular structures. In addition, this organism is also widely studied for biotechnological reasons, from enzymes and antibiotic production to vaccine delivery.

I am particularly interested in this latter aspect and, therefore, decided to focus my attention on the mechanism of assembly within the spore coat of the coat components used as carriers of heterologous antigens. A better understanding of how CotB and CotC are assembled and of the factors required for their assembly within the coat may provide essential information on the most appropriate way to display heterologous proteins fused to CotB or CotC.

I, initially, worked on the assembly of the CotC, showing taht is assembles on the coat in various forms and that it interacts with another coat component, CotU. CotU ia recently identified coat component taht shares with CotC over 70% of homology. Genetic and biochemical experiments, reported in Chapter 2, show that CotC and CotU directly interact and that such interaction does not occur in heterologous hosts. Based on this, I proposed that a specific *B. subtilis* factor is needed to promote the interaction between CotC and CotU.

In Chapter 3 are reported the experiments I performed to identify the specific factor needed to mediate the CotC-CotU interaction. I, first, noticed that the interaction never occurred in *cotE* null mutants and, then, showed by pull down experiments that both CotC and CotU directly interact with CotE *in vitro*. In addition, I also showed that the interaction did not occur when CotC and CotU were expressed together in *Escherichia coli* while it did occur when also *cotE* was co-expressed.

In Chapter 4 studied the mechanism of assembly of CotB. This protein is probably assembled as a dimer (CotB-66), most likely a heterodimer with CotG (Zilhão et al., 2004). Since CotB has a particular primary structure characterized by the presence of serine-lysine rich repeats (SKR) in its C-terminal part, I decided to investigate whether the SKR repeats were involved in CotB assembly and in the formation of the mature protein of 66 kDa. To this aim I constructed a *cotB* deletion mutant (*cotB*Δ*SKR*), and studied its assembly.

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Chapter 2

"CotC-CotU Heterodimerization during Assembly of the *Bacillus subtilis* Spore Coat"

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Abstract

We report evidence that CotC and CotU, two previously identified components of the *Bacillus subtilis* spore coat, are produce concurrently in the mother cell chamber of the sporulating cell under the control of σ^K and GerE and immediately assembled around the forming spore. In the coat, the two proteins interact to form a coat component of 23kDa. The CotC-CotU interaction was not detected in two heterologous hosts, suggesting that it occurs only in *B. subtilis*. Monomeric forms of both CotC and CotU failed to be assembled at the surface of the developing spore and accumulated in the mother cell compartment of cells mutant for *cotE*. In contrast, neither CotU nor CotC accumulated in the mother cell compartment of cells mutant for *cotH*. These results suggest that CotH is required to protect both CotU and CotC in the mother cell compartment of the sporangium and that CotE is needed to allow their assembly and subsequent interaction at the spore surface.

Introduzione

In *Bacillus subtilis*, the spore coat is a complex multiprotein structure that plays an important role in spore germination and resistance to toxic chemicals (8, 11, 13). Recently, new functions have been assigned to the coat, from sensing the external environment through active enzymes present on its surface (4, 12, 24, 40) to protecting the spore from predaction by phagocytic protozoans (21). In addition, the coat is a novel system for the display at the spore surface of heterologous antigens (16, 18, 25), enzymes (22), and bioactive molecules (20).

The coat is dynamic structure, able to adapt to changes in the spore volume by expanding and contracting in response to dehydratation and rehydration occurring during the *B. subtilis* life cycle (3). A heterogeneous group of over 50 polypeptides form the three main structural layers of the coat: a diffuse undercoat, a laminated lightly staining inner layer, and a thick electron-dense outer coat (11, 13). Several of these polypeptides have been studied, and their structural genes (*cot* genes) have been identified. Expression of all *cot* genes is governed by a cascade of four transcription factors acting specifically in the mother cell compartment of the sporangium in the sequence SigmaE-SpoIIID-SigmaK-GerE; SigmaE and SigmaK are RNA

polymerase sigma factors, and SpoIIID and GerE are DNA-binding proteins acting in conjunction with SigmaE- and SigmaK-driven RNA polymerase (8, 11, 13). An additional transcription factor, GerR, has recently been implicated in the control of some coat genes (10).

In addition to the transcriptional regulation, control exerted at the protein level seems to be particularly important for the assembly of the multiprotein structure of the coat. A variety of posttranslational modifications have been shown to occur during coat formation: some coat-associated polypeptides appear to be glycosylated (11, 13), others are derived from proteolytic processing of larger precursors (1, 6, 34, 37), and some others are highly cross-linked as a result of reactions that take place at the spore surface (12, 40). In addition, a small subset of coat proteins, known as morphogenetic proteins, play an important role in controlling the assembly of most of the coat. These proteins have no effects on coat protein synthesis but act post-translationally to guide the assembly of the various coat components around the forming spore (19). SpoVM, a 26-aminoacid peptide, is believed to adhere to the outer forespore membrane and to allow the localization of SpoIVA around the forming pore (30). The SpoIVA layer then directs the assembly of the morphogenetic protein CotE in a ring-like structure around the forespore (9). Inner coat components are thought

to infiltrate through the CotE, while outer coat proteins assemble on the outside of the E ring (8, 11, 13). Additional proteins with morphogenetic functions are needed at later stages of coat formation. For instance, SpoVID has the dual role of directing SafA to the forming spore and maintaining the CotE ring around the forespore (4, 28). Another example is CotH, which plays a role in the assembly of various outer coat components, partially controls assembly of CotE, and is required for the development of the normal morphological features of spores (19, 26, 41). A recent study has shown that CotH controls the assembly of the coat proteins CotB, CotC, CotG, CotS, CotSA, CotQ, CotU, CotZ, and YusA (19). In different studies, it has been proposed that the role of CotH in the assembly of CotC, CotG, and CotB is to stabilize CotC (17) and CotG, which in turn is needed for the assembly and dimerization of CotB (41). In particular, CotC does not accumulate in the mother cell compartment, where it is synthesized, but is immediately assembled around the forming spore (17). Assembly of CotC requires expression of both *cotH* and *cotE*, does not accumulate in the mother cell compartment when is assembly is prevented by mutation of *cotH* (17). In contrast, overexpression of CotH allows the accumulation of CotC in the mother cell compartment, suggesting that CotH, or a CotH-dependent factor, acts

to prevent degradation of CotC in the mother cell and then allows it assembly within the coat (2). The mechanism of assembly of CotC is of interest, as the abundant CotC protein has been used as a vehicle for the display of heterologous proteins at the spore surface (18).

Here, we report that CotU, a recently identified structural homologue of CotC (23), intercats with CotC, forming an alkali-soluble coat protein of 23kDa in a CotE- and CotH-dependent manner. CotC and CotU share almost identical N-terminal regions, with 23 out to 24 identical amino acid residues, and less conserved C-terminal parts (Fig 1A) (7). In addition, both CotU and CotC contain high numbers of tyrosine, lysine, and aspartic acid residues that account for over 70% of their total numbers of amino acids. This peculiar primary structure likely causes the unusual migration of the two proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with CotC and CoU having deduced masses of 8.8 and 11.4 kDa and apparent masses of 12 and 17 kDa, respectively.

We show that like CotC, the CotU protein does not accumulate in the mother cell compartment of a *cotH* mutant. In contrast both CotC and CotU (but not the 23-kDa species) accumulated in the mother cell of a *cotE* mutant but failed to be assembled. These results reinforce the view that CotH has a role in the stabilization of certain coat proteins in

the mother cell cytoplasm. The results also indicate that formation of the 23-kDa CotC-CotU species takes place at the spore surface, following the assembly of both proteins.

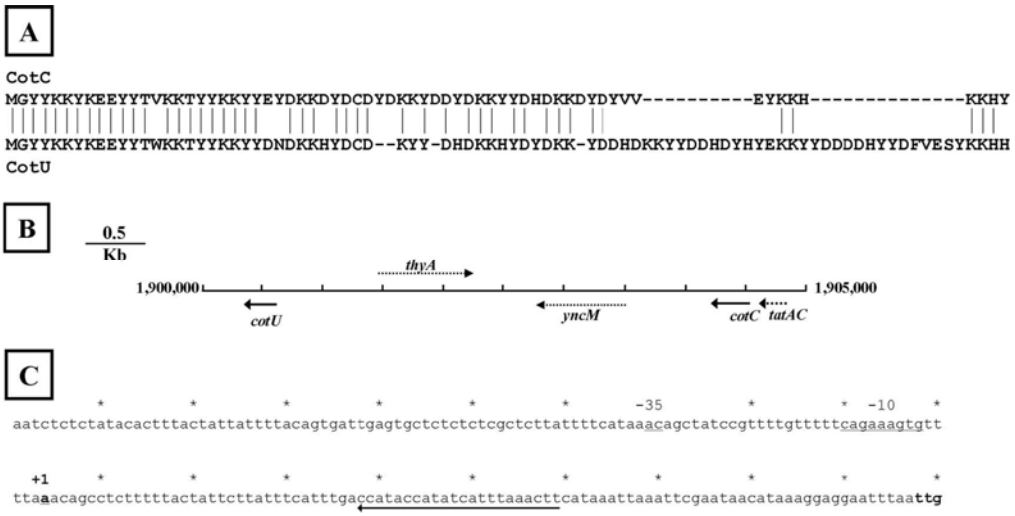

Fig. 1. (A) CotC and CotU amino acid alignement. (B) Schematic representation of the *cotC-cotU* chromosomal region. The arrows and numbers indicate the directions of transcription and the *B. subtilis* chromosome, respectively. (C) *cotU* promoter region. The translational start site (TTG) is in boldface, the transcriptional start site is indicate as +1, and the putative promoter sequences are underlined. The arrow indicates oligonucleotidi U-pr-Anti, used for the primer extension experiment shown in Fig. 2B.

Materials And Methods

Bacterial strains and transformation. The *B. subtilis* strains are listed in Table 1. Plasmid amplification for nucleotide sequencing, subcloning experiments, and transformation of *Escherichia coli* competent cells were performed with *E. coli* strain DH5α (33). Bacterial strains were transformed by previously described procedures: $CaCl_2$-mediated transformation of *E. coli* competent cells (33) and two-step transformation of *B. subtilis* (5).

Plasmid and strain construction. Isolation of plasmids, restriction digestion, and ligation of DNA were carried out by standard methods (33). Chromosomal DNA from *B. subtilis* was isolated as described elsewhere (5). Fragments of *cotC* and *cotU* DNAs were PCR amplified from the *B. subtilis* chromosome; the amplification was primed with the synthetic oligonucleotides listed in Table 2. The *cotU::lacZ* translational fusion was obtained as follows. A DNA fragment containing 476 bp upstream of the *cotU* translational start site was PCR amplified using chromosomal DNA as a template and oligonucleotides Y3s and Ua (Table 2) and inserted into plasmid pJM783 (40) upstream of the promoterless *lacZ* gene.

TABLE 1. *B. subtilis* strains

Strain	Relevant genotype	Source
PY79	Wild type	36
BD063	*cotA*::*cat*	7
RH248	Δ*amyE*::*cotU-lacZ* Cm^r	This work
RH249	*gerE* Δ*amyE*::*cotU-lacZ* Cm^r	This work
AZ153	*gerE36*	Lab collection
AZ198	$SP\beta_{C2}$ *cotC*::*lacZ* Cm^r	Lab collection
RH228	*cotC*::His_6 *cotC*::*spc*	This work
RH208	*cotU*::*neo* *cotU*::His_6	This work
RH209	*cotU*::*neo* *cotC*::*cat*	18
RH210	*cotU*::*neo* *cotC*::*cat* *cotU*::His_6	This work
RH225	Δ*amyE*::*cotC*-His_6 Cm^r	This work
RH232	Δ*amyE*::*cotC*-His_6 Erm^r	This work
RH254	*cotU*::*neo* *cotC*::*cm* Δ*amyE*::*cotU*-His_6	This work
RH256	*oxdD*::*cat*	This work
RH255	*sodA*::*cat*	This work
RH233	*tgl*::*spc*	40
ER203	*cotG*::Δ*erm*	32
RH101	*cotC*::*spc*	17
RH202	*cotU*::*neo*	17

The resulting plasmid, pRH92, carrying the *cotU::lacZ* fusion, was introduced by single reciprocal (Campbell-like) recombination between *B. subtilis* DNA sequences in pRH92 and corresponding region of homology in the chromosome I the PY79 wild-type strain of *B. subtilis*. Several chloramphenicol resistant (Cm^r) clones were analyzed by PCR, and one of them, RH248, was used for further studies. Chromosomal DNA containing the integrated fusion-bearing plasmid was then used to transform a congenic collection of sporulation mutants. *cotU::*His_6 and *cotC::*His_6 gene fusions were constructed by using the gene splicing by overlap extension technique

as described by Horton et al. (14). The *B. subtilis* chromosome was used as a template, and the synthetic oligonucleotides Y3s/Y2anti and Y-His 1/Y-His2 for *cotU::*His_6 and CotCp/C-His1 and C-His2/Cstop for *cotC::*His_6 were used as primers (Table 2). The 680- and 402-bp PCR products obtained for *cotU::*His_6 and *cotC::*His_6 were cloned into the pGEM-T easy vector (Promega). The inserts in the resulting plasmids were analyzed by DNA sequencing to verify the absence of unwanted mutations, released with SphI and SalI, and introduced into pDG364 (5) previously digested with the same two enzymes. *E. coli* competent cells were transformed with the ligation mixture, and the selected ampicillin-resistant clones were screened by restriction analysis of their plasmids.

The obtained plasmids, pRH42 (*cotU::*His_6) and pRH48 (*cotC::*His_6) were linearized and used to transform competent cells of the *B. subtilis* strains RH202 and RH101 (Table 1), yielding strains RH208 (*cotU::*His_6) and RH228 (*cotC::*His_6), respectively. Cm^r clones were the result of double-crossover recombination, due to the interruption of the nonessential *amyE* gene on the *B. subtilis* chromosome. Several Cm^r clones were tested by PCR using chromosomal DNA as a template and oligonucleotides AmyA and AmyS (Table 2). Two

clones, one from each transformation, were named RH208 (*cotU::His6*) and RH228 (*cotC::His6*) and kept for further studies.

To obtain a *B. subtilis* strain carrying both *cotC::*His_6 and *cotU::*His_6 fusions, the Cm^r determinant (*cat*) of the strain RH225 was replaced with an erythromycin resistance gene cassette (*erm*) by using plasmid pECE72 (Bacillus Genetic Stock center, Columbus, OH). Chromosomal DNA of the resulting strain, RH232, was used to transform competent cells of strain RH210 (*cotU::*His_6). Several clones resistant to erythromycin were tested by PCR, and one, RH254, was selected for further studies.

sodA null mutation was obtained by PCR amplifying an internal part of the gene using *B. subtilis* chromosomal DNA as a template and oligonucleotides SodA-3F and SodA-445R (_Table 2). The 445-bp PCR product was cloned into plasmid pER19 (31), and the resulting plasmid, pRH97, was used to transform competent cells of the *B. subtilis* strain PY79, yielding strain RH255.

An *oxdD* null mutation was obtained by PCR amplifying an internal part of the gene using *B. subtilis* chromosomal DNA as a template and oligonucleotides OxdD-3F and OxdD-647R (Table 2). The 647-bp PCR product was cloned into plasmid pER19 (31), and the resulting

plasmid, pRH121, was used to transform competent cells of the *B. subtilis* strain PY79, yielding strain RH256.

Primer extension analysis. Total RNA was extracted from a wild-type strain 8h after the onset of sporulation using the Qiagen Mini Kit (Qiagen, Milan Italy) according to the manufacture's instructions. Total RNAs were dissolved in 50 µl of RNase-free water and stored at -80°C. The final concentration and quality of the RNA samples were estimated either spectrophotometrically or by agarose gel electrophoresis with ethidium bromide staining. Total RNAs were treated with RNase-free DNase (1 U/µg of total RNA; turbo DNA-free; Ambion) for 30 min at 37°C, and the reaction was stopped with DNase inactivation reagent. For primer extension experiments, 10 µg of total RNA was used with [γ32-P]dATP (GE Heathcare)-labeled oligonucleotidi U-pr-Anti (Table 2), deoxynucleoside triphosphate, and avian myeloblastosis virus reverse transcriptase (BRL) to prime cDNA synthesis, as previously described (26). The reaction products were fractionated on 6 M urea-6% polyacrylamide gels, along with DNA-sequencing reactions using pRH85 (pGEM-T easy/cotU) as the template primed with the same oligonucleotide.

Expression in *E. coli*. The *cotU* coding region was amplified from *B. subtilis* chromosomal DNA with primers Ycoding and Y2a (Table 2). The 302-bp PCR product was cleaved with XhoI and EcoRI and ligated into the expression vector pRSETA, previously digested with the same restriction enzymes (Invitrogen). The recombinant plasmid carrying an in-frame fusion of the 3' end of the *cotU* coding region to six histidine codons under the transcriptional control of a T7 promoter was used to transform competent cells of *E. coli* BL21 (DE3) (Invitrogen), yielding strain RH59. This strain was grown in ampicillin-supplemented (50 μg/ml) tryptone-yeast extract medium to an optical density of 0.7 at 600 nm. The T7 promoter was then induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside; 0.5 mM final concentration) to the culture and incubating it for 2 h at 37 °C.

The His_6-tagged CotU protein was purified under denaturing conditions via Ni^{2+}-nitrilotriacetic acid affinity chromatography as recommended by the manufacturer (Qiagen, Inc.) and used to raise specific antibodies (IGtech, Salerno, Italy).

TABLE 2. Synthetic oligonucleotides

Oligonucleotide	Sequence (5′–3′)	Restriction site	Position of annealing[b]
Y3s	gtcgacGATTTAATGCATTGTATTTTACC	SalI	-494/-471
Ua	aagcttTTCCAAGTATAATACTCTTC	HindIII	+43/+24
SodA-3F	ggtaccGCTTACGAACTTCC	KpnI	+4/+18
SodA-445R	ggatccGGTTTGGCGTGC	BamHI	+446/+434
OxdD-3F	ggtaccCTGTTGGAACAA	KpnI	+4/+20
OxdD-647R	ggatccCGGCACATTCCC	BamHI	+648/+635
Ycoding	ctcgagTTGGGTTATTATAAA	XhoI	+1/+15
Ya2	gcatgcTTATAAATAGGGGAAGGC	SphI	+449/+430
CotCp	ACATgcatgcTGTAGGATAAATCGTTTG	SphI	-181/-63
CotCSTOP	gtcgacTTATTAGTAGTGTTTTTTATGC	SalI	+357/+338
C-His1	GTCATCGTCATGGTGGTGATGATGATGCATATATACTCCTCC		-13/+1
C-His2	CATCACCACCATGACGATGACGATAAGATGGGTTATTACAAA		+15/+1
Y-His1	CCATGATGATGATGATGATGCAATTAAATTCCTCC		+3/-12
Y-His2	TTGCATCATCATCATCATCATGGTTATTATAAAAAA		+4/+18
AmyA	CGAGAAGCTATCACCGCCCAGC		+2128/+2150
AmyS	CCAATGAGGTTAAGAGTATTCC		+569/+590
U-pr-Anti	AAGTTTAAATGATATGGTATGG		+39/+60

[a] Uppercase and lowercase letters indicate nucleotides complementary to corresponding cotU or cotC DNA and unpaired flanking sequences carrying a restriction site, respectively.

[b] Positions refer to cotU, cotC, or amyE sequences, considering the first nucleotide of the translational start site as _1.

Western blotting. *B. subtilis* sporulation of wild-type and recombinant strains was induced by the exhaustion method (5, 27). After a 30-h incubation at 37°C, spores were collected, washed four times, and purified by lysozyme treatment as previously described (5, 27). The number of purified spores obtained was measured by direct counting with a Bürker chamber under an optical microscope (Olympus BH-2 with 40x lenses). Aliquots of 10^{10} spores suspended in 0.3 ml of distilled water were used to extract coat proteins by 0.1 N NaOH treatment at 4°C as previously reported (2). The concentration of the extract coat proteins was determined by the Bio-Rad DC (Detergent Compatible) Protein Assay to avoid potential interference by the NaOH present (0.2 to 0.6 mN, final concentration) in the extraction buffer and 15 μg of total proteins fractionated on 18% denaturing polyacrylamide gels. The proteins were electrotransferred to nitrocellulose filters (Bio-Rad) and used for Western blot analysis by standard procedures. For the analysis of sporulating cells, samples were harvested at various times during sporulation and distrupted by sonication in 25 mM Tris (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 15% (vol/vol) glycerol, and 0.1 mg/ml of phenylmethylsulfonyl fluoride. The sonicated material was then fractionated by centrifugation at 10,000 rpm for 20 min. The pellet, containing the forming spores

resistant to the sonication treatment, was solubilised by 0.1 NaOH treatment at 4°C, and the total protein concentration was determined as describe above. Fifty micrograms (mother cell extract) or 15 μg (forespore extract) of total proteins was fractionated on 18% denaturing polyacrylamide gels. Western blot filters were visualized by the SuperSignal West Pico Chemiliminescence (pierce) method as specified by the manufacturer.

Results

The *cotU* gene of *B. subtilis* is under σ^K-GerE control. The control (formerly *ynzH*) and *cotC* genes, coding for CotU and CotC, respectively, are located about 4 kb apart in the *B. subtilis* chromosome (Fig. 1B). Previous reports (15, 39) showed that *cotC* transcription is driven by σ^K-containing RNA polymerase and that the DNA-binding protein GerE acts as a transcriptional activator of *cotC* expression. Recently, studies using DNA arrays have suggested that *cotU* is also transcribed under the control of σ^K-driven RNA polymerase (10, 35). To analyze *cotU* expression in more detail, we constructed a transcriptional gene fusion between the *cotU* promoter region and the *lacZ* gene of *E. coli* and measured the activities of β-galactosidase at various times after the onset of sporulation in an otherwise wild-type strain and a collection of congenic sporulation mutants. In all cases, the fusion was integrated at the *cotU* locus as a result of a single reciprocal crossover event (see Materials and Methods). In agreement with the expectation that *cotU* is under the control of σ^K, we observed that β-galactosidase production commenced between 6 and 7 h after the onset of sporulation (Fig. 2A), at the same time as expression of a *cotC::lacZ* fusion in a congenic

strain (39) (Fig. 2A). Moreover, expression of *cotU::lacZ* was severely reduced in a σ^K mutant (*spoIIIC*) (data not shown), as well as in mutants (*spoIIG*, *spoIIID*, *spoIIIG*, and *spoIVF*) (data not shown) known to be impaired in σ^K production. *cotU::lacZ*-driven synthesis of β-galactosidase was also impaired in a *gerE* mutant (Fig. 2A), indicating that efficient transcription of *cotU* requires the presence of the *gerE*-encoded DNA-binding protein.

By analogy with the case of *cotC* (15, 39) and in extension of previous observations (10, 35), we inferred that *cotU* is transcribed by σ^K-containing RNA polymerase acting in conjunction with GerE.

A primer extension experiment was performed to map the *cotU* promoter and transcriptional start site. The extension product obtained (Fig. 2B) allowed us to localize the 5' terminus of *cotU* mRNA 91 bp upstream of the beginning of the open reading frame (Fig. 1C). Sequences upstream of a 5' terminus (+1) resembled the conserved features of a σ^K promoter, matching in four of six positions the consensus -10 (consensus, CATANNNTA; *cotU*, CAgANNNTg; differences are in lowercase) and in both positions the consensus -35 (AC) (fig. 1C).

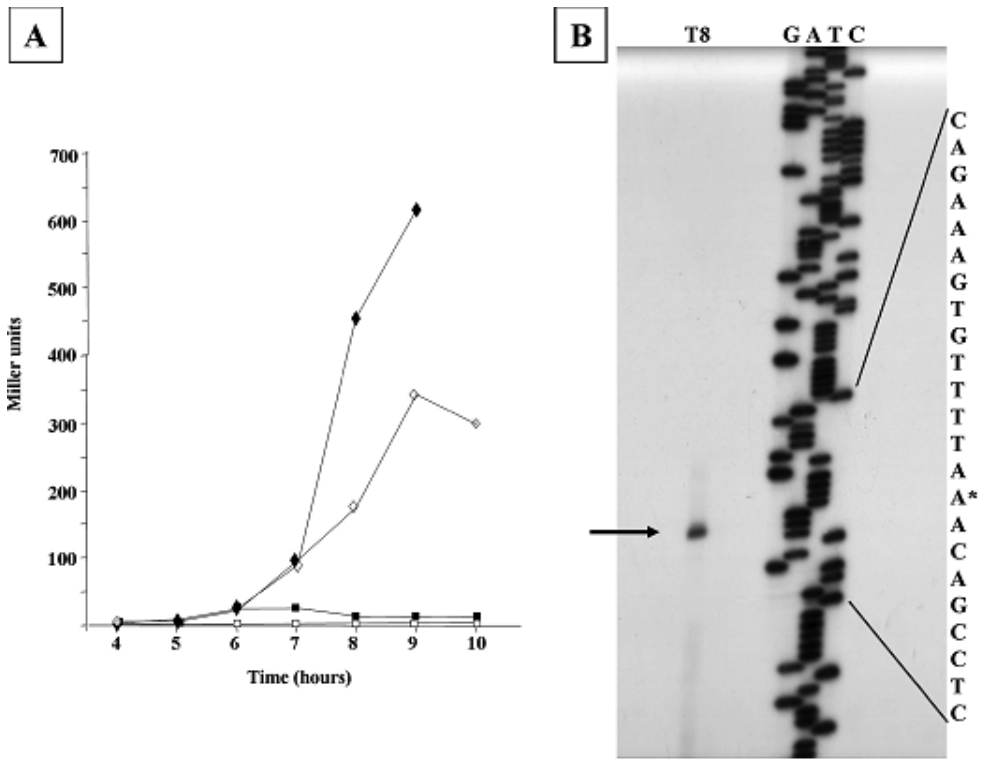

FIG. 2. (A) Expression of a *cotU::lacZ* transcriptional fusion during sporulation in a otherwise wild-type (open diamonds) or a *gerE* null mutant (closed square) strain and of a *cotC::lacZ* transcriptional fusion in a otherwise wild-type strain (closed diamonds). Background levels of β-galactosidase activity were determined in a wild-type strain bearing no *lacZ* gene (open square). Samples were collected at various times after the onset of sporulation. Enzymes activity is expressed in Miller units. The data are the means of three independent experiments. (B) Primer extension analysis of the *cotU* promoter region performed with total RNA extracted from sporulating cells 8 hours (T8) after the onset of sporulation. Primer extension and sequencing reactions were primed with the synthetic oligonucleotidi U-pr-Anti (Table 2).

CotU and CotC directly interact in the spore coat. In order to study CotU assembly within the spore coat, we overexpressed a His_6-tagged version of *cotU* in *E. coli* and used the protein partially purified by Ni^{2+} affinity chromatography to raise a polyclonal antibody (see Materials and Methods). Due to the high similarity between CotU and CotC, our antibody reacted to the gene products of both *cotU* and *cotC*, and this recognition appeared to be specific, since no proteins were recognized in a *cotC cotU* double-null mutant (Fig. 3). Our anti-CotU antibodies recognized six polypeptides in the coat protein fractions of wild-type spores; four of them (of 12, 12.5, 21, and 30 kDa) were also detected in the coat extracts from *cotU* mutant spores (Fig. 3) and thus corresponded to the previously identified products of *cotC* expression (17). The 17-kDa protein was absent from the coats of *cotU* null mutant spores but present in the coats of *cotC* null mutant spores (Fig. 3), suggesting that it was the product of *cotU* expression. The conclusion that the 17-kDa protein is the product of *cotU* is also supported by the results of a previous study (7), in which N-terminal amino acid sequence analysis revealed a perfect match with the deduced sequence of CotU for the first 20 N-terminal positions of the 17-kDa protein.

The remaining polypeptide of 23 kDa was detected only among the proteins extracted from the coats of wild-type spores (Fig. 3), suggesting that it is dependent on the expression of both *cotC* and *cotU* (17). The genetic dependence of the 23-kDa protein on the *cotC* and *cotU* expression obviously suggests the possibility that it is the result of an interaction between CotC and CotU.

To test this possibility, we constructed two recombinant strains of *B. subtilis* carrying a His-tagged version of either *cotC* (RH228; see below) or *cotU* (RH208) as the only copy of *cotC* or *cotU* present in those strains. As shown in Fig. 4A, the 23-kDa proteins of strains RH208 and RH228 showed similarity slower migrations on SDS-PAGE than the 23-kDA protein of the congenic wild-type strain. The slower migration was most likely due to the presence of the His tag, as suggested by the observation that other tagged proteins also displayed altered electrophoretic properties. For instance, in strain RH228 (CotC-His6) the presence of the His_6 tag slowed the migration of the four CotC forms and of the 23-kDa protein, although in the experiment shown in Fig. 4A, this effect is observable only for the higher-molecular-mass proteins (21,23, and 30 kDa); in strain RH208 (CotU-His_6), the presence of the His_6 tag reduced the migration of both *cotU*-dependent proteins of 17 and 23 kDa (Fig. 4A). Moreover,

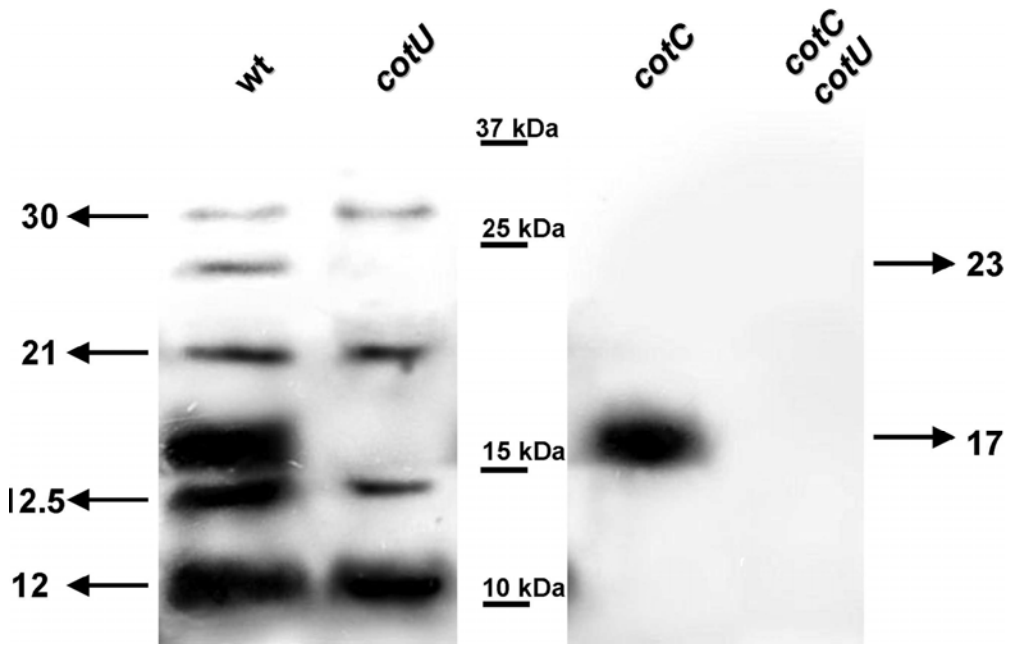

FIG. 3. Western blot analysis with anti-CotU antibody of coat proteins extracted from a wild type (wt) and congenic strains with null mutations in *cotU*, *cotC*, and *cotC cotU*. The proteins were fractionated on an 18% polyacrylamide gel and, upon electrotransfer onto nitrocellulose membranes, reacted with CotU-specific rabbit antibodies and then with peroxydase-conjugated secondary antibodies and visualized by the Pierce method. The arrows indicate the apparent molecular weights of the observed proteins. The molecular masses of a marker are also indicated. Identical results were obtained with anti-CotC antibody.

when spore coat proteins were purified on Ni^{2+} columns, fractionated on SDS-PAGE, and analyzed by Western blotting with anti-CotC (17) or anti-CotU antibodies, the RH228 (CotC-His_6) (Fig. 4B) or RH208 (CotU-His_6) (Fig.4C) but not those from a congenic wild-type strain

(not shown). The variation in the relative abundances of the 23-kDa protein in the Western blots in Fig. 4 (compare panel A with panels B and C) most likely depended on poor recovery of this less well represented protein species from Ni^{2+} columns. Thus, the 23-kDa polypeptide was purified by Ni^{2+} affinity chromatography when the His_6 tag was joined to either CotC or CotU.

The genetic dependence and the biochemical evidence shown in Fig. 4 together indicate that CotC and CotU directly interact to form the 23-kDa polypeptide. To gain insight into the interaction between CotC and CotU, we constructed gene fusions in which the His_6 tag was placed in frame to the 3' or 5' end of both the *cotC* and *cotU* genes. All fusions were integrated on the chromosome of strain RH209, carrying null mutations in both *cotC* and *cotU* (18), and the extracted coat proteins were analyzed by Western blotting. A wild-type pattern of CotC- and CotU-dependent proteins was observed when CotU was tagged at either end (not shown) and when CotC was tagged at its N-terminal end (Fig. 5A).

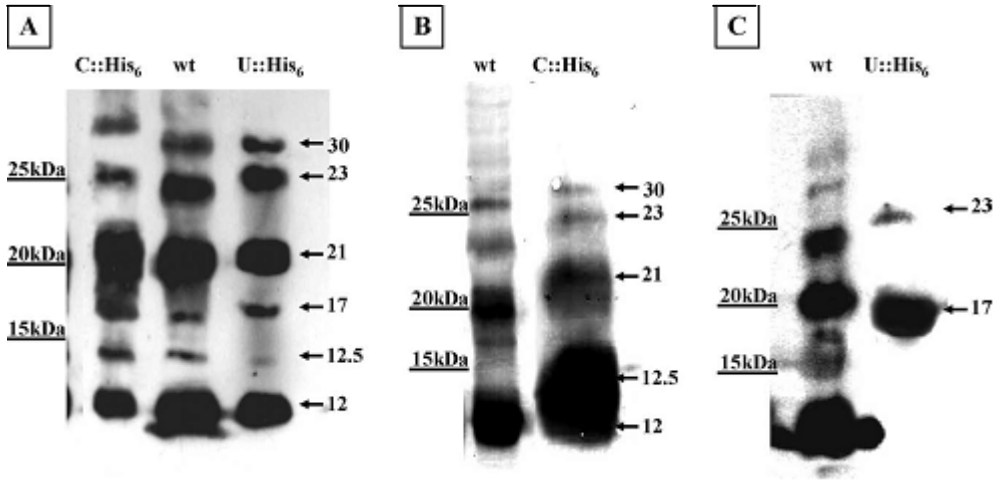

FIG. 4. Western blot analysis wih anti-CotU (a and C) or anti-CotC (B) antibody. Coat proteins were extracted from a wild-type (t) strain and a congenic strain carrying a *cotC*::His_6 (C::His_6) or a *cotU*::His_6 (U::His_6) fusion as indicated. Proteins were fractionated and then blotted (A) or purified through an Ni column and then blotted (B andC). In panels B and C, the wt lane contains unpurified proteins from wild-type spores. The arrows indicate the apparent molecular weights of the observed proteins. Molecular mass are also indicated.

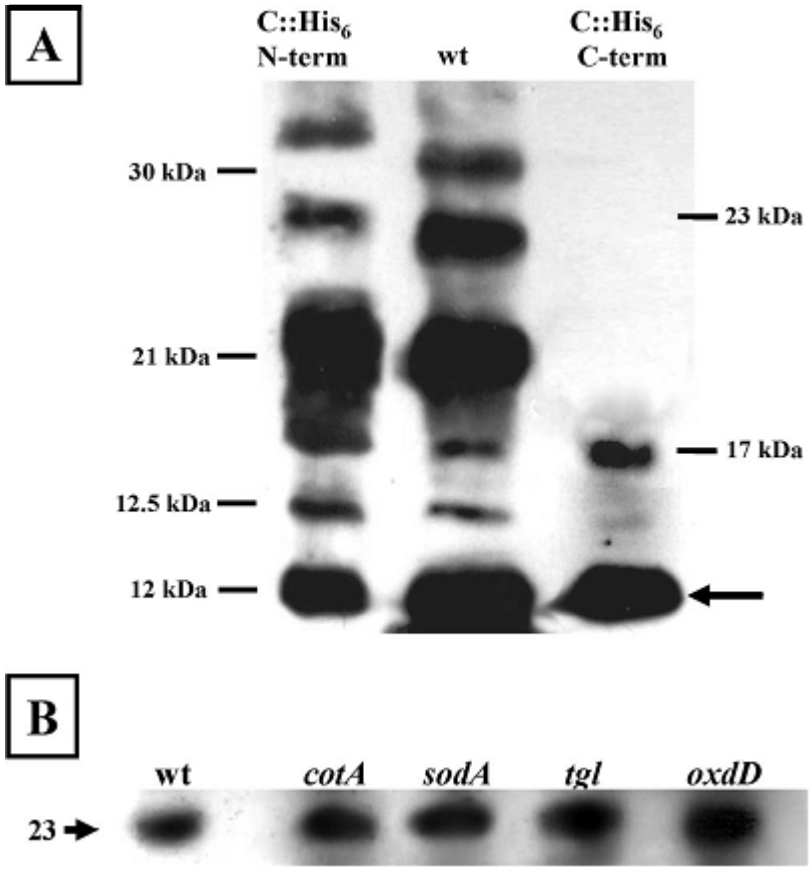

FIG. 5. Western blot analysis with anti-CotC antibody. (A) proteins extracted from recombinant *B. subtilis* strains containing the His_6 tag fused at the 5' (C::His_6 N-term) or 3' (C.::His_6 C-term) end of the *cotC* gene and from a congenic wild-type strain (wt). The indicated molecular masses refer to the various CotC- and CotU-dependent proteins from a wilde-type strain (without the His_6 tag). The arrow indicates the 12-kDa species of CotC. (B) Proteins were extracted from spores of a wild-type *B. subtilis* strain and of a collection of congenic strains with null mutations in *cotA*, *oxdD*, *sodA*, or *tgl*. Identical results were obtained with anti-CotU antibody.

The presence of the His_6 tag at the C terminus of CotC did not impair assembly of the CotC species of 12 kDa (presumably the CotC monomer) (Fig. 5A): the level of CotC (12 kDa) was not reduced relative to the amount found in the coats of wild-type spores (Fig. 5A), yet formation of all other forms of CotC and of the 23-kDa CotC-CotU species was severely reduced. These results, therefore, suggest that the C-terminal end of CotC is involved in the formation of both the CotC-CotC homodimer and the CotC-CotU heterodimer. Moreover, these results strongly support the idea that multimerization of CotC and CotU occurs at the spore surface, following assembly of both proteins.

CotU and CotC do not interact in heterologous hosts. Yeast two-hybrid experiments were performed using *cotU* and *cotC* coding sequences fused to the activation or the DNA-binding domain of the *Saccharomyces cerevisiae* transcriptional activator GAL4, as previously described (17, 28). The gene fusions were then introduced into the yeast reporter strains Y187/Y190 (28), and the expression of a *lacZ* reporter gene followed. As previously reported, an interaction was detected between CotC and itself, indicating that CotC molecules self-interact (17). In contrast, and under similar conditions, no

interaction was detected between CotC and CotU (data not shown), indicating that the two proteins do not interact when produced in yeast cells. To verify whether CotU and CotC were able to interact in a different heterologous host, we co-expressed *cotU* and *cotC* in *E. coli*. Plasmid pRH51 (17), carrying the *cotC* coding region His_6 tagged at its 5' end under the transcriptional control of the T7*lac* promoter, was engineered to replace the ampicillin resistance gene with a kanamycin resistance gene cassette, and the resulting plasmid was used to transform strain BL21 (DE3) (Novagen), already containing plasmid pRH59 with a version of *cotU*::His_6 tagged at its 3' end. Upon IPTG induction, three bands corresponding in size to those produced by expressing *cotU* and *cotC* separately were produced and recognized by anti-CotU, anti-CotC, and anti-His_6 antibodies. No additional proteins due to the interaction of CotC and CotU were observed (data not shown). In contrast, the 23-kDa CotC-CotU species was detected by both anti-CotC and anti-CotU antibodies in coat extracts of a *B. subtilis* strain expressing both His_6-CotC (His_6 at the 5' end) and cotU-His_6 (His_6 at the 3' end) (not shown). Therefore, the same proteins that failed to interact in *E. coli* were able to interact in *B. subtilis*, thus excluding the possibility that the interaction was inhibited in *E. coli* by the simultaneous presence of the His_6 tag in both partners involved in

the interaction. Since the CotC-CotU interaction occurs in *B. subtilis* but not in two heterologous hosts (*S. cerevisiae* and *E. coli*), we hypothesized that a specific factor might be needed to mediate that interaction.

CotC-CotU interaction does not require CotA, OxdD, Tgl, or SodA. CotA, OxdD, SodA, and Tgl are coat components with laccase (24), oxalate decarboxylase (4), superoxide dismutase(12), and transglutaminase (40) activities, respectively. It has been proposed that spore coat-associated enzymatic activities may be involved in mediating specific protein-protein interactions, including protein cross-linking reactions within the coat (11). Based on this, we used a collection of congenic strains of *B. subtilis* mutant for *cotA* (BD063), *oxdD* (RH256), *sodA* (RH255), or *tgl* (Rh233) to analyzed whether the laccase, oxalate decarboxylase, superoxide dismutase, or transglutaminase activity was involved in mediating the CotC-CotU interaction. The 23-kDa protein (Fig5B), as well as all other CotC- and CotU-dependent proteins (not shown), was present in the coat fractions of spores of all mutant strains analyzed. Therefore, none of the enzymatic activities tested is required for formation of the 23-kDa CotC-CotU species.

Assembly of CotU and of the CotU-CotC heterodimer. Western blotting of proteins extracted from the mother cell or the forespore compartment of sporulating cells had indicated that none of the CotC forms accumulated in the mother cell, suggesting their rapid assembly onto the forming spore (17). Here, we have extended this analysis to the assembly of CotU and of the CotU-CotC polypeptide of 23-kDa. Sporulating cells of a wild-type strain were harvested at various times during sporulation and lysed by sonication as described in Materials and Methods, and the forming spores (forespore fraction) were separated from the mother cell cytoplasm (mother dell fraction). The forming spores were then extracted by alkali treatment, and the released proteins were compared with those present in the mother cell cytoplasm. For each time point, both protein fractions were analyzed by Western blotting with anti-CotU antibodies. At all time points analyzed, we detected no *cotU*-dependent polypeptides in the mother cell fraction (not shown). In the forespore fraction, we observed the four CotC forms (12, 12.5, 21, 30 kDa) from hour 6 onward; as previously reported (17), CotU (17 kDa) and the CotU-CotC species (23 kDa) were detected from hour 8 onward (fig. 6A). In the time course experiment shown in Fig. 6A, CotU, as well as the 23-kDa protein, appeared 1 hour later than CotC. Since the analysis of the

cotU::*lacZ* transcriptional fusion has shown that the timing of expression of *cotU* is identical to that of *cotC* (Fig. 2), the delayed appearence of CotU could be due to a posttranscriptional control on *cotU* expression. Alternatively, it could be that the high concentration of CotC in wild-type spores would not allow a sufficiently long exposure of the membrane, needed to visualize the less abundant CotU. To discriminate between the two possibilities, we repeated a similar time course experiment with a *cotC* null strain. As shown in Fig. 6B, when CotC was not present and the membrane could be exposed for enough time, CotU was detected 8h after the onset of sporulation, at the same time as the appearance of CotC (Fig. 6A). thus, although CotC and CotU accumulate at different rates, the onsets of their syntheses coincide.

The absence of CotC and CotU forms from the mother cell fraction suggests their rapid assembly at the surface of the developing spore. This again suggests that formation of the 23-kDa species takes place only at the spore surface.

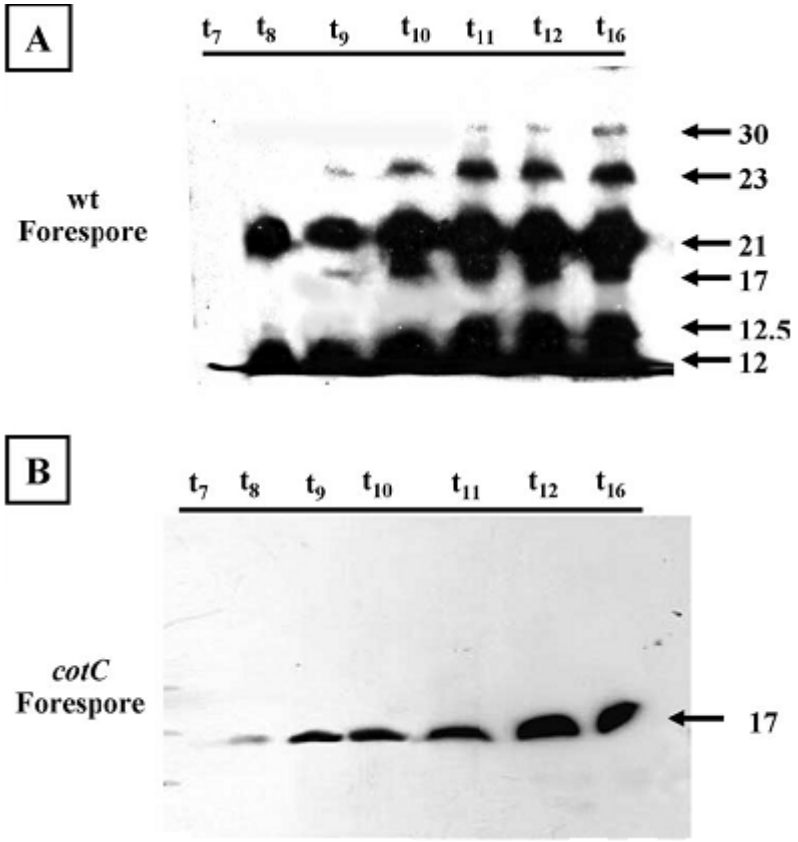

FIG. 6. Western blot of proteins extracted at various times (t_7 to t_{16}) after the onset of sporulation from the forespores of sporulating cells of a wild-type (wt) (A) and a *cotC* null mutant (B) strain of *B. subtilis*. The proteins were fractionated on a 15% polyacrylamide gel and, upon electrotransfer onto nitrocellulose membranes, reacted with CotU- specific rabbit antibodies and then with peroxidise-conjugated secondary antibodies and visualized by the Pierce method. The estimated size of CotC- and CotU-dependent polypetides are indicated in kilodaltons.

CotU assembly and CotU-CotC interaction depend on CotE and CotH. To analyze the requirements for CotU assembly and for the CotC-CotU interaction, we performed a Western blot analysis with anti-CotU antibody and proteins extracted from mature spores of the wild-type and spores of various congenic strains deficient in the CotU-CotC proteins of 23 kDa were found in the coats of wild-type spores but were not found in *cotE* or *cotH* spores (Fig. 7A). Therefore, CotU assembly and CotC-CotU interaction appear to be CotE and CotH dependent. Since CotE and CotH do not affect *cotC* or *cotU* expression (26, 38), we predicted that in *cotE* and *cotH* null mutants, CotU and CotC would accumulate in the mother cell. This was true for the *cotE* null mutant, in which CotU and the two main forms of CotC (12 and 21 kDa) (17) were found in the mother cell compartment (Fig. 7B).

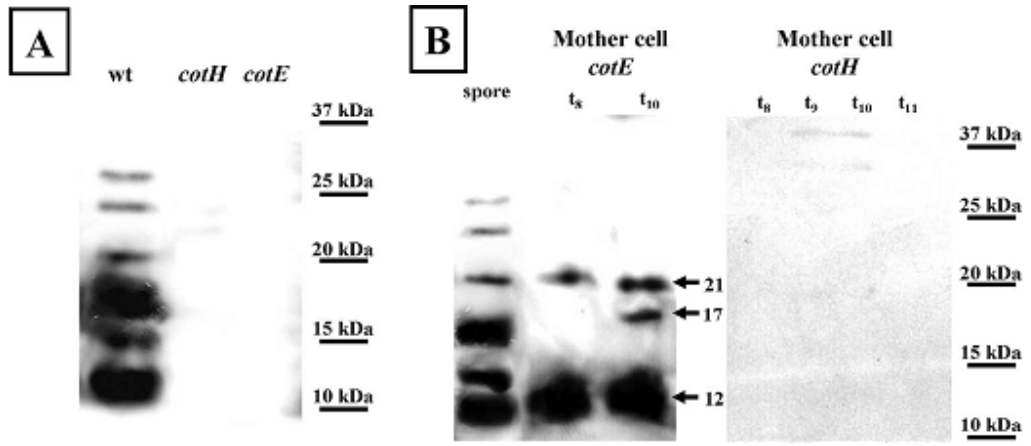

FIG. 7. Western blot analysis with anti-CotU antibody. (A) Coat proteins were extracted from a wild-type (wt) and congenic strains with null mutations in *cotH* or *cotE*. (B) Proteins were extracted at the indicated times (t_8 to t_{11}) during sporulation from the mother cell compartments of sporulating cultures of a *cotE* or a *cotH* mutant strain. The lane labelled "spore" contains coat proteins extracted from spores of a wild-type strain.

However, the CotC-CotU protein of 23 kDa was not found in the mother cell compartment of a *cotE* mutant (Fig. 7B) (17). This is in agreement with the idea that the 23-kDa CotC-CotU protein is formed at the spore surface. The absence of the two additional forms of CotC (12.5 and 30 kDa) in the mother cell of a *cotE* null mutant as been previously reported and discussed (17). In the *cotH* null mutant, CotU and the CotU-CotC form, as well as all CotC forms, were not found to accumulate in the mother cell (Fig. 7B). it has been previously

proposed that the assembly of all CotC forms depends on the presence of CotH, which protects and/or stabilizes them in the mother cell compartment, allowing their assembly around the forming spore (2, 17). By analogy, we propose here that CotH is also involved in the stabilization/protection of CotU in the mother cell and possibly in its assembly on the coat.

Therefore, formation of the 23-kDa CotC-CotU species is under dual control: CotH stabilizes and/or protects both interaction partners in the mother cell compartment, whereas CotE governs their assembly and subsequent interaction at the spore surface.

Discussion

In the present work, we have shown that the *cotU* gene is transcribed during sporulation under the joint control of the σ^K factor of RNA polymerase and the transcriptional factor GerE. Therefore, *cotU* and *cotC*, both of which code for highly similar tyrosine-rich components of the coat, are expressed coincidentally in the mother cell compartments of sporulating cells. The interaction of GerE with the *cotU* promoter region was not directly observed but was inferred on the basis of the effect of a *gerE* mutation on *cotU*-driven β-galactosidase activity.

We have also studied the assembly of CotU and expanded previous work on the assembly of the related protein CotC. We present genetic and biochemical evidence that CotU and CotC specifically interact, forming a protein of 23 kDa extractable from the coats of mature spores.

The CotC-CotU species appears shorter than expected on SDS-PAGE if the apparent sizes of CotC and CotU are added to each other. A possible explanation is that a fragment of one of the two components is cleaved when the interaction occurs. Our data do not allow us to establish whether such a proteolytic event actually occurs. However,

the data in Fig. 4 showing that the CotC-CotU species can be purified on an Ni^{2+} column when a His tag is placed at the C-terminal end of CotU or at the N-terminal end of CotC allow us to exclude the possibility that those two regions are involved in a potential proteolytic cleavage. Several lines of evidence suggest that the interaction of CotC and CotU to form the CotU-CotC species of 23 kDa takes place at the spore surface. First, neither CotU nor CotC is detected in the mother cell compartment of wild-type cells, suggesting that their assembly occurs rapidly following their synthesis (reference 17 and this work). Second, a *cotE* null mutation prevented the assembly of both CotU and CotC, causing the accumulation of the monomeric forms of both proteins in the mother cell but not the accumulation of the 23-kDa CotU-CotC form. Finally, expression of an allele of *cotC* fused at its 3' end to the sequence for the His_6 tag resulted in the assembly of wild-type amounts of monomeric CotC but drastically impaired the formation of all the multimeric forms of CotC and of the CotC-CotU heterodimer. Although specific interactions between coat components have often been suggested as an important aspect of coat formation (19), only a few cases of direct interaction have been reported for coat proteins. In this respect, the observation that the CotU-CotC interaction involves an additional factor active at

the spore surface. It is tempting to speculate that this factor could be a cross-linking enzyme, possibly assembled under CotE control (formation of the CotC-CotU species of 23 kDa was not detected in the mother cell of *cotE* mutant cells). The CotU-CotC interaction is not affected by extraction under alkaline conditions or by boiling in the presence of SDS and reducing agents. Both CotU and CotC are tyrosine rich, and formation of irreversible peroxydase-catalyzed o,o-dityrosine cross-links, which may occur within the coats, could account for the stability of the 23-kDa species. We note that cross-linking reactions have been shown to take place at the spore surface even after spore release from the mother cell, including the multimerization of another tyrosine-rich abundant coat protein, CotG (29, 40). However, we also note that work is needed to characterize the nature of the CotU-CotC interaction and the putative factor involved. In any case, our results also indicate that the C-terminal end of CotC is likely to be involved in interaction with itself and with CotU. It may be that the C-terminal region of CotC represents a nucleation point for multimer formation, because the presence of the His_6 tag in this region drastically reduces formation of all multimeric forms of CotC (including the CotC-CotU species).

Our results also expand on previous observations related to the role of the morphogenetic protein CotH in assembly of the coat structure. CotU does not accumulate in the mother cell cytoplasm of a *cotH* mutant. This cannot be due to reduced stability of the protein in the mother cell cytoplasm, since it was detected by Western blotting in the cytoplasm of a *cotE* mutant, as well as in *E. coli*. We believe it is more likely that a specific factor (a protease?) degrades CotU in the absence of CotH (or a *cotH*-dependent protein). Similarity to what has been proposed for CotC (17) and CotG (42), we hypothesize that in a wild-type strain, CotH (or a *cotH*-dependent protein) would prevent CotU degradation either by interacting in a chaperone-like manner with CotU or its specific protease in the mother cell or by immediately recruiting CotU into the coat of the forming spore.

In a recent paper, CotU assembly within the coat was analyzed by fluorescence microscopy and found to be only partially dependent on CotE and CotH (19). In the present study, our Western blotting experiments indicated that CotU assembly is totally dependent on both morphogenetic proteins. As previously proposed for other coat components (19), this discrepancy could be explained by the presence of CotU in both the soluble (extractable) and insoluble (resistant to extraction) fractions of coat proteins. Fluorescence microscopy would

then allow the detection of all CotU molecules, while Western blotting would detect only soluble CotU molecules. An alternative possibility is that CotU and the chimeric protein CotU-green fluorescent protein used in the previous study (19) have different stability and/or assembly properties. Since none of the mutants described here has any obvious phenotypic consequences for the properties of the coat, the functionality of a CotU-green fluorescent protein fusion is difficult to assess.

Future work will aim at characterizing in detail the interaction between CotC and CotU, including the identification and role of the putative factor involved, and the role of CotH in the process.

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Chapter 3

"CotE binds to and mediates the interaction of CotC and CotU during spore coat formation in *Bacillus subtilis*"

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Abstract

CotE is a morphogenic protein that controls the assembly of the coat, the proteinaceous structure that surrounds and protects the spore of *Bacillus subtilis*. On the base of genetic experiments, CotE has long been inferred to interact with several outer coat components but such interactions have never been directly demonstrated.

To assay the interaction of CotE with other coat components we used CotC and CotU, two outer coat protein known to be under CotE control and to form a heterodimer. We report here pull-down experiments that provide the first direct evidence that CotE contacts other coat components. In addition, co-expression experiments demonstrate that CotE is needed and sufficient to allow formation of the CotC-CotU heterodimer in a heterologous host.

Introduction

The spore of *Bacillus subtlis* is a dormant cell, resistant to harsh conditions and able to survive extreme environmental conditions (25). Spores are produced in a sporangium that consists of an inner cell, the forespore, that will become the mature spore and an outer cell, the mother cell, that will lyse, liberating the mature spore (18, 26). Resistance of the spore to noxious chemicals, lytic enzymes and predation by soil protozoans is in part due to the coat, a complex, multilayered structure of more than 50 proteins that encases the spore (5, 13, 8). Proteins that constitute the coat are produced in the mother cell and deposited around the outer membrane surface of the forespore in an ordered manner (8).

A small subset of coat proteins have a regulatory role on the formation of the coat. Those proteins, referred to as morphogenic factors, do not affect the synthesis of the coat components but drive their correct assembly outside of the outer forespore membrane (8). Within this subset of regulatory coat proteins, SpoIVA and CotE play a crucial role. SpoIVA (20, 23, 6) is assembled into the basement layer of the coat and is anchored to the outer membrane of the forespore through its C terminus that contacts SpoVM, a small, amphipathic peptide embedded in the forespore membrane (16, 21, 22). A *spoIVA* null mutation impairs the assembly of the coat around the forming spore

and, as a consequence, coat material accumulates in the mother cell cytoplasm (23).

CotE (28) assembles into a ring and surrounds the SpoIVA basement structure. The inner layer of the coat is then formed between the SpoIVA basement layer and the CotE ring by coat components produced in the mother cell that infiltrate through the CotE ring, while the outer layer of the coat is formed outside of CotE (6). However, not all CotE molecules are assembled into the ring-like structure and CotE molecules are also found in the mother cell cytoplasm, at least up to eight hours after the start of sporulation (3). CotE was first identified as a morphogenic factor in a seminal study in which an ultrastructural analysis indicated that a *cotE* null mutation prevented formation of the electron-dense outer layer of the coat while didn't affect inner coat formation (28). A subsequent mutagenesis study has revealed that CotE has a modular structure with a C terminal domain involved in directing the assembly of various coat proteins, an internal domain involved in targeting of CotE to the forespore and a N terminal domain that, together with the internal domain, directs the formation of CotE multimers (17). More recently, formation of CotE multimers has been also confirmed by a yeast-two-hybrid approch (14). In a global study of protein interactions in the *B. subtilis* coat, performed by a fluorescence microscopy analysis of a collection of strains

carrying *cot-gfp* fusions, CotE has been proposed to interact with most outer coat compenents (12).

From those and other studies the interactions of CotE with coat structural components have been exclusively inferred on the base of genetic experiments, i.e. *cotE* mutants that failed to assemble one or more coat components. Evidence of a direct interaction between CotE and another coat component have never been provided. We addressed this issue by using as a model two coat components, CotC and CotU, known to be controlled by CotE and to form a heterodimer (28, 10).

CotC is an abundant, 66 amino acids protein known to assemble in the outer coat in various forms: a monomer of 12 kDa, a homodimer of 21 kDa and two less abundant forms of 12.5 and 30 kDa, probably due to post-translational modifications of CotC (9). CotU is a structural homolog of CotC of 86 amino acids. The two proteins, that share an almost identical N terminus and a less conserved C terminus (Fig. 1A) (10), interact originating an heterodimer of 23 kDa. Heterodimer formation most likely requires a *B. subtilis* specific factor since it does not occur in *Escherichia coli* or *Saccharomyces cerevisiae* (10). CotC and CotU are synthesized in the mother cell compartment of the sporulating cell but do not accumulate there since are immediately assembled around the forming spore (Fig. 1B) (10). In a strain carrying a *cotE* null mutation CotC and CotU, together with all other

outer coat components, do not assemble around the forming spore (10). CotC and CotU are also dependent on CotH, an additional morphogenic factor involved in coat formation (9). Since a *cotH* null mutation prevents CotC and CotU assembly in the coat as well as their accumulation in the mother cell cytoplasm (10), while a mutation causing *cotH* over-expression allows CotC and CotU accumulation in the mother cell cytoplam (1), it has been proposed that CotH acts by stabilizing CotC and CotU in the mother cell cytoplasm (1, 10).

Here we provide the first direct evidence that CotE interacts with two other coat components, CotC and CotU, and show that CotE is essential and sufficient to mediate CotC-CotU interaction to form a heterodimer.

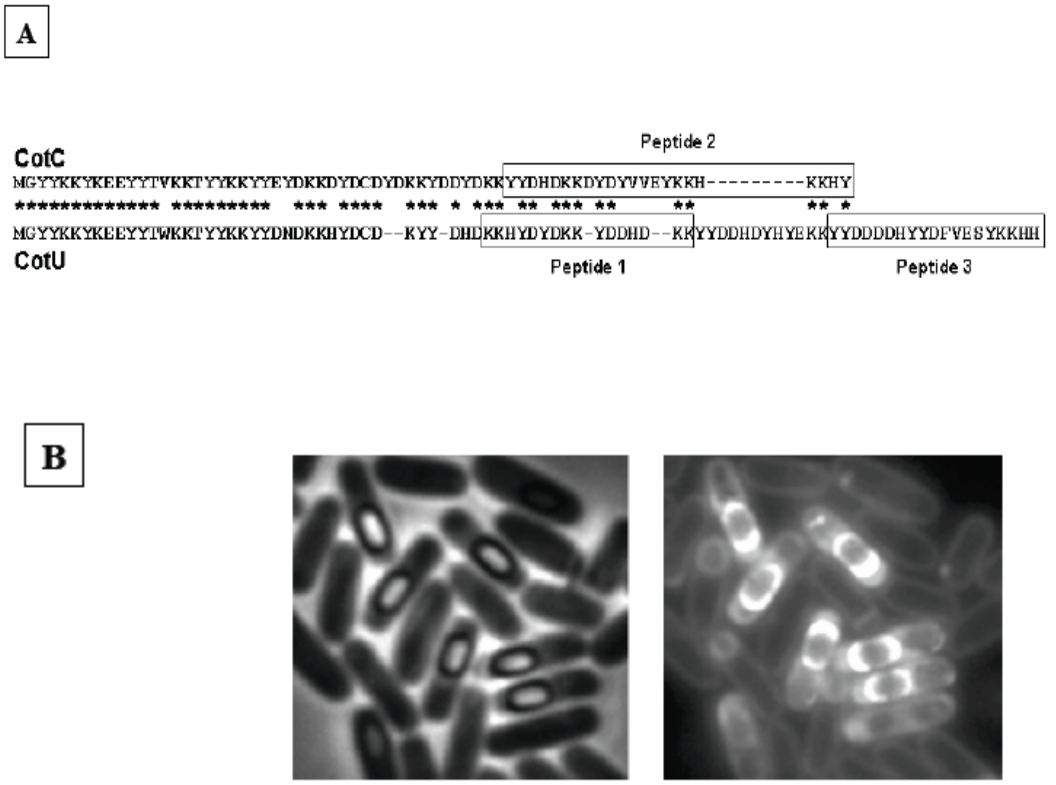

FIG. 1**:** (A) Alignment of the amino acid sequences of CotC and CotU. Asterisks indicate identical residues. Boxed are peptides identified as diagnostic of CotC or CotU (see below). (B) Microscopy analysis of sporulating cells of *Bacillus subtilis* carrying the *cotC::gfp* fusion collected eight hours after the onset of sporulation. The same microscopy fiels was observed by phase contrast (left panel) or by fluorescence microscopy after vital staing with FM4-64 to label the membranes (right panel). In cells containing a prespore the green fluorescent signal was exclusively localized around the forming spore.

Materials and Methods

Bacterial strains and transformation. *B. subtilis* strains used in this study are listed in Table 1. Plasmid amplification for subcloning experiments, nucleotide sequence and transformation of *Escherichia coli* competent cells were performed with *E. coli* strain DH5α (24). *E. coli* strain BL21 (DE3) (Novagene) was used for protein over-expression. Bacterial strains were transformed by previously described procedures: CaCl2-mediated transformation of *E. coli* competent cells (24) and two-step transformation of *B. subtilis* (4).

Genetic and molecular procedures. Isolation of plasmids, restriction digestion and ligation of DNA were carried out by standard methods (24). Chromosomal DNA from *B. subtilis* was isolated as described elsewhere (4). A *spoIVA cotE* double mutant was obtained by transforming competent cell of strain 67 (*spoIVA*) (Table 1) with chromosomal DNA extracted from strain BZ213 (*cotE::cat*) generating strain RH263.

Table 1. *Bacillus subtilis* and *Escherichia coli* strains used.

B. subtilis		
Strain name	**Relevant genotype**	**Source**
PY79	prototrophic	27
BZ213	*cotE::cat*	28
67	*spoIVA*	7
RH263	*spoIVA cotE::cat*	This work
E. coli [a]		
Strain name	**Relevant genotype of contained plasmid**	**Source**
RH52	*cotC::his*	9
RH59	*cotU::his*	10
RH62	*cotC::his*	10
RH63	*cotC::his cotU::his*	10
RH125	*cotC::his cotU::his cotE*	This work
RH136	*cotE*	This work
AP19	*cotE::his*	This work

[a]All *E. coli* strains are derivative of strain BL21(D3) transformed with various plasmids.

Sporulating cells lysates and immunoblot analysis. Sporulation of all *B. subtilis* strains was induced by the exhaustion method (4). Sporulating cells were harvested after eight and ten hours from the onset of sporulation and mother cells and forespore fractions isolated as described before (10). Whole-cell lysates of sporulating cells were prepared by sonication (10) followed by detergent treatment (62.5mM Tris-Hcl, pH6.8, 4%SDS, 5% glycerol, 2%B-mercaptoethanol, 0.003% bromophenol blue) at 100°C for 7 minutes. 50 μg (mother cell extract or whole-cell lysates) or 15 μg (forespore extract) of total proteins were subjected to immunoblot analysis with the anti-CotC or anti-CotU antibodies as described previously (10), except that

polyvinylidene difluoride membranes were used instead of nitrocellulose.

Over-production of 6xHis-tagged and untagged CotE. To over-express CotE in *E. coli*, the coding region of the *cotE* gene was PCR amplified using the *B. subtilis* chromosomal DNA as a template and oligonucleotides E-rbs-PstI-F (ctgcagtttaAGAAGGAGAtatacatatgtct gaatacagggaat; *Pst*I and *Nde*I restriction sites are underlined, the ribosome binding site is in uppercase) and E-STOP (ccaagcttattcttcaggatctcccac; *Hind*III restriction site is underlined) as primers. The amplification product of 582 bp was digested with *Nde*I and *Hind*III and ligated to the same sites of the expression vector pRSETB (Invitrogen), to obtain pRH134. By digesting pRSETB with *Nde*I the His tag present on the vector was removed.

To over-express a 6xHis tagged copy of CotE in *E. coli,* the coding region of the *cotE* gene was amplified by PCR using *B. subtilis* chromosomal DNA as template and oligonucleotides E-NdeI-F (taggaattccatatgtctgaatacagggaat; underlined is the*Eco*RI restriction site) and E-STOP (ccaagcttattcttcaggatctcccac; underlined is the *Hind*III restriction site) as primers. The amplified fragment of 564 bp was digested with *EcoRI* and *Hind*III and ligated to plasmid pRSETB (Invitrogen), previously digested with the same enzymes. The recombinant plasmid, pAP18, carried the *cotE* coding region fused to

a 6xHis tag under the transcriptional control of a T7 inducible promoter.

Plasmids pRH134 and pAP18 were checked by nucleotide sequence analysis and used to transform competent cells of *E. coli* BL21 (DE3) (Novagen), to create RH136 and AP19, respectively (Table1). CotE and CotE-His were produced by auto-induction by growing cells at 37°C for 18 hours with orbital shaking (150 rpm) and by using the Overnight Express Autoinduction System1, following manufacturer's instructions (Novagen). CotE-His protein was purified under denaturing conditions via Ni-nitrilotriacetic acid affinity chromatography as recommended by the manufacturer (Qiagen, Inc) and used to raise specific antibodies in mice by PriMM srl (Italy).

Co-expression of *cotC, cotU* and *cotE* in *E.coli*. The coding part of *cotE*, amplified by PCR using oligonucleotides E-rbs-PstI-F and E-STOP as described above, was digested with *Pst*I and *Hind*III and inserted in the same sites of pRH62 (10), immediately downstream of *cotC::his* to create pRH122. In this plasmid both genes were under the control of the same T7 promoter and formed a single transcriptional unit. The new recombinant plasmid was used to transform the competent cells of *E. coli* strains RH59 (10), harboring *cotU::his* under the control of T7 promoter, to create strain RH125. Proteins

were produced from these *E. coli* strains by the auto-induction procedure, as describe above.

Pull-down experiments. Strains RH52, RH59 and RH136 (Table1) were grown for 18 hours at 37°C in auto-induction medium (see above). Samples (14 ml) were collected by centrifugation and resuspendend in 1.5 ml of Lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM Imidazole, 2mg/ml Lysozyme and 0.01mg/ml RNase). After 30 min at 4°C, the lysates were sonicated (20 min pulses at 20 Hz with a Sonicator Ultrasonic Liquid Processor; Heat System Ultrasonic Inc., NY, USA). The suspension was clarified by centrifugation at 13.000g at 4°C for 20 min and those of strains RH52 and RH59 applied to Ni-NTA Magnetic Agarose Beads (Qiagen), separately. After 1h of incubation at room temperature with shake, the beads were washed with 2.5 ml of Wash Buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM Imidazole), the extract of RH136 strains added to both samples and incubated for 1 h at room temperature with shake. Unbound proteins were removed by washing with Wash Buffer at three different concentration of imidazole (40mM, 100mM and 250mM). Bound proteins were eluted using the Wash Buffer at increasing concentration of imidazole (500mM and 1M). Eluted proteins were resolved on SDS-12.5% PAGE gels and subjected to immunoblot analysis.

Preparation of samples for MALDI TOF analysis. Strain RH125 was grown for 18 hours at 37°C in auto-induction medium (see above). Cells (100 ml) were washed with 10 ml of 1x phosphate-buffered saline (PBS), suspended with 5 ml of 1x PBS and 5 ml of 2x Cracking Buffer (120mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 2% β-mercaptoethanol) and heated at 100°C for 10 min. The suspension was clarified by centrifugation at 13.000g for 20 min and the supernatant diluted 1:10 using Binding buffer (20 mM Sodium Phosphate, 500 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol, 25 mM imidazole pH 7.4). The diluted sample was applied to a His Trap HP (GE Healthcare, Healthcare Europe, GmbH, Milan, Italy) equilibrated with 10 ml of Binding buffer. The column was washed with 10 ml of Binding buffer and proteins eluted with the same buffer, supplemented with increasing concentrations of imidazole (50 mM, 100 mM, 250 mM, 500 mM). Purified proteins were resolved on SDS-15% PAGE gels and bands sent for MALDI- mass spectrometry analysis.

Reduction and Alkylation of coat proteins. Coat proteins extracted from mature spore by 0.1N NaOH treatment at 4°C (4) were treated with various reducing conditions (10mM or 80mM dithiothreitol or 1% B-mercaptoethanol, for either 60 min at 80°C or over night at 37°C) and then alkylated by adding 50 mM iodoacetic acid (IAA), 1 N

NaOH for 30 min on ice in the dark or directly alkylated. The proteins were washed in an Amicon Ultra-15 Centricon (Millipore) with 10 volumes 1xPBS, resolved on SDS-15% PAGE gels and subjected to immunoblot analysis.

Fluorescence Microscopy. For CotC-GFP localization, 500 µl of cells were collected after eight hours from the onset of sporulation, briefly pelleted and resuspended in 30 µl of 1x PBS containing 1.5µg of FM4-64 (SIGMA) per ml. The concentrated cells (3 µl) were immediately spotted onto microscope slides and firmly covered with poly L-lysine (0.1% solution, Sigma) treated coverslips. Fluorescence microscopy was performed with an Olympus BX51 microscope equipped with a 100x UPlanF1 objective, a U-WIBA filter cube (excitation filter 460–490 nm, barrier filter 515–550 nm) for GFP visualization and a U-WG filter cube (excitation filter 510–550 nm, barrier filter >590nm) for FM4-64 visualization. Exposure times were typically in the range of 500–1000 ms. Images were captured and cropped with analySIS software (SIS). Finally, images were exported as TIF files to the Adobe Photoshop software for minor adjustments of brightness and contrast.

Results

***cotE* expression is required to allow the interaction between CotC and CotU.** It has been previously reported that CotC assembles in the spore coat as a monomer and homodimer and that it forms with CotU a heterodimer of 23 kDa (10). In western blot experiments performed on sporulating cells of a wild type strain of *B. subtilis* eight and ten hours after the onset of sporulation CotC, CotU and the CotC-CotU heterodimer were found only in the forespore compartment (Fig. 2A), indicating that they are assembled immediately after their synthesis in the mother cell compartment (10). In a similar experiment performed with an isogenic strain carrying a *cotE* null mutation, CotC and CotU were found in the mother cell compartment while the heterodimer was not formed, indicating that CotC and CotU are normally produced, that they are not assembled around the forming spore and that they fail to interact (Fig. 2B).

A possible explanation for the lack of interaction in the *cotE* mutant is that the heterodimer can only be formed when the two partners of the interaction are already assembled on the forming coat.

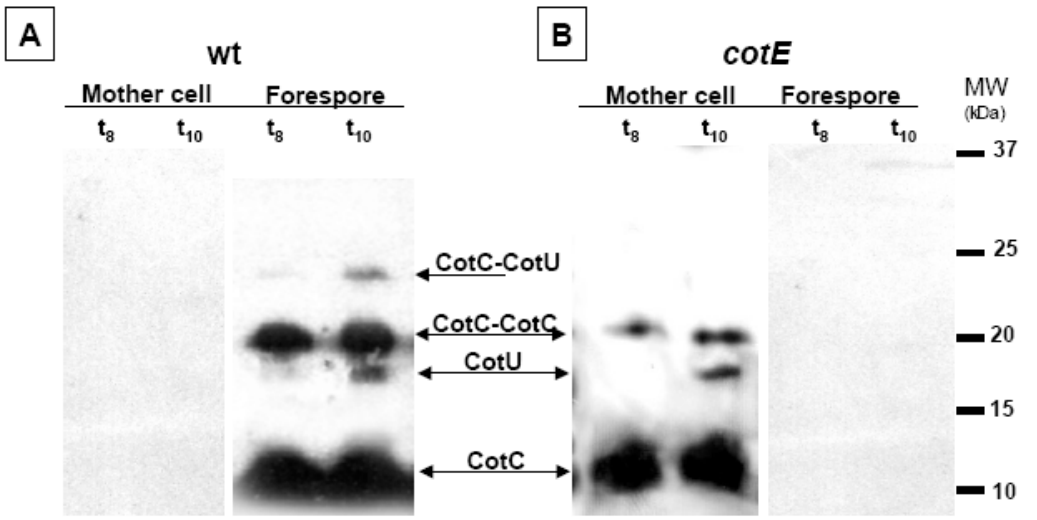

FIG. 2: Western blot of proteins extracted from the Mother cell or Forespore fractions of sporulating wild type (A) or isogenic *cotE* (B) mutant cells eight and ten hour after the onset of sporulation. Proteins were fractionated on 15% polyacrylamide gels, electrotransferred to membranes and reacted with anti-CotC antibody. Identical results were obtained with anti-CotU antibody

Two lines of evidence indicate that this is not the case and that the heterodimer forms also when the two partners are not assembled: i) a western blot analysis of extracts of sporulating cells of a *B. subtilis* strain mutated in the *spoIVA* genes, in which the entire coat is not around the spore (23), showed that the CotC-CotU heterodimer is formed but only in a *cotE*-dependent way (Fig. 3A); ii) a western blot analysis with extracts of the mother cell fraction of sporulating cells of a *B. subtilis* mutant having an increased expression of the *cotH* gene (1), confirms that the 23 kDa heterodimer is formed in the mother cell cytoplasm in a *cotE*-dependent way (Fig. 3B).

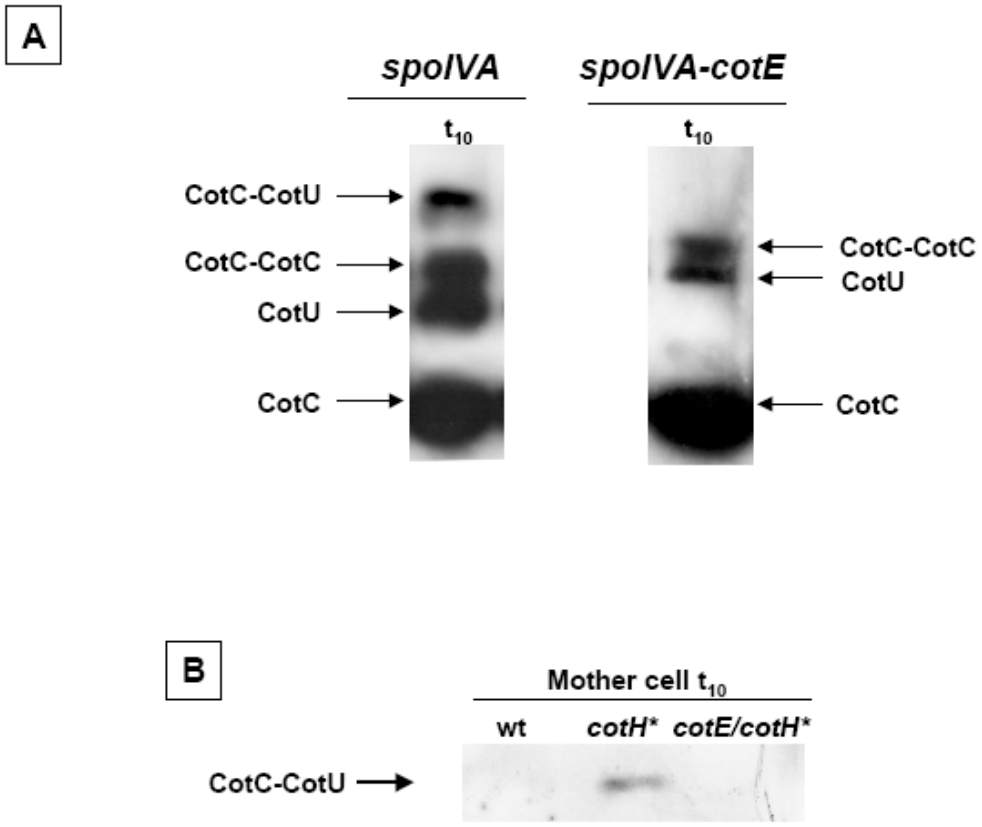

FIG. 3: Western blot of proteins extracted ten hours after the onset of sporulation from (A) sporulating cells of a *spoIVA* mutant or a *spoIVA cotE* double mutant and (B) the mother cell fraction of a strain over-expressing *cotH* (indicated as *cotH**). Proteins were fractionated on 15**%** polyacrylamide gels, electrotransferred to membranes and reacted with anti-CotU antibody. Identical results were obtained with anti-CotC antibody.

Taken together these data support the hypothesis that CotE or a CotE-dependent protein is needed to allow the interaction between CotC and CotU and that such interaction can occur independently from the assembly of CotC and CotU on the spore coat.

Formation of CotC homodimer and CotC-CotU heterodimer does not involve cysteine residues. CotC-CotU heterodimer as well as CotC-CotC homodimer, were observed on SDS-PAGE (Fig. 2 and 3) after extraction from sporulating cells or mature spores by treatment with 0.1N NaOH at 4°C (19), indicating that both dimers are resistant to reducing and denaturing conditions.

Since both CotC and CotU have a cysteine residue at position 32 (Fig. 1A), we decided to verify whether those cysteines were involved in the formation of the homo- and/or hetero-dimer. To this aim we exposed proteins extracted from a wild type strain to extreme reducing conditions (Materials and Methods) and to subsequent alkylation with iodoacetic acid (IAA) (15). IAA binds to reduced cysteines and prevents them from re-forming sulfur bridges. None of the tested conditions impaired formation of the dimers after either one hour of incubation at 60 °C (Fig. 4) or an overnight incubation at 37°C (not shown), thus excluding that they were depended on cysteine-mediated sulfur bridges.

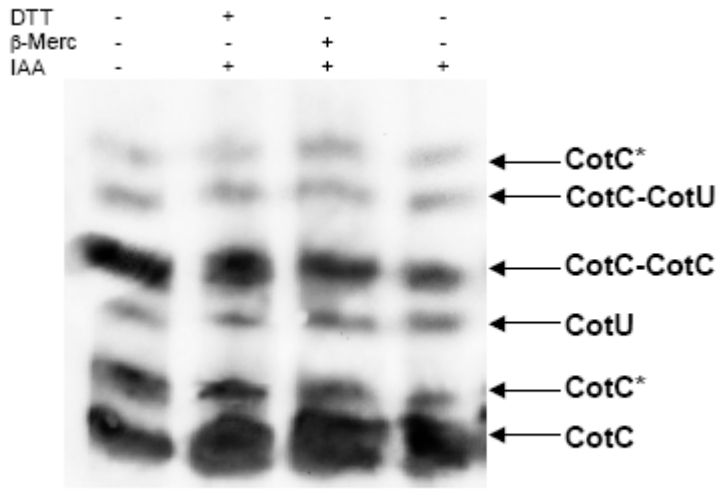

FIG. 4**:** Effect of reduction and alkylation on CotC homodimer and CotC-CotU heterodimer. Coat proteins extracted from wild type spores were incubated for 60 min at 60°C in the absence or the presence of different reducing agents. After reduction, coat proteins were alkylated by adding 50mM IAA. Treated proteins were fractionated on 15% polyacrylamide gels, electrotransferred to membranes and reacted with anti-CotC. CotC* indicates the two so far uncharacterized forms of CotC (Isticato et al., 2004).

CotE binds to CotC and CotU. To verify whether CotE directly interacts with CotC and/or CotU, we over-expressed in *E. coli* a his-tagged version of *cotC* (CotC-His), a his-tagged version of *cotU* (CotU-His) or an untagged version of *cotE* and performed a *in vitro* His-tag pull-down assay. After auto-induction, *E.coli* cells were lysed by sonication and Ni-NTA magnetic beads incubated with extracts of cells expressing CotC-His or CotU-His. Beads were then washed and incubated with the extract of cells expressing untagged CotE. After

additional washes proteins were eluted and used for western blot experiments with anti-CotC, anti-CotU, anti-His or anti-CotE antibodies. As shown in Fig. 5, untagged CotE bound Ni-NTA beads when CotC-His (panel A) or CotU-His (panel B) was present. In the absence of CotC-His or CotU-His, untagged CotE was not able to bind to the Ni-NTA beads (Fig. 5C).

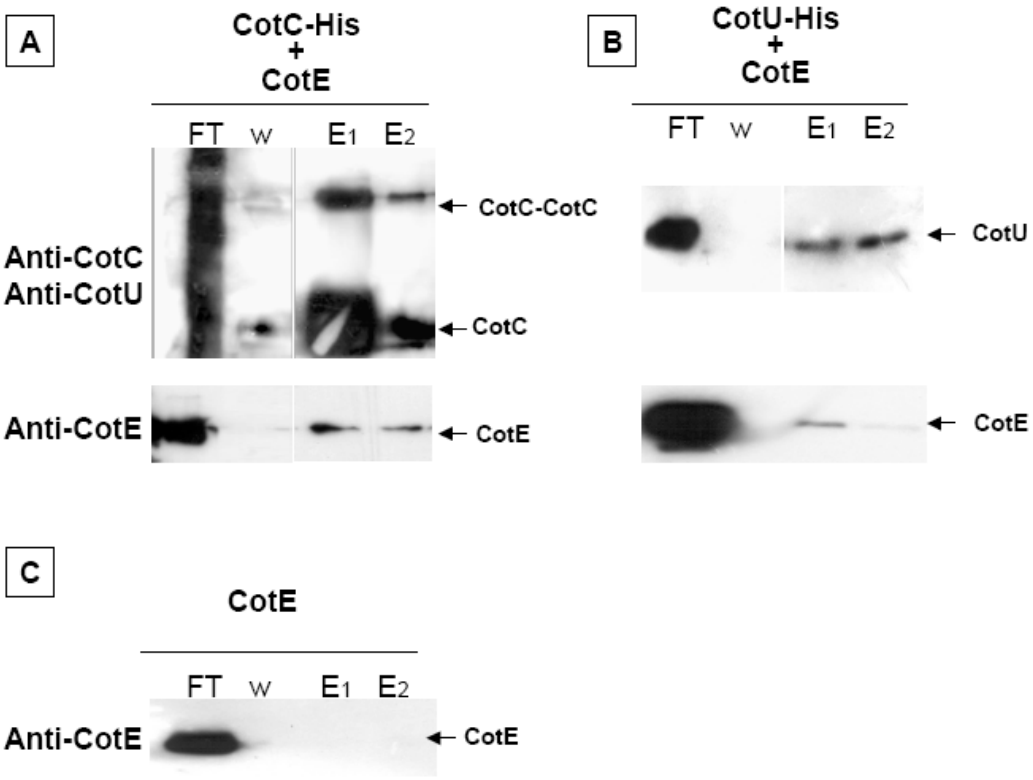

FIG. 5: Pull-down experiment performed by binding CotC-His (A) or CotU-His (B) to Ni-NTA magnetic beads. Untagged CotE was then added and flowthrough (FT), washes (W) and eluted (E1 and E2) proteins collected. Proteins were fractionated on 12.5% polyacrylamide gels, electrotransferred to membranes and reacted with anti-CotC, anti.CotU and anti-CotE antibodies. The same experiment was also performed without CotC-His or CotU-His (C).

These *in vitro* results demonstrated that CotE directly interacts with CotC and CotU and are the first direct proof of an interaction between CotE and other components of the *B. subtilis* spore coat.

CotE mediates the interaction of CotC and CotU. To investigate whether the interaction with CotE was sufficient to induce the formation of the CotC-CotU heterodimer, we over-expressed all three proteins together in the same *E. coli* strain and used the extracted proteins for western blot analysis with anti-CotC, anti-CotU, anti-His and anti-CotE antibodies. As previously reported (10), when only *cotC* and *cotU* were over-expressed together in *E. coli* three proteins were recognized by anti-CotC and anti-CotU antibodies (Fig. 6A, lane1). The three bands corresponded in size to CotC, CotU and CotC homodimer (10). When also *cotE* was expressed in the same cells an additional protein was formed (Fig. 6A, lane 2). The additional protein did not contain CotE since when the same gel was reacted against anti-CotE antibody, a CotE-specific signal was observed at a different position on the gel (Fig. 6B). It is unlikely that the additional protein is an unspecific signal since it was not present in lane 1 (CotC+CotU) or lane 3 (CotE) of Fig. 6A and identical results were obtained with anti-CotU and anti-His antibodies (not shown).

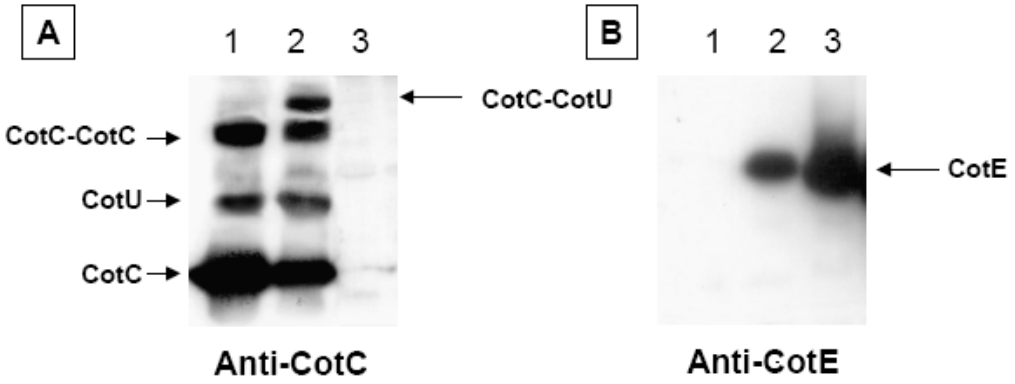

FIG. 6**:** Co-expression experiment. Extracts from *E. coli* cells over-producing CotC and CotU (lane 1), CotC, CotU and CotE (lane2) and CotE (lane3) were fractionated on 15**%** polyacrylamide gels, electrotransferred to membranes and reacted with anti-CotC antibody. Identical results were obtained with anti-CotU antibody.

To determine the nature of the proteins formed when CotC, CotU and CotE were over-expressed together in *E. coli*, we performed a MALDI TOF mass spectrometry analysis. The four proteins shown in Fig. 6A (lane 2) were excised from the SDS-polyacrylammide gel and subjected to MALDI TOF using a MALDI-TOF micro MX (Waters Co., Manchester, UK), as describes previously (2). Due to the high percentage of identity between CotC and CotU amino acid sequences (Fig. 1B), it was necessary to identify diagnostic peptides to discriminate between the two coat proteins. In particular, three

peptides at the C-terminal end of the two proteins were found to be diagnostic: peptide 1 and 3 of CotU and peptide 2 of CotC (Fig. 1A). As reported in Table 2, peptides 1 and 3 (diagnostic of CotU) were found in the protein tentatively assigned as CotU while only peptide 2 (dignostic of CotC) was found in proteins indicated as CotC monomer and CotC-CotC homodimer. All three peptides were found in the additional protein of Fig. 6A (lane2), confirming that it was the CotC-CotU heterodimer (Table 2).

These results confirm the nature of the proteins expressed in *E. coli* and indicate that no additional factors other than CotE are needed to mediate CotC-CotU heterodimer formation.

Table 2. Molecular mass values obtained by MALDI-TOF mass spectrometry of tryptic digests of band CotC, CotU and CotC-U. Theoretical and experimental mass values together with peptide sequence positions are reported

Peptide	Sequence position[a]	Theoretical Mr [M+H]+	Experimental Mr [M+H]+ Band "CotC"	Experimental Mr [M+H]+ Band "CotU"	Experimental Mr [M+H]+ Band "CotC-CotC"	Experimental Mr [M+H]+ Band "CotC-CotU"
1	40-54	1869.83	-	1869.78	-	1869.78
2	45-66	2964.42	2964.43	-	2964.46	2964.46
3	68-86	2539.04	-	2539.01	-	2538.91

[a] Refers to the amino acid sequence of CotC (peptide 2) and CotU (peptides 1 and 3) as reported in Fig. 1A.

Discussion

Main result of this work is the first direct evidence that CotE interacts with a coat component other than CotE itself. CotE of *B. subtilis* is a morphogenic factor required for outer coat formation (28), known to form multimers organized in a ring-like structure assembled around the forming spore (6, 17 , 14). CotE has been proposed as a major regulatory factor of outer coat assembly and its interaction with several components of the coat inferred on the base of genetic experiments (12). We provide here a direct evidence that CotE interacts with CotC and with CotU, two outer coat components whose assembly around the spore has been previously indicated as CotE-dependent (28, 10). The pull-down experiments of Fig. 5, demonstrate that CotE is retained on Ni-NTA magnetic beads only when CotC-His or CotU-His have been previously bound to the beads, indicating that CotE directly binds CotC and CotU. A future challange will be to define these interactions by identifying the amino acid residues involved in CotE-CotC and CotE-CotU contacts.

A second important result of this work is that CotE is sufficient to mediate CotC-CotU interaction in *E. coli* cells. The co-expression experiment of Fig. 6 and the MALDI TOF analysis of the proteins produced in *E. coli* clearly indicate that only in the presence of CotE the two coat components interact forming the heterodimer previously

observed in the coat protein fraction of *B. subtilis* spores (10). However, at this stage we can not explain how CotE mediates CotC-CotU interaction and can only hypothesize that CotE either recognizes CotC and CotU as substrates and catalyzes their interaction or acts as a platform to which CotC and CotU bind. In the latter case, CotE role would be to hold the two coat components close to each other allowing their spontaneous or self-catalyzed interaction. Although an enzymatic activity associated to CotE has never been demonstrated, we observed that CotE contains a region of homology with the consensus of the Walker A domain of ATPases (conseusus: A/GXXXXGKT (11); CotE: A_{47} - - - G_{51} K_{52} T_{53}). However, so far we have been unable to detect any ATPase activity associated to *E. coli*-purified CotE (data not shown). Additional experiments will be needed to clarify CotE role in CotC-CotU interaction and discriminate between the two hypothesis.

An additional result of this work is that CotC-CotC homodimers and CotC-CotU heterodimers are resistant to denaturing and reducing conditions and are not dependent on the single cysteine residue that both proteins have at their position 32 (Fig. 1A). While CotC-CotC homodimers form spontaneously in *E. coli* (9), CotC-CotU heterodimers require CotE to form. Preliminary results indicate that amino acids at the C terminus of CotC are involved in the formation

of both the homo- and the hetero-dimer (Isticato and Ricca, unpublished results), but, also in this case, additional experiments will be needed to clarify the nature of those interactions.

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Chapter 4

“Interactions during the assembly of CotB into the *Bacillus subtilis* spore coat”

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Manuscript

Abstract

Assembly of the *Bacillus subtilis* spore coat involves over 60 protein components, which self-organize into an inner lamellar coat and a striated electrondense outer coat. CotB is a surface-exposed component of the outer coat that has been used as a vehicle for the presentation of heterologous antigens at the spore surface. CotB is synthesized as a 46 kDa protein (CotB-46) that is converted into a 66 kDa species (CotB-66), in a *cotG* dependent manner. CotB (380 residues) consist of a N-terminal region (residues 15 to 145) which bears similarity to another coat protein, YwrJ; a region (residues 252 to 330) formed by 4 serine- and lysine rich repeats (the SKR region); a C-terminal tail (residues 330 to 380). We have previously shown that CotB interacts with itself and with CotG, and that this latter also self-interacts. Here we show that the N-terminal region of CotB interacts with itself, but not with its C-terminal region, or with CotG. In contrast, the C-terminal region of CotB interacts with CotG. Deletion of the SKR region of CotB results in the accomulation of a 43 kDa protein that undergoes assembly but does not forms multimers of itself or with wild type CotB. These results suggest that the SKR region of CotB is involved in the interaction with CotG which is needed for the formation of CotB-66. We further show that the

multimerization of both CotB and CotG occurs at the spore surface. Since the assembly of CotB-46 is *cotG*-independent, we infer that CotG promotes the formation of CotB-66 at the spore surface. Also, since both CotB-46 and CotG are found in *cotE* spores, we infer that CotE or a CotE-dependent protein is needed in addition to CotG, for the formation of CotB-66. Lastly, we show that the C-terminal end of CotG is important for the formation of CotB-66, but not for the multimerization of CotG.

Introduction

The *Bacillus subtilis* spore is encased in a multilayered protein coat that confers resistance to peptidoglycan-breaking enzymes and noxious chemicals and also influences the germination response. The coat is formed by over 60 polypeptides, ranging in size from about 6 to about 70 kDa, which are assembled into a lamellar inner coat and a thick electron-dense outer coat (5,6). Biogenesis of the spore coat is the result of a complex process of macromecular assembly that is controlled at different levels. It involves intricate genetic regulation, with the sequential participation of at least five mother cell-specific transcription factors in the order σ^E, SpoIIID, σ^K, GerE and GerR. The transcriptional control guarantees that the production of coat structural components, as well as the morphogenetic proteins that guide their assembly, occurs in the mother cell chamber of the sporulating cell in a defined temporal order. However, assembly of the inner and outer coat layers does not closely reflect the order of transcription of coat structural genes (*cot*) but rather is largely dependent on a topological plan that is laid down early in the process and requires the products of three morphogenetic genes, *spoIVA*, *spoVID* and *cotE* (1, 22, 24, 25). The localization of SpoIVA is required for the assembly of another morphogenetic protein, CotE (4, 27) at a distance of about 75nm from SpoIVA (7). The gap defined by the positions of SpoIVA and CotE, or

matrix, is of unknown composition. When σ^K becomes active, the matrix is thought to be converted into the inner coat.

Activation of σ^K results in the expression of several genes coding for spore coat proteins and for GerE (2), which is an ambivalent transcriptional regulator of coat gene expression. GerE acts together with σ^K to activate a late class of *cot* genes, but it also represses transcription of other *cot* genes(16, 17, 26, 27). These regulatory circuits suggest that the time and level of expression of the genes coding for coat structural components are important for the correct assembly of the coat structure (5, 6, 14). Proper assembly of the coat further relies on mechanisms such as translational control (21) and posttranslational modifications, including proteolytical processing of larger precursors, protein secretion, and protein cross-linking. These modifications may provide an additional level of control over the timing of assembly of specific components.

An aboundant outer coat component is CotB, a 46-kDa polypeptide (CotB-46) consisting of a N-terminal region (residues 15 to 145) which bears similarity to another coat protein, YwrJ; a region (residues 252 to 330) formed by 4 serine- and lysine rich repeats (the SKR region); a C-terminal tail (residues 330 to 380) (Fig. 1).

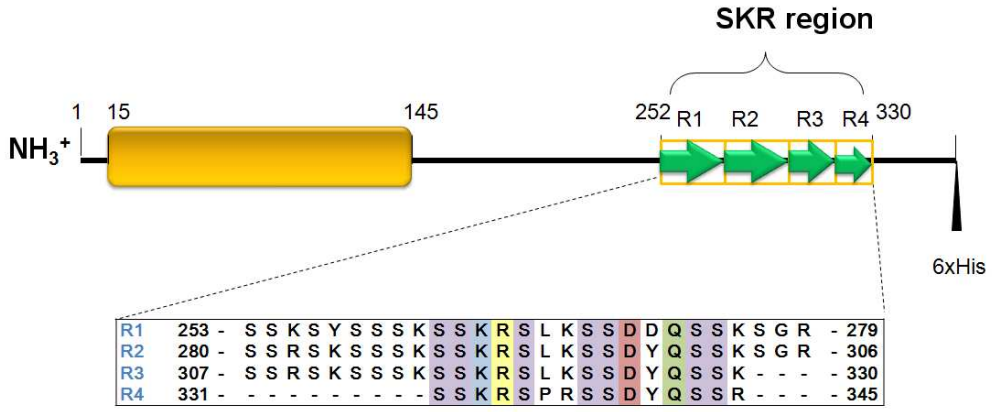

FIG. 1: CotB primary structure. The yellow part represent the N-terminal region, which show a similarity with YwrJ, another coat component; the green arrows indicate the 4 Serine- and lysine rich repeats; the black line the remaining amino-acidic sequence and the C-terminal region, bringing the fusion with 6xHis tag, utilized for protein purification.

CotB-46 is posttranslationally converted into a form of about 66 kDa (CotB-66). Formation of CotB-66 requires both *cotG* and *cotH* because CotG does not accumulate in a *cotH* mutant. Since CotB has been utilized as a vehicle for the presentation of heterologous antigens at the spore surface, suggesting its potential use in vaccine development (18, 19), its mechanism of assembly is also relevant to obtain a more efficient heterologous display.

In this work we show that the N-terminal region of CotB interacts with itself, but not with its C-terminal region or with CotG. In contrast, the C-terminal region of CotB interacts with CotG. Deletion of the SKR region of CotB results in the accumulation of a 43 kDa

protein that undergoes assembly but does not forms multimers of itself or with wild type CotB. These results suggest that the SKR region of CotB is involved in the interaction with CotG which is needed for the formation of CotB-66. We further show that the multimerization of both CotB and CotG occurs at the spore surface. Since both CotB-46 and CotG are found in *cotE* spores, we infer that CotE or a CotE-dependent protein is needed in addition to CotG, for the formation of CotB-66. Lastly, we show that the C-terminal end of CotG is important for the formation of CotB-66, but not for the multimerization of CotG.

Materials And Methods

Bacterial strains, media, and general techniques. The *B. subtilis* strains used in this study are listed in Table 1. Sporulation was induced by nutrient exhaustion in Difco sporulation medium (20). *Pfu* polymerase (Stratagene) was used in all PCRs, and the cloned products were sequenced to ensure that no mutations were introduced.

TABLE 1. Bacterial strains used in this study

Strain	Genotype/phenotype	Reference or source
MB24	*trp*C2 *met*C3/wild type/Spo$^+$	Laboratory stock
AH2055	*trp*C2 *met*C3 *cotB*::ΩpRZ29/Nm^r, Sp^r	Zilhão et al., 2004
AH2143	*trp*C2 *met*C3 *cotG*::*erm*/Erm^r	Laboratory stock
AH2707	*trp*C2 *met*C3 *cotE*::*sp*/Sp	Laboratory stock
AH2253	*trp*C2 *met*C3 *cotB*::ΩpAH2252/ Cm^r	This work
AH2255	*trp*C2 *met*C3 *cotB*::ΩpAH2252/ Δ*amyE*::*cotB*/Cm^r	This work
AH2256	*trp*C2 *met*C3 *cotB*::*sp*/ Δ*amyE*::*cotB*/Sp^r, Cm^r	This work
AH2257	*trp*C2 *met*C3 *cotB*::*sp*/ Δ*amyE*::*cotB*Δ*42*/Sp^r, Cm^r	This work
AH2258	*trp*C2 *met*C3 *cotB*::*sp*/ Δ*amyE*::*cotB*Δ*105*/Sp^r, Cm^r	This work
AH2259	*trp*C2 *met*C3 *cotB*::*sp*/ Δ*amyE*::*cotB*Δ*129*/Sp^r, Cm^r	This work
AH2260	*trp*C2 *met*C3 *cotB*::*sp*/ Δ*amyE*::*cotB*Δ*50*/Sp^r, Cm^r	This work
AH2261	*trp*C2 *met*C3 *cotB*::*sp*/ Δ*amyE*::*cotB*Δ*74*/Sp^r, Cm^r	This work
AH2264	*trp*C2 *met*C3 *cotG*::*erm*/Δ*amyE*::*cotG-6His*/ Cm^r, Erm^r	This work

Construction of strains with deletion of the SK region. Plasmid pRZ24, bringing the coding region of *cotB* (28), was used as template to perform a PCR with primers *cotB*-1038D and *cotB*-756R (Table 2). Those two primers were divergent and not overlapping and extension

reactions from them synthesized a *cotB* allele deleted of the SK region. The resulting PCR mixture was ligated and then digested with restriction enzyme *BglII*, cleaving only inside the SK region, to eliminate the template DNA. Amplified DNA was then used to transform *E. coli* competent cells yielding plasmid pAH2252. Then, the plasmid pAH2252 was introduced by single reciprocal (Campbell-like) recombination between *B. subtilis* DNA sequences in pAH2252 and the corresponding region of homology in the chromosome in the MB24 wild-type strain of *B. subtilis*. Several chloramphenicol resistant clones were analyzed by PCR, and one of them, AH2253, was used for further studies. Next, to create a *B. subtilis* strain expressing a copy of $cotB_{wt}$ and a *cotB* gene with the deletion, a 1,424-bp PCR product encompassing the *cotB* promoter and coding region was generated with primers *cotB*-13D and *cotB*-1437R (Table 2) and cloned into the pGemT-easy vector (Promega). The insert in the resulting plasmids was analyzed by DNA sequencing to verify the absence of unwanted mutations, released with *HindIII* and *EcoRI*, and entroduced into pDG364 (3) previously digested with the same two enzymes. *Escherichia coli* competent cells were transformed with the ligation mixture, and the selected ampicillin-resistant clones were screened by restriction analysis of their plasmids. One plasmid, pAH2254, was linearized and used to transform competent cells of the

B. subtilis strain AH2253, yelding strain AH2255. Cm^r clones were the result of double crossover recombination, due to the interruption of the nonessential *amyE* gene on the *B. subtilis* chromosome. Several Cm^r clones were tested by PCR using chromosomal DNA as a template and oligonucleotides AmyA and AmyS as primers (Table 2).

Yeast Two-hybrid analysis. The complete coding region of *cotG* was PCR amplified with primers *cotG*-5/Nco and *cotG*-Bam/3 (Table 2). The 5' (coding for residues 1-213) and 3'-end regions (encoding amino acids 195-380) of *cotB* were independently amplified using primers *cotB*-5/Nco and *cotB*-OMO/6 and *cotB*-Nco/ID and *cotB*-OMO/3, respectively (Table 2). The same pair of primers used to amplify the 3'-end regions of *cotB*, was used also to amplify the 3'-end regions of *cotB*Δ*SKR*. The *cotB* N- and C-terminal regions, the *cotB*Δ*SKR* C-terminal region, and *cotG* PCR products were digested by *Nco*I and *Bam*HI and inserted between the *Nco*I and *Bam*HI sites of both pAS2-1 and pACT2 (Clontech), to create *in frame* fusions to the GAL4 DNA-binding (DNA-BD) or activation domains (AD). The resulting plasmids are listed in Table 3. Yeast strains Y187 (MATa, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4, met-, gal80, URA3::GAL1UAS-GAL1TATA-lacZ) and Y190 (MATa, ura3-52, his3-200, ade2-101, lys2-801, trp-901, leu2-3, 112, gal4, gal80cyhr2,

LYS2::URA::GAL1UAS- HIS3TATA-HIS3, URA3::GAL1UAS-GAL1TATA-lacZ) (Matchmaker Two-hybrid system, Clontech) were transformed independently with the pAS2-1 and pACT-2 vectors and/or each of these constructs respectively. The resulting clones were used in pairwise matings selecting for LEU+ and TRP+. Colony lift assays for detection of β-galactosidase activity were essentially as previously described (21).

Partial deletions of SK region. The *cotB* promoter and coding region was PCR amplified from *B. subtilis* chromosomal DNA as template and primers B1 and B4 (Table 2). The 1,406-bp PCR product was cloned in pGemT-easy vector, forming pNS1. Next, the insert was released with *Sph*I and *Sal*I and cloned between the same restriction sites of pDG364, yielding pRH31. From the pNS1, the fragment was digested again, using the restriction enzymes *EcoR*I and *Bgl*II and cloned in pDG364 previously digested with the restriction enzymes *EcoR*I and *Bam*HI, to obtain a deleted form of *cotB* gene, called *cotB*Δ*42*, and the plasmid pRH32. Then, the 1,090-bp PCR fragment generated with primers B1 and B3 (Table 2), using as template the chromosomal DNA of *B. subtilis* was cloned in the pGemT-easy vector, yielding pNS3. The fragment of 1,116-bp (bringing same restriction sites from the polycloning sites of pGemT-easy vector) was

digested with *Sph*I and *Sal*I and cloned in pDG364 digested with the same restriction sites, yielding pRH33. The 1,024-bp PCR product encompassing the *cotB* promoter and coding region deleted of 384-bp at the 3' end, corresponding to the entire SK region, with primers B1 and Bstop, was cloned in pGemT-easy vector, to form pRH34 and then digested with *Sph*I and *Sal*I and inserted between the same restriction sites in pDG364, yielding pRH35. The plasmids formed, pRH31, pRH32, pRH33 and pRH35 were linearized and used to transform the competent cells of *B. subtilis* strain AH2055 (*cotB::sp*), to obtain the recombinant strains AH2256, AH2257, AH2258 and AH2259 (Table 1), respectively, through double-crossover recombination at the locus *amyE*.

Construction of a $CotG_{wt}$/CotG-6His strain. To tag *cotG* at its 3' end, a PCR product of 585-bp encompassing the *cotG* promoter and coding region was generated with forward primer *cotG*-D and as reverse primer *cotG*-stopR, containing at 5' end a sequence code for 6 His residues (Table 2). The product of amplification was cloned in pGemT-easy vector, to yield the pAH2262. The fragment of *cotG* gene fused to six His-tag was released from pAH2262 by digestion with *Sph*I and *Sal*I, and cloned in pDG364, previously digested with the same restriction enzymes. The Cm^r clones were tested by PCR

using chromosomal DNA as a template and oligonucleotides AmyA and AmyS (Table 2). A positive clone, AH2263, was linearized and used to transform competent cells of strain wild-type MB24, yielding the strain AH2264.

TABLE 2. Primers used in this study

Primer	Sequence (5'-3')/restriction site or six-His tag[a]
cotB-1038D	TCACCAGGATATTCAAGTTCAATAAAAAGTTCAGG
cotB-756R	AATGAACGAATTGCTGTCCTTATCATTATTGTCTTCC
AmyA	CGAGAAGCTATCACCGCCCAGC
AmyS	CCAATGAGGTTAAGAGTATTCC
cotB-13D	GCGAGTATATTAAAAAGCTTTCACAATACC/*HindIII*
cotB-1437R	CCTCAACATCTGTGTTAAGGAATTCATTCAAAC/*EcoRI*
B/5/Nco	GAGCCATGGGAATGAGCAAGAGGAGA/*NcoI*
B/OMO/3	GCCTAGGATCCGGGCATCACTTTATC/*BamHI*
B/OMO/6	GCCTAGGATCCGATGCGAAGCACCTC/*BamHI*
B/Nco/ID	GTAGATAATGCCATGGGCCATTATAC/*NcoI*
G/5/Nco	GAGCCATGGAATTGGGCCACTATTCC/*NcoI*
G/Bam/3	TACCTCCGCCGGGATCCTATTGAAAC/*BamHI*
B1	ACATGCATGCACGGATTAGGCCGTTTGTCC/*SphI*
B3	GAAAGATCTGGATGATTGATCATCTGAAG/*BglII*
B4	GAAAGATCTAAATTTACGTTTCCAGTGATAG/*BglII*
B5	GTCGACTTTGATGATTGATAATCTGAAG/*SalI*
B6	GTCGACGATGATTGATAATCC/*SalI*
BSTOP	CTACTAAATGAACGAATTGCTGTCC
cotG-D	ACATGCATGTTGGGCCACTATTCC/*SphI*
cotG-stopR	CCATGATGATGATGATGATGTTATTTGTATTTC

Preparation of *B. subtilis* whole-cell extracts and immunoblotting. *B. subtilis* whole-cell lysates were prepared, and immunoblotting experiments were conducted as described previously (24) except that

12,5% or 15% polyacrylamide gels were used. Antibodies were used at the following concentrations: anti-CotB, 1:2,000; anti-CotG, 1:20,000; and anti-His (Novagen) (monoclonal), 1:5,000. Secondary anti-rabbit or anti-mouse antibodies (Sigma) were used at concentrations of 1:10,000 or 1:5,000, respectively.

Spore purification and extraction of spore coat proteins. Spores were harvested 24 h after the onset of sporulation and purified by density gradient centrifugation as described previously (11, 13). Proteins were extracted from purified spores and fractionated on SDS-12,5% or 15% PAGE gels. The gels were stained with Coomassie blue and then transferred to nitrocellulose for immunoblotting or to polyvinylidene difluoride membranes for N-terminal sequence analysis (11, 13).

Results

CotB-66 formation requires the presence of SK region. It has been previously reported that *cotB* gene encodes for a 46 kDa polypeptide (CotB-46), that is immediately converted in CotB-66, in CotG-dependent manner (28). Two lines of evidence suggest that the SK region is involved in the interaction with CotG:

i) a phylogenetic analysis showed that a CotB homolog is present in many spore formers, however in same isolates two copies of *cotB* are found, both lacking the SK region. In this latter case a copy of the *cotG* gene is not present on the chromosome (data not shown).

ii) the analysis of a recombinant strain of *B. subtilis* carrying a copy of *cotB* deleted of the SK region (AH2253), as the only copy of *cotB* indicated that the mature 66 kDa form of CotB was never found. Sporulating cells of strain AH2253 were harvested at various times during sporulation and lysed by sonication as described in Materials and Methods. The forming spores were separated from the mother cell cytoplasm, protein extracted and compared with those present in extracts of mature spores by western blot with anti-CotB antibodies (Fig. 2A). At all time points analyzed, we detected the CotB monomer of 43 kDa (because the absence of SK region), but never the CotB-66 form.

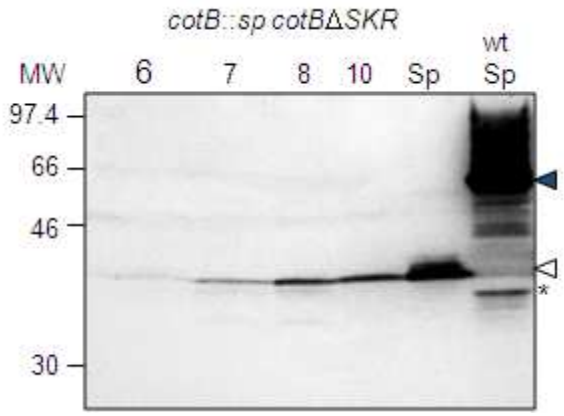

FIG 2: Western blot analysis using anti-CotB antibody, of proteins extracted at various times (t_6, t_7, t_8, t_{10}) after the onset of sporulation from the forespores of sporulating cells of a *cotB::spc* and a *cotBΔSKR* strains and from mature spores of wild-type (wt) and *cotBΔSKR* strains.

The C-terminal end of CotB and the SK region are involved in the CotB-66 formation. To investigate if regions of CotB other than the SK region were involved in CotB-66 formation, we performed an yeast two-hybrid analysis (8, 9, 10) on fragments of CotB. We fused the coding sequence for the N- and the C-terminal regions of CotB and of CotBΔSKR to either the activation domain (AD) in pACT-2 or to the DNA-binding domain (DNA-BD) in pAS2-1 of the yeast transcriptional activator GAL4. Combinations of the fusion plasmids and/or vectors were introduced into appropriate yeast reporter strains (21) (table 3). Interaction of the fusion proteins in the yeast strains resulted in the expression of a *lacZ* reporter gene and was detected by a colony lift assay (see Materials and Methods). As shown in Table 3,

no β-galactosidase activity was detected when individual fusions were expressed with the corresponding control vector. In addition, background levels (yeast cells carrying both vectors) were found to be negligible (Table 3). By this system we detected interactions between the N-terminal region of CotB and itself, between CotG and itself, between CotG and the C-terminal region of CotB. All of the interactions were found when the involved proteins were fused to either the GAL4 AD and GAL4 BD (table 3). In contrast, no interaction was found between the C-terminal region and the N-terminal region of CotB and between CotG and the C-terminal region of CotBΔSKR. These results suggest that CotB is capable of self interaction through its N-terminal part and of associating with CotG through its C-terminal end. This latter interaction does not occur when the SK region is lacking.

Table 3. Detection of *lacZ* transcription by colony lift assay of yeast strains expressing fusions of CotG, CotB (N- and C-terminal regions) and CotBΔSKR (C-terminal region) to the GAL4 activation and binding domain

AD fusion[a]	DNA- BD fusion[b]				
	pAS2-1 (BD)	pAS-Bn (CotBn-BD)	pAS-Bc (CotBc-BD)	pAS-BΔc (CotBΔc-BD)	pAS-G (CotG-BD)
pACT-2 (AD)	–[c]	–	–	–	–
pAC-Bn/(CotBn-AD)	–	++	–	–	–
pAC-Bc/(CotBc-AD)	–	–	–	–	+
pAC-BΔc/(CotBΔc-AD)	–	–	–	–	–
pAC-G/(CotG-AD)	–	–	+	–	+

[a] Constructs based on pACT-2 which carry in-frame fusions of CotG, CotB (N- and C-terminal regions) and CotBΔSKR (C-terminal region) to the GAL4 AD. The plasmids were transformed into yeast strain Y190 (Clontech) before mating.

[b] Constructs based on pAS2-1 which carry fusions of CotG, CotB (N- and C-terminal regions) and CotBΔSKR (C-terminal region) to the GAL4 BD. The plasmids were transformed into yeast strain Y187 (Clontech) before mating.

[c] the time of development of blue color on a colony lift assay for the detection of *lacZ* expression is indicated as follows: ++, development of color in 1h; +, development of color in up to 5 h; the symbol – indicates no color development in incubations of up to 24 h. Six independent colonies were tested for each combination.

CotB-46 and CotBΔSKR do not form an heterodimer. To independently test whether CotBΔSKR could interact to $CotB_{wt}$, we constructed a *B. subtilis* strain carrying both a wild type copy of *cotB* and the deleted version, *cotBΔSKR*. To this aim a wild type allele of *cotB* was inserted at the *amyE* locus (strain AH2255). Protein extracts

from this strain were compared by Coomassie-Blue stained SDS-PAGE and by western blot with protein extracts from isogenic wild type, *cotG* or *cotB* null mutant strains and with the recombinant strain *cotB::sp/cotB*Δ*SKR*. As reported in Fig. 3, the 66 kDa form of CotB is observed in extracts of the recombinant strain *cotB::sp/cotB*Δ*SKR*, both in SDS-PAGE (panel A) and western blot (panel B) experiments. When the only copy of *cotB* present lacks of the SK region a protein of approximately 44 kDa (corresponding to a monomer of CotBΔSKR) accumulates (Fig. 3B). The same 44 kDa protein is also found when a wild type and a deleted form of *cotB* are present.

This results suggest that the 66 kDa protein is formed only by wild type CotB while the 44 kDa is formed by the deleted allele, supporting the hypothesis that CotBwt and CotBΔSKR do not interact and that the SK region is needed for the formation of CotB-66.

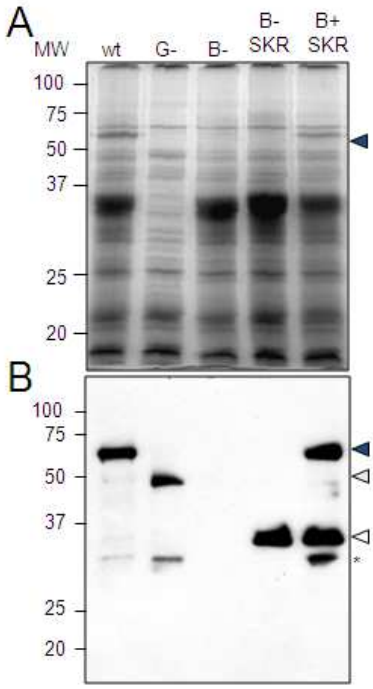

FIG. 3: (A) Coomassie gel showing the different patterns of proteins in the null mutants *cotG* and *cotB*, and in the recombinant mutants *amyE::cotB/cotB*Δ*SKR*, compared with wt. (B) The same strains were analyzed in a western blot, using antibody anti-CotB.

The entire SK region is involved in the formation of CotB-66. To test whether the entire SK region is involved in formation of CotB-66 and consequently in interaction with CotG, I decided to make partial deletions of the SK region. To this aim I deleted from the C-terminal end of the SK region: i) seven amino acids (CotBΔ42); ii) fifteen amino acids (CotBΔ50); iii) thirtynine amino acids (CotBΔ74); iv) seventy amino acids (CotBΔ105); and ninentyfour amino acids, corresponding to the entire SK region (CotBΔ129).

Different fragments were obtained by PCR reactions with the primers specified in Table 2. All deleted forms of *cotB* were inserted on the *B. subtilis* chromosome of *cotB* null mutant strain and were tested by western blotting analysis using anti-CotB antibodies.

A deletion of fifteen amino acids from the C-terminal end of the SK region (CotBΔ50) totally impaired the formation of the 66 kDa form of CotB (Fig. 4). As a consequence the 66 kDa form of CotB was found in CotBΔ42 but not in either one of the more extended deletions (Fig. 4). All deletions except CotBΔ42 did not allow the formation of the CotB dimer but only of a CotB monomer, supporting the previous conclusion that the SK region is essential for the production of the mature for of the protein.

The C-terminal end of CotG is necessary to form CotB-66. In order to study the region of CotG involved in interaction with CotB to form CotB-66, we constructed a *B. subtilis* strain, AH2039, bringing two copies of the *cotG* gene, a wild type copy and a copy carrying at its 3' end a His_6-tag.

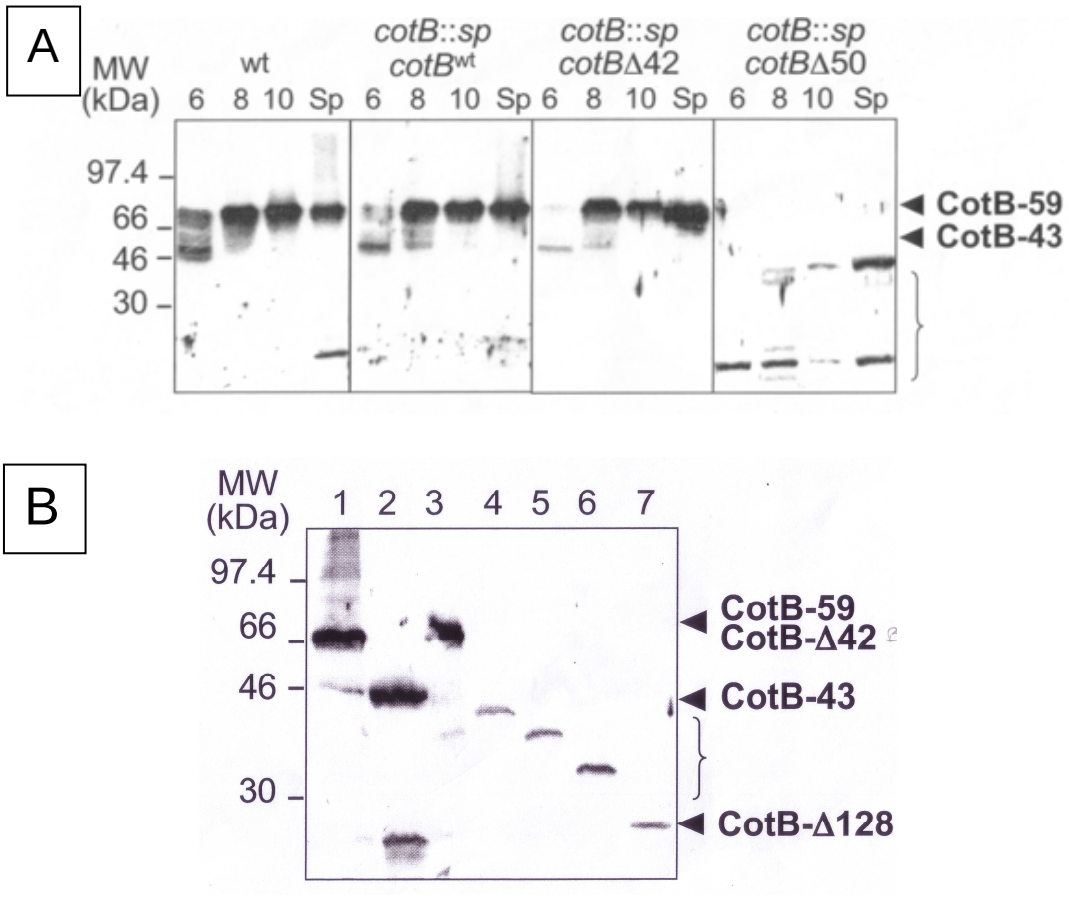

FIG. 4: western blotting analysis of samples harvested at various point time during sporulation (t_6, t_8, t_{10}) of wild-type, AH2256, AH2257, AH2258, AH2259, AH2260, and AH2261(A) and the coat extract from spores of all *cotB* deleted, wild-type and *cotG* null mutant strains (B) with anti-CotB antibody.

Then, we used a polyclonal antiserum to follow the assembly of CotB-66 in a wild type (MB24) and in strain AH2039, during sporulation (Fig. 5A).

In whole-cell extracts prepared from strain MB24 cells harvested from hour 6 to 10 of sporulation, the antibody detected mostly the CotB-66,

and same degradation products of smaller molecular weight. With strain AH2039 the antibody detected two different species of about 46 and 30 kDa, corresponding to monomer CotB-46 and to a degradation product of CotB, respectively.

The result demonstrated that when the C-terminal end of CotG is blocked by a 6xHis-tag, the CotB-66 can not form, suggesting that this region of CotG protein is necessary for its interaction with CotB.

Western blot experiments were also performed with coat proteins extracted from mature spores and reacted against anti-CotG (Fig. 5B) or anti-CotB (Fig. 5C) antibodies. The anti-CotG antibody reacted against two proteins, absent in spore coat extracts of a *cotG* null mutant strain AH2143 (*cotG::erm*) (Fig. 5B). The anti-CotB antibody weakly reacted against a protein of 66 kDa in both a *cotG* null mutant and $cotG_{wt}$/*cotG::6xHis* strains, but strongly reacted against a 44 kDa protein, suggesting that the 44 kDa form of CotB is present but unable to generate the 66 kDa form. In the light of these results we can propose that the presence of 6xHis-tag to the C-terminal end of CotG prevents the interaction between CotG and CotB-46, and therefore formation of the CotB-66.

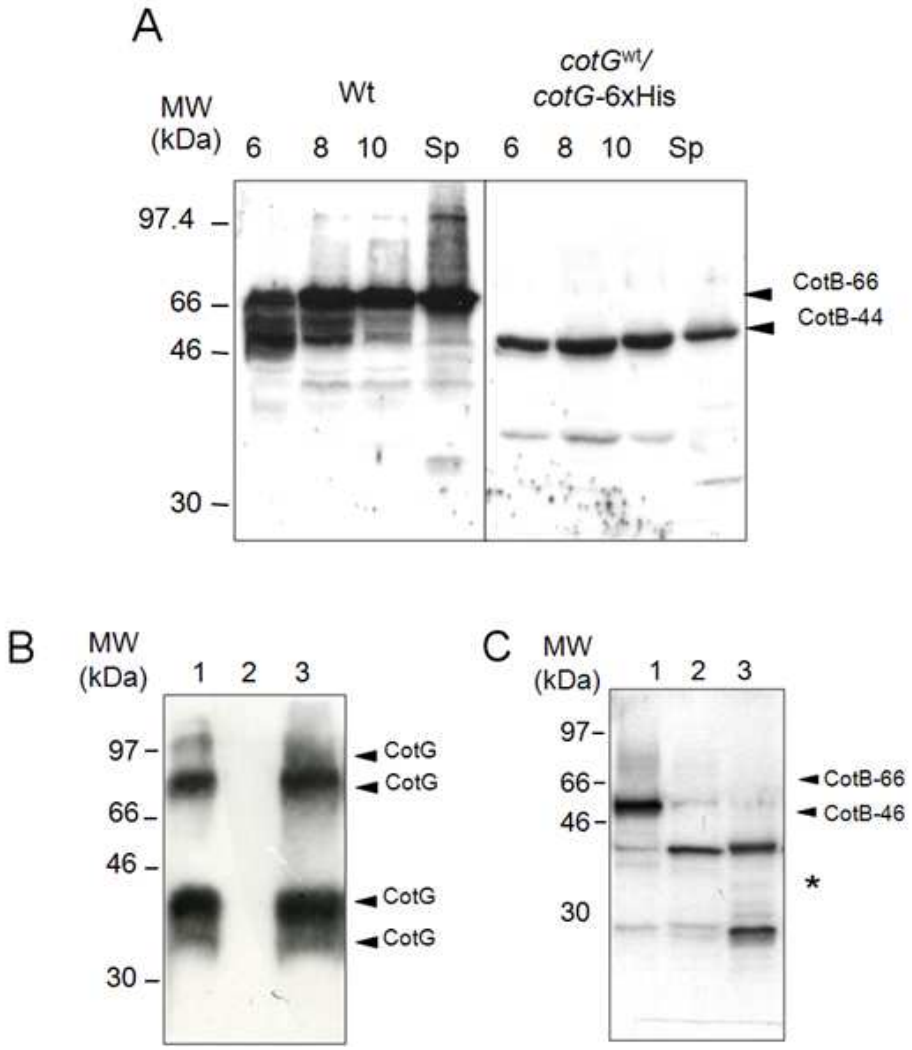

FIG. 5: (A) Accumulation of CotB in sporulating *B. subtilis* expressing two copy of CotG ($CotG_{wt}$ and CotG-6xHis). The figure depicts the immunoblot analysis of the accumulation of CotB at the indicated times (in hours) after the onset of sporulation in spore coat extracts in the wild-type and *cotGwt/cotG::6xHis* strains. (B) western blot with anti-CotG antibody. Coat proteins were extracted from wild-type strain, *cotG* null mutant strain and recombinant strain *cotGw/cotG::6xHis*. (C) western blotting analysis with anti-CotB antibody of coat extracts of wild-type strain, *cotG* null mutant strain and recombinant strain *cotGw/cotG::6xHi.* Black arrowheads on the right side of the panels indicate the positions of proteins attempt. Molecular mass markers are also indicated.

Assembly of CotG, CotB and CotB-66. None of the CotB and CotG forms accumulate in the mother cell of a wild type strain, suggesting that they immediately assemble on the surface of the forming spore. Here, we analyzed the assembly of CotG and CotB forms in a *cotE* null mutant strain, in which most coat components fail to assemble. Sporulating cells of strain AH2707 (*cotE::sp*) were divided into mother cell and a forespore fractions and analyzed for the presence of CotB and CotG.

As shown in Fig. 6A, CotB was assembled as a monomer (CotB-46) while the mature form of 66 kDa was never found, suggesting that its formation occurs at the spore surface and that it is strongly dependent on the presence of the CotE protein or of a CotE-dependent factor. Moreover, the absence of CotE does not have effect on the formation of CotG multimers, that are detected into the forespore extracts (Fig. 6C). Thus, in conclusion the CotB and CotG assembly is independent from CotE while their interaction is CotE-dependent.

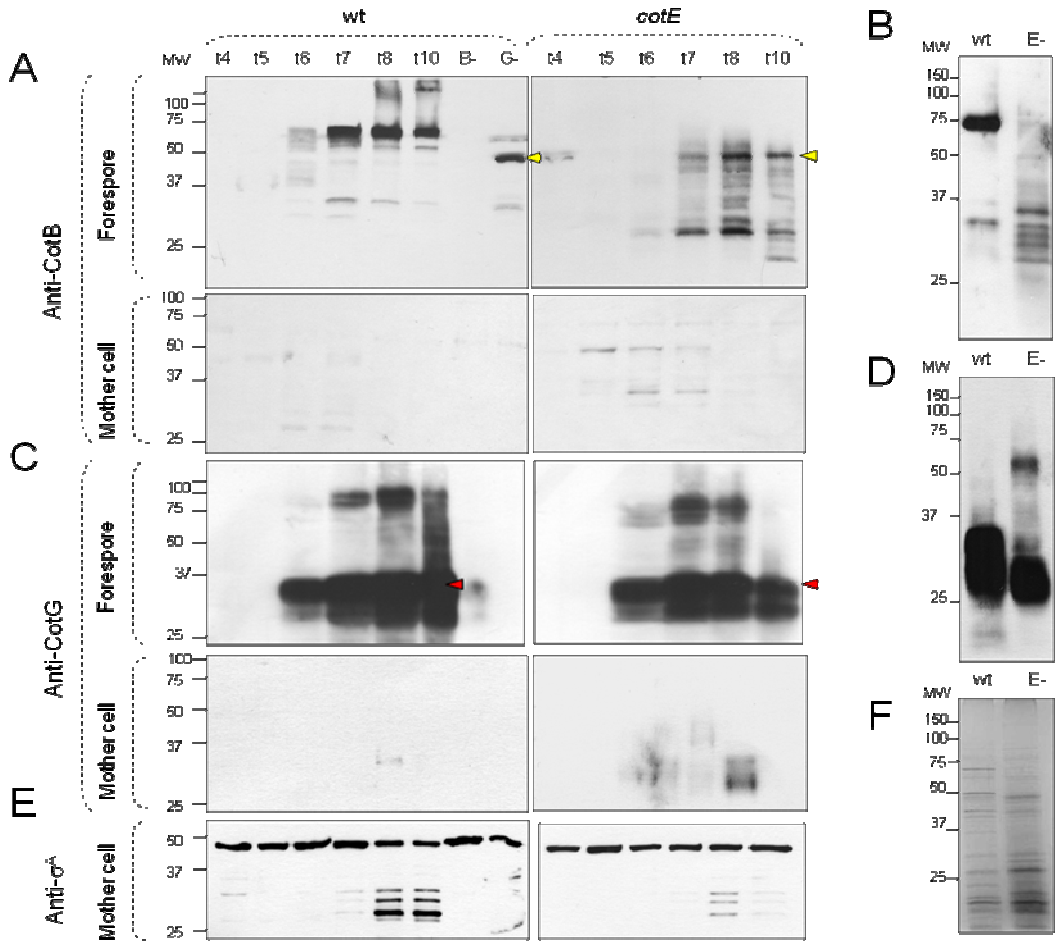

FIG. 6: Western blotting analysis with anti-CotB, anti-CotG and anti- σ^A antibodies. Samples are collected from sporulating cultures of a wild-type strain (panels A, C, E, on the left) and *cotE* null mutant strain (panels A, C, E on the right) at various time (t_4, t_5, t_6, t_7, t_8, t_{10}) in hours from the onset of sporulation. The cells were lysed and fractionated into mother cell and forespore fractions (see Materials and Methods), and subjected to immunoblot analysis, using all antibodies. The coat extracts from mature spore of wild-type and *cotE* null mutant strain were also analyzed using anti-CotB and anti-CotG antibodies and by Coomassie staining (panels B, D, F). The yellow arrows indicate the CotB-46 band, while the red arrows indicate the CotG monomer.

Discussion

CotB is an abundant component of the spore coat of *Bacillus subtilis* characterized by a particular primary structure, with a central region formed by 4 serine-lysine rich repeats (SK region). In the present work, we have shown that the SK region is involved in interaction between CotB and CotG during formation of the mature form of CotB, CotB-66.

A combination of Yeast-Two-Hybrid, deletion analysis and western blot data helped us to formulate an interaction model for the formation of CotB-66 (Fig.7). In this model, we propose that CotG, which is presumed to work as a dimer or a higher order multimer, is required to bring two molecules of CotB-46 together. Then, a third protein (presumably CotE or a protein under CotE control) is required to cross-link CotB-46 into the CotB-66 form. Because CotB is able to interact with itself, it could be possible that this interaction occurs to form a CotB-66 chain, during assembly around the spore. Anyway the orientation of CotB or CotG in the complexes is still not known.

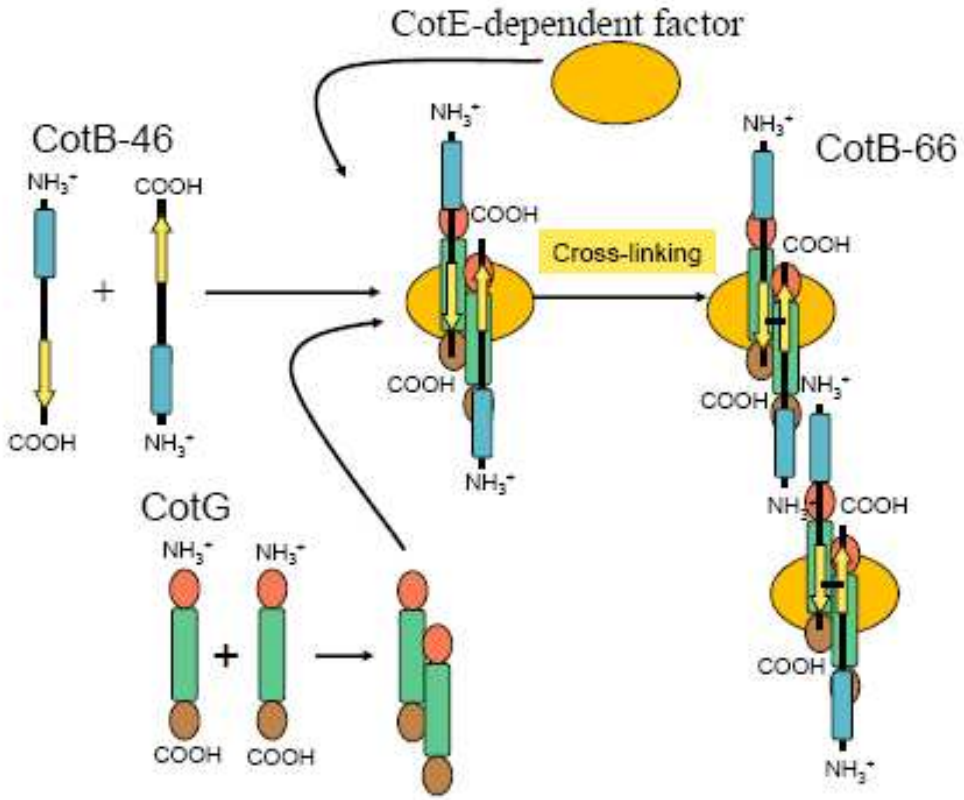

Fig. 7: Interaction model to explain the probable interactions among the component of CotB-66 complex.

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Chapter 5

Summary and discussion

Summary

Bacterial spore are highly differentiated cell types, specifically designed to survive adverse conditions. Their structure is very different from the structure of normal vegetative bacterial cells and consists mainly of three different parts: the core, the cortex and the coat.

Spore coat is formed by two different layers: inner coat and outer coat. It confers to the spore the capacity to resist to several physico-chemical agents and also contributes to the response of spores to the presence of germinants.

The coat is formed by over 60 polypeptides, which are assembled into a lamellar inner coat and the thick electron-dense outer coat. Due to the safety and stability properties of the spore and to the availability of molecular tools to genetically manipulate them in recent years it has been proposed to use the spore as a mucosal vaccine vehicle. To this aim, gene fusions between the gene coding for a coat component and heterologous DNA coding for an antigen, have been obtained. So far, two outer coat components, CotC and CotB, have been used. However, both CotC and CotB, form protein complexes with other coat component when assembled in the coat and this may affect antigens display.

Aim of my Thesis work has been to study the mechanism of assembly of CotB and CotC.

CotC assembly

CotC is assembled around the spore outer coat layer, as monomer (12 kDa), dimer (21 kDa), modified monomer (12.5 kDa), modified dimer (30 kDa), and as an heterodimer (23 kDa), interacting with another coat component CotU (Isticato et al., 2004; Isticato et al., 2008). The formation of this heterodimer was the object of my work. In *Chapter 2*, I initially focused my attention on the characterization of the structural gene coding for CotU, on the timing of CotU assembly and on the factors required for CotU assembly. Then, I focused on the interaction between CotC and CotU and observed that when expressed in heterologous hosts, such as *Escherichia coli* and *Saccharomyces cerevisiae*, the two proteins do not interact. This observation suggested that the interaction requires a specific *B. subtilis* factor to occur. I tested the possibility that this factor could be a coat component with a known catalytic activity, and to this aim I constructed strains of *B. subtilis* with null mutations in *sodA*, *oxdD*, *cotA* and *tgl* genes. Those genes code for superoxide dismutase, oxalate decarboxylase, laccase and transglutaminase, respectively, and

are known to be involved in spore coat assembly. However, none of these four enzymes resulted involved in the interaction between CotC and CotU.

Successively, I analyzed the possibility that the specific factor mediating CotC-CotU interaction was CotE, a known morphogenetic factor involved in the assembly of several coat components (*Chapter 3*). Co-purification experiments showed that CotE directly interacts with CotC or CotU. This is an important results, since it is the first time that CotE is shown to directly interact with another coat protein. So far, that CotE contacts other coat components has been suggested on the base of genetic experiments but never demonstrated biochemically. In addition, I also showed that the presence of CotE is sufficient to mediate the formation of the CotC-CotU heterodimer: while the heterodimer is not formed when CotC and CotU are co-expressed in *E. coli*, when also CotE is expressed in the same cells the heterodimer is formed. A MALDI-TOFF analysis was used to prove that the protein observed by western blot was the CotC-CotU heterodimer.

On the base of these results, I suggested two possible model: i) CotE acts as a platform on which CotC and CotU accomodate and where they, being held close to each other, interact forming an heterodimer;

and ii) CotE has an enzymatic activity and catalyzes the interaction between CotC and CotU.

CotB assembly

In the last part of my work (*Chapter 4*) I focused my attention on CotB assembly. It is already known (Zilhão et al., 2004) that CotB is assembled on the spore surface as a dimer of 66 kDa (CotB-66) and also that CotG, another coat component, is required for the formation of the 66 kDa form of CotB. It has been hypothesized that the 66 kDa form of CotB is an heterodimer formed by a CotB monomer (46 kDa) and by a CotG monomer (24 kDa). During my Thesis I tried to clarify the interaction between CotB and CotG, identifying regions in both proteins involved in the interaction.

CotB has a peculiar primary structure consisting of repeats rich in serine and lysine (SK region). I constructed a *B. subtilis* mutant strain containing as the only copy of *cotB*, an allele with a deletion of the SKR region. A western blot analysis indicate that the SK region is important for the formation of the mature form of CotB (CotB-66). This conclusion also confirmed by a yeast-two-hybrid analysis. In addition, CotG and CotB were shown to assemble also in a *cotE* null

mutant strain, but CotB was not found in the 66 kDa form suggesting that CotE could be involved in the CotB-66 formation.

A possible model is that two monomers of CotB (CotB-46), are bound one to each other through their C-terminal ends, and that a CotG favour the interaction. After that, another factor, either CotE or a CotE-dependent protein, stabilize the complex. In this way, each molecule of CotB-66 is bound to another through the N-terminal region of CotB, to form a chain, that is localized around the spore.

Appendix

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CotC-CotU Heterodimerization during Assembly of the *Bacillus subtilis* Spore Coat▽

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We report evidence that CotC and CotU, two previously identified components of the *Bacillus subtilis* spore coat, are produced concurrently in the mother cell chamber of the sporulating cell under the control of σ^K and GerE and immediately assembled around the forming spore. In the coat, the two proteins interact to form a coat component of 23 kDa. The CotU-CotC interaction was not detected in two heterologous hosts, suggesting that it occurs only in *B. subtilis*. Monomeric forms of both CotU and CotC failed to be assembled at the surface of the developing spore and accumulated in the mother cell compartment of cells mutant for *cotE*. In contrast, neither CotU nor CotC accumulated in the mother cell compartment of cells mutant for *cotH*. These results suggest that CotH is required to protect both CotU and CotC in the mother cell compartment of the sporangium and that CotE is needed to allow their assembly and subsequent interaction at the spore surface.

In *Bacillus subtilis*, the spore coat is a complex multiprotein structure that plays an important role in spore germination and resistance to toxic chemicals (8, 11, 13). Recently, new functions have been assigned to the coat, from sensing the external environment through active enzymes present on its surface (4, 12, 24, 40) to protecting the spore from predation by phagocytic protozoans (21). In addition, the coat is a novel system for the display at the spore surface of heterologous antigens (16, 18, 25), enzymes (22), and bioactive molecules (20).

The coat is a dynamic structure, able to adapt to changes in the spore volume by expanding and contracting in response to dehydration and rehydration occurring during the *B. subtilis* life cycle (3). A heterogeneous group of over 50 polypeptides form the three main structural layers of the coat: a diffuse undercoat, a laminated lightly staining inner layer, and a thick electron-dense outer coat (11, 13). Several of these polypeptides have been studied, and their structural genes (*cot* genes) have been identified. Expression of all *cot* genes is governed by a cascade of four transcription factors acting specifically in the mother cell compartment of the sporangium in the sequence SigmaE-SpoIIID-SigmaK-GerE; SigmaE and SigmaK are RNA polymerase sigma factors, and SpoIIID and GerE are DNA-binding proteins acting in conjunction with SigmaE- and SigmaK-driven RNA polymerase (8, 11, 13). An additional transcription factor, GerR, has recently been implicated in the control of some coat genes (10).

In addition to the transcriptional regulation, control exerted at the protein level seems to be particularly important for the assembly of the multiprotein structure of the coat. A variety of posttranslational modifications have been shown to occur during coat formation: some coat-associated polypeptides appear to be glycosylated (11, 13), others are derived from proteolytic processing of larger precursors (1, 6, 34, 37), and some others are highly cross-linked as a result of reactions that take place at the spore surface (12, 40). In addition, a small subset of coat proteins, known as morphogenetic proteins, play an important role in controlling the assembly of most of the coat. These proteins have no effects on coat protein synthesis but act posttranslationally to guide the assembly of the various coat components around the forming spore (19). SpoVM, a 26-amino-acid peptide, is believed to adhere to the outer forespore membrane and to allow the localization of SpoIVA around the forming spore (30). The SpoIVA layer then directs the assembly of the morphogenetic protein CotE in a ringlike structure around the forespore (9). Inner coat components are thought to infiltrate through the CotE ring, while outer coat proteins assemble on the outside of the E ring (8, 11, 13). Additional proteins with morphogenetic functions are needed at later stages of coat formation. For instance, SpoVID has the dual role of directing SafA to the forming spore and maintaining the CotE ring around the forespore (4, 28). Another example is CotH, which plays a role in the assembly of various outer coat components, partially controls assembly of CotE, and is required for the development of the normal morphological features of spores (19, 26, 41). A recent study has shown that CotH controls the assembly of the coat proteins CotB, CotC, CotG, CotS, CotSA, CotQ, CotU, CotZ, and YusA (19). In different studies, it has been proposed that the role of CotH in the assembly of CotC, CotG, and CotB is to stabilize CotC (17) and CotG, which in turn is needed for the assembly and dimerization of CotB (41). In particular, CotC does not accumulate in the mother cell compartment, where it is synthesized, but is immediately assembled around the forming spore (17). Assembly of CotC requires expression of

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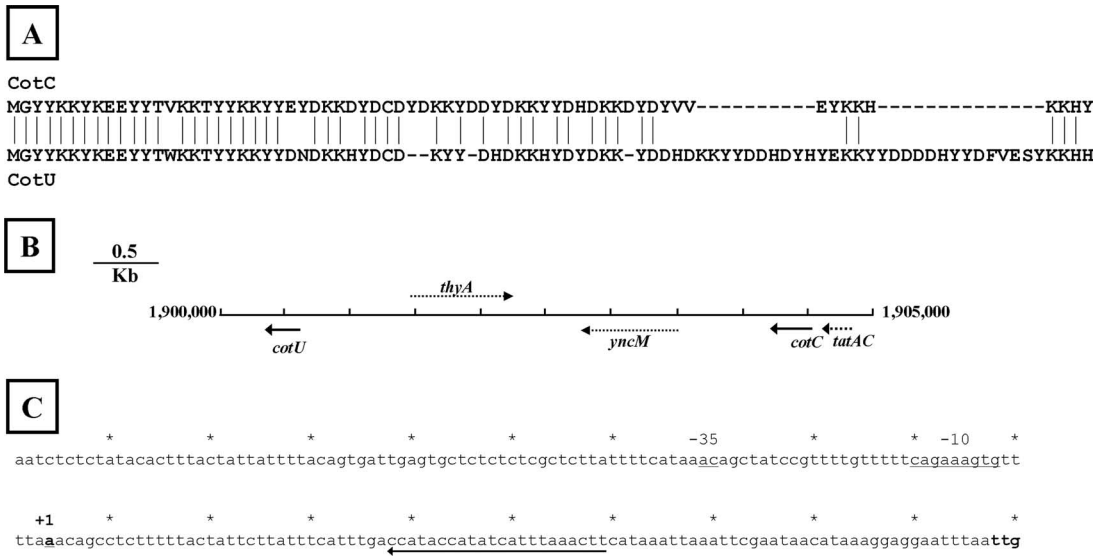

FIG. 1. (A) CotC and CotU amino acid alignment. (B) Schematic representation of the *cotC-cotU* chromosomal region. The arrows and numbers indicate the directions of transcription and the positions on the *B. subtilis* chromosome, respectively. (C) *cotU* promoter region. The translational start site (TTG) is in boldface, the transcriptional start site is indicated as +1, and the putative promoter sequences are underlined. The arrow indicates oligonucleotide U-pr-Anti, used for the primer extension experiment shown in Fig. 2B.

both *cotH* and *cotE*, but CotC does not accumulate in the mother cell compartment when its assembly is prevented by mutation of *cotH* (17). In contrast, overexpression of *cotH* allows the accumulation of CotC in the mother cell compartment, suggesting that CotH, or a CotH-dependent factor, acts to prevent degradation of CotC in the mother cell and then allows its assembly within the coat (2). The mechanism of assembly of CotC is of interest, as the abundant CotC protein has been used as a vehicle for the display of heterologous proteins at the spore surface (18).

Here, we report that CotU, a recently identified structural homologue of CotC (23), interacts with CotC, forming an alkali-soluble coat protein of 23 kDa in a CotE- and CotH-dependent manner. CotC and CotU share almost identical N-terminal regions, with 23 out of 24 identical amino acid residues, and less conserved C-terminal parts (Fig. 1A) (7). In addition, both CotU and CotC contain high numbers of tyrosine, lysine, and aspartic acid residues that account for over 70% of their total numbers of amino acids. This peculiar primary structure likely causes the unusual migration of the two proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with CotC and CotU having deduced masses of 8.8 and 11.4 kDa and apparent masses of 12 and 17 kDa, respectively.

We show that like CotC, the CotU protein does not accumulate in the mother cell compartment of a *cotH* mutant. In contrast both CotC and CotU (but not the 23-kDa species) accumulated in the mother cell of a *cotE* mutant but failed to be assembled. These results reinforce the view that CotH has a role in the stabilization of certain coat proteins in the mother cell cytoplasm. The results also indicate that formation of the 23-kDa CotC-CotU species takes place at the spore surface, following the assembly of both proteins.

MATERIALS AND METHODS

Bacterial strains and transformation. The *B. subtilis* strains utilized are listed in Table 1. Plasmid amplification for nucleotide sequencing, subcloning experiments, and transformation of *Escherichia coli* competent cells were performed with *E. coli* strain DH5α (33). Bacterial strains were transformed by previously described procedures: $CaCl_2$-mediated transformation of *E. coli* competent cells (33) and two-step transformation of *B. subtilis* (5).

Plasmid and strain construction. Isolation of plasmids, restriction digestion, and ligation of DNA were carried out by standard methods (33). Chromosomal DNA from *B. subtilis* was isolated as described elsewhere (5). Fragments of *cotC* and *cotU* DNAs were PCR amplified from the *B. subtilis* chromosome; the amplification was primed with the synthetic oligonucleotides listed in Table 2.

The *cotU*::*lacZ* translational fusion was obtained as follows. A DNA fragment containing 476 bp upstream of the *cotU* translational start site was PCR amplified using chromosomal DNA as a template and oligonucleotides Y3s and Ua (Table

TABLE 1. *B. subtilis* strains

Strain	Relevant genotype	Source
PY79	Wild type	36
BD063	*cotA*::*cat*	7
RH248	Δ*amyE*::*cotU-lacZ* Cm^r	This work
RH249	*gerE* Δ*amyE*::*cotU-lacZ* Cm^r	This work
AZ153	*gerE36*	Lab collection
AZ198	$SP\beta_{C2}$ *cotC*::*lacZ* Cm^r	Lab collection
RH228	*cotC*::His_6 *cotC*::*spc*	This work
RH208	*cotU*::*neo cotU*::His_6	This work
RH209	*cotU*::*neo cotC*::*cat*	18
RH210	*cotU*::*neo cotC*::*cat cotU*::His_6	This work
RH225	Δ*amyE*::*cotC*-His_6 Cm^r	This work
RH232	Δ*amyE*::*cotC*-His_6 Erm^r	This work
RH254	*cotU*::*neo cotC*::*cm* Δ*amyE*::*cotU*-His_6	This work
RH256	*oxdD*::*cat*	This work
RH255	*sodA*::*cat*	This work
RH233	*tgl*::*spc*	40
ER203	*cotG*::Δ*erm*	32
RH101	*cotC*::*spc*	17
RH202	*cotU*::*neo*	17

TABLE 2. Synthetic oligonucleotides

Oligonucleotide	Sequence (5′–3′)[a]	Restriction site	Position of annealing[b]
Y3s	gtcgacGATTTAATGCATTGTATTTTACC	SalI	−494/−471
Ua	aagcttTTCCAAGTATAATACTCTTC	HindIII	+43/+24
SodA-3F	ggtaccGCTTACGAACTTCC	KpnI	+4/+18
SodA-445R	ggatccGGTTTGGCGTGC	BamHI	+446/+434
OxdD-3F	ggtaccCTGTTGGAACAA	KpnI	+4/+20
OxdD-647R	ggatccCGGCACATTCCC	BamHI	+648/+635
Ycoding	ctcgagTTGGGTTATTATAAA	XhoI	+1/+15
Ya2	gcatgcTTATAAATAGGGGAAGGC	SphI	+449/+430
CotCp	ACATgcatgcTGTAGGATAAATCGTTTG	SphI	−181/−63
CotCSTOP	gtcgacTTATTAGTAGTGTTTTTTATGC	SalI	+357/+338
C-His1	GTCATCGTCATGGTGGTGATGATGATGCATATATACTCCTCC		−13/+1
C-His2	CATCACCACCATGACGATGACGATAAGATGGGTTATTACAAA		+15/+1
Y-His1	CCATGATGATGATGATGATGCAATTAAATTCCTCC		+3/−12
Y-His2	TTGCATCATCATCATCATCATGGTTATTATAAAAAA		+4/+18
AmyA	CGAGAAGCTATCACCGCCCAGC		+2128/+2150
AmyS	CCAATGAGGTTAAGAGTATTCC		+569/+590
U-pr-Anti	AAGTTTAAATGATATGGTATGG		+39/+60

[a] Uppercase and lowercase letters indicate nucleotides complementary to corresponding *cotU* or *cotC* DNA and unpaired flanking sequences carrying a restriction site, respectively.

[b] Positions refer to *cotU, cotC*, or *amyE* sequences, considering the first nucleotide of the translational start site as +1.

2) and inserted into plasmid pJM783 (40) upstream of the promoterless *lacZ* gene. The resulting plasmid, pRH92, carrying the *cotU*::*lacZ* fusion, was introduced by single reciprocal (Campbell-like) recombination between *B. subtilis* DNA sequences in pRH92 and the corresponding region of homology in the chromosome in the PY79 wild-type strain of *B. subtilis*. Several chloramphenicol-resistant (Cm^r) clones were analyzed by PCR, and one of them, RH248, was used for further studies. Chromosomal DNA containing the integrated fusion-bearing plasmid was then used to transform a congenic collection of sporulation mutants.

cotU::His_6 and *cotC*::His_6 gene fusions were constructed by using the gene splicing by overlap extension technique as described by Horton et al. (14). The *B. subtilis* chromosome was used as a template, and the synthetic oligonucleotides Y3s/Y2anti and Y-His1/Y-His2 for *cotU*::His_6 and CotCp/C-His1 and C-His2/Cstop for *cotC*::His_6 were used as primers (Table 2). The 680- and 402-bp PCR products obtained for *cotU*::His_6 and *cotC*::His_6 were cloned into the pGEM-T easy vector (Promega). The inserts in the resulting plasmids were analyzed by DNA sequencing to verify the absence of unwanted mutations, released with SphI and SalI, and introduced into pDG364 (5) previously digested with the same two enzymes. *E. coli* competent cells were transformed with the ligation mixture, and the selected ampicillin-resistant clones were screened by restriction analysis of their plasmids.

The obtained plasmids, pRH42 (*cotU*::His_6) and pRH48 (*cotC*::His_6), were linearized and used to transform competent cells of the *B. subtilis* strains RH202 and RH101 (Table 1), yielding strains RH208 (*cotU*::His_6) and RH228 (*cotC*::His_6), respectively. Cm^r clones were the result of double-crossover recombination, due to the interruption of the nonessential *amyE* gene on the *B. subtilis* chromosome. Several Cm^r clones were tested by PCR using chromosomal DNA as a template and oligonucleotides AmyA and AmyS (Table 2). Two clones, one from each transformation, were named RH208 (*cotU*::His_6) and RH228 (*cotC*::His_6) and kept for further studies.

To obtain a *B. subtilis* strain carrying both *cotC*::His_6 and *cotU*::His_6 fusions, the Cm^r determinant (*cat*) of strain RH225 was replaced with an erythromycin resistance gene cassette (*erm*) by using plasmid pECE72 (Bacillus Genetic Stock Center, Columbus, OH). Chromosomal DNA of the resulting strain, RH232, was used to transform competent cells of strain RH210 (*cotU*::His_6). Several clones resistant to erythromycin were tested by PCR, and one, RH254, was selected for further studies.

sodA null mutation was obtained by PCR amplifying an internal part of the gene using *B. subtilis* chromosomal DNA as a template and oligonucleotides SodA-3F and SodA-445R (Table 2). The 445-bp PCR product was cloned into plasmid pER19 (31), and the resulting plasmid, pRH97, was used to transform competent cells of the *B. subtilis* strain PY79, yielding strain RH255.

An *oxdD* null mutation was obtained by PCR amplifying an internal part of the gene using *B. subtilis* chromosomal DNA as a template and oligonucleotides OxdD-3F and OxdD-647R (Table 2). The 647-bp PCR product was cloned into plasmid pER19 (31), and the resulting plasmid, pRH121, was used to transform competent cells of the *B. subtilis* strain PY79, yielding strain RH256.

Primer extension analysis. Total RNA was extracted from a wild-type strain 8 h after the onset of sporulation using the Qiagen Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. Total RNAs were dissolved in 50 μl of RNase-free water and stored at −80°C. The final concentration and quality of the RNA samples were estimated either spectrophotometrically or by agarose gel electrophoresis with ethidium bromide staining. Total RNAs were treated with RNase-free DNase (1 U/μg of total RNA; Turbo DNA-free; Ambion) for 30 min at 37°C, and the reaction was stopped with DNase inactivation reagent. For primer extension experiments, 10 μg of total RNA was used with [γ^{32}-P]dATP (GE Healthcare)-labeled oligonucleotide U-pr-Anti (Table 2), deoxynucleoside triphosphate, and avian myeloblastosis virus reverse transcriptase (BRL) to prime cDNA synthesis, as previously described (26). The reaction products were fractionated on 6 M urea-6% polyacrylamide gels, along with DNA-sequencing reactions using pRH85 (pGEM-T easy/*cotU*) as the template primed with the same oligonucleotide.

Expression in *E. coli*. The *cotU* coding region was PCR amplified from *B. subtilis* chromosomal DNA with primers Ycoding and Y2a (Table 2). The 302-bp PCR product was cleaved with XhoI and EcoRI and ligated into the expression vector pRSETA, previously digested with the same restriction enzymes (Invitrogen). The recombinant plasmid carrying an in-frame fusion of the 3′ end of the *cotU* coding region to six histidine codons under the transcriptional control of a T7 promoter was used to transform competent cells of *E. coli* BL21(DE3) (Invitrogen), yielding strain RH59. This strain was grown in ampicillin-supplemented (50 μg/ml) tryptone-yeast extract medium to an optical density of 0.7 at 600 nm. The T7 promoter was then induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside; 0.5 mM final concentration) to the culture and incubating it for 2 h at 37°C. The His_6-tagged CotU protein was purified under denaturing conditions via Ni^{2+}-nitrilotriacetic acid affinity chromatography as recommended by the manufacturer (Qiagen, Inc.) and used to raise specific antibodies (IGtech, Salerno, Italy).

Western blotting. *B. subtilis* sporulation of wild-type and recombinant strains was induced by the exhaustion method (5, 27). After a 30-h incubation at 37°C, spores were collected, washed four times, and purified by lysozyme treatment as previously described (5, 27). The number of purified spores obtained was measured by direct counting with a Bürker chamber under an optical microscope (Olympus BH-2 with 40× lenses). Aliquots of 10^{10} spores suspended in 0.3 ml of distilled water were used to extract coat proteins by 0.1 N NaOH treatment at 4°C as previously reported (2). The concentration of the extracted coat proteins was determined by the Bio-Rad DC (Detergent Compatible) Protein Assay to avoid potential interference by the NaOH present (0.2 to 0.6 mN, final concentration) in the extraction buffer and 15 μg of total proteins fractionated on 18% denaturing polyacrylamide gels. The proteins were then electrotransferred to nitrocellulose filters (Bio-Rad) and used for Western blot analysis by standard procedures. For the analysis of sporulating cells, samples were harvested at various times during sporulation and disrupted by sonication in 25 mM Tris (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 15% (vol/vol) glycerol, and 0.1 mg/ml of

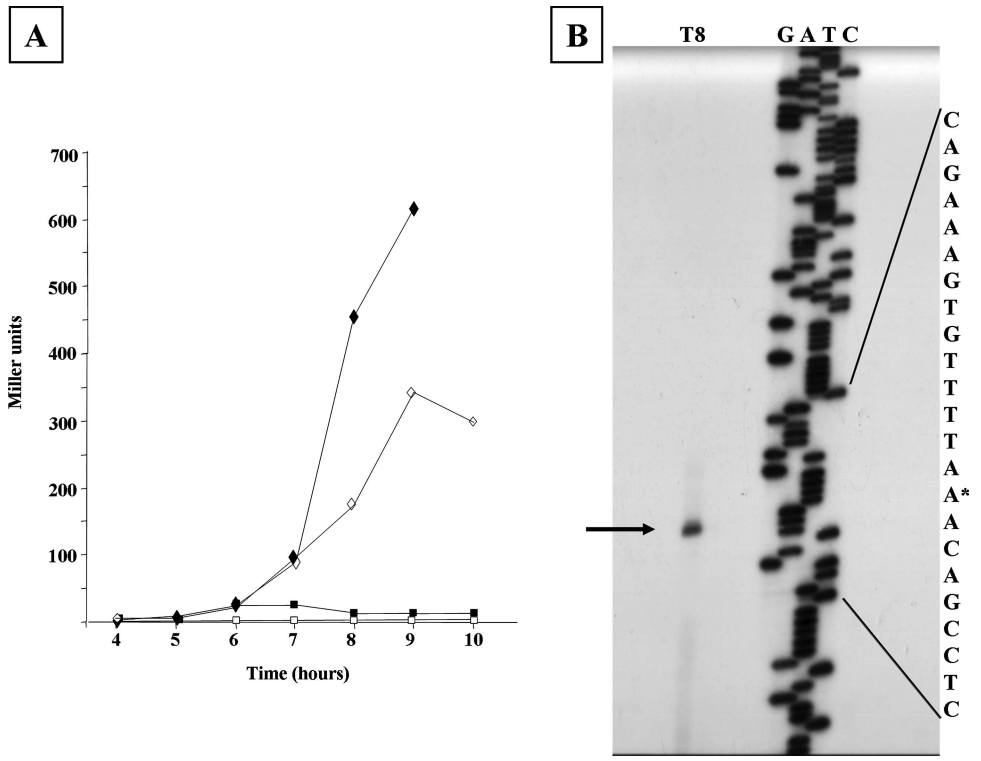

FIG. 2. (A) Expression of a *cotU*::*lacZ* transcriptional fusion during sporulation in an otherwise wild-type (open diamonds) or a *gerE* null mutant (closed squares) strain and of a *cotC*::*lacZ* translational fusion in an otherwise wild-type strain (closed diamonds). Background levels of β-galactosidase activity were determined in a wild-type strain bearing no *lacZ* gene (open squares). Samples were collected at various times after the onset of sporulation. Enzyme activity is expressed in Miller units. The data are the means of three independent experiments. (B) Primer extension analysis of the *cotU* promoter region performed with total RNA extracted from sporulating cells 8 hours (T8) after the onset of sporulation. Primer extension and sequencing reactions were primed with the synthetic oligonucleotide U-pr-Anti (Table 2).

phenylmethylsulfonyl fluoride. The sonicated material was then fractionated by centrifugation at 10,000 rpm for 20 min. The pellet, containing the forming spores resistant to the sonication treatment, was solubilized by 0.1 N NaOH treatment at 4°C, and the total protein concentration was determined as described above. Fifty micrograms (mother cell extract) or 15 μg (forespore extract) of total proteins was fractionated on 18% denaturing polyacrylamide gels. Western blot filters were visualized by the SuperSignal West Pico Chemiluminescence (Pierce) method as specified by the manufacturer.

RESULTS

The *cotU* gene of *B. subtilis* is under σ^K-GerE control. The *cotU* (formerly *ynzH*) and *cotC* genes, coding for CotU and CotC, respectively, are located about 4 kb apart in the the *B. subtilis* chromosome (Fig. 1B). Previous reports (15, 39) showed that *cotC* transcription is driven by σ^K-containing RNA polymerase and that the DNA-binding protein GerE acts as a transcriptional activator of *cotC* expression. Recently, studies using DNA arrays have suggested that *cotU* is also transcribed under the control of σ^K-driven RNA polymerase (10, 35). To analyze *cotU* expression in more detail, we constructed a transcriptional gene fusion between the *cotU* promoter region and the *lacZ* gene of *E. coli* and measured the activities of β-galactosidase at various times after the onset of sporulation in an otherwise wild-type strain and a collection of congenic sporulation mutants. In all cases, the fusion was integrated at the *cotU* locus as a result of a single reciprocal crossover event (see Materials and Methods). In agreement with the expectation that *cotU* is under the control of σ^K, we observed that β-galactosidase production commenced between 6 and 7 h after the onset of sporulation (Fig. 2A), at the same time as expression of a *cotC*::*lacZ* fusion in a congenic strain (39) (Fig. 2A). Moreover, expression of *cotU*::*lacZ* was severely reduced in a σ^K mutant (*spoIIIC*) (data not shown), as well as in mutants (*spoIIG*, *spoIIID*, *spoIIIG*, and *spoIVF*) (data not shown) known to be impaired in σ^K production. *cotU*::*lacZ*-driven synthesis of β-galactosidase was also impaired in a *gerE* mutant (Fig. 2A), indicating that efficient transcription of *cotU* requires the presence of the *gerE*-encoded DNA-binding protein.

By analogy with the case of *cotC* (15, 39) and in extension of previous observations (10, 35), we inferred that *cotU* is transcribed by σ^K-containing RNA polymerase acting in conjunction with GerE.

A primer extension experiment was performed to map the *cotU* promoter and transcriptional start site. The extension product obtained (Fig. 2B) allowed us to localize the 5′ terminus of *cotU* mRNA 91 bp upstream of the beginning of the open reading frame (Fig. 1C). Sequences upstream of the 5′ terminus (+1) resembled the conserved features of a σ^K promoter, matching in four of six positions the consensus −10 (consensus, CATANNNTA; *cotU*, CAgANNNTg; differences are in lowercase) and in both positions the consensus −35 (AC) (Fig. 1C).

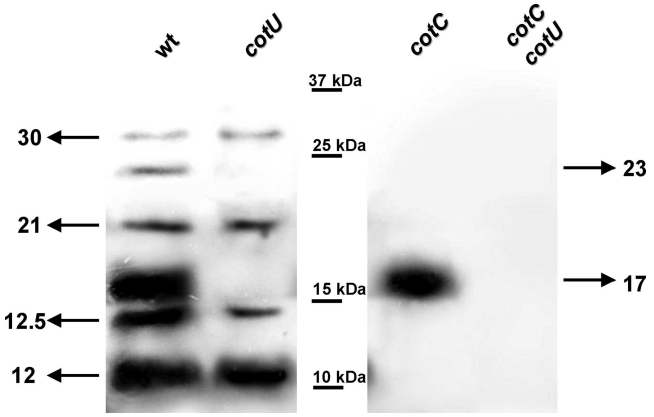

FIG. 3. Western blot analysis with anti-CotU antibody of coat proteins extracted from a wild type (wt) and congenic strains with null mutations in *cotU*, *cotC*, and *cotU cotC*. The proteins were fractionated on an 18% polyacrylamide gel and, upon electrotransfer onto nitrocellulose membranes, reacted with CotU-specific rabbit antibodies and then with peroxidase-conjugated secondary antibodies and visualized by the Pierce method. The arrows indicate the apparent molecular weights of the observed proteins. The molecular masses of a marker are also indicated. Identical results were obtained with anti-CotC antibody.

CotU and CotC directly interact in the spore coat. In order to study CotU assembly within the spore coat, we overexpressed a His_6-tagged version of *cotU* in *E. coli* and used the protein partially purified by Ni^{2+} affinity chromatography to raise a polyclonal antibody (see Materials and Methods). Due to the high similarity between CotU and CotC, our antibody reacted to the gene products of both *cotU* and *cotC*, and this recognition appeared to be specific, since no proteins were recognized in a *cotC cotU* double-null mutant (Fig. 3). Our anti-CotU antibodies recognized six polypeptides in the coat protein fractions of wild-type spores; four of them (of 12, 12.5, 21, and 30 kDa) were also detected in the coat extracts from *cotU* mutant spores (Fig. 3) and thus corresponded to the previously identified products of *cotC* expression (17). The 17-kDa protein was absent from the coats of *cotU* null mutant spores but present in the coats of *cotC* null mutant spores (Fig. 3), suggesting that it was the product of *cotU* expression. The conclusion that the 17-kDa protein is the product of *cotU* is also supported by the results of a previous study (7), in which N-terminal amino acid sequence analysis revealed a perfect match with the deduced sequence of CotU for the first 20 N-terminal positions of the 17-kDa protein.

The remaining polypeptide of 23 kDa was detected only among the proteins extracted from the coats of wild-type spores (Fig. 3), suggesting that it is dependent on the expression of both *cotC* and *cotU* (17). The genetic dependence of the 23-kDa protein on *cotC* and *cotU* expression obviously suggests the possibility that it is the result of an interaction between CotC and CotU. To test this possibility, we constructed two recombinant strains of *B. subtilis* carrying a His-tagged version of either *cotC* (RH228; see below) or *cotU* (RH208) as the only copy of *cotC* or *cotU* present in those strains. As shown in Fig. 4A, the 23-kDa proteins of strains RH208 and RH228 showed similarly slower migrations on SDS-PAGE than the 23-kDa protein of the congenic wild-type strain. The slower migration was most likely due to the presence of the His tag, as suggested by the observation that other tagged proteins also displayed altered electrophoretic properties. For instance, in strain RH228 (CotC-His_6) the presence of the His_6 tag slowed the migration of the four CotC forms and of the 23-kDa protein, although in the experiment shown in Fig. 4A, this effect is observable only for the higher-molecular-mass proteins (21, 23, and 30 kDa); in strain RH208 (CotU-His_6), the presence of the His_6 tag reduced the migration of both *cotU*-dependent proteins of 17 and 23 kDa (Fig. 4A). Moreover, when spore coat proteins were purified on Ni^{2+} columns, fractionated on SDS-PAGE, and analyzed by Western blotting with anti-CotC (17) or anti-CotU antibodies, the 23-kDa species was found in extracts from strain RH228 (CotC-His_6) (Fig. 4B) or RH208 (CotU-His_6) (Fig. 4C) but not those from a congenic wild-type strain (not shown). The variation in the relative abundances of the 23-kDa protein in the Western blots in Fig. 4 (compare panel A with panels B and C) most likely depended on poor recovery of this less well represented protein species from Ni^{2+}

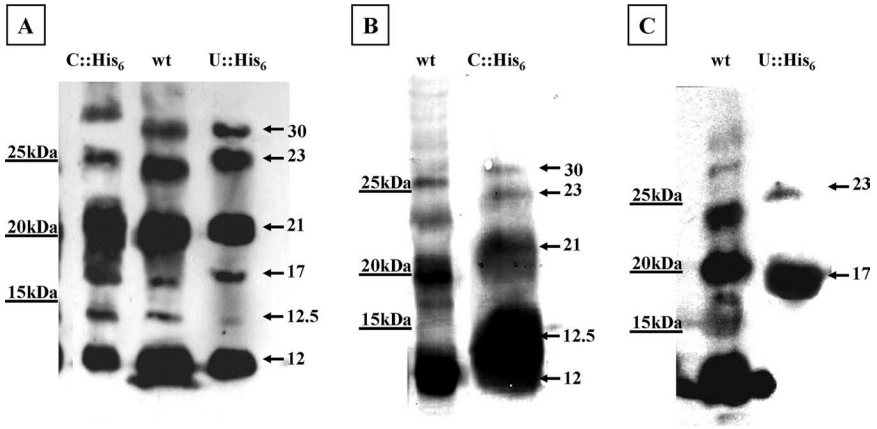

FIG. 4. Western blot analysis with anti-CotU (A and C) or anti-CotC (B) antibody. Coat proteins were extracted from a wild-type (wt) strain and a congenic strain carrying a *cotC*::His_6 (C::His_6) or a *cotU*::His_6 (U::His_6) fusion as indicated. Proteins were fractionated and then blotted (A) or purified through an Ni column and then blotted (B and C). In panels B and C, the wt lane contains unpurified proteins from wild-type spores. The arrows indicate the apparent molecular weights of the observed proteins. Molecular mass markers are also indicated.

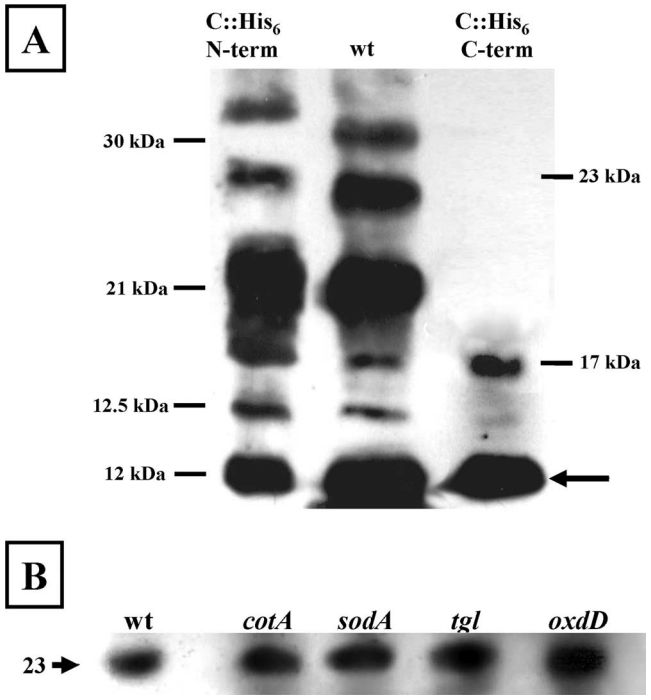

FIG. 5. Western blot analysis with anti-CotC antibody. (A) Proteins extracted from recombinant *B. subtilis* strains containing the His_6 tag fused at the 5′ (C::His_6 N-term) or 3′ (C::His_6 C-term) end of the *cotC* gene and from a congenic wild-type strain (wt). The indicated molecular masses refer to the various CotC- and CotU-dependent proteins from a wild-type strain (without the His_6 tag). The arrow indicates the 12-kDa species of CotC. (B) Proteins were extracted from spores of a wild-type *B. subtilis* strain and of a collection of congenic strains with null mutations in *cotA*, *oxdD*, *sodA*, or *tgl.* Identical results were obtained with anti-CotU antibody.

columns. Thus, the 23-kDa polypeptide was purified by Ni^{2+} affinity chromatography when the His_6 tag was joined to either CotC or CotU.

The genetic dependence and the biochemical evidence shown in Fig. 4 together indicate that CotC and CotU directly interact to form the 23-kDa polypeptide. To gain insight into the interaction between CotC and CotU, we constructed gene fusions in which the His_6 tag was placed in frame to the 3′ or 5′ end of both the *cotC* and *cotU* genes. All fusions were integrated on the chromosome of strain RH209, carrying null mutations in both *cotC* and *cotU* (18), and the extracted coat proteins were analyzed by Western blotting. A wild-type pattern of CotC- and CotU-dependent proteins was observed when CotU was tagged at either end (not shown) and when CotC was tagged at its N-terminal end (Fig. 5A). The presence of the His_6 tag at the C terminus of CotC did not impair assembly of the CotC species of 12 kDa (presumably the CotC monomer) (Fig. 5A): the level of CotC (12 kDa) was not reduced relative to the amount found in the coats of wild-type spores (Fig. 5A), yet formation of all other forms of CotC and of the 23-kDa CotC-CotU species was severely reduced. These results, therefore, suggest that the C-terminal end of CotC is involved in the formation of both the CotC-CotC homodimer and the CotC-CotU heterodimer. Moreover, these results strongly support the idea that multimerization of CotC and CotU occurs at the spore surface, following assembly of both proteins.

CotU and CotC do not interact in heterologous hosts. Yeast two-hybrid experiments were performed using *cotU* and *cotC* coding sequences fused to the activation or the DNA-binding domain of the *Saccharomyces cerevisiae* transcriptional activator GAL4, as previously described (17, 28). The gene fusions were then introduced into the yeast reporter strains Y187/Y190 (28), and the expression of a *lac*Z reporter gene followed. As previously reported, an interaction was detected between CotC and itself, indicating that CotC molecules self-interact (17). In contrast, and under similar conditions, no interaction was detected between CotC and CotU (data not shown), indicating that the two proteins do not interact when produced in yeast cells.

To verify whether CotU and CotC were able to interact in a different heterologous host, we coexpressed *cotU* and *cotC* in *E. coli*. Plasmid pRH51 (17), carrying the *cotC* coding region His_6 tagged at its 5′ end under the transcriptional control of the T7*lac* promoter, was engineered to replace the ampicillin resistance gene with a kanamycin resistance gene cassette, and the resulting plasmid was used to transform strain BL21(DE3) (Novagen), already containing plasmid pRH59 with a version of *cotU* His_6 tagged at its 3′ end. Upon IPTG induction, three bands corresponding in size to those produced by expressing *cotU* or *cotC* separately were produced and recognized by anti-CotU, anti-CotC, and His_6 antibodies. No additional proteins due to the interaction of CotC and CotU were observed (data not shown). In contrast, the 23-kDa CotC-CotU species was detected by both anti-CotC and anti-CotU antibodies in coat extracts of a *B. subtilis* strain expressing both His_6-*cotC* (His_6 at the 5′ end) and *cotU*-His_6 (His_6 at the 3′ end) (not shown). Therefore, the same proteins that failed to interact in *E. coli* were able to interact in *B. subtilis*, thus excluding the possibility that the interaction was inhibited in *E. coli* by the simultaneous presence of the His_6 tag in both partners involved in the interaction.

Since the CotC-CotU interaction occurs in *B. subtilis* but not in two heterologous hosts (*S. cerevisiae* and *E. coli*), we hypothesized that a specific factor might be needed to mediate that interaction.

CotC-CotU interaction does not require CotA, OxdD, Tgl, or SodA. CotA, OxdD, SodA, and Tgl are coat components with laccase (24), oxalate decarboxylase (4), superoxide dismutase (12), and transglutaminase (40) activities, respectively. It has been proposed that spore coat-associated enzymatic activities may be involved in mediating specific protein-protein interactions, including protein cross-linking reactions within the coat (11).

Based on this, we used a collection of congenic strains of *B. subtilis* mutant for *cotA* (BD063), *oxdD* (RH256), *sodA* (RH255), or *tgl* (RH233) to analyze whether the laccase, oxalate decarboxylase, superoxide dismutase, or transglutaminase activity was involved in mediating the CotC-CotU interaction. The 23-kDa protein (Fig. 5B), as well as all other CotC- and CotU-dependent proteins (not shown), was present in the coat fractions of spores of all mutant strains analyzed. Therefore, none of the enzymatic activities tested is required for formation of the 23-kDa CotC-CotU species.

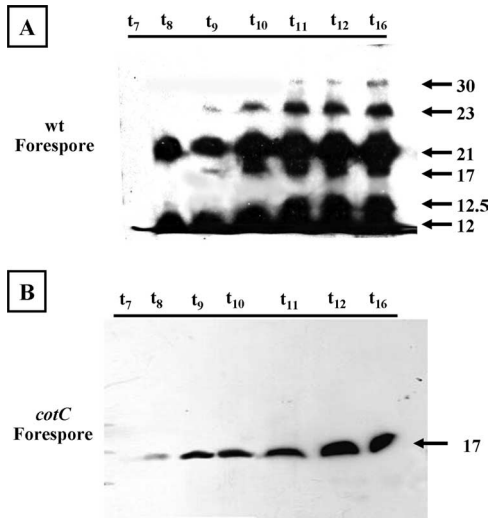

FIG. 6. Western blot of proteins extracted at various times (t_7 to t_{16}) after the onset of sporulation from the forespores of sporulating cells of a wild-type (wt) (A) and a *cotC* null mutant (B) strain of *B. subtilis*. The proteins were fractionated on a 15% polyacrylamide gel and, upon electrotransfer onto nitrocellulose membranes, reacted with CotU-specific rabbit antibodies and then with peroxidase-conjugated secondary antibodies and visualized by the Pierce method. The estimated sizes of CotC- and CotU-dependent polypeptides are indicated in kilodaltons.

Assembly of CotU and of the CotU-CotC heterodimer. Western blotting of proteins extracted from the mother cell or the forespore compartment of sporulating cells had indicated that none of the CotC forms accumulated in the mother cell, suggesting their rapid assembly onto the forming spore (17). Here, we have extended this analysis to the assembly of CotU and of the CotU-CotC polypeptide of 23 kDa. Sporulating cells of a wild-type strain were harvested at various times during sporulation and lysed by sonication as described in Materials and Methods, and the forming spores (forespore fraction) were separated from the mother cell cytoplasm (mother cell fraction). The forming spores were then extracted by alkali treatment, and the released proteins were compared with those present in the mother cell cytoplasm. For each time point, both protein fractions were analyzed by Western blotting with anti-CotU antibodies. At all time points analyzed, we detected no *cotU*-dependent polypeptides in the mother cell fraction (not shown). In the forespore fraction, we observed the four CotC forms (12, 12.5, 21, and 30 kDa) from hour 6 onward; as previously reported (17), CotU (17 kDa) and the CotU-CotC species (23 kDa) were detected from hour 8 onward (Fig. 6A).

In the time course experiment shown in Fig. 6A, CotU, as well as the 23-kDa protein, appeared 1 hour later than CotC. Since the analysis of the *cotU*::*lacZ* transcriptional fusion has shown that the timing of expression of *cotU* is identical to that of *cotC* (Fig. 2), the delayed appearance of CotU could be due to a posttranscriptional control on *cotU* expression. Alternatively, it could be that the high concentration of CotC in wild-type spores would not allow a sufficiently long exposure of the membrane, needed to visualize the less abundant CotU. To discriminate between the two possibilities, we repeated a similar time course experiment with a *cotC* null strain. As shown in Fig. 6B, when CotC was not present and the membrane could be exposed for enough time, CotU was detected 8 h after the onset of sporulation, at the same time as the appearance of CotC (Fig. 6A). Thus, although CotC and CotU accumulate at different rates, the onsets of their syntheses coincide.

The absence of CotC and CotU forms from the mother cell fraction suggests their rapid assembly at the surface of the developing spore. This again suggests that formation of the 23-kDa species takes place only at the spore surface.

CotU assembly and CotU-CotC interaction depend on CotE and CotH. To analyze the requirements for CotU assembly and for the CotC-CotU interaction, we performed a Western blot analysis with anti-CotU antibody and proteins extracted from mature spores of the wild type and spores of various congenic strains deficient in the production of key coat morphogenetic factors. CotU and the CotU-CotC proteins of 23 kDa were found in the coats of wild-type spores but were not found in *cotE* or *cotH* spores (Fig. 7A). Therefore, CotU assembly and CotC-CotU interaction appear to be CotE and CotH dependent. Since CotE and CotH do not affect *cotC* or *cotU* expression (26, 38), we predicted that in *cotE* and *cotH*

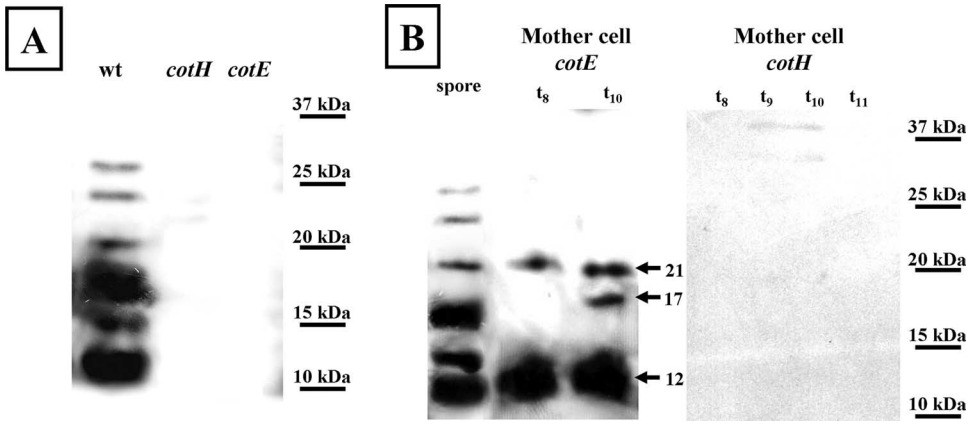

FIG. 7. Western blot analysis with anti-CotU antibody. (A) Coat proteins were extracted from a wild-type (wt) and congenic strains with null mutations in *cotH* or *cotE*. (B) Proteins were extracted at the indicated times (t_8 to t_{11}) during sporulation from the mother cell compartments of sporulating cultures of a *cotE* or a *cotH* mutant strain. The lane labeled "spore" contains coat proteins extracted from spores of a wild-type strain.

null mutants, CotU and CotC would accumulate in the mother cell. This was true for the *cotE* null mutant, in which CotU and the two main forms of CotC (12 and 21 kDa) (17) were found in the mother cell compartment (Fig. 7B). However, the CotC-CotU protein of 23 kDa was not found in the mother cell compartment of a *cotE* mutant (Fig. 7B) (17). This is in agreement with the idea that the 23-kDa CotC-CotU protein is formed at the spore surface. The absence of the two additional forms of CotC (12.5 and 30 kDa) in the mother cell of a *cotE* null mutant has been previously reported and discussed (17). In the *cotH* null mutant, CotU and the CotC-CotU form, as well as all CotC forms, were not found to accumulate in the mother cell (Fig. 7B). It has been previously proposed that the assembly of all CotC forms depends on the presence of CotH, which protects and/or stabilizes them in the mother cell compartment, allowing their assembly around the forming spore (2, 17). By analogy, we propose here that CotH is also involved in the stabilization/protection of CotU in the mother cell and possibly in its assembly on the coat.

Therefore, formation of the 23-kDa CotC-CotU species is under dual control: CotH stabilizes and/or protects both interaction partners in the mother cell compartment, whereas CotE governs their assembly and subsequent interaction at the spore surface.

DISCUSSION

In the present work, we have shown that the *cotU* gene is transcribed during sporulation under the joint control of the σ^K factor of RNA polymerase and the transcriptional factor GerE. Therefore, *cotU* and *cotC*, both of which code for highly similar tyrosine-rich components of the coat, are expressed coincidentally in the mother cell compartments of sporulating cells. The interaction of GerE with the *cotU* promoter region was not directly observed but was inferred on the basis of the effect of a *gerE* mutation on *cotU*-driven β-galactosidase activity.

We have also studied the assembly of CotU and expanded previous work on the assembly of the related protein CotC. We present genetic and biochemical evidence that CotU and CotC specifically interact, forming a protein of 23 kDa extractable from the coats of mature spores. The CotC-CotU species appears shorter than expected on SDS-PAGE if the apparent sizes of CotC and CotU are added to each other. A possible explanation is that a fragment of one of the two components is cleaved when the interaction occurs. Our data do not allow us to establish whether such a proteolytic event actually occurs. However, the data in Fig. 4 showing that the CotC-CotU species can be purified on an Ni^{2+} column when a His tag is placed at the C-terminal end of CotU or at the N-terminal end of CotC allow us to exclude the possibility that those two regions are involved in a potential proteolytic cleavage. Several lines of evidence suggest that the interaction of CotC and CotU to form the CotU-CotC species of 23 kDa takes place at the spore surface. First, neither CotU nor CotC is detected in the mother cell compartment of wild-type cells, suggesting that their assembly occurs rapidly following their synthesis (reference 17 and this work). Second, a *cotE* null mutation prevented the assembly of both CotU and CotC, causing the accumulation of the monomeric forms of both proteins in the mother cell but not the accumulation of the 23-kDa CotU-CotC form. Finally, expression of an allele of *cotC* fused at its 3′ end to the sequence for the His_6 tag resulted in the assembly of wild-type amounts of monomeric CotC but drastically impaired the formation of all the multimeric forms of CotC and of the CotC-CotU heterodimer. Although specific interactions between coat components have often been suggested as an important aspect of coat formation (19), only a few cases of direct interaction have been reported for coat proteins. In this respect, the observation that the CotU-CotC interaction occurs in *B. subtilis* but not in two heterologous hosts is particularly intriguing. It seems to suggest that the interaction involves an additional factor active at the spore surface. It is tempting to speculate that this factor could be a cross-linking enzyme, possibly assembled under CotE control (formation of the CotC-CotU species of 23 kDa was not detected in the mother cell of *cotE* mutant cells). The CotU-CotC interaction is not affected by extraction under alkaline conditions or by boiling in the presence of SDS and reducing agents. Both CotU and CotC are tyrosine rich, and formation of irreversible peroxidase-catalyzed *o*,*o*-dityrosine cross-links, which may occur within the coats, could account for the stability of the 23-kDa species. We note that cross-linking reactions have been shown to take place at the spore surface even after spore release from the mother cell, including the multimerization of another tyrosine-rich abundant coat protein, CotG (29, 40). However, we also note that a coat-associated peroxidase has not yet been found. Further work is needed to characterize the nature of the CotU-CotC interaction and the putative factor involved. In any case, our results also indicate that the C-terminal end of CotC is likely to be involved in mediating the interaction of CotC with itself and with CotU. It may be that the C-terminal region of CotC represents a nucleation point for multimer formation, because the presence of the His_6 tag in this region drastically reduces formation of all multimeric forms of CotC (including the CotC-CotU species).

Our results also expand on previous observations related to the role of the morphogenetic protein CotH in assembly of the coat structure. CotU does not accumulate in the mother cell cytoplasm of a *cotH* mutant. This cannot be due to reduced stability of the protein in the mother cell cytoplasm, since it was detected by Western blotting in the cytoplasm of a *cotE* mutant, as well as in *E. coli*. We believe it is more likely that a specific factor (a protease?) degrades CotU in the absence of CotH (or a *cotH*-dependent protein). Similarly to what has been proposed for CotC (17) and CotG (42), we hypothesize that in a wild-type strain, CotH (or a *cotH*-dependent protein) would prevent CotU degradation either by interacting in a chaperone-like manner with CotU or its specific protease in the mother cell or by immediately recruiting CotU into the coat of the forming spore.

In a recent paper, CotU assembly within the coat was analyzed by fluorescence microscopy and found to be only partially dependent on CotE and CotH (19). In the present study, our Western blotting experiments indicated that CotU assembly is totally dependent on both morphogenetic proteins. As previously proposed for other coat components (19), this discrepancy could be explained by the presence of CotU in both the soluble (extractable) and insoluble (resistant to extraction) fractions of coat proteins. Fluorescence microscopy would then

allow the detection of all CotU molecules, while Western blotting would detect only soluble CotU molecules. An alternative possibility is that CotU and the chimeric protein CotU-green fluorescent protein used in the previous study (19) have different stability and/or assembly properties. Since none of the mutants described here has any obvious phenotypic consequences for the properties of the coat, the functionality of a CotU-green fluorescent protein fusion is difficult to assess.

Future work will aim at characterizing in detail the interaction between CotC and CotU, including the identification and role of the putative factor involved, and the role of CotH in the process.

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CotE binds to CotC and CotU and mediates their interaction during spore coat formation in *Bacillus subtilis*

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CotE is a morphogenic protein that controls the assembly of the coat, the proteinaceous structure that surrounds and protects the spore of *Bacillus subtilis*. CotE has long been thought to interact with several outer coat components but such interactions were hypothesized from genetic experiments and have never been directly demonstrated.

To study the interaction of CotE with other coat components we focused our attention on CotC and CotU, two outer coat proteins known to be under CotE control and to form a heterodimer. We report here pull-down experiments that provide the first direct evidence that CotE contacts other coat components. In addition, co-expression experiments demonstrate that CotE is needed and sufficient to allow formation of the CotC-CotU heterodimer in a heterologous host.

Introduction

The spore of *Bacillus subtlis* is a dormant cell, resistant to harsh conditions and able to survive extreme environmental conditions (25). Spores are produced in a sporangium that consists of an inner cell, the forespore, that will become the mature spore and an outer cell, the mother cell, that will lyse, liberating the mature spore (18, 26). Resistance of the spore to noxious chemicals, lytic enzymes and predation by soil protozoans is in part due to the coat, a complex, multilayered structure of more than 50 proteins that encases the spore (5, 13, 8). Proteins that constitute the coat are produced in the mother cell and deposited around the outer membrane surface of the forespore in an ordered manner (8).

A small subset of coat proteins have a regulatory role on the formation of the coat. Those proteins, referred to as morphogenic factors, do not affect the synthesis of the coat components but drive their correct assembly outside of the outer forespore membrane (8). Within this subset of regulatory coat proteins, SpoIVA and CotE play a crucial role. SpoIVA (20, 23, 6) is assembled into the basement layer of the coat and is anchored to the outer membrane of the forespore through its C terminus that contacts SpoVM, a small, amphipathic peptide embedded in the forespore membrane (16, 21, 22). A *spoIVA* null mutation impairs the assembly of the coat around the forming spore and, as a consequence, coat material accumulates in the mother cell cytoplasm (23).

CotE (28) assembles into a ring and surrounds the SpoIVA basement structure. The inner layer of the coat is then formed between the SpoIVA basement layer and the CotE ring by coat components produced in the mother cell that infiltrate through the CotE ring, while the outer layer of the coat is formed outside of CotE (6). However, not all CotE molecules are assembled into the ring-like structure and CotE molecules are also found in the mother cell cytoplasm, at least up to eight hours after the start of sporulation (3). CotE was first identified as a morphogenic factor in a seminal study in which an ultrastructural analysis indicated that a *cotE* null mutation prevented formation of the electron-dense outer layer of the coat while

didn't affect inner coat formation (28). A subsequent mutagenesis study has revealed that CotE has a modular structure with a C terminal domain involved in directing the assembly of various coat proteins, an internal domain involved in targeting of CotE to the forespore and a N terminal domain that, together with the internal domain, directs the formation of CotE multimers (17). More recently, formation of CotE multimers has been also confirmed by a yeast-two-hybrid approch (14). In a global study of protein interactions in the *B. subtilis* coat, performed by a fluorescence microscopy analysis of a collection of strains carrying *cot-gfp* fusions, CotE has been proposed to interact with most outer coat compenents (12).

From those and other studies the interactions of CotE with coat structural components have been exclusively inferred on the base of genetic experiments, i.e. *cotE* mutants that failed to assemble one or more coat components. Evidence of a direct interaction between CotE and another coat component have never been provided. We addressed this issue by using as a model two coat components, CotC and CotU, known to be controlled by CotE and to form a heterodimer (28, 10).

CotC is an abundant, 66 amino acids protein known to assemble in the outer coat in various forms: a monomer of 12 kDa, a homodimer of 21 kDa and two less abundant forms of 12.5 and 30 kDa, probably due to post-translational modifications of CotC (9). CotU is a structural homolog of CotC of 86 amino acids. The two proteins, that share an almost identical N terminus and a less conserved C terminus (Fig. 1A) (10), interact originating an heterodimer of 23 kDa. Heterodimer formation most likely requires a *B. subtilis* specific factor since it does not occur in *Escherichia coli* or *Saccharomyces cerevisiae* (10). CotC and CotU are synthesized in the mother cell compartment of the sporulating cell but do not accumulate there since are immediately assembled around the forming spore (Fig. 1B) (10). In a strain carryng a *cotE* null mutation CotC and CotU, together with all other outer coat components, do not assemble around the forming spore (10). CotC and CotU are also dependent on CotH, an additional morphogenic factor involved in coat formation (9). A *cotH* null mutation

prevents CotC and CotU assembly in the coat as well as their accumulation in the mother cell cytoplasm (10). Since a mutation causing *cotH* over-expression allows CotC and CotU accumulation in the mother cell cytoplam (1), it has been proposed that CotH acts by stabilizing CotC and CotU in the mother cell cytoplasm (1, 10).

Here we provide the first direct evidence that CotE interacts with two other coat components, CotC and CotU, and show that CotE is essential and sufficient to mediate CotC-CotU interaction to form a heterodimer.

Methods

Bacterial strains and transformation. *B. subtilis* strains used in this study are listed in Table 1. Plasmid amplification for subcloning experiments, nucleotide sequence and transformation of *Escherichia coli* competent cells were performed with *E. coli* strain DH5α (24). *E. coli* strain BL21 (DE3) (Novagene) was used for protein over-expression. Bacterial strains were transformed by previously described procedures: CaCl2-mediated transformation of *E. coli* competent cells (24) and two-step transformation of *B. subtilis* (4).

Genetic and molecular procedures. Isolation of plasmids, restriction digestion and ligation of DNA were carried out by standard methods (24). Chromosomal DNA from *B. subtilis* was isolated as described elsewhere (4). A *spoIVA cotE* double mutant was obtained by transforming competent cell of strain 67 (*spoIVA*) (Table 1) with chromosomal DNA extracted from strain BZ213 (*cotE::cat*) generating strain RH263.

Sporulating cells lysates and immunoblot analysis. Sporulation of all *B. subtilis* strains was induced by the exhaustion method (4). Sporulating cells were harvested after eight and ten hours from the onset of sporulation and mother cells and forespore fractions isolated as described before (10). Whole-cell lysates of sporulating cells were prepared by sonication (10) followed by detergent treatment (62.5mM Tris-Hcl, pH6.8, 4%SDS, 5% glycerol, 2%B-mercaptoethanol, 0.003% bromophenol blue) at 100°C for 7 minutes. 50 µg (mother cell extract or whole-cell lysates) or 15 µg (forespore extract) of total proteins were subjected to immunoblot analysis with the anti-CotC or anti-CotU antibodies as described previously (10), except that polyvinylidene difluoride membranes were used instead of nitrocellulose.

Over-production of 6xHis-tagged and untagged CotE. To over-express CotE in *E. coli*, the coding region of the *cotE* gene was PCR amplified using the *B. subtilis* chromosomal DNA as a template and oligonucleotides E-rbs-PstI-F (ctgcagtttaAGAAGGAGAtatacatatgtctgaatacagggaat; *Pst*I and *Nde*I restriction sites are

underlined, the ribosome binding site is in uppercase) and E-STOP (ccaagcttattcttcaggatctcccac; *Hind*III restriction site is underlined) as primers. The amplification product of 582 bp was digested with *Nde*I and *Hind*III and ligated to the same sites of the expression vector pRSETB (Invitrogen), to obtain pRH134. By digesting pRSETB with *Nde*I the His tag present on the vector was removed.

To over-express a 6xHis tagged copy of CotE in *E. coli,* the coding region of the *cotE* gene was amplified by PCR using *B. subtilis* chromosomal DNA as template and oligonucleotides E-NdeI-F (taggaattccatatgtctgaatacagggaat; underlined is the*Eco*RI restriction site) and E-STOP (ccaagcttattcttcaggatctcccac; underlined is the *Hind*III restriction site) as primers. The amplified fragment of 564 bp was digested with *EcoRI* and *Hind*III and ligated to plasmid pRSETB (Invitrogen), previously digested with the same enzymes. The recombinant plasmid, pAP18, carried the *cotE* coding region fused to a 6xHis tag under the transcriptional control of a T7 inducible promoter.

Plasmids pRH134 and pAP18 were checked by nucleotide sequence analysis and used to transform competent cells of *E. coli* BL21 (DE3) (Novagen), to create RH136 and AP19, respectively (Table1). CotE and CotE-His were produced by auto-induction by growing cells at 37°C for 18 hours with orbital shaking (150 rpm) and by using the Overnight Express Autoinduction System1, following manufacturer's instructions (Novagen). CotE-His protein was purified under denaturing conditions via Ni-nitrilotriacetic acid affinity chromatography as recommended by the manufacturer (Qiagen, Inc) and used to raise specific antibodies in mice by PriMM srl (Italy).

Co-expression of *cotC*, *cotU* and *cotE* in *E.coli*. The coding part of *cotE*, amplified by PCR using oligonucleotides E-rbs-PstI-F and E-STOP as described above, was digested with *Pst*I and *Hind*III and inserted in the same sites of pRH62 (10), immediately downstream of *cotC::his* to create pRH122. In this plasmid both genes were under the control of the same T7 promoter and formed a single transcriptional unit. The new recombinant plasmid was used to transform the competent cells of *E. coli* strains RH59 (10), harboring *cotU::his* under

the control of T7 promoter, to create strain RH125. Proteins were produced from these *E. coli* strains by the auto-induction procedure, as describe above.

Pull-down experiments. Strains RH52, RH59 and RH136 (Table1) were grown for 18 hours at 37°C in auto-induction medium (see above). Samples (14 ml) were collected by centrifugation and resuspendend in 1.5 ml of Lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM Imidazole, 2mg/ml Lysozyme and 0.01mg/ml RNase). After 30 min at 4°C, the lysates were sonicated (20 min pulses at 20 Hz with a Sonicator Ultrasonic Liquid Processor; Heat System Ultrasonic Inc., NY, USA). The suspension was clarified by centrifugation at 13.000g at 4°C for 20 min and those of strains RH52 and RH59 applied to Ni-NTA Magnetic Agarose Beads (Qiagen), separately. After 1h of incubation at room temperature with shake, the beads were washed with 2.5 ml of Wash Buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM Imidazole), the extract of RH136 strains added to both samples and incubated for 1 h at room temperature with shake. Unbound proteins were removed by washing with Wash Buffer at three different concentration of imidazole (40mM, 100mM and 250mM). Bound proteins were eluted using the Wash Buffer at increasing concentration of imidazole (500mM and 1M). Eluted proteins were resolved on SDS-12.5% PAGE gels and subjected to immunoblot analysis.

Preparation of samples for MALDI TOF analysis. Strain RH125 was grown for 18 hours at 37°C in auto-induction medium (see above). Cells (100 ml) were washed with 10 ml of 1x phosphate-buffered saline (PBS), suspended with 5 ml of 1x PBS and 5 ml of 2x Cracking Buffer (120mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 2% β-mercaptoethanol) and heated at 100°C for 10 min. The suspension was clarified by centrifugation at 13.000g for 20 min and the supernatant diluted 1:10 using Binding buffer (20 mM Sodium Phosphate, 500 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol, 25 mM imidazole pH 7.4). The diluted sample was applied to a His Trap HP (GE Healthcare, Healthcare Europe, GmbH, Milan, Italy) equilibrated with 10 ml of Binding buffer. The column was washed with 10 ml of Binding

buffer and proteins eluted with the same buffer, supplemented with increasing concentrations of imidazole (50 mM, 100 mM, 250 mM, 500 mM). Purified proteins were resolved on SDS-15% PAGE gels and bands sent for MALDI- mass spectrometry analysis.

Reduction and Alkylation of coat proteins. Coat proteins extracted from mature spore by 0.1N NaOH treatment at 4°C (4) were treated with various reducing conditions (10mM or 80mM dithiothreitol or 1% B-mercaptoethanol, for either 60 min at 80°C or over night at 37°C) and then alkylated by adding 50 mM iodoacetic acid (IAA), 1 N NaOH for 30 min on ice in the dark or directly alkylated. The proteins were washed in an Amicon Ultra-15 Centricon (Millipore) with 10 volumes 1xPBS, resolved on SDS-15% PAGE gels and subjected to immunoblot analysis.

Fluorescence Microscopy. For CotC-GFP localization, 500 µl of cells were collected after eight hours from the onset of sporulation, briefly pelleted and resuspended in 30 µl of 1x PBS containing 1.5µg of FM4-64 (SIGMA) per ml. The concentrated cells (3 µl) were immediately spotted onto microscope slides and firmly covered with poly L-lysine (0.1% solution, Sigma) treated coverslips. Fluorescence microscopy was performed with an Olympus BX51 microscope equipped with a 100x UPlanF1 objective, a U-WIBA filter cube (excitation filter 460–490 nm, barrier filter 515–550 nm) for GFP visualization and a U-WG filter cube (excitation filter 510–550 nm, barrier filter >590nm) for FM4-64 visualization. Exposure times were typically in the range of 500–1000 ms. Images were captured and cropped with analySIS software (SIS). Finally, images were exported as TIF files to the Adobe Photoshop software for minor adjustments of brightness and contrast.

185 **Results**

186 ***cotE* expression is required to allow the interaction between CotC and CotU.** It has
187 been previously reported that CotC assembles in the spore coat as a monomer and
188 homodimer and that it forms with CotU a heterodimer of 23 kDa (10). In western blot
189 experiments performed on sporulating cells of a wild type strain of *B. subtilis* eight and ten
190 hours after the onset of sporulation CotC, CotU and the CotC-CotU heterodimer were found
191 only in the forespore compartment (Fig. 2A), indicating that they are assembled immediately
192 after their synthesis in the mother cell compartment (10). In a similar experiment performed
193 with an isogenic strain carrying a *cotE* null mutation, CotC and CotU were found in the
194 mother cell compartment while the heterodimer was not formed, indicating that CotC and
195 CotU are normally produced, that they are not assembled around the forming spore and that
196 they fail to interact (Fig. 2B).

197 A possible explanation for the lack of interaction in the *cotE* mutant is that the heterodimer
198 can only be formed when the two partners of the interaction are already assembled on the
199 forming coat. Two lines of evidence indicate that this is not the case and that the heterodimer
200 forms also when the two partners are not assembled: i) a western blot analysis of extracts of
201 sporulating cells of a *B. subtilis* strain mutated in the *spoIVA* genes, in which the entire coat
202 is not around the spore (23), showed that the CotC-CotU heterodimer is formed but only in a
203 *cotE*-dependent way (Fig. 3A); ii) a western blot analysis with extracts of the mother cell
204 fraction of sporulating cells of a *B. subtilis* mutant having an increased expression of the *cotH*
205 gene (1), confirms that the 23 kDa heterodimer is formed in the mother cell cytoplasm in a
206 *cotE*-dependent way (Fig. 3B).

207 Taken together these data support the hypothesis that CotE or a CotE-dependent protein is
208 needed to allow the interaction between CotC and CotU and that such interaction can occur
209 independently from the assembly of CotC and CotU on the spore coat.

210

211 **Formation of CotC homodimer and CotC-CotU heterodimer does not involve cysteine**
212 **residues.** CotC-CotU heterodimer as well as CotC-CotC homodimer, were observed on

SDS-PAGE (Fig. 2 and 3) after extraction from sporulating cells or mature spores by treatment with 0.1N NaOH at 4°C (19), indicating that both dimers are resistant to reducing and denaturing conditions.

Since both CotC and CotU have a cysteine residue at position 32 (Fig. 1A), we decided to verify whether those cysteines were involved in the formation of the homo- and/or hetero-dimer. To this aim we exposed proteins extracted from a wild type strain to extreme reducing conditions (Methods) and to subsequent alkylation with iodoacetic acid (IAA) (15). IAA binds to reduced cysteines and prevents them from re-forming sulfur bridges. None of the tested conditions impaired formation of the dimers after either one hour of incubation at 60 °C (Fig. 4) or an overnight incubation at 37°C (not shown), thus excluding that they were depended on cysteine-mediated sulfur bridges.

CotE binds to CotC and CotU. To verify whether CotE directly interacts with CotC and/or CotU, we over-expressed in *E. coli* a his-tagged version of *cotC* (CotC-His), a his-tagged version of *cotU* (CotU-His) or an untagged version of *cotE* and performed a *in vitro* His-tag pull-down assay. After auto-induction, *E.coli* cells were lysed by sonication and Ni-NTA magnetic beads incubated with extracts of cells expressing CotC-His or CotU-His. Beads were then washed and incubated with the extract of cells expressing untagged CotE. After additional washes proteins were eluted and used for western blot experiments with anti-CotC, anti-CotU, anti-His or anti-CotE antibodies. As shown in Fig. 5, untagged CotE bound Ni-NTA beads when CotC-His (panel A) or CotU-His (panel B) was present. In the absence of CotC-His or CotU-His, untagged CotE was not able to bind to the Ni-NTA beads (Fig. 5C). These *in vitro* results demonstrated that CotE directly interacts with CotC and CotU and are the first direct proof of an interaction between CotE and other components of the *B. subtilis* spore coat.

CotE mediates the interaction of CotC and CotU. To investigate whether the interaction with CotE was sufficient to induce the formation of the CotC-CotU heterodimer, we over-

241 expressed all three proteins together in the same *E. coli* strain and used the extracted
242 proteins for western blot analysis with anti-CotC, anti-CotU, anti-His and anti-CotE
243 antibodies. As previously reported (10), when only *cotC* and *cotU* were over-expressed
244 together in *E. coli* three proteins were recognized by anti-CotC and anti-CotU antibodies (Fig.
245 6A, lane1). The three bands corresponded in size to CotC, CotU and CotC homodimer (10).
246 When also *cotE* was expressed in the same cells an additional protein was formed (Fig. 6A,
247 lane 2). The additional protein did not contain CotE since when the same gel was reacted
248 against anti-CotE antibody, a COtE-specific signal was observed at a different position on the
249 gel (Fig. 6B). It is unlikely that the additional protein is an unspecific signal since it was not
250 present in lane 1 (CotC+CotU) or lane 3 (CotE) of Fig. 6A and identical results were obtained
251 with anti-CotU and anti-His antibodies (not shown).
252 To determine the nature of the proteins formed when CotC, CotU and CotE were over-
253 expressed together in *E. coli*, we performed a MALDI TOF mass spectrometry analysis. The
254 four proteins shown in Fig. 6A (lane 2) were excised from the SDS-polyacrylammide gel and
255 subjected to MALDI TOF using a MALDI-TOF micro MX (Waters Co., Manchester, UK), as
256 describes previously (2). Due to the high percentage of identity between CotC and CotU
257 amino acid sequences (Fig. 1B), it was necessary to identify diagnostic peptides to
258 discriminate between the two coat proteins. In particular, three peptides at the C-terminal end
259 of the two proteins were found to be diagnostic: peptide 1 and 3 of CotU and peptide 2 of
260 CotC (Fig. 1A). As reported in Table 2, peptides 1 and 3 (diagnostic of CotU) were found in
261 the protein tentatively assigned as CotU while only peptide 2 (dignostic of CotC) was found in
262 proteins indicated as CotC monomer and CotC-CotC homodimer. All three peptides were
263 found in the additional protein of Fig. 6A (lane2), confirming that it was the CotC-CotU
264 heterodimer (Table 2).
265 These results confirm the nature of the proteins expressed in *E. coli* and indicate that no
266 additional factors other than CotE are needed to mediate CotC-CotU heterodimer formation.
267
268

Discussion

Main outcome of this work is that it provides the first direct evidence that CotE interacts with a coat component other than CotE itself. CotE of *B. subtilis* is a morphogenic factor required for outer coat formation (28), known to form multimers organized in a ring-like structure assembled around the forming spore (6, 17 , 14). CotE has been proposed as a major regulatory factor of outer coat assembly and its interaction with several components of the coat inferred on the base of genetic experiments (12). We provide here a direct evidence that CotE interacts with CotC and with CotU, two outer coat components whose assembly around the spore has been previously indicated as CotE-dependent (28, 10). The pull-down experiments of Fig. 5, demonstrate that CotE is retained on Ni-NTA magnetic beads only when CotC-His or CotU-His have been previously bound to the beads, indicating that CotE directly binds CotC and CotU. A future challange will be to define these interactions by identifying the amino acid residues involved in CotE-CotC and CotE-CotU contacts.

A second important result of this work is that CotE is sufficient to mediate CotC-CotU interaction in *E. coli* cells. The co-expression experiment of Fig. 6 and the MALDI TOF analysis of the proteins produced in *E. coli* clearly indicate that only in the presence of CotE the two coat components interact forming the heterodimer previously observed in the coat protein fraction of *B. subtilis* spores (10). However, at this stage we can not explain how CotE mediates CotC-CotU interaction and can only hypothesize that CotE either recognizes CotC and CotU as substrates and catalyzes their interaction or acts as a platform to which CotC and CotU bind. In the latter case, CotE role would be to hold the two coat components close to each other allowing their spontaneous or self-catalyzed interaction. Although an enzymatic activity associated to CotE has never been demonstrated, we observed that CotE contains a region of homology with the consensus of the Walker A domain of ATPases (conseusus: A/GXXXXGKT (11); CotE: A_{47} - - - G_{51} K_{52} T_{53}). However, so far we have been unable to detect any ATPase activity associated to *E. coli*-purified CotE (data not shown).

296 Additional experiments will be needed to clarify CotE role in CotC-CotU interaction and
297 discriminate between the two hypothesis.
298
299 An additional result of this work is that CotC-CotC homodimers and CotC-CotU heterodimers
300 are resistant to denaturing and reducing conditions and are not dependent on the single
301 cysteine residue that both proteins have at their position 32 (Fig. 1A). While CotC-CotC
302 homodimers form spontaneously in *E. coli* (9), CotC-CotU heterodimers require CotE to form.
303 Preliminary results indicate that amino acids at the C terminus of CotC are involved in the
304 formation of both the homo- and the hetero-dimer (Isticato and Ricca, unpublished results),
305 but, also in this case, additional experiments will be needed to clarify the nature of those
306 interactions.
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310
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Figures legend

Fig. 1 (A) Alignment of the amino acid sequences of CotC and CotU. Asterisks indicate identical residues. Boxed are peptides identified as diagnostic of CotC or CotU (see below). (B) Microscopy analysis of sporulating cells of *Bacillus subtilis* carrying the *cotC::gfp* fusion collected eight hours after the onset of sporulation. The same microscopy fiels was observed by phase contrast (left panel) or by fluorescence microscopy after vital staing with FM4-64 to label the membranes (right panel). In cells containing a prespore the green fluorescent signal was exclusively localized around the forming spore.

Fig. 2 Western blot of proteins extracted from the Mother cell or Forespore fractions of sporulating wild type (A) or isogenic *cotE* (B) mutant cells eight and ten hour after the onset of sporulation. Proteins were fractionated on 15**%** polyacrylamide gels, electrotransferred to membranes and reacted with anti-CotC antibody. Identical results were obtained with anti-CotU antibody.

Fig. 3 Western blot of proteins extracted ten hours after the onset of sporulation from (A) sporulating cells of a *spoIVA* mutant or a *spoIVA cotE* double mutant and (B) the mother cell fraction of a strain over-expressing *cotH* (indicated as *cotH**). Proteins were fractionated on 15**%** polyacrylamide gels, electrotransferred to membranes and reacted with anti-CotU antibody. Identical results were obtained with anti-CotC antibody.

Fig. 4 Effect of reduction and alkylation on CotC homodimer and CotC-CotU heterodimer. Coat proteins extracted from wild type spores were incubated for 60 min at 60°C in the absence or the presence of different reducing agents. After reduction, coat proteins were alkylated by adding 50mM IAA. Treated proteins were fractionated on 15% polyacrylamide gels, electrotransferred to membranes and reacted with anti-CotC. CotC* indicates the two so far uncharacterized forms of CotC (Isticato et al., 2004).

Fig. 5 Pull-down experiment performed by binding CotC-His (A) or CotU-His (B) to Ni-NTA magnetic beads. Untagged CotE was then added and flowthrough (FT), washes (W) and eluted (E1 and E2) proteins collected. Proteins were fractionated on 12.5**%** polyacrylamide gels, electrotransferred to membranes and reacted with anti-CotC, anti.CotU and anti-CotE antibodies. The same experiment was also performed without CotC-His or CotU-His (C).

Fig. 6 Co-expression experiment. Extracts from *E. coli* cells over-producing CotC and CotU (lane 1), CotC, CotU and CotE (lane2) and CotE (lane3) were fractionated on 15**%** polyacrylamide gels, electrotransferred to membranes and reacted with anti-CotC antibody. Identical results were obtained with anti-CotU antibody.

Table 1. *Bacillus subtilis* and *Escherichia coli* strains used.

B. subtilis		
Strain name	**Relevant genotype**	**Source**
PY79	prototrophic	27
BZ213	*cotE::cat*	28
67	*spoIVA*	7
RH263	*spoIVA cotE::cat*	This work
E. coli [a]		
Strain name	**Relevant genotype of contained plasmid**	**Source**
RH52	*cotC::his*	9
RH59	*cotU::his*	10
RH62	*cotC::his*	10
RH63	*cotC::his cotU::his*	10
RH125	*cotC::his cotU::his cotE*	This work
RH136	*cotE*	This work
AP19	*cotE::his*	This work

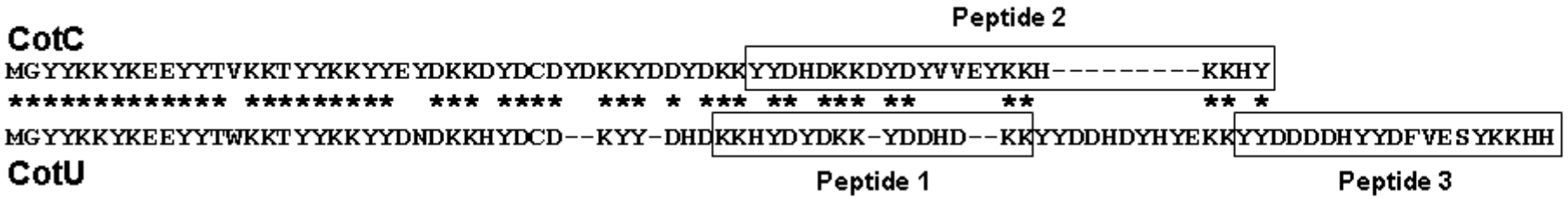
[a]All *E. coli* strains are derivative of strain BL21(D3) transformed with various plasmids.

Table 2. Molecular mass values obtained by MALDI-TOF mass spectrometry of tryptic digests of band CotC, CotU and CotC-U. Theoretical and experimental mass values together with peptide sequence positions are reported

Peptide	Sequence position[a]	Theoretical Mr $[M+H]^+$	Experimental Mr $[M+H]^+$ Band "CotC"	Experimental Mr $[M+H]^+$ Band "CotU"	Experimental Mr $[M+H]^+$ Band "CotC-CotC"	Experimental Mr $[M+H]^+$ Band "CotC-CotU"
1	40-54	1869.83	-	1869.78	-	1869.78
2	45-66	2964.42	2964.43	-	2964.46	2964.46
3	68-86	2539.04	-	2539.01	-	2538.91

[a] Refers to the amino acid sequence of CotC (peptide 2) and CotU (peptides 1 and 3) as reported in Fig. 1A.

A


Peptide 2
CotC
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MGYYKKYKEEYYTWKKTYYKKYYDNDKKHYDCD--KYY-DHDKKHYDYDKK-YDDHD--KKYYDDHDYHYEKKYYDDDDHYYDFVESYKKHH
CotU
Peptide 1
Peptide 3

B

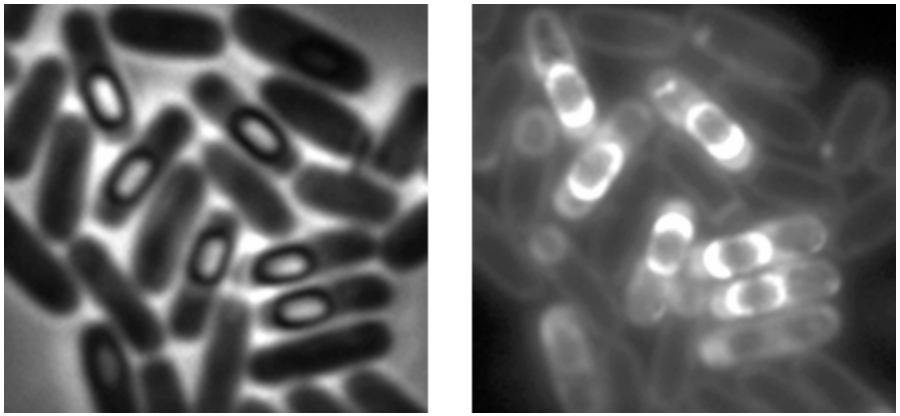

Figure 1

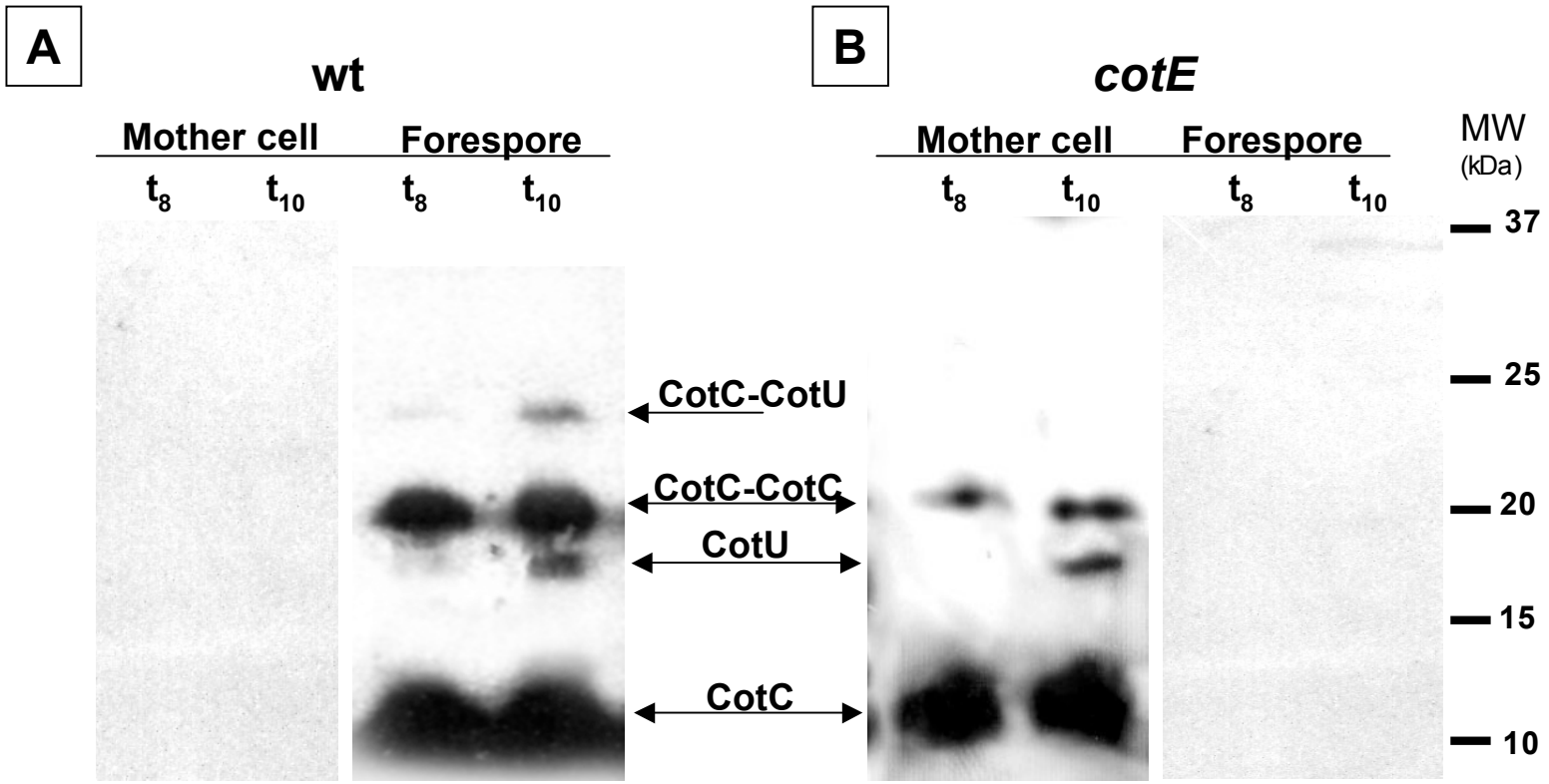
A
wt
Mother cell
Forespore
t_8
t_{10}
t_8
t_{10}
CotC-CotU
CotC-CotC
CotU
CotC
B
cotE
Mother cell
Forespore
t_8
t_{10}
t_8
t_{10}
MW
(kDa)
37
25
20
15
10

Figure 2

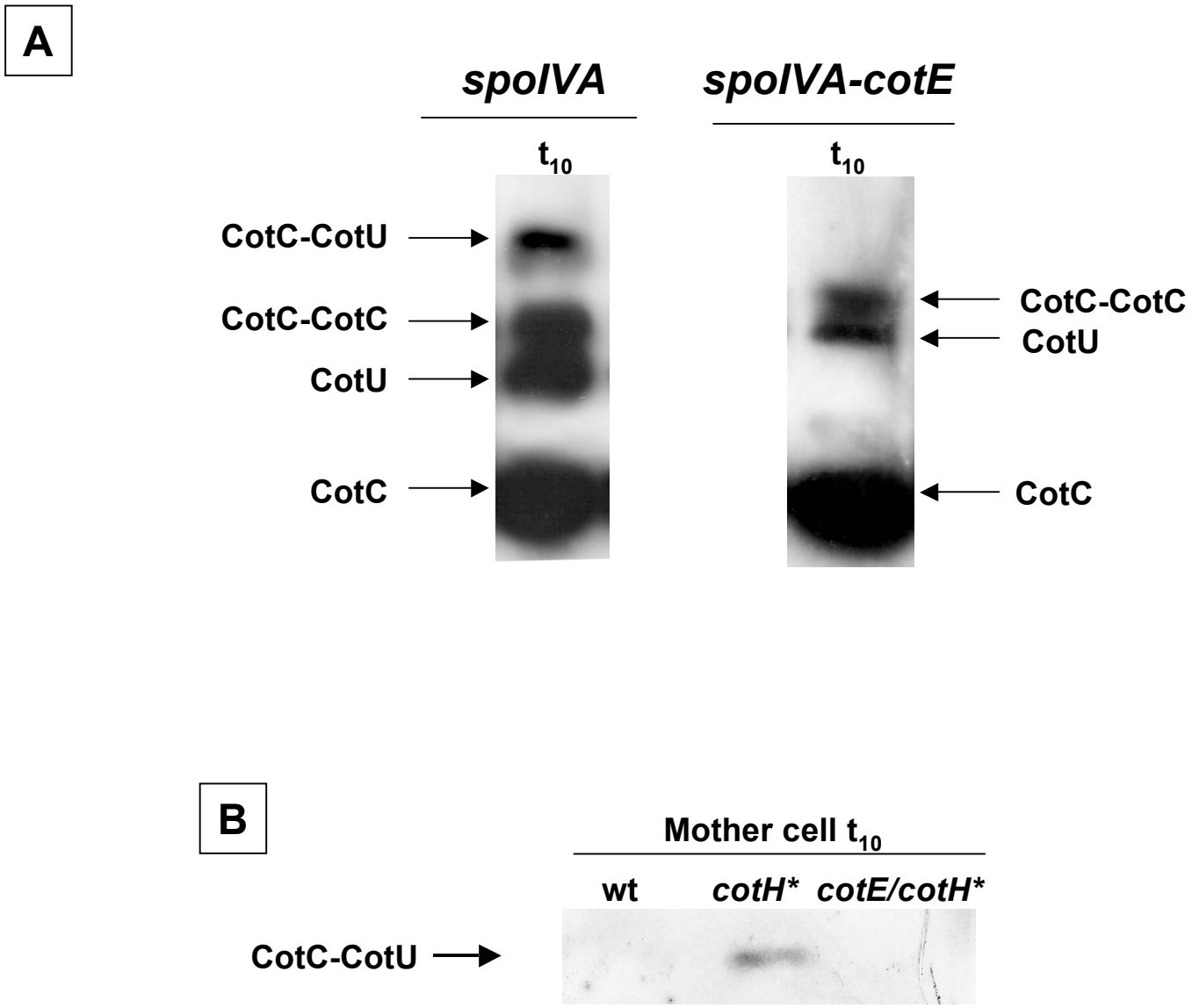
A
spoIVA
spoIVA-cotE
t10
t10
CotC-CotU
CotC-CotC
CotU
CotC
CotC-CotC
CotU
CotC
B
Mother cell t10
wt
cotH*
cotE/cotH*
CotC-CotU

Figure 3

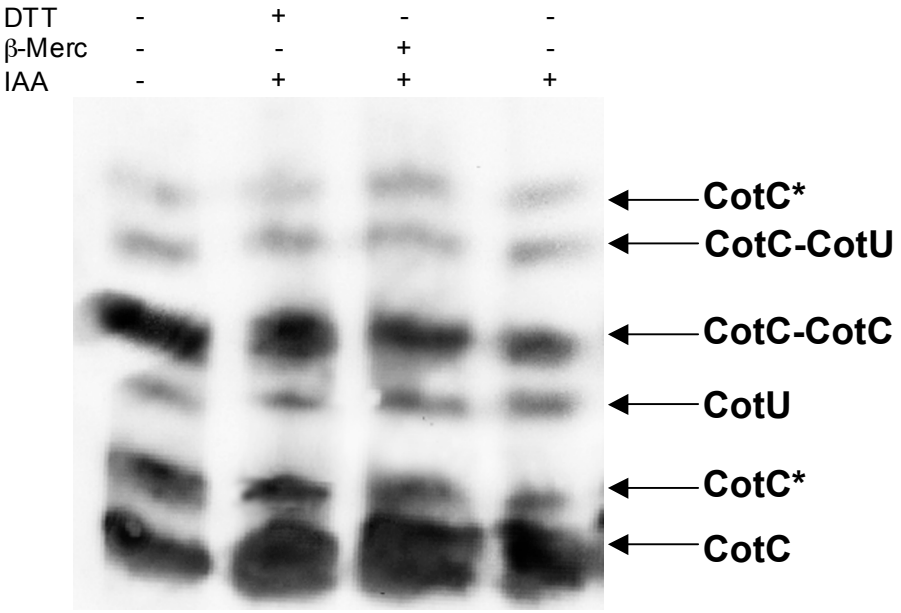

Figure 4

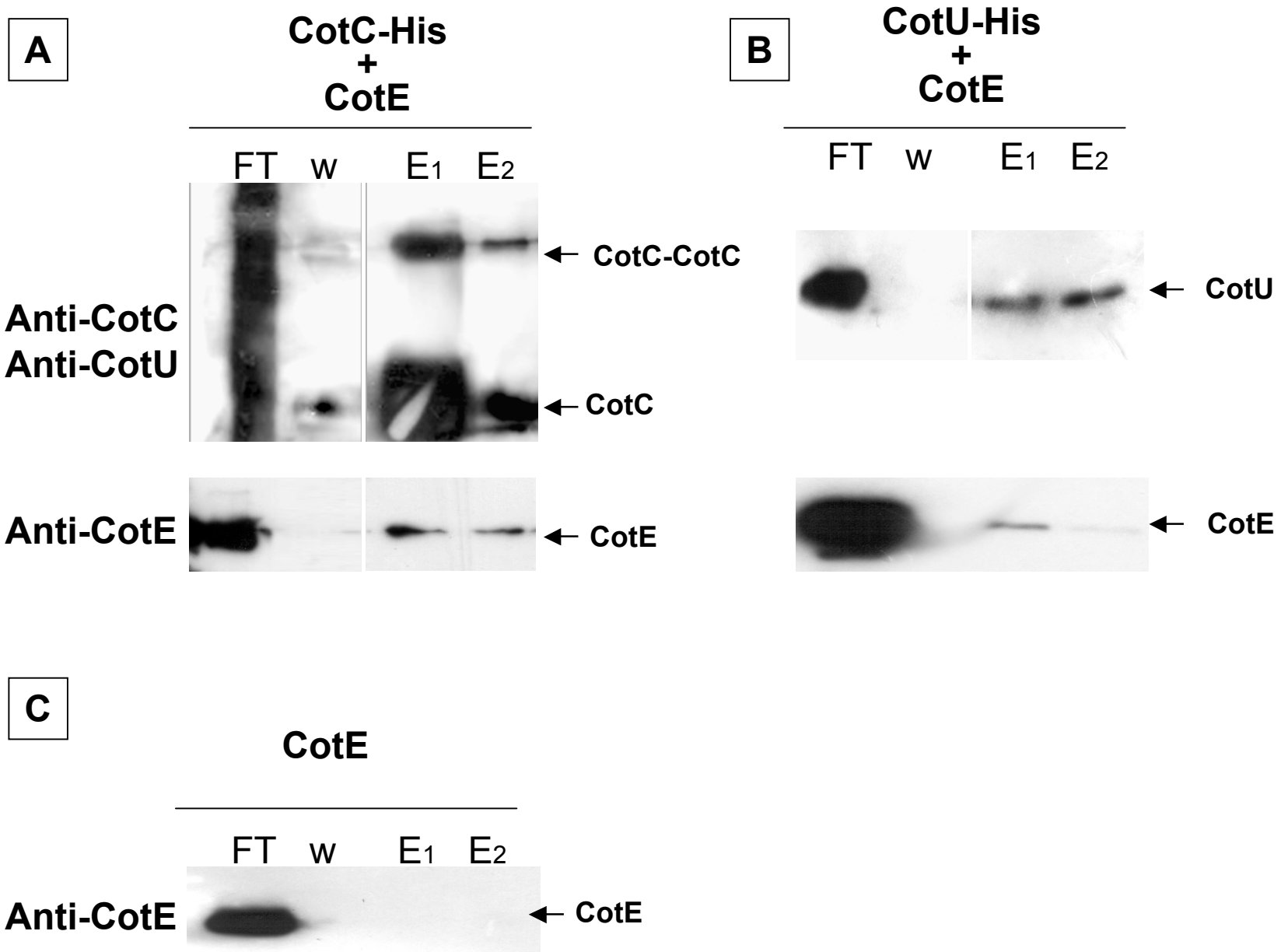
A
CotC-His
+
CotE
FT
W
E1
E2
Anti-CotC
Anti-CotU
CotC-CotC
CotC
Anti-CotE
CotE
B
CotU-His
+
CotE
FT
W
E1
E2
CotU
CotE
C
CotE
FT
W
E1
E2
Anti-CotE
CotE

Figure 5

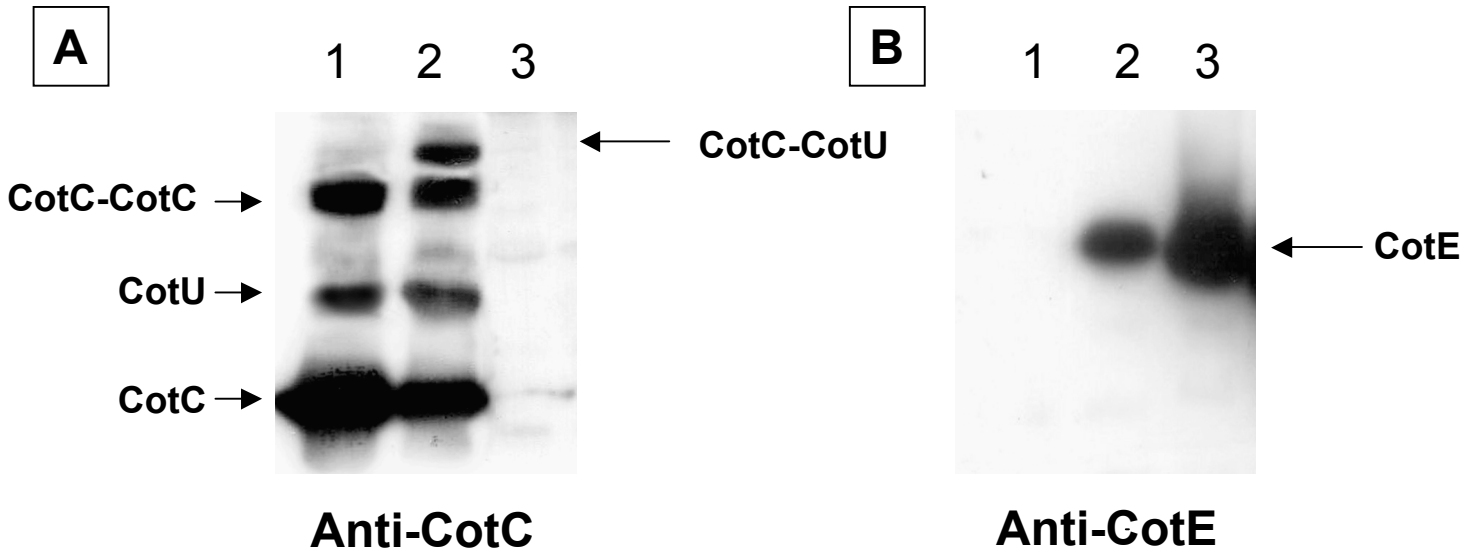
A
1 2 3
CotC-CotC
CotU
CotC
CotC-CotU
Anti-CotC
B
1 2 3
CotE
Anti-CotE

Figure 6