"Federico II" University of Naples

DOCTORATE IN BIOLOGY
(XXIX Cycle)

Analysis of the spore structure in the model species *Bacillus subtilis* and *Bacillus megaterium*

Tutor
Prof. Loredana Baccigalupi

Ph.D student
Saggese Anella

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CHAPTER 1

Introduction

1 Introduction

1.1 Bacterial spore formers

Spore formers are organisms able to form an endospore (spore), a quiescent cell type with a peculiar structure that allows survival at harsh environmental conditions such as starvation, high temperatures, ionizing radiations, mechanical abrasion, chemical solvents, detergents, hydrolytic enzymes, desiccation, pH extremes and antibiotics (Nicholson, *el al*., 2000). These species commonly belong to two genera, *Bacillus* (aerobic) and *Clostridium* (anaerobic). In both cases the process of spore formation (sporulation) is induced by a variety of environmental conditions that limit cell growth and/or block DNA replication and occurs through a series of generally similar morphological changes. However, substantial differences in the regulation of the process and in the structure of the protective layers of the mature spore have been observed between various species (Onyenwoke *et al*., 2004; Paredes *et al*., 2005; De Hoon *et al.*, 2010; Galperin *et al*., 2012). Genomic analysis have shown that a common set of 56 key sporulation genes are present in the genomes of all spore formers while an approximately additional 60 genes are found in all *Bacilli* but are absent in all *Clostridia* (Galperin *et al*., 2012).

1.2 Sporulation

The mechanism of spore formation has been finely characterized in the model species *Bacillus subtilis*. In response to nutritional starvation or a variety of environmental conditions that limit cell growth and/or block DNA replication, the intracellular levels of the master regulator Spo0A-P increase and the sporulation pathway is activated. The first morphological evidence of the induction of sporulation is the formation of an asymmetrically cell division septum that produces a large mother cell and a small forespore (figure 1). The mother cell contributes to the spore development and at the end of sporulation process will lyse releasing the spore in the environment. Soon after the asymmetric cell division, the septum membrane migrates around the forespore, that results surrounded by a double membrane as result of the engulfment process (figure 1). A series of protective layers (cortex, coat, crust and in some species, exosporium) are then synthesized in the mother cell cytoplasm and assembled around the forming spore. The cortex is a peptidoglycan layer chemically different from that of the vegetative cells that is deposited between the two membranes and 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 proteinaceus coat is deposited around the outer surface of the outer membrane. Two major coat layers can be observed by electron microscope analysis: a darkly stained outer coat and a more lightly stained lamellar inner coat. At the end of the development process the mature spore is characterized by a dehydrated cytoplasm containing a condensed and inactive chromosome and surrounded by various protective layers. The final step is the lysis of the mother cell and the release of the formed spore. Because of such a peculiar structure the spore can survive in the absence of water and nutrients and in the presence of unfavourable conditions (extremes of heat and pH, UV radiations, solvents, hydrogen peroxide and lytic enzymes) for very long periods.

When environmental conditions are suitable, the spore can germinate and there by convert back into a growing cell. When this occurs, first the spore core rehydrates and swells and then cortex and coat crack,

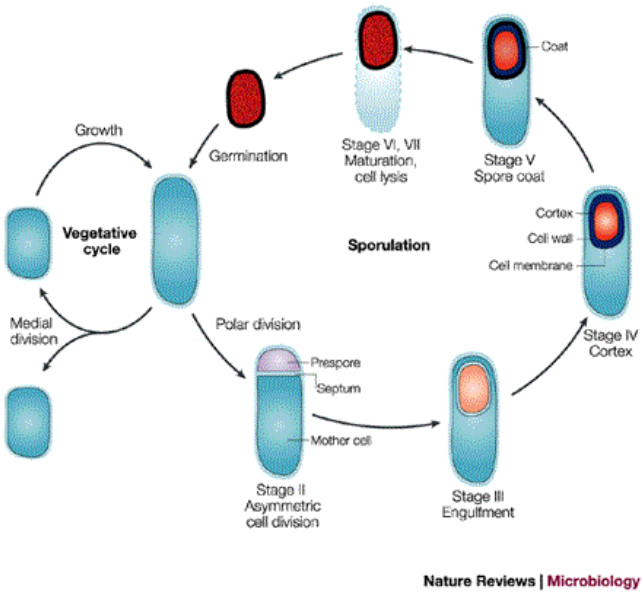

Figure 1: schematic representation of sporulation and germination in *Bacillus subtilis*.

releasing the nascent cell that can eventually, re-sporulate (figure 1). The processes of sporulation and germination have been recently reviewed (Higgins and Dworkin, 2012; Dworkin and Shah, 2010).

1.3 Genetic control of sporulation

The sporulation represents an example of cell differentiation in bacteria because two cells with an identical chromosome, the forespore and the mother cell, follow different gene expression programmes. The main mechanisms responsible for the establishment of cell-specific and time specific gene is due 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 (spo genes), (Losick and Stragier, 1992).

Two of these sigma factors (σ^F and σ^G) are specifically expressed in the forespore and two (σ^E and σ^K) are

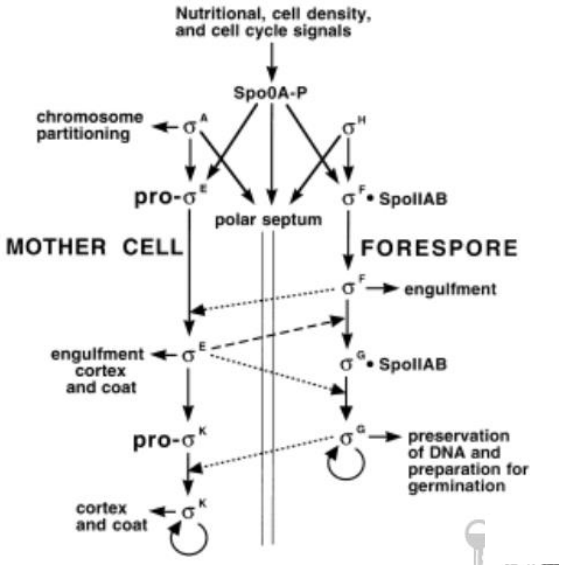

Figure 2: C*riss-cross regulation*. The thin arrows indicate the transcriptional control in the two different cells, while the thick arrows indicate the three checkpoints that coordinate the gene expression of one cell with the orphological changes of the other.

only expressed in the mother cell determining a differentiated gene expression in the two cell compartments. Anyway, the gene expression in one cell is coordinated with the gene expression in the other one. According to the *criss-cross* model (figure 2), σ^F in the prespore is responsible of the activation of the gene coding for σ^G in the same compartment but also of σ^E in the mother cell. Similarly σ^E in the mother cell, is responsible of the expression of σk but also activates σ^G in the prespore that in turn activates the last sigma factor, σ^k in the mother cell. This model, confirmed by several experimental data, ensures the spatially and temporary controlled activation of the four sigma factors that causes the formation of a cascade of gene expression that guides the spore development.

1.4 Germination and outgrowth

The spore is a metabolically inactive cell but remains able to sense the presence of nutrient thanks to the presence of specific sensors recognizing mainly amino acids and sugars, located in the inner membrane (Hornstra *et al*., 2005).

When the environmental conditions became favorable to the vegetative growth, the spore is able to germinate, returning to active growth. The process is called germination and is an irreversible process (Parker *et al*., 1996) that involves a series of morphological and biochemical changes that lead to degradation of the spore layers, cytoplasm rehydration and loss of spore dormancy and resistance. The germination takes few minutes to complete and is followed by the cell enlargement process termed outgrowth. The amino-acids necessary for the protein synthesis during the outgrowth are provided by the degradation of the coat components and of the SASP (Small Acid Soluble Proteins, DNA binding proteins responsible for UV resistance). In laboratory conditions, the germination is efficiently induced by L-alanine or L-asparagine and a mixture of molecules known as GFK (glucose, fructose and KCl). The correct assembly of the coat is important to guarantee proper germination efficiency.

1.5 Spore Structure

The bacterial spore is characterized by a core surrounded by several layer that in sequence are: the inner membrane, the cortex, the outer membrane, the spore coat and the spore crust (Figure 3). The core is the innermost part of the spore. It contains the spore cytoplasm with all cellular components, such as cytoplasmic proteins, ribosomes and DNA associated to a large amount of Small Acid Soluble Proteins (SASPs) which protects the DNA against many types of damage. The core cytoplasm has a water content of only 30 – 50 % instead of the 70 – 88 % of the vegetative cytoplasm (Setlow, 1994). This dehydratated state plays an important role in spore longevity, dormancy and resistance. The core is surrounded by the inner membrane, containing the germination receptors, that in turn is surrounded by the cortex, a modified peptidoglycan layer. The cortex is important for the maintenance of spore core dehydration, resistance and dormancy. The outer membrane, the second membrane layer that derives from the engulfment process and that has opposite polarity with respect to the inner membrane. The most external structure of the spore is the coat, a complex multilayered structure composed of more than 70 proteins, which plays roles in spore resistance, germination, and apparently possesses enzymatic functions that may possibly permit interactions with other organisms in the environment. It consists of two main layers (Figure 3): the inner layer (IC, thick 20 - 40 nm) is formed by the juxtaposition of three to six lamellae

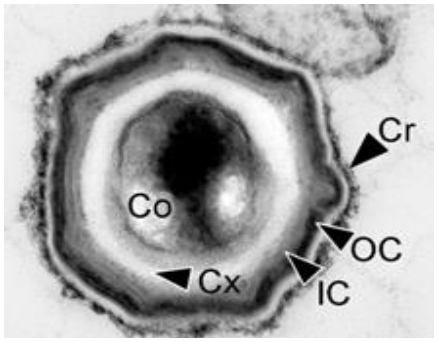

Figure 3: *Bacillus subtilis* spore ultrastructure. The figure shows the spore core (Co), the cortex peptidoglycan layer (Cx), the inner coat (IC) and the outer coat (OC) and the crust (Cr) (McKenney *et al*, 2010).

aligned along the periphery of the spore, and the outer layer (OC, thick 40 - 90 nm) that appears more electrons dense. Another recently characterized layer is the crust (Cr) mainly composed by glycoproteins (McKenney *et al*., 2010).

It has been proposed that the crust could represent a rudimentary exosporium, a layer found in other *Bacillus* species such as *B. antracis* and *B. megaterium* (see figure 3 of chapter 5, pag 76). This glycosylated layer is intimately connected to the rest of the coat and both coat layers are packed closely together and appear thicker at the spore poles and thinner along its sides (Driks, 1999). Till date it was shown that CgeA, CotZ and CotY are major structural components of the crust (Imamura *et al*., 2011).

About the exosporium, some features have been elucidated in the species of the *Bacillus cereus* group, *B.cereus, B. anthracis*, and *B. thuringiensis* (Terry *et al*, 2017; Maes *et al*, 2016). It is composed of an external hair-like nap and a paracrystalline basal layer and it contains approximately 20 different proteins (Steichen *et al*, 2003; Steichen *et al* 2005*;* Redmond *et al.,* 2004), which are deposited around the spore in a progressive encasement process (Qi Peng *et al.* 2016) . The exosporium acts as the outer permeability barrier of the spore and contributes to spore survival and virulence.

1.6 Regulation of coat assembly

Analysis of localization of various coat proteins and their timing of appearance, suggest that the assembly of the 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 proteins; and at the post-translational level, with the involvement of a series of morphogenetic proteins controlling the assembly of other coat components. Moreover a series of post-translational modifications, including phosphorylation and glycosylation, have been reported to occur (Driks *et al*., 1994; Ricca *et al*., 1997).

1.6.1 Transcriptional regulation of cot genes

Coat assembly is mainly a function of the mother cell and covers a period of about 6 hours, beginning with asymmetric division of the sporangial cell. Thus, the expression of genes coding for coat components (*cot* genes) is under the control of the two sigma factors that regulate the mother cell gene expression: σ^E and σ^K; in addiction, three DNA binding proteins, SpoIIID, GerR and GerE, act as transcriptional regulators, activating and/or repressing the gene expression in the mother cell. Due to the action of these transcriptional factors it is possible to identify four classes of *cot* genes (Figure 4). The gene expression program in the mother cell compartment involves the activation of 383 genes that

represent about 9% of *Bacillus subtilis* genome. The first transcription factor acting in the mother cell is σ^E that is responsible of the expression of genes coding for the coat morphogenetic proteins SpoIVA and SpoVID, and for the transcriptional factors SpoIIID and GerR. SpoIIID acts together with σ^E to repress or to activate the gene expression while GerR, seems to work only as a repressor of σ^E –activated genes (Eichenberger *et al*., 2004). After engulfment, σ^K is activated and directs the expression of a large group genes coding for the other coat proteins (Figure 4). The σ^K –controlled regulon is composed of *cotA, cotD, cotF, cotH, cotM, cotP, 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 comprising the genes *cotB, cotC, cotG, cotV, cotW, cotX, cotY,* and *cotZ*. GerE can act as repressor or activator of the expression some σ^K –controlled genes. It down regulates *cotA* and *cotM* and activates *cotD,cotG, 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 (Zhang *et al*. 1999). 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 timing of σ^K activation is altered and the coat components of classes III and IV, are synthesized one hour earlier than in the wild type cells, produce spores impaired in their germination efficiency (Cutting *et al*., 1991; Ricca *et al*., 1992).

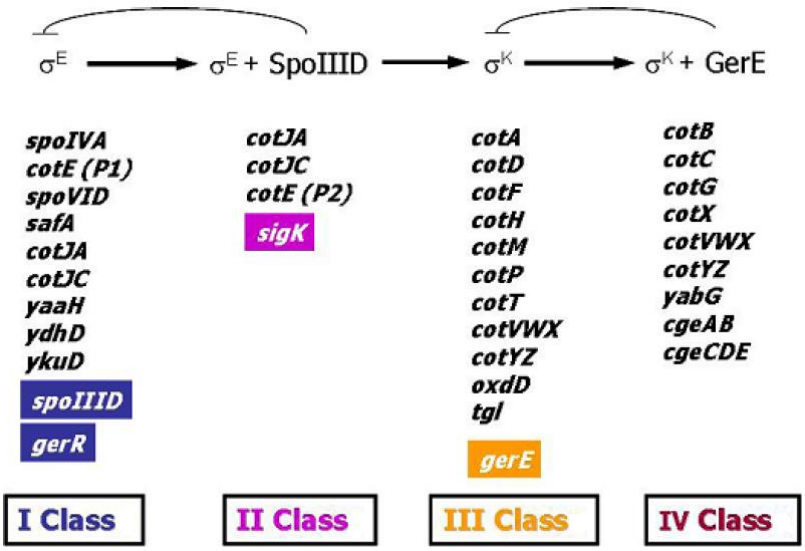

Figure 4: Program of mother cell gene expression

1.6.2 Post-translational regulation of Cot proteins assembly

The coat assembly is also regulated by the action of proteins with morphogenetic activity, i.e. structural coat components that control the assembly of other coat components within the coat layers without influencing their structural gene expression. The most important morphogenetic proteins are: SpoIVA, SpoVID, CotE and 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 near to 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. Studies from Ramamurthi and Losick (2008) have demonstrated that SpoIVA is an ATPase and that this activity allows itself assembly. It has been proposed that two interaction sites are involved in SpoIVA assembly: 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 (below) and as the site of coat attachment (above). Mutants carrying a *spoIVA null* allele, show a coat correctly formed with inner and outer parts 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 coat to the forespore surface from the mother cell side (Driks *et al.*, 1994). SpoIVA is also required for the proper localization of another key 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 proper localization of SpoIVA to the forespore outer membrane is a prerequisite also for the localization of another morphogenetic protein, CotE (Zheng *et al*., 1990). CotE is a 24 kDa protein found in several *Bacillus* species and also in a *Geobacillus* and an *Oceanobacillus* (Henriques and Moran, 2007). Expression of *cotE* relies on two promoters, designed P1 and P2. Transcription from P1 initiates soon after the asymmetric division and is turned off by the repressive action of SpoIIID. Transcription from P2 appears to be under the joined control of σ^E and SpoIIID, but remains active also after the activation of σ^K, to be repressed in the final stages of sporulation by GerE (Costa, *et al.*, 2006). CotE localizes about 75 nm from the forespore outer membrane and then encircles the engulfed forespore. Electron microscope analysis has shown that *cotE* mutants totally lack the outer coat layer (Zheng *et al.*, 1990). The region delimited by the SpoIVA and the CotE rings is referred 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 electron dense outer layer (Figure 5). Assembly of the outer coat involves the cooperation between CotE and an additional morphogenetic protein CotH (Naclerio *et al*., 1996). A 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 the targeting of CotE to the forespore, and the N-terminal domain that, together with the internal domain, directs the formation of CotE homo-multimers (Little and Driks, 2001). Also Krajcikova *et al.* (2009) confirmed CotE multimerization, moreover it was demonstrated that CotE physically interacts with many other spore coat components (Kim *et al*., 2006, Figure 6) and is essential for formation of CotC-CotU hetero-oligomers (Isticato *et al*., 2010).

CotH is a 42.8-kDa protein found in several *Bacillus* and also in some *Clostridium* species (Henriques and Moran, 2007). CotH plays a morphogenetic role in the assembly of at least 9 other coat components: CotB, CotC, CotG, CotS, CotSA, CotQ, CotU, CotZ and YusA (Kim *et al*., 2006, Zilhao *et al.*, 2004) and in the development of the lysozyme resistance and the germination efficiency of the mature spore (Naclerio *et al.*, 1996, Zilhao *et al*., 1999). Moreover, CotH directs deposition of a subset of the CotE-dependent coat proteins and is, itself, at least partially CotE dependent (Naclerio *et al*., 1996; Zilhao *et al*., 1999; Little and Driks, 2001). CotH may function in part in the mother cell cytoplasm, perhaps as a protease inhibitor or as a chaperone (Baccigalupi *et al*., 2004; Isticato *et al.*, 2004), as far as it is required for the stabilization of CotG and CotC. Its important role in protein assembly during late stages of sporulation was observed also in TEM images, which reveal that absence of CotH severely affects spore surface (Figure 7).

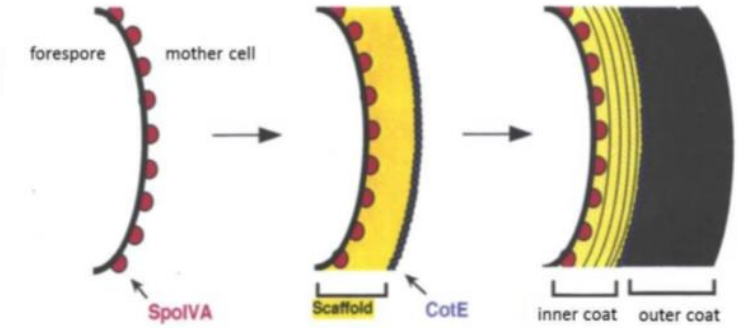

Figure 5: spore coat assembly model

The structural gene coding for CotH is clustered together with *cotG* on the *Bacillus subtilis* chromosome that is divergently transcribed (Naclerio *et al.*, 1996, Giglio *et al*, 2011). CotG is another morphogenetic protein important in late stages of sporulation. It is a 24 kDa protein produced in the mother cell compartment of the sporangium around hour 8 of sporulation under the control of the mother cell specific

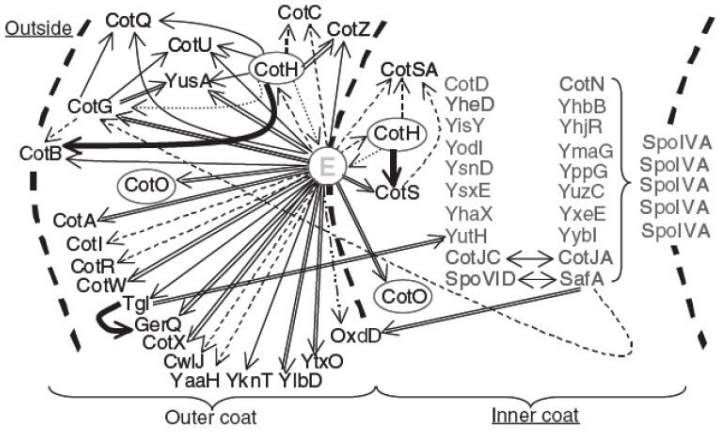

Figure 6: Model of the coat protein interaction network. An arc of the spore coat is indicated; E is CotE. The interior of the spore is to the right of the diagram. The inner and outer coat layers are indicated, as well as the outside of the spore (on the left). Directions of the arrows indicate the assembly dependencies. In some cases, the dependencies are partial. A double line indicates that the dependency was detected by fluorescence microscopy. A thicker line indicates that the dependency was detected by SDS-PAGE, but not by fluorescence microscopy. A dashed line indicates that the dependency was detected by SDS-PAGE. (Kim, *et al.*, 2006).

factor σ^K and of transcriptional regulator GerE. The *cotG* expression is indirectly controlled by another transcriptional regulator GerR, through the activation of SpoVIF, which positively acts on GerE and on GerE-dependent genes (Cangiano *et al.*, 2010). CotG is assembled around the forming spore as two main forms of about 32 and 36 kDa. The 32-kDa form most likely represents the unmodified product of the *cotG* gene (24 kDa) whose abnormal migration may be attributed to its unusual primary structure characterized by the presence of 7 tandem repeats of 7 and 6 amino acids followed by 5 repeats of 7 amino acids (Giglio *et al.*, 2011). It has been proposed that the modular structure of *cotG* is the outcome of several rounds of gene elongation events of an ancestral module (Giglio *et al*., 2011). It is interesting to note that in all CotG-containing *Bacilli* CotG has a modular structure although the number and the length of the repeats differ in the various microorganisms (Giglio *et al*., 2011). The other CotG form of 36-kDa could be due to extensive cross-linking of the protein as it is assembled into the spore coat. That CotG is able to form cross-linked forms has been suggested on the basis of the analysis of the coat structure in *sodA* mutant cells (Henriques *et al.*, 1998). Spores produced by *cotG* mutants are not affected in their resistance to lysozyme or germination properties (Sacco *et al*., 1995). CotG strictly requires *cotH* expression for its assembly and none of the CotG forms is assembled in the coat of *cotH* spores (Naclerio *et al*., 1996). CotG has also a morphogenetic role on the assembly of CotB and controls the conversion of the CotB-46 form into the mature form of 66 kDa (CotB-66) extracted from wild type spores (Zilhao *et al*., 2004). The interactions between various coat components, inferred only on the base of by genetic dependence, form a complex network and are schematically reported in Figure 6 (Kim *et al.*, 2006).

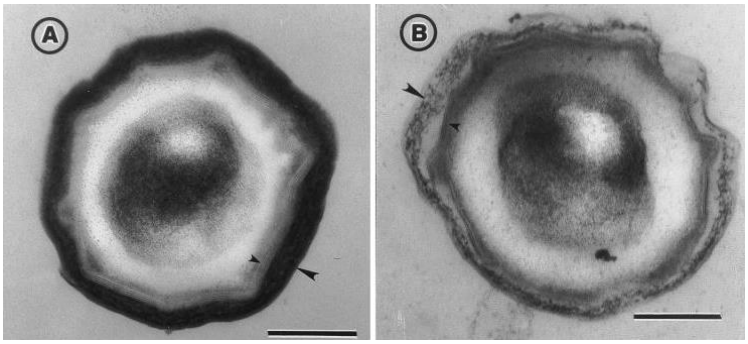

Figure 7: EM analysis of spores purified from wild-type strain (A) and congenic *cotH* deletion mutant (B)

1.7 Bacterial Spores as Biotechnological tool

The bacterial spore has been proposed as a platform to display heterologous proteins, with potential applications ranging from the development of mucosal vaccines to re-usable biocatalysts, diagnostic tools, and bioremediation devices (Knecht *et al.,* 2011; Isticato and Ricca, 2014; Ricca *et al.,* 2014). The remarkable and well documented resistance of the spore (McKenney *et al.,* 2012), the amenability of several spore-forming species to the genetic manipulation (Harwood and Cutting, 1990) and the safety record of several species (Cutting, 2011) support the use of the spore as a display and delivery system. Two strategies have been developed to display heterologous proteins on the spore surface and *Bacillus*

subtilis has been used as model. A recombinant strategy, based on the construction of gene fusions between DNA coding for a selected spore surface protein and DNA coding for the protein to be displayed, has been used over the years to display a variety of heterologous proteins (Isticato and Ricca, 2014). The strains carrying the gene fusions are induced to sporulate and the chimeric proteins formed by a structural component of the spore coat and a heterologous part are naturally assembled on the surface of the forming spore as spore coat components. With this approach a variety of antigens and enzymes have been successfully exposed on the spore surface permitting to propose the recombinant spore as mucosal vaccine or nano catalyzer.

However this promising strategy has as huge drawback the release into nature of the genetically modified microorganisms. Recently, to overcome this obstacle, a non-recombinant approach has been proposed. It is based on the spontaneous adsorption between purified spores and purified proteins and it appears particularly well suited for applications involving the delivery of active molecules to human or animal mucosal surfaces.

The first evidence suggesting that heterologous proteins can be spontaneously adsorbed on the spore surface comes from a study by Yim *et al.* (2009). They over-expressed in *Bacillus subtilis* the gene coding for NADPH-cytochrome P450 reductase (CPR) and induced the cells to sporulate. Purified spores contained the enzyme attached on the surface and showed CPR activity. This result suggested that the spore is able to bind heterologous proteins and that they conserve the proper conformation. Using the beta galactosidase as model enzyme (Sirec T. *et al.* 2012) it has been shown that the binding efficiency is pH-dependent and that the immobilized enzyme is more stable respect to the soluble form (unbound sample). The same non-recombinant approach has been successfully used also to bind antigenic molecules obtaining spores able to induce specific and protective immune responses in mice (Huang *et al.*, 2010).

The molecular mechanism at the base of spores ability to bind external proteins is not well understood. It has been proposed that hydrophobic and electrostatic forces are involved in the process. Experiments performed with the beta galactosidase enzyme indicated that Bacillus *subtilis* spores lacking the outermost structures, the crust and the outer coat layer, have an adsorption efficiency even higher than wild type spores, suggesting that those structures, mainly formed by proteins and glycoproteins, have an inhibitory effect on the adhesion.

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Outline of the Thesis

Outline of the Thesis

This PhD Thesis reports the results of my research work in the laboratory of Prof. Loredana Baccigalupi at the Department of Biology of the Federico II University of Naples, Italy. During these three years I focused on two bacteria of the same genus, *Bacillus subtilis* and *Bacillus megaterium*. These organisms are Gram-positive, aerobic, spore formers and, therefore, share the ability to undergo a complex developmental cell differentiation process and produce highly resistant spores (Tan IS and Ramamurthi KS, 2014). As reported in the Introduction, the process is induced by unfavorable environmental conditions and starts with an asymmetric cell division that produces a large mother cell and a small forespore. The mother cell contributes to forespore maturation and undergoes autolysis at the end of the process, allowing the release of the mature spore into the environment. The peculiar structure of the spore, characterized by a dehydrated cytoplasm surrounded by various protective layers, is responsible of the resistance of the spore to extremes of heat and pH, to UV radiations, and to the presence of solvents, hydrogen peroxide and lytic enzymes. In the presence of water, nutrients and favorable environmental conditions the mature spore can germinate generating a cell able to grow and, eventually, to re-sporulate (Setlow P., 2003).

Spore resistance is in part due to the presence of the spore coat, a multilayered structure composed of more than 70 proteins that surrounds the spore (McKenney *et al*. 2013). The biogenesis of the spore coat is finely regulated at multiple levels as reported in chapter 1 (paragraph 1.6 of the Introduction) and represents an interesting model system to study mechanisms of gene expression and of protein-protein interaction in bacteria.

The spore surface of the two bacterial species I used for my PhD work, substantially differs from each other for the presence around the spore of *B. megaterium* of an exosporium, a poorly characterized outermost spore layer not present around the *B. subtilis* spore. Furthermore, while *B. subtilis* is the model system for spore formers and the surface of its spore is extremely well characterized, not much is known about the surface of the *B. megaterium* spore.

My PhD project focuses on the molecular characterization of the spore surface of these two bacterial species. This Thesis is organized in two parts: the **first part**, Chapters 2-4, focuses on the role of three coat components, CotG, CotH and CotE, in the assembly of the spore coat in *Bacillus subtilis*. The **second part,** Chapters 5 and 6, focuses on the spore coat of *B. megaterium* and on the use of its spore as a platform to display heterologous proteins.

In particular, **CHAPTER 2**, focuses on the coat proteins CotH and CotG. Previous work in prof. Baccigalupi's lab showed that the structural genes coding for these two proteins are adjacent but divergent on the *B. subtilis* chromosome and that *cotG* is entirely contained between the promoter and the coding part of *cotH* (Giglio *et al*., 2011). A consequence of this peculiar gene organization is that all so far characterized knock-out *cotG* mutants were also impaired in *cotH* expression and were then double *cotG cotH* mutants. Therefore, I decided to construct a *cotG* mutant in which *cotH* expression was not affected. The analysis of the single *cotG* mutant and of the *cotG cotH* double mutant showed that CotG negatively affects the efficiency of spore germination and the assembly of at least three outer coat proteins, CotC, CotU and CotS. However, this negative action of CotG is counteracted by CotH suggesting that the two proteins have an antagonistic role.

Data reported in Chapter 2 have been published in 2014:

> **Saggese A.**, Scamardella V., Sirec T., Cangiano G., Isticato R., Pane F., Amoresano A., Ricca E., Baccigalupi L. 2014. *Antagonistic role of CotG and CotH on spore germination and coat formation in Bacillus subtilis.* PLoS ONE 9(8):e104900.

In **CHAPTER 3**, I addressed a different aspect of CotG. This protein has a peculiar primary structure, characterized by a central region formed by positively charged and random coiled tandem repeats. CotG is not widely conserved in spore formers but it is only present in 3 *Bacillus* and 2 *Geobacillus* species (Giglio *et al.*, 2011). I performed a more accurate bioinformatic analysis of the genomes of all entirely sequenced *Bacillus* species and observed that they all encode a CotG-like protein. These proteins do not have a conserved amino acid sequence with respect to CotG of *B. subtilis* but they all share the same structural properties: a modular organization with the central module composed of random coiled repeats of positively charged amino acids. In order to understand the role of these modules, I constructed and analyzed a series of deletion mutants lacking only one or two of the modules of CotG. The analysis of these mutants allowed me to conclude that the external C- and N-terminal modules are sufficient to ensure CotB maturation and that the central module is essential for the CotG negative effect on germination and CotC, CotU and CotS assembly (see Chapter 2). Data reported in Chapter 3 have been published in 2016:

> **Saggese A.**, Isticato R., Cangiano G., Ricca E. and Baccigalupi L. 2016. *CotG of Bacillus subtilis is a modular protein of spore forming Bacilli.* J Bacteriol. 198(10):1513-20.

In **CHAPTER 4** I focused on CotH and on its interaction with a major morphogenetic regulator of the spore coat assembly, CotE (Zheng L. *et al.* 1988). Previous studies have demonstrated that CotE regulates CotH assembly which in turn controls the assembly of at least nine outer coat proteins (Naclerio *et al.*, 1996; Zilhao *et al.*, 1999). I have observed that CotH also controls the assembly of CotE and this mutual dependency is due to a direct interaction between the two proteins. A collection of *cotE* deletion mutants were first used to show that the C terminus of CotE is involved in the interaction with CotH. Then, the C terminus of CotE has been dissected and new mutants constructed to precisely define the amino acids of CotE involved in the interaction with CotH. Data reported in Chapter 4 have been published in 2015:

> Isticato R., Sirec T., Vecchione S., Crispino A., **Saggese A.**, Baccigalupi L., Notomista E., Driks A. and Ricca E. 2015. *The direct interaction between two morphogenetic proteins is essential for spore coat formation in Bacillus subtilis*. PLoS ONE. 10(10):e0141040.

In **CHAPTER 5** I analyzed the spore coat of *B. megaterium* SF185, a strain isolated from ileal biopsy of human volunteers (Fahkry *et al.*, 2008). A spore surface protein was selected for its relative abundance, its N-terminal sequence was determined and the coding gene identified by reverse genetics. To study the function of this protein I constructed a knock out mutant and analyzed the mutant spores by thin section electron microscopy (TEM). Mutant spores presented a normal spore structure but totally lacked the exosporium, therefore suggesting that the abundant protein plays an essential role in exosporium formation. To perform some experiments of this part of my Thesis work I visited for three months the laboratory of Prof. Graham Christie at the Department of Chemical Engineering and Biotechnology of the

University of Cambridge, UK, a leader in the genetic manipulation of *B. megaterium.*
A manuscript reporting these results is in preparation.

In **CHAPTER 6** I used the spores of *B. megaterium* to verify if they can be used as a platform to display heterologous proteins. The *B. subtilis* spore has been extensively characterized as display system by both a recombinant (Isticato and Ricca, 2014) and a non-recombinant approach (Ricca *et al.*, 2014). Cells and spores of *B. megaterium* are significantly larger than those of other species, including the model species *B. subtilis* (Di Luccia *et al.* 2016). In addition, spores of *B. megaterium* are surrounded by an exosporium, a poorly characterized structure composed of proteins and carbohydrates (Di Luccia *et al.* 2016). Aim of this part of my Thesis was to verify whether spores surrounded by an exosporium were able to display heterologous proteins like spores that do not have such additional surface layer (*B. subtilis* spores). To this focus I followed the non-recombinant display approach and used the monomeric form of the Red Fluorescent Protein (mRFP) of the coral *Discosoma* sp. (Campbell *et al*., 2002) as a model heterologous protein. Non-recombinant adsorption of mRFP to spores was monitored by western- and dot-blotting and by fluorescence microscopy. My results showed that *B. megaterium* spores were more efficient than *B. subtilis* spores in tightly adsorbing mRFP with over 100 μg of mRFP adsorbed by each spore. I found that the exosporium of *B. megaterium* is permeable to mRFP molecules that infiltrates through it and fill up the space between the outercoat and the exosporium. These data propose the *B. megaterium* spore as an ideal vehicle to bind and deliver heterologous proteins.
Data reported in Chapter 6 have been published in 2016:

> Lanzilli M., Donadio G., Addevico R., **Saggese A.**, Cangiano G., Baccigalupi L., Christie G., Ricca E. and Isticato R. 2016. *The exosporium of Bacillus megaterium QM B1551 is permeable to the red fluorescence protein of the coral Discosoma sp.* Front. Microbiol. 7:1752.

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CHAPTER 2

Antagonistic role of CotG and CotH on spore germination and coat formation in *Bacillus subtilis*

Anella Saggese[1], Veronica Scamardella[1], Giuseppina Cangiano[1], Rachele Isticato[1], Francesca Pane[2], Angela Amoresano[2], Ezio Ricca[1] and Loredana Baccigalupi[1]

[1]Department of Biology, University of Naples Federico II-MSA-Via Cinthia 26-80126 Naples, Italy

[1]Department of Chemistry, University of Naples Federico II-MSA-Via Cinthia 26-80126 Naples, Italy

Published in August 2014 on *PLoS ONE* 9(8):e104900

2.1 Abstract

Spore formers are bacteria able to survive harsh environmental conditions by differentiating a specialized, highly resistant spore. In *Bacillus subtilis*, the model system for spore formers, the recently discovered crust and the proteinaceous coat are the external layers that surround the spore and contribute to its survival. The coat is formed by about seventy different proteins assembled and organized into three layers by the action of a subset of regulatory proteins, referred to as morphogenetic factors. CotH is a morphogenetic factor needed for the development of spores able to germinate efficiently and involved in the assembly of nine outer coat proteins, including CotG. Here we report that CotG has negative effects on spore germination and on the assembly of at least three outer coat proteins. Such negative action is exerted only in mutants lacking CotH, thus suggesting an antagonistic effect of the two proteins, with CotH counteracting the negative role of CotG.

2.2 Introduction

Spore formers are Gram-positive bacteria belonging to different genera and including more than 1,000 species [1]. The common feature of these organisms is the ability to differentiate a spore, a dormant cell type that can survive for long periods in the absence of water and nutrients and resisting to a vast range of stresses (high temperature, dehydration, absence of nutrients, presence of toxic chemicals) [2]. When the environmental conditions ameliorates the spore germinates originating a cell able to grow and eventually sporulate [3]. Spore resistance to lytic enzymes and toxic chemicals is in part due to the presence of the spore coat, a multilayered structure composed by more than 70 proteins that surrounds the spore [4, 5]. Development of the mature spore is finely controlled through different mechanisms acting at various levels. The synthesis of coat proteins (Cot proteins) is regulated by a cascade of transcription factors controlling the timing of expression of their structural genes (cot genes) while coat assembly is controlled by a subset of Cot protein with a morfogenetic role [5]. Among the morphogenetic proteins, CotH plays a role in the assembly of at least 9 other coat components, including CotG, CotC/U and CotS, [6-9]. In addition, CotH contributes to the formation of spores able to germinate efficiently and to resist to lysozyme treatment [9]. CotH action is strictly connected with that of the major outer coat regulator CotE and mutant spores lacking both CotH and CotE germinate less efficiently and showed an increased sensitivity to lysozyme than single cotE null spores [9]. A recent report has shown that, when over-expressed, CotH bypasses the requirement for CotE, and suggests that CotE acts by localizing CotH on

the spore coat and thus allowing its activity. In the presence of high CotH concentrations, due to the gene over-expression, CotH does not require CotE anymore and is able to drive the assembly of CotH-dependent proteins in a CotE-independent way [10].

The cotH structural gene is clustered with two other cot genes: cotB, transcribed in the same direction, and cotG divergently oriented with respect to cotH. A recent paper [11] has shown that the cotH promoter maps more than 800 bp upstream of its coding region, that this region is not translated and entirely contains the divergently transcribed cotG gene. A direct consequence of this peculiar chromosomal organization is that cotG insertion/deletion mutations so far analyzed [12], should also affect cotH expression leading to double cotG cotH mutants. If this is the case, then, the role of CotG has never been studied in an otherwise wild type strain and induces us to reconsider some previously reported results. Indeed, cotG spores have been previously reported as identical to isogenic wild type spores for both germination efficiency and lysozyme-resistance [12], while cotH spores have been shown to be about 35% less efficient than isogenic wild type spores upon induction of germination [8]. However, if an insertion-deletion within cotG impairs also the expression of cotH [11], those data imply that when both CotG and CotH are both lacking spores germinate normally but when only CotH is lacking spore germination is defective. In order to clarify the role CotG and its interaction with CotH, we first verified that CotH is not produced in a strain with an insertion/deletion mutation in cotG and then constructed for the first time a single cotG null mutant. The phenotypic analysis of the mutant spores is reported.

2.3 Results and Discussion

2.3.1 Construction of a *cotG* mutant

To verify whether a strain with an insertion/deletion mutation in cotG produced CotH, coat proteins extracted from a wild type strain (PY79) and of two isogenic mutants in cotG (ER203) or in cotH (ER220) were compared. As previously reported [8], both mutants have on SDS-PAGE a strongly altered pattern of coat proteins with several minor differences characteristic of the two strains [8] (Fig.1A). A western blot analysis with anti-CotH antibody of the coat proteins of the three strains confirmed that CotH is not produced in a strain with an insertion/deletion mutation in cotG (Fig. 1B).

In order to obtain a cotG null mutation that does not affect cotH transcription, we introduced a single nucleotide in the cotG coding region by gene-soeing [13], thus causing the formation of a stop codon 21 bp downstream of the cotG translation start site (Fig. 2A). The entire cotGstopcotH region was PCR

amplified, cloned into an integrative vector and inserted at the amyE locus on the B. subtilis chromosome of strain AZ603 carrying a deletion of the entire cotG cotH locus, yielding strain AZ604. An identical strategy was followed to PCR amplify, clone, integrate at the amyE locus and transfer into strain AZ603 a wild type copy of the cotG cotH region (AZ608).

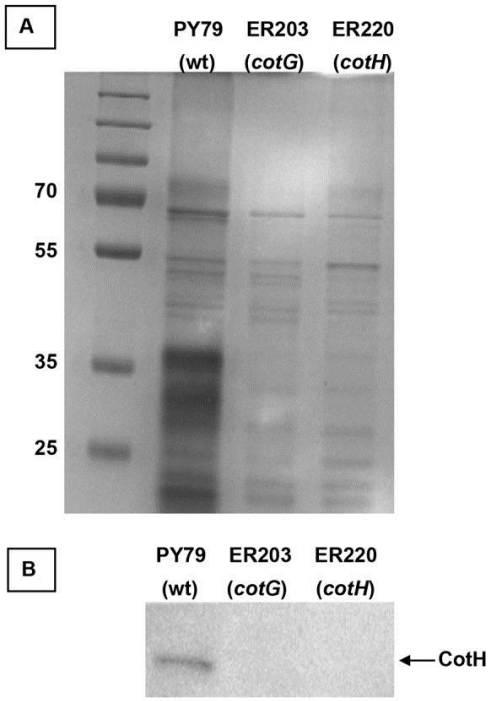

Figure 1: Production of CotH in a cotG null mutant.
(A) SDS-PAGE fractionation of coat proteins from a wild type strain (PY79) and isogenic strains carrying null mutations in *cotG* (ER203) or in *cotH* (ER220). A molecular weight marker is also present and the size of relevant bands indicated. (B) Western blot with anti-CotH antibody of the same three strains analyzed in panel A. The arrow points to the CotH specific band.

To verify the production of CotG and CotH in AZ604 (ΔcotG ΔcotH amyE::cotGstopcotH) and AZ608 (ΔcotG ΔcotH amyE::cotGcotH) western blots with anti-CotG or anti-CotH antibodies were performed. As shown in Fig. 2BC, the ectopic expression of a wild type copy of the cotG cotH region (lane 4 in both panels) in strain AZ603 complemented the deletion of the cotG cotH locus (lanes 2 in both panels). As expected, the ectopic expression of cotGstopcotH in strain AZ603 did not affect CotH production (panel B, lane 3) and did not produce CotG (panel C, lane 3).

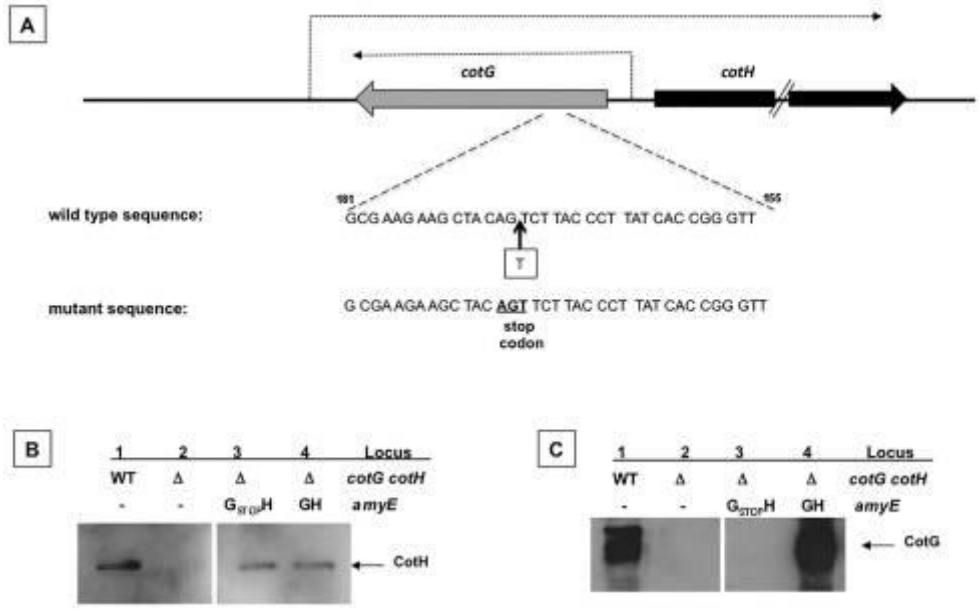

Figure 2: Construction of a single cotG mutant.
(A) Thick gray and black arrows indicate the coding parts of *cotG* and *cotH*, respectively. Dashed arrow indicates the mRNA produced from the *cotG* and *cotH* promoters, as already reported. Site of insertion of the additional base in the *cotG* coding sequence (wild type sequence) that causes the formation of a premature stop codon (mutant sequence). Western blot analysis with anti-CotH (B) and anti-CotG (C) antibodies of proteins extracted by SDS treatment from wild type and isogenic mutant spores. The mutants genotype relative to the *cotG cotH* and *amyE* loci is indicated. Arrows point the CotH and CotG specific bands.

2.3.2 Role of CotG on spore germination and resistance to lysozyme

We used the single *cotG* null mutant strain (AZ604) to analyze the efficiency of germination and the resistance to lysozyme. Together with AZ604 we considered for our analysis spores of three other isogenic strains: a wild type (PY79) containing both CotG and CotH [8, 12], *cotH* null (ER220) containing only CotG [7] and *cotH cotG* null (AZ603) lacking both proteins. As shown in Fig. 3A, AZ604 spores (*cotG*) showed an efficiency of germination identical to that of wild type spores (white and gray circles in the figure). As previously reported [8], spores of the *cotH* null strain were slightly less efficient in germination than wild type spores (white squares in Fig. 3A). With spores of strain AZ603 (*cotG cotH*) the germination efficiency was restored to wild type levels (black squares in Fig. 3A). These results indicate that the germination defect observed with spore lacking only CotH was rescued in spores lacking both CotH and CotG. As a consequence they suggest that the germination impairment is not directly due to the absence of CotH as previously believed [8] but instead to the presence of CotG in a *cotH* null background. This finding also suggest a protective role for CotH in counteracting the CotG negative effect. The same four strains were also used to analyze the spore resistance to lysozyme and were all identical to wild type spores (Fig. 3B).

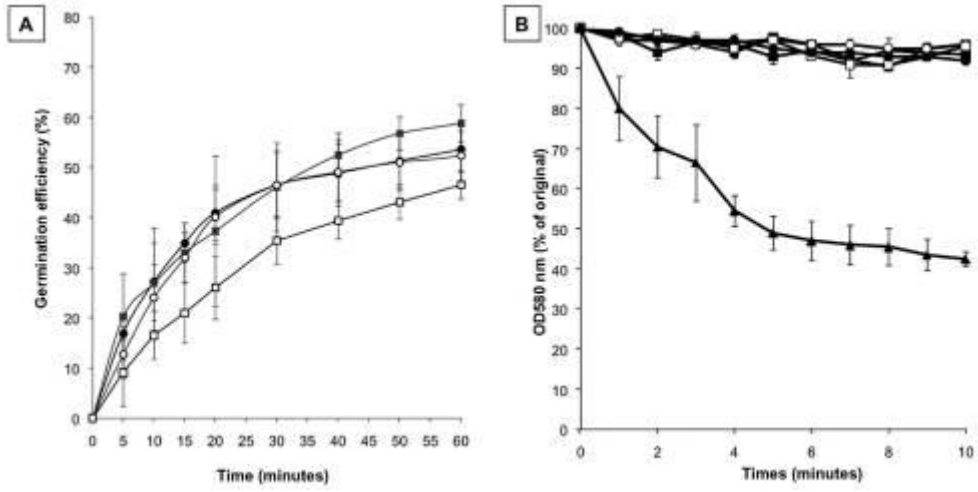

Figure 3: Germination efficiency and lysozyme-resistance assays.
Spores derived from wild type (PY79, black circles), *cotG* null (AZ604, white circles), *cotH* null (ER220, white squares) and *cotGcotH* null (AZ603, black squares) were tested for germination efficiency (A) and for lysozime resistance (B). Germination was induced by Asn-GFK and measured as percentage of loss of optical density at 580 nm. Similar results were obtained by using L-Ala to induce germination. A *cotE* null strain (black triangles) known to be sensitive to lysozyme has been used as positive control during the lysozime treatment. Error bars are based on the standard deviation of 4 independent experiments.

2.3.3 Role of CotG on coat protein assembly

We then analyzed the assembly of various coat proteins in the presence and in the absence of CotG and/or CotH. For our analysis we compared by western blot a wild type strain (PY79) and isogenic strains with an insertion/deletion in cotH (ER220, *cotH::spc*), or deleted of the entire *cotH cotG locus* (AZ603) and expressing either a *wild type* (AZ608) or a *cotG mutant* (AZ604) copy of the *cotH cotG locus*.

As shown in Fig. 4, our analysis confirmed that levels of CotA (a CotH-independent protein) is not affected by CotH and/or CotG and that CotB maturation is dependent on the presence of both CotG and CotH [14]. Indeed, in spores of strains lacking CotG or CotH or both, CotB is assembled within the coat in its immature 43 kDa form. Only when both CotG and CotH are present the mature protein of 66kDa is formed (Fig. 4A).

CotC and CotU are two CotH-dependent proteins that are homologous and recognized by both anti-CotC and anti-CotU antibodies [15]. CotC is present within the spore coat as a monomer (12 kDa), homodimer (21 kDa) and as two additional forms of 12.5 and 30 kDa (16]. CotU is found as a 17 kDa monomer [15] and as a heterodimer with CotC of 23 kDa [17]. As expected, all the CotC/CotU forms are found when both CotG and CotH are present (Fig. 4B, lanes 1 and 3) and none of them is observed when CotH is not

expressed (Fig. 4B, lane 5). However, when both CotH and CotG are lacking (Fig. 4B, lane 2) as well as when only CotG is lacking (Fig. 4B, lane 4) all CotC/CotU proteins are normally assembled on the spore. These data indicate that, as for the germination phenotype, CotG has a negative role on CotC/CotU assembly and that its role is counteracted by CotH.

To confirm the negative effect of CotG in a *cotH background*, we inserted an ectopic copy of *cotG* allele at *amyE locus* in the double *cotGcotH* mutant and also in this case all the CotC/CotU forms are no more assembled in the coat (Fig 4B, lane 6). CotS is 41 kDa, cotH-dependent spore coat protein [18], clearly identified by SDS-PAGE and western blot [19]. As shown in Fig. 5A, a protein absent in the *cotS null mutant* (AZ541, lane 2), is not present in the cotH mutant (ER220, lane 5) but is present in both the single *cotG mutant* (AZ604, lane 4) and in the double *cotH cotG mutant* (AZ603, lane 3). To confirm this SDS-PAGE analysis we constructed a *cotS::gfp* fusion and integrated it on the chromosome of a wild type strain (PY79). By chromosomal DNA-mediated transformation we then moved the fusion into strains AZ603 (*ΔcotG ΔcotH*), AZ604 (*ΔcotG ΔcotH amyE::cotGstopcotH*) and ER220 (*cotH::spc*) and analyzed all resulting strains by fluorescence microscopy.

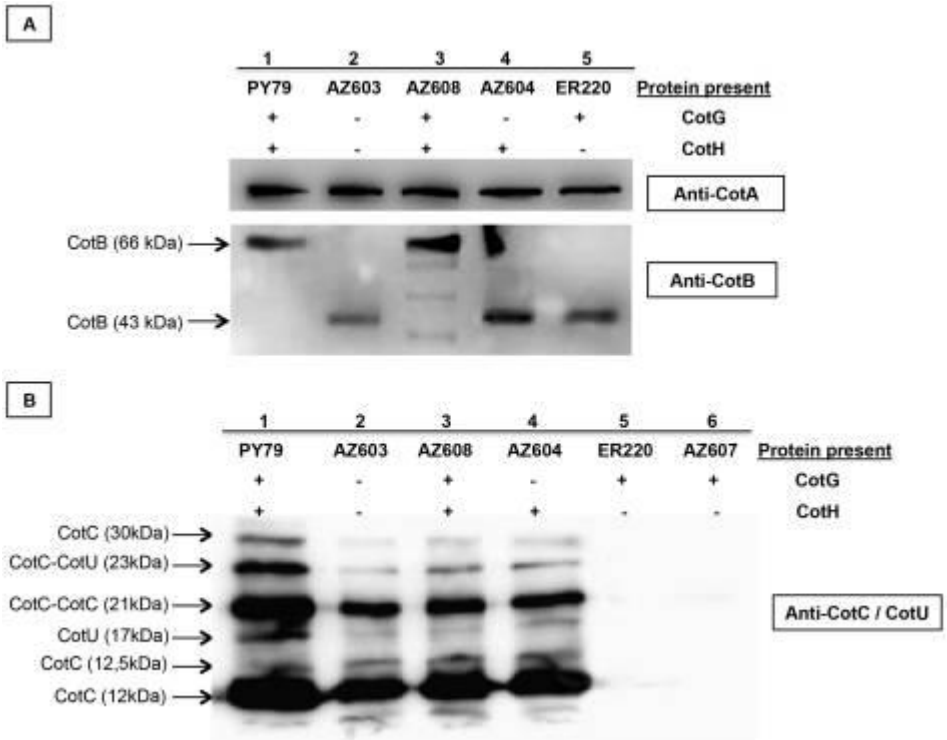

Figure 4: Western blot analysis.
Western blot analysis of proteins extracted from mature spores of wild type (PY79, lane 1), ΔcotGΔcotH (AZ603, lane 2), ΔcotGΔcotH amyE::cotGcotH (AZ608, lane 3), ΔcotGΔcotH amyE::cotGstopcotH (AZ604, lane 4), cotH::spc (ER220, lane 5) and ΔcotGΔcotH amyE::cotG (AZ607, lane 6 of panel B) strains. For CotA and CotB detection (panel A) the proteins have been extracted by SDS treatment while for CotC and CotU detection (panel B) the NaOH treatment has been used. Proteins (25 µg) were reacted with CotA, CotB and CotC specific rabbit antibodies and then with peroxidase-conjugated secondary antibodies and visualized by the Pierce method. The estimated size of CotB, CotC and CotU is indicated.

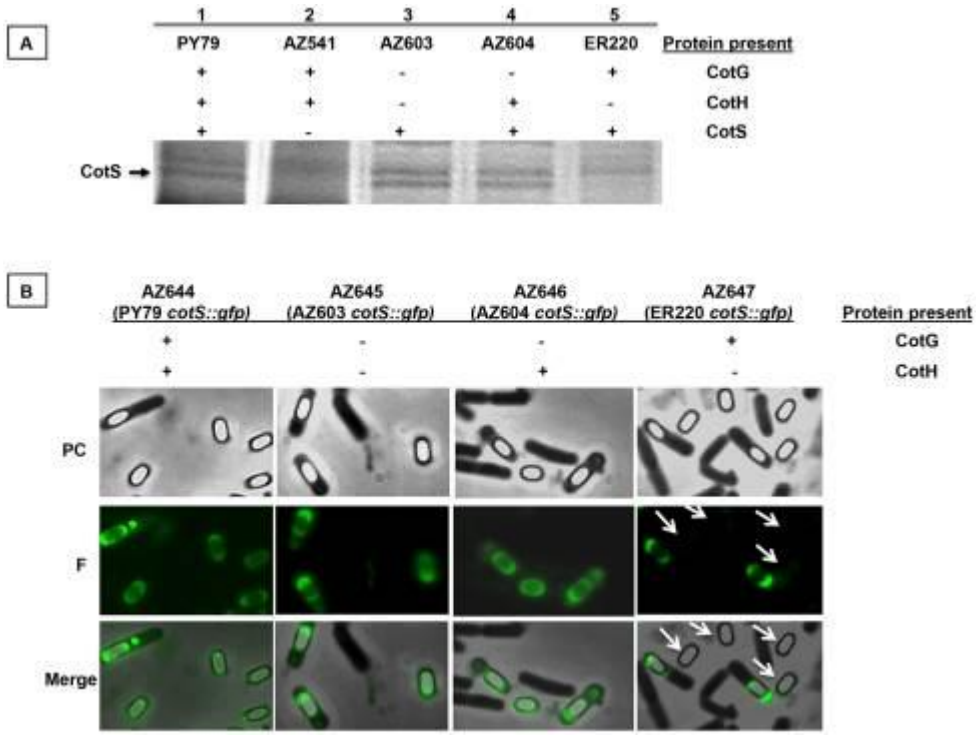

Figure 5: SDS-PAGE and Fluorescence analysis.
(A) Proteins released after treatment with SDS of spores of the indicated strains were fractionated on a 12,5% polyacrilamide gel. The arrow indicates the 41 kDa band correspoding to CotS (18). The gel was stained with Coomassie brilliant blue. (B) Strains carrying the *cotS::gfp* fusion were analyzed by phase-contrast (PC) and fluorescence (F) microscopy. The bottom panel reports a merge of the two images. Exposure time was 588 ms in all cases.

A fluorescence signal was observed around mature and forming spores in a wild type strain and in isogenic strains lacking both CotH and CotG (AZ603) or lacking only CotG (AZ604) (Fig. 5B). However, when CotG is present and CotH is lacking (ER220) [7] a fluorescence signal was observed around forming spores but never around mature, free spores (Fig. 5B). This result is in agreement with the SDS-PAGE of Fig. 5A, performed with proteins extracted from mature spores, and indicates that, also for CotS assembly, CotG has a negative role antagonized by CotH.

2.3.4 On the nature of CotG-CotH interaction

The nature of the antagonistic action of CotH on CotG negative role, suggested by results of Fig. 3, 4 and 5, is not clear. However some hints come from a recent bioinformatic analysis that has identified CotH as a putative kinase [20]. In addition, another previous report has shown that a *B. anthracis* protein with some similarities with CotG of *B. subtilis* is highly phosphorylated [21]. These literature data induced us to hypothesize that CotH is a kinase and CotG one of its substrates. To partially support this hypothesis

we performed a mass spectrometry analysis of CotG. Coat proteins extracted from wild type spores were fractionated on SDS polyacrylamide gel and a region of the gel containing CotG used to reduce, alkylate and digest the proteins *in situ* with trypsin (see Material and Methods). The peptide mixture was divided in two aliquots and submitted to MALDIMS and nanoLCMSMS analyses and then directly analyzed by nanoHPLC-chip MS/MS. Due to the low resolution of the SDS-PAGE, more than one protein was identified in the same region of the gel but CotG exhibited the highest MASCOT score (not shown).

Several phosphorylation sites were identified within CotG, some detected in the MALDIMS runs and some by a manual interpretation of the MS/MS spectra (Table S2). Fig. 6 reports a summary of the phosphorylation sites identified in CotG. The occurrence of phosphorylation sites at level of Ser15, Ser39 and Thr147 was unambiguous and suggests that a kinases belonging to Serine-threonine kinase family is involved in CotG modification. Other phosphorylation sites occurred in amino acid sequences repeated several times within the CotG central region (for example, the tripeptides SYK underlined or SYR double-underlined in Fig. 6), thus impairing the exact localization of the modifications. Although we cannot definitely conclude that all of the underlined and double-underlined tripeptides are phosphorylated, the absence of the same tryptic fragments among the unmodified peptides strongly suggests that most, if not all of them are phosphorylated and that serine, always present in those tripeptides, is the most probable amino acid interested by the post-translational modification.

While Ser15 is in the N-terminal part of CotG, Ser39, Thr147 and all the other possible sites of phosphorylation are located in the repeated central region (Fig. 6 and Table S2). This region is composed by random coiled repeats [11], each containing serine residues surrounded by positively charged amino acids (Fig. 6). In a bioinformatic analysis of known phosphorylated proteins [22] all these features have been indicated as typical of intrinsically disordered structures and have been identified as predictor of phosphorylation substrates.

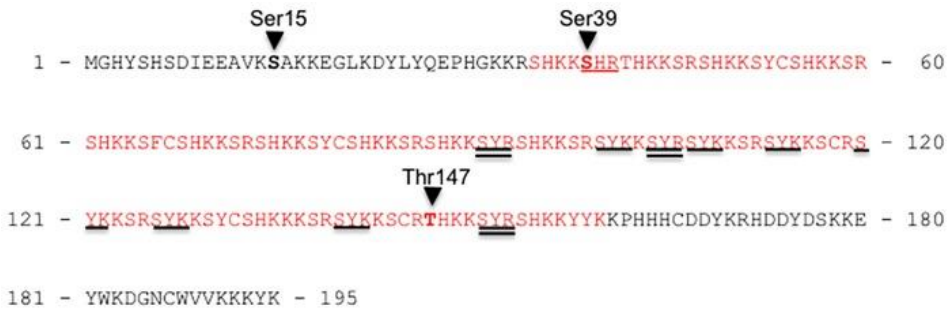

Figure 6: CotG and phosphorylation sites.
Results of a mass spectrometry analysis of peptides derived from trypsine digestion of CotG are reported. Unambiguosly identified sites of phosphorylation are indicated. Tripeptides containing a phosphate moiety are underlined; the random coiled tandem repeats region is in red.

In a *cotH* mutant CotG is not present around both the mature and the forming spore [8] but accumulates in the mother cell compartment of the sporulating cell [7]. However, its peculiar structure has so far impaired CotG isolation from the mother cell compartment of sporulating *B. subtilis* cells as well as from a heterologous host (*E. coli*), therefore not allowing further analysis. Although additional experiments, beyond the aims of this manuscript, will be needed to confirm that CotH is a kinase and CotG one of its substrates, we speculate that in a wild type strain CotG would be mainly present in a phosphorylated form and that, in this form, it plays its structural role as a coat component. In a *cotH* mutant, we predict that CotG would not be phosphorylated and have a negative effect on the assembly of some coat proteins and on spore germination.

2.4 Conclusions

Because of the peculiar chromosomal organization of the *cotG cotH* locus [11], in a *cotG* null mutant also the expression of the *cotH* gene is impaired and, as a consequence, the presumed *cotG* mutant is a double mutant lacking both CotG and CotH. In this work we constructed for the first time a *cotG* null mutant in which CotH is produced. A phenotypic analysis of this mutant has shown that it does not differ significantly from the isogenic wild type strain but has also shown that phenotypes previously attributed to the lack of CotH are only observed when in the *cotH* strain is present CotG. When both CotH and CotG are absent the defects observed in the single *cotH* mutant are completely restored and the double mutant is indistinguishable from the isogenic wild type strain. This is the case of the germination defect of *cotH* spores that is rescued in a *cotG cotH* double mutant; is the case of CotC/U and CotS assembly within the coat. CotG has a peculiar primary structure: it has several repeats in its central part and has a high positive charge (pI 10.26). In a wild type strain CotG is highly phosphorylated and this post-translational modification is probably important to neutralize the positive charges and, consequently to guarantee protein stability and ability to interact with other coat components. The kinase responsible of this modification has not been identified yet. A recent bioinformatic data has indicated that CotH has some homology with eukaryotic Ser-Thr kinases [20] and our results functionally linking CotG to CotH, point to CotH as the kinase responsible of CotG phosphorylation. Future site-directed mutagenesis experiments will be needed to support this hypothesis.

2.5 Methods

2.5.1 Bacterial strains and transformation

B. subtilis strains are listed in Table 1. Plasmid amplification for nucleotide sequencing, subcloning experiments, and transformation of *E. coli* competent cells were performed with *Escherichia coli* strain DH5α [23]. Bacterial strains were transformed by previously described procedures: $CaCl_2$-mediated transformation of *E. coli* competent cells [23] and two-step transformation of *B. subtilis* [24].

Table 1: *Bacillus subtilis* strains used in this study.

Strain	Relevant genotype	Reference
PY79	wild type	[32]
ER220	*cotH::spec*	[8]
AZ541	*cotS::cm*	[33]
AZ603	*ΔcotG ΔcotH::neo*	This work
AZ604	*ΔcotG ΔcotH::neo amyE::cotG$_{stop}$cotH*	This work
AZ608	*ΔcotG ΔcotH::neo amyE::cotGcotH*	This work
AZ607	*ΔcotG ΔcotH::neo amyE::cotG*	This work
AZ644	*cotS::gfp*	This work
AZ645	*ΔcotG ΔcotH::neo cotS::gfp*	This work
AZ646	*ΔcotG ΔcotH::neo amyE::cotG$_{stop}$cotH cotS::gfp*	This work
AZ647	*cotS::gfp cotH::spec*	This work

2.5.2 Genetic and molecular procedures

Isolation of plasmids, restriction digestion and ligation of DNA, were carried out by standard methods [23]. Chromosomal DNA from *B. subtilis* was isolated as described elsewhere [24].

2.5.3 Deletion of the *cotG cotH* locus

The *cotG cotH* locus was entirely deleted and substituted by a neomycin-resistance (*neo*) gene cassette. Chromosomal DNA of strain PY79 was used as a template and oligonucleotide pairs Del3-H18 and H29-B-anti (Table S1) were used to prime the PCR amplification of two DNA fragments of 361 bp and 704 bp, respectively located upstream and downstream of the *cotH* gene. The two DNA fragments were separately cloned in the pBEST501 vector [25] at 5' or 3' ends of the *neo* gene. The resulting plasmid, pVS6, was then linearized by restriction digestion with *Sca*I and used to transform competent cells of the PY79 strain of *B. subtilis*. Replacement of the *cotH cotG* locus on the chromosome with the *neo* gene occurred by double cross-over between homologous DNA sequences originating strain AZ603 (*ΔcotG*

ΔcotH) and was verified by PCR.

2.5.4 Construction of a single *cotG* mutant

The entire *cotH cotG* locus was PCR amplified using oligonucleotides Del5 and H28 (Table S1) to prime the reaction and PY79 chromosomal DNA as a template. The resulting DNA fragment was cloned into plasmid pDG364 [24], yielding plasmid pVS8. To insert a single nucleotide within the cotG coding part (at position +22, considering as +1 the first nucleotide of the first *cotG* codon) we used a *gene soeing* approach [13]. Two partially overlapping DNA fragments were PCR amplified priming the reaction with oligonucleotide pairs Gstop/Del5 (743 bp) and Gstop-anti/H (317 bp) (Table S1) and using chromosomal DNA of PY79 as a template. The obtained PCR products were used as templates to prime a third linear PCR of 7 cycles using only the external primers Del5 and H (Table S1). The single-strand products thus obtained were mixed and used to perform a standard PCR program of 20 cycles that led to their cohesion. The recombinant fragment was cloned in pGemT easy vector (Promega) and controlled by sequencing to confirm the presence of the point mutation resulting in the substitution of the 8th *cotG* codon with a stop codon. The mutant *cotG* allele (here called $cotG_{stop}$) was digested with *Bam*HI-*Bgl*II and cloned into pVS8 to replace the wild type *cotG* allele, yielding plasmid pVS7. Both plasmids pVS7 (carrying the $cotG_{stop}$*cotH* locus) and pVS8 (carrying the wild type *cotG cotH* locus) were separately used to transform competent cells of AZ603 (*ΔcotG ΔcotH*). The occurrence of a single reciprocal (Campbell-like) recombination event between homologous DNA on the plasmids and on the chromosome (*amyE* locus) was verified by PCR.

2.5.5 Ectopic expression of cotG

The entire *cotG* gene was PCR amplified priming the reaction with oligonucleotide pairs G22 and H19 (774 bp), cloned in pGEM-T Easy vector (Promega), controlled by sequencing and transferred into the integrative vector pDG364 [24] using *Eco*RI and *Bam*HI restriction sites.

The plasmid was used to transform the double mutant AZ603 (*ΔcotG ΔcotH*). The occurrence of a single reciprocal (Campbell-like) recombination event between homologous DNA sequences present on the plasmid and on the chromosome (*amyE* locus) was verified by PCR and yielded strain AZ607 (*ΔcotG ΔcotH, amyE::cotG*).

2.5.6 Construction of *cotS::gfp* fusion

The *gfp mut3a* gene, encoding the green fluorescent protein (GFP) [26] was PCR amplified using plasmid pAD123 (Bacillus Genetic Stock Center, BGSC, www.bgsc.org) as a template and priming the reaction with oligonucleotides GFPfor and GFPrev (Table S1). The *gfp mut3a* gene was cloned in pGEM-T Easy vector (Promega), controlled by sequencing and transferred into the integrative vector pER19 [27] using *Pst*I and *Bam*HI restriction sites. The region containing the entire *cotS* gene except the stop codon, was PCR amplified using chromosomal DNA of strain PY79 as a template and priming the reaction with oligonucleotides cotS-for and cotS-rev (table S1), and cloned in frame with *gfp* using the *Sph*I restriction site located at 5' end of *gfp*. The resulting plasmid pcotS-gfp was used to transform competent cells of strain PY79. The occurrence of a single reciprocal (Campbell-like) recombination event between homologous DNA sequences present on the plasmid and on the chromosome (*cotS* locus) yielded strain AZ644 (*cotS::gfp*) was verified by PCR. Chromosomal DNA of strain AZ644 was then used to transfer the *cotS-gfp* fusion into strains AZ603 (*ΔcotG ΔcotH*), AZ604 ($cotG_{stop}$) and ER220 (*cotH::spec*), yielding respectively AZ645 (*ΔcotG ΔccotH cotS::gfp*), AZ646 ($cotG_{stop}$ *cotS::gfp*), AZ647 (*cotH::spec cotS::gfp*). Fluorescence microscopy analysis was performed with an Olympus BX51 fluorescence microscope using a Fluorescein-Isothiocyanate (FITC) filter as previously reported [28]. Typical acquisition times were 588 ms and the Images were captured using a Olympus DP70 digital camera and processed.

2.5.7 Spore purification, extraction of spore coat proteins and western blot analysis

Sporulation was induced by exhaustion by growing cells in DSM (Difco Sporulation Medium) as described elsewhere [24]. After a 30 hours of incubation at 37°C, spores were collected, washed four times, and purified as described by Nicholson and Setlow [29] using overnight incubation in H_2O at 4°C to lyse residual sporangial cells. Spore coat proteins were extracted from a suspension of spores by SDS-dithiothreitol (DTT) [24], or NaOH [29] treatment as previously described. The concentration of extracted proteins was determined by using Bio-Rad DC protein assay kit (Bio-Rad), and 20 µg of total spore coat proteins were fractionated on 12,5% SDS polyacrylamide gels and electrotransferred to nitrocellulose filters (Bio-Rad) for Western blot analysis following standard procedures. CotH-, CotA-, CotC-, CotB- and CotG-specific antibodies were used at a working dilutions of 1:150 for CotH detection and 1:7000 for CotA, CotC, CotB and CotG detection. Then an horseradish peroxidase (HRP)-conjugated

anti-rabbit secondary antibody was used (Santa Cruz). Western blot filters were visualized by the SuperSignal West Pico chemiluminescence (Pierce) method as specified by the manufacturer.

2.5.8 Germination efficiency and lysozyme resistance

Purified spores were heat activated as previously described [24] and diluted in 10 mM Tris-HCl (pH 8.0) buffer containing 1 mM glucose, 1 mM fructose, and 10 mM KCl. After 15 min at 37°C, germination was induced by adding 10 mM L-alanine or 10 mM L-asparagine and the optical density at 580 nm was measured at 5-min intervals for 60 minutes [24].

Sensitivity to lysozyme was measured as described by Zheng *et al.* [30]. Spores were prepared as previously described [24], omitting the lysozyme step and eliminating vegetative cells by heat treatment (10 min at 80°C). Purified spores were then suspended in 10 mM Tris-HCl (pH 7.0) buffer containing lysozyme (50 mg/ml), and the decrease in optical density was monitored at 595 nm at 1-min intervals for 10 min. Spore viability was measured after 30 min as CFU on TY agar plates.

2.5.9 In situ digestion and mass spectral analyses

Protein bands corresponding to CotG were excised from the gel and destained by repetitive washes with 0.1 M NH_4-HCO_3 pH 7.5 and acetonitrile. Samples were then submitted to in situ trypsin digestion and analyzed by MALDI mass spectrometry and LCMSMS as previously described [31]. The acquired MS/MS spectra were transformed in *mzData* (.XML) format and used for protein identification with a licensed version of MASCOT software (www.matrixscience.com) version 2.4.0. Raw data from nanoLC-MS/MS analysis were used to query the NCBInr database NCBInr 20121120 (21,582,400 sequences; 7,401,135,489 residues). Mascot search parameters were: trypsin as enzyme; 3, as allowed number of missed cleavage; carboamidomethyl as fixed modification; oxidation of methionine; phosphorylation of serine/threonine/tyrosine; pyro-Glu N-term Q as variable modifications; 10 ppm MS tolerance and 0.6 Da MS/MS tolerance; peptide charge from +2 to +3. Peptide score threshold provided from MASCOT software to evaluate quality of matches for MS/MS data was 25.Spectra with MASCOT score of < 25 having low quality were rejected.

2.6 Acknowledgements

We thank L. Di Iorio for technical support.

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2.8 Supporting Information

Table S1. Oligonucleotides used in this study

Primer	Sequence 5' - 3'[(a)]	Restriction site	Position of annealing[(b)]
Gstop	GCTTCTTCGATGTCAAGAATGGGAATAG		-175/-147 **(***cotH*)
Gstop-anti	CTATTCCCATTCTTGACATCGAAGAAGC		-147/-175 **(***cotH*)
Del5	ggatccGCCTTTATCGTTAGGAT	BamHI	-884/-867 **(***cotH*)
H	ggatccgcgccggaattcAGCGATATCAATATCCAG	BamHIEcoRI	+126/+144 **(***cotH*)
H28	gcatgcAATTCAATAGCCTAATTGTC	SphI	+1140/+1120 **(***cotH*)
H29	ctgcagGCCGGATGTGATCTGCGAG	PstI	+1019/+1038 **(***cotH*)
B-anti	aagcttCGTCGGCATTATCTACAAGG	HindIII	+1814/+1794 **(***cotH*)
Del3	ggatccCAAATTCTCCGTTCTCC	BamHI	-1145/-1128 **(***cotH*)
H18	ggatccATTTGCCCTGTATTAGATATATG	BamHI	-805/-828 **(***cotH*)
G22	TTCCGTACCTCCGCCGGCAGCC		+610/+632 (*cotG*)
H19	ggatccCCATAATCCTCCTTACAAATT	BamHI	-142/-121 (cotG)
CotS-for	gcatgcATGCCGAACGTATCAATG	SphI	-313/-295 **(***cotS*)
CotS-rev	gcatgcATTCGCCTCCCGATACG	SphI	+883/+900 (*cotS*)
GFP for	ctgcagATGAGTAAAGGAGAAGAAC	PstI	+1/+19 (*gfp*)
GFP rev	ggatccTTATTTGTATAGTTCATCCATGCC	BamHI	+694 /+717 **(***gfp*)

[a] Capital and lowercase letters indicate bases of DNA and of an unpaired tail carrying a restriction site (underlined).
[b] Considering as +1 the first base of the first codon in *cotH*, *cotS*, *cotG* or *gfp* as indicated in brackets.

Table S2. Mass spectral analyses of CotG trypsin digest

Observed m/z value	Post-translational Modification	Aminoacid Position	Sequence	Mass spectral technique
473.61	-	158-160	YYK	MALDI
477.43	phosphorilation	100-102	SYK	MALDI
477.43	phosphorilation	107-109	SYK	MALDI
477.43	phosphorilation	113-115	SYK	MALDI
477.43	phosphorilation	120-122	SYK	MALDI
477.43	phosphorilation	126-128	SYK	MALDI
477.43	phosphorilation	140-142	SYK	MALDI
479.1	phosphorilation	39-41	SHR	MALDI
505.26	phosphorilation	91-93	SYR	MALDI
505.26	phosphorilation	104-106	SYR	MALDI
505.26	phosphorilation	151-153	SYR	MALDI
518.3	-	136-139	KKSR	MALDI
720.24	phosphorilation	98-102	SRSYK	MALDI
720.24	phosphorilation	111-115	SRSYK	MALDI
720.24	phosphorilation	124-128	SRSYK	MALDI
720.24	phosphorilation	138-142	SRSYK	MALDI
893.42	-	64-70	KSFCSHK	MALDI
893.42	-	64-71	SFCSHKK	MALDI
893.42	-	38-44	KSHRTHK	MALDI
893.42	-	39-45	SHRTHKK	MALDI
909.16	-	51-57	KSYCSHK	MALDI
909.16	-	77-83	KSYCSHK	MALDI
909.16	-	129-135	KSYCSHK	MALDI
909.16	-	52-58	SYCSHKK	MALDI
909.16	-	78-84	SYCSHKK	MALDI
909.16	-	130-136	SYCSHKK	MALDI
1007.44	-	172-179	HDDYDSKK	MALDI
1676.42	-	19-32	EGLKDYLYQEPHGK	MALDI
976.45	-	184-191	DGNCWVVK	LCMSMS
1104.54	-	184-192	DGNCWVVKK	LCMSMS
1582.72	-	180-191	EYWKDGNCWVVK	LCMSMS
1675.81	-	19-32	EGLKDYLYQEPHGK	LCMSMS
1675.82	-	19-32	EGLKDYLYQEPHGK	LCMSMS
710.82	-	180-192	EYWKDGNCWVVKK	LCMSMS
1710.82	-	179-191	KEYWKDGNCWVVK	LCMSMS
1803.92	-	18-32	KEGLKDYLYQEPHGK	LCMSMS
1964.88	phosphorilation	2-18	GHYSHSDIEEAVKSAKK	LCMSMS
2170.05	phosphorilation	15-32	SAKKEGLKDYLYQEPHGK	LCMSMS
2571.15	-	172-191	HDDYDSKKEYWKDGNCWVVK	LCMSMS

CHAPTER 3

CotG-Like modular proteins are common among spore-forming Bacilli

Anella Saggese[1], *Rachele Isticato*[1], *Giuseppina Cangiano*[1], *Ezio Ricca*[1] *and Loredana Baccigalupi*[1]

[1]*Department of Biology, University of Naples Federico II-MSA-Via Cinthia 26-80126 Naples, Italy*

Published in April 2016 on *JOURNAL OF BACTERIOLOGY* 198(10):1513-20.

3.1 Abstract

CotG is an abundant protein initially identified as an outer component of the *Bacillus subtilis* spore coat. It has an unusual structure characterized by several repeats of positively charged amino acids that are probably the outcome of multiple rounds of gene elongation events of an ancestral minigene. CotG is not highly conserved and its orthologues are present only in two *Bacillus* and two *Geobacillus* species. In *B. subtilis* CotG is the target of extensive phosphorylation by a still unidentified enzyme and has a role in the assembly of some outer coat proteins. We report now that most spore-forming Bacilli contain a protein not homologous to CotG of *B. subtilis* but sharing a central 'modular' region defined by a pronounced positive charge and random coiled tandem repeats. Conservation of the structural features in most spore-forming Bacilli suggests a relevant role for the CotG-like protein family for the structure and function of the bacterial endospore. To expand our knowledge on the role of CotG we dissected the *B. subtilis* protein by constructing deletion mutants that express specific regions of the protein and observed that they have different roles on the assembly of other coat proteins and on spore germination.

3.1.1 Importance

CotG of *B. subtilis* is not highly conserved in the *Bacillus* genus however, a CotG-like protein with a modular structure and chemical features similar to those of CotG, is common in spore-forming Bacilli, at least when CotH is also present. Conservation of CotG-like features when CotH is present suggests that the two proteins act together and may have a relevant role in the structure and function of the bacterial endospore. Dissection of the modular composition of CotG of *B. subtilis* by constructing mutants that express only some of the modules has allowed a first characterization of CotG modules and will be the basis for a more detailed functional analysis.

3.2 Introduction

Bacillus subtilis is the model system for the study of endospore-forming bacteria. The processes of spore formation and germination and also endospore (spore) structure have all been studied in detail in *B. subtilis* and then confirmed in other species. Spore formation starts when cell growth is restricted by nutrient starvation or other harsh environmental conditions (1, 2). The first morphological step of spore formation is asymmetric cell division that produces a large mother cell and a small forespore. The mother cell contributes to forespore maturation and undergoes autolysis at the end of the process, allowing the release of the mature spore (1, 2). The spore is stable and resistant to conditions that would be lethal for most other cells. Spores survive for extended periods of time in the absence of water and nutrients, in extremes of heat and pH, and in the presence of UV radiation, as well as after exposure to solvents, hydrogen peroxide, and lytic enzymes (3, 4). The spore is, however, able to sense the environment and respond to the presence of water and nutrients, generating a vegetative cell that is able to grow and, eventually, resporulate (5).

The resistance properties of the spore are due to its unusual structure. The dehydrated cytoplasm, containing a copy of the chromosome, is surrounded by a series of protective layers. A peptidoglycan

cortex is the first shell, the encasement layer, and is surrounded by a multilayered protein coat and finally a thin crust (6). The coat and crust are together composed of at least 70 different proteins and glycoproteins. Several coat and crust proteins have been identified and characterized, but little is known about the identities of the sugars present on the spore surface. It is, however, known that their presence on the spore surface modulates the relative hydrophobicity of the spore (7,- 9).

Coat formation is finely controlled by a variety of mechanisms acting at various levels. The synthesis of coat proteins is regulated by at least two mother cell-specific sigma subunits of RNA polymerase and at least three additional transcriptional regulators. These transcription factors act in a temporal sequence, controlling the expression of the coat structural genes (*cot* genes) in the mother cell (6). At least some coat proteins are subject to posttranslational maturation events, including proteolytic cleavage, cross-linking, phosphorylation, and glycosylation reactions (10). An important subset of coat proteins, referred to as morphogenetic proteins, controls the assembly of other coat components and the formation of coat and crust layers (11,–15). The subset of morphogenetic coat proteins includes major members such as SpoIVA, SafA, SpoVID, and CotE, which are needed to drive coat and crust formation, and minor members responsible for the maturation or assembly of some other coat proteins (6, 10).

CotG belongs to the second group of morphogenetic proteins. It was initially characterized as an abundant component of the outer coat layer (16) needed, together with CotH, for the maturation of another outer coat protein, CotB (16, 17). CotG is a 195-amino-acid protein characterized by a central region of 126 residues formed by positively charged and randomly coiled tandem repeats. It has been proposed that the repeats are the outcome of multiple rounds of gene elongation events in an ancestral minigene (18). CotG is not widely conserved in spore formers, and its homologues can be found in only two *Bacillus* and two *Geobacillus* species (10, 18). The structural gene coding for CotG is also unusual in that it is entirely contained between the promoter and the coding part of another gene, *cotH*, also coding for a morphogenetic coat protein (18, 19). More recently, CotG was identified as the target of extensive phosphorylation by a still unidentified enzyme (20). In that same study, it was also observed that CotG has a negative effect on the assembly of at least three coat proteins: CotC, CotU, and CotS (20). This negative effect is counteracted by CotH, and strains lacking both CotG and CotH assemble CotC, CotU, and CotS like the isogenic wild-type strain. When CotH is absent, CotG plays its negative role, and CotC, CotU, and CotS are not assembled (20). A model showing the interactions among these Cot proteins is presented in Fig. 1.

In the present work, we have analyzed the genomes of the entirely sequenced *Bacillus* species and observed that, in most cases, they encode a CotG-like protein. These proteins do not have a conserved amino acid sequence with respect to CotG of *B. subtilis*, but they all share the same structural properties, and at least some of them share the unusual chromosomal organization of the *cotG-cotH* locus.

To gain insight into the role of such modular structures, we constructed and analyzed a series of deletion mutants expressing discrete modules of *B. subtilis* CotG, providing the first functional analysis of this novel class of modular morphogenetic proteins.

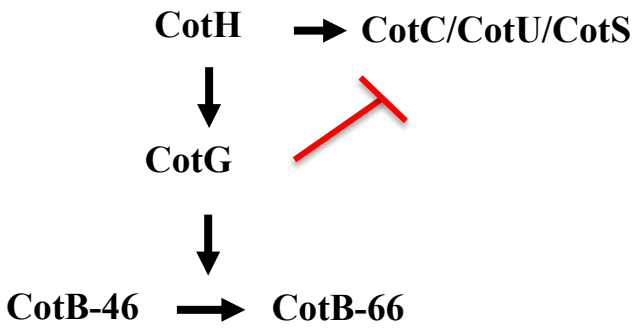

Figure 1: Model of the CotH-dependent protein interaction network. Shown is a working model of the interactions among the indicated proteins. CotH has a positive effect on the assembly of CotG, CotC, CotU, and CotS (arrows). CotG in turn controls CotB maturation from the immature 46-kDa CotB form to the mature 66-kDa CotB form. The gray line indicates the negative effect of CotG on CotC, CotU, and CotS assembly (17, 20, 30).

3.3 MATERIALS AND METHODS

3.3.1 Bioinformatic analysis

Orthologues of CotH were identified by a BLAST analysis (http://blast.ncbi.nlm.nih.gov/) using the CotH sequence of *Bacillus subtilis* strain 168 (GenBank accession number NP_391487.1) as a query against *Bacillales* (taxonomic ID 1385). Completely sequenced *Bacillus* species containing a CotH orthologue (sharing a minimum of 40% identity with respect to the query sequence) were considered for analysis of the gene upstream of *cotH*. A list of species with completely sequenced genomes is available in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (http://www.genome.jp/kegg/catalog/org_list.html) and was used for genomic analysis.

3.3.2 Bacterial strains and transformation

B. subtilis PY79 was used as the recipient strain for transformation procedures. Plasmid amplification for nucleotide sequencing, subcloning experiments, and transformation of *Escherichia coli* competent cells was performed with *Escherichia coli* strain DH5α (21). Transformation was performed by using previously described procedures: CaCl2-mediated transformation of *E. coli* (20) and two-step transformation of *B. subtilis* competent cells (22).

3.3.3 Genetic and molecular procedures

Isolation of plasmids, restriction digestion, and ligation of DNA were carried out by using standard methods (21). Chromosomal DNA from *B. subtilis* was isolated as described previously (22).

Construction of a *cotG* internal deletion mutant.

DNA coding for the internal repeats of *cotG* was deleted by using the gene splicing by overlap extension (gene SOEing) technique (23). Briefly, two partially overlapping DNA fragments were obtained with oligonucleotide couples H19/Gsoe1 (to amplify the promoter and 5′ coding part of *cotG*) and X2/Gsoe2 (to amplify the 3′ coding region of *cotG* and its transcription terminator). The obtained PCR products

were separately used as the templates for a linear PCR of 7 cycles using only the respective external primers H19 and X2, thus obtaining single-stranded fragments partially overlapping at their 3′ regions. The single-stranded products were mixed and used to perform a standard PCR program of 20 cycles, which led to their fusion. The recombinant fragment was cloned into the pGEM-T Easy vector (Promega) and sequenced to confirm the correct gene fusion. The modified version of *cotG* (here called *cotG*Δ) was then moved by BamHI-EcoRI digestion into the pDG1731 integrative vector commonly used to integrate cloned genes at the *thrC* locus of the *B. subtilis* chromosome. The resultant plasmid, pVS11, was linearized with ScaI and used to transform AZ603 (Δ*cotG* Δ*cotH*) (20) competent cells to generate strain AZ612, which carried a double-crossover recombination event at the nonessential *thrC* gene on the *B. subtilis* chromosome and carried the *cotG*Δ allele in a *cotH* background. Chromosomal DNA of *B. subtilis* AZ612 (Δ*cotG* Δ*cotH thrC*::*cotG*Δ) was used to transform competent cells of the AZ604 mutant (Δ*cotG* Δ*cotH amyE*::*cotG*stop-*cotH*), thus generating strain AZ613 expressing the deleted *cotG*Δ gene in the presence of wild-type *cotH* (Δ*cotG* Δ*cotH amyE*::*cotG*stop *cotH thrC*::*cotG*Δ).

To construct the *cotG*-Nterm and *cotG*-Cterm genes expressing only the N-terminal and C-terminal regions of CotG, respectively, the same gene SOEing procedure was used. Oligonucleotide couples H19/Gsoe3 and X2/Gsoe4 were used to amplify the 5′ region of *cotG* (promoter plus the N-terminal coding sequence) and the transcription terminator, and oligonucleotide couples H19/Gsoe5 and X2/Gsoe6 were used to amplify the *cotG* promoter and the 3′ region of *cotG* (C-terminal coding region plus the terminator). By using the same procedure as the one described above, we obtained recombinant genes expressing the N-terminal or the C-terminal region of CotG by PCR. The genes were subcloned into the pGEM-T Easy vector, confirmed by DNA sequencing, and then cloned into the BamHI/EcoRI sites of pDG1731, yielding plasmids pVS13 (containing *cotG*-Nterm) and pVS12 (containing *cotG*-Cterm). Both plasmids were separately used to transform competent cells of *B. subtilis* strain AZ603 (Δ*cotG* Δ*cotH*). The double-crossover recombination event at the *thrC* locus originated from strains AZ616 (Δ*cotG* Δ*cotH thrC*::*cotG*-Nterm) and AZ614 (Δ*cotG* Δ*cotH thrC*::*cotG*-Cterm), expressing the CotG-Nterm and CotG-Cterm forms, respectively, in a *cotH* background. Chromosomal DNA of these strains was used to transform competent cells of AZ604 to obtain AZ617 (Δ*cotG* Δ*cotH amyE*::*cotG*stop *cotH thrC*::*cotG*-Nterm) and AZ615 (Δ*cotG* Δ*cotH amyE*::*cotG*stop *cotH thrC*::*cotG*-Cterm), expressing the CotG-Nterm and CotG-Cterm forms, respectively, in the presence of *cotH*.

Construction of strains expressing the *cotS*::*gfp* fusion.

Chromosomal DNA of strain AZ644 (*cotS*::*gfp*) (19) was used to transfer the *cotS-gfp* fusion into strains AZ612 (Δ*cotG* Δ*cotH thrC*::*cotG*Δ), AZ614 (Δ*cotG* Δ*cotH thrC*::*cotG*-Cterm), and AZ616 (Δ*cotG* Δ*cotH thrC*::*cotG*-Nterm), yielding AZ649, AZ660, and AZ661, respectively.

3.3.4 Transcriptional analysis

Total RNA was extracted from *Bacillus licheniformis* ATCC 14580 and *Bacillus cereus* ATCC 10987 5 h after the onset of sporulation by using an RNeasy Plus minikit (Qiagen, Milan, Italy) according to the manufacturer's instructions. Total RNAs were dissolved in 50 µl of RNase-free water and stored at −80°C. The final concentration and quality of the RNA samples were estimated 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 reverse transcription-PCR (RT-PCR) analysis, samples containing 2 μg of DNase-treated RNAs of *B. licheniformis* and *B. cereus* were incubated with oligonucleotide H1 (H1-lich and H1-cereus, respectively) at 65°C for 5 min and slowly cooled to room temperature (RT) to allow primer annealing. RNAs were then retrotranscribed by incubating the mixture at 50°C for 1 h in the presence of 1 μl AffinityScript multitemperature reverse transcriptase (Stratagene), 4 mM deoxynucleoside triphosphates (dNTPs), 1× reaction buffer (Stratagene), and 10 mM dithiothreitol (DTT). The enzyme was then inactivated at 70°C for 15 min. The cDNA was PCR amplified with oligonucleotide H1 (H1-lich or H1-cereus) coupled with H2 (H2-lich or H2-cereus), annealing in the *cotH* coding region, and H3 (H3-lich or H3-cereus), annealing in the *cotH* 5′ region, at the end of the divergent gene. As a control, PCRs were carried out with RNA not subjected to reverse transcription to exclude the possibility that the amplification products were derived from contaminating genomic DNA.

Spore purification, extraction of spore coat proteins, and Western blot analysis.

Sporulation was induced by exhaustion by growing cells in DSM (Difco sporulation medium) as described previously (22). After 30 h of incubation at 37°C, spores were collected, washed four times, and incubated overnight in H2O at 4°C to lyse residual sporangial cells, as described previously by Nicholson and Setlow (24). Spore coat proteins were extracted from a suspension of spores by SDS-DTT (22) or NaOH (24) treatment as described previously. The concentration of extracted proteins was determined by using a Bio-Rad DC protein assay kit (Bio-Rad), and 20 μg of total spore coat proteins was fractionated on 12.5% SDS-polyacrylamide gels. The proteins were then electrotransferred onto a nitrocellulose filter (Bio-Rad) for Western blot analysis according to standard procedures. CotC- and CotB-specific antibodies were used at working dilutions of 1:7,000. The horseradish peroxidase (HRP)-conjugated anti-rabbit antibody was used (Santa Cruz) as a secondary antibody. Western blot filters were visualized by using the SuperSignal West Pico chemiluminescence (Pierce) method as specified by the manufacturer.

3.3.5 Fluorescence microscopy

CotS assembly was monitored by fluorescence microscopy using an Olympus BX51 fluorescence microscope with a fluorescein isothiocyanate (FITC) filter. Samples expressing the *cotS*::*gfp* fusion and containing both sporangia and mature spores were obtained after overnight incubation in DSM in order to induce sporulation. Exposure times were 588 ms, and the images were captured by using an Olympus DP70 digital camera.

Germination efficiency.

Purified spores were heat activated (20 min at 70°C) and diluted in 10 mM Tris-HCl (pH 8.0) buffer containing 1 mM glucose, 1 mM fructose, and 10 mM KCl (GFK). After 15 min at 37°C, germination was induced by adding 10 mM asparagine to the mixture, and the optical density at 600 nm was measured at 5-min intervals for 60 min (22).

3.4 RESULTS AND DISCUSSION

3.4.1 The CotG-like protein family

In *B. subtilis*, the *cotG* and *cotH* genes are adjacent on the chromosome and divergently transcribed and encode two spore coat proteins: CotH, conserved in several *Bacillus* and *Clostridium* species, and CotG, found in only three *Bacillus* and two *Geobacillus* species (10, 18). In all three CotG-containing bacilli, *B. subtilis*, *B. amyloliquefaciens*, and *B. atrophaeus*, the chromosomal organization of the *cotG-cotH* locus is conserved, with the two genes being adjacent and divergent and with *cotG* being entirely contained between the promoter and the coding region of *cotH* (18). We have now expanded and updated the analysis of the *cotH-cotG* locus in the chromosomes of all completely sequenced *Bacillus* genomes listed in the KEGG database (http://www.genome.jp/kegg/catalog/org_list.html), selecting one representative strain for each species. We observed that 24 out of 35 entirely sequenced *Bacillus* species contain a CotH orthologue. Only 7 of the CotH-containing species also contain a CotG orthologue, and in all cases, *cotG* is adjacent and divergently transcribed with respect to *cotH* (see strains 1 to 7 in Table S1 in the supplemental material). The analysis of the 17 other CotH-containing species indicated that 15 of them have an open reading frame (ORF) adjacent and divergent with respect to *cotH* (see strains 8 to 22 in Table S1 in the supplemental material). The product of the divergent ORF is known to code for exosporium protein B (ExsB) in *B. anthracis* (25), while in all other species, it codes for a protein of unknown function. Although not homologous to CotG, these proteins share some peculiar structural features with CotG. The most striking of these features is the presence of a central region consisting of several repeats (Fig. 2). In various species, the numbers and amino acid sequences of such repeats are highly variable (Fig. 2). However, all these putative proteins share a list of common traits, as summarized in Table 1: (i) a high isoelectric point (pI), ranging from 9.28 for *B. anthracis* to 12.95 for *B. megaterium*; (ii) an elevated percentage of positively charged amino acids, ranging from 22.5% for *Bacillus* sp. JS to 44.6% for *B. pumilus*; and (iii) an elevated percentage of serine and threonine residues, ranging from 9.1% for *B. licheniformis* to 39% for *B. megaterium*. The only exception is the sequence found in *Bacillus* sp. 1NLA3E, which has a low percentage of positively charged amino acids and, as a consequence, a low pI. Our bioinformatic analysis also predicted that these putative proteins contain many potential phosphorylation sites (Table 1) and that most of them have an unfoldability index of <0 and an instability index of >40 (Table 1). These two parameters are indicative of intrinsically disordered proteins (IDPs), characterized by a nonordered, three-dimensional structure that can adopt a fixed structure after binding to a ligand or to another protein (26).

Although these proteins have different amino acid sequences and therefore do not share significant homologies with CotG of *B. subtilis* or among themselves, based on their chromosomal location and their structural features, we refer to them here as CotG-like proteins.

In B. subtilis and in two CotG-containing *Bacillus* species analyzed previously (18), cotG is entirely contained between the promoter and the coding region of cotH. An RT-PCR approach was used to verify whether the same situation also occurred in two of the other strains considered in this study and available at the BGSC (*Bacillus Genetic Stock Center*), *B. cereus* ATCC 10987 and *B. licheniformis* ATCC 14580.

Figure 2: Repeats of CotG and CotG-like proteins. The tandem repeats of CotG of *B. subtilis* (18) and of CotG-like proteins found in the indicated species are shown. *B. subtilis* CotG modules are boxed. Positively charged amino acids are in red.

Species (strain, protein identification)[d]	No. of residues	pI	% positively charged residues	% Ser-Thr residues	No. (%) of phosphorylated Thr or Ser residues[a]	Unfoldability index[b]	Instability index[c]
B. subtilis (168, BSU36070)	195	10.26	47.2	21.0	28 (68)	−0.716	77.91
B. anthracis (Sterne, BAS1898)	186	9.28	29.6	13.4	8 (33)	−0.174	62.30
B. cereus (ATCC 10987, BCE_2114)	180	9.40	27.8	14.4	6 (26)	−0.167	58.53
B. cytotoxicus (NVH39198, Bcer98_1534)	182	9.73	35.1	12.6	9 (39)	−0.351	44.37
B. licheniformis (ATCC 14580, BL01346)	168	9.62	39.8	9.1	2 (13)	−0.536	26.21
B. megaterium (QM B1551, BMQ_1411)	195	12.95	43.0	39.0	50 (66)	−0.829	201.18
B. pumilus (SAFR032, ND)	216	10.95	44.5	21.0	40 (83)	−0.760	90.75
B. thuringiensis (BMB171, BMB171_C1814)	186	9.33	29.6	13.9	11 (42)	−0.175	61.96
B. weihenstephanensis (KBAB4, BcerKBAB4_1895)	196	9.62	31.9	15.1	8 (27)	−0.218	58.65
B. mycoides (219298, DJ92_4596)	233	10.22	35.6	20,2	24 (51)	−0.288	56.40
B toyonensis (ND, Btoyo_4601)	193	9.59	32.1	16.06	10 (32)	−0.225	65.58
Bacillus sp. JS (ND, MY9_3666)	154	10.66	22.5	18.8	7 (24)	+0.024	14.92
Bacillus sp. 1NLA3E (ND, B1NLA3E_12550)	220	6.36	1.8	35.4	1 (1)	+0.340	86.25
B. smithii (ND, BSM4216_1708)	220	12.43	34.1	35.9	65 (76)	−0.613	183.13
B. bombysepticus (ND, CY96_09240)	192	9.64	31.2	15.1	10 (34)	−0.208	63.86
Bacillus sp. strain YP1 (ND, QF06_16450)	159	12.16	22.6	20.1	6 (18)	−0.002	5.79

Table 1: Features of CotG-like proteins in Bacillus spp

[a]Phosphorylation prediction was done by using the NetPhosBac 1.0 server (http://www.cbs.dtu.dk/services/NetPhosBac-1.0/). For each Ser and Thr residue, a score between 0 and 1 was calculated. When the score is ≥0.5, the residue is predicted to be a phosphorylation site.

[b]Disorder prediction was done by using Foldindex (http://bip.weizmann.ac.il/fldbin/findex). Positive values indicate a structured polypeptide, whereas negative values indicate a disordered protein.

[c]The instability index was determined by using ProtParam (http://web.expasy.org/protparam/). Values of ≥40 predict that the protein is unstable.

[d]Protein identification is the protein code in the KEGG database. ND, not determined (the strain name is not available in the KEGG list or the protein is not available [the divergent ORF upstream of *cotH* is not annotated in the NCBI database]).

Total RNA was extracted from sporulating cells of both species 5 h after the beginning of sporulation and used as a template to produce a specific cDNA with synthetic oligonucleotide H1 (see Table S2 in the supplemental material), mapping in the cotH coding region (Fig. 3A).

The cDNA was then PCR amplified with oligonucleotide H1 coupled with H2 (see Table S2 in the supplemental material), annealing in the *cotH* coding region, and H3 (see Table S2), annealing in the *cotH* upstream region, at the end of the divergent gene (Fig. 3A). In both organisms, we observed an amplification product of the expected size (Fig. 3B and andC)C) with both oligonucleotides pairs (H1/H2 and H1/H3), indicating that, as in *B. subtilis*, *cotH* is transcribed from a distal promoter and that the *cotG*-like gene is located between the *cotH* promoter and its coding region. The conservation of the unusual transcriptional organization also found in these two species supports the idea that these proteins of unknown function may have a role similar to that of *B. subtilis* CotG and may be considered a protein family typical of *Bacillus* species.

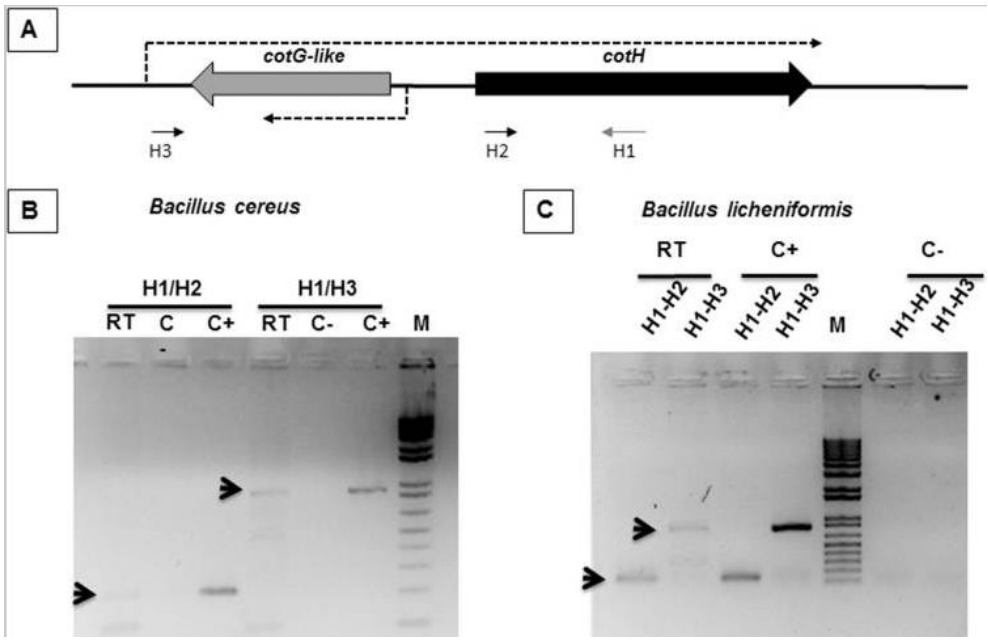

Figure 3: *cotH* transcription in *B. cereus* and *B. licheniformis*. (A) Schematic representation of the *cotH-cotG* locus. Arrows indicate the positions of the synthetic oligonucleotides used for RT-PCR. Dashed arrows indicate the direction of transcription. (B and C) Reverse transcription reactions were performed by using total RNA from sporulating cells of *B. cereus* (B) or *B. licheniformis* (C) as a template and were primed with oligonucleotide H1. Amplification reactions were performed by using cDNA as the template and oligonucleotide pair H1/H2 or H1/H3, as indicated. Negative controls (C−) and positive controls (C+) were RNA samples treated and not treated with DNase, respectively. Arrows indicate the amplification products of the expected size, and M indicates the molecular weight marker.

3.4.2 Construction of cotG mutant alleles

The presence of positively charged repeats in the central region of all CotG-like proteins and of C- and N-terminal regions prompted us to investigate the function of such modules. To address this question, we used B. subtilis to construct three cotG deletion mutants (Fig. 4) either lacking all central repeats (cotGΔ) or expressing only the N-terminal part (cotG-Nterm) or only the C-terminal region (cotG-Cterm) of CotG.

Each mutant allele of cotG was independently integrated at the thrC locus on the B. subtilis chromosome of parental strain PY79 and then moved by chromosomal DNA-mediated transformation into strain AZ604 (ΔcotG-ΔcotH amyE::cotGstop cotH) not expressing the wild-type copy of cotG (20). It is known that CotG action is mediated by CotH when it exerts either its positive role on CotB maturation or its negative role on CotC, CotU, and CotS assembly and on spore germination (20). Therefore, by using chromosomal DNA-mediated transformation, we moved all mutant cotG alleles in strain AZ603 (ΔcotG-ΔcotH) also lacking the cotH gene (20). Strains in both mutant backgrounds are indicated in Fig. 4 and are described in Table S3 in the supplemental material. Spores of all strains indicated in Fig. 4 were then analyzed to assess the role of the various CotG modules in the assembly of some CotG-controlled proteins and in spore germination.

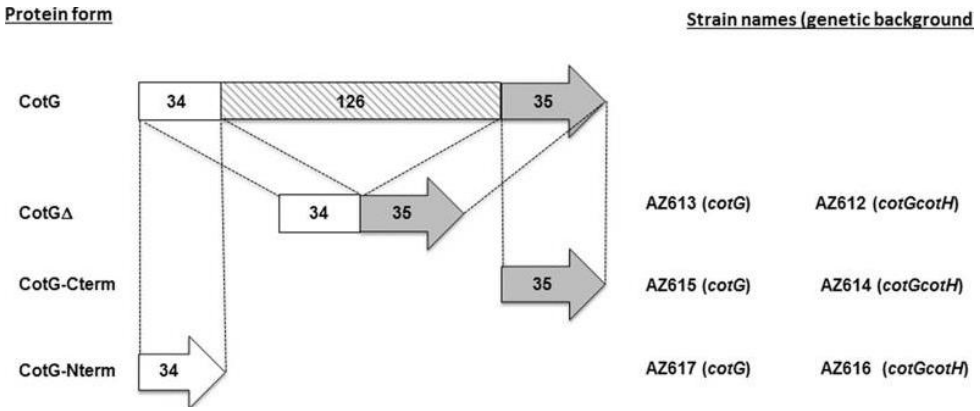

Figure 4: CotG versions. The wild-type protein (CotG) is represented as being composed of three domains: the N- and C-terminal regions of 34 and 35 amino acids, respectively, and a central domain of 126 amino acids organized into tandem repeats (18). CotGΔ lacks the central domain, and CotG-Cterm and CotG-Nterm contain only the C- and N-terminal domains, respectively. All constructs were integrated into the chromosomes of strains carrying either a null mutation in *cotG* or a double-null mutation in *cotG* and *cotH* (indicated in parentheses). The names of strains carrying the different *cotG* alleles in the two genetic backgrounds are shown.

3.4.3 The C- and N-terminal modules of CotG are essential for CotB maturation

CotB is extracted from wild-type spores in its 66-kDa mature form (17). CotB maturation from the 46-kDa form requires the presence of both CotH and CotG (17, 20). The Western blot shown in Fig. 5A confirms that without CotG (lane 2) or CotH (lane 3), only the immature CotB form is assembled onto the spore, showing that all three mutant versions of CotG allowed CotB maturation in a CotH-dependent way. Therefore, all three versions of CotG were able to cooperate with CotH and convert the 46-kDa CotB form (CotB-46) into CotB-66. However, CotGΔ was clearly more efficient than CotG-Cterm or CotG-Nterm and was able to convert all CotB-46 molecules into CotB-66 to the same extent as that found for the wild type (Fig. 5A). CotG-Cterm or CotG-Nterm was able to produce some CotB-66, but the major part of the CotB molecules were in the 46-kDa form (Fig. 5A). Based on this, we speculate that both the C- and N-terminal regions of CotG cooperate with CotH and act somehow synergistically when both are present. The results shown in Fig. 5A also suggest that the central repeats of CotG are not required for CotB maturation.

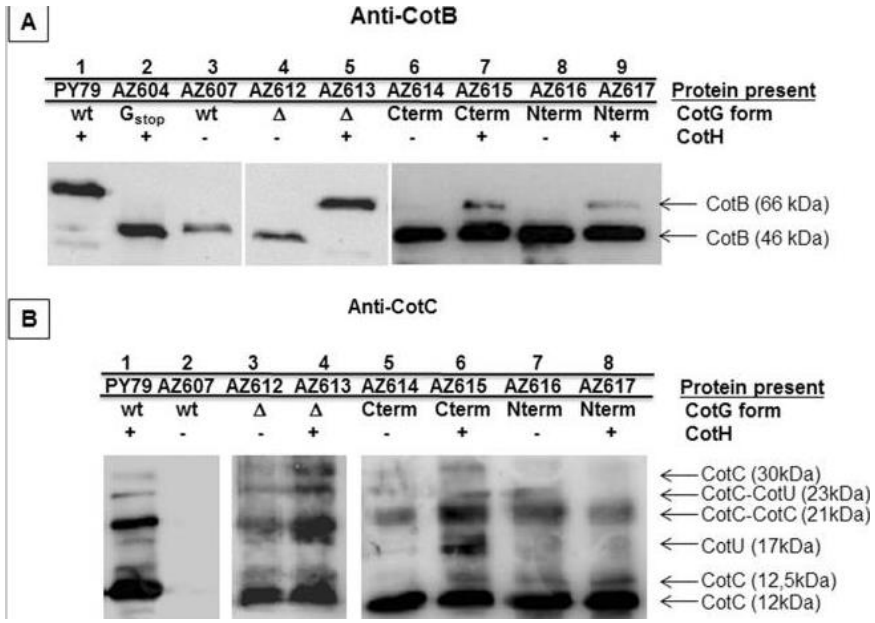

Figure 5: Effects of CotG on CotB, CotC, and CotU assembly. Western blot analysis of coat proteins extracted from purified spores of the indicated strains was performed. Proteins were fractionated on 15% SDS-PAGE gels, electrotransferred onto a membrane, and incubated with anti-CotB (A) and anti-CotC (B) antibodies. The type of CotG allele expressed in each strain (CotG form) in the presence (+) or in the absence (−) of CotH is also indicated. wt, wild type.

3.4.4 The internal repeats are responsible for the negative effect of CotG on the assembly of CotC/CotU and CotS.

CotC and CotU share significant homologies and are both recognized by anti-CotC and anti-CotU antibodies (27, 28). They are assembled in several forms, including a CotC homodimer of 21 kDa and a CotC-CotU heterodimer of 23 kDa (29). It was recently reported (20) that CotH counteracts a not-understood negative role played by CotG on CotC and CotU assembly on the spore. In mutant strains lacking only CotG or both CotG and CotH, all CotC/CotU forms are normally present and assembled, but when CotG is present and CotH is not present, all forms of CotC and CotU are not found around the forming spore or in the mother cell cytoplasm (Fig. 5B, lanes 1 and 2) (20). As shown in Fig. 5B, none of the three mutant forms of CotG, CotGΔ, CotG-Cterm, and CotG-Nterm, had a negative effect on CotC or CotU, which were normally assembled around the forming spore, independently from the presence of CotH. This suggests that the internal repeats of CotG are directly involved in the negative role of CotG in CotC/CotU assembly. CotS is a 41-kDa protein whose assembly is negatively controlled by CotG when CotH is not present (20). A *cotS*::*gfp* fusion (19) was used to monitor the effects of CotG modules on CotS assembly. As shown in Fig. 6, only the wild-type version of CotG had a negative effect on CotS that was not assembled on mature spores. Instead, a normal CotS-dependent fluorescence signal was observed in mature spores as well as in sporulating cells of strains expressing each of the mutant CotG forms (CotGΔ, CotG-Cterm, and CotG-Nterm). As in the case of CotC/CotU, this suggests that the internal repeats of CotG are responsible for the negative role of CotG in CotS assembly. Taken together, the results shown in Fig. 5 and 6 indicate that (i) the N-terminal and C-terminal modules of CotG are

responsible for the positive effect of CotG on CotB maturation, acting synergistically, and (ii) the internal repeats of CotG are responsible for the negative effects of CotG on the assembly of CotC, CotU, and CotS.

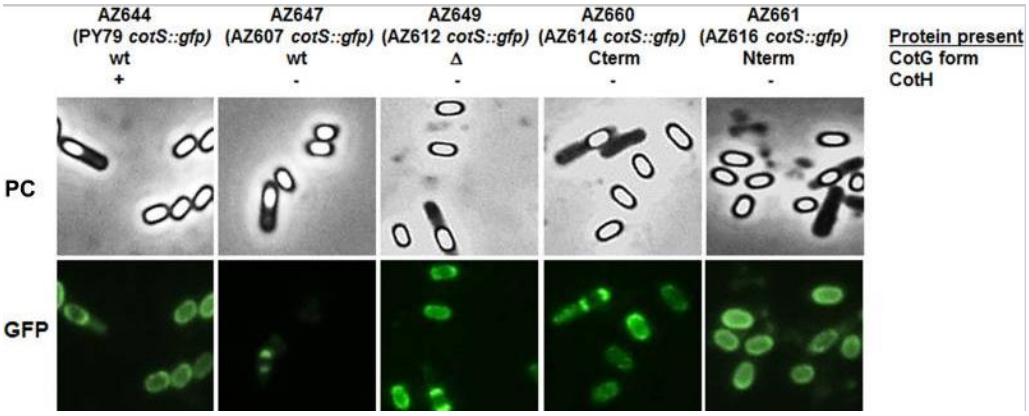

Figure 6: Effects of CotG on CotS assembly. A *cotS*::*gfp* fusion was introduced into a wild-type strain (PY79) and into *cotH* strains expressing wild-type CotG (AZ607), CotGΔ (AZ612), CotG-Cterm (AZ614), and CotG-Nterm (AZ616). Representative fields using phase-contrast microscopy (PC) and fluorescence microscopy (green fluorescent protein [GFP]) are shown. The exposure time was 588 ms in all cases.

3.4.5 The internal repeats are responsible for the negative effect of CotG on the efficiency of germination.

It was previously reported that a *cotH*-null mutation causes a small defect in the efficiency of spore germination in response to asparagine (19). More recently, we showed that such a defect is actually due not to the lack of CotH but rather to the presence of CotG that, in the absence of CotH, exerts its negative effect (20). Indeed, mutant spores lacking only CotG or both CotG and CotH have a germination efficiency similar to that of an isogenic wild-type strain (20). To verify whether the internal repeats are also involved in this negative effect of CotG, we analyzed the germination efficiency of strains expressing the wild-type and mutant *cotG* alleles in a *cotH* background. As shown in Fig. 7, the only strain showing a defect in the efficiency of spore germination was the strain expressing the wild-type allele of *cotG*. The efficiency of germination of spores of all other strains was identical to that of wild-type spores (Fig. 7, dashed line). The results shown in Fig. 7 then indicate that the internal repeats of

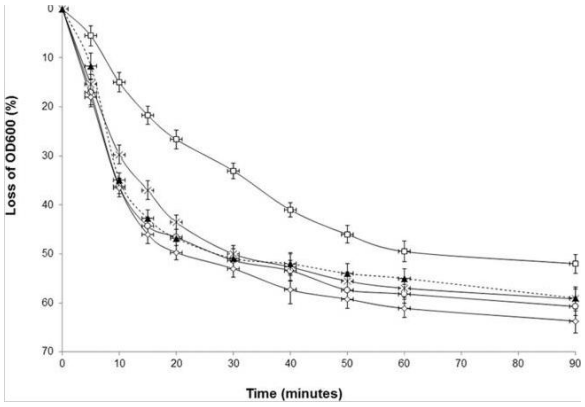

Figure 7: **Effects of CotG on germination efficiency**. Spores derived from strains expressing wild-type CotG (AZ607) (squares), CotGΔ (AZ612) (crosses), CotG-Cterm (AZ614) (circles), and CotG-Nterm (AZ616) (diamonds) in a *cotH* background were tested for germination efficiency and compared with those of a wild-type strain (PY79) (dashed line). Germination was induced by Asn-GFK and measured as the percent loss of the optical density at 600 nm. Error bars are based on the standard deviations of data from four independent experiments. OD600, optical density at 600 nm.

CotG are involved in the negative effect of this protein on spore germination.

3.5 Conclusions

CotG of *B. subtilis* is not highly conserved in the *Bacillus* genus; however, a CotG-like protein with a modular structure and chemical features similar to those of CotG is common in spore-forming bacilli, at least when *cotH* is also present. The conservation of CotG-like proteins in almost all species containing a CotH orthologue suggests that the two proteins act together and may have a relevant role in the structure and function of the *Bacillus* spore. To address the function of the various modules of CotG of *B. subtilis*, we have constructed mutants expressing CotG-deleted forms lacking the central modular region (CotGΔ) or expressing only the N- or the C-terminal part of CotG. Analysis of the various mutants allowed us to propose that the N- and C-terminal modules are able to both interact with CotH and mediate CotB maturation and that this interaction is synergistic. The central part of CotG, containing the repeats of positively charged amino acids, is not involved in CotB maturation but is instead responsible for the negative effect of CotG on the assembly of at least three coat components, CotC, CotU, and CotS, and on the efficiency of spore germination. CotH counteracts this negative effect, ensuring the correct assembly of the spore coat. Our findings indicate that CotG and CotH are functionally linked to each other and support the idea that the entire *cotH-cotG* locus and not only *cotH* has been conserved during the evolution of spore-forming bacilli.

3.6 Acknowledgements

We thank L. Di Iorio for technical support.

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3.7 Supplemental material:

Table S1: Analysis of *cotH* locus in *Bacillus* species with completely sequenced genomes

Species[a]	CotH orthologue[b]	CotG orthologue[b]	Divergent gene[c]	Tax ID[d]	Accession number[e]
1. *B. subtilis spizizenii*	+	+	+	655816	NC_014479.1
2. *B. amyloliquefaciens*	+	+	+	326423	NC_014551.1
3. *B. atropheus*	+	+	+	720555	NC_014639.1
4. *B. methylotroficus*	+	+	+	326423	NC_009725.1
5. *B. sp. Pc3*	+	+	+	1446792	CP010406.1
6. *B. sp. BH072*	+	+	+	1570330	CP009938.1
7. *B. sp. BS34A*	+	+	+	1639654	LN680001.1
8. *B. licheniformis*	+	-	+	279010	NC_006270.3
9. *B. anthracis*	+	-	+	1392	NC_005945.1
10. *B. cereus*	+	-	+	396343	NC_003909.8
11. *B. thuringiensis*	+	-	+	714359	NC_014171.1
12. *B. cytotoxicus*	+	-	+	315749	NC_009674.1
13. *B. weihenstephanensis*	+	-	+	315730	NC_010184.1
14. *B. toyonensis*	+	-	+	155322	NC_022781.1
15. *B. mycoides*	+	-	+	1405	CP007626.1
16. *B. pumilus*	+	-	+	536229	NC_009848.1
17. *B. megaterium*	+	-	+	545693	NC_014019.1
18. *B. smithii*	+	-	+	1479	CP012024.1
19. *B. bombysepticus*	+	-	+	658666	CP007512.1
20. *B. sp.JS*	+	-	+	1127744	NC_017743.1
21. *B. sp. 1NLA3E*	+	-	+	666686	NC_021171.1
22. *B. sp. YP1*	+	-	+	1574141	CP010014.1
23. *B. sp. WP8*	+	-	-	756828	CP010075.1
24. *B. sp. LM 4-2*	+	-	-	1628753	CP011101.1
25. *B. sp. X1*	-	ND	ND	498382	CP008855.1
26. *B. sp. OxB-1*	-	ND	ND	98228	AP013294.1
27. *B. halodurans*	-	ND	ND	86665	NC_002570.2
28. *B. clausii*	-	ND	ND	79880	NC_006582
29. *B. pseudofirmus*	-	ND	ND	79885	NC_013791.2
30. *B. cellulolyticus*	-	ND	ND	1413	CP002394.1
31. *B. coagulans*	-	ND	ND	1121088	CP009709.1
32. *B. infantis*	-	ND	ND	324767	NC_022524.1
33. *B. iehensis*	-	ND	ND	300825	CP003923.1
34. *B. methanolicus*	-	ND	ND	1471	CP007739.1
35. *B. endophyticus*	-	ND	ND	135735	CP011974.1

[a] List of Bacillus species whose genomes have been completely sequenced (source KEGG, Kyoto Encyclopedia of Genes and Genomes database http://www.genome.jp/kegg/catalog/org_list.html);
[b] presence (+) or absence (-) of a protein sharing a minimum of 40% sequence identity respect to CotH and CotG of B. subtilis 168
[c] presence (+) or absence (-) of an adjacent and divergent orf respect to cotH; [d] Taxonomy ID from NCBI (http://www.ncbi.nlm.nih.gov/taxonomy) e accession numbers of the respective genomic sequences
ND, not determined

Table S2: Oligonucleotides used in this study

Primer	Sequence 5' - 3'	Restriction site	Position of annealing[c]
X_2	gaatccGCCTTTATCGTTAGGAT[a]	EcoRI	-891/-874 (*cotH*)
H_{19}	ggatccCCATAATCCTCCTTACAAATT[a]	BamH1	-142/-121 (*cotG*)
Gsoe-1:	GGTGGTGCGGTTTGCGTTTTTTTCCATGAGGCTCTTGG[a]		+77/+102 (*cotG*)
Gsoe-2:	catggaaaaaaacgcAAACCGCACCACCACTGCG[b]		+480/+499 (*cotG*)
Gsoe-3:	CAATGAAAACTTAGATTAGCGTTTTTTTCCATGAGGCTCTTGG[b]		+77/+102 (*cotG*)
Gsoe-4:	tcatggaaaaaaacgcTAATCTAAGTTTTCATTGTTTCAAT[b]		+585/610 (*cotG*)
Gsoe-5:	GTGGTGGTGCGGTTTCAATTTGAAATCCTCCTTTTAAAAATATG[b]		-26/+3 (*cotG*)
Gsoe-6:	ggaggatttcaaattgAAACCGCACCACCACTGCGACGAC[b]		+480/504 (*cotG*)
H1-lich	GTATGACACTGCGACAGG		+126/+144 (*cotH*)
H2-lich	TGATGATGAGTGCCACTGCC		-5/+15 (*cotH*)
H3-lich	GGCTCACCATGCAGCAAGCC		-699/-680 (*cotH*)
H1-cereus	CGGCTTGTAAAACATAACATG		+171/+192 (*cotH*)
H2- cereus	GAAGAGAACTGAGAAGGGATG		-27/-7 (*cotH*)
H3- cereus	GTATGAAAAACTAGCAATTGC		-733/-712 (*cotH*)

[a] Capital and lowercase letters indicate bases of DNA and of an unpaired tail carrying a restriction site, respectively.
[b] lowercase letters in Gsoe2, Gsoe4, Gsoe6 indicate the sequences complementary to the 5' region (underlined) of oligonucleotides Gsoe1, Gsoe3, Gsoe5, respectively
[c] Considering as +1 the first base of the first codon in cotH or cotG as indicated in brackets.

Table S3: *Bacillus subtilis* strains used in this study

Strain	Relevant genotype	Reference
PY79	Wild type	[Youngman et al, 1984]
AZ603	*ΔcotG ΔcotH::neo*	[Saggese et al., 2014]
AZ604	*ΔcotG ΔcotH::neo amyE::cotGstop,cotH*	[Saggese et al., 2014]
AZ607	*ΔcotG ΔcotH::neo amyE::cotG*	[Saggese et al., 2014]
AZ612	*ΔcotG ΔcotH::neo thrC::cotGΔ*	This work
AZ613	*ΔcotG ΔcotH::neo amyE::cotGstop,cotH thrC::cotGΔ*	This work
AZ614	*ΔcotG ΔcotH::neo thrC::cotG-Cterm*	This work
AZ615	*ΔcotG ΔcotH::neo amyE::cotGstop,cotH thrC::cotG-Cterm*	This work
AZ616	*ΔcotG ΔcotH::neo thrC::cotG-Nterm*	This work
AZ617	*ΔcotG ΔcotH::neo amyE::cotGstop,cotH thrC::cotG-Nterm*	This work
AZ644	*cotS::gfp*	This work
AZ647	*ΔcotG ΔcotH::neo amyE::cotG cotS::gfp*	This work
AZ649	*ΔcotG ΔcotH::neo thrC::cotGΔ cotS::gfp*	This work
AZ660	*ΔcotG ΔcotH::neo thrC::cotG-Cterm cotS::gfp*	This work
AZ661	*ΔcotG ΔcotH::neo thrC::cotG-Nterm cotS::gfp*	This work

CHAPTER 4

The direct interaction between two morphogenetic proteins is essential for spore coat formation in Bacillus subtilis.

Rachele Isticato[1], Teja Sirec[1], Stefano Vecchione[1], Anna Crispino[1], Anella Saggese[1], Loredana Baccigalupi[1], Eugenio Notomista[1], Adam Driks[2], Ezio Ricca[1]

[1]Department of Biology, University of Naples Federico II-MSA-Via Cinthia 26-80126 Naples, Italy

[2]Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois, USA

Published in October 2015 on *PLoS ONE*; 10(10):e0141040

4.1 Abstract

In *Bacillus subtilis* the protective layers that surround the mature spore are formed by over seventy different proteins. Some of those proteins have a regulatory role on the assembly of other coat proteins and are referred to as morphogenetic factors. CotE is a major morphogenetic factor, known to form a ring around the forming spore and organize the deposition of the outer surface layers. CotH is a CotE-dependent protein known to control the assembly of at least nine other coat proteins. We report that CotH also controls the assembly of CotE and that this mutual dependency is due to a direct interaction between the two proteins. The C-terminal end of CotE is essential for this direct interaction and CotH cannot bind to mutant CotE deleted of six or nine C-terminal amino acids. However, addition of a negatively charged amino acid to those deleted versions of CotE rescues the interaction.

4.2 Introduction

Many biological systems depend on molecular self-assembly to create complex supramolecular structures that carry out diverse functions. Those complex structures are generally based on noncovalent interactions between the forming molecules and require strict regulatory mechanisms. The spore coat of the gram-positive bacterium *Bacillus subtilis* is an example of such supramolecular structure and, because of the amenability of this microorganism to genetic and molecular analysis, is a model to study and improve our knowledge on the formation of self-assembled structures.

Spore formation starts when cell growth is no longer allowed by nutrient starvation or other environmental conditions affecting DNA replication [1, 2]. First morphological step of spore formation is an asymmetric cell division that produces a large mother cell and a small forespore. The mother cell contributes to forespore maturation and undergoes autolysis at the end of the process allowing the release of the mature spore into the environment [1, 2]. The mature spore is extremely stable and resistant to harsh conditions. It can survive for extended periods of time in the absence of water and nutrients and to extremes of heat and pH, to UV radiation, and to the presence of solvents, hydrogen peroxide and lytic enzymes [3, 4]. The spore is, however, able to sense the environment and respond to the presence of water and nutrients generating a cell able to grow and, eventually, to re-sporulate [5].

The resistance properties of the spore are due to its unusual structure and physiology. The dehydrated cytoplasm, containing a copy of the chromosome, is surrounded by a series of protective layers. A peptidoglycan-like cortex is the first shield, surrounded by a multilayered coat and a crust [6]. Coat and crust together are composed of at least 70 different proteins and glycoproteins. While several proteins of both coat and crust have been identified and characterized, little is known about which of them is glycosylated and the identities of the sugars on the spore surface. It is, however, known that the presence of glycoproteins on the spore surface modulates the relative hydrophobicity of the spore [7, 8]. Coat formation is finely controlled by a variety of mechanisms acting at various levels. The synthesis of coat proteins is regulated by at least two mother cell-specific sigma subunits of RNA polymerase and at least three additional transcriptional regulators. These transcription factors act in a temporal sequence, and differentially in the forespore and mother cell, thereby controlling the time and cell type of expression of the coat structural genes (*cot* genes) [6]. At least some coat proteins are subject to post-translation maturation events, including proteolytic cleavage, cross-linking, phosphorylation and glycosylation

reactions [9]. An important subset of coat proteins, referred to as morphogenetic coat proteins, have especially important roles in coat formation, in that these proteins direct the assembly of others in the coat, forming a complex network of interactions [10–14]. Within this subset of regulatory coat proteins, CotE plays a crucial role in the assembly of outer coat and crust: without it, these layers are not assembled [15]. Consistent with this role, CotE is present between the inner and outer coat layers in the mature spore [16]. Interestingly, CotE is also found in the mother cell cytoplasm, up to at least eight hours after the start of sporulation [17]. A mutagenesis study showed that CotE has a modular structure: a C terminal domain that directs the assembly of various coat proteins including some known to be in the outer coat layer, an internal domain involved in targeting CotE to the forespore and a N terminal domain that, together with the internal domain, directs the formation of CotE multimers [18, 19]. More recently, formation of CotE multimers was confirmed by yeast-two-hybrid analysis [20]. A global study of the coat protein interaction network in *B. subtilis* suggested that CotE interacts, directly or indirectly, with most outer coat proteins [10, 12]. A direct interaction with CotE has been demonstrated for SpoVID [14], CotC and CotU [21]. In the case of CotC and CotU, the interaction with CotE is essential for CotC-CotU heterodimerization [21].

CotH is a CotE-dependent (i.e., CotH assembly depends on CotE) [22, 23] morphogenetic protein responsible of the assembly of at least 9 other coat proteins, including CotG, CotC, CotU and CotS [12]. In the absence of CotH, the coat is severely altered and spore resistance and germination is severely impaired [22, 23] At least in part, these phenotypes are likely the result of the lack of an important subset of outer coat proteins [10, 22]. CotH assembly can be engineered to be CotE independent, by over-expression of the *cotH* gene [24]. This suggests that CotE facilitates CotH assembly but is not necessarily essential for this event. There is also evidence that at least some degree of CotE assembly is CotH-dependent [12], suggesting a role for CotH in stabilizing CotE at the spore surface after the initial deposition of CotE. Mutant spores lacking both CotH and CotE germinate less efficiently and showed an increased sensitivity to lysozyme than single *cotE* mutant spores [22]. This suggests additional roles for CotH that are CotE-independent. Taken as a whole, these prior studies establish that CotH plays a major role in coat assembly and, as a result, in key spore properties. However, they leave unclear the mechanism(s) by which CotE directs CotH assembly into the coat. In particular, it remains unknown whether additional coat proteins mediate this pivotal interaction.

In the present work, we focus on the CotE-CotH interaction and on its role in coat formation. We further characterize the dependency of CotE assembly on CotH, and provide evidence arguing that the CotE-CotH interaction is independent of any other morphogenetic coat proteins. Based on our results, we propose a revised model for the assembly of the CotH-dependent portion of the outer coat.

4.3 Materials and Methods

4.3.1 General methods, analysis of spore proteins and immunoassays

Strains and primers used for polymerase chain reaction (PCR) are listed in S1 and S2 Tables, respectively. Manipulations of *B. subtilis* were performed as described previously [25]. Sporulation was induced by the by exhaustion method in Difco Sporulation Medium (DSM). Ten milliliters of sporulating cells were harvested at various times during sporulation and mother cells and forespore fractions isolated

as described before [26]. Whole-cell lysates of sporulating cells were prepared by sonication [26] followed by detergent treatment (62.5 mM Tris-HCl (pH 6.8), 4% SDS, 5% glycerol, 2% beta-mercaptoethanol, 0.003% bromophenol blue) at 100°C for 7 min. 50 μg (mother cell extract or whole-cell lysates) or 20 μg (forespore extract) of total proteins was used for western blot analysis. Extraction of proteins from mature spores (from fifteen milliliters culture) was performed with treatment at 65°C in SDS-DTT extraction buffer or at 4°C in 0.1 M NaOH [24]. Western blot analysis were performed by standard procedures. For electrotransfer was used nitrocellulose membrane and the proteins were then hybridized with either anti-CotH, anti- CotE anti-CotB, anti-CotG or anti-CotC antibodies as described previously [26].

4.3.2 Strain construction and recombinant DNA procedure

Recombinant DNA procedures were carried out as described previously [27] unless otherwise indicated. CotE mutants were obtained by amplifying the *cotE* gene by PCR using the *B. subtilis* chromosomal DNA as a template and the CotE-P synthetic oligonucleotide (S2 Table) as forward primer. Four different oligonucleotides, CotE 525-9EE-R, CotE 525-6D-R, CotE 525-6E-R, CotE 525-6K-R (S2 Table) were independently used as reverse primers. All amplification fragments (all of about 920 bp) were firstly cloned in pGem-T (*Promega*) and then excised by enzymatic digestion using *BamH*I and *EcoR*I restriction enzymes and finally cloned into pDG364 vector, previously digested with the same enzymes. By this strategy plasmids pTS25 (9EE), pTS22 (6D), pTS23 (6E) and pTS24 (6K) were obtained. All four plasmids were sequenced and then used to transform a *B. subtilis cotE* null mutant strain (RH211), obtaining RH401 (-9EE), RH402 (-6D), RH403 (-6E) and RH404 (-6K) mutant strains.

4.3.3 Overproduction of proteins in *E. coli* and pull-down experiments

cotH coding region was PCR amplified using the *B. subtilis* chromosomal DNA as a template and oligonucleotides H34 and H35 (S2 Table) to prime the reaction. The obtained fragment of 1,100 bp was digested *SacI* and *KpnI* restriction enzymes and cloned in frame with poly-His tag into the pBAD-B expression vector (*Life Technologies*) previously digested with the same enzymes. To over-produce CotE -9EE, -6D or -6E, the *cotE* coding regions were amplified by PCR using the oligonucleotides E-NdeI-F and pDG364 720R-HindIII (S2 Table) to prime the reactions and chromosomal DNA of strains RH401, RH402 or RH403 as templates. All fragments were digested with *Nde*I and *Hind*III restriction enzymes and then cloned the pRSET-B expression vector (*Invitrogen*) previously digested with the same enzymes. All the resulting plasmids were checked by nucleotide sequence analysis and used to transform *E. coli* strain BL21(DE3) (Novagen) to create the strains RH406 (-9EE), RH405 (-6D) and RH407 (-6E) (S1 Table).

For the His tag pull down assays, CotH-His and all the untagged modified versions of CotE were produced using the *Overnight Express Autoinduction Media* as previoulsy described [21]. Briefly, after an over-night incubation at 37°C in 15 mL of autoinduction medium (*Novagen*), the cells of all strains were collected by centrifugation and resuspended in 1.5 ml of lysis buffer (50 mM NaH_2PO4, 300 mM NaCl, 10 mM imidazole, 2 mg/ml lysozyme, and 0.01 mg/ml RNase). After 30 min at 4°C, the lysates were sonicated and the suspension was clarified by centrifugation at 13,000 g at 4°C for 20 min. 300μg of

extract from strain expressing CotH-His was applied to Ni-NTA magnetic agarose beads (*Qiagen*). After 1 h of incubation at room temperature with shaking, the beads were washed with 2.5 ml of wash buffer (50 mM NaH_2PO4, 300 mM NaCl, 10 mM imidazole), and 300 μg of extract from strain RH134 (or RH404, or RH405 or RH406) was added to the beads and incubated for 1 h at room temperature with shaking to facilitate binding. Unbound proteins were removed by washing with wash buffer at three different concentrations of imidazole (40 mM, 100 mM, and 250 mM. Bound proteins were eluted using the wash buffer at increasing concentrations of imidazole (500 mM and 1 M) Eluted proteins were resolved on 12.5% SDS-PAGE gels and subjected to immunoblot analysis [26].

4.4 Results and Discussion

4.4.1 CotE assembly depends on CotH

A western blot approach with anti-CotE-antibody was used to investigate the degree to which CotE coat assembly within the coat is dependent on CotH. Fig 1A shows that the amount of extracted CotE is higher in mature spores of a wild type *B. subtilis* strain than in spores of a congenic *cotH* mutant strain (strain ER220). Consistent with the possibility that CotE assembly is due, at least in part, to direct interactions with CotH, we found that the amount of extracted CotE is higher in spores engineered to over express *cotH* (strain RG24) than in wild type spores (Fig 1A). We conclude from these experiments that the assembly of CotE in mature spores depends on the level of CotH.

To more fully characterize the consequences of CotH on CotE assembly, we measured the levels of CotE in each of the two compartments of sporulating cells. To do this, we applied western blot analysis with anti-CotE-antibody on proteins extracted from either the mother cell or the forespore compartment at various times after the onset of sporulation. As previously reported [17], in a wild type strain, three hours after the onset of sporulation (T_3), CotE was mostly found in the mother cell compartment (Fig 1B). After that it was found in both compartments (T_7) and, somewhat later (T_9), CotE was mostly in the forespore. In a congenic strain lacking CotH, at all time points tested the majority of CotE was found in the mother cell cytoplasm (Fig 1B). These results suggest that CotH acts at the level of CotE incorporation into the coat, and not at the level of CotE synthesis or stability. These data provide strong confirmation of previous results suggesting that CotE is a CotH-controlled protein [12].

Recent work suggests that CotH counteracts a negative role played by CotG [28]. In that study, it was shown that the assembly of at least three coat proteins, CotC, CotU and CotS, requires CotH only when CotG is present. Indeed, although all three proteins fail to assemble in spores of a strain lacking CotH, the defect is eliminated when CotG is also lacking. Therefore, we addressed the hypothesis that CotG contributes to CotH-dependent CotE assembly, by measuring CotE assembly in a strain lacking both CotH and CotG (strain AZ603). As shown in Fig 1C, the amount of CotE assembled around mature spores of a strain lacking only CotH or both CotH and CotG is similar, demonstrating that CotE assembly is not negatively affected by CotG.

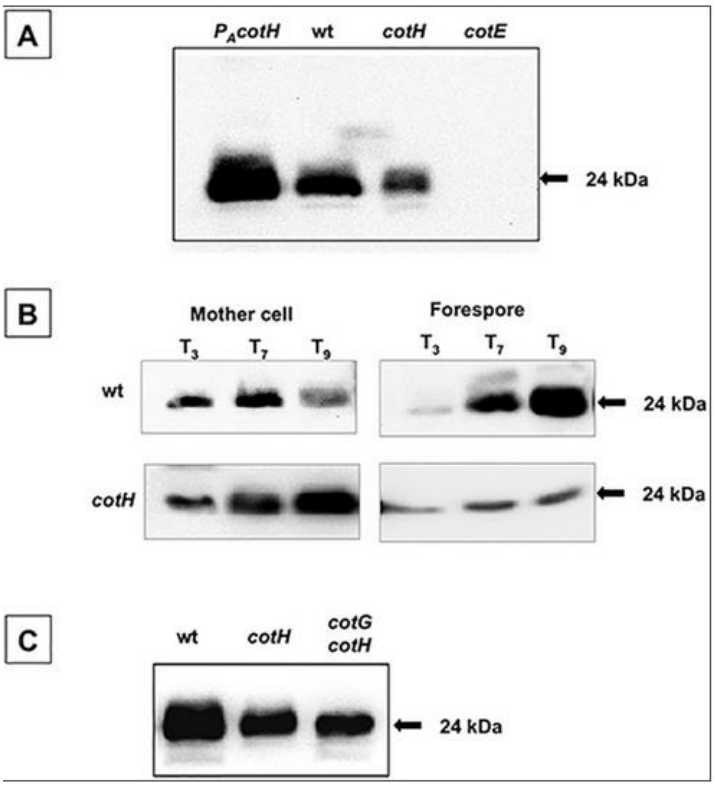

Figure 1: CotE *in vivo* assembly in mature spores and during sporulation. Proteins for western blot analysis were extracted from: (A) mature spores of a wild type strain (wt), or isogenic strains lacking CotH (*cotH;* ER220) or CotE (*cotE;* RH211) or over-producing CotH (P_A*cotH;* RG24) (24); (B) mother cell or forespore compartments of sporulating cells of a wild type strain (wt), or an isogenic strain lacking CotH (*cotH;* ER220), 3 (T_3), 7 (T_7), and 9 (T_9) hours after the initiation of sporulation; (C) mature spores of a wild type strain (wt), or isogenic strains lacking CotH (*cotH;* ER220), or CotG and CotH (*cotG cotH;* AZ603). Proteins were fractionated on 15% SDS-PAGE, electrotransfered on a membrane and reacted with anti-CotE antibody.

4.4.2 CotE and CotH interactions *in vitro*

To address the key question of whether any *B. subtilis* coat proteins mediate the interaction between CotE and CotH, we overproduced in *E. coli* a His-tagged version of CotH (CotH-His) and an untagged version of CotE, and performed an *in vitro* His tag pull-down assay. To do this, we lysed *E. coli* cells producing CotH-His, incubated them with Ni-NTA magnetic beads and then incubated the beads with an extract of cells producing untagged CotE. We then eluted proteins from the beads, fractionated them by SDS-PAGE and performed blot analysis, with anti-CotH (Fig 2A), anti-CotE (Fig 2B and 2C) or anti-His (not shown) antibodies. A small fraction of untagged CotE was able to bind Ni-NTA beads when CotH-His was present (Fig 2B). In the absence of CotH-His, untagged CotE was not able to bind to the Ni-NTA beads (Fig 2C). These data argue against the view that a *B. subtilis* coat protein is required for the CotE-CotH interaction. The simplest interpretation of these data is that the interaction between these two proteins is direct.

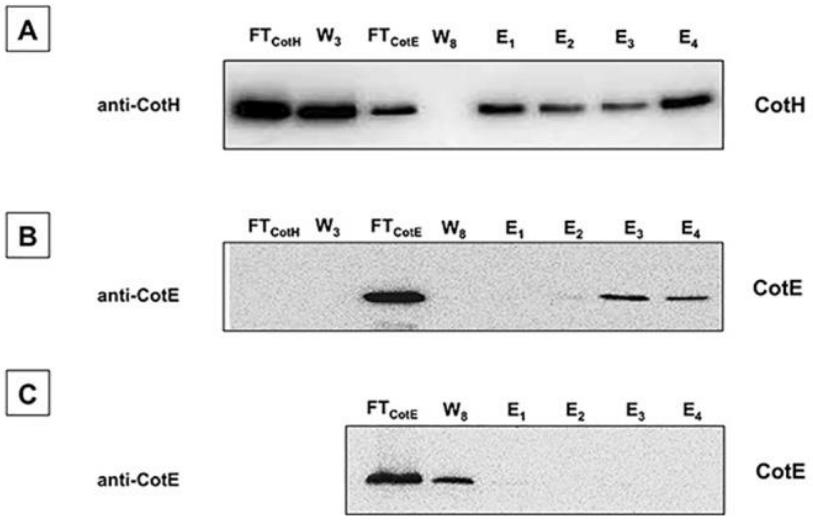

Figure 2: Immunoprecipitation analysis of the *in vitro* CotH-CotE interactions.
CotH-His was bound to a Ni-NTA column and the flowthrough (FT_{CotH}) and washes (W1-W3, here only W3 is shown) were collected. Untagged CotE was then added, and flow through (FT_{CotE}), washed (W1—W8, here only W8 is shown), and eluted (E1—E4) proteins collected as described in Materials and Methods. Proteins were fractionated on 12.5% polyacrylamide gels, electrotransferred to membranes, and reacted with anti-CotH (A), anti-CotE (B) antibodies. The same experiment was also performed without CotH-His (C).

4.4.3 The C terminus of CotE protein is involved in the interaction with CotH

Previous studies demonstrated that the CotE sequence is organized into functional modules, where the N- and C-termini have roles in the assembly of the outer coat proteins, and an internal block of residues directs the assembly of CotE to the developing forespore [18, 19]. To identify regions of CotE that mediate interactions with CotH, we analyzed a collection of congenic *cotE* mutants (Fig 3A; [17]), each harboring a different 20 amino acid deletion in the resulting protein. Western blot analysis showed that CotE was present in spores from all the mutant strains (Fig 3B). As expected [18], the levels of CotE present in the various strains is lower than in wild type strain. Also as expected, CotE was only barely detectable in spores of strain TB95, due to the functions of the missing amino acids (58 to 75) (Fig 3B) [18]. Western blot analysis with anti-CotH antibodies demonstrated the presence of similar levels of CotH in spores of all the mutant strains except TB95 (presumably because of the low level of CotE) and in strain SL484 (missing amino acids 162–181) (Fig 3C). We conclude, therefore, that amino acids within 162–181 participate in CotH assembly. This finding is consistent with previous work, showing that that the absence of these residues results in low levels of assembly of the CotH-dependent protein CotG [19]. Our finding is also consistent with the view that the interaction between CotE and CotH in the coat is direct.

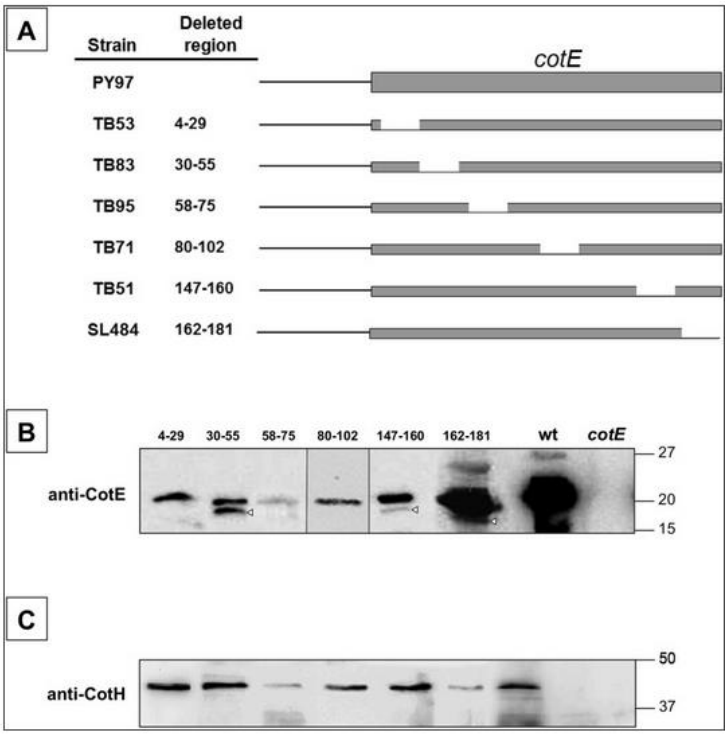

Figure 3: Effects of deletions in CotE on *in vivo* CotH assembly.
(A) CotE and various deletion mutant versions of the protein are indicated. To the left of each construct is the strain name and the deleted amino acids. Coat proteins were extracted from mature spores of wild type and mutants, and analyzed by western blot with anti-CotE (B) or anti-CotH (C) antibodies. White triangles indicate likely CotE degradation products. Molecular masses are indicated in kilodaltons.

4.4.4 Role of a negatively charged amino acid at the C terminus of CotE

To identify more precisely the amino acids in CotE that direct CotH assembly, we analyzed CotH assembly in spores of strains TB126, SL483, TB124 and SL507 lacking the last 3, 6, 9 or 12 residues at the C terminus of CotE, respectively [19] (Fig 4A). Western blot with anti-CotE antibody and SDS-PAGE analyses showed that in spores of all mutant strains, CotE was present, and at levels consistent with those reported previously (Fig 4B and 4C) [19]. Western blot analysis with anti-CotH antibody revealed that CotH was present when the 3 C terminal-most amino acids were missing (in strain TB126) but was absent when the 6 or 9 C terminal-most amino acids were missing (in strains SL483 and TB124) (Fig 4D). Interestingly, CotH was detected when twelve (strain SL507 in Fig 4D) or twenty (strain SL484 in Fig 3) amino acids were deleted. These data are striking in light of previous data indicating that the deletion of 6 or 9 amino acids did not prevent assembly of the CotH-dependent proteins, including CotG [19].

While seeking an explanation of the data just described, we noted that in all the versions of CotE that successfully assembled CotH (wild type, and those missing 3, 12 or 20 C terminal amino acids), but not the versions that failed to assemble CotH, the final residue was negatively charged (Fig 4A). To test the hypothesis that to direct CotH assembly CotE must have a negatively charged C terminus, we generated a strain bearing a version of CotE lacking the C-terminal 9 amino acids and with the addition of two glutamic acid (EE) residues (strain RH401). Western blot analysis showed that spores from this strain do assemble CotH (Fig 5A).

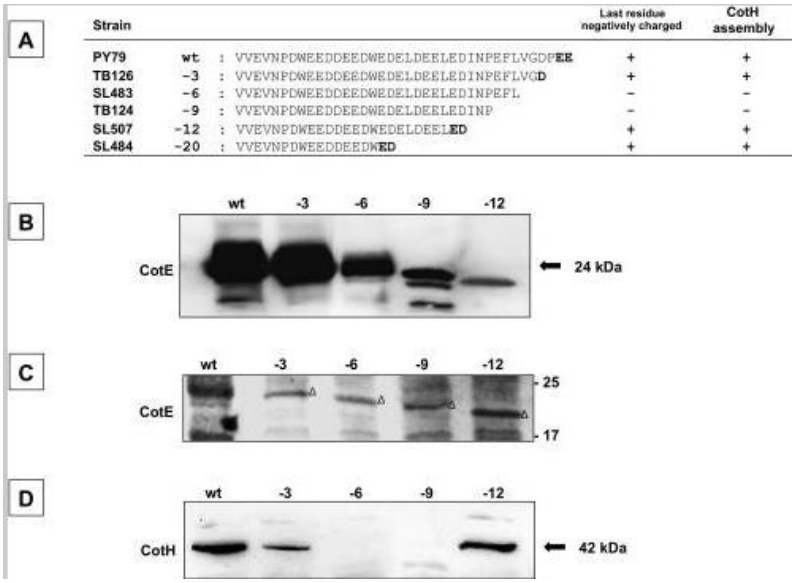

Figure 4: Effects of C-terminal deletions of CotE on *in vivo* CotH assembly.
(A) The amino acid sequences of the C termini of CotE and various mutant versions. To the left of each sequence are the strain name and the number of deleted amino acids. Negatively charged amino acids when present at the C terminus are indicated in bold. Western blot with anti-CotE antibody (B), SDS-PAGE (C) and western blot with anti-CotH- antibody (D) of coat proteins extracted from spores of the strain indicated in panel A. Triangles in panel C indicate the predicted CotE bands. Proteins were fractionated on 12.5% polyacrylamide gels, electrotransferred to membranes, and reacted with anti-CotH and anti-CotE antibodies.

Previous experiments showed that CotH assembly to the spore becomes strictly dependent on CotE only at late times during sporulation (after T11) (24). Therefore, we asked whether the presence of a positive amino acid to the CotE C terminus (strain TB124 lacking 9 C-terminal residues) would affect this early phase of apparently CotE-independent CotH assembly. We found that the pattern of CotH assembly was similar to that previously seen in a *cotE* null mutant strain (strain RG25 in [24]); CotH was found around the spore at early times during sporulation (T6 and T10) but not at a late time (T18) (Fig 6A) or in mature spores (Fig 5). When a negatively charged amino acid was added a wild type pattern of CotH assembly was rescued (Fig 6A). Therefore, the presence of a positive residue to the CotE C-terminus does not prevent early CotH assembly.

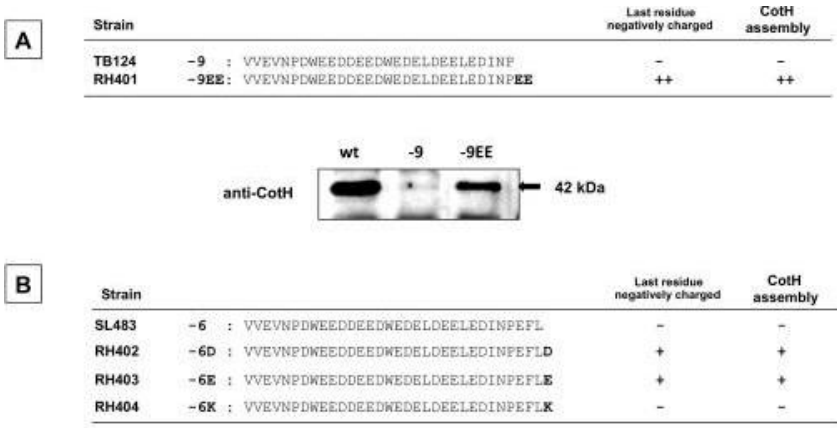

Figure 5: Rescue of *in vivo* CotH assembly by addition of negatively charged residues.
Strains bearing versions of CotE lacking nine (A) or six (B) C-terminal amino acids were modified by adding the indicated C terminal residue and analyzed by western blot. Proteins were fractionated on 12.5% polyacrylamide gels, electrotransferred to membranes, and reacted with anti-CotH antibody.

We then conclude that during sporulation CotH initially assembles around the spore in a CotE-independent way and that the interaction with CotE is essential to stabilize CotH presence around the forming spore. Such interaction strictly requires the presence of a negatively charged amino acid as C terminus of CotE.

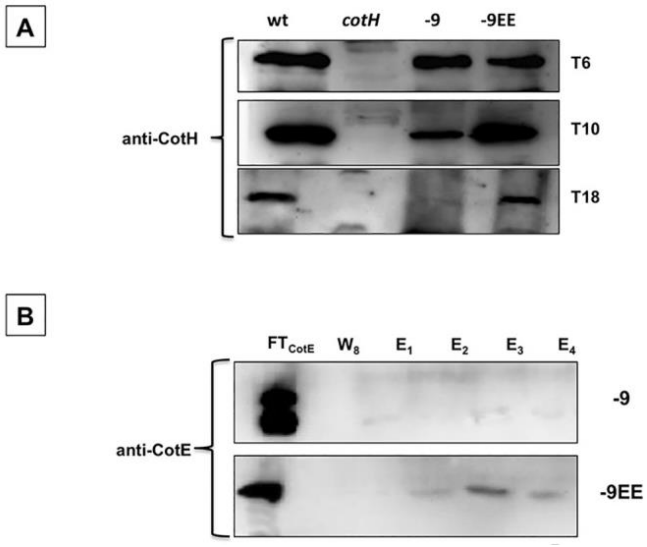

Figure 6: Time course of *in vivo* CotE-CotH interaction and *in vitro* pull down experiments.
(A) Western blot of proteins extracted from the forespore compartment of sporulating cells of a wild type strain (wt), or isogenic strains lacking CotH (*cotH;* ER220), carrying a deleted version of CotE (-9; TB124) or carrying a deleted version with a negatively charged C terminus (-9EE). Proteins were extracted 6 (T_6), 10 (T_{10}), and 18 (T_{18}) hours after the initiation of sporulation. (B) Untagged CotE -9 and -9EE versions were independently added to a Ni-NTA column bound to CotH-His as in Fig 2A. Flow through (FT_{CotE}), washed (W1—W8, here only W8 is shown), and eluted (E1—E4) proteins collected as described in Materials and Methods. Proteins were fractionated on 12.5% polyacrylamide gels, electrotransferred to membranes, and reacted with anti-CotH and anti-CotE antibodies.

4.4.5 CotH interactions *in vitro* with CotE missing 9 amino acids but with an additional negatively charged residue

Our studies so far suggested the possibility that a negatively charged amino acid on the C terminus of CotE interacts directly with CotH. If so, then we would expect an appropriately engineered version of CotE to interact with CotH in the *in vitro* assay already described. To address this, we analyzed interactions between CotH and overproduced versions of CotE with deletions of 6 or 9 amino acids, and with or without the addition of an acidic residue. As shown in Fig 6B, CotE lacking 9 amino acids was unable to pull down CotH-His, but was able to when a negatively charged residue was added to the C terminus. The same result was obtained using the version of CotE lacking the 6 C-terminal residues: this version of CotE did not pull down CotH-His, but the pull down was successful when a glutamic acid (E) residue was added (not shown).

4.4.6 The interaction of CotH with a truncated version of CotE is functional

In the experiment just described, we showed that addition of a negatively charged amino acid to a version of CotE missing either the C-terminal -6 and -9 amino acids rescued CotH assembly. To further characterize the degree of rescue of CotH function by these versions of CotE, we next tested whether they directed assembly of the CotH-controlled proteins CotB, CotC, CotG, and CotU. CotG is an abundant protein extracted from wild type spores in a 32 and a 36 kDa form both strictly dependent on CotH for coat assembly [12, 28, 29] (Fig 7A). The removal of the C-terminal 6 and 9 amino acids from CotE results in the loss of the 36 kD but not the 32 kD CotG species (Fig 7A and data not shown). The addition of a negatively charged residue to the C terminus of the truncation restored the presence of the 36 kDa species (Fig 7A and data not shown). The simplest interpretation of these results is that assembly of the 36 kDa species depends on an interaction between CotE and CotH. Our data also argue that assembly of the 32 kDa species requires CotH but it does not depend on a CotE-CotH interaction, implying that CotH can participate in assembly of CotG-32 even if it is in the mother cell cytoplasm and not bound into the coat (Fig 6A).

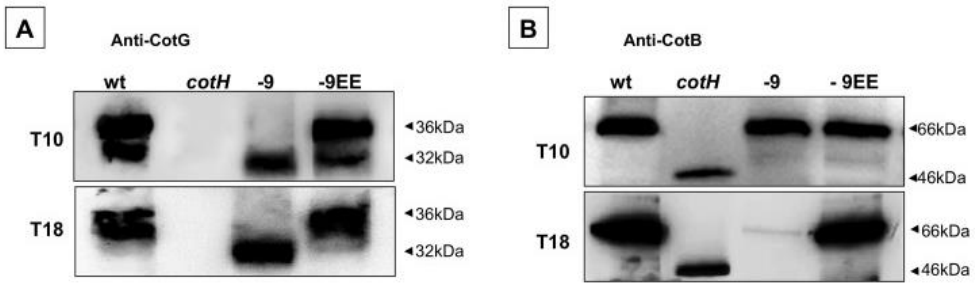

Figure 7: Effects of CotE mutations on CotB and CotG assembly during sporulation.
Western blot with anti-CotG (A) and anti-CotB (B) antibodies of proteins extracted from mature spores of a wild type strain (wt) or of isogenic strains lacking CotH (*cotH*; ER220) or carrying modified version of CotE (-9; TB124, -9EE; RH401). Proteins were fractionated on 12.5% polyacrylamide gels, electrotransferred to membranes, and reacted with the anti-CotG (A) and anti-CotB (B) antibodies.

CotB is extracted from wild type spores in its 66 kDa mature form [30]. However, CotB is produced as a 46 kDa species whose maturation requires CotH and CotG [30]. Prior to the point in coat assembly when CotH deposition is independent of CotE (T10) (Fig 6A), the mature, 66 kDA form of CotB is present in the spore of a strain containing either the 6 or 9 amino acid truncation version of CotE, (Fig 7B and data not shown). In the same strain but at a later time (T18), CotH is no longer assembled within the coat (Fig 6A and data not shown) and also the mature form of CotB is no longer present in the coat (Fig 7B and data not shown). The presence of a negatively charged amino acids to either CotE truncation construct rescues CotH assembly and also assembly of the mature form of CotB (Fig 7B and data not shown).

CotC and CotU share significant sequence identity and are both recognized by anti-CotC and anti-CotU antibodies [31, 32]. They are assembled in several forms including a CotC-CotU heterodimer of 23 kDa whose formation requires a direct interaction with CotE [21]. CotH plays a role in this event, by counteracting a still poorly understood negative role played by CotG on CotC and CotU assembly [28]. Assembly of CotU and CotC monomer forms, and CotC dimers, was not significantly affected by deletion of the CotE C terminus (Fig 8A). However, we did detect significant changes in the levels of the

23 kDa CotC-CotU heterodimer and the 30 kDa form of CotC [31, 32], that appear both to be strongly reduced in spores bearing either CotE truncation construct (Fig 8A). Assembly of these species were not rescued by the addition of a negatively charged amino acid (data not shown).

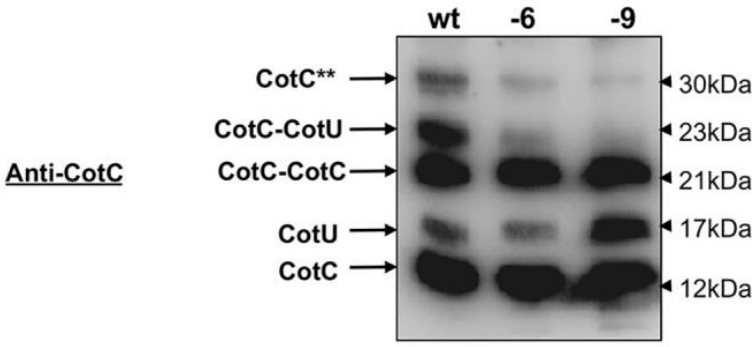

Figure 8: Effects of CotE mutations on assembly of CotC and CotU during sporulation.
Western blot with anti-CotC antibody of proteins extracted from mature spores of a wild type strain (wt), or isogenic strains carrying either deleted version of CotE (-6; SL483, -9; TB124). The various forms of CotC and CotU are indicated. CotC** indicates a post-translationally modified form of CotC [31]. Proteins were fractionated on 12.5% polyacrylamide gels, electrotransferred to membranes, and reacted with the indicated antibodies.

4.5 Conclusions

The assembly of CotH and CotE is mutually dependent. CotH dependence on CotE has been established in several previous studies [23, 24, 30].The dependence of CotE on CotH is much less well characterized [12]. Here we further characterize this interaction and use the results to generate a refined model for coat assembly. Our principal finding is that both the CotE-dependent assembly of CotH, and the CotH-dependent assembly of CotE, require amino acids at the CotE C-terminus, and the effects of removal of these residues is overridden by the addition of a negatively charged amino acid to the end of the construct. We suggest that the simplest interpretation of these data is that the mutually dependent assembly of CotE and CotH is due to a direct interaction between these proteins. CotH has at least two roles. First, it directs the assembly of a large subset of coat proteins [12]. Second, CotH stabilizes the already deposited CotE.

Our results also provide insights into the mechanism by which CotG negatively effects assembly of CotC and CotU [28]. Our data suggest a model in which the 32 kDa form of CotG (whose assembly is CotH-independent) does not have the ability to exert the negative affect on CotC or CotU assembly (that CotG usually produces in *cotH* mutant spores [28]), but the 36 kDa form does. This result is striking because it is, to our knowledge, the first evidence for a difference in function in assembly between isoforms of a coat protein. We suggest, therefore, that in addition to the functions already described for CotH, a key role is, directly or indirectly, to suppress a deleterious effect of the 36 kDa form of CotG on coat assembly. This possible role underscores what is likely to be an important general feature of coat protein assembly; the need to suppress certain possible interactions which, left unchecked, lead to misassembled coats. We speculate that there are other interactions between coat proteins whose major or even primary

function is to prevent maladaptive assembly. Possibly, the potential for deleterious interactions among a relatively large number of coat proteins has led to a correspondingly large number of such "prophylactic" interactions and, therefore, a surprisingly high degree of biochemical complexity to the coat as a whole.

4.6 Acknowledgments

We thank L. Di Iorio for technical support.

4.7 References

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4.8 Supporting Information

S1 Table: List of strains used in this study.

Strain	Genotype	Source[a]
Bacillus subtilis		
PY79	wild type	reference below
RH211	*cotE::spc*	[21]
ER220	*cotH::spc*	[22]
RG24	*pAH::cat*	[24]
AZ603	*ΔcotG ΔcotH::neo*	[25]
TB70	*cotE::cat::spc amyE::cotEΔ159-181*	[18]
TB51	*cotE::cat::spc amyE::cotEΔ147-160*	[18]
TB71	*cotE::cat::spc amyE::cotEΔ80-102*	[18]
TB95	*cotE::cat::spc amyE::cotEΔ58-75*	[18]
TB83	*cotE::cat::spc amyE::cotEΔ30-55*	[18]
TB126 (-3)	*cotE::cat::spc amyE::cotEΔ179–181*	[19]
SL483 (-6)	*cotE::cat::spc amyE::cotEΔ176–181*	[19]
TB124 (-9)	cotE::*cat::spc amyE::cotEΔ173–181*	[19]

SL507 (-12)	*cotE::cat::spc amyE::cotEΔ170–181*	[19]
SL484 (-20)	*cotE::cat::spc amyE::cotEΔ162–181*	[19]
RH401 (-9EE)	*cotE::spc::cat* amyE::*cotEΔ173–181EE*	This study
RH402 (-6D)	*cotE::spc::cat* amyE:: *cotEΔ176–181D*	This study
RH403 (-6E)	*cotE::spc::cat* amyE::*cotEΔ176–181E*	This study
RH404 (-6K)	*cotE::spc::cat* amyE::*cotEΔ176–181K*	This study
Escherichia coli [b]		
CotH-His	*cotH::6his*	This study
RH134	*cotE*	[21]
RH405	*cotEΔ173–181EE*	This study
RH406	*cotEΔ176–181D*	This study
RH407	*cotEΔ176–181E*	This study

[a] Numbers refer to references in the text. [b] All *E. coli* strains are derivatives of strain BL21(DE3) transformed with various plasmids. The relevant genotypes shown for *E. coli* strains are those of the contained plasmid.

Reference:

Youngman P, Perkins JB, Losick R. A novel method for the rapid cloning in *Escherichia coli* of *Bacillus subtilis* chromosomal DNA adjacent to Tn917 insertion. Mol. Gen. Genet. 1984; 195:424-433.

S2 Table. List of primers

Oligonucleotide	Sequence[a, b]	Restriction site	Position of annealing[c]
CotE-P	CGggatccCGAGCTCGTTGCACACACC	BamHI	-370/-388 (*cotE*)
CotE 525-9EE-R	aagcttATTCCTCCGGGTTGATGC	HindIII	+505/+519(*cotE*)
CotE 525-6D-R	aagcttAGTCTAAAAACTCCGGGTTG	HindIII	+510/+526(*cotE*)
CotE 525-6E-R	aagcttATTCTAAAAACTCCGGGTTG	HindIII	+510/+526(*cotE*)
CotE 525-6K-R	aagcttACTTTAAAAACTCCGGGTTG	HindIII	+510/+526(*cotE*)
E-NdeI-F	TAGGcatatgTCTGAATACAGGGAAATT	NdeI	+1/+21(*cotE*)
PDG364 720R-H3	aagcttGGTAATGGTAGCGACCGG	HindIII	+702/+720 (pDG364)
H34	gagctcGATGAAGAATCAATCCAATTTACCG	SacI	-1/+24 (*cotH*)
H35	ggtaccTCATAAAATACTTAAATGATCTTTGAGG	KpnI	+1062/+1086 (*cotH*)

[a] Capital and lowercase letters indicate nucleotides complementary to corresponding gene DNA and unpaired flanking sequences carrying a restriction site, respectively. [b] Underlined letters indicate codons which have been inserted. [c] Referred to *cotE* sequences, taking the first nucleotide of the initiation codon as +1.

CHAPTER 5

SF185_1531 *protein of Bacillus megaterium is essential for exosporium formation*

Anella Saggese, *Graham Christie, Ezio Ricca, Loredana Baccigalupi*

manuscript in preparation

5.1 Abstract

Bacillus megaterium is a Gram-positive, spore forming bacterium characterized by the large dimensions of cells and spores, significantly larger than those of other species, including the model species *B. subtilis*. In addition to the dimensions, spores of *B. megaterium* differ from those of *B. subtilis* also for the presence of an exosporium, a still poorly characterized outermost spore layer mainly composed of proteins and carbohydrates. We report here the identification and characterization of SF185_1531, an abundant spore surface protein of *B. megaterium and, by reverse genetics, of its structural gene.* An electron microscopy analysis of a mutant strain lacking SF185_1531 showed that its spore was not surrounded by an exosporium, indicating SF185_1531 as an essential component of this outermost layer. A physiological analysis of the mutant lacking SF185_1531 indicated that spores were defective in germination and impaired in their resistance to lysozyme.

5.2 Introduction

Bacterial endospores (spores) are one of the most resilient cell structures observed in nature. They are formed in response to nutrient starvation by gram-positive bacteria of the *Bacillus* and *Clostridium* genera, which are both members of the Phylum *Firmicutes* (Setlow, 2014). Spore formation is a strategy used by some bacteria to withstand environmental challenges and ensure cell survival over extended periods of time (Driks, 1999; Henriques & Moran, 2007; McKenney *et al*., 2013). Sporulation is initiated when the vegetative cell encounters unfavourable conditions, such as nutrient limitation, and follows a strictly regulated sequence of morphological, biochemical and genetical steps which are highly conserved among spore formers (Henriques & Moran, 2007).

In response to starvation the vegetative cell undergoes an asymmetric cell division and originates two cell compartments separated by a septum. In a process superficially resembling phagocytosis, the larger compartment, the mother cell, then engulfs the pre-spore, isolating it from the surrounding environment with a double membrane. The primordial germ cell wall is assembled at the exterior of the innermost membrane, which serves as precursor for the new cell wall after the spore germinates to resume the vegetative state. In addition, a thick layer of structurally unique peptidoglycan, referred to as the cortex, which is crucial in maintaining spore dormancy, is deposited between the primordial germ cell wall and the outer membrane. At the same time, the multi-layered, proteinaceous spore coat is synthesized in the mother cell and deposited onto the developing spore surface. While the core and cortex structure is widely conserved among spores of differing species, the composition and morphology of the outer layers can vary considerably, it is strain and species specific, and may be adapted for the diverse niches in which spores are found (Henriques & Moran, 2007). In addition, in some species including *B. cereus*, *B. anthracis* and *B. megaterium* (Todd *et al.* 2003; Redmond *et al.* 2004; Beaman *et al.* 1972), the spore coat is surrounded by an additional layer, the exosporium, still poorly characterized but formed mainly by proteins and carbohydrates.

When present, the exosporium is the first point of contact with the environment, while at the same time isolates the spore internal teguments from their immediate surroundings (Kailas *et al.*, 2011). By altering the surface hydrophobicity, the exosporium also plays an important role in spore adherence to various surfaces (Koshikawa *et al.*, 1989; Driks, 2009; Faille *et al.*, 2010).

B. megaterium SF185 was isolated by ileal biopsies of healthy human volunteers (Fakhry *et al*., 2008) and was shown to produce and secrete bioactive molecules exerting a cytoprotective functions on colonic epithelial cells (Di Luccia *et al.* 2016). SF185 secreted factors induce Hsp27, promote PKB/Akt and enhance the phosphorylation of p38 MAPK, required in the prevention of oxidant-induced intestinal epithelial cell injury and loss of barrier function (Di Luccia *et al.* 2016). Based on this SF185 has been proposed as a new probiotic bacterium with potential beneficial properties. We report here the characterization of an abundant spore surface protein of SF185 as a starting point of the characterization of the spore of this potentially interesting strain.

5.3 Materials and Methods

5.3.1 Bacterial strains

Bacterial strains used in this study are listed in Table 1. *Bacillus megaterium* was cultured on LB agar or broth at 30 °C, supplemented with antibiotics (5 μg mL^{-1} kanamycin, 1.25 μg mL^{-1} tetracycline), where appropriate. Polyethylene glycol mediated transformation of *B. megaterium* protoplasts, and subsequent isolation of transformant colonies that had undergone single crossover recombination event was achieved as described previously (Gupta *et al*., 2013). Plasmid constructs were prepared using standard molecular biology techniques and propagated in *Escherichia coli* DH5α in LB medium.

Table 1 *Bacillus megaterium* strains used in this study.

Strain	Relevant genotype	Source
SF185	Wild-type strain	Fakhry *et al.* 2008
OSMeg2	ΔSF185_1531 (Km^r; Tet^r)[a]	This study
OSmeg2Rev	revertant	This study

[a] Km^r, kanamycin resistance (5 μg mL^{-1}); Tet^r, tetracycline resistance (1.25 μg mL^{-1})

5.3.2 Molecular biology procedures

In order to obtain a *B. megaterium* SF_1531 null mutant strain we cloned in pUCTV2 plasmid 5′ (promoter and first codon were not included) and 3′ fragments of the SF185_1531 ORF flanking a kanamycin resistance cassette. The resultant plasmid (pSOCam1) was prepared by PCR and Gibson Assembly (NEB) and it was used to transform *B. megaterium* SF185. As a result of the single homologous recombination at the *SF185_1531* locus, it was obtained an insertion of the entire plasmid, involving a first copy of gene interrupted by kanamycin cassette, and a second not functional copy because lacking of a functional promoter. A Tc^r Km^r transformant was isolated following procedures described previously (Gupta *et al.,* 2013) and it was controlled by PCR using specific oligonucleotides to prime the reactions.

To obtain a revertant strain, null mutant cells were incubated on LB plate without antibiotics at 42°C for a night; then colonies (several hundreds) were replicated on plates with either tetracycline, kanamycin or no

antibiotics. Colonies growing only in the absence of both antibiotics were considered potential revertants and used for further analysis by PCR.

Oligonucleotide sequences are reported in supplementary material (Table S1).

5.3.3 Spore purification, and analysis of spore coat proteins

Bacillus megaterium spores were prepared in supplemented nutrient broth (SNB) in a shaking incubator (typically 72 h at 30 °C, 225 r.p.m.) and purified and stored as described previously (Gupta *et al*., 2013). Spore coat proteins were extracted from a suspension of spores by SDS-DTT (dithiothreitol) treatment (Cutting S, Vander Horn PB, 1990). The concentration of extracted proteins was determined by using Bio-Rad DC protein assay kit (Bio-Rad), and 20 µg of total extracted proteins were fractionated on SDS-PAGE (12% Bis-Tris gel) and visualized by Coomassie blue staining. The band was excised and submitted to Edman degradation reaction to detect the N-terminal sequence of the protein of interest.

5.3.4 Microscopy

Fluorescence and thin-section transmission electron microscopy (TEM) analyses were conducted as described previously (Manetsberger *et al* 2014). Negatively stained intact spores were imaged using a Philips CM100 transmission electron microscope operated at an accelerating voltage of 100 kV. Micrographs were collected at a size of 1024 × 1024 pixels using a Gatan Multiscan 794 1k × 1k CCD camera and analyzed with Gatan Digital Micrograph software (DM, Gatan Inc.).

5.3.5 Spore germination and lysozyme resistance

The progress of spore germination of various *B. megaterium* strains used in this work was monitored by determining the absorbance (at 580 nm) of heat-shocked (70°C, 10 min) spores suspended (OD600 ≈ 0.4) in 5 mM Tris-HCl, pH 7.8, plus 10 mM glucose, 10 mM proline, 10 mM leucine, and 50 mM potassium bromide (KBr). Germination assays were conducted in 96-well plates incubated at 30°C in a Multiskan spectrum plate reader (Thermo).

Sensitivity to lysozyme was measured as described by Zheng *et al.*1988. Spores were prepared as previously described and eliminating vegetative cells by heat treatment (10 min at 80°C). Purified spores were then suspended in 10 mM Tris-HCl (pH 7.0) buffer containing lysozyme (50 mg/ml) and the decrease in optical density was monitored at 580 nm at 1-min intervals for 10 min. Spore viability was measured after 30 min as CFU on TY agar plates.

All experiments were conducted in triplicate with at least two independently prepared batches of spores.

5.3.6 Bioinformatic analysis

Bioinformatic analysis was conducted through BLAST program using SF_1531 protein sequence of SF185 as query against *B. megaterium* genome (taxid: 1404). We considered only completely sequenced genomes sharing a minimum of 40% identity with respect to the query sequence.

5.4 Results and Discussion

5.4.1 SF185_1531 is a spore surface protein of *B. megaterium*

B. megaterium SF185 spores were purified as reported in Materials&Methods and used to extract surface proteins by a previously reported alkaline extraction procedure (Cutting, Vander Horn, 1990). Extracted proteins were then fractionated by SDS-PAGE and several proteins detected (Fig. 1A). We focused our attention on the protein arrowed in Fig. 1A, that was smaller than 20 kDa, abundant and apparently well separated from other proteins. The protein was used to detect the N-terminal sequence by the Edman degradation reaction and the sequence of 10 N-terminal amino acids determined. A BlastP analysis indicated that the N-terminal part of the protein corresponded to the product of *SF185_1531* gene of the *B. megaterium* SF185 chromosome (Supplementary Material). This gene codes for a 128-residue protein with a predicted mass of 16 kDa, therefore corresponding well to the apparent mass deduced by the SDS-PAGE of Fig. 1A. The primary sequence of SF185_1531 contains several repeats in its central part, has a high isoelectric point and a high unfoldability index. All these features resembled those of the CotG-like family of Bacilli (Saggese *et al*. 2016). However, differently from members of the CotG-like family of Bacilli, SF185_1531 is not indicated as a potentially phosphorylated protein and its coding gene is not adjacent and divergently oriented with respect to a *cotH* homolog (Saggese *et al*. 2016). All together SF185_1531 has only some of the properties of the CotG-family and cannot be considered a member of such protein group (table S2, supplementary material).

To study the role of SF185_1531 we constructed a null mutant by cloning a kanamycin-resistance gene cassette between the 5′ and 3′ regions of *SF185_1531* in plasmid pUCTV2. The recombinant plasmid was then used to transform cells of *B. megaterium* SF185 as described in the Materials&Methods section (Fig. 1B). Kanamycin and tetracycline resistant cells were analyzed by PCR to confirm the interruption of the *SF185_1531* gene and the clone indicated as OSmeg2 selected for further studies.

Cells of the mutant strain were then used to obtain a revertant by excision of the inserted plasmid DNA from the chromosome (Fig. 1B). To this aim cells were grown without any antibiotic selection and then analyzed for antibiotics resistance. Clones able to grow only in the absence of both kanamycin and tetracycline were considered as potential revertant and analyzed by PCR. Strain OSmeg2Rev carried a wild type version of the *SF185_1531* locus and was considered for further analysis.

Spores of OSmeg2 and OSmeg2Rev were purified, surface proteins extracted and analyzed by SDS-PAGE as described above. The analysis of the extracted proteins indicated that the SF185_1531 was not present in OSmeg2 spores while it was present in OSmeg2Rev spores (Fig. 1A), confirming that *SF185_1531* is the structural gene for a spore surface protein, that OSmeg2 carries a null mutation in *SF185_1531* and that OSmeg2Rev is an excision revertant of the mutant.

The analysis of the SDS-PAGE of Fig. 1A also indicated that other proteins in addition to SF181_1531 were lacking in the mutant strain. These proteins, indicated with a red star in Fig. 1A, were all rescued in the revertant strain. An effect of the *SF185_1531* gene on several proteins could be explained hypothesizing either a regulatory role of the SF_1531 protein on the assembly of other surface components or that the other proteins lacking in the mutant strain are modified version of SF185_1531 or protein complexes containing SF185_1531.

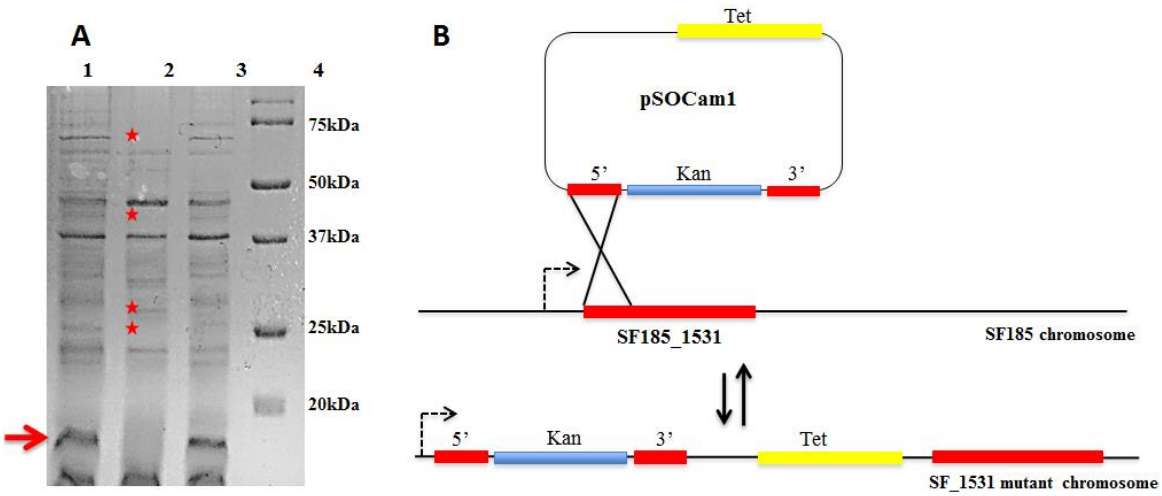

Figure 1: (A) pattern of spore surface proteins extracted from *Bacillus megaterium* strain SF185 (lane 1), ΔSF185_1531 (lane 2) and SF185_1531R (lane 3). Molecular markers are shown in lane 4. The arrow indicates the band corresponding to the 1531 protein. The red stars indicate bands absent in the mutant spores. **(B)** Schematic representation of the cloning strategy used to obtain the SF185_1531 null mutant strain and its revertant.

5.4.2 SF185_1531 is needed for spore germination and resistance to lysozyme

In order to understand the physiological role of SF185_1531 we analyzed two typical spore-associated phenotypes: the efficiency of germination and the resistance to lysozyme digestion. As shown in Fig. 2, both phenotypes were altered in the mutant spores and rescued in the revertant. Fig. 2A shows that mutant spores, OSmeg2, were about 10-15 minutes delayed in the start of the germination process and that after 60 minutes the efficiency of germination was less than 30%, compared to the 60% of the wild type and of the revertant. Fig. 2B shows that OSmeg2 spores are less resistant than wild type and revertant spores to lysozyme digestion.

Taken together, results of Fig. 1 and 2 suggest that SF185_1531 has an important structural and functional role for spores of strain SF185.

5.4.3 SF185_1531 is essential for exosporium formation

To analyze the spore surface of SF185, of the mutant lacking SF185_1531 and of its revertant, we followed a transmission electron microscopy (TEM) approach. As shown in the Fig. 3, spores of SF185 are surrounded by an exosporium that gives to the spore a "walnut" shape, typical of the *B. megaterium* species (Manetsberger *et al*., 2015). Surprisingly the exosporium was totally lacking from around the OSmeg2 spores and rescued in its revertant. Total lack of the exosporium as a consequence of inactivation of the *SF185_1531* gene clearly indicates the SF185_1531 protein as essential for exosporium formation. Results of Fig. 3 suggest that the various proteins lacking in OSmeg2 spores (Fig. 1A) are probably other protein components of the exosporium of SF185 and that the exosporium has a role in spore germination and resistance to lysozyme (Fig. 2).

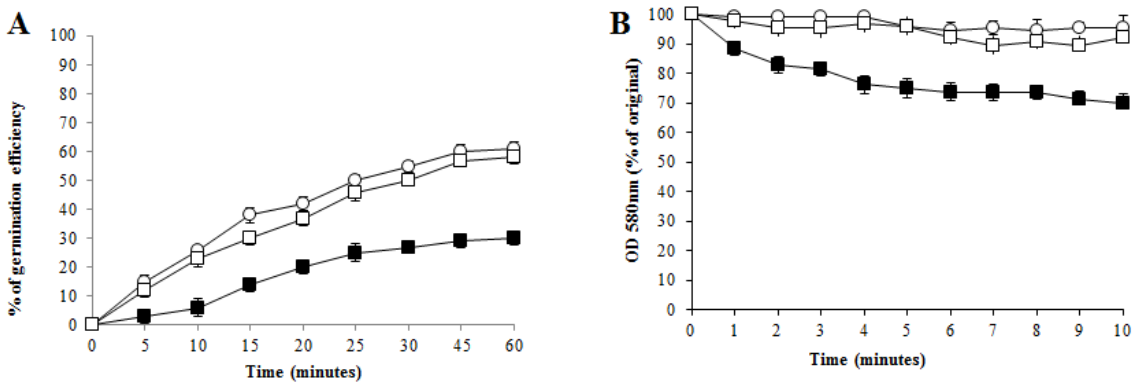

Figure 2: Effects of SF185_1531 on germination efficiency (A) and lysozyme resistance (B). Spores of strains SF185 spores (white circles), OSmeg2 (black squares) and OSmeg2Rev (white squares) were either (A) induced by 5 mM Tris-HCl, pH 7.8, plus GPLK (10 mM glucose, proline, leucine, 50 mM K bromide), the decrease of optical density followed over time and reported as percentage of germination efficiency; or (B) exposed to lysozyme and the decrease of optical density followed over time. The data are averages of three independent experiments.

5.5 Conclusions

This study reports the characterization of a spore surface protein of a *B. megaterium* strain isolated from the human intestine and shown to induce stress response pathways *in vitro* in human epithelial model cells. A first important result of this report is that the identified protein is essential for exosporium formation, since a strain lacking the SF185_1531 protein does not form the exosporium. This drastic effect could be due to either a regulatory role of SF185_1531 that would be needed to allow the assembly of other exosporium components or to an essential structural role of the protein. A bioinformatic analysis indicated that SF185_1531 is not conserved across the *B. megaterium* group. A SF185_1531 homolog was found only in strain QM B1551, the best studied strain of *B. megaterium* (Table S3). However, this homolog is annotated as a putative spore surface protein of unknown function.

Another relevant result of this report is the observation that the exosporium of SF185 is involved in spore germination and resistance to lysozyme digestion. This result confirms previous data obtained with another *B. megaterium* strain. Indeed, a mutant of *B. megaterium* QM B1551 lacking some plasmids and producing spores without the exosporium is slightly defective in spore germination (Christie and Lowe, 2007) and only partially resistant to lysozyme (Gerhardt *et al.* 1984).

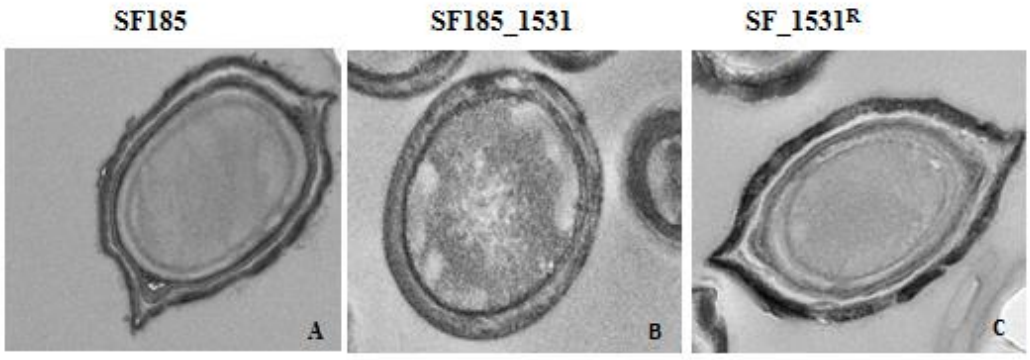

Figure 3: Thin-section TEM analysis of *Bacillus megaterium* spores of the SF185 (A), OSmeg2 (B) and OSmeg2Rev (C) strains.

Our results leave open interesting questions on the chromosomal or plasmid location of the *SF185_1531* genes in SF185 and on the role of the SF185_1531 homolog identified in the genome of the model strain QM B1551. Addressing these questions will be a challenging task of future research.

5.6 References

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5.7 Supplementary material

Table S1: list of oligonucleotides used in this study

Oligonucleotides	Sequence
os01	5' cagctatgaccatgattacgaattcagtaaatcttataaaaacaaaaaag 3'
os02	5' gagcgcctacgaggaatttgtatcgatcgtatttgtctttttgtgcttg 3'
os03	5' caagcacaaaaaagacaaatacgatcgatacaaattcctcgtaggcgctc 3'
os04	5' cttttatatttacatttatcctttgataaacccagcgaaccatttgagg 3'
os05	5' cctcaaatggttcgctgggtttatcaaaaggataaatgtaaatataaaag 3'
os06	5' ccactgttccaatggaaaaggttgggaattcgctatgttactagaggaac 3'

Table S2. Evaluation of SF_1531 protein as CotG-like protein.

Species (Strain / protein code)	Number of residues	pI	% positively charged residues	% Ser-Thr residues	Phosphorylated Thr or Ser residues (%) [a]	Unfoldability index [b]	Instability index [c]
B. subtilis **(168 / BSU36070)**	195	10.26	47.2	21.0	28	-0.716	77.91
B. megaterium **(SF185/ SF_1531)**	128	9,32	42.9	7	0	-0.697	26.63

[a] Phosphorylation prediction was done by using the NetPhosBac 1.0 server (http://www.cbs.dtu.dk/services/NetPhosBac-1.0/). For each Ser and Thr residue, a score between 0 and 1 was calculated. When the score is ≥0.5, the residue is predicted to be a phosphorylation site.

[b] Disorder prediction was done by using Foldindex (http://bip.weizmann.ac.il/fldbin/findex). Positive values indicate a structured polypeptide, whereas negative values indicate a disordered protein.

[c] The instability index was determined by using ProtParam (http://web.expasy.org/protparam/). Values of ≥40 predict that the protein is unstable.

Table S3: a bioinformatic analysis to investigate of the presence of SF185_1531 homolog across the *B. megaterium* group.

Bacillus megaterium **strain completely sequenced[a]**	**SF_1531 protein orthologue[b]**	**Identity (%)**
B. megaterium QMB1551	+	**80%**
B. megaterium DSM 319	-	
B. megaterium ATCC 14581	-	
B. megaterium WSH-002	-	
B. megaterium Q3	-	

[a] List of *Bacillus megaterium* strains whose genomes have been completely sequenced (source NCBI genome, https://www.ncbi.nlm.nih.gov/genome/genomes/945).

[b] presence (+) or absence (-) of a protein sharing a minimum of 40% sequence identity with SF_1531 protein of *B. megaterium* SF185.

CHAPTER 6

The exosporium of Bacillus megaterium QM B1551 is permeable to the red fluorescence protein of the coral Discosoma sp.

*Mariamichela Lanzilli[1], Giuliana Donadio[1], Roberta Addevico[1], **Anella Saggese**[1], Giuseppina Cangiano[1], Loredana Baccigalupi[1], Graham Christie[2], Ezio Ricca[1], Rachele Isticato[1].*

[1]Department of Biology, University of Naples Federico II-MSA-Via Cinthia 26-80126 Naples, Italy

[2]Department of Chemical Engineering and Biotechnology, University of Cambridge Cambridge, UK.

Front. Microbiol., 2016 Nov 4;7:1752

6.1 Abstract

Bacterial spores spontaneously interact and tightly bind heterologous proteins. A variety of antigens and enzymes have been efficiently displayed on spores of *Bacillus subtilis*, the model system for spore formers. Adsorption on *B. subtilis* spores has then been proposed as a non-recombinant approach for the development of mucosal vaccine/drug delivery vehicles, biocatalysts, bioremediation, and diagnostic tools. We used spores of *B. megaterium* QM B1551 to evaluate their efficiency as an adsorption platform. Spores of *B. megaterium* are significantly larger than those of *B. subtilis* and of other *Bacillus* species and are surrounded by the exosporium, an outermost surface layer present only in some *Bacillus* species and lacking in *B. subtilis.* Strain QM B1551 of *B. megaterium* and a derivative strain totally lacking the exosporium were used to localize the adsorbed monomeric Red Fluorescent Protein (mRFP) of the coral *Discosoma* sp., used as a model heterologous protein. Our results indicate that spores of *B. megaterium* adsorb mRFP more efficiently than *B. subtilis* spores, that the exosporium is essential for mRFP adsorption, and that most of the adsorbed mRFP molecules are not exposed on the spore surface but rather localized in the space between the outer coat and the exosporium.

Keywords: surface display, protein delivery, spores, *Bacillus megaterium*, exosporium

6.2 Introduction

Gram-positive bacteria of the *Bacillus* and *Clostridium* genera can differentiate to form an endospore (spore), a metabolically quiescent cell produced in response to conditions that do not allow cell growth (McKenney *et al*., 2012). Once released in the environment, the spore survives in its dormant state for extremely long periods, resisting to a vast range of stresses such as high temperatures, dehydration, absence of nutrients and the presence of toxic chemicals (McKenney *et al*., 2012). However, the quiescent spore is able to continuously monitor the environment and respond to the presence of water and nutrients by germinating and originating a vegetative cell that is able to grow and sporulate (McKenney *et al*., 2012). Resistance to non-physiological conditions is, in part, due to the spore surface structures. In *Bacillus subtilis*, the model system for spore formers, the spore surface is organized in a multilayered coat and in a crust, a recently discovered layer that surrounds the spore coat (McKenney *et al.*, 2012). *B. subtilis* spores are negatively charged (Huang *et al*., 2010; Pesce *et al.*, 2014) and have a relative hydrophobicity that is in part due to the glycosylation of some spore surface proteins (Cangiano *et al*., 2014; Rusciano *et al*., 2014). In several *Bacillus* and *Clostridium* species, including *B. cereus*, *B. anthracis*, *B. megaterium*, and *C. difficile*, the outermost spore structure is the exosporium, a morphologically distinct layer composed of proteins and glycoproteins that surrounds the coat (Díaz-González *et al.*, 2015; Manetsberger *et al.*, 2015b; Stewart, 2015).

The bacterial spore has been proposed as a platform to display heterologous proteins, with potential applications ranging from the development of mucosal vaccines to re-usable biocatalysts, diagnostic tools, and bioremediation devices (Knecht *et al.*, 2011; Isticato and Ricca, 2014; Ricca *et al.*, 2014). The remarkable and well documented resistance of the spore (McKenney *et al.*, 2012), the amenability of several spore-forming species to the genetic manipulation (Harwood and Cutting, 1990) and the safety record of several species (Cutting, 2011; Baccigalupi *et al.*, 2015) support the use of the spore as a

display and delivery system. Two strategies have been developed to display heterologous proteins on the spore surface. A recombinant strategy, based on the construction of gene fusions between DNA coding for a selected spore surface protein and DNA coding for the protein to be displayed, has been used over the years to display a variety of heterologous proteins (Isticato and Ricca, 2014). A non-recombinant approach, based on the spontaneous adsorption between purified spores and purified proteins, has been also used to display various enzymes and antigens (Ricca *et al.*, 2014). The molecular details controlling spore adsorption have not been fully elucidated. It is known that the adsorption is more efficient when the pH of the binding buffer is acidic (pH 4) (Huang *et al.*, 2010; Sirec *et al.*, 2012) and that a combination of electrostatic and hydrophobic interactions are likely involved in the interaction (Huang *et al.*, 2010; Sirec *et al.*, 2012). It is also known that mutant spores with severely altered spore surfaces interact more efficiently than isogenic wild type spores with model proteins (Sirec *et al.*, 2012; Donadio *et al.*, 2016).

Here, we used a fluorescent protein, the monomeric form of the Red Fluorescent Protein (mRFP) of the coral *Discosoma* sp. (Campbell *et al*., 2002), to evaluate whether spores of *B. megaterium* are able to interact with and adsorb a model heterologous protein. *B. megaterium* comprises a number of morphologically distinct strains sharing the unusual large dimensions of both cells (length up to 4 μm and a diameter of 1.5 μm) and spores (length up to 3 μm and diameter of 1 μm) (Di Luccia *et al.*, 2016). Spores of some strains of *B. megaterium* are surrounded by an exosporium, and since so far only spores of species that lack an exosporium have been considered as adsorption platforms, no data are available on the impact of the exosporium in the interaction with heterologous proteins.

The QM B1551 strain is the best-characterized strain of *B. megaterium*. This strain carries about 11% of its genome on seven indigenous plasmids (Rosso and Vary, 2005; Vary *et al.*, 2007; Eppinger *et al*., 2011), two of which – pBM500 and pBM600 – have been identified as carrying genes that are essential to the formation of this strain's distinctive "walnut-shaped" exosporium (Manetsberger *et al.*, 2015a). The protein composition of the QM B1551 exosporium is as yet poorly characterized, with only a few genes encoding orthologs of recognized exosporium protein in spores of other species being identified by genomic analyses. These include genes encoding BclA nap proteins, which form a localized nap in *B. megaterium* QM B1551 spores, plus an ortholog of the BxpB protein that forms the basal layer of the exosporium in *B. cereus* family spores. The latter appears to fulfill a different role in *B. megaterium* QM B1551 spores, since a null mutant strain retained an apparently normal exosporium (Manetsberger *et al.*, 2015a).

In this paper, we present data that demonstrates that spores of *B. megaterium* QM B1551 can efficiently adsorb mRFP, and provide evidence that protein molecules are able to cross the permeability barrier presented by the exosporium to localize in the inter-coat space.

6.3 Materials and Methods

6.3.1 Bacterial Strains, Spore, and RFP Production

The *B. megaterium* strains employed in this study are QM B1551 and its plasmid-less derivative PV361 (Rosso and Vary, 2005). The *B. subtilis* strain used in this study was PY79 (Youngman *et al*., 1984). Sporulation of all *Bacillus* strains was induced by the exhaustion method (Cutting and Vander Horn,

1990). After 30 h of growth in Difco Sporulation (DS) medium at 37°C with vigorous shaking spores were collected and purified as described by Nicholson and Setlow (1990) using overnight incubation in H_2O at 4°C to lyse residual sporangial cells. The number of purified spores obtained was measured by direct counting with a Bürker chamber under an optical microscope (Olympus BH-2 with 40× lens).

For mRFP production, cells of *Escherichia coli* strain RH161 (Donadio et al., 2016), bearing the expression vector pRSET-A carrying an in-frame fusion of the 5′ end of the *rfp* coding region to six histidine codons under the transcriptional control of a T7 promoter, were grown for 18 h at 37°C in 100 ml of autoinduction medium to express the heterologous protein (Isticato *et al.*, 2010). The His_6-tagged RFP protein was purified under native conditions using a His-Trap column as recommended by the manufacturer (GE Healthcare Life Science). Purified protein was desalted using a PD10 column (GE Healthcare Life Science) to remove high NaCl and imidazole concentrations.

6.3.2 Adsorption Reaction

Unless otherwise specified 5 μg of purified recombinant mRFP was added to a suspension of spores (5×10^8) in 50 mM Sodium Citrate pH 4.0 at 25°C in a final volume of 200 μl. After 1 h of incubation, the binding mixture was centrifuged (10 min at 13,000 g) to fractionate mRFP bound-spores in the pellet from free mRFP in the supernatant.

6.3.3 Western and Dot-Blot Analysis

Spore pellets from adsorption reactions were resuspended in 40 μl of spore coat extraction buffer (Nicholson and Setlow, 1990; Giglio et al., 2011), incubated at 68°C for 1 h to solubilize spore coat proteins and loaded onto a 12% SDS-PAGE gel. The proteins were then electro-transferred to nitrocellulose filters (Amersham Pharmacia Biotech) and used for Western blot analysis as previously reported (Isticato et al., 2008) using monoclonal mRFP-recognizing anti-His antibody (Sigma). A quantitative determination of the amount of mRFP was obtained by dot blot experiments comparing serial dilutions of purified mRFP and binding assay supernatant. Filters were then visualized by the ECL-prime method (Amersham Pharmacia Biotech) and subjected to densitometric analysis by Quantity One 1-D Analysis Software (Bio-Rad). Dot blot and relative densitometric analyses were performed three times to verify the significance of the results.

6.3.4 Fluorescence and Immunofluorescence Microscopy

Post-adsorption spores were resuspended in 50 μl of 1x PBS pH 4.0 and 5 μl of the suspension placed on microscope slides and covered with a coverslip previously treated for 30 s with poly-L-lysine (Sigma). Immunofluorescence was performed as described by Isticato et al. (2013), with the following modifications: 2.0×10^6 RFP-adsorbed spores of QM B1551 and PV361 *B. megaterium* strains were pretreated with 1% bovine serum albumin (BSA) – 1x PBS pH 4.0 for 30 min prior to 2 h-incubation at 4°C with the anti-polyHistidine antibodies (mouse; Sigma) diluted 1:20 in 1x PBS pH 4.0–1% BSA. As a control of the specificity of this technique, non-adsorbed spores were directly treated with anti-His antibodies. After three washes, the samples were incubated with a 64-fold diluted anti-mouse secondary antibody conjugated with fluorescein isothiocyanate, FITC (Santa Cruz Biotechnology, Inc.) and washed

four times with 1x PBS pH 4.0. Washed samples were resuspended in 20 µl of 1x PBS pH 4.0 and 10 µl were analyzed. All samples were observed with an Olympus BX51 fluorescence microscope fitted with a 100× UPlan F1 oil objective; U-MNG or U-MWIBBP cube-filters were used to detect the red fluorescence emission of mRFP or the green emission of FITC-conjugated antibodies, respectively. Exposure times were in the range between 200 and 3000 ms. Images were captured using an Olympus DP70 digital camera and processed with Image Analysis Software (Olympus) for minor adjustments of brightness, contrast and color balance (McCloy *et al.*, 2014). ImageJ (v1.48, NIH) was used to draw an outline around 80 spores for each strain and minimum, maximum and mean fluorescence values per pixel were recorded for each spore. Values of fluorescence intensity were displayed subsequently as box-plots with 5–95% confidence intervals (McCloy *et al.*, 2014).

6.3.5 Statistical Analysis

Results from dot blot and fluorescence microscopy analysis are the averages from three independent experiments. Statistical significance was determined by the Student *t*-test, and the significance level was set at $P < 0.05$.

6.4 Results

6.4.1 mRFP of *Discosoma* sp. is Adsorbed by *B. megaterium* Spores

To verify whether spores of *B. megaterium* QM B1551 were able to adsorb mRFP, 5 µg of the purified protein (Materials and Methods) were incubated with 5.0×10^8 purified spores. The adsorption reaction was performed in 50 mM sodium citrate at pH 4.0, as previously described (Sirec *et al*., 2012). After the reaction, spores were extensively washed with 1x PBS pH 4.0, spore surface proteins were extracted as described in Materials and Methods and analyzed by western blotting with anti-polyHistidine-Peroxidase antibody (Sigma), which recognizes the histidine-tagged N terminus of mRFP. As shown in Figure Figure1A1A, mRFP was extracted from spores, indicating that it was absorbed during the reaction and then released by the extraction treatment. To evaluate the efficiency of adsorption, we analyzed the amount of mRFP left unbound, i.e., post-adsorbed spores were collected by centrifugation and the supernatant serially diluted and analyzed by dot blotting (Figure 1B). A densitometric analysis of the dot blot (Supplementary Table 1) showed that when 5 µg of mRFP was used in the adsorption reaction less than 1% was left unbound, indicating that about 99% of the heterologous protein was adsorbed to *B. megaterium* spores. To analyze whether adsorbed mRFP molecules were tightly bound to the spore surface, post-adsorption reaction spores were washed twice with PBS buffer at pH 3.0 or pH 7.0, or with a 1M NaCl, 0.1% Triton X-100 solution as previously described (Donadio *et al.*, 2016). As shown in Figure 1C, and supported by densitometric analysis of the dot blot (Supplementary Table 2), the washes at pH 3.0 did not cause any release of the adsorbed mRFP, while the washes at pH 7.0 or with 1M NaCl, 0.1% Triton X-100 caused a minimal, less than 1%, release of mRFP molecules. Therefore, results presented in Figure 1 suggest that mRFP was efficiently adsorbed and tightly bound to *B. megaterium* spores. To assess whether spore-adsorbed mRFP molecules retained their fluorescence properties, we performed a fluorescence microscopy analysis. As shown in Figure 2, post-adsorption reaction spores were associated with a strong fluorescence signal visible around the entire spore surface.

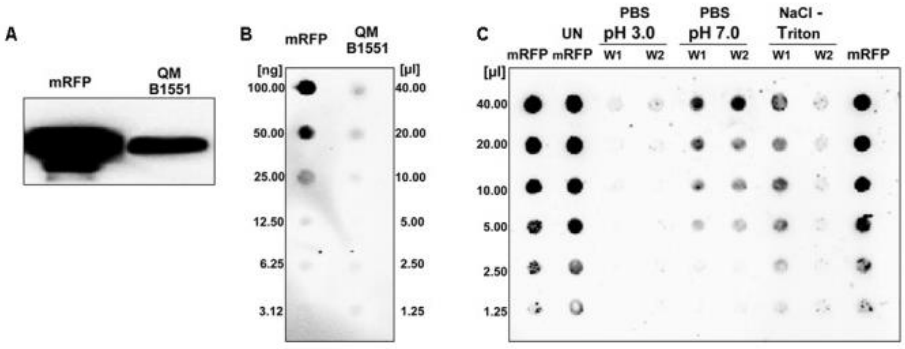

Figure 1: *Bacillus megaterium* QM B1551 spores adsorb mRFP. 5×10^8 spores were incubated with 5 µg of purified mRFP and then the samples subject to centrifugation. **(A)** Spore surface proteins were extracted from the pellet fraction by SDS-DTT treatment, fractionated on SDS-PAGE and analyzed by Western blot. Purified mRFP (20 µg) was used as a marker. **(B)** The supernatant, containing the unbound mRFP, was serially diluted and analyzed by dot blot (QM B1551). Serial dilutions of purified mRFP (mRFP) were used as a standard. **(C)** Spores adsorbed with mRFP were washed twice (W1 and W2) with PBS buffer at pH 3.0 or pH 7.0, or with a 1M NaCl, 0.1% Triton X-100 solution. Serial dilutions of purified mRFP and unbound mRFP (UN-mRFP) were used as standards. Immuno-reactions in all panels were performed with mRFP-recognizing anti-His antibody conjugated with horseradish peroxidase.

6.4.3 The Exosporium has an Essential Role in mRFP Adsorption

Strain QM B1551 of *B. megaterium* contains seven indigenous plasmids (Rosso and Vary, 2005; Eppinger *et al*., 2011) and plasmid-encoded genes are essential for exosporium formation (Manetsberger *et al.*, 2015a). PV361 is a QM B1551-cured strain lacking all seven plasmids and, as a consequence, totally lacking the exosporium (Manetsberger *et al.*, 2015a). We used spores of strain PV361 to analyze the role of the exosporium in mRFP adsorption. In parallel, we also used spores of *B. subtilis* PY79 that in a previous study have been shown to adsorb mRFP (Donadio *et al.*, 2016). To compare the adsorption efficiency of spores of the *B. subtilis* PY79 and *B. megaterium* QM B1551 and PV361 strains, we adsorbed 5 µg of purified mRFP with 5.0×10^8 spores of each of the three strains. After the adsorption reactions spores were collected by centrifugation, proteins extracted by SDS-DTT treatment and analyzed by western blotting with mRFP-recognizing anti-His antibody. As shown in Figure 3A, mRFP was apparently extracted in larger amounts from spores of QM B1551 than from spores of the other two strains. In an attempt to quantify these apparent differences, unbound mRFP from the adsorption reactions was serially diluted and analyzed by dot blotting (Figure 3B). A densitometric analysis of the dot blot of Figure 3B (Supplementary Table 3) indicated that PY79 and PV361 spores adsorbed about 90% of the total mRFP while QM B1551 spores adsorbed almost all (over 99%) purified mRFP.

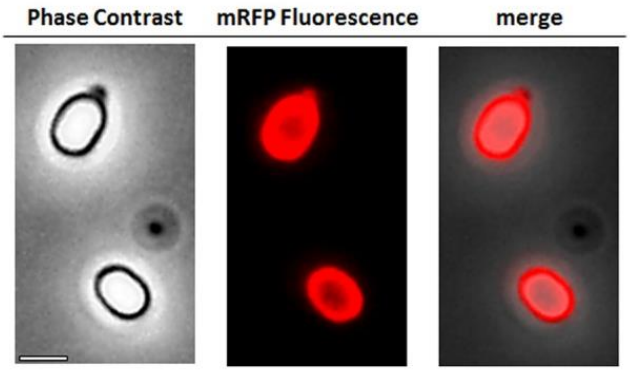

Figure 2: Fluorescence microscopy analysis of B. megaterium QM B1551-mRFP spores. QM B1551 spores incubated with mRFP (5 µg), and subsequently washed, were analyzed by fluorescence microscopy. The same microscopy field was observed by phase contrast and fluorescence microscopy. Scale bar 1 µm. The merge panel is reported. The exposure time was 200 ms.

Based on the results of Figures 3 and 4, we conclude that the exosporium, present in QM B1551 and lacking in PV361 has a relevant role in the adsorption of mRFP.

In addition, results of Figure 4 indicated that *B. subtilis* PY79 spores are more efficient than *B. megaterium* PV361 spores in adsorbing mRFP, whereas dot blotting reported in Figure 3B indicated similar adsorption efficiencies for the two strains. We believe that this discrepancy is due to a strong reduction of fluorescence when mRFP is bound to PV361 but not to PY79 or QM B1551 spores (see below).

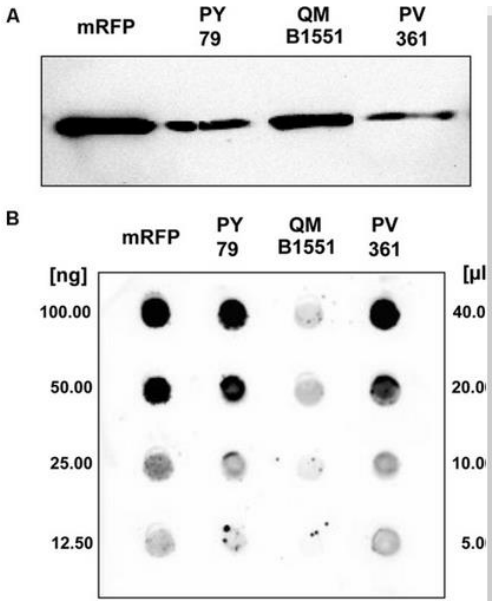

Figure 3: Monomeric Red Fluorescent Protein adsorption to spores of B. megaterium QM B1551 and PV361 and B. subtilis PY79. 5 × 108 spores of each strain were incubated with 5 µg of purified mRFP and then the samples subject to centrifugation. Spores in the pellet fractions were used to extract surface proteins that were subsequently analyzed by western blot (A), while the supernatants were serially diluted and analyzed by dot blot (B). Serial dilutions of purified mRFP were used as standards. Immuno-reactions in both panels were performed with anti-His antibody conjugated with horseradish peroxidase.

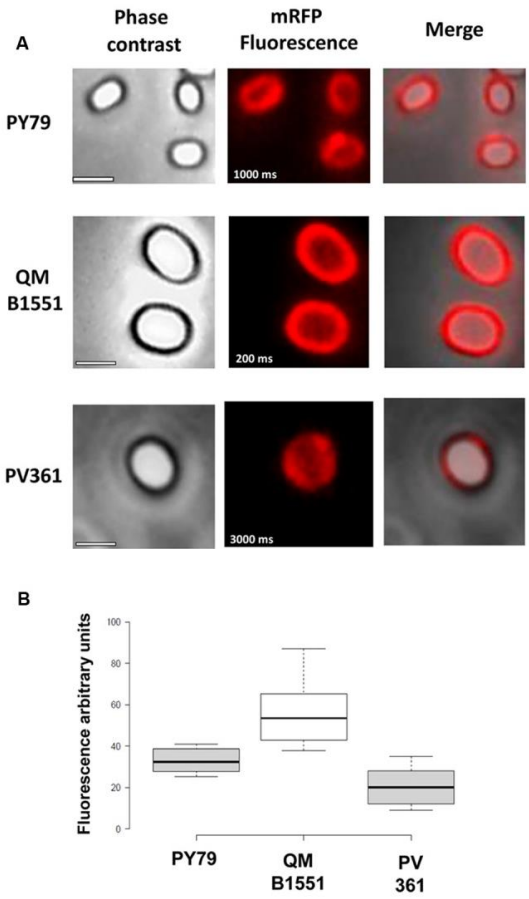

Figure 4: Efficiency of adsorption of mRFP to spores of *B. megaterium* QM B1551 and PV361 and *B. subtilis* PY79. (A) Fluorescence microscopy images of PY79, QM B1551, and PV361 spores following mRFP adsorption and washing. Exposure times are indicated. Phase contrast and red fluorescence overlays are shown (merge panel). Scale bars 1 μm. **(B)** Box plots displaying the fluorescence intensity of eighty different spores of each strain. Limits of each box represent the first and the third quartile (25 and 75%) and the values outside the boxes represent the maximum and the minimum values. The line dividing the box indicates the median value for each strain. *P* value is less than 0.0001.

6.4.4 Quantitative Assessment of mRFP Adsorption to QM B1551 Spores

Dot blot experiments (**Figure 3B**) indicated that when 5 μg of purified mRFP was used in adsorption reactions with 5.0×10^8 spores of the QM B1551 strain almost all heterologous protein was bound to the spore. In order to define the maximal amount of mRFP that can be adsorbed to QM B1551 spores, we repeated the reactions with increasing concentrations of purified mRFP, i.e., 5.0×10^8 QM B1551 spores were reacted with 5, 10, 20, 40, 80, and 160 μg of purified mRFP. After the reactions spores were collected by centrifugation and the supernatants containing unbound mRFP were serially diluted and analyzed by dot blotting (Figure 5A). Figure 5B displays the results of a densitometric analyses of the dot blot, which indicates that when 5–80 μg of mRFP was reacted with 5×10^8 spores, the percentage of protein bound to spores was over 90%. A decrease of bound mRFP was observed when 160 μg of purified protein were used in the reaction. However, even when 160 μg of purified mRFP was used over 60% of the protein was absorbed, indicating that 5.0×10^8 spores of QM B1551 can adsorb about 100 μg of mRFP.

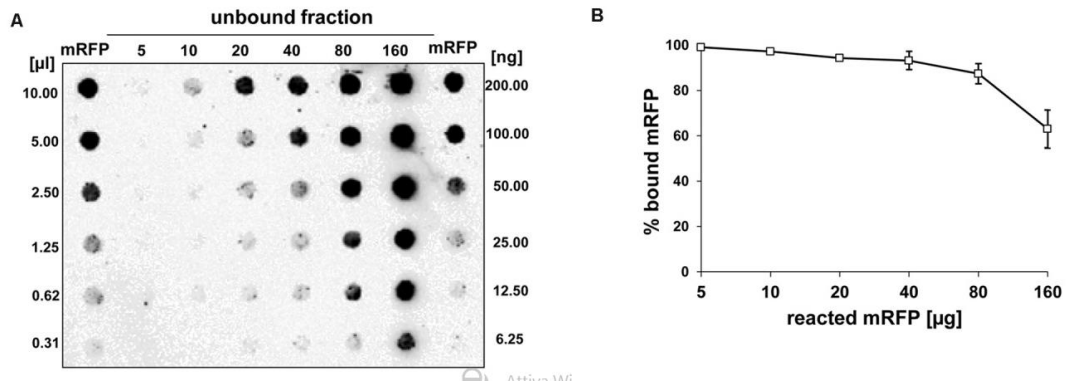

Figure 5: Quantitative assessment of mRFP adsorption to *B. megaterium* QM B1551 spores. 5×10^8 spores were incubated with 5, 10, 20, 40, 80, and 160 µg of purified mRFP. The reaction mixtures were subsequently subject to centrifugation and the supernatants serially diluted and analyzed by dot blot **(A)**. Serial dilutions of purified mRFP were used as standards. Immuno-reactions in both panels were performed with anti-His antibody conjugated with horseradish peroxidase. (**B**) Percentage of mRFP adsorbed to spores after reaction with defined amounts of endogenous mRFP. Error bars show the standard errors of the mean from three experiments and the *P* value never above 0.0025.

6.4.5 mRFP Localizes to the Inter-Coat Space in *B. megaterium* QM B1551 Spores

An immuno-fluorescence microscopy approach was employed to assess whether adsorbed mRFP molecules were exposed on the surface of *B. megaterium* QM B1551 spores. QM B1551 spores adsorbed with various amounts of mRFP were reacted with monoclonal anti-His antibody recognizing the recombinant mRFP, then with fluorescent anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc.) and observed under the fluorescence microscope (Figure 6). With the lowest amount of mRFP used in this experiment (2 µg) the mRFP fluorescence signal (red) was observed all around the spore while the immunofluorescence signal (green) was weak and mainly concentrated at the spore poles, suggesting that only in those points mRFP was exposed on the spore surface. Increasing the amount of mRFP used in the reaction the number of green spots increased (5 and 10 µg) and with highest amount of mRFP used in the reaction (20 µg) an almost complete green ring was observed around the spores. Based on the results presented in Figure 6, we hypothesized that mRFP molecules infiltrate through the exosporium and localizes in the inter-coat space between the outer coat and the exosporium, i.e., when a low amount of mRFP is used almost all protein molecules are internal to the exosporium and are available to the antibody at only a few locations. Increasing amounts of mRFP in adsorption reactions results in the inter-coat space "filling up," until ultimately more mRFP molecules are available to the antibody on the spore surface. This hypothesis implies that if the exosporium is lacking then all mRFP should be available to the antibody. To test this, we compared by immunofluorescence microscopy equal numbers of spores of QM B1551 (with exosporium) and of PV361 (without exosporium) incubated with the same amount of mRFP (5 µg). When the exosporium was present (QM B1551) mRFP was only partially available to the antibody and green spots were observed **(**Figure **7** and Supplementary Figure S2). When the exosporium was not present (PV361) adsorbed mRFP was available to the antibody all around the spore and a complete green ring was formed, supporting the hypothesis that mRFP is internal to the exosporium in QM B1551 spores.

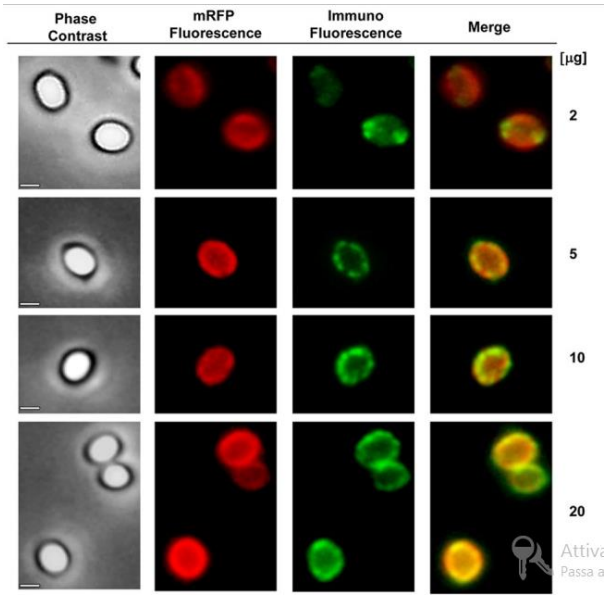

Figure 6: Immunofluorescence analysis of mRFP-adsorbed *B. megaterium* QM B1551 spores. Aliquots of 5×10^8 QM B1551 spores were incubated with variable concentrations of mRFP and were subsequently analyzed by phase contrast, fluorescence and immunofluorescence microscopy, as described in the Materials and Methods. The same microscopy field for each reaction is reported together with the merge panel. The exposure time was 200 ms for all images. Scale bar, 1 µm.

While QM B1551 spores used in the experiments of Figure 7 showed a complete red fluorescent ring as in Figure 2, PV361 spores showed a very weak red fluorescent signal. With PV361 spores a red signal was only observed using long exposure times at the fluorescence microscope (Figure 4). Since mRFP is present around PV361 spores (Figures 3 and **7**), we conclude that mRFP fluorescence is weakened when the protein is adsorbed to PV361 spores. Further experiments will be needed to fully address this point.

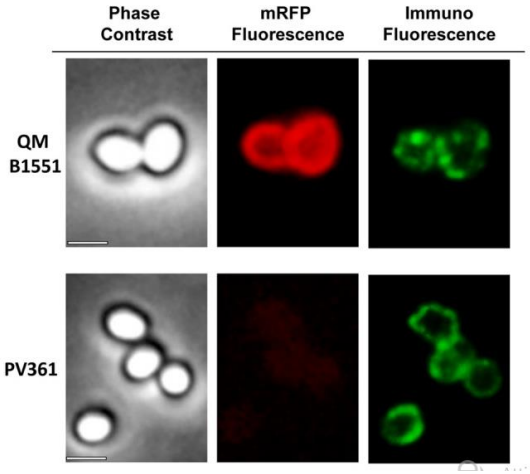

Figure 7: Immunofluorescence of mRFP adsorbed to *B. megaterium* QM B1551 and PV361 spores. 5×10^8 QM B1551 and PV361 spores were incubated with 5 µg mRFP and then analyzed by immunofluorescence microscopy, as described in the Materials and Methods. For each field phase contrast and immunofluorescence microscopy are shown. The exposure time was 200 ms for all images. Scale bar, 1 µm.

6.5 Discussion

The main findings of this report are that spores of *B. megaterium* are extremely efficient in adsorbing the heterologous model protein mRFP, that the exosporium has an important role in this process, and that mRFP molecules infiltrate through the exosporium localizing between the outer coat and the exosporium. These results expand previous work performed on spores of *B. subtilis* and demonstrate that spores of a different species may also be used to deliver heterologous proteins via the adsorption method. The high efficiency of adsorption observed with *B. megaterium* spores is in part due to the large size of its spore compared with that of *B. subtilis.* Indeed, the *B. megaterium* spore surface area is about 2.5-fold larger than the *B. subtilis* spore, with a surface of 5.33 μm^2 (h: 1.60 ± 0.16 w: 0.84 ± 0.07) vs. 1.98 μm^2 (h: 1.07 ± 0.09 w: 0.48 ± 0.03). The large dimensions allow the adsorption of up to 100 μg of mRFP when 160 μg of protein are reacted with spores.

The observation that mRFP crosses the exosporium indicates that it is permeable to mRFP, a 27 kDa protein. Permeability of the exosporium is not totally surprising since germinants present in the environment have to cross the external layers of the spore to reach their receptors, albeit germinants are typically small molecules with molecular masses typically <200 Da. Additionally, the mRFP data are broadly in agreement with the results of previous studies conducted with labeled dextrans and related molecules (Koshikawa *et al.*, 1984; Nishihara *et al.*, 1989). In those studies, the *B. megaterium* QM B1551 exosporium was suggested to represent a permeability barrier to molecules with molecular weights greater than 100 KDa, while influencing the passage of molecules with masses somewhere between 2 and 40 kDa (Koshikawa *et al*., 1984; Nishihara *et al.*, 1989).

An interesting challenge for future work will be to establish the mechanism or route of infiltration that mRFP, and by inference other heterologous proteins of interest, takes to enter the inter-coat space. Examination by transmission electron microscopy of sectioned *B. megaterium* QM B1551 spores indicates that the exosporium comprises two identical "shells" (Manetsberger *et al*., 2015a), and it may be that the interface between each of these structures (described as "apical openings" in early papers) permits ingress of relatively large molecules. Discerning the basis for the apparent loss of mRFP fluorescence upon adsorption to PV361 spores, and whether mRFP molecules are able to infiltrate the outer coat layers, as observed for *B. subtilis* spores (Donadio *et al.*, 2016), will also be of interest.

In the current study, we hypothesize that mRFP molecules preferentially cross the exosporium and accumulate in the inter-coat space between the outer coat and the exosporium. In this model, mRFP molecules are only adsorbed and displayed on the spore surface once adsorption sites (or volumetric capacity?) in the inter-coat space are sufficiently occupied. This implies that the adsorption approach to surface display can be used with *B. megaterium* QM B1551 spores, although the system is dependent on the spore to protein ratio used in adsorption reactions. Since various strains of *B. megaterium* have long been used industrially for the production of enzymes such as amylases and dehydrogenases, vitamins and antimicrobial molecules (Vary *et al.*, 2007), our data suggest a new biotechnological application for the *B. megaterium* spore as a vehicle to bind and deliver heterologous proteins.

6.6 References

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6.7 Supplementary Material

TABLE S1: Densitometric analysis of dot blot experiments with the supernatants of the adsorption reaction with QM B1551 spores (Figure 1B).

Densitometric analysis of dot blot experiments with the supernatants of the adsorption reaction with QM B1551 spores (fig.1B)

mRFP source	Amount of sample used	Density (OD/mm2) [a]	Amount of mRFP (ng) [b]	mRFP µg (% total)
Purified	100.00 ng	565.31	NA	NA
mRFP	50.00 ng	279.67	NA	NA
	25.00 ng	139.18	NA	NA
	12.50 ng	56.84	NA	NA
	6.25 ng	38.73	NA	NA
unbound	40.0 µl	89.00	16.69	0.50 (0.55%)
mRFP	20.0 µl	77.31	11.05	
	10.0 µl	47.30	6.95	

[a] Density measured by optical density (OD) per square millimeter and obtained by ChemiDocXRS apparatus with Quantity-One software (Bio-Rad).
[b]Calculated from signals (density OD/mm2) obtained with purified mRFP.
NA, not applicable.

TABLE S2: Densitometric analysis of dot blot experiments with the supernatants of various washes after the adsorption reaction (Figure 1C).

mRFP source	Amount of sample used	Density (OD/mm2) [a]	Amount of mRFP (ng) [b]	mRFP µg (% total)
Purified	50.0 ng	989205.3	NA	NA
mRFP	25.0 ng	487172.1	NA	NA
	12.5 ng	281015.5	NA	NA
	6.25 ng	170208.0	NA	NA
unbound	10.0 µl	1083584.0	55.17	1.125 (11%)
mRFP	5.0 µl	639823.0	31.83	
	2.5 µl	263354.4	12.40	
Wash 1	10.0 µl	14893.5	NA	NA
PBS pH3	5.0 µl	7695.1	NA	NA
Wash 2	40.0 µl	20188.1	NA	NA
PBS pH3	20.0 µl	9235.0	NA	NA
Wash 1	20.0 µl	252555.0	11.50	0.059 (0.59%)
PBS ph7	10.0 µl	147062.6	6.00	
	5 µl	68093.6	2.12	
Wash 2	10.0 µl	154130.0	6.53	0.055 (0.55%)
PBS pH7	5 µl	68093.6	2.45	
Wash 1	40.0 µl	374963.4	18.00	0.046 (0.46%)
0.1% Triton	20.0 µl	212687.3	9.30	
0.1M NaCl				
Wash 2	40 µl	65720.4	1.70	0.0037 (0.37%)
0.1% Triton	20 µl	46127.0	0.62	
0.1M NaCl				

[a] Density measured by optical density (OD) per square millimeter and obtained by ChemiDocXRS apparatus with Quantity-One software (Bio-Rad).
[b]Calculated from signals (density OD/mm2) obtained with purified mRFP.
NA, not applicable.

TABLE S3: Densitometric analysis of dot blot experiments with the supernatants of the adsorption reaction performed with spores of strains PY79, QM B1551, and PV361 (Figure 3B).

Densitometric analysis of dot blot experiments with the supernatants of the adsorption reaction performed with spores of strains PY79, QM B1551 and PV361 (fig. 3B)

mRFP source	Amount of sample used	Density (OD/mm2) [a]	Amount of mRFP (ng) [b]	mRFP µg 200 µl (% total)
Purified	100.0 ng	344.07	NA	NA
mRFP	50.0 ng	167.22	NA	NA
	25.0 ng	86.72	NA	NA
	12.5 ng	42.70	NA	NA
PY79	40.0 µl	642.27	189.5	4.81 (9.62%)
	20.0 µl	322.82	97.02	
	10.0 µl	180.45	55.83	
QMB155	40.0 µl	32.17	10.92	0.27 (0.55%)
	20.0 µl	14.80	5.64	
PV361	20.0 µl	344.95	96.15	5.23 (10.46%)
	10.0 µl	192.56	52.32	
	5.0	89.75	30.52	

[a] Density measured by optical density (OD) per square millimeter and obtained by ChemiDocXRS apparatus with Quantity-One software (Bio-Rad).
[b]Calculated from signals (density OD/mm2) obtained with purified mRFP.
NA, not applicable.

TABLE S4: Densitometric analysis of dot blot experiments with the supernatants of the adsorption reaction performed with different amounts of mRFP.

Densitometric analysis of dot blot experiments with the supernatants of the adsorption reaction performed with different amounts of mRFP

mRFP source	Amount of sample used	Density (OD/mm2) [a]	Amount of mRFP (ng) [b]	mRFP µg (% total)
Purified	200.0 ng	497377.00	NA	NA
mRFP	100.0 ng	234815.52	NA	NA
	50 ng	130591.46	NA	NA
	25 ng	39776.74	NA	NA
	12.5 ng	21699.47	NA	NA
	6.25 ng	12754.88	NA	NA
	3.12 ng	7494.43	NA	NA
5 µg	10.0 µl	3336.70	NA	NA
	5.0 µl	1942.96	NA	
10 µg	10.0 µl	15553.08	12.76	0.28 (2.8%)
	5.0 µl	8226.25	7.66	
20 µg	10.0 µl	98835.80	58.70	1.14 (5.7%)
	5.0 µl	34504.54	23.28	
	2.5 µl	17289.19	13.80	
40 µg	1.25 µl	16725.41	13.35	2.72 (6.8%)
	0.625 µl	12065.26	10.34	
80 µg	2.5 µl	223133.86	127.45	10.88 (13.6%)
	1.25 µl	122514.80	72.36	
160 µg	2.5 µl	903113.64	502.31	58.8 (37%)
	1.25 µl	422611.05	238.22	
	0.625 µl	220020.65	123.15	

[a] Density measured by optical density (OD) per square millimeter and obtained by ChemiDocXRS apparatus with Quantity-One software (Bio-Rad).
[b]Calculated from signals (density OD/mm2) obtained with purified mRFP.
NA, not applicable.

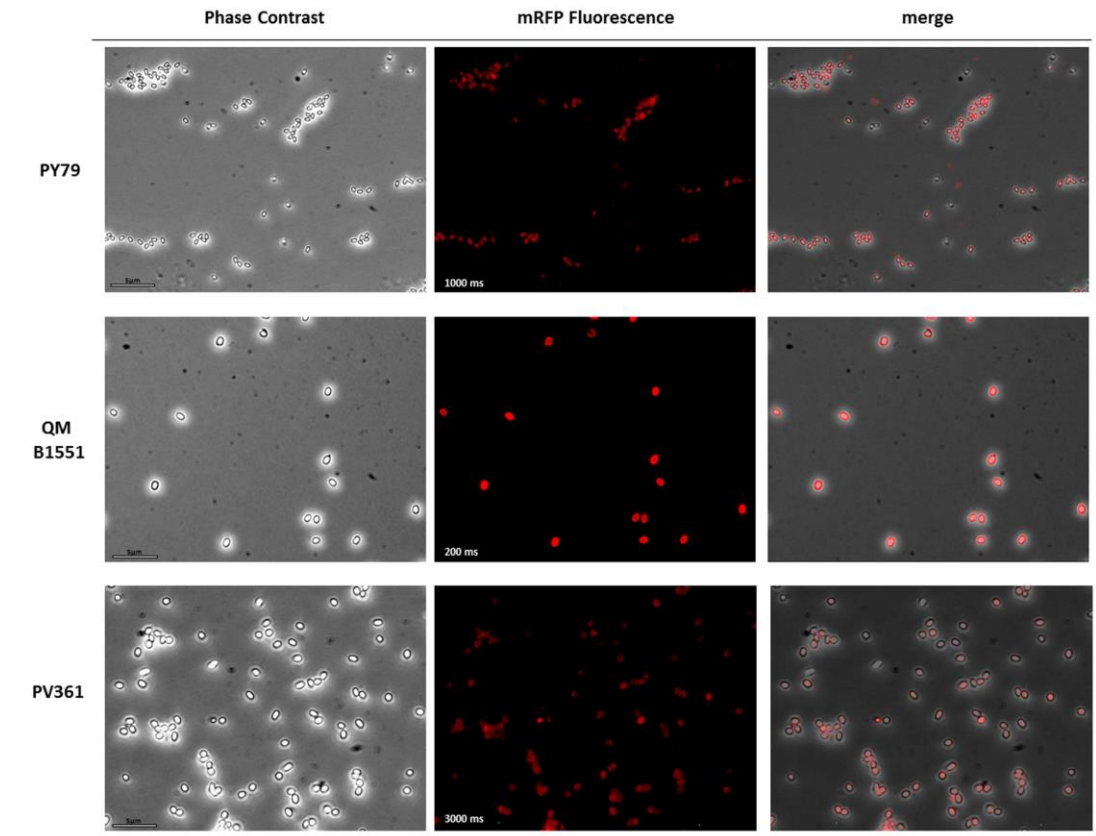

FIGURE S1: Whole field images of fluorescence microscopy analysis reported in Figure 4. The same microscopy field was observed by phase contrast and fluorescence microscopy. Scale bar 5 μm. The merge panel is reported. The exposure tie is indicated.

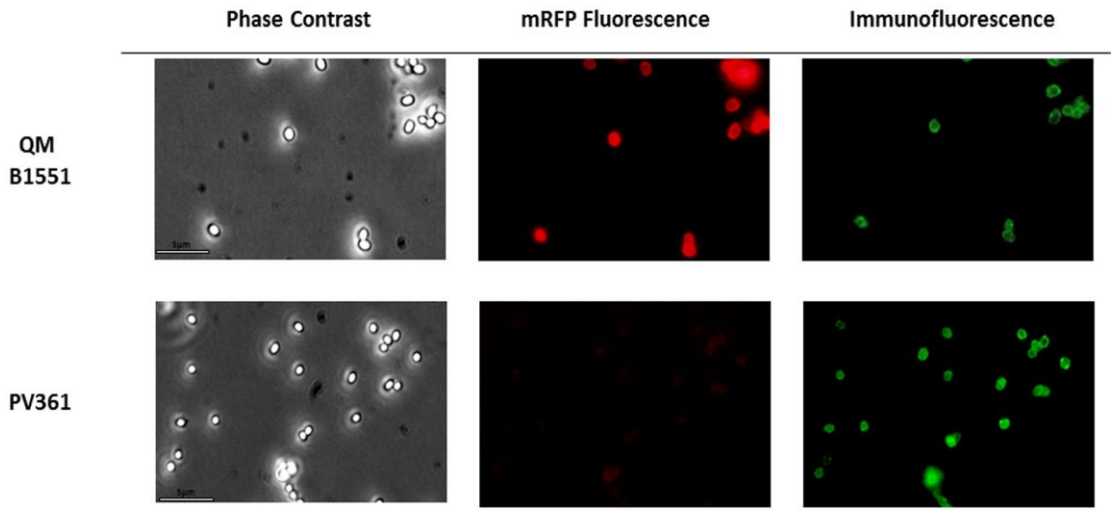

FIGURE S2: Whole field images of Immunofluorescence analysis reported in Figure 7. The same microscopy field was observed by phase contrast and fluorescence microscopy. Scale bar 5 μm. The exposure time was 200 ms for all images.

CHAPTER 7

Conclusions

7. Conclusions

My PhD work, summarized in this Thesis, allows me to draw several conclusions. The first part of the Thesis (Chapters 2-4) clarified some aspects of the structure and function of the spore surface of *B. subtilis*. In particular I worked on the antagonistic role of the proteins CotG and CotH for the efficiency of germination and for the assembly of other coat components (Chapter 2), on the structure of CotG and the identification of a CotG-like family of proteins, conserved in all spore-forming *Bacilli* (Chapter 3) and on the interaction between CotH and a major morphogenetic regulator of the spore coat, CotE (Chapter 4). This part of the Thesis clearly indicates that the structure of the spore coat of *B. subtilis* and the mechanisms controlling its assembly, although studied for several years, are still far from being totally solved. This, in turn, indicates that *B. subtilis* and the surface of its spore are still valid model systems to study gene expression, protein-protein interactions and assembly of sub-cellular structures in bacteria. In more details, Chapter 2 shows that two *B. subtilis* genes, adjacent but divergent on the chromosome, code for two products with a strictly related function, with one, CotG, having a negative effect on the spore structure and function and the other, CotH, counteracting this effect. My work showed that CotG is a highly phosphorylated protein and suggested that CotH could be the kinase responsible of CotG phosphorylation. This hypothesis has been then confirmed by *in vitro* and *in vivo* studies published after the publication of the results reported in Chapter 2 (Nguyen *et al.* 2016). It is now possible to hypothesize that the unphosphorylated form of CotG, present when CotH is lacking, has a negative effect on germination and assembly of some spore coat components. Chapter 3 shows that a CotG-like protein is present in all spore-forming Bacilli. Although the primary structure is not conserved, all the typical structural properties found in CotG are conserved. This suggests that members of this protein family have an essential function for the spore structure. We hypothesize that such function could be related to the ability of spores to respond to humidity levels (Sahin *et al.* 2012). Experiments are currently in progress to verify this hypothesis in the lab where I performed my Thesis work. Chapter 4 reports a detailed analysis of the interaction between two proteins with a regulatory role for spore formation, CotH and CotE. Pull-down experiments firstly showed that the two proteins directly interact, and then the analysis of deletion and point mutations allowed me to dissect CotE and identify the amino acid residues responsible of the interaction with CotH.

The second part of the Thesis (Chapters 5 and 6) focused on *B. megaterium* a spore-forming species far less studied than *B. subtilis*. Spores of this species are characterized by the presence of an exosporium, an additional protective layer lacking in *B. subtilis* spores. My work allowed the characterization of a protein present on the surface of *B. megaterium* spores that has an essential role for exosporium formation and the definition of the role of the exosporium in ensuring an efficient spore germination and resistance to toxic chemicals (Chapter 5). This part of my work opens to a better understanding of the structure and function of the exosporium, a structure still poorly characterized. The final chapter of this Thesis explored the possibility of using spores of *B. megaterium* to display heterologous proteins. These spores were proved to be much more efficient than spores of *B. subtilis*, opening extremely interesting perspectives for the biotechnological use of this bacterial species.

Summary

This PhD Thesis reports the results of my research work in the laboratory of Prof. Loredana Baccigalupi at the Department of Biology of the Federico II University of Naples, Italy. During these three years I focused on two bacteria of the same genus, *Bacillus subtilis* and *Bacillus megaterium*. These organisms are Gram-positive, aerobic, spore formers and, therefore, share the ability to undergo a complex developmental cell differentiation process that led to the production of highly resistant spores (Tan and Ramamurthi, 2014). When cells of this genus can not grow vegetatively because of nutrient starvation or other unfavorable environmental conditions, enter the irreversible program of spore formation (sporulation). The start of this differentiation process is an asymmetric cell division that produces a large mother cell and a small forespore. The mother cell contributes to forespore maturation and undergoes autolysis at the end of the process, allowing the release of the mature spore into the environment. The peculiar structure of the spore, characterized by a dehydrated cytoplasm surrounded by various protective layers, is responsible of the resistance of the spore to extremes of heat and pH, to UV radiations, and to the presence of solvents, hydrogen peroxide and lytic enzymes. In the presence of water, nutrients and favorable environmental conditions the mature spore can germinate generating a cell able to grow and, eventually, to re-sporulate (Setlow P., 2003).

Spore resistance is in part due to the presence of the spore coat, a multilayered structure composed of more than 70 proteins, that surrounds the spore (McKenney *et al*. 2013). Transcription of genes coding for coat components is controlled by a cascade of transcription factors including two sigma factors of the RNA polymerase, SigE and SigK, and at least three regulators, SpoIIID, GerR and GerE, all active in the mother cell (Henriques and Moran, 2007; Cangiano *et al.*, 2010). In addition, a subset of coat protein with both a structural and a regulatory (morphogenetic) role controls the assembly of the various coat components (Henriques and Moran, 2007; McKenney *et al*., 2013). The spore coat is then a complex subcellular structure and is an interesting model system to study mechanisms of gene expression and of protein-protein interaction in bacteria.

The spore surface of the two bacterial species I used for my PhD work substantially differ from each other. *B. subtilis* is the model system for spore formers and the surface of its spore is extremely well characterized, while not much is known about the surface of the *B. megaterium* spore. One main difference between the spores of the two species is the presence in *B. megaterium* of an exosporium, a poorly characterized outermost spore layer not present around the *B. subtilis* spore.

My PhD project focused on the molecular characterization of the spore surface of *B. subtilis* and of *B. megaterium* and this Thesis is organized in two parts: the **first part**, **Chapters 2-4**, focused on the role of three coat components, CotG, CotH and CotE, in the assembly of the spore coat in *Bacillus subtilis*. The **second part** of the Thesis, **Chapters 5** and **6,** focused on the spore coat of *B. megaterium* and on the use of its spore as a platform to display heterologous proteins.

PART 1: Spore Coat Assembly in *Bacillus subtilis*

CHAPTER 2 and **3** reported the study of the role of two coat components, CotH and CotG. CotH has been previously identified and partially characterized in prof. Baccigalupi's lab. It has a structural and also a regulative role being responsible of the assembly of at least nine other coat components and

important to ensure the development of spores able to germinate efficiently (Naclerio *et al.* 1996; Zilhao *et al.* 1999). CotG is one of the CotH-dependent proteins that, in turn, regulates the assembly of the mature form of CotB. The working model reporting the genetic dependency between these Cot proteins is reported in figure 1.

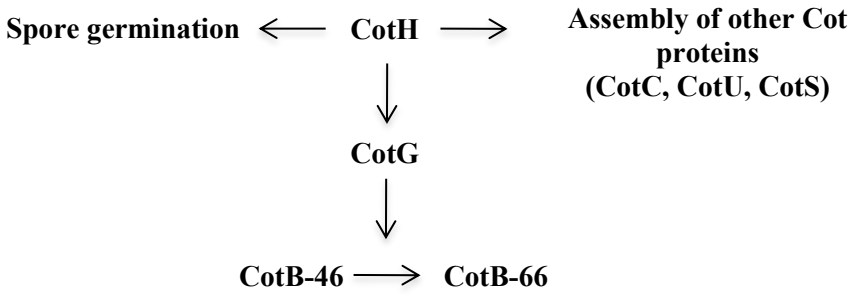

Figure 1: Working model showing the interaction among the indicated proteins. CotH has a positive effect on the germination efficiency and on the assembly of CotG, CotC, CotU and CotS. CotG in turn controls CotB maturation from the immature CotB 46 kDa to the mature CotB-66 form. (Isticato R. *et al*., 2004; Zilhao R. *et al*, 2004; Baccigalupi L. *et al*., 2004)

The genes *cotG* and *cotH* are adjacent and divergently transcribed. Moreover, it has been previously shown by this research group that the two genes have an unusual transcriptional organization with *cotH* promoter located more than 800 bp upstream from its coding region and *cotG* entirely comprised in this untranslated *cotH* region (figure 2 , Giglio *et al.* 2011).

A consequence of this peculiar gene organization is that all so far characterized knock-out *cotG* mutants were also impaired in *cotH* expression and were then double *cotG cotH* mutants. Therefore, I decided to construct a *cotG* mutant in which *cotH* expression was not affected. The analysis of the single *cotG* mutant showed that in absence of CotG the spore had a normal germination efficiency and that CotB is assembled in its immature CotB46 form (see figure 1),

indicating that CotG was necessary for CotB maturation, as already reported. The unexpected result was that while the *cotH* single mutant showed a series of defects in spore germination and coat proteins assembly, the *cotG cotH* double mutant did not show any of the defects associated with the absence of CotH and the spore was similar to the wild type. This results indicated that all the phenotypes so far attributed to the absence of CotH (and visible in the single *cotH* mutant) were actually due to the presence of CotG that only in absence of CotH exerted its negative effect. Therefore we suggested that

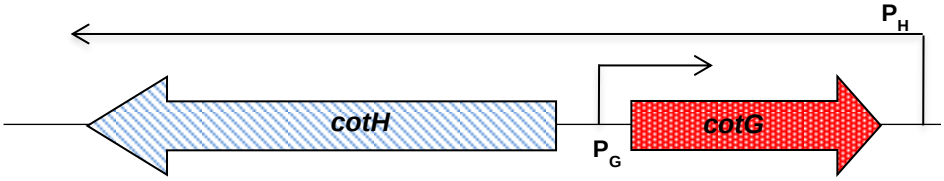

Figure 2: Schematic representation of the *cotH-cotG* locus in *Bacillus subtilis*

CotH had a protective role counteracting the negative effect of CotG and proposed the new working model reported in the figure 3. These data are reported in Chapter 2 and have been published in 2014:

> **Saggese A.**, Scamardella V., Sirec T., Cangiano G., Isticato R., Pane F., Amoresano A., Ricca E., Baccigalupi L. 2014. *Antagonistic role of CotG and CotH on spore germination and coat formation in Bacillus subtilis.* PLoS ONE 9(8):e104900.

The mechanism by which CotH counteracts CotG negative effect is not known. Other results of my work, reported in chapter 2, showed that CotG is a peculiar protein containing positively charged aminoacid repeats in its central part that are highly phosphorylated. We hypothesized that CotH could be the kinase responsible of such CotG phoshorilation, on the base of CotH homology with Ser-Thr Kinases family. A recent paper by Nguyen *et al* (2016) effectively demonstrated with *in vitro* and *in vivo* data, that CotH is a kinase and that CotG is one of its targets. We proposed that the unphosphorilated form of CotG, as it is in the *cotH* mutant, exerts the negative effect on coat proteins assembly and on germination. Therefore, in a *wt* strain, CotH is able to modify CotG by phosphorilation, counteracting its negative action.

Then we asked if also in *Bacillus* species other than *B. subtilis* could be hypothesized a similar mechanism of interaction between the two coat proteins. A bioinformatic analysis performed aligning the amino acid sequences of CotH and CotG with the available databank showed that CotH is highly conserved among all *Bacillus* species while CotG is conserved only in 5 species (Giglio *et al.* 2011). I decided to analyze the transcriptional organization of the *cotH* locus in the available genomes of *Bacilli* that do not contained a *cotG* homologue. The analysis evidenced that in all cases, they contained a divergent gene upstream to *cotH* (Saggese *et al.* 2014). The genes coded for a protein with a primary sequence not homologous to CotG but conserving a series of typical features of CotG and in particular: i) the presence of a central region composed by several random-coiled repeats similar to each other; ii) a high isoelectric point due to the presence of positively charged aminoacids; iii) a disordered structure and an aminoacid sequence that resulted a good substrate of phosphorylation, as suggested by bioinformatic analysis.

Beside the absence of a homologous primary amino acid sequence, because of the structural similarities and of the common chromosomal organization, we referred to these proteins as belonging to a new family of CotG-like proteins highly conserved in the *Bacillus* genera (figure 4).

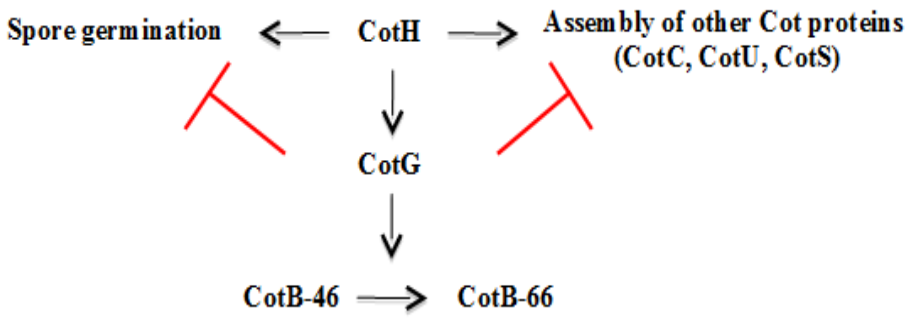

Figure 3: New model representing the Cot proteins interaction network. The red lines indicate the negative effect of CotG on CotC, CotU, and CotS assembly and on the spore germination efficiency.

Figure 4: Repeats of CotG and CotG-like proteins. The tandem repeats of CotG of *B. subtilis* (18) and of CotG-like proteins found in the indicated species are shown. *B. subtilis* CotG modules are boxed. Positively charged amino acids are in red.

The presence of a common structure of the CotG-like proteins, prompted me to investigate about the function of such modules and I addressed the point in our model species *Bacillus subtilis*. I constructed and analyzed a series of deletion mutants:

1) expressing the N-terminal and the C-terminal regions of CotG, lacking the central repeats;
2) expressing only the N-terminal region of CotG;
3) expressing only the C-terminal region of CotG.

The analysis of these mutants allowed me to conclude that:

1. the N- and the C- terminal regions were sufficient to guarantee the maturation of CotB-46 (immature form) into CotB-66 (mature form);
2. the central region composed by the aminoacid repeats was responsible of the CotG negative effect on germination and on CotC, CotU and CotS assembly (see Chapter 2).

These data are reported in Chapter 3 and published in 2016:

> **Saggese A.**, Isticato R., Cangiano G., Ricca E. and Baccigalupi L. 2016. *CotG of Bacillus subtilis is a modular protein of spore forming Bacilli.* J Bacteriol. 198(10):1513-20.

The CotH protein also interacts with CotE, another morphogenetic protein involved in the assembly of the outer surface

layer around the spore. CotE plays a crucial role in the assembly of outer coat and crust: without it, these layers are not assembled (Zheng *et al.* 1988). Several previous studies demonstrated that CotE regulates CotH assembly which in turn controls the assembly of other coat proteins (Nacleiro *et al.* 1996; Zilhao *et al.* 1999).

I reported here that CotH also controls the assembly of CotE and this mutual dependency is due to a direct interaction between the two proteins. A collection of deletion mutants of *cotE* were used to show

that the C-terminus of CotE is responsible of the interaction with CotH and new mutants have been constructed in order to define the aminoacids involved.

These results are reported in **chapter 4** and published in the paper:

> Isticato R., Sirec T., Vecchione S., Crispino A., **Saggese A.**, Baccigalupi L., Notomista E., Driks A. and Ricca E. 2015. *The direct interaction between two morphogenetic proteins is essential for spore coat formation in Bacillus subtilis.* PLoS ONE. 10(10):e0141040.

PART 2: Spore Coat Structure in *Bacillus megaterium*

Part of my work focused on the spore coat of *B. megaterium* and on the use of its spore as a platform to display heterologous proteins. As already mentioned in the introduction the spores produced by *B. megaterium* present an additional layer outside the spore coat, called exosporium (Di Luccia *et al.* 2016), that is absent in *B. subtilis*. The strain QM B1551 represents the best studied strain of *B. megaterium* and is considered as a model for this species. Several studies aimed at the characterization of the QM B1551 spore coat and exosporium have been published but a full elucidation of the mechanisms and the proteins controlling the process is still needed. In my studies on the composition, architecture and assembly of the *B. megaterium* spore coat and exosporium I considered the *B. megaterium* strain SF185, isolated by this research group from ileal biopsy of human volunteers (Fahkry *et al.* 2008).

As first I conducted a proteomic analysis to identify major *B.megaterium* SF185 coat and exosporium proteins. To this aim the spores have been treated with SDS-dithiothreitol (DTT) to extract external proteins that have been fractionated on SDS-PAGE and visualized by Coomassie blue staining (figure 5A).The most intense band, of about 16 kDa, has been purified, was used to detect the N-terminal sequence by the Edman degradation reaction and found to correspond to a hypothetical protein encoded by *SF185_1531* gene. In order to investigate on its role in the spore surface assembly I constructed a knock out mutant introducing the plasmid pSOCam1 in its structural gene by a single crossing over event causing the interruption of the *SF185_1531* gene (Figure 5B). Then the purified mutant spores

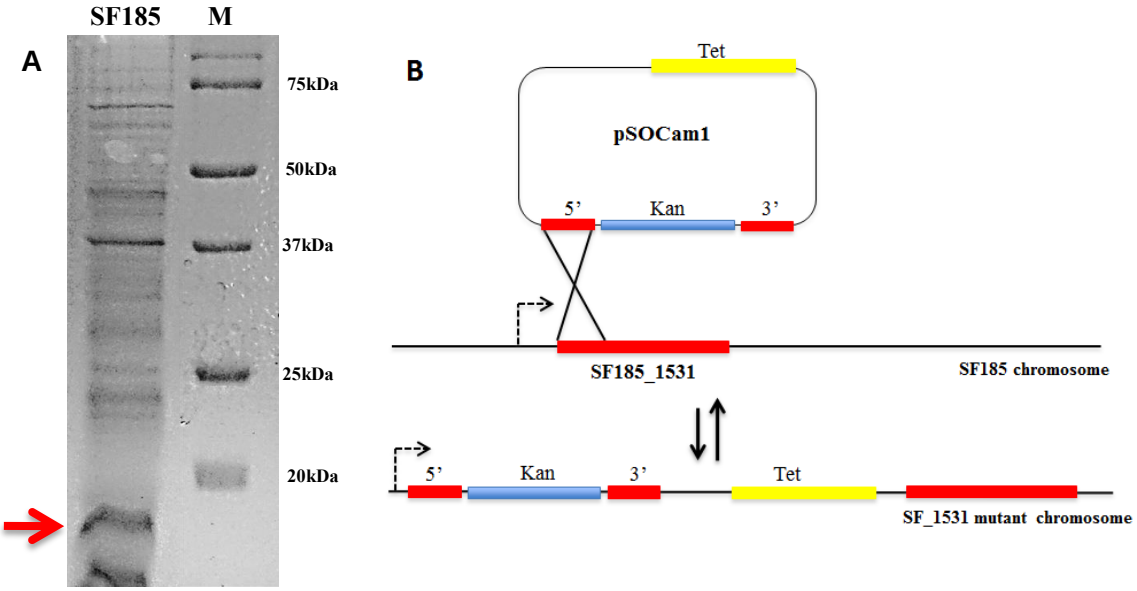

Figure 5A: pattern of spore surface proteins extracted from *Bacillus megaterium* strain SF185 (lane 1); molecular marker are shown in lane 2. The red arrow indicates the band corresponding to the SF_1531 protein. **Figure 5B:** Schematic representation of the cloning strategy used to obtain the SF185_1531 null mutant strain.

were analyzed by thin section microscopy (Figure 6A and 6B).

In comparison to the wt, the mutant spores presented an intact spore coat but they totally lacked the exosporium. This result suggested that the protein had a central role in controlling directly or indirectly the exosporium assembly. To verify that the observed phenotype was due to the inactivation of the single SF_1531 gene and to exclude the presence of secondary unexpected mutation, I decided to obtain a revertant strain inducing the excision of the plasmid from the *SF_1531* mutant chromosome.

As reported in figure 6C the revertant strain produced spores similar to the wt and normally able to produce the exosporium indicating that the protein SF_1531 plays a crucial role in the exosporium formation in *B. megaterium* strain SF185. A manuscript reporting these results is in preparation (see chapter 5).

In the chapter 6 of the Thesis I investigated on the possibility to use *B. megaterium* spores as support for the binding of external molecules with diverse biological activities. Until now *Bacillus subtilis* spores have been proposed as a platform to display heterologous proteins, with potential applications ranging from the development of mucosal vaccines to re-usable biocatalysts, diagnostic tools and bioremediation devices (Knecht *et al*., 2011; Isticato and Ricca, 2014; Ricca *et al*., 2014). To test the binding ability of *B. megaterium* spores, I used the Red Fluorescent Protein (mRFP) of the coral *Discosoma sp.* (Campbell *et al*., 2002), and *B. megaterium* QM B1551 as models. The data indicated that mRFP was efficiently adsorbed and tightly bound to *B. megaterium* spores.

The spores of *B. megaterium* are structurally different respect to the spores produced by *B. subtilis*: they are significantly bigger and, as mentioned before, are surrounded by an additional layer, the exosporium. To analyze the binding efficiency and characterize the process of *B. megaterium* I compared the results obtained with *B. subtilis* and with a *B. megaterium* mutant producing spores without the exosporium (Manetsberger *et al.* 2015). The results suggested that the binding efficiency of *B. megaterium* spores was higher respect to *B. subtilis* spores and that the presence of the exosporium is essential for mRFP adsorption in *B. megaterium*. These data also suggest a new biotechnological application for the *B. megaterium* spores as a vehicle to bind and deliver heterologous proteins. These results are reported in Chapter 6 and have been published in paper:

> Lanzilli M., Donadio G., Addevico R., **Saggese A.**, Cangiano G., Baccigalupi L., Christie G., Ricca E., Isticato R. 2016. *The exosporium of Bacillus megaterium QM B1551 is permeable to the red fluorescence protein of the coral Discosoma sp.* Front. Microbiol., 7:1752.

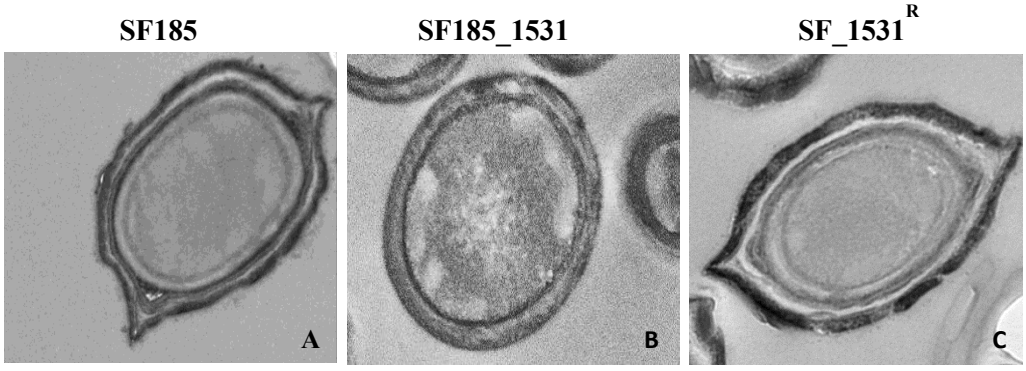

Figure 6: Thin-section TEMs of *Bacillus megaterium* spores obtained from the SF185 wild type strain (A), the SF_1531 mutant (B) and the SF_1531^{R} revertant strain (C).

Riassunto

Durante la mia tesi di dottorato ho lavorato nel laboratorio di microbiologia della prof. Baccigalupi, presso il dipartimento di Biologia dell'Università Federico II di Napoli, dove mi sono occupata dello studio di batteri sporigeni appartenenti al genere *Bacillus*, in particolare delle 2 specie *B. subtilis* e *B. megaterium*.

Il processo di sporulazione rappresenta un esempio di differenziamento cellulare in quanto due cellule con identico cromosoma seguono programmi di espressione genica differenziati. Se le condizioni ambientali sono favorevoli alla moltiplicazione cellulare, la cellula va incontro ad un classico ciclo vegetativo durante il quale essa si accresce, replica il suo cromosoma e si divide in modo simmetrico originando due cellule identiche tra loro ed alla cellula madre che le ha originate. Quando le condizioni ambientali non sono ottimali per la crescita microbica, i batteri sporigeni seguono invece un ciclo vitale alternativo, detto "sporulazione", che comporta la formazione di due cellule differenti tra loro, la cellula madre e la prespora. Il processo di sporulazione necessita di circa 10 ore per completarsi e dare origine ad una spora matura che viene rilasciata nell'ambiente per lisi della cellula madre (Tan and Ramamurthi. 2014). La spora può rimanere in questo stato metabolicamente inerte per un periodo di tempo indefinito, resistendo a condizioni ambientali anche estreme (quali temperatura, pH o esposizione a sostanze chimiche tossiche), ma qualora le condizioni ritornassero favorevoli, la spora è in grado di germinare, dando origine ad una nuova fase di crescita vegetativa (Setlow, 2003).

Le spore batteriche devono la loro estrema resistenza alla presenza di un abbondante strato proteico costituito da più di 70 proteine (proteine Cot) detto tunica sporale (McKenney *et al*. 2013). La formazione della tunica è un processo altamente controllato sia a livello trascrizionale che post-traduzionale. A livello trascrizionale intervengono 2 fattori sigma dell'RNA polimerasi SigE e SigK e 3 regolatori trascrizionali SpoIIID, GerR e GerE (Henriques A. Moran, 2007; Cangiano *et al*., 2010). Il controllo post-traduzionale è svolto da alcune proteine che hanno sia ruolo strutturale che morfogenetico in quanto regolano l'assemblaggio dei vari componenti strutturali della tunica. Alla luce di ciò, la formazione della tunica sporale rappresenta un ottimo sistema modello per lo studio sia dei meccanismi di controllo dell'espressione genica che delle interazioni proteiche nei batteri.

Relativamente alla superficie esterna della spora, le due specie batteriche oggetto di studio differiscono per più aspetti. La tunica del sistema modello *B. subtilis* è stata ampiamente studiata e caratterizzata mentre molto meno conosciuta è la struttura delle spore di *Bacillus megaterium*. Inoltre è noto che le spore di *B. megaterium* presentano uno strato esterno aggiuntivo chiamato esosporio, assente invece in *B. subtilis*.

Il mio progetto di dottorato è consistito nella caratterizzazione molecolare della superficie delle spore di *B. subtilis* e *B. megaterium* e la Tesi è stata organizzata in 2 parti: la **prima parte**, (Capitoli 2, 3 e 4), è focalizzata sul ruolo che le 3 proteine CotG, CotH e CotE, svolgono nell'assemblaggio della tunica sporale in *B. subtilis.* La **seconda parte**, suddivisa nei capitoli 5 e 6, è focalizzata sullo studio della superficie sporale di *B. megaterium* e sull'utilizzo delle sue spore come piattaforma per l'esposizione di proteine eterologhe.

PARTE 1: Assemblaggio della tunica sporale di *Bacillus subtilis*

I capitoli **2** e **3** riportano il ruolo che le proteine CotH e CotG svolgono durante l'assemblaggio della tunica sporale. La proteina CotH è stata già precedentemente studiata e in parte caratterizzata presso il laboratorio dove ho svolto la Tesi di Dottorato. La proteina è un componente della tunica che influenza la germinazione e che regola l'assemblaggio di almeno altri 9 componenti della tunica tra cui la proteina CotG (Naclerio *et al*. 1996; Zilhao *et al.* 1999). CotG a sua volta è necessaria per la maturazione di CotB, un'altra proteina della tunica che, solo in presenza di CotG, viene convertita da una forma di 46 kDa in una forma matura di 66kDa che si trova assemblata sulla spora (figura 1, pag. 98). I 2 geni strutturali che codificano per *cotG* e *cotH* mappano nella stessa regione cromosomica e sono adiacenti e trascritti divergentemente. Esperimenti effettuati nel mio laboratorio avevano dimostrato che il promotore di *cotH* è posizionato circa 800 bp a monte della regione codificante e di conseguenza il gene *cotG* è interamente compreso tra il promotore e il *coding* di *cotH* (figura 2, pag. 98; Giglio *et al.* 2011). Come conseguenza di tale peculiare organizzazione trascrizionale, qualsiasi inserzione o delezione nel gene *cotG* comporta anche una inattivazione del gene *cotH.* Allo scopo di chiarire il fenotipo del singolo mutante *cotG* ho introdotto una mutazione puntiforme che comportasse la formazione di un codone di stop nella regione codificante per *cotG*, senza alterare in alcun modo l'espressione del gene divergente. Dall'analisi del singolo mutante è emerso che in assenza della proteina CotG, come atteso da dati già riportati, non si verifica la maturazione CotB che si assembla nella sua forma immatura da 46kDa. Le spore mutanti non presentano altri difetti e sono perfettamente in grado di germinare. Il mutante *cotH*, come già noto in letteratura, mostrava una serie di difetti sia per quanto riguarda l'assemblaggio di diverse proteine Cot (CotG, CotU, CotS, CotC), sia per quanto riguarda l'efficienza di germinazione. Un risultato inaspettato è emerso in seguito all'analisi del doppio mutante *cotGcotH*: nonostante l'assenza di CotH esso non ha mostrato alcun difetto né di germinazione, né di assemblaggio delle proteine della tunica sporale. Questo risultato ci ha permesso di concludere che i fenotipi sinora attribuiti all'assenza di CotH, sono in realtà dovuti alla presenza di CotG, la quale, in assenza dell'CotH che ha funzione antagonista, esercita una serie di effetti negativi sulla formazione della tunica e sulla germinazione. Sulla base di questi dati ho proposto il nuovo modello riportato schematicamente in figura 3 (pag. 99). Questi dati sono riportati nel capitolo 2 e sono stati pubblicati nel seguente paper:

> **Saggese A.**, Scamardella V., Sirec T., Cangiano G., Isticato R., Pane F., Amoresano A., Ricca E., Baccigalupi L. 2014. *Antagonistic role of CotG and CotH on spore germination and coat formation in Bacillus subtilis.* PLoS ONE 9(8):e104900.

Il meccanismo con cui CotH antagonizza l'effetto negativo di CotG non è ancora noto. Un altro risultato del mio lavoro, riportato nel capitolo 2, mostra che CotG ha una peculiare struttura proteica costituita da una serie di moduli aminoacidici ripetuti in tandem, carichi positivamente e ampiamente fosforilati. Sulla base del fatto che CotH ha una certo grado di omologia con la famiglia delle Serin-treonin chinasi eucariotiche, ho ipotizzato che CotH potesse essere la chinasi responsabile della fosforilazione di CotG. Un recente lavoro pubblicato da Nguyen e collaboratori (2016) ha dimostrato mediante esperimenti in vitro e in vivo che effettivamente CotH è una chinasi e che CotG è uno dei suoi target. Sulla base di questi dati ho quindi ipotizzato che in assenza di CotH, CotG non viene

fosforilata ed in questa forma esercita l'effetto negativo da me evidenziato. L'attività chinasica di CotH è dunque in grado di mascherare le cariche positive di CotG, stabilizzando la proteina e rendendola incapace di svolgere la sua azione negativa.

Successivamente mi sono chiesta se anche nelle altre specie del genere *Bacillus* è presente un simile meccanismo di interazione tra queste 2 proteine. Per chiarire ciò è stata condotta un'analisi bioinformatica allineando le sequenze proteiche di CotG e CotH con tutti i genomi dei Bacilli completamente sequenziati disponibili in banca dati. Da tale analisi è emerso che mentre CotH è una proteina molto conservata in tutte le specie del genere *Bacillus*, CotG è conservata solo in 5 specie (Giglio *et al.* 2011). Mi sono quindi chiesta quale fosse l'organizzazione trascrizionale del locus *cotH* nelle specie che risultavano non contenere CotG. Dall'analisi dei genomi è emerso che in realtà in tutte le specie è sempre presente un gene divergente a *cotH* che codifica per una proteina che pur non avendo una sequenza aminoacidica primaria omologa a CotG, conserva tutte le sue caratteristiche peculiari (Saggese *et al.* 2014):

1) presenza di una regione centrale costituita dalla ripetizione di moduli ripetuti in tandem pressocché identici tra loro (figura 4, pag. 100) ;
2) alta % di aminoacidi carichi positivamente con conseguente elevato punto isoelettrico;
3) una sequenza aminoacidica che, da un'analisi bioinformatica, risulta sempre intrinsicamente disordinata e un buon substrato di fosforilazione

Sulla base di queste osservazioni, possiamo dire di aver identificato una nuova famiglia di proteine altamente conservate, chiamate *CotG-like*, che nonostante l'assenza di una omologia di sequenza con la proteina CotG, conservano le stesse caratteristiche strutturali e sono codificate da un gene con la stessa localizzazione cromosomica.

La conservazione evolutiva della struttura modulare di CotG e delle proteine CotG-like, mi ha spinto ad investigare sul ruolo che tali moduli svolgono e, a tale scopo, ho costruito una serie di mutanti per delezione di CotG di *B. subtilis*:

1) il primo che esprime le due regioni N-terminale e C-terminale di CotG, ma deleto dell'intera porzione modulare centrale;
2) il secondo che esprime solo la regione N-terminale;
3) il terzo che esprime solo la regione C-terminale.

L'analisi di tali mutanti ha mostrato che:

- le regioni N-terminale e C-terminale sono sufficienti per garantire la maturazione di CotB nella forma da 66kDa;
- la regione centrale costituita dai moduli ripetuti in tandem è responsabile dell'effetto negativo di CotG sull'assemblaggio di CotC, CotU e CotS e sulla germinazione (vedi capitolo 2).

Questi risultati confermano l'ipotesi secondo cui il ruolo di CotH è quello di fosforilare i moduli ripetuti mascherando le cariche positive degli aminoacidi basici e consentendo alla proteina di assumere una corretta conformazione.

Questi dati sono riportati nel capitolo 3 e pubblicati nel seguente paper:

> **Saggese A.**, Isticato R., Cangiano G., Ricca E. and Baccigalupi L. 2016. *CotG of Bacillus subtilis is a modular protein of spore forming Bacilli.* J Bacteriol. 198(10):1513-20.

La proteina CotH interagisce anche con CotE, un'altra proteina morfogenetica che svolge un ruolo cruciale per consentire il corretto assemblaggio della tunica sporale e in particolare di tutto lo strato più esterno della spora (Zheng L. *et al.* 1988). Tra le varie proteine CotE-dipendenti c'è la proteina CotH che, a sua volta regola l'assemblaggio di altri componenti della tunica (Nacleiro *et al.* 1996; Zilhao *et al.* 1999). I nostri studi hanno evidenziato che, in maniera reciproca, anche CotH controlla l'assemblaggio di CotE, in seguito a una diretta interazione tra le 2 proteine. Mediante l'analisi di una serie di mutanti di *cotE*, abbiamo dimostrato che la regione C-terminale di CotE è responsabile dell'interazione con CotH e, mediante la costruzione di mutanti puntiformi, abbiamo identificato gli specifici aminoacidi coinvolti in tale interazione.
I risultati ottenuti sono riportati nel capitolo 4 e pubblicati nel seguente paper:

> Isticato R., Sirec T., Vecchione S., Crispino A., **Saggese A.**, Baccigalupi L., Notomista E., Driks A. and Ricca E. 2015. *The direct interaction between two morphogenetic proteins is essential for spore coat formation in Bacillus subtilis.* PLoS ONE. 10(10):e0141040.

PARTE 2: Studio della struttura della spora di *Bacillus megaterium*

Una parte del mio progetto di Tesi ha riguardato lo studio della spora di *B. megaterium* e del suo utilizzo come piattaforma per l'esposizione di proteine eterologhe. Come già menzionato nell'introduzione, le spore prodotte da *B. megaterium* presentano una strato esterno detto esosporio (Di Luccia *et al.* 2016), assente in *B. subtilis*. Il sistema modello per la specie di *B.megaterium* è il ceppo QM B1551, sul quale sono stati già compiuti diversi studi relativi alla composizione della tunica sporale e dell'esosporio, ma non è ancora disponibile una completa caratterizzazione. Al fine di contribuire ad ampliare le conoscenze in merito, ho deciso di investigare sulla composizione, la struttura e l'assemblaggio dell'esosporio, considerando un ceppo SF185 isolato dall'ileo di volontari sani (Fahkry *et al.* 2008). Per prima cosa ho condotto un'analisi proteomica per identificare le più abbondanti proteine che costituiscono gli strati più esterni della spora. A tale scopo le proteine estratte sono state trattate con SDS-DTT, frazionate su un gel di poliacrilammide e successivamente colorate mediante Blu di Comassie. Tra le diverse proteine presenti (figura 5A, pag. 101), una di quelle più abbondanti corrispondeva alla banda di circa 16kDa. La proteina è stata estratta e l'analisi della sequenza della regione N-terminale ha evidenziato che si trattava di una proteina a funzione sconosciuta codificata dal gene *SF185_1531*. Al fine di identificare il ruolo che la proteina svolge, ho costruito un mutante *knock out* trasformando il ceppo con il plasmide pSOCam1 (figura 5B, pag. 101) che, in seguito a un evento di singolo crossing over a livello del gene strutturale in questione *SF_1531*, ne causava l'interruzione; le spore mutanti sono state quindi osservate mediante microscopia elettronica a trasmissione. Le immagini riportate in figura 6 (pag. 102) mostrano che rispetto alle spore wt (figura 6A), quelle mutanti mancano totalmente dell'esosporio (figura 6B). Questo risultato suggerisce che la proteina SF_1531 svolge, in maniera diretta o indiretta, un ruolo cruciale nell'assemblaggio dell'esosporio. Per verificare che effettivamente tale fenotipo fosse dovuto realmente all'inattivazione del singolo gene *SF_1531* e non alla presenza di eventuali mutazioni secondarie, è stata indotta l'escissione del plasmide dal ceppo mutante al fine di ottenere la

ricostruzione del gene selvatico con un fenotipo "revertante". Anche in questo caso le spore sono state analizzate al TEM. Come si può osservare in figura 6C il ceppo revertante presenta una spora identica a quella del ceppo *wt*, con un esosporio perfettamente intatto.

I dati ottenuti mi hanno permesso di confermare che la proteina SF_1531 effettivamente è indispensabile per il corretto assemblaggio dello strato più esterno che avvolge le spore di *B. megaterium.* I risultati ottenuti sono riportati nel capitolo 5 e sono raccolti in un lavoro in fase di stesura. Infine, nel capitolo 6, è riportato l'utilizzo delle spore di *B. megaterium* come vettore per l'esposizione di molecole eterologhe con attività antigenica o enzimatica. Sino ad oggi solo le spore dell'organismo modello *B. subtilis* sono state utilizzate con questo scopo per diverse applicazioni biotecnologiche, ottenendo vaccini mucosali, bio-catalizzatori o sistemi per la diagnostica e la *bioremediation*. Durante il mio progetto di tesi abbiamo deciso di verificare se anche le spore di *B. megaterium* fossero in grado di legare molecole eterologhe. Queste, rispetto alle spore di *B. subtilis* sono più grandi ed inoltre, come già detto precedentemente, possiedono l'esosporio come strato esterno aggiuntivo. A tale scopo ho utilizzato la proteina fluorescente mRFP di *Discosoma coral* (Campbell *et al*., 2002) per valutare se le spore del ceppo QM B1551 di *B. megaterium* fossero in grado di adsorbirla sulla propria superficie. I risultati ottenuti hanno dimostrato che effettivamente mRFP viene legata sulla spora di *B. megaterium* in modo più efficiente rispetto alle spore di *B. subtilis*. Abbiamo inoltre effettuato una parziale caratterizzazione del meccanismo di adesione valutando se e in che modo l'esosporio influenza il processo. A questo scopo abbiamo utilizzato un ceppo di *B. megaterium* mutante che produce spore mancanti di esosporio (Manetsberger *et al.* 2015) ed abbiamo confrontato l'efficienza di legame della mRFP rispetto a quella ottenuta con le spore wt. Dall'analisi è emerso che la presenza dell'esosporio è essenziale per consentire l'adsorbimento di mRFP in *B. megaterium*. In conclusione i dati ottenuti suggeriscono che le spore di *B. megaterium* possono essere utilizzate come veicolo per legare proteine eterologhe in quanto in grado di legarle stabilmente e con elevata efficienza. I risultati ottenuti sono riportati nel capitolo 6 e sono stati pubblicati nel seguente paper:

> Lanzilli M., Donadio G., Addevico R., **Saggese A.**, Cangiano G., Baccigalupi L., Christie G., Ricca E., Isticato R. 2016. *The exosporium of Bacillus megaterium QM B1551 is permeable to the red fluorescence protein of the coral Discosoma sp.* Front. Microbiol., 7:1752.

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